LEVEL II

AD

RCS MEDDH-288(R1)

RESEARCH IN BIOLOGICAL AND MEDICAL SCIENCES

INCLUDING

BIOCHEMISTRY, COMMUNICABLE DISEASE AND IMMUNOLOGY, INTERNAL MEDICINE, PHYSIOLOGY, PSYCHIATRY, SURGERY, AND VETERINARY MEDICINE,

(PROJECTS AND WORK UNITS ARE LISTED IN TABLE OF CONTENTS)

ANNUAL PROGRESS REPORT
1 OCTOBER 1978 – 30 SEPTEMBER 1979

VOLUME I

WALTER REED ARMY INSTITUTE OF RESEARCH
WALTER REED ARMY MEDICAL CENTER
WASHINGTON, D. C. 20012

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The various subjects covered in this report are listed in the Table of Contents. Abstracts of the individual investigations are included on the DD Form 1498 introducing each work unit report.

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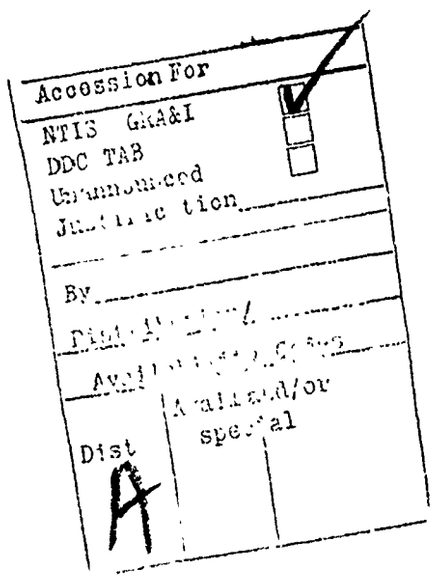
FORWARD

IN CONDUCTING THE RESEARCH DESCRIBED IN THIS REPORT, THE INVESTIGATORS ADHERED TO THE "GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS" AS PROMULGATED BY THE COMMITTEE ON CARE AND USE OF LABORATORY ANIMALS OF THE INSTITUTE OF LABORATORY ANIMAL RESOURCES, NATIONAL ACADEMY OF SCIENCES - NATIONAL RESEARCH COUNCIL.



SUMMARY

THE VARIOUS SUBJECTS COVERED IN THIS REPORT ARE LISTED IN THE TABLE OF CONTENTS. ABSTRACTS OF THE INDIVIDUAL INVESTIGATIONS ARE INCLUDED ON THE DD FORM 1498 INTRODUCING EACH WORK UNIT REPORT, AND NAMES OF THE INVESTIGATORS ARE GIVEN AT THE BEGINNING OF EACH REPORT.

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VOLUME I

VOLUME II

Project 3A161101A91C

IN-HOUSE LABORATORY INDEPENDENT RESEARCH

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
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78 10 01	H. Termination	U	U	NA	NL	☐ YES ☒ NO	A. WORK UNIT

10. NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	095
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)*

(U) Investigations of the Owl Monkey as a Laboratory Animal

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

001700 Animal Husbandry 002600 Biology 012900 Physiology

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76 01	79 09 30	DA			C. In-House

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18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
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19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME:

NAME

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Aotus trivirgatus; (U) Owl monkey; (U) Breeding; (U) Karyotype

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To obtain biologic and physiologic data of the owl monkey essential for optimizing colony management procedures, recognizing disease states and breeding and raising Aotus for studies of falciparum malaria, for which it is the best nonhuman primate host. Aotus are no longer commercially available in sufficient numbers to meet the Army's requirements for its malaria drug development research program. Physiologic data, presently unavailable or unconfirmed for the owl monkey, is critically needed to assure the success of a domestic breeding effort to supply these monkeys for malaria research.

24. (U) Physiologic data was obtained from infants to fully mature adults to include the following measurements: growth and development patterns, serum biochemical and enzyme values, and hematologic values. In addition breeding information was obtained. A standard Subhuman Primate Pregnancy Test Kit was evaluated as a simplified means of pregnancy diagnosis in owl monkeys.

25. (U) 78 10 - 79 09 During the year there were 22 live births and 12 abortions or still births in the colony. The Subhuman Primate Pregnancy Test Kit was determined to be a safe and useful method for pregnancy determination. The overall accuracy of the test could be greatly increased by performing a second independent test. To reduce the death loss in these delicate animals, various management procedures have been evaluated. The death rate among newborn animals has been reduced from 50% to less than 20%. To further define this animal model, serum protein electrophoretic patterns were studied. Differences were noted among the various subspecies, some of which appeared to be karyotype specific. This information should be evaluated to explain the difference in response to malaria infection of the various subspecies, and as a potential use for subspecies identification. For technical report see Walter Reed Army Institute of Research Annual Progress Report. 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 095 Investigations of the Owl Monkey as a Laboratory Animal

Investigators.
Principal: Robert D. Hall, LTC, VC
Associates: M. J. Reardon, MAJ, VC; R. J. Beattie, LTC, VC

Description:

To obtain biologic and physiologic data on the owl monkey (Aotus trivirgatus) which are needed to optomize colony management, recognize disease states, and successfully breed and rear these monkeys for falciparum malaria studies, for which it is the best nonhuman primate host. Physiologic data, presently unavailable or unconfirmed for the owl monkey, is critically needed to assure the success of a domestic breeding effort to supply these monkeys for malaria research.

During the reporting period, research activities have included analyses of: 1) a hemagglutination inhibition test for early detection of pregnancy, 2) weight gains and dental eruptions in infant monkeys, 3) management techniques utilized, and 4) serum protein electrophoretic patterns of owl monkeys.

Progress

1. Pregnancy Diagnosis in Owl Monkeys: Evaluation of the Hemagglutination Inhibition Test for Urinary Chorionic Gonadotropin.

The purposes of this study were to define the pattern of excretion of urinary chorionic gonadotropin during gestation of the owl monkey and to evaluate the application of the Subhuman Primate Pregnancy Test kit for detecting pregnancy in owl monkeys using a practical management technique.

The Subhuman Primate Pregnancy Test kit has been used for detecting pregnancy in several species of nonhuman primates. This hemagglutination inhibition tube test for uriniary chorionic gonadotropin uses an antiserum (H-26) to the β-subunit of ovine luteinizing hormone which cross-reacts with the chorionic gonadotropins of a variety of primate species and provides definitive results within 2 hours using neat urine. In addition, this same antiserum has been employed in a radioimmunoassay system

for quantification of chorionic gonadotropins.

During this study, five monkeys became pregnant and delivered live offspring. Urine specimens collected throughout these pregnancies were assayed by the Subhuman Primate Pregnancy Test kit and radioimmunoassay for Aotus chorionic gonadotropin.

Results of the hemagglutination inhibition test for Aotus gonadotropin using the Subhuman Primate Pregnancy Test kit between the sixteenth and fourteenth week prepartum, only half of the specimens showed a positive reaction; however, between the fourteenth week and the last week, the Subhuman Primate Pregnancy Test results were highly reliable for detecting pregnancy, exceeding 90% accuracy. Equivocal results bracketed or followed by a series of at least three positive reactions were considered positive. These equivocal results were seen most frequently at the beginning and end of the gestation period when chorionic gonadotropin levels were near the minimum levels detectable by the Subhuman Primate Pregnancy Test kit.

Detecting abortions by monitoring urinary chorionic gonadotropin levels was essential information when forming a reproductive profile for owl monkey breeders. Data from this study showed that owl monkeys continue to produce chorionic gonadotropin at detectable levels until the week of delivery. This made the Subhuman Primate Pregnancy Test particularly useful for monitoring continuation of gestation in owl monkeys. The pregnancy test kit allowed detection of spontaneous abortions rapidly and accurately, and eliminated the need for frequent handling to perform uterine palpation, which, itself, may contribute to fetal wastage.

2. Weight Gains and Sequence of Dental Eruptions in Infant Owl Monkeys.

Data on the time and sequence of eruption of deciduous and permanent teeth of owl monkeys were expanded by examining newborn monkeys twice weekly until all deciduous teeth had erupted and biweekly until all permanent teeth had erupted. The age at eruption of the deciduous teeth was observed and recorded for 28 infants. Owl monkeys are born without teeth. Eruptions usually began with the central incisors (I_1) at 1-3 weeks of age and were completed at 4-7 weeks of age when the posterior premolars (P_3) emerged.

A general eruption sequence of I_1,I_2, P_1, P_2, C and P_3 was observed; however, 42% of the animals had minor variations in this sequence. P, and P_2 or I_2 and P_1 frequently erupted

A general eruption sequence of I_1, I_2, P_1, P_2, C and P_3 was observed; however, 42% of the animals had minor variations in this sequence. P_1 and P_2 or I_2 and P_1 frequently erupted simultaneously. The eruption of mandibular I_2 incisors preceded the eruption of the ipsilateral maxillary I_2 incisors on 39 of 56 (70%) occasions; both erupted concurrently on the other 17 occasions. Otherwise, there was no consistency in maxillary or mandibular teeth erupting first.

The age at eruption of the permanent teeth was also observed. The anterior molars (M_1) were the first permanent teeth to erupt at 3.5-5 months of age. The eruption sequence of permanent teeth was M_1, M_2, I_1, M_3, I_2, P_3, P_2, P_1, and C for mandibular teeth and M_1, M_2, I_1, I_2, M_3, P_3, P_2, P_1, and C for maxillary teeth. Mandibular molars (M_1, M_2, and M_3) usually erupted before their maxillary counterparts while maxillary incisors (I_1 and I_2) and middle premolars (P_2) usually erupted before their mandibular counterparts. In 8 of 9 monkeys observed, all permanent teeth had erupted by 14.5-16 months of age.

In our experience, monitoring body weight was the simplest method for evaluating the health and monitoring the development of infant Aotus. Body weights were obtained when dental examinations were performed. The body weight of 36 apparently normal, 1-to-3 day old owl monkeys ranged from 69 to 114 grams with an average of 90 $\pm$ 11.2 grams ($\pm$ 1 standard deviation). The rate of weight gain was approximately 16 grams per week from birth through the 15th week of age and 10 grams per week thereafter through the 52nd week. This weight gain provided a valuable standard with which to evaluate the health status and observed versus expected growth performance for all other infant Aotus. No such standard has been here-to-fore available for any breeding colony.

3. Management techniques.

Data on the nutritional, environmental, and social requirements of Aotus are minimal. Therefore, colony management procedures were developed based upon: (a) the published experiences of other investigators; (b) the known requirements of other South American primates, and (c) experience acquired at the WRAIR over a 5 year period of time. This experience included quarantining and conditioning wild caught Aotus, as well as long term housing of Aotus on research projects and breeding experiments.

Monkeys are housed as breeding pairs or indivudally within 75 x 75 x 75 cm (0.4 cubic meters) wire mesh cages, as previously described (Schmidt, 1973). Two metal perches located 25 cm and 43 cm respectively, above the grid floor of each cage help keep the monkeys' tails from becoming contaminated with fecal matter. Stainless steel trays which contain heat treated hardwood chips are placed under the grid floor of each cage to collect animal wastes.

Little is known about the specific nutrient requirements of the owl monkey. The current diet represents a subjective attempt to satisfy all nutritional requirements until more definitive data are available. The basic diet is a commerically prepared high protein monkey biscuit (Purina Monkey Chow 25 TM, Ralston Purina Co., St Louis, MO). Biscuits are supplemented 5 days each week with either 1/2 apple or 1/2 orange per monkey, and on the remaining 2 days (Tuesday and Thursday) with 35 to 40 ml of a liquid dietary supplement per monkey. Results of a 90 day feed trial with 44 pairs of monkeys established that 8 biscuits per day was an optimum quantity of commercial food to offer each monkey.

Experience has demonstrated that the owl monkey exhibits a poor tolerance to handling or other forms of environmental stress. For example, abortions occurred in 7 Aotus within several days following tranquilization and physical examination. Trained veterinary technicians, protected with heavy leather gloves, atraumatically captured and restrained the monkeys. Monkeys were chemically restrained with ketamine HCl (Vetalar TM, Park Davis & Co., Detroit, MI) at a dosage rate of 5 mg/kg intramuscularly for all painful procedures, including venipuncture, or any other procedure that required the animal to be restrained for longer than 1 or 2 minutes. We feel that chemical restraint is safe and reduces stress to the animal. Clearly, for maximum breeding efficiency, handling and other forms of environmental stress should be kept to a minimum.

4. Serum Electrophoretic Patterns of Karyotypically Defined Owl Monkeys.

Alloalbuminemia, the presence of types of albumin migrating electrophoretically faster or slower than the common albumin for a species, has been reported. Bisalbuminemia, a unique type of alloalbuminemia, is characterized by two distinct albumin fractions with different electrophoretic mobilities, each representing one-half of the normal amount of albumin for the species. Bisalbuminemia has been reported in man, horses, sheep, cattle

and Japanese quail, and it is thought to be governed by codominant alleles. Published reports also indicate that some owl monkeys (Aotus trivirgatus) have bisalbuminemia. Some owl monkeys also have an additional and distinctly separate third alpha globulin.

Two hundred thirty-eight karyotyped A trivirgatus and 29 unkaryotyped offspring were studied. Of this number, 37 pairs and their colony-born offspring were available for study. Both sexes and a wide spectrum of ages from juvenile to old adults were present in the karyotyped groups.

There were distinct albumin and alpha globulin differences observed between individuals and in some instances a pattern was limited to one or two karyotypes. Three phenotypic forms of albumin were observed and designated as fast (F), double (D), and slow (S). The fast albumin was the common form for the species; all other forms were grouped together as slow. Karyotypes II, III, and IV were characterized by a heterogeneous albumin pattern with F, D and S phenotypes represented. These groups also had alpha 1 and alpha 2 globulin bends exclusively. Karyotype I, sometimes referred to as A trivirgatus trivirgatus, had F and S albumin phenotypes and three distinct alpha globulin bands. The third alpha globulin was electrophoretically faster than alpha 1 and alpha 2 under the conditions of this study and was designated as fast alpha. The only other karyotypes with a fast alpha were VII and hybrids involving Karyotype I. Karyotypes V, VI, and VII were represented by only a few animals in our colony but were observed to have the S albumin phenotype exclusively and V and VI had only alpha 1 and alpha 2 globulins.

The results of the present study indicate that there are definite serum protein differences involving the albumin component and the alpha globulins among different karyotypes of Aotus monkeys. The electrophoretic separation of serum proteins is a simple inexpensive method of screening for certain phenotypes which suggest that the indivudal is of particular genotype.

The mobility of the alpha globulin protein fraction is ahead of the alpha 1 globulin fraction and does not constitute alpha 3 globulin, as such, although it is a third band in the combined alpha region. The expression of the fast alpha trait appears to follow a dominant transmission pattern since all offspring of pairs in which one parent possessed the trait also have the fast alpha. The possible role of this protein fraction in malaria susceptibility is intriguing since Karyotype I animals (A trivirgatus trivirgatus) have been shown to be resistant to Plasmodium falciparum.

Conclusion.

During the year there were 22 live births and 12 abortions or still births in the colony. The Subhuman Primate Pregnancy Test Kit was determined to be a safe and useful method for pregnancy determination. The overall accuracy of the test could be greatly increased by performing a second independent test. To reduce the death loss in these delicate animals, various management procedures have been evaluated. The death rate among newborn animals has been reduced from 50% to less than 20%. To further define this animal model, serum protein electrophoretic patterns were studied. Differences were noted among the various subspecies, some of which appeared to be karyotype specific. This information should be evaluated to explain the difference in response to malaria infection of the various subspecies, and as a potential use for subspecies identification.

Management techniques for optimal production and physiological parameters to evaulate these techniques were obtained. These include various housing and husbandry techniques and evaluation of weight gains to monitor health status. From this information, the foundation for a successful breeding colony of owl monkeys has been developed.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 095 Investigations of the Owl Monkey as a Laboratory Animal

Literature Cited.

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16. Walter Reed Army Institute of Research Annual Progress Report, 1 July 1976 - 30 September 1977.

17. Laurell, C.B., and Nilehn. A New Type of Inherited Serum Albumin Anomaly. J Clin Invest 45:1935-1945. 1966.

Publications:

1. Hall, R.D., Beattie, R.J. and Wyckoff, G.H., Jr. Weight Gains and Sequence of Dental Eruptions in Infant Owl Monkeys (Aotus trivirgatus). Nursery Care of Nonhuman Primates, G.C. Ruppenthal and D.J. Reese editors, Plenum Publishing Corp. 1979.

2. Hall R.D. and Hodgen, G.D. Pregnancy Diagnosis in Owl Monkeys (Aotus trivirgatus): Evaluation of the Hemagglutination Inhibition Test for Urinary Chorionic Gonadotropin. Lab Anml Sci 29:3 1979.

3. Reardon, M.J., Hall, R.D., and Davidson, C.E. Serum Electrophoretic Patterns of Karyotypically Defined Owl Monkeys (Aotus trivirgatus). (Accepted for publication) Lab Anml Sci. October 1979.

4. Hall, R.D., Renquist, D.M., Montrey, R.D., Beattie, R.J. and Wyckoff, G.H., Jr. Management of an Experimental Breeding Colony of Aotus Monkeys. (Prepared for publication)

Presentations:

1. Hall, R.D. et al. Weight Gains and Sequence of Dental Eruptions in Infant Owl Monkeys (Aotus trivirgatus). Conference of Nursery Care of Nonhuman Primates, Seattle, Washington. May, 1977.

2. Beattie, R.J. Panel Discussion on Management of Breeding Colonies in Laboratory Animals. 7th Annual Seminar, National Capital Area Branch of American Association of Laboratory Animal Science, Cockeysville, MD. September, 1977.

3. Hall, R.D. and Hodgen, G.D. Pregnancy Diagnosis in Owl Monkeys: Evaluation of the Hemagglutination Inhibition Test for Urinary Chorionic Gonadotropin. 29th Annual Session for American Association of Laboratory Animal Science, New York, NY. September, 1978.

4. Reardon, M.J. and Hall R.D. Electrophoretic Study of Serum Protein and Hemoglobin in Karyotypically Defined Owl Monkeys. 29th Annual Session of American Association of Laboratory Animal Science, New York, NY. September 1978.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6448	79 10 01	DD DRAE(AR)636

3 DATE PREV SUMRY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 REGRADING	8 DISP'N INSTR	8a SPECIFIC DATA-CONTRACTOR ACCESS	9 LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10 NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	096
b. CONTRIBUTING				
c. CONTRIBUTING				

11 TITLE (Precede with Security Classification Code)

(U) Biochemical and Immunological Characterization of Rickettsial Proteins

12 SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMANCE METHOD
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18 RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	4	163
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19 RESPONSIBLE DOD ORGANIZATION

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21 GENERAL USE

Foreign intelligence not considered

22 KEYWORDS (Precede EACH with Security Classification Code)

(U)Biochemistry; (U)Structure-Function Relationship; (U)Structure-Antigenicity Relationship

23, 24, 25 TECHNICAL OBJECTIVE, APPROACH, PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Isolate and purify the subcellular rickettsial components responsible for eliciting immunologic protection. Localize and identify the rickettsial surface proteins that affect virulence. Antigenically characterize the peripheral proteins of rickettsiae. These studies will aid in the development of inactivated vaccines capable of protecting deployed troops.

24. (U) Isolate and evaluate the peripheral rickettsial macromolecules as experimental immunogens. Analyze the rickettsial proteins using polyacrylamide gel electrophoresis and immunoelectrophoresis with specific radiolabeling by extrinsic radioiodination or sodium borohydride reduction. Evaluate the potential roles of activity for each protein isolated. Compare the composition of isolated proteins from representative strains of the different rickettsial groups. Develop the rickettsial plaque reduction assay. Determine the antigenic interrelationships among strains of scrub typhus rickettsiae using plaque reduction, complement fixation, immunofluorescent, cross-neutralization, and mouse virulence tests with specific rickettsial antibody prepared in laboratory animals.

25. (U) 78 10 - 79 09 Antigens of the typhus and scrub rickettsial groups were analyzed using enzyme-linked immunosorbent assay and polyacrylamide gel electrophoresis. Of the 6 major structural proteins of typhus rickettsiae, the major group specific antigenic activity migrated with proteins 3 and 4, a peripheral cell envelope protein. The broad peak of reactivity indicated that this antigen was most likely a complex moiety, perhaps a glycoprotein. Analysis of scrub typhus prototype strains Gilliam and Karp showed the presence of 8 antigens reactive with mouse antisera. Heterologous antisera identified antigenic variations in the Karp strain. A plaque reduction assay was developed. Homologous and heterologous tests demonstrated the test will be useful for strain differentiation and characterization. Eight strains of R tsutsugamushi have been cloned for characterization. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78 - 30 Sep 79.

*Available to contractors upon offeror's approval

DD FORM 1498 (MAR 68) PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 096 Biochemical and Immunological Characterization of Rickettsial Proteins

Investigators:

Principals: Joseph V. Osterman, PhD; MAJ Stanley C. Oaks, Jr., MSC; Christine S. Eisemann, MS

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Description:

Investigations to isolate and purify the subcellular rickettsial components responsible for eliciting immunologic protection, localize and identify the rickettsial surface proteins that affect virulence, and antigenically characterize the peripheral proteins of rickettsiae. These studies will aid in the identification of rickettsial strains and in the development of inactivated vaccines capable of protecting deployed military troops.

During the reporting period, research activities have included: (1) analysis of the antigens of the typhus and scrub typhus rickettsial groups, (2) evaluation of strains of mice as model systems for typhus and spotted fever group infections, (3) development of a plaque reduction assay, and (4) characterization of strains of Rickettsia tsutsugamushi by various techniques.

Progress:

Previous investigations of typhus group rickettsiae, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), demonstrated the presence of 6 major cell envelope proteins (1). The topographical orientation of these molecules within the cell envelope was ascertained by selective extrinsic radioidination. The most peripheral proteins (2) were of the greatest interest, since they are most accessible to the immune system of the host and may have a role in stimulating protective immunity. The current studies were undertaken to examine the antigenicity and immunogenicity of these peripheral proteins.

Antigenicity of the proteins from R prowazeki and R typhi was examined by a modified enzyme-linked immunosorbent assay (ELISA). Proteins (100-200 ug) from intact organisms were separated by SDS-PAGE and the gels were sectioned as previously described (1). Each gel slice was placed in a polystyrene tube and eluted with 0.05M carbonate buffer, pH 9.6. These conditions favored the binding of the eluted protein to the surface of the tube, and

residual gel material could then be removed by washing with 0.01M phosphate buffered saline containing 0.05% Tween 20 and 0.003M sodium azide (pH 7.2). Rickettsial antigen (protein) bound to the tube was exposed to either homologous or heterologous mouse anti-rickettsial serum. The colorimetric portion of the ELISA reaction was completed by adding anti-mouse IgG globulin conjugated to alkaline phosphatase, washing, then indroducing Sigma phosphatase substrate. Figure 1 illustrates the ELISA profile obtained after electrophoresis of R prowazeki proteins and reaction with homologous antiserum. A small quantity of ^{125}I-labeled organisms was co-electrophoresed in the gel to serve as a marker for the position of the 6 major proteins. A broad peak of antigenic activity was apparent (fractions 27-36) in the same region of the gel which contained proteins 3 and 4. When heterologous, anti-R typhi serum was employed, a similar pattern of antigenic reactivity was observed (Fig. 2). In this latter experiment, protein 4 of the marker rickettsiae was selectively radioiodinated.

It seems clear that the serum-reactive antigen peak contains group-specific determinants, since binding of both homologous and heterologous antisera was observed. However, electrophoretic mobility of the antigen peak was not coincident with any single rickettsial protein. The antigen peak seemed to include both proteins 3 and 4, but individual protein reactivity could not be distinguished.

Scrub typhus rickettsiae also were electrophoresed and examined by the ELISA technique. Rickettsia tsutsugamushi, strains Gilliam and Karp, were propagated in irradiated L-929 cells, partially purified by differential centrifugation, and chromatographed in a Sepharose 4B column. The resulting ELISA profiles for the 2 strains of R tsutsugamushi are shown in Figs. 3a and 3b. At least 8 reactive antigens were detected for both strains. Each of the antigens from Gilliam strain rickettsiae reacted with both homologous Gilliam and heterologous Karp antisera (Fig. 3a). By contrast, only Karp antigens 2, 3, and 7 were reactive with heterologous Gilliam antiserum (Fig. 3b). Of the 8 peaks of antigenic reactivity for Gilliam ricketteiae, 2 could be correlated to specific rickettsial proteins (Fig. 4).

Three of the serum-reactive antigens apparently contained group-specific determinants, as evidenced by the binding of both homologous and heterologous antisera. The deviation of reactivity between strains of the remaining 5 antigens could not be explained with these studies; however, the observations made coincided with the characteristics of Gilliam and Karp strains defined by the plaque reduction assay (3). Thus, analysis of scrub typhus rickettsiae by SDS-PAGE-ELISA provides an additional parameter for evaluating antigenic relatedness among strains of this rickettsial group.

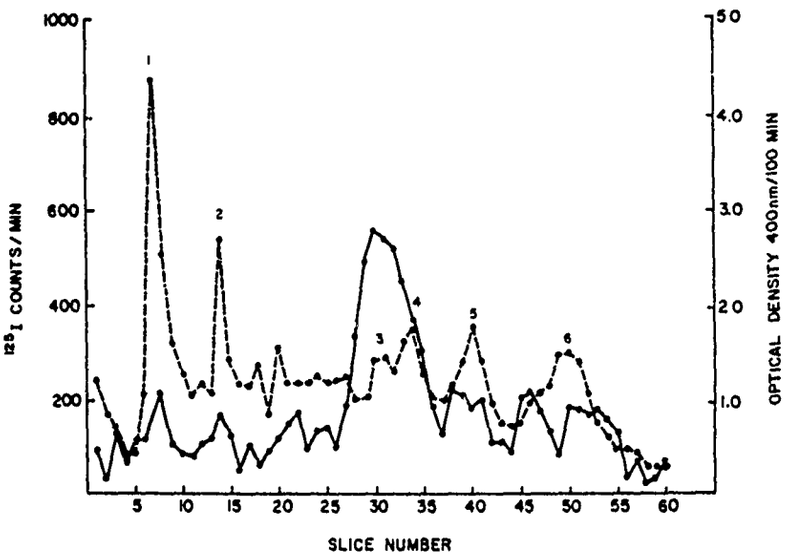

Fig. 1. Electropherogram of proteins of R prowazeki analyzed by an ELISA using homologous antisera. ELISA = o; ^{125}I counts/min = ●.

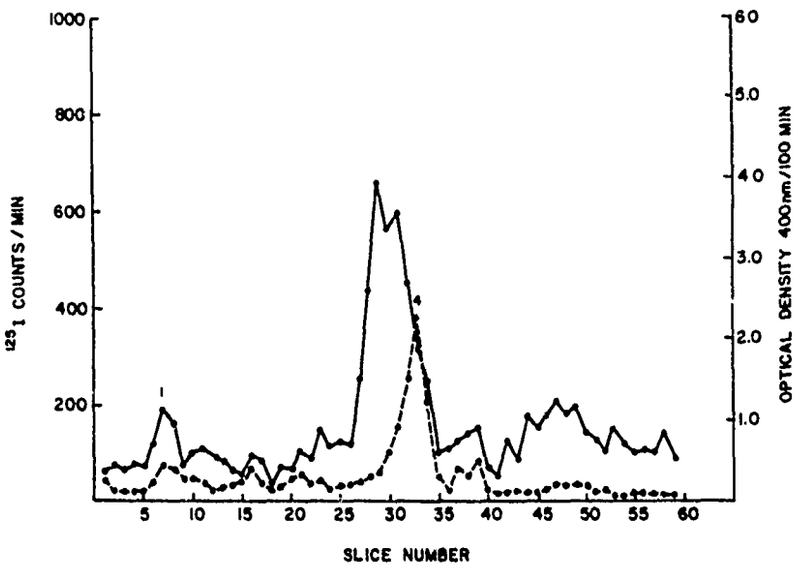

Fig. 2. Electropherogram of proteins of R prowazeki analyzed by an ELISA using heterologous, R typhi antisera. ELISA = o; ^{125}I counts/min = ●.

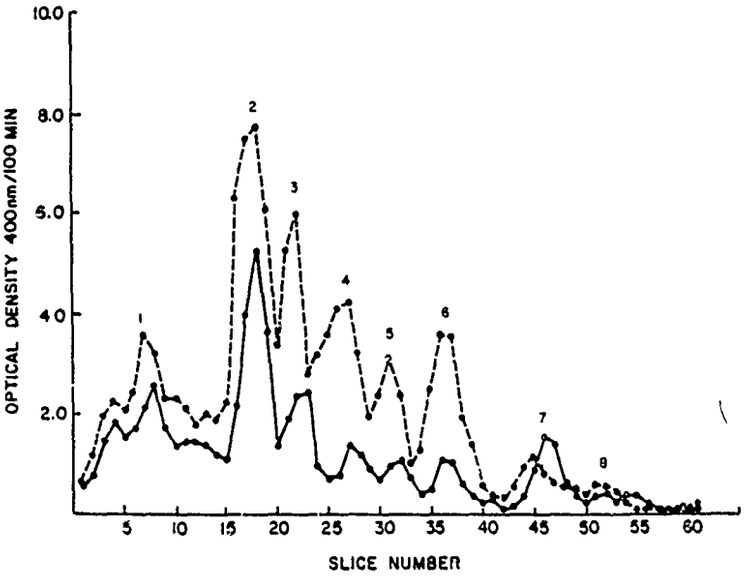

Fig. 3a. Electropherograms of proteins of R tsutsugamushi, strain Gilliam, analyzed by an ELISA using homologous Gilliam antiserum (o) and heterologous Karp antiserum (●).

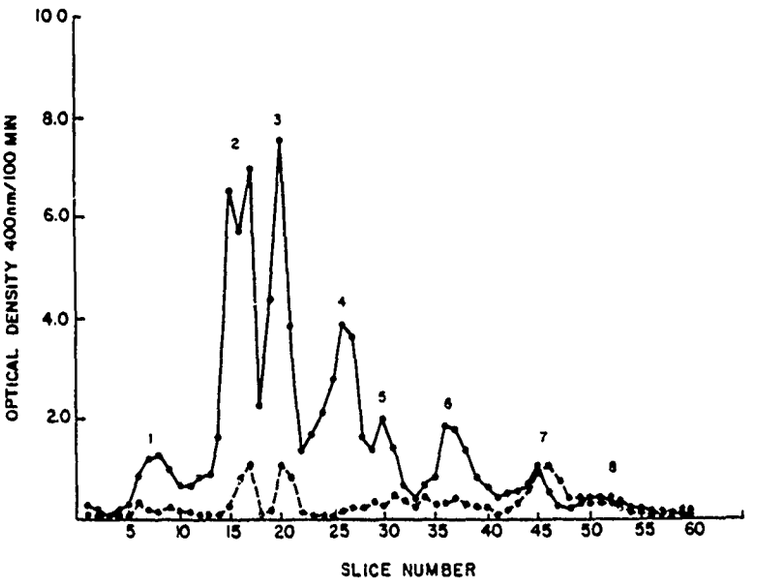

Fig. 3b. Electropherograms of proteins of R tsutsugamushi, strain Karp, analyzed by an ELISA using homologous Karp antiserum (o) and heterologous Gilliam antiserum (•).

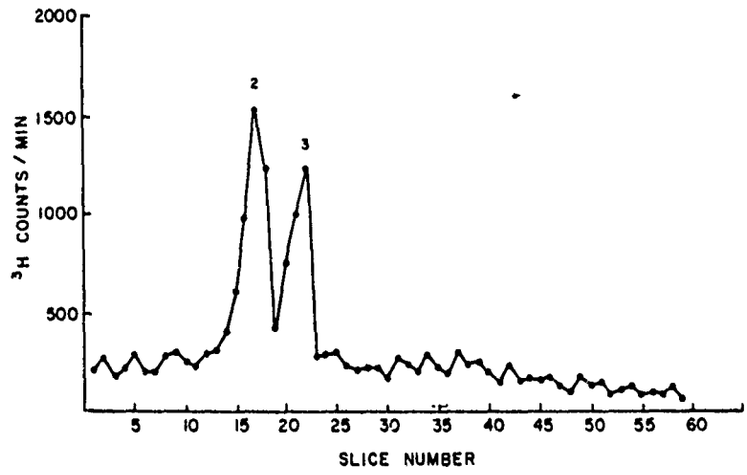

Fig. 4. Electropherogram of R tsutsugamushi, strain Gilliam, intrinsically labeled with ^{3}H-amino acids.

2. Evaluation of various strains of mice for a model animal system for typhus group and spotted fever group rickettsiae.

Typhus and spotted fever group rickettsiae do not induce lethal infections in the commonly used species of laboratory animals. Evaluation of the virulence of these organisms, therefore, has been limited to detection of a febrile response and/or demonstration of a specific humoral antibody response. Availability of an animal model system that exhibited a lethal response to infection with either typhus or spotted fever group rickettsiae would enhance significantly the study of these organisms, and would provide a better means of evaluating potential immunogens. Recent studies of Rickettsia tsutsugamushi (4) showed that resistance or susceptibility to infection with the different scrub typhus strains is dependent upon the genetic background of the strain of mouse used. These studies were designed to evaluate various strains of mice for susceptibility to lethal infection of typhus and spotted fever group rickettsiae.

Rickettsia typhi (Wilmington strain) and R conorii (Malish strain) were used as the representatives for the typhus and spotted fever groups, respectively. The number of viable rickettsiae in each stock suspension was determined by plaque assay.

Fourteen inbred mouse strains were studied (Table 1). All mice were tested at 8 to 12 weeks of age and were females. Mice were conditioned for a minimum of 7 days before being used in experiments.

The 50% mouse lethal dose (MLD_{50}) was determined by intraperitoneal injection of groups of 5 mice with 0.2 ml each of 10-fold dilutions of the appropriate inoculum. MLD_{50} values were calculated by the method of Reed and Muench at 28 days after inoculation. Three response patterns were defined: (a) resistant, (b) selectively resistant, and (c) susceptible. Selectively resistant mice were characterized by extremely erratic death and survival patterns that could not be clearly correlated with the concentration of rickettsiae given.

Results of the plaque assays and of the titrations in mice are shown in Table 1. The inoculum dilutions injected, as indicated by the PFU data, encompassed the range of 0.1 to 10^5 rickettsiae/mouse. Ten strains of mice were resistant ($\log_{10}$ MLD_{50} = $\leq$ 3.2) and 2 were selectively resistant to infection with R typhi. Similarly 8 strains of mice were resistant to R conorii. Three strains of mice (CBA/J, DBA/1J, and DBA/2J) exhibited moderate susceptibility to infection with R conorii, while a 4th strain (C3H/HeJ) was markedly susceptible. The MLD_{50} of 6.7 for R conorii in C3H/HeJ mice corresponded to approximately 2 PFU.

Table 1. Response of mouse inbred strains to infection with R typhi or R conorii

	Log_{10} titers per milliliter					
	R typhi			R conorii		
Mouse strain	response[a]	MLD_{50}	PFU	response	MLD_{50}	PFU
A/HeJ	ND[b]	ND	ND	r	$\leq$ 2.2	7.8
A/J	r	$\leq$ 3.2	8.1	ND	ND	ND
AKR/J	r	$\leq$ 3.2	8.1	r	$\leq$ 2.2	8.0
Balb/cJ	r	$\leq$ 3.2	9.0	r	$\leq$ 2.2	7.8
Balb/cByJ	r	$\leq$ 3.2	8.1	r	$\leq$ 2.2	ND
B10.D2/nSn	r	$\leq$ 3.2	9.0	r	$\leq$ 2.2	7.8
C3H/HeJ	ND	ND	ND	s	6.7	7.0
C3Heb/FeJ	r	$\leq$ 3.2	8.1	ND	ND	ND
CBA/J	sr	ND	8.4	s	3.9	7.0
DBA/1J	r	$\leq$ 3.2	8.5	s	4.2	7.3
DBA/2J	r	$\leq$ 3.2	8.6	s	4.2	6.9
RF/J	r	$\leq$ 3.2	8.4	r	$\leq$ 3.2	7.0
Sec/1ReJ	sr	ND	8.4	r	$\leq$ 2.2	8.0
SWR/J	r	$\leq$ 3.2	ND	r	$\leq$ 2.2	8.2

[a] s, susceptible; r, resistant; sr, selectively resistant
[b] ND, not determined

Of the mouse strains evaluated, the C3H/HeJ strain may, after further study, prove to be an acceptable animal model for R conorii, and possibly other spotted fever group rickettsiae. None of the mouse strains was suitable for typhus group investigations. Further investigations are in progress to extend the evaluation procedure to other strains of mice.

3. Plaque reduction assay for studying antigenic relationships among strains of Rickettsia tsutsugamushi.

The antigenic heterogeneity of strains of scrub typhus rickettsiae isolated from infected human, animal, and chigger sources has been well documented using the complement fixation (5-8), cross-neutralization (9-11), corss-vaccination (12), toxin neutralization (13, 14), and immunofluorescence (15,16) tests. The toxin neutralization and cross-neutralization tests are the only ones which provide any insight into the in vivo protective capacity of circulating antibody to scrub typhus rickettsiae. Use of the toxin neutralization test is limited, however, to those strains of Rickettsia tsutsugamushi for which highly concentrated suspensions required to elicit a toxic effect can be prepared (13,14). Both cross-neutralization and toxin neutralization tests require large numbers of mice, which can be logistically prohibitive when screening many strains of R tsutsugamushi at one time.

Recent studies on the genetics of mouse susceptibility to scrub typhus infection (4,17) have demonstrated that the genetic background of the strain(s) of mice must be considered when designing and analyzing in vivo tests. An in vitro means of assessing antigenic differences among strains of scrub typhus rickettsiae would be of definite value, as such a test would preclude host susceptibility as a factor in interpretation of the results.

The work reported here is concerned with the development of a plaque reduction assay for studying the effect of homologous and heterologous antibody on strains of R tsutsugamushi. Homogeneity of the strains used in testing the efficacy of the assay was assured by the use of plaque-purified rickettsiae (18) and antiserum prepared against these cloned strains.

Yolk sac-propagated rickettsiae. R tsutsugamushi strains Karp (egg passage 52), Gilliam (egg passage 164), and Kato (egg passage 162) were propagated in pathogen-free, embryonated chicken eggs. Stocks were stored at -70 C as 20% yolk sac suspensions (w/v) in sucrose-phosphate-glutamate buffer. (19).

Cell culture-propagated rickettsiae. Cloned Karp, Gilliam, and Kato strains of R tsutsugamushi (18) were propagated in monolayers of irradiated L-929 cells. The infected L-929 cells were harvested after 9 to 11 days by vigorous shaking of the containers, and the resulting cell suspensions were centrifuged at 1,000 x g for 10 min. The growth medium was poured off and the infected cells were resuspended in cold (4 C) Snyder I diluent (20), blended 3 x 30 sec at 45,000 rpm in a microchamber attachment, dispensed into vials, frozen in an ethanol-dry ice bath, and stored at -70 C.

Cell culture. L-929 cells (CCL 1) were routinely propagated as monolayer cultures at 37 C. Growth medium was Medium 199 with Earle's balanced salts supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS).

Cell irradiation. The procedure was as previously described (18). Culture dishes (60 x 15 mm) were seeded with 5 ml of growth medium containing 2.5 x 10^6 irradiated cells, allowed to stand undisturbed for 20 min at room temperature on a level surface, and then incubated overnight at 34 C in a humidified atmosphere of 5% CO_2 in air.

Plaquing. The procedure was as previously described (18). Rickettsial stocks were assayed using this method and the results expressed as the titers in plaque forming units (PFU)/ml.

Serum preparation. New Zealand white, male rabbits were pre-bled, the sera separated by standard methods (21), and tested by the microimmunofluorescence (micro-IF) test (22). After determining that each rabbit had no detectable scrub typhus antibodies, several rabbits were bled by cardiac puncture to obtain non-immune serum for use in controls and as diluent in the plaque reduction assay. Rabbit antiserum to each of the cloned scrub typhus strains was prepared by injecting 1 ml of cell culture propagated rickettsiae (approximately 2.5 x 10^7 PFU) into the ear vein. All rabbits were bled by cardiac puncture after 28 days. Sera were separated, and filter sterilized by passage through an 0.8 u membrane filter followed by filtration through a sterile 0.2 u filter. The sterile serum samples were distributed into vials and stored at -20 C. Sera, both immune and non-immune, were inactivated at 56 C for 30 min prior to each experiment. Immune sera were tested by the micro-IF method to determine titers to each of the scrub typhus strains used.

Female BALB/cDub mice (Flow Laboratories, Dublin, VA), 18 to 25 g, were inoculated subcutaneously with 0.2 ml containing approximately 10^3 PFU of cell culture-propagated, cloned rickettsiae diluted in cold Snyder I diluent (4 C). These mice were bled by excision of the left axillary artery after 28 days. Sera were processed,

sterilized, and stored in the same manner as described for the rabbit sera. Non-immune serum was collected from uninoculated mice of the same lot. All mouse sera were tested by the micro-IF test for titers to the Karp, Gilliam, and Kato strains.

Serum fractionation. Immune rabbit serum was diluted 1:2 with physiological saline and 1 ml samples were layered onto 10 ml sucrose velocity gradients with a density range of 10 to 40% (w/v) sucrose (density gradient grade) prepared in TEN buffer (1). Gradients were centrifuged at 150,000 x g for 21 hr at 4 C and then 0.5 ml fractions were collected from the bottom of the tubes. Protein determinations were done on each fraction (23) with optical density readings taken at 750 nm. The reference index of every fifth fraction also was determined.

Serum fraction diafiltration. Diafiltration using Minicon^{TM} concentrators was performed on pooled sucrose gradient fractions to remove the sucrose and concentrate the samples 10X. This was done by concentrating the samples 10X, then reconstituting it with TEN buffer to the original volume. This procedure was repeated 3X without the addition of buffer after the third 10X concentration. The final product was stored at -20 C.

Immunodiffusion. A modification of the Ouchterlony method of gel diffusion was used (24). Four ml of 0.6% agarose (SeaKem^{R}) prepared in pH 7.2, 0.07 M phosphate buffered saline (PBS) containing 0.05% sodium azide were pipetted into 60 mm culture dishes and allowed to solidify. Wells, 1.5 mm in diameter, were cut using a plastic template and the agarose plugs were removed by suction. To prevent reagent leakage form the wells, a drop of melted agarose was added to each well and the excess removed by suction. Wells were filled with 5 ul of the appropriately diluted antigen or antiserum and the dishes placed in a humidity chamber at 4 C for 4 days.

Microimmunofluorescence test. The method used was a modification of the procedure developed by Robinson et al (22) for the detection of scrub typhus antibodies in human serum. Cloned Karp, Gilliam, and Kato strains of R tsutsugamushi were propagated in irradiated L-929 cells and harvested as described above, except that the cell pellet was resuspended in PBS. After blending, this was further diluted 1:5 with PBS to yield approximately 10^3 organisms/400X microscopic field. Antigen spots were applied with a pen nib to microscope slides positioned over a template. Each slide received 12 sets of spots, each set composed of 1 spot each of the cloned Karp, Gilliam, and Kato strains. Slides were air dried, acetone-fixed for 10 min, and stored for up to 3 weeks at -20 C. Prior to use, antigen slides were brought to room temperature with a warm air blower to remove condensation.

Serum samples to be tested were diluted 2-fold in PBS in a microtiter plate, beginning at a 1:20 dilution. Five ul of each dilution were pipetted onto appropriate sets of antigen spots. A negative control consisting of a 1:20 dilution of non-immune serum from the homologous species, was included on each slide. Slides were incubated in a humidity chamber at 34 C for 30 min, rinsed in PBS, and washed twice in PBS for periods of 5 min each. Slides were air dired after a 1 min rinse in distilled water. Fluorescein-conjugated anti-rabbit globulin was diluted 1:32 in normal yolk sac diluent (20% normal yolk sac in Snyder I diluent). The dilution of conjugate was determined by a prior conjugate titration. Each set of antigen spots was covered with 5 ul of the diluted conjugate, the slides were incubated and washed as before, were air dired, and a drop of mounting medium (90% glycerin/10% PBS, adjusted to pH 8.4 with 0.2 N NaOH) was placed on each slide prior to the addition of a coverslip. Slides were examined at 400X magnification using vertical fluorescence. A spot was scored as positive if there was obvious fluorescence of rickettsiae when compared to controls. Titers against each antigen were considered as the reciprocal of the highest positive serum dilution.

Development of the plaque reduction assay. Heat-inactivated rabbit antiserum was diluted 4-fold in non-immune rabbit serum and 0.4 ml portions of each dilution were transferred to sterile tubes. A yolk-sac grown suspension of the homologous strain of rickettsiae was diluted in cold (4 C) BHI broth to 400 PFU/0.1 ml, and 0.4 ml portions were distributed to each tube containing antiserum dilutions and to a control tube containing a 1:2 dilution of non-immune rabbit serum. Tubes were shaken and then incubated at 34 C for 15 min. After incubation, the antigen-serum mixtures were gently mixed by pipetting and 0.1 ml aliquots were plaqued. Uninfected control dishes received either BHI broth or non-immune serum only. The results of duplicate experiments using the cloned Kato strain reacted with homologous antiserum are illustrated in Figure 5. The 1:4 antiserum dilution resulted in a 70 to 75% reduction in plaque numbers. A comparison of the results of the separate experiments indicated good reproducibility and showed a direct relationship, at the lowest 4 dilutions, between the percent plaque reduction and the dilution of antiserum.

Homologous and heterologous assays using cloned and parental prototype strains of rickettsiae. To determine the feasibility of using the plaque reduction assay to differentiate among strains of scrub typhus rickettsiae, each of the 3 cloned strains was reacted with immune sera prepared against cell culture propagated stocks of the same strains. Figures 6 to 8 contain the results of the 9 assays. In Figures 6 and 7, homologous plaque reduction was much greater throughout the entire antiserum dilution range than that

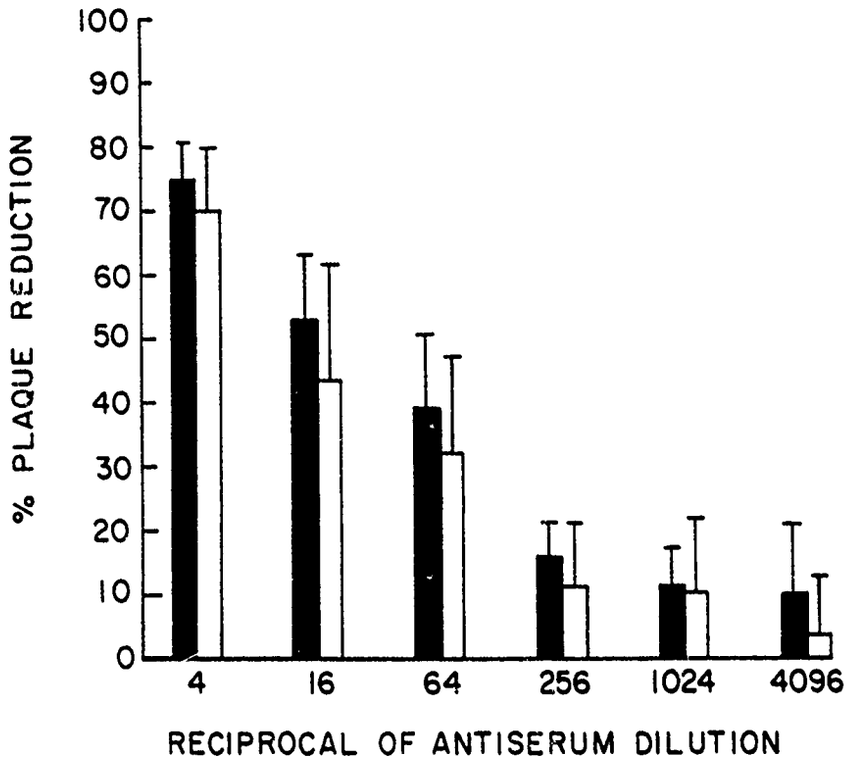

Figure 5. Rabbit antiserum plaque reduction assay. Duplicate experiments using rabbit anti-Kato serum vs. the cloned Kato strain of R tsutsugamushi.

Experiment #1 (■), #2 (□). Results are calculated using averages of counts from 4-6 dishes at each dilution.

of the heterologous systems. In comparison, the relationships in Figure 8 are somewhat erratic, with the only clear cut results exhibited by the 1:4 dilution of antiserum. Duplication of the experiments gave similar results. An interpretation of the results in Figures 6 to 8 would suggest that the Karp and Gilliam strains exhibited the least antigenic similarity, while the Kato strain appeared to contain antigens that were cross-reactive with both the Gilliam and Karp strains.

A second series of 9 assays also was done using the parental Karp, Gilliam, and Kato strains of R tsutsugamushi. The antisera used were the same lots from the preceeding 9 assays in order to compare the relationships among the cloned and parental strains. To conserve antisera, only the initial 3 dilutions were used, since the other dilutions were unnecessary for interpretation of the assays. These results are depicted in Figures 9 to 11. A comparison with the results in Figures 6 to 8 suggested that the cloned strains were antigenically representative of the parental stocks from which they were selected and that the parental stocks were each antigenically homogeneous and not a mixture of antigenic types.

Sucrose velocity gradient study. Antisera from rabbits bled 5 and 28 days post-inoculation with the Gilliam strain were each diluted 1:2 with normal saline and layered onto sucrose gradients. The IgM and IgG fractions were separated by velocity centrifugation and subsequent fractionation of the gradients. Immunodiffusion against goat anti-rabbit IgM and goat anti-rabbit IgG confirmed the identity of the fractions and the absence of cross-contamination of the immunoglobulins.

Antibody titers of immune and non-immune sera and the IgM and IgG fractions were determined by microimmunofluorescence (Table 2). The single intravenous injection of antigen produced potent antisera when the rabbits were bled 28 days post-infection, the titers ranging from 1,280 to 10,240. In contrast, antiserum obtained 5 days after infection yielded a low titer of 80. The titer of the IgM fraction from the 28 day antiserum was low, as expected, with most of the antibody response residing in the IgG fraction. The titer obtained with the IgM fraction from the 5 day antiserum was 80, with no detectable scrub typhus-specific antibody in the IgG fraction.

Plaque reduction assays using the cloned Gilliam strain reacted against the IgM and IgG fractions of the 5 day rabbit anti-Gilliam serum are illustrated in Figure 12. Unfractionated 5 day antiserum was used as a control. Both the whole antiserum and IgM fraction caused a reduction in plaques. There was negligible reduction elicited by the IgG fraction. IgM and IgG fractions of 28 day rabbit anti-Gilliam serum were reacted against the cloned

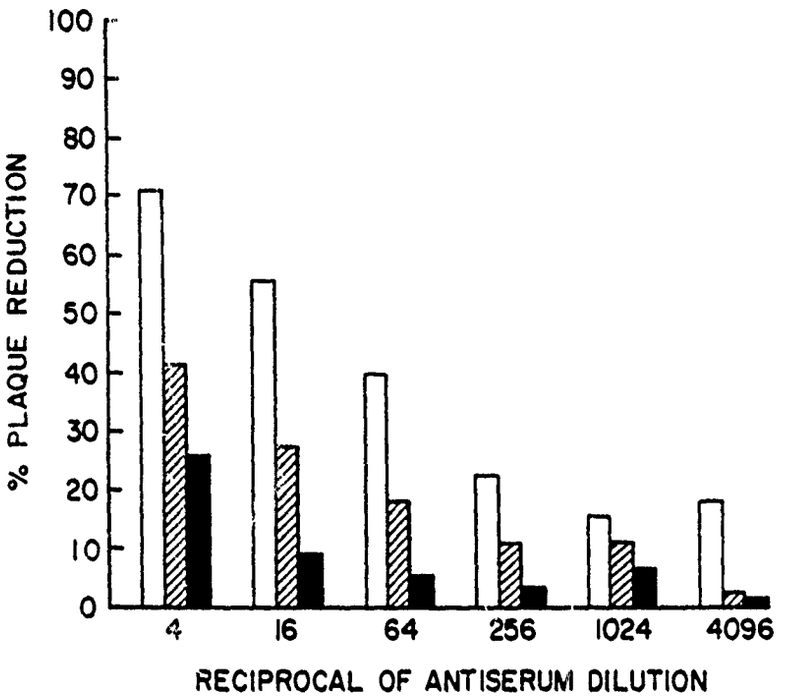

Figure 6. Effect of rabbit anti-Karp serum on plaque formation by cloned Karp, Kato, and Gilliam strains of R tsutsugamushi.

Karp (□), Kato (▨), and Gilliam (■) antigens. Results are calculated using averages of counts from 4-6 dishes at each dilution.

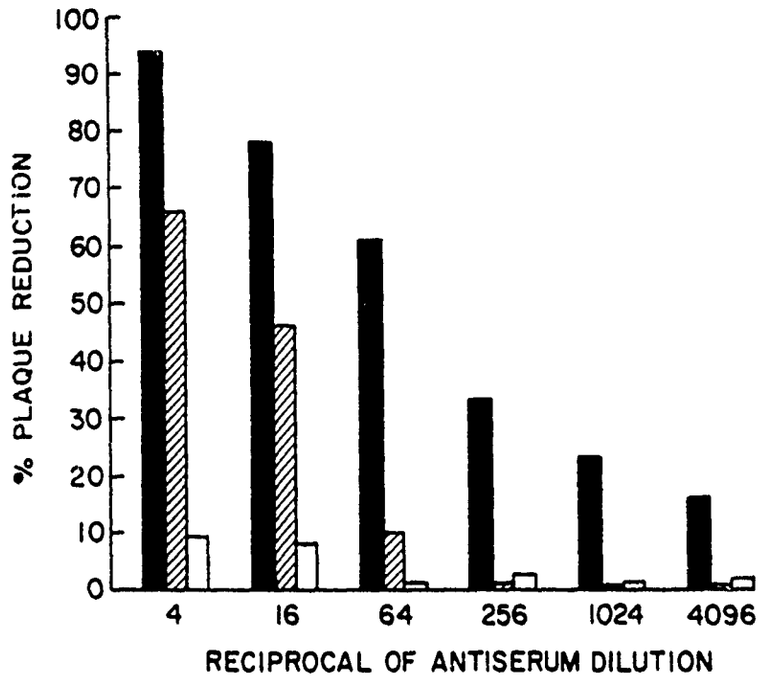

Figure 7. Effect of rabbit anti-Gilliam serum on plaque formation by cloned Gilliam, Kato, and Karp strains of R tsutsugamushi.

Gilliam (■), Kato (▨), and Karp (□) antigens. Results are calculated using averages of counts from 4-6 dishes at each dilution.

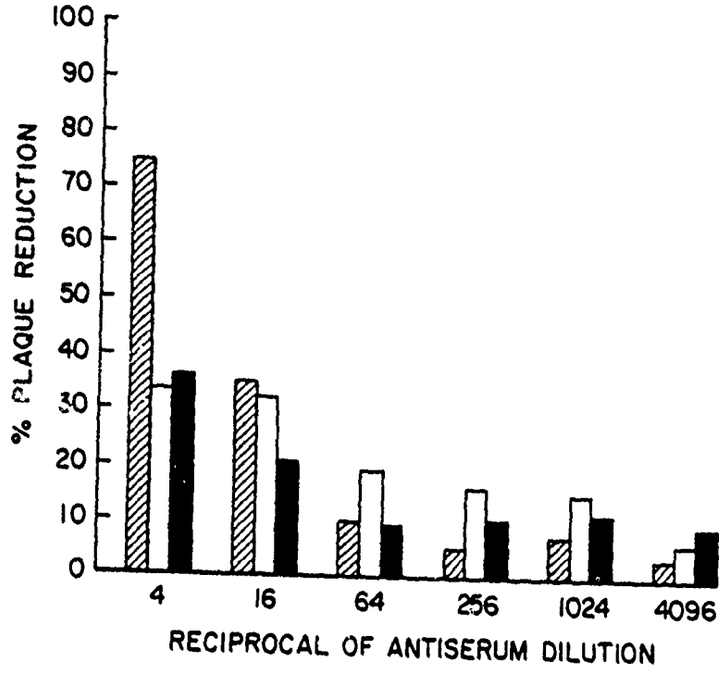

Figure 8. Effect of rabbit anti-Kato serum on plaque formation by cloned Kato, Karp, and Gilliam strains of R tsutsugamushi.

Kato (▨), Karp (□), and Gilliam (■) antigens. Results are calculated using averages of counts from 4-6 dishes at each dilution.

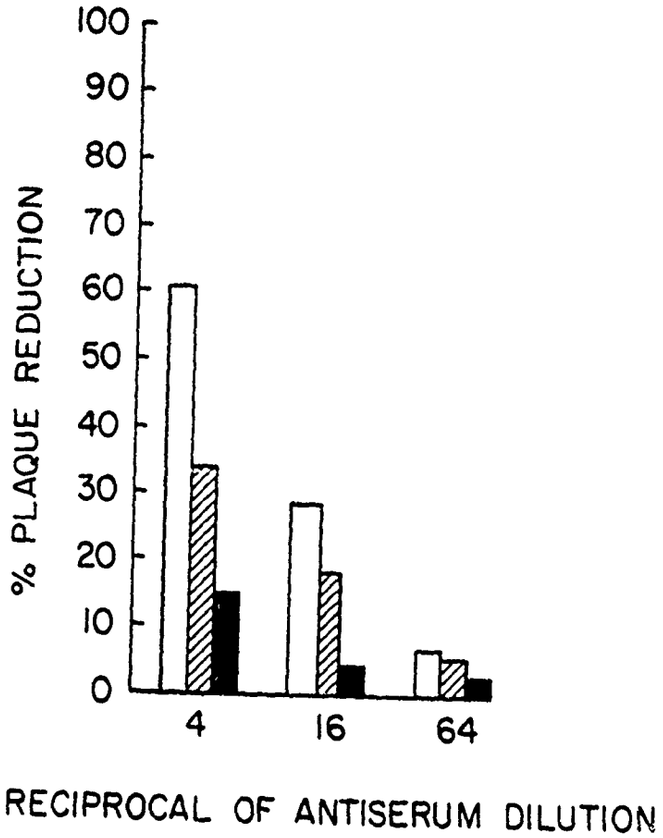

Figure 9. Effect of rabbit anti-Karp serum on plaque formation by parental Karp, Kato, and Gilliam strains of R tsutsugamushi.

Karp (□), Kato (▨), and Gilliam (■) antigens. Results are calculated using averages of counts from 4-6 dishes at each dilution.

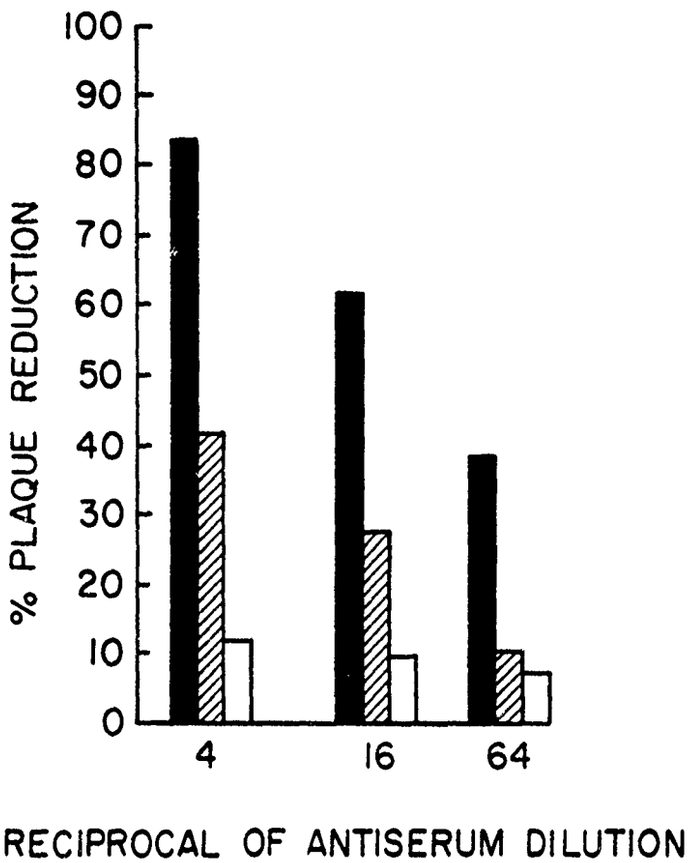

Figure 10. Effect of rabbit anti-Gilliam serum on plaque formation by parental Gilliam, Kato, Karp strains of R tsutsugamushi.

Gilliam (■), Kato (▨), and Karp (□) antigens. Results are calculated using averages of counts from 4-6 dishes at each dilution.

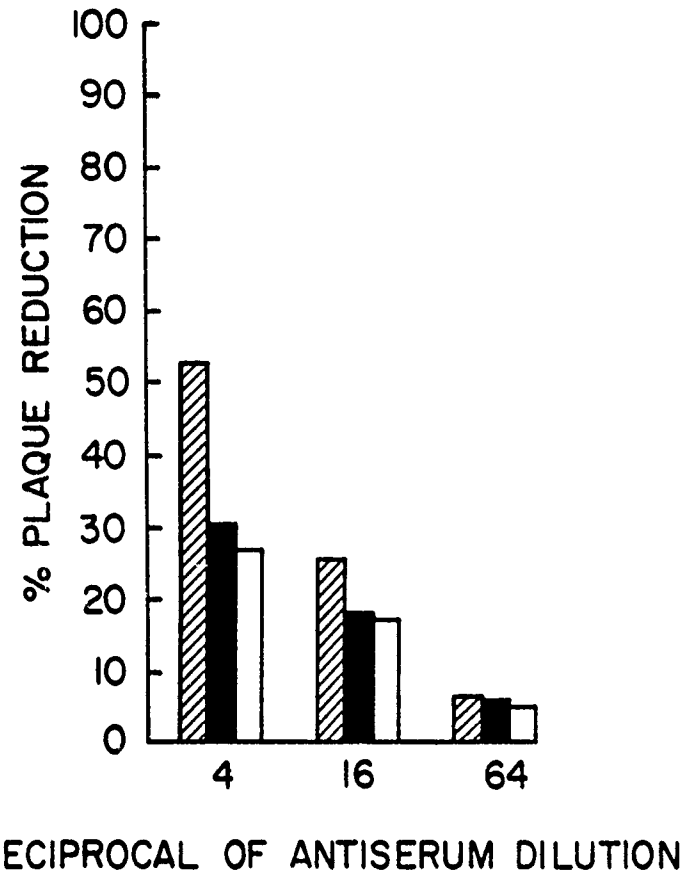

Figure 11. Effect of rabbit anti-Kato serum on plaque formation by parental Kato, Gilliam, and Karp strains of R tsutsugamushi.

Kato (▨), Gilliam (■), and Karp (□) antigens. Results are calculated using averages of counts from 4-6 dishes at each dilution.

Table 2. Immunofluorescene titers of unfractionated immune and non-immune sera, and sucrose gradient fraction pools.

Serum	IF antigen titer		
	Karp	Gilliam	Kato
Non-immune	-[a]	-	-
Kato immune-28 day[b]	640[c]	40	1,280
Karp immune-28 day	2,560	80	640
Gilliam immune-28 day	160	10,240	80
IgM fraction	-	40	-
IgG fraction	160	5,120	160
Gilliam immune-5 day	-	80	-
IgM fraction	-	80	-
IgG fraction	-	-	-

[a] Negative at 1:20 or greater.

[b] Days after infection on which rabbits were bled to obtain antiserum.

[c] Reciprocal of highest dilution showing positive immunofluorescene.

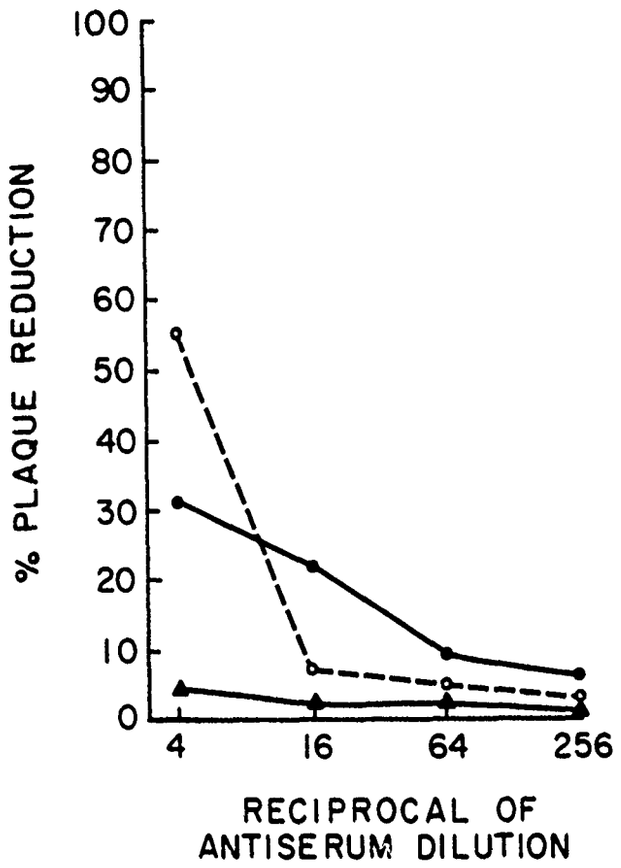

Figure 12. Effect of IgM and IgG fractions from rabbit anti-Gilliam serum obtained 5 days post-infection on plaque formation by the cloned Gilliam strain of R tsutsugamushi.

IgM fraction (o), IgG fraction (▲), and unfractionated control (●). Results are calculated using averages of counts from 4-6 dishes as each dilution.

Gilliam strain in the plaque reduction assay. These results are illustrated in Figure 13. Unfractionated 28 day antiserum was used as a control. Both IgM and IgG fractions caused a reduction in plaque numbers, but the reduction was only one-third of that elicited by the whole antiserum.

Initial experiments in the development of the plaque reduction assay used antiserum prepared in BALB/c mice, since the mouse has long been the animal of choice for studying scrub typhus infection. Although substantial plaque reduction was observed at low dilutions of antiserum, the mouse system was rejected because of erratic results. This prompted consideration of guinea pigs and rabbits for use in antiserum production. The rabbit was selected because of previous experience in this laboratory indicating that rabbits generally produce more potent antisera than guinea pigs when only a single injection of antigen is used. The results of preliminary experiments with rabbit anti-Kato serum demonstrated that an assay using antiserum prepared in rabbits gave excellent, reproducible results. This was somewhat unexpected, as other investigators (25) had reported that plaque reduction with spotted fever group rickettsiae required the addition of an antiglobulin.

Since the plaque reduction assay was intended for use in differentiating among strains of R tsutsugamushi, a series of experiments was performed using homologous and heterologous antiserum in conjunction with the cloned prototype strains. The results demonstrated that it is possible to discriminate among the 3 strains of scrub typhus rickettsiae on the basis of their reactions with known antisera. In no instance was there 100% reduction in the number of plaques. This could be the result of insufficient antibody or possibly the existence of a non-neutralizable fraction, such as has been demonstrated in viral systems (26).

The present study has demonstrated a level of antigenic relatedness between the Karp, Gilliam, and Kato strains that has not been observed in most other investigations. The Gilliam strain, long thought to be antigenically unrelated to the other 2 strains, was strongly cross-reactive with the Kato strain and demonstrated minor cross-reactivity with the Karp strain. The substantial reactivity observed between the Karp and Kato strains reinforced the previous observations of other workers. The pattern and magnitude of cross-reactivity between the 3 strains as demonstrated by the plaque reduction assay are almost identical to those reported by Shishido (6) using the CF test. A particulate antigen was used in his studies, with antisera obtained from guinea pigs after a single injection of rickettsiae. Others, however, have demonstrated more heterogeneity between these strains. Bengtson (5)

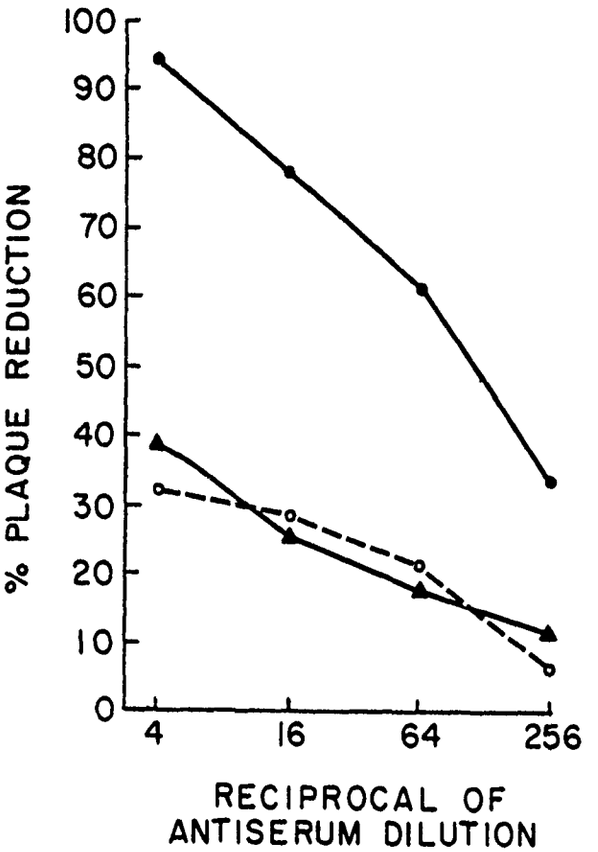

Figure 13. Effect of IgM and IgG fractions from rabbit anti-Gilliam serum obtained 28 days post-infection on plaque formation by the cloned Gilliam strain of R tsutsugamushi.

IgM fraction (o), IgG fraction (▲), and unfractionated control (●). Results are calculated using averages of counts from 4-6 dishes at each dilution.

compared the Karp and Gilliam strains using a soluble CF test with human and guinea pig antisera and consistently found homologous titers to be 3- to 5-fold greater than heterologous titers. The heterogeneity was so marked that the author (5) suggested that future diagnostic testing of sera from suspected scrub typhus patients should include both the Karp and Gilliam antigens in the CF procedure. Even more remarkable results were reported by Elisberg et al (27) using guinea pig antisera in the CF test with particulate Karp, Kato, and Gilliam antigens. Homologous titers ranged from 320 to 1,280, while results with heterologous antigens were negative at the initial 1:10 dilutions of antisera.

The results from the preceding investigations, in which the CF test was used, illustrate the problem encountered when one attempts to compare the test results among laboratories. This is due to the lack of standardization in the preparation of antigens for the CF test. Not only does the method of antigen preparation differ, but some laboratories use a soluble antigen and others a particulate antigen. The plaque reduction assay, on the other hand, does not require any manipulation of the rickettsiae that might change the antigenic properties and employs a continous cell line that is readily available from cell repositories and other sources.

The IF (15), cross-neutralization (10,11) and toxin neutralization (14) tests also have been used to study the antigenic relationship of scrub typhus strains. Results with the IF test using antiserum from laboratory workers who had become infected with either the Karp, Gilliam, or Kato strains (15) were similar to those of the present study. Cross reactions detected by the IF test were not as strong, however, as those experienced in the plaque reduction assays.

A comparison of cross-neutralization test results (10,11) with those of the plaque reduction assays only can be made with the Karp and Gilliam strains, as the Kato strain was not used in the neutralization studies. Appreciable protection was offered by the homologous antisera to both of the strains in the cross-neutralization test. Heterologous test results indicated there was little similarity between these two strains, as was shown also by the plaque reduction assay. Neutralization indices were of limited value in interpreting the results due to the inherent variability caused by the irregular susceptibility of the outbred mice used for the tests.

The toxin neutralization test (14) was very specific in comparison to those tests discussed previously. These workers reported that the toxins of each of the 3 prototype strains were neutralized by

homologous immune sera, but not by heterologous sera. The absence of heterologous reactions prevented comparison with the plaque reduction results.

A comparison of the results of the series of plaque reduction assays using the parent strains demonstrated that the cross-reactivity patterns were the same as those observed with cloned strains. If the parent stocks were mixtures of strains, then cloning should have resulted in a decrease in cross-reactivity. This did not occur and indicated that the antigenic composition of the cloned prototype strains were representative of those of the parent strains, implying that the parent strains were each homogeneous in their antigenic composition.

Evidence concerning the mechanism of plaque reduction was obtained from immunodiffusion and plaque reduction assays with IgM and IgG fractions of immune serum. The immunodiffusion results demonstrated the effectiveness of sucrose velocity gradient centrifugation in separating IgM and IgG fractions of 5 and 28 day post-infection sera. Cross-contamination of the IgM and IgG fraction pools was not detected by immunodiffusion using commercially-prepared goat anti-rabbit IgM and goat anti-rabbit IgG. Plaque reduction assays using the IgM and IgG fractions, as well as unfractionated sera, indicated that both classes of immunoglobulin were capable of causing a reduction in plaque numbers. However, when the results of these assays were compared with the antibody titers obtained by the micro-IF assay, it became apparent that IgM may be more efficient than IgG in plaque neutralization.

An earlier study of the neutralization of R tsutsugamushi in a cell culture system (28) indicated that homologous hyperimmune rabbit serum neutralized rickettsiae only at low serum dilutions (less than 1:20), whereas heterologous antisera had no detectable effect. Their system relied upon the microscopic observation of a rickettsial cytopathogenic effect on the host cells. It was difficult to make any comparison between the study of Barker et al (28) and the present study as there was no reference point from which to start, such as the antibody titers of the sera used. The reason for the difference in results may be that the plaque reduction assay was more sensitive than was detection of rickettsial cytopathogenicity by microscopy.

The major contribution of the plaque reduction assay will be its usefulness as a means of studying the antigenic composition of the numerous strains of scrub typhus rickettsiae. The advantage that the plaque reduction assay has over serologic tests is that it offers a possible in vitro correlate of the in vivo protective capacity of scrub typhus antibody. A serologic titer does not

necessarily reflect the effect that the antibody will have on the infectivity of the organism; whereas, a biological assay such as is presented here clearly demonstrates the effect of that antibody.

Another potential use for the plaque reduction assay is in the selection of candidate strains for the preparation of vaccines to prevent scrub typhus infection. Strains of R tsutsugamushi selected must be able to elicit a broad base of immunity. Initial studies of candidate vaccine strains formerly were done using the cross-vaccination and cross-neutralization tests. In comparison, the plaque reduction assay not only is more precise, but costs about one-fourth as much as either of the above tests and requires less space. The lack of precision exhibited by the cross-neutralization and cross-vaccination tests, as well as the toxin neutralization test, is due to the irregular susceptibility of the outbred mouse stocks generally used in these tests.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 096 Biochemical and Immunological Characterization of Rickettsial Proteins

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OC 6459	79 10 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NI	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	097
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Antigenic Characterization of Sandfly Fever Viruses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 002600 Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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17. CONTRACT/GRANT

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b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

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FISCAL YEAR CURRENT 80	2	190

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ASSOCIATE INVESTIGATORS
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NAME: DALRYMPLE, Dr. Joel M.

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Arbovirus; (U) Antigen; (U) Immunology; (U) Immunopathology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)

23 (U) To define the antigenicity and immunogenicity of the proteins of Sandfly Fever viruses, to develop rapid means of specific diagnosis, to study the immune response to infection and to develop means for reducing disability. Sandfly fever affected both American and German troops during World War II. Antigenically distinct strains of these viruses exist in the Mediterranean Basin, Central America and Asia.

24 (U) Contemporary virological and immunological methods are used to identify and compare the antigens of distinct strains of Sandfly Fever viruses. Replication of viruses in cell culture systems is studied to determine methods of producing high titered virus for biochemical analysis.

25 (U) 77 10 - 78 09 The most sensitive cells for plaque assay of Sandfly Fever viruses under agar containing DMSO were chick embryo cells for Karimabad, baby hamster kidney (BHK) cells for Itaporanga and Candiru, and Vero cells for Chagres, Punta Toro, Naples and Sicilian viruses. Maximum virus production (10^6 to 10^9 plaque-forming units/ml) was obtained from infected Vero cells cultured under liquid medium for all viruses except Karimabad which replicated to higher titers in BHK cells. Analysis of structural proteins of the viruses was accomplished by radiolabelling them with ^{35}S methonine in Actinomycin D inhibited cells. Virus concentration and purification was accomplished with polyethylene glycol 6000, equilibrium centrifugation in potassium tartrate-glycerol gradients, and rate-zonal centrifugation through sucrose gradients. The seven viruses differed markedly in their stability to purification. The number and molecular weight of the structural proteins of each virus was unique, e.g. only 3 polypeptides (58,000,52,000, 28,100) were detected in Punta Toro whereas Chagres had five (90,000, 56,000,54,000,52,000,20,000). Structural protein analysis may prove valuable in Phlebotomus Fever virus identification when antigenic analysis alone does not provide definitive information. See WRAIR Annual Progress Report 1 Oct 78 to 30 Sep 79.

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 097, Antigenic Characterization of Sandfly Fever Viruses

Investigators:

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Description

To define the characteristics and antigenicity of the proteins of Sandfly Fever Viruses, to develop improved means of specific identification, to study the immune response to infection and to develop means of reducing disability. Emphasis is placed on Sandfly Fever Viruses that are capable of infecting humans.

Progress

I Structural Proteins of Sandfly Fever Viruses.

A. Determination of Optimal Conditions for Virus Replication and Radiolabeling.

Seven Sandfly Fever virus strains: Karimabad, Itaporanga, Chagres, Punta Toro, Candiru, Naples, and Sicilian have been examined in experiments directed towards the biochemical and biophysical characterization of the antigenic viral components, particularly the structural proteins. To accomplish this goal, sufficient radiolabeled and purified virion is required. Methods for the propagation, radiolabeling, purification, and assaying of the viruses has been the subject of this research effort.

All seven strains replicate in H-Vero cells (a specific line of VERO cells obtained from Dr. Robert Tesh, Honolulu, Hawaii), but differ greatly in virus yield.

Certain strains; i.e. Candiru, Sicilian, and Naples, still replicate poorly. Five cell lines were tested for virus propagation; one primary vertebrate line (chick embryo cells), one invertebrate line (C6/36 Aedes albopictus cells), and three continuous vertebrate lines (H-VERO described above, P-VERO from Panama, and the CER and BHK continuous baby hamster kidney cell lines). Virus was assayed by plaque assay on H-Vero cells (Table 1). Multiplicity of infection was either 1.0 or 0.1 depending upon the optimal yield results of growth curves previously obtained in H-Vero cells.

Techniques for the efficient incorporation of radioactive amino acids into the viral proteins have been examined. Variables studied included the use of actinomycin D as an inhibitor of host cell protein synthesis, and the time and quantity of label added. Optimal conditions for the propagation of viruses with high specific activities are detailed below. Chagres and Itaporanga which are propagated in P-VERO cells were greatly enhanced by the addition of 0.5 µg/ml of actinomycin D at 4 hrs, radiolabel addition at 8-12 hrs with 10 µCi/ml of ^{35}S methionine, and replacing supernatant with methionine deficient media (10% normal). Incorporation of isotope was increased tenfold over previous attempts without these modifications and radioactivity of the cellular debris was decreased providing lower background during analysis and purification. Karimabad was propagated in BHK cells, with no actinomycin D, using methionine deficient media and, 10 µCi/ml of ^{35}S-methionine added at 24 hours. The other four strains were grown in H-VERO cells (which did not appear to be sensitive to actinomycin D) methionine deficient media and higher concentrations of isotope were required. Low yields and purification problems have hindered progress on these viruses but virus yields with high specific activities are anticipated.

B. Virus Purification

Virus concentration and purification included the

slow speed centrifugation of infected cell supernatants followed by a high-speed clarification at 10,000 x g. Virus was precipitated by polyethylene glycol and resuspended in about one-hundredth the original volume. Karimabad, Chagres, Itaporanga and Punta Tora virus concentrates were first purified by rate-zonal centrifugation on sucrose gradients and subsequently centrifuged to equilibrium on potassium tartrate (50%)--glycerol (30%) gradients. Candiru, Sicilian and Naples viruses suffered enormous losses on rate-zonal sucrose gradients, therefore, only the density tartrate-glycerol gradients were used. This procedure still presents purification problems for these viruses because the major peak of contaminating cellular components is separated by only two fractions from peak virus activity. All viruses banded at a density of 1.18 gms/cc under these conditions.

C. Virus Assay(s)

The standard plaque assay on H-VERO cells using an overlay containing 1% DMSO has been described (FY 78 WRAIR Annual Report). Several cell lines were compared for plaquing efficiency using this overlay medium (Table 2). A single virus suspension for each virus was compared on different cell monolayers and the estimated titer of this original suspension was recorded for each cell type used for assay. The H-VERO cells appeared to be the most sensitive overall with Karimabad on CER and Candiru on BHK being exceptions.

The solid-phase radioimmunoassay (RIA) routinely used for alphavirus and flavivirus antigens was successfully applied to the Sandfly Fever group viruses. The distribution of virus antigens on density gradients detected by RIA closely paralleled the intrinsic radiolabeled virion.

A hemagglutination assay was developed which gives increased HA titers with purified preparations of these agents. Procedures were essentially the same as those

Table 1. Infectious Sandfly Fever Virus Yields from a Variety of Cell Substrates.

Virus	Cell Line CER	H-VERO	P-VERO	CEC	A. albo
Karimabad	$5x10^{7}$*	$7.5x10^{6}$	———	$4.5x10^{5}$	$>5x10^{5}$
Punca Tora	$2.35x10^{8}$	$1.25x10^{9}$	$2.55x10^{8}$	$2.8x10^{8}$	$>5x10^{5}$
Itaporanga	$4.4x10^{8}$	$5x10^{8}$	$8x10^{8}$	$5.5x10^{6}$	$1.3x10^{8}$
Chagres	$>5x10^{5}$	$2.6x10^{8}$	$5.8x10^{8}$	$>5x10^{3}$	$2x10^{7}$
Sicilian	$8.5x10^{6}$	$8x10^{7}$	$8.5x10^{6}$	$>5x10^{3}$	$>5x10^{5}$
Candiru	$5x10^{6}$	$1.6x10^{7}$	$2.5x10^{6}$	$>5x10^{3}$	$>5x10^{5}$
Naples	$2.5x10^{5}$	$4.25x10^{7}$	$1.5x10^{7}$	$>5x10^{3}$	$>5x10^{5}$

*PFU/ml of supernatants harvested at time of maximal CPE.

Table 2. Plaquing Efficiency of Sandfly Fever Viruses on Various Cell Substrates

Virus	Cell line CER	H-VERO	P-VERO	BHK
Punta Tora	$6x10^{7}$*	$1.1x10^{8}$	$2.5x10^{7}$	$7.5x10^{7}$
Karimabad	$2x10^{8}$	$6.5x10^{7}$	———	$4.5x10^{5}$
Candiru	$2x10^{6}$	$3x10^{6}$	$1.5x10^{6}$	$7.5x10^{6}$
Sicilian	$>1x10^{5}$	$1.5x10^{6}$	$>1x10^{5}$	$>1x10^{5}$
Itaporanga	$8x10^{6}$	$1.1x10^{7}$	$>1x10^{5}$	$4x10^{7}$
Chagres	$>1x10^{5}$	$1.8x10^{7}$	$>1x10^{5}$	$>1x10^{5}$
Naples	$>1x10^{5}$	$1x10^{6}$	$>1x10^{5}$	$>1x10^{5}$

*PFU/ml

for the classical togavirus HA with the notable exception that the NaCl concentration was increased to 0.4 molar. All viruses exhibited a pH 5.8 optimum for hemagglutination.

Polyacrylamide gel electrophoretic analysis of virion structural polypeptides is still in progress but sufficiently encouraging to expect a complete description of the proteins for each of the viruses in the near future.

II Karimabad Virus.

A. Production of Defective Interfering (DI) Particles.

1. Background

The continued serial undiluted passage of a number of RNA viruses has been shown to lead to the production of DI particles which can interfere with the replication of infectious virions. The production of DI particles may be important in the determination of the animal virulence of certain virus preparations. The objective of this study was to determine if Karimabad virus, a member of the Phlebotomus fever group in the Bunyaviridae family, produces DI particles and, if so, to begin characterization of these DI particles.

2. Cycling of Virus Titers in Serial Undiluted Viral Passages.

When Karimabad virus was passed serially using undiluted inoculum from the previous passage, a cyclic pattern of virus yields was easily discernible (Figure 1). This cycling of virus yield is characteristic of the production of DI particles; infectivity titer varies inversely to the concentration of DI particles. Virus grown by serial undiluted passage was able to interfere with the replication of homologous virus grown at low multiplicity of infection (MOI) (Table 3).

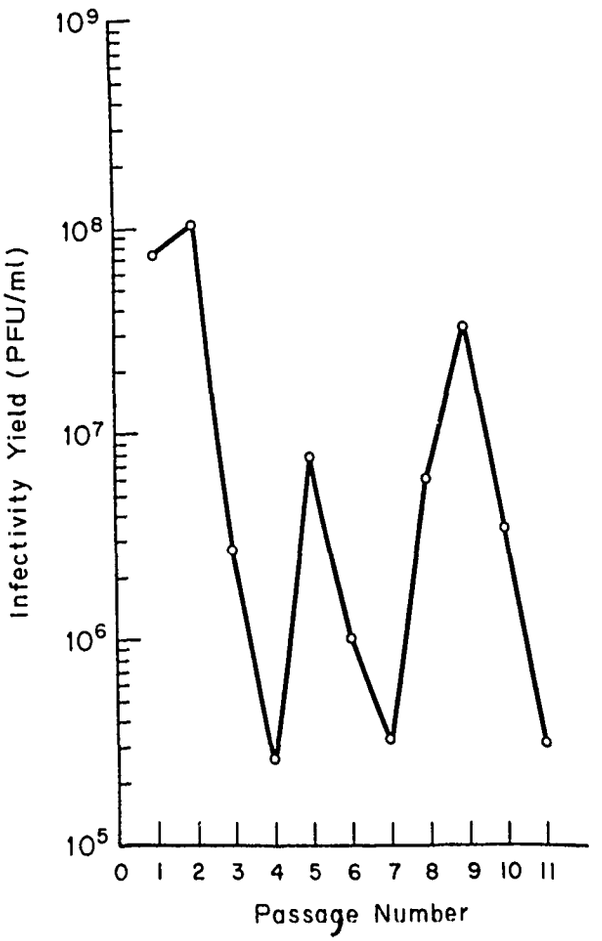

Figure 1. Cycling of Karimabad virus yields following high MOI passage in VERO cells. Titers indicated represent the maximal virus titer achieved during each serial undiluted passage.

Table 3. Interference of Karimabad DI Particles with Replication of Phlebotomus Fever Group Viruses.

Phlebotomus Fever Group Virus	Virus Titer (pfu/ml) Alone	With Karimabad DI Particles*	Interference (%)
Karimabad (low MOI)	9.75×10^7	5.25×10^5	99.5
Itaporanga	1.42×10^7	1.60×10^6	88.7
Candiru	5.2×10^7	5.8×10^7	0.0

*Each sample was coinfected with 5.25×10^4 pfu/ml, high MOI passage 4 Karimabad virus.

Karimabad populations containing interference activity were able to interfere with certain heterologous Phlebotomus Fever group viruses.

3. Characterization of Viral Populations with High Concentrations of DI Particles.

Karimabad virus populations containing interfering activity have been compared with cloned Karimabad virus propagated at low MOI. Resuspended polyethylene glycol precipitates of released virus were subjected to equilibrium density centrifugation on glycerol-potassium tartrate gradients. Virus grown at low MOI exhibited a single peak of hemagglutinin activity (HA) which corresponded to infectious virion at a density of 1.21 gm/ml (Figure 2, top panels). Virus preparations containing interfering activity had an additional HA particle which sedimented at 1.24 gm/ml (Figure 2, bottom panels). This particle cosedimented with the interference activity (Table 4). Unfortunately, this additional particle cosediments with a non-hemagglutinating particle (Figure 3). This non-hemagglutinating particle appears to be a nucleocapsid since it contains only virion RNA (Figure 3, top panels) and the nucleocapsid protein (Figure 4).

4. Conclusions.

Populations of Karimabad virus grown in serial undiluted passages contain interfering activity. This activity cosediments on equilibrium density gradients with nucleocapsid structures produced in low MOI infections.

Experiments are in progress to characterize the interfering particles and separate them from non-interfering nucleocapsid structures.

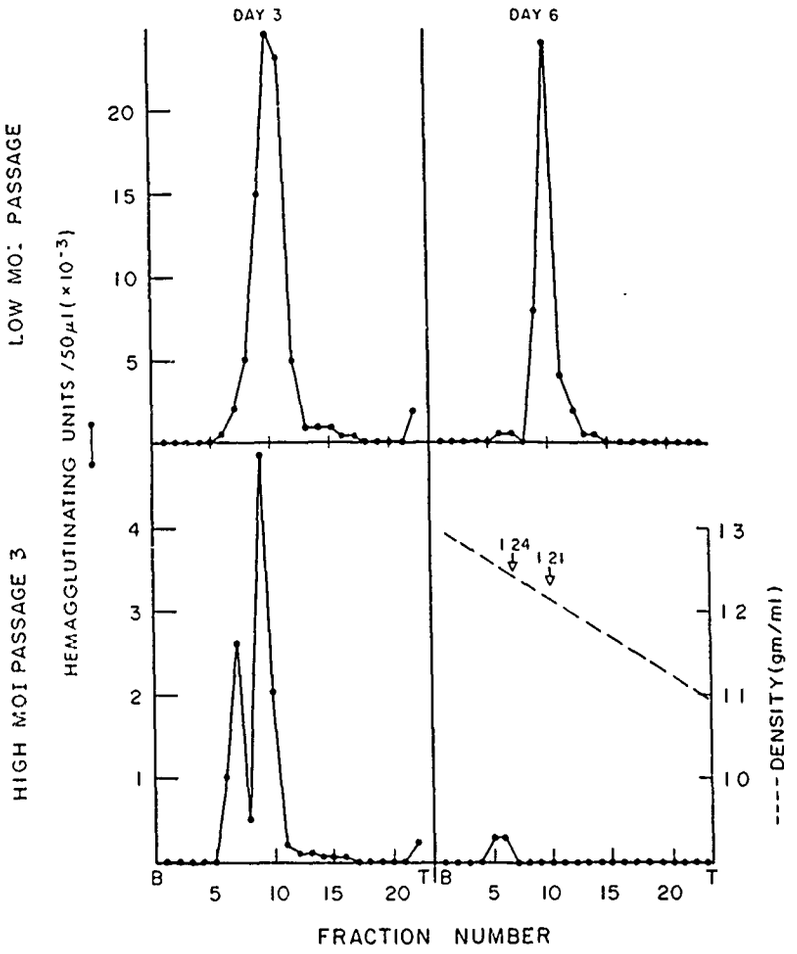

Figure 2. Equilibrium density centrifugation of Karimabad virus grown at low or high MOI. Distribution of hemagglutinating particles in virus grown at high MOI (containing DI particles) as compared to low MOI virus.

Table 4. Interference by Partially Purified Defective Particles with Replication of Low Passage Karimabad Virus+

Virus Titer		Fraction 7 Low MOI	Fraction 7 High MOI	Fraction 10 Low MOI	Fraction 10 High MOI
A.	Gradient Fraction Alone	2.2×10^2	11.2×10^1	4.0×10^8	6.0×10^6
B.	Fraction Plus Low MOI Kar.	9.9×10^7	3.5×10^4	5.1×10^8	8.3×10^6
	% Interference by Fraction*	0.0%	99.99%	0.0%	19.8%

+titer 9.75×10^7 pfu/ml $\equiv$ C

* % Interference = 100% -

$$\frac{\text{Titer of Gradient Fraction with Low MOI Kar}}{\text{Gradient Fraction Alone + Low MOI Kar Alone}} \times 100$$

$$= 100\% - \left(\frac{B}{A + C} \times 100\right)$$

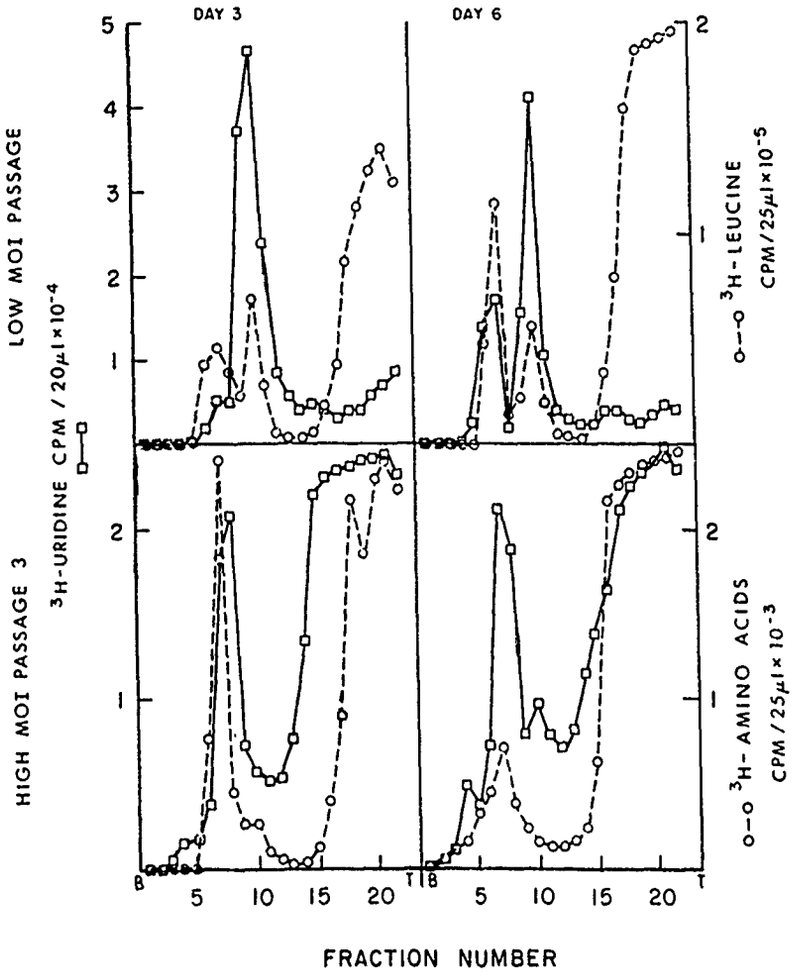

Figure 3. Equilibrium density centrifugation of Karimabad virus grown at low or high MOI. Distribution of particles with labeled RNA or protein in virus grown at high MOI (containing DI particles) as compared to low MOI virus.

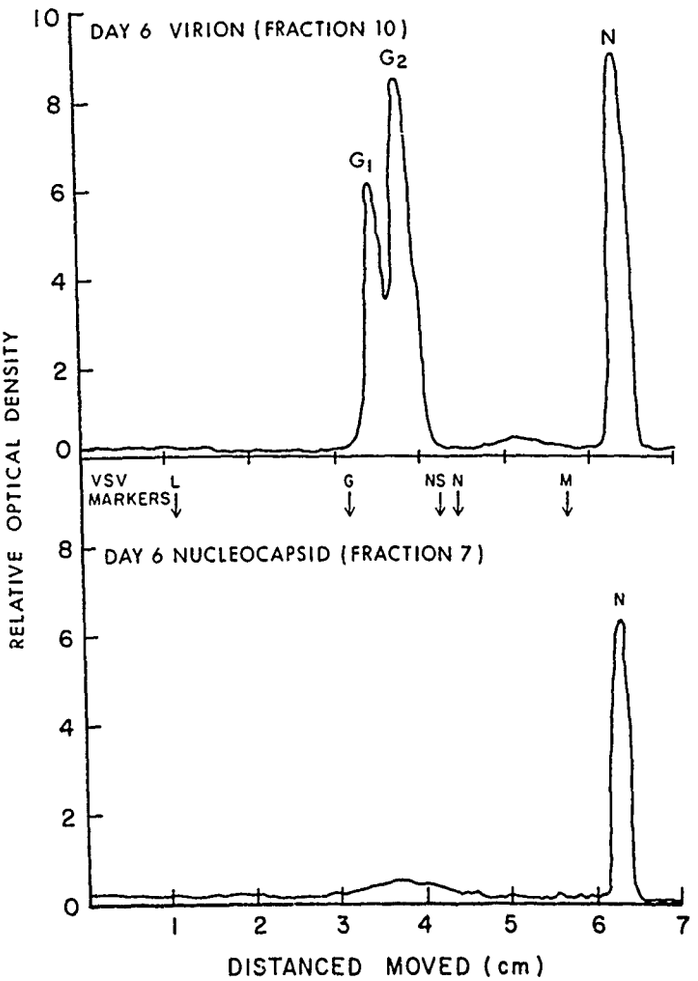

Figure 4. SDS - Polyacrylamide gel electrophoresis of ^{35}S-methionine labeled virion and nucleocapsid proteins. Samples are from low MOI gradient shown in Figures 2 and 3.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 097, Antigenic Characterization of Sandfly Fever Viruses

Publications.

1. McCown, J.M.; Brandt, W.E.; Bancroft, W.H.; and Russell, P.K. Dimethylsulfoxide Enhancement of Phlebotomus Fever Virus Plaque Formation. Am. J. Trop. Med. Hyg. 28:733-739, 1979.

2. Robeson, G.; El Said, L.H.; Brandt, W.; Dalrymple, J.; and Bishop, D.H.L. Biochemical Studies on the Phlebotomus Fever Group Viruses (Bunyaviridae Family) J. Virol. 30:339-350, 1979.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DAOC6460	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	098
b. CONTRIBUTING				
c. [illegible]				

11. TITLE (Precede with Security Classification Code)

(U) Immunology of Plasmodium falciparum in vitro

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE d. AMOUNT:
e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	1	155
FISCAL YEAR CURRENT 80	1	170

19. RESPONSIBLE DOD ORGANIZATION

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ADDRESS: Division of CD&I

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)

NAME: Carter L. Diggs, COL

TELEPHONE: (202) 576-3544

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Haynes, J.D., MAJ

NAME: Chulay, H, LTC

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immunity, (U) Malaria, (U) Tropical Medicine, (U) Antigens (U) Antibodies

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) The objective of this work unit is to exploit newly developed methods of culture of human malaria parasites (Plasmodium falciparum) in order to elucidate the mechanisms of immunity to the organism and explore the feasibility of developing a vaccine against this major military infectious disease problem.

24 (U) The approach is to improve existing culture technology to produce parasite antigens; to characterize these antigens and evaluate them as vaccine candidates by immunizing experimental animals; and to develop in vitro tests which are predictive of protective immunity.

25 (U) 78 10-79 09 Synchronization of the parasite life cycle and density gradient separation techniques have been used to increase the yield of mature parasites. Multiple proteins obtained from radioisotopically labelled cultured parasites have been demonstrated by SDS-PAGE. Antigen has been prepared from such cultures for experimental immunization, analysis of cross reactivity with other parasites, and comparative studies of parasite and host enzymes and their inhibitors. Parasite growth was inhibited in vitro by immune monkey serum. Most of the inhibition was due to immunze IgG. Studies using synchronized cultures have demonstrated that merozoites are the targets of the immune serum with resulting blockade of penetration of uninfected erythrocytes. Electron microscopy reveals immune precipitates coating the merozoites. Similar precipitates form on erythrocytes infected with mature parasites. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78-30 Sept 79.

* Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 098 Immunology of Plasmodium falciparum In vitro

Investigators:
Jeffrey Chulay. LTC, MC; Carter Diggs, COL, MC; Rufus Gore; Cynthia Hall; David Haynes; MAJ, MC; Teresa Jareed; Lois Simonton; Pramaun Tapchaisri; Andre Toussaint; Karen Wabnitz; Joseph Williams.

Objective: The problem under investigation is the evaluation of feasibility of development of a vaccine against the human malaria caused by Plasmodium falciparum.

Description: This problem is of importance because malaria is the single greatest cause of morbidity among all infectious diseases. It has been the major cause of combat days lost in many military campaigns. The development of drug resistant parasites and the inadequacy of vector control measures have recently resulted in a world wide resurgence of this disease.

The problem has been approached through the development and continual improvement of methods for the culture of P. falciparum for the production and characterization of antigens. These are used in experimental immunization and for the development of assays which will be predictive of protective immunity in experimentally immunized animals or humans.

Our current work is based on methods for the continuous culture of P. falciparum developed in our laboratory and at the Rockefeller University. Infected erythrocytes are introduced into suspensions of normal human erythrocytes in culture medium with 10% human serum under reduced oxygen tension. Typically, a six to ten-fold increase in parasites is obtained in 48 hours; the organisms can be maintained indefinitely by subculture.

We have studied and improved several aspects of the system and have utilized it for a number of investigations into the immunology and biochemistry of P. falciparum. For example, the receptors on the erythrocyte to which the merozoites of the parasite attach have been studied through the use of mutart erythrocytes and enzymatic treatment; the receptor is best correlated with a normal glycophorin content of the cell. Cultured parasites have also been used as a source of antigen for serologic tests for serum antibodies against the parasite.

Progress: Tritiated hypoxanthine is avidly incorporated into P. falciparum grown in vitro. Radioisotope incorporation gives results comparable to morphological assessment of growth but markedly increases the number of variables which can be conveniently studied. Factors influencing the amount of incorporation include parasites concentration, erythrocyte concentration, parasite stage, the use of serum vs plasma, duration of culture, duration of exposure to the radioisotope, and the presence of other purines in the culture medium. When these parameters are controlled, the amount of radioisotope incorporation, measured by a semi-automated technique (MASH), is directly proportional to the number of parasites present. This system hs been useful in a variety of assays, including detection of inhibition of parasite growth by immune serum (Figure 1) and by antimalarial drugs (Figure 2), and in further defining culture requirements. Parasites showed good growth only when initial pH was between 7.30 and 7.45 (Figure 3). Growth in deficient media demonstrated a critical role for isoleucine and a lesser requirement for glutamine and several other amino acids (Figure 4) Work continues on attempts to develop a culture media which will require much less human serum for large scale cultures. One week was spent at the W. Alton Jones Cell Science Center learning techniques and principles of serum-free tissue culture with W. McKeehan. Neither MCBD 202 nor F-12k media allowed any further reduction of human serum in the medium. Medium RPMI 1640 could support some growth with only 1% serum. Since the quality of distilled water is critical with low serum concentrations, our source of distilled water (GIBCO) was compared with their standard in a mammalian diploid cell line cloning assay and found to be adequate. Another research assistant is needed to carry on this work.

Some biochemical studies were performed in collaboration with the Department of Hematology. Examination of purine salvage enzymes in Plasmodium falciparum obtained from in vitro cultures revealed high levels of a parasite specific adenosine deaminase (ADA; E.C. 3.5.4.4) (Table 1). The partially purified enzyme from P. falciparum was distinguished from erythrocyte ADA by its greater electrophoretic mobility in starch gel (Figure 5) and its lack of sensitivity to erythro-9- -(2-hydroxy-3-nonyl) adenine (EHNA) (Figure 6), an

inhibitor of erythrocyte ADA. When both ADAs were inhibited by deoxycoformycin, low concentrations of adenosine or deoxyadenosine inhibited growth of the parasite (Figures 7 and 8). Similarly, the toxicity of a purine nucleoside analogue with antimalarial activity (cordycepin) was greatly enhanced when ADA was inhibited (Figure 9). Thus, in addition to participation in the purine salvage pathways, parasite ADA detoxifies adenine nucleosides and analogues. The results show that there are major differences between the host and parasite enzymes, which might be exploited in the development of purine analogues with selective toxicity for the parasite.

We have improved methods for the enhanced production of the mature forms of the parasite through culture synchronization. Appropriately timed treatment with sorbitol solutions (adapted from C. Lambros and L. Vanderberg) lyse erythrocytes infected with mature parasites and result in a relatively homogeneous population of intraerythrocytic immature parasites. These then grow up to give high yields of mature parasites. These parasites have been concentrated by density gradients (colloidal silica or Metrizamide), and by taking the supernatant from a sorbitol lysis and centrifuging at a high speed. These are being used as a source of antigen for immunization experiments, for the analysis of cross reactivity with other parasites, and as a source of extracts to study the biochemistry of the parasite. Analysis of 3H-isoleucine metabolically labeled parasite proteins reveals many bands on SDS gels (Figure 10). Some parasite antigens cross react antigenically with another species of malaria parasite (collaboration with Lou Miller). Experimental animals are being immunized with mature parasites from synchronized cultures; the immune sera and cells from these animals will be examined for their ability to inhibit the growth of the parasite in vitro. As specific antigens are purified, these will be examined for their role in protective immunity.

Serum from owl monkeys immune to P. falciparum by virtue of infection followed by chemotherapy (Figures 11 and 12) has been found to inhibit the development of parasite growth in

vitro (Table 2). IgG isolated from the serum by affinity chromatography using staphylococcal protein A contained most of the inhibitory activity. Studies of the changing character of the populations of the various stages of the parasites in synchronized cultures treated with immune serum demonstrated that growth inhibition is probably due to failure of merozoites to penetrate uninfected erythrocytes. (Figure 13) When immune serum (IS) was added to partially synchronized cultures of P. falciparum, parasites mature normally during the first day, from ring forms to trophozoites and schizonts. In contrast, the number of new rings detected after two days (following reinvasion) was reduced by 90%, compared with cultures containing normal serum (NS). Clusters of merozoites, often around clumps of malarial pigment, were seen frequently in cultures growth with IS but rarely in cultures with NS. Electron microscopy confirmed the normal development of intraerythrocytic parasites. Electron microscopy revealed immune precipitates coating free and aggregated merozoites (Figure 14) and the surface of erythrocytes infected with mature parasites (Figure 15) (collaboration with M. Aikawa). This phenomenon may form the basis for an in vitro assay predictive of protective immunity. Although antisera from monkeys immune to one strain of P. falciparum cross react with other strains in an indirect immunofluorescent assay, preliminary results show little cross reactivity in the in vitro growth inhibition assay, suggesting that strain specific antigens play an important role in protective immunity.

Discussion and Recommendations: Our work is presently oriented to 6 areas:

1) In vitro culture conditions. The production of moderate amounts of antigen presently requires excessive effort (eg. media changes at midnight). Automation is needed to obtain larger amounts of antigen. The 1L 410 Cell Culture Center would supply the necessary monitoring and control of p02, pC02, pH and media flow. Since we are now near the limits of our ability to supply human serum (2 units per week) for culture on a moderate scale, the development of a serum-free medium has a high priority. We need a new laboratory assistant to help carry out this work.

2) In vitro assays. The assay for inhibition of parasite growth by immune serum seems to correlate sometimes but not always with protection against challenge with parasites. Further work is necessary to establish the significance of this mechanism of immunity and to identify and purify the antigen(s) (possibly some strain specific) involved. Work with cell mediated killing of parasites, thus far negative, will continue.

3) Synchronization. Synchronization to within a 4 hour span is now easily achieved with 2 sequential sorbitol lysis treatments. However further work is needed to achieve synchronization within a 30 minute span, thought to be desirable if healthy, antigenic merozoites are to be obtained.

4) Concentration. Of the various methods tried, concentration by simple centrifugation from sorbitol lysis supernatants seems the best.

5) Stabilization. Since merozoites can not yet be obtained within a 30 minute span, healthy merozoites at "time 0" have not been obtained for a comparison of different methods for stabilization. When "time 0" merozoites are obtained, protease inhibitors and other agents and treatments will be compared with regard to their effects on morphological (light and electron microscopy), biological (reinvasion capacity), and biochemical (molecular weights of labeled proteins on SDS-PAGE) integrity.

6) Purification and analysis of antigens. This needs much work. We hope to use in vitro assays which correlate with protective immunity to help identify relevant antigens. Once an antigen is identified, there are many alternatives open for subsequent purification and immunological analysis. We hope to employ strategies involving the following tactics which are in varying stages of readiness:

(a) Metabolic labeling of parasite antigens with radioactive amino acids and sugars.

(b) External labeling merozoite surface antigens.

(c) Identification of stage-specific antigens by using short pulses of radioactive precursors at different stages of synchronous cultures.

(d) The use of various immune antibodies from humans and experimental animals to precipitate antigen, and their use in sequence to remove antigen of host red cell origin or of immature stage parasite origin or of cross-reactive species or strain origin.

(e) The use of immunofluorescence of living merozoites, and immunoferritin labeling in electron microscopy to localize antigens.

(f) The use of monoclonal antibodies obtained from hybridomas.

(g) Purification of antigen by methods utilizing differences in molecular weight, isoelectric point, charge, and affinity adsorption.

(h) The use of purified antigens to absorb immune antisera before testing in an *in vitro* growth inhibition assay.

(i) Immunization of experimental animals with purified antigen.

(j) Eventually, following the identification of a relevant antigen, the cloning of the corresponding parasite DNA sequence into *E. coli*, for mass antigen production.

Our immediate need is for another investigator with a Ph.D. in chemistry and experience in membrane protein chemistry.

Table 1

Purine salvage enzymes in parasitized erythrocytes.

Sample	N	Mean parasitemia (%)	Mean enzyme activity (U/g protein)				
			APRT	HGPRT	AK	PNP	ADA
Camp	10	4.2±0.5	0.37±0.03	1.38±0.13	0.19±0.01	30.8±1.7	2.00±0.19
FCR-3	7	10.1±1.9	0.43±0.03	1.60±0.14	0.18±0.01	28.8±1.5	2.92±0.49
Control	8	(0)	0.40±0.04	1.44±0.09	0.19±0.01	28.8±2.0	1.15±0.11

Table 2

Reproducibility of inhibition assay.*

Animal	Serum	Percent inhibition at 1:10 dilution	Titer (50% inhibition)
A091-046	1	30, 25, 24	—
A076-049	2	80, 90, 84	29, +, 30
	3	58, 61	11, 1:14
A072-037	4	73, 72, 80	1:27, 1:23, 1:27

*Test sera were obtained from each animal 28-37 days after challenge with 200-500 million parasitized erythrocytes. Aliquots Aliquots of each serum were tested on separate occasions.

+Tested only at 1:10 dilution in this experiment.

Figure 1.

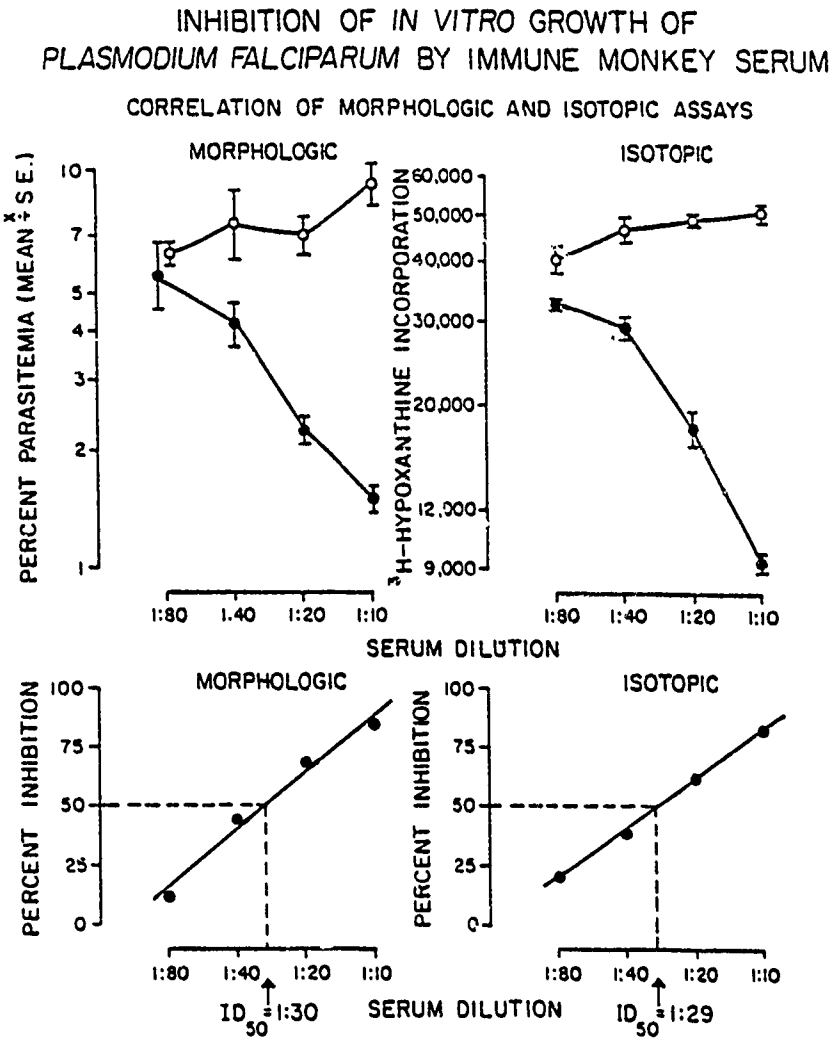

INHIBITION OF IN VITRO GROWTH OF
PLASMODIUM FALCIPARUM BY IMMUNE MONKEY SERUM
CORRELATION OF MORPHOLOGIC AND ISOTOPIC ASSAYS
MORPHOLOGIC
ISOTOPIC
PERCENT PARASITEMIA (MEAN ± S.E.)
^{3}H-HYPOXANTHINE INCORPORATION
10
7
5
4
3
2
1
60,000
50,000
40,000
30,000
20,000
12,000
9,000
1:80
1:40
1:20
1:10
SERUM DILUTION
MORPHOLOGIC
ISOTOPIC
PERCENT INHIBITION
100
75
50
25
0
ID50 = 1:30
ID50 = 1:29
SERUM DILUTION

Figure 2.

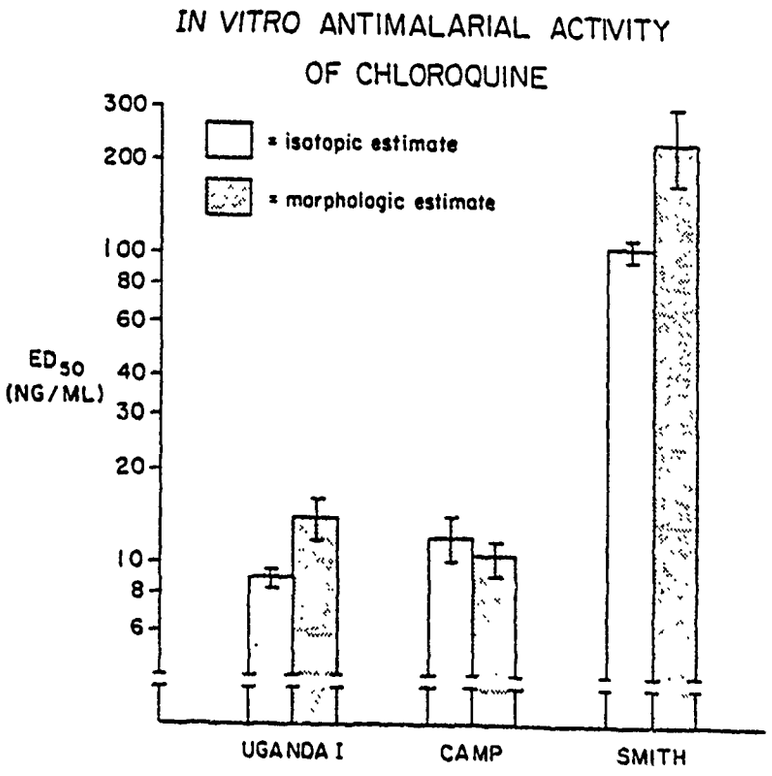

IN VITRO ANTIMALARIAL ACTIVITY
OF CHLOROQUINE
= isotopic estimate
= morphologic estimate
ED50 (NG/ML)
300
200
100
80
60
40
30
20
10
8
6
UGANDA I
CAMP
SMITH

Figure 3.

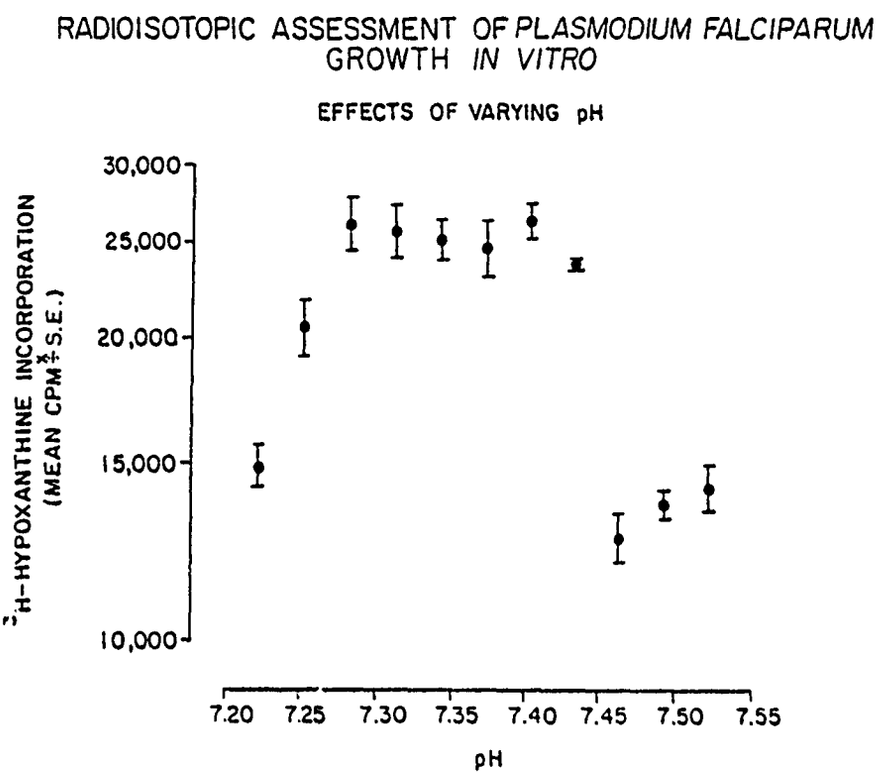

RADIOISOTOPIC ASSESSMENT OF PLASMODIUM FALCIPARUM GROWTH IN VITRO
EFFECTS OF VARYING pH
^{3}H-HYPOXANTHINE INCORPORATION (MEAN CPM ± S.E.)
30,000
25,000
20,000
15,000
10,000
7.20
7.25
7.30
7.35
7.40
7.45
7.50
7.55
pH

Figure 4.

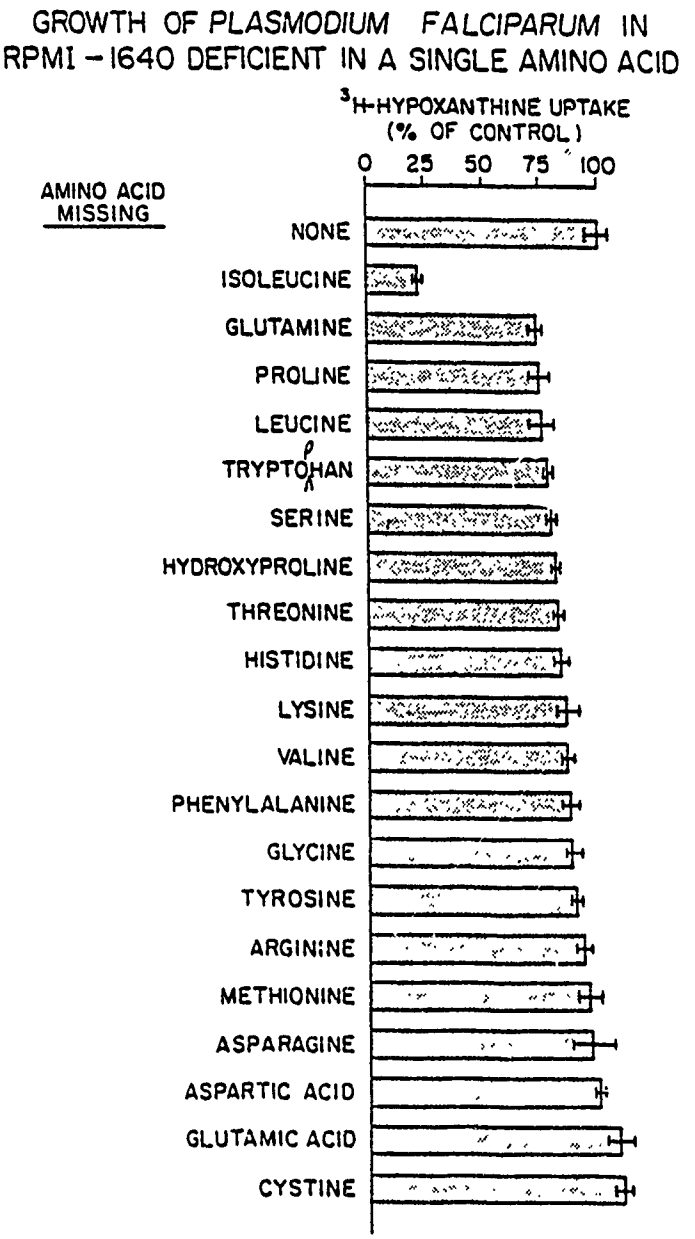

GROWTH OF PLASMODIUM FALCIPARUM IN RPMI – 1640 DEFICIENT IN A SINGLE AMINO ACID
^{3}H-HYPOXANTHINE UPTAKE (% OF CONTROL)
0 25 50 75 100
AMINO ACID MISSING
NONE
ISOLEUCINE
GLUTAMINE
PROLINE
LEUCINE
TRYPTOPHAN
SERINE
HYDROXYPROLINE
THREONINE
HISTIDINE
LYSINE
VALINE
PHENYLALANINE
GLYCINE
TYROSINE
ARGININE
METHIONINE
ASPARAGINE
ASPARTIC ACID
GLUTAMIC ACID
CYSTINE

Figure 5.

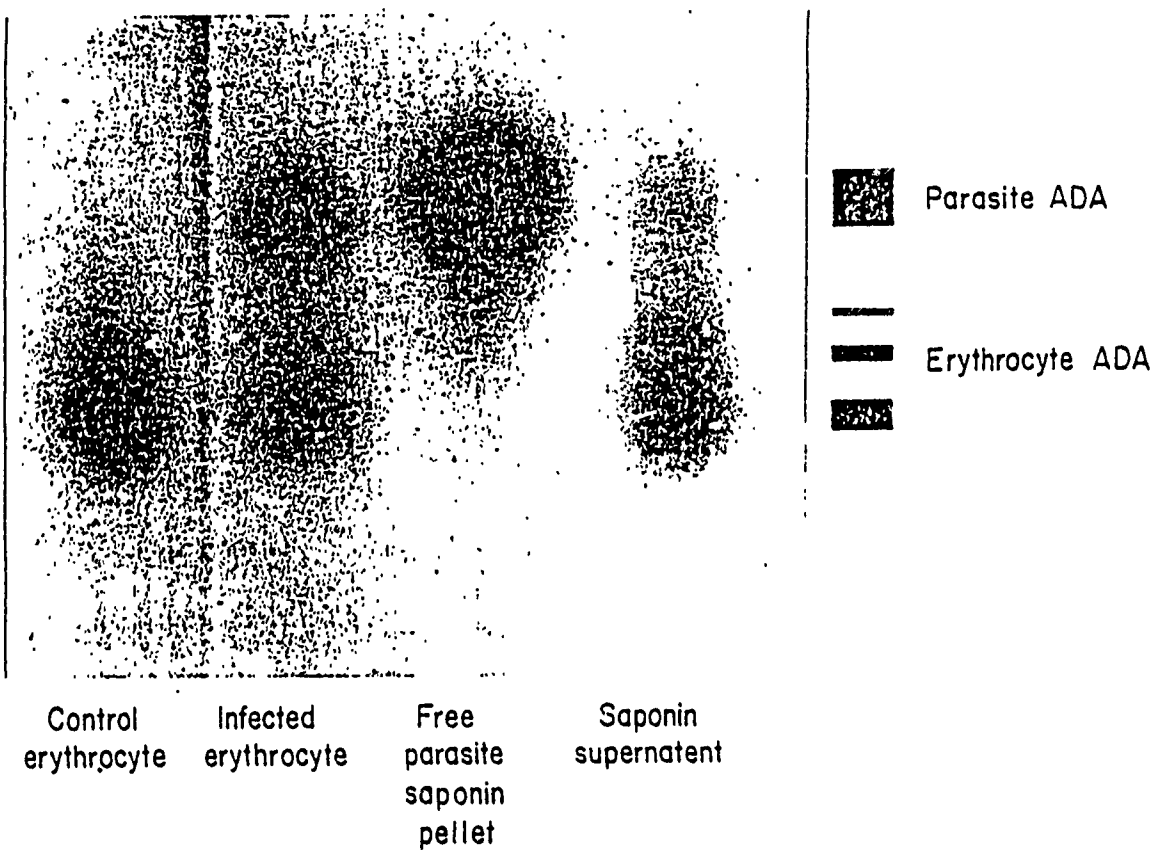

Parasite ADA
Erythrocyte ADA
Control erythrocyte
Infected erythrocyte
Free parasite saponin pellet
Saponin supernatent

Figure 6.

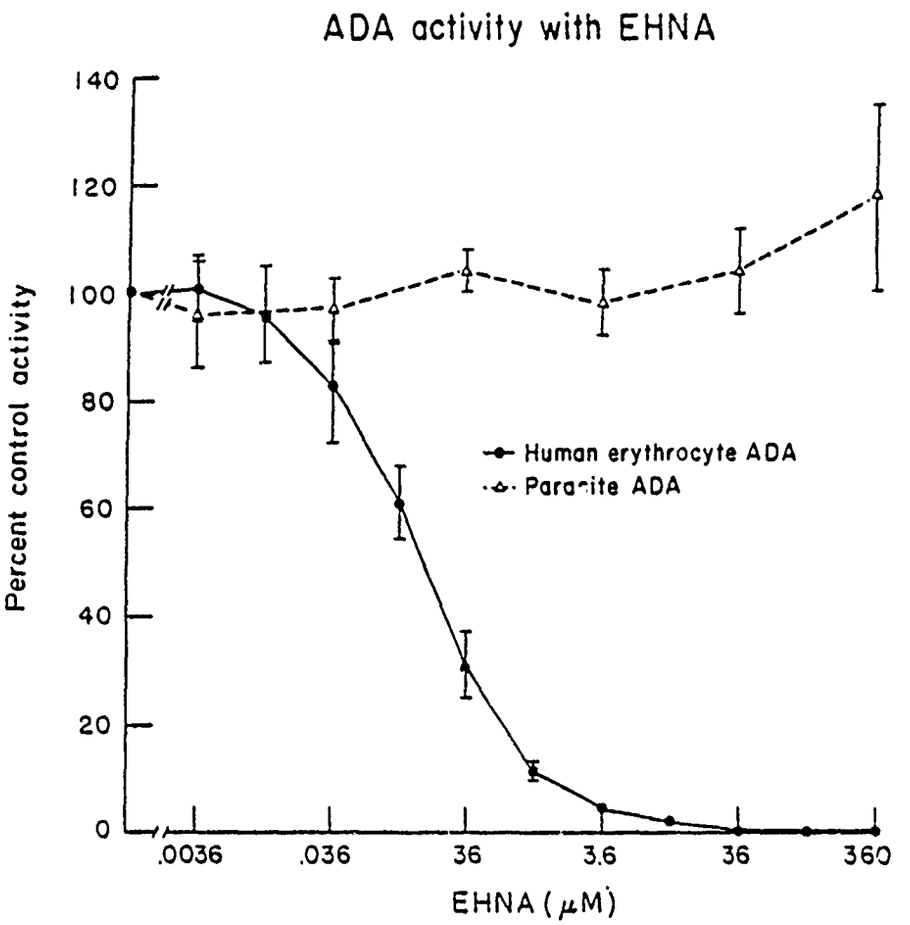

ADA activity with EHNA
Percent control activity
140
120
100
80
60
40
20
0
.0036
.036
36
3.6
36
360
EHNA (μM)
Human erythrocyte ADA
Parasite ADA

Figure 7.

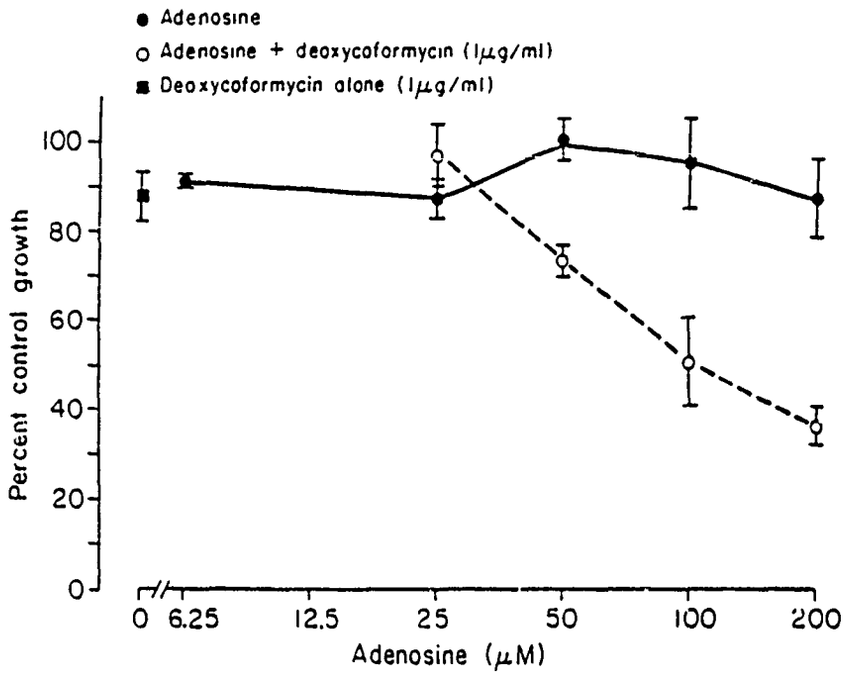

● Adenosine
○ Adenosine + deoxycoformycin (1μg/ml)
■ Deoxycoformycin alone (1μg/ml)
Percent control growth
100
80
60
40
20
0
0 6.25 12.5 25 50 100 200
Adenosine (μM)

Figure 8.

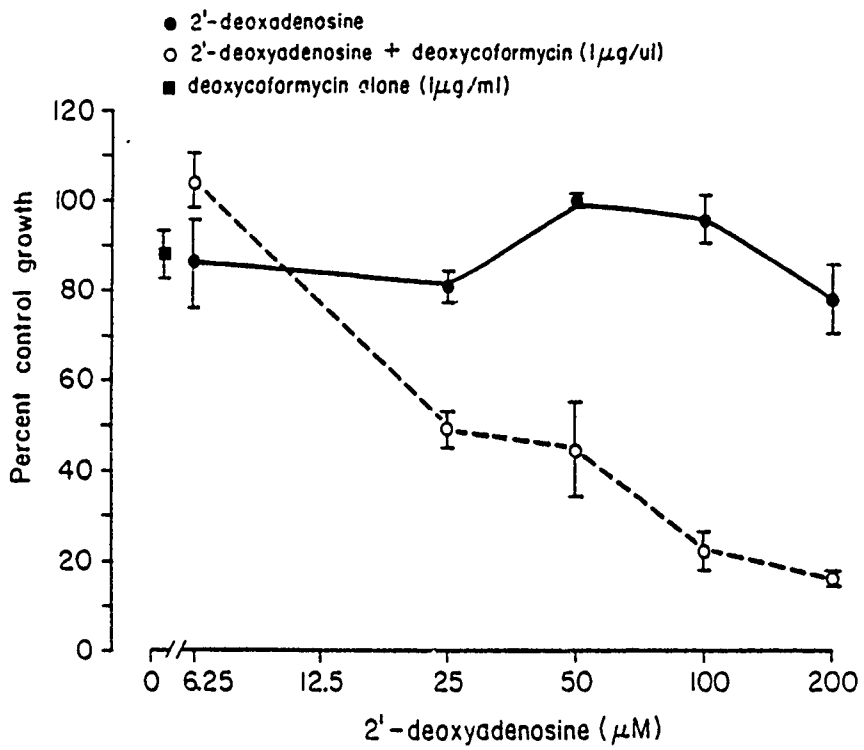

● 2'-deoxadenosine
○ 2'-deoxyadenosine + deoxycoformycin (1μg/ul)
■ deoxycoformycin alone (1μg/ml)
Percent control growth
120
100
80
60
40
20
0
0
6.25
12.5
25
50
100
200
2'-deoxyadenosine (μM)

Figure 9.

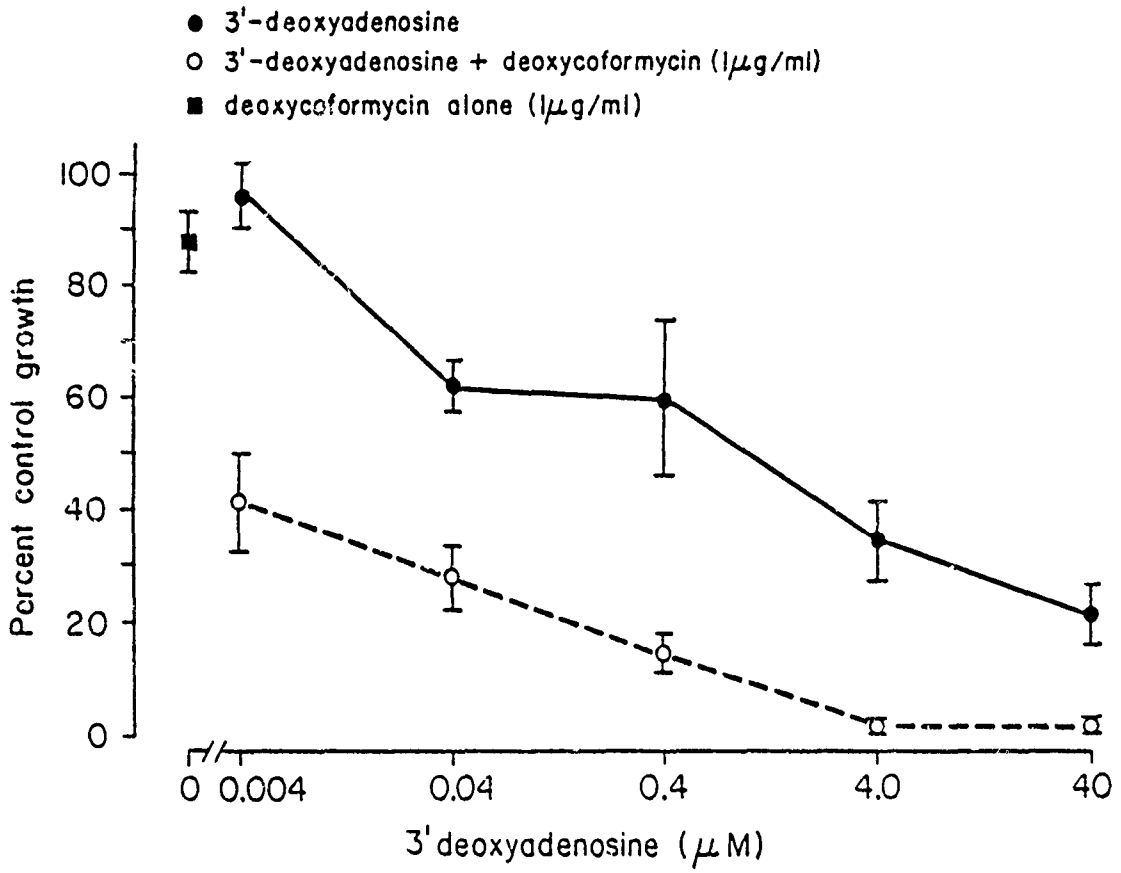

● 3'-deoxyadenosine
○ 3'-deoxyadenosine + deoxycoformycin (1μg/ml)
■ deoxycoformycin alone (1μg/ml)
Percent control growth
100
80
60
40
20
0
0
0.004
0.04
0.4
4.0
40
3'deoxyadenosine (μM)

Figure 10.

Scintillant enhanced autoradiograph of ^{3}H-isoleucine labelled trophozoite proteins run on SDS-gradient PAGE.

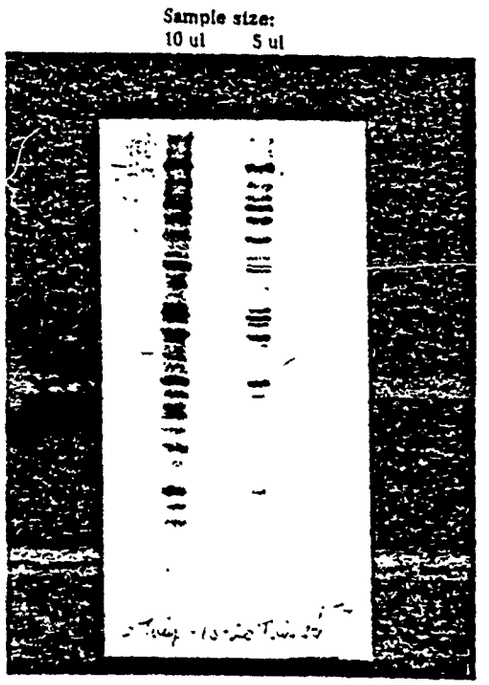


Figure 11.

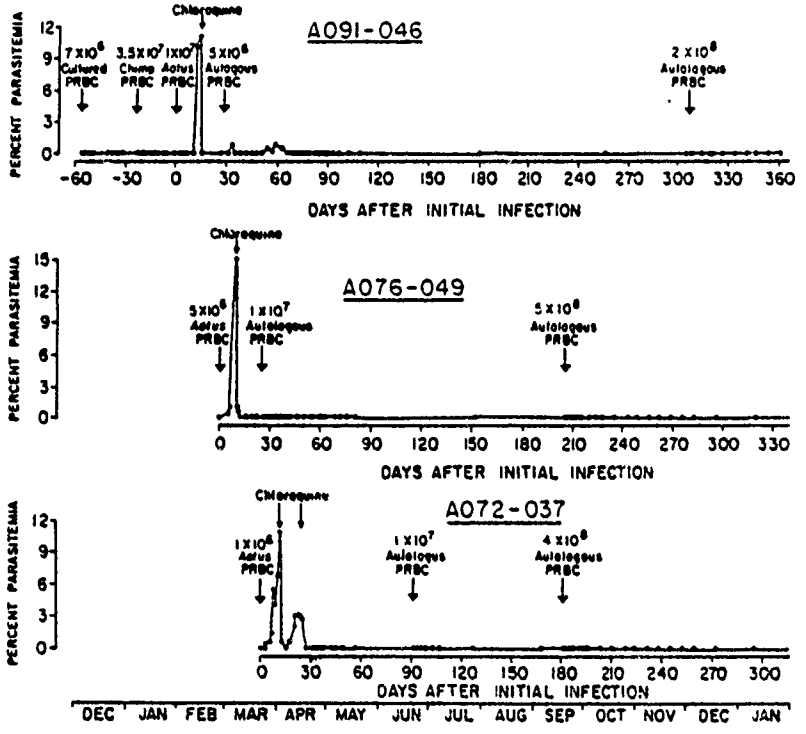
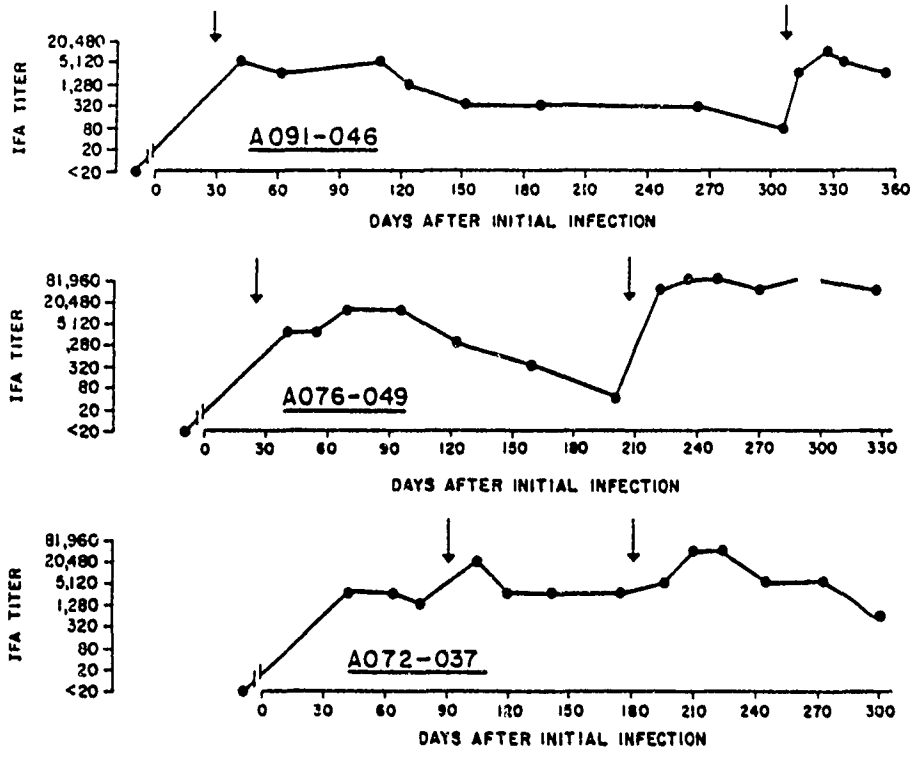

A091-046
Chloroquine
7 X 10^6 Cultured PRBC
3.5 X 10^7 Chimp PRBC
1 X 10^7 Aotus PRBC
5 X 10^6 Autologous PRBC
2 X 10^8 Autologous PRBC
PERCENT PARASITEMIA
DAYS AFTER INITIAL INFECTION
A076-049
Chloroquine
5 X 10^6 Aotus PRBC
1 X 10^7 Autologous PRBC
5 X 10^8 Autologous PRBC
PERCENT PARASITEMIA
DAYS AFTER INITIAL INFECTION
A072-037
Chloroquine
1 X 10^6 Aotus PRBC
1 X 10^7 Autologous PRBC
4 X 10^8 Autologous PRBC
PERCENT PARASITEMIA
DAYS AFTER INITIAL INFECTION
DEC JAN FEB MAR APR MAY JUN JUL AUG SEP OCT NOV DEC JAN

Figure 12.


IFA TITER
20,480
5,120
1,280
320
80
20
<20
A091-046
0 30 60 90 120 150 180 210 240 270 300 330 360
DAYS AFTER INITIAL INFECTION
IFA TITER
81,960
20,480
5,120
1,280
320
80
20
<20
A076-049
0 30 60 90 120 150 180 210 240 270 300 330
DAYS AFTER INITIAL INFECTION
IFA TITER
81,960
20,480
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<20
A072-037
0 30 60 90 120 150 180 210 240 270 300
DAYS AFTER INITIAL INFECTION

Figure 13.

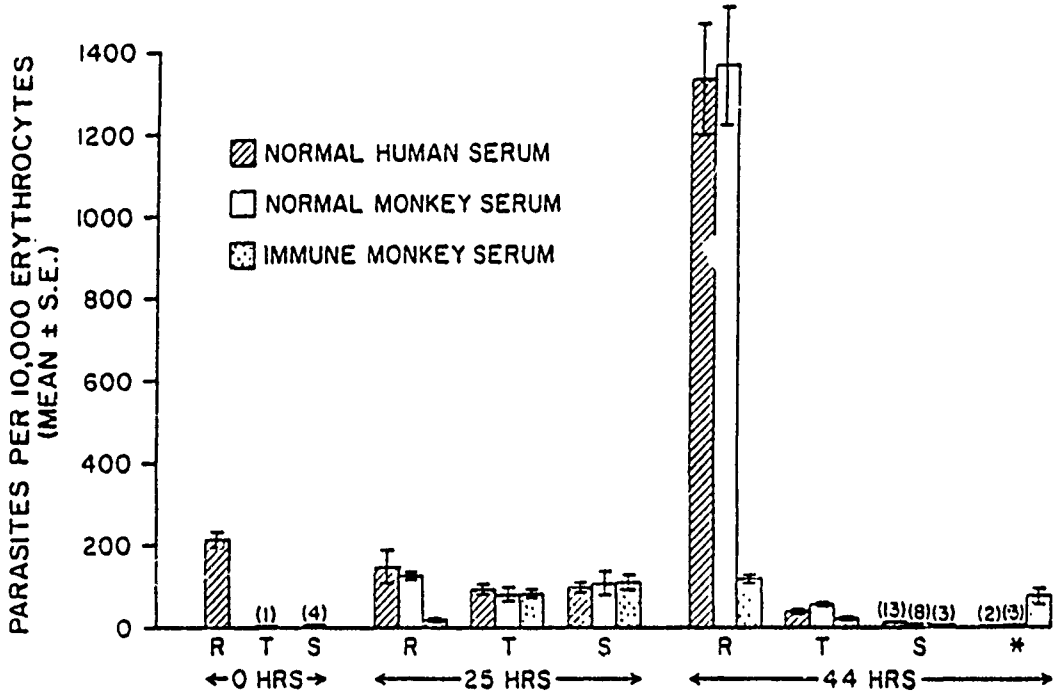

PARASITES PER 10,000 ERYTHROCYTES (MEAN ± S.E.)
1400
1200
1000
800
600
400
200
0
NORMAL HUMAN SERUM
NORMAL MONKEY SERUM
IMMUNE MONKEY SERUM
(1)
(4)
(13)(8)(3)
(2)(3)
R T S R T S R T S *
←0 HRS→
←25 HRS→
←44 HRS→

Figure 14.

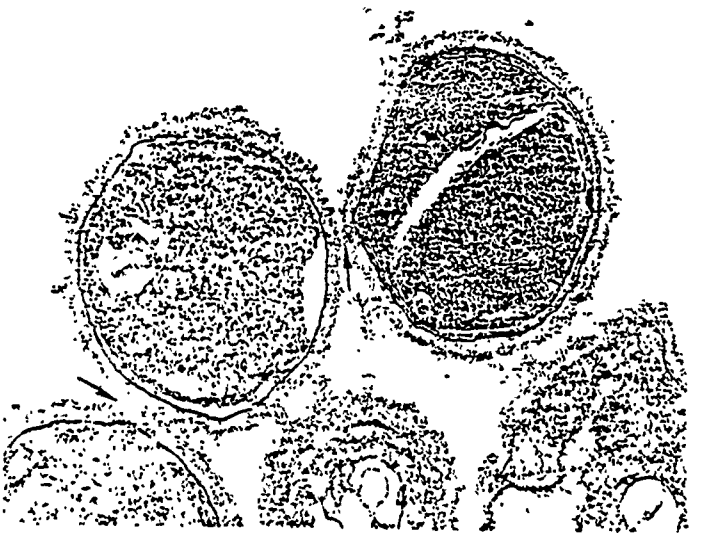
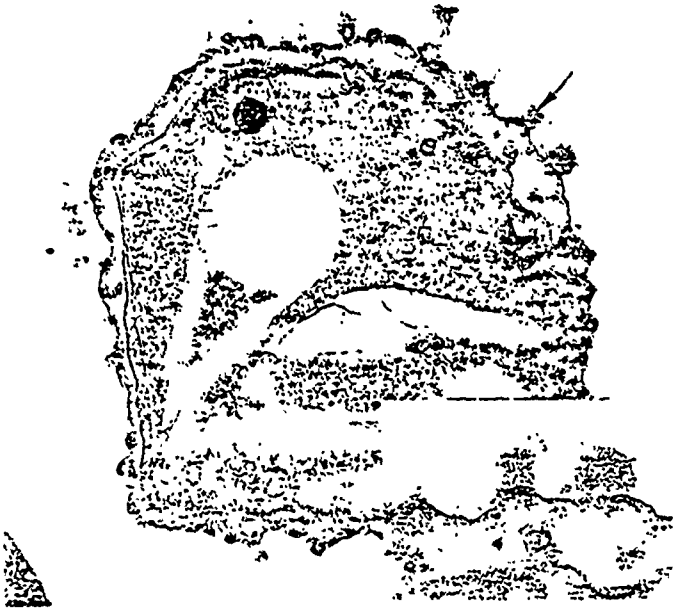


Figure 15.



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6462	79 09 30	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	H. Term	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A 91C	00	100
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Procede with Security Classification Code)

(U) Host Defense Mechanism Responses to Stress and Shock

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1	109
FISCAL YEAR CURRENT 79	1	80

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Surgery

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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)

NAME: Fleming, A. LTC (P)

TELEPHONE: (202) 576-3791

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Formeister, J. MAJ*

NAME: Shatney, C. MAJ

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Host defense mechanisms; (U) Hemorrhagic shock; (U) Barbituate anesthesia; (U) Stress

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)

23 (U) To evaluate the effect of experimental shock and the stress of surgery on the competence of host defense mechanisms of individual subjects. These studies will provide insight to the effects of trauma on the combat casualties ability to resist infection. It is possible that therapeutic approaches will result to reduce infection rate in combat casualties.

24 (U) Various tests of cell-mediated immunity were used to assess the competence of the host defense system. Using canines subjected to sodium pentobarbital anesthesia and hemorrhagic shock, the ability of the small lymphocyte to proliferate as measured by a mitogen-induced (stimulation of mitosis) blastogenesis assay was monitored.

25 (U) 78 10 - 79 09 Twenty-one canines were administered Hg anesthetic doses of sodium pentobarbital and placed in hemorrhagic shock at 50 mm Hg for one hour. The total lymphocyte count was depressed in most animals on the first day following shock and did not return to normal until seven to nine days after recovering from shock. The presence of nucleated red blood cells in the cell cultures caused significant interference in the interpretation of the blastogenesis assay. Bacterial contamination, possible from invasion of the blood stream by bacteria from the gastrointestinal tract, also caused significant interference with many of the assays, and required the addition of antibiotics to the cultures to eliminate the problem. Other tests of host defense mechanisms are currently being sought. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78 - 30 Sept 79.

*Expired on 7 February 1979 - Previously the principal investigator.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6462	79 09 30	DD DR&E (AR)636

3 DATE PREV SUM RY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 REGRADING	8A DISP N INSTR N	8b SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM
78 10 01	H. Term	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10 NO /CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A 91C	00	100
b. CONTRIBUTING				
c. CONTRIBUTING				

11 TITLE (Precede with Security Classification Code)

(U) Host Defense Mechanism Responses to Stress and Shock

12 SCIENTIFIC AND TECHNOLOGICAL AREAS

016200 Stress Physiology

13 START DATE	14 ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	CONT	DA	C. In-House

17 CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER

c. TYPE d. AMOUNT:

e. KIND OF AWARD f. CUM. AMT.

18 RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING FISCAL YEAR 78	1	109
CURRENT FISCAL YEAR 79	1	80

19 RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL

NAME: Russell, Philip K, COL

TELEPHONE: (202) 576-3551

20 PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Surgery

ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)

NAME: Fleming, A. LTC (P)

TELEPHONE: (202) 576-3791

SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME: Formeister, J. MAJ*

NAME: Shatney, C. MAJ

21 GENERAL USE

Foreign intelligence not considered

22 KEYWORDS (Precede EACH with Security Classification Code) (U) Host defense mechanisms; (U) Hemorrhagic shock; (U) Barbituate anesthesia; (U) Stress

23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To evaluate the effect of experimental shock and the stress of surgery on the competence of host defense mechanisms of individual subjects. These studies will provide insight to the effects of trauma on the combat casualties ability to resist infection. It is possible that therapeutic approaches will result to reduce infection rate in combat casualties.

24 (U) Various tests of cell-mediated immunity were used to assess the competence of the host defense system. Using canines subjected to sodium pentobarbital anesthesia and hemorrhagic shock, the ability of the small lymphocyte to proliferate as measured by a mitogen-induced (stimulation of mitosis) blastogenesis assay was monitored.

25 (U) 78 10 - 79 09 Twenty-one canines were administered Hg anesthetic doses of sodium pentobarbital and placed in hemorrhagic shock at 50 mm Hg for one hour. The total lymphocyte count was depressed in most animals on the first day following shock and did not return to normal until seven to nine days after recovering from shock. The presence of nucleated red blood cells in the cell cultures caused significant interference in the interpretation of the blastogenesis assay. Bacterial contamination, possible from invasion of the blood stream by bacteria from the gastrointestinal tract, also caused significant interference with many of the assays, and required the addition of antibiotics to the cultures to eliminate the problem. Other tests of host defense mechanisms are currently being sought. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78 - 30 Sept 79.

*Expired on 7 February 1979 - Previously the principal investigator.

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DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE DD FORMS 1498A 1 NOV 68 AND 1498 1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3A161101A91C IN HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 100 Host Defense Mechanism Responses to Stress and Shock

Investigator
Principal: LTC (P) Arthur Fleming, MC
Co: MAJ Clayton Shatney, MC
MAJ Joseph Formeister, MC

I. Effects of Hemorrhagic Shock on Cell Mediated Immunity

A. Background and Statement of the Problem. It is not known to what extent host defense mechanisms are compromised following shock and the stress of anesthesia and surgery, and hence responsible for permitting bacterial invasion to go unchecked. Because of the prevalence of sepsis in combat casualties, we would like to determine to what extent host defense mechanisms are altered during experimental shock and anesthesia and how these alterations might be reversed.

Infection is a leading cause of morbidity and mortality in combat casualties. In a survey of patients dying in hospitals in Vietnam during the period between 1 January to 31 December 1969, it was found that hemorrhagic shock accounted for 24% of deaths and septic shock (i.e., shock secondary to an overwhelming infection) was the cause of death in 11% of cases. Thus, hemorrhagic and septic shock accounted for greater than one-third of the deaths and was exceeded only by head injuries which accounted for 42.5% of deaths. This mortality occurred despite a rapid evacuation time of the combat casualty in Vietnam and the availability of various antibiotics. The infection rate following combat injuries in Thailand has recently been documented over a three month period of time (July-Sept 1978) in 50 casualties. In casualties with lower extremity injuries, there was a 20% incidence of infection on admission to the hospital; and with upper extremity injuries, there was a 80% incidence of infection. The conditions that prevail at the time of injury such as weather, terrain and distance from medical support may be additive to any suppression of host defense mechanisms and thus contribute to the increased infection rate that the combat casualty faces. It is possible that therapeutic approaches will result from these studies to reduce the infection rate in injured soldiers.

B. Experimental Approach. Initially, the ability of lymphocytes to proliferate (multiply or reproduce) was evaluated both *in vitro* and *in vivo* as an index of the competence of the host defense system. The ability of lymphocytes to proliferate was determined by a blastogenesis assay in animals subjected to anesthesia alone. The second step was to determine the combined effect of anesthesia and hemorrhagic shock on the ability of the lymphocyte to proliferate. The third and most important step will

be to determine the influence of various fluids used for resuscitation of hemorrhagic shock on host defense parameters. In addition to lymphocyte rate of proliferation, function will be determined by a lectin induced cellular cytotoxicity test (agents which induce lymphocytes to kill target cells); and the levels of serum proteins, immunoglobulins (IgG), complement (C3) and total opsonic activity will be determined.

C. Results and Discussion. Two hours after an anesthetic dose of sodium pentobarbital (28.6 mg/kg) canine lymphocytes were significantly suppressed as determined by blastogenesis assays (two different assays were used - tritiated thymidine uptake using erythroagglutinating mitogenic fraction of phytohemagglutinin P (E-PHA) and leukoagglutinating mitogenic fraction of phytohemagglutinin P (L-PHA). Recovery as measured by a progressive return to pre-anesthetic values was initiated in most animals at twenty-four hours. By 72 hours, the rate of proliferation as measured by the above test had returned to 75% of control determinations. These results lend support to the concept that sodium thiopental used to induce clinical anesthesia may be an important agent in the suppression of host defense mechanisms after major surgery and may be additive to any suppression caused by or induced by shock. The above assays were repeated before, during and after the induction of anesthesia with sodium pentobarbital and one hour of shock at 50 mm Hg.* Depression of the proliferative response was present on the day following surgery and there was evidence of a return toward normal by 72 hours. At one week following shock, the proliferative response of the lymphocytes was again depressed, possibly related to the nucleated red blood cells which occurred in the cell cultures at this point. The decreased lymphocyte proliferation that occurred two hours after administration of sodium pentobarbital was not seen in the shock animal, however, suggesting that during the early stages of shock, there may have been a enhancement of stimulation of the immune response, this overriding the effect of anesthesia. The depression of lymphocyte proliferation that occurs at 24 hours suggest a direct relationship to the shock state. Test of lymphocyte function as well as the other test of host defense parameters are planned for the coming year.

*The initial principal investigator became ill after the completion of the first step of this study and expired prior to starting the second step. The second phase of this study was carried out by college students and the current principal investigator during the past three months and was thus impeded significantly by restarting after many months of delay.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6463	79 09 30	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	H. Term	U	U	NA	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A 91C	00	101
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Thrombotic Mechanisms in Stress

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

016200 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE — EXPIRATION:
b. NUMBER
c. TYPE — d. AMOUNT:
e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 78	1	99
FISCAL YEAR CURRENT 79	1	86

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
NAME: Russell, Philip K, COL
TELEPHONE: (202) 576-3551

20. PERFORMING ORGANIZATION

NAME: Walter Reed Army Institute of Research
Division of Surgery
ADDRESS: Washington, D.C. 20012

PRINCIPAL INVESTIGATOR (Provide last if U.S. academic institution)
NAME: Clagett, G. MAJ
TELEPHONE: (202) 576-3391
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Platelet survival; (U) Thrombotic events; (U) Platelet inhibitors; (U) Vascular trauma; (U) Wound healing

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To assess the influence of platelet interaction with prosthetic surfaces on subsequent platelet function and reactivity in vitro and in vivo. To document the participation of platelets in wound healing. The influence of varying aspirin dosages on in vitro platelet function and in vivo platelet thrombogenesis will be tested. These studies are militarily relevant in terms of: a) modifying thromboembolism associated with blood in contact with prosthetic surfaces, b) documenting a physiologic role of platelets in normal wound healing and thus potentially improving healing following trauma, and c) reducing arterial and venous thromboembolism associated with vascular trauma.

24 (U) Multiple in vitro and in vivo platelet functions are assessed before and after inserting thoracoabdominal prosthetic aortic bypass grafts. Rabbits, rendered severely thrombocytopenic by injection with sheep anti-rabbit platelet serum, are compared to normal controls in terms of wound strength and healing characteristics. In vitro and in vivo platelet function are assessed under varying dosages of aspirin.

25 (U) 78 10 - 79 09 Platelets were documented to reversibly interact with prosthetic surfaces leading to biochemical function changes in platelets. Wounds from rabbits with low circulating platelet counts had significantly impaired wound tensile strength compared to animals with normal platelet counts. A dose-response relationship was found to exist with aspirin and in vivo thrombosis. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78 - 30 Sept 79.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 66 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE.

Project 3A161101A91C IN HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 101 Thrombotic Mechanisms in Stress

Investigator
Principal: MAJ G. Patrick Clagett, MC

I. In Vivo Interactions Between Platelets and Prosthetic Arterial Surfaces: Platelet Serotonin Dynamics

A. Background and Statement of the Problem. Previous studies and reports from our laboratory have documented marked biochemical and functional changes in blood platelets of animals following placement of prosthetic surfaces in the arterial circulation. Platelets had shortened survival, became more sensitive to aggregating agents, had impaired ability to participate in in vivo thrombogenesis, and had reduced serotonin levels. Our recent work has been directed towards elucidating the mechanism underlying the reduction in platelet serotonin.

B. Experimental Approach. As in previous studies, thoracico-abdominal aortic bypass grafts of woven Dacron were placed in dogs creating an animal model in which arterial blood would be continuously and chronically exposed to a prosthetic surface. Platelet survival time using ^{51}Cr labelled autologous platelets, platelet serotonin levels, and in vitro platelet ^{14}C-serotonin uptake and release were all determined before and at intervals following placement of arterial grafts. Additionally, urinary excretion of 5-hydroxy indoleacetic acid (5HIAA) (the major metabolite of serotonin) was measured for 2 months before and after graft placement. In vivo release of serotonin was assessed by labelling autologous platelets with ^{51}Cr (a non-releasable cytoplasmic label) and ^{14}C-serotonin (a releasable storage granule label), re-infusing them into animals with and without arterial grafts, and measuring ^{51}Cr and ^{14}C labelled platelets' survival. Finally, the ability of antiplatelet agents, aspirin and dipyridamole, to inhibit platelet interaction with the prosthetic surface was assessed by treating animals with these drugs and measuring the above parameters.

C. Results and Discussion. Following placement of grafts, platelet serotonin levels decreased from 2.01 ± 0.22 to 1.37 ± 0.17 mg/10^9 platelets ($p<.005$). Serotonin levels remained low for up to 6 months after graft placement and returned to normal when platelet survival time began to normalize. Maximum in vitro uptake of ^{14}C serotonin was depressed after graft placement (89.0 ± 0.71 before vs 72.0 ± 1.11% after, $p<.001$). In vivo release of serotonin was documented by the double labelling experiments. In animals with grafts, platelet survival time was shortened but ^{14}C label

survived 1.55 ± 0.10 times longer than ^{51}Cr label. This was significantly greater ($p<.02$) than control animals with normal platelet survival time in whom ^{14}C label survived 1.21 ± 0.05 times longer than ^{51}Cr. The ratio of $^{14}C/^{51}Cr$ activity over 5 days following infusion of double labelled platelets increased from 3.16 ± 0.52 (day 1) to 5.85 ± 1.18 (day 5), $p<.001$. Among controls, this ratio increased only slightly (1.99 ± 0.34 to 2.26 ± 0.42) and was significantly less on days 2 thru 5 than in animals with grafts ($p<.05$-$.005$). These data document platelet release and reutilization of ^{14}C serotonin in animals with grafts. Urinary excretion of 5HIAA increased slightly but not significantly. Treatment of animals having arterial grafts with platelet inhibitors, aspirin and dipyridamole, had no influence on the shortened platelet survival time or the reduction in platelet serotonin.

D. Conclusion and Recommendations. In these animals, we conclude that platelets adhere to the prosthetic surface, release serotonin and recirculate. Reutilization of released serotonin occurs but may be limited by depressed platelet uptake. The net effect is a reduction in platelet serotonin. These data further support our contention that platelets reversibly interact with prosthetic surfaces. Furthermore, the interaction between platelets and prosthetic surfaces in this animals model is very strong since conventional platelet inhibitors fail to normalize platelet survival and platelet serotonin levels. These findings were reported in London, July 1979 at the VIIth International Congress on Thrombosis and Hemostasis and will be presented at the Annual Meeting of the Association for Academic Surgery in November, 1979.

These studies raise several questions about the mechanism underlying shortened platelet survival time in the presence of prosthetic arterial surfaces. The accepted mechanism is that platelets adhere and aggregate on the prosthetic surface and are thereby "consumed" by the prosthetic surface. Our studies, however, indicate that the interaction is reversible with platelets returning to the circulation in an altered form. A reasonable hypothesis would be that the platelets returning to the circulation have reduced survival and are removed from the circulation by the reticuloendothelial system. We plan further studies to elucidate the mechanism of shortened platelet survival and to assess the participation of the reticuloendothelial system.

II. Aspirin Dosage and Antithrombotic Effect

A. Background and Statement of the Problem. The clinical utility of aspirin in reducing arterial thromboembolism from prosthetic surfaces, preventing stroke and transient cerebral ischemia, and preventing venous thromboembolism in patients under-

going major orthopedic surgery is established. The most efficacious antithrombotic dose of this drug, however, is controversial and not established. The controversy centers on the mechanism of action of aspirin in inhibiting prostaglandin metabolic pathways. Aspirin acts to acetylate and thus inhibit the action of cyclo-oxygenase, an enzyme responsible for converting arachidonic acid to labile endoperoxide intermediates. In the platelet, endoperoxides are rapidly converted to thromboxanes which trigger platelet aggregation. In the vessel wall (primarily the endothelium), the endoperoxides are converted to prostacyclin which inhibits platelet aggregation and thus prevents platelet deposition and thrombous formation on vessel surfaces. Cyclo-oxygenase in platelets is much more sensitive to aspirin than cyclo-oxygenase in the vessel wall. Also, since platelets do not have nuclei they cannot rephicate and replenish acetylated cyclo-oxygenase. Thus, the effects of aspirin on platelets last for the lifespan of the acetylated platelets. Endothelium, on the other hand, can replenish cyclo-oxygenase so the effect of aspirin is temporary.

Since small and large doses of aspirin equivalently suppress platelet function measured by in vitro platelet function tests, it is generally accepted that there is no correlation between aspirin dosage and antithrombotic effect. Furthermore, because of the differential effect by aspirin on platelet and vessel wall cyclo-oxygenase, it is possible that low doses of aspirin infrequently administered would be more efficacious in preventing thromboembolism than large doses. These hypotheses were tested in the following studies by comparing in vivo antithrombotic activity, in vitro platelet function tests, and vessel wall prostacyclin activity in animals given different doses of aspirin.

B. Experimental Approach. A standardized animal model of arterial thrombosis consisting of an arteriovenous shunt with flow rate controlled at 200 ml/min was devised. The occlusion time in seconds (T-O) of this device was found to be dependent upon formation of a platelet-rich thrombus confirmed by electron microscopy. Three groups of dogs were treated with 3 doses of ASA for 4 days and mean T-O under conditions of treatment was divided by mean T-O during a control period to obtain an index of prolongation of T-O by ASA in each animal. Two weeks were allowed to elapse between control and treatment periods. In 3 separate animals, mean T-O during treatment with a low dose (160 mg BID) and a high dose (650 mg BID) were compared. In all animals, in vitro platelet function tests were performed and salicylate levels measured. In 3 additional animals, the effect of the different ASA doses on vessel wall prostacyclin (potent inhibitor of platelet aggregation produced by endothelium) generation was assessed by bioassay of anti-aggregating activity of venous rings obtained

during control and ASA treatment periods.

C. Results and Discussion. ASA in all doses prolonged arteriovenous shunt T-O. The highest dose, however, produced a significantly greater index prolongation of T-O than either of the lower doses. Prolongation of T-O was correlated with ASA dose in mg/kg/24 hrs ($r = 0.82$, $P<0.01$) and salicylate levels ($r = 0.77$, $p<0.01$). In contrast, there were no correlations evident with in vitro studies. ASA in all doses produced equivalent suppression of platelet aggregation, ^{14}C serotonin release and platelet prostaglandin synthesis as measured by malonyldialhyde production. In the 3 animals in which mean T-O was compared following low and high dose ASA, high dose ASA produced significantly longer mean T-O in each. Vessel wall prostacyclin activity was abolished by all doses of ASA used in this study.

D. Conclusion and Recommendations. These results suggest an in vivo antithrombotic dose dependency of ASA not apparent from in vitro platelet function tests. This occurs despite inhibition by ASA of prostacyclin activity from the vessel wall. These studies raise concern about the importance of vessel wall prostacyclin in preventing thrombosis. Furthermore on the clinical level, high doses of aspirin may be more antithrombotic than lower doses. Clinical trials will be necessary to test this hypothesis.

These findings were presented at the VIIth International Congress on Thrombosis and Hemostasis in July and will be presented on the Surgical Forum of the American College of Surgeons in October.

III. The Participation of Platelets in Wound Healing

A. Background and Statement of the Problem. When traumatic injuries or surgical wounding occurs platelets aggregate at the ends of severed vessels to staunch bleeding. In performing this hemostatic function, platelets undergo the release reaction whereby they secrete a wide variety of substances. Among them are included serotonin, platelet factor 4 (or antiheparin factor), and platelet mitogenic factor. These substances may be important in wound healing. Serotonin stimulates fibroblastic growth and augments scar formation when injected subcutaneously into experimental animals. Platelet factor 4 has been shown to inhibit collagenase and therefore may aid wound repair. Platelet mitogenic factor is a potent stimulator of fibroblastic and smooth muscle cell growth in in vitro cell culture systems. These substances, then, released by platelets during tissue injury and hemostasis might be important in modulating wound repair and healing.

B. Experimental Approach. Sheep were immunized against rabbit platelets and anti-rabbit platelet serum (APS) was harvested. Normal sheep serum (NSS) was collected from non-immunized sheep. Two groups of rabbits were studied. In the experimental group the animals were rendered thrombocytopenic by injecting APS; the control group was injected with NSS and maintained normal platelet counts. Following the first day injections, all animals underwent surgery to produce a standard dorsal cutaneous wound 20 cm in length which was sutured with interrupted nylon sutures 1 cm apart. The injections in both groups of animals were continued for 5-7 days.

At intervals, 10 and 20 days after wounding, all animals were anesthetized and 1 x 4 cm wound strips were harvested. The wound tensile strength was then measured by a tensiometer. Samples of the wound were also fixed in formalin for histologic analysis.

C. Results and Discussion. These studies are incomplete and still in progress. Preliminary analysis of the data demonstrates the following. In experimental animals administered APS, platelet counts immediately dropped from 300,000 - 500,000/µl to 5,000 - 10,000/µl. These animals remained severely thrombocytopenic throughout the period they were administered the APS (5-7 days). Control animals, given NSS, maintained normal platelet counts. Wound tensile strength was significantly less ($p<.001$) at 20 days in the experimental group. There was no difference at 10 days. Histologic analysis has not, as yet, been performed on the wound samples.

D. Conclusion and Recommendations. Platelets have an important role in normal wound healing. In the presence of severe thrombocytopenia wound healing is impaired. Further studies are necessary to determine exact mechanisms whereby platelets enhance wound healing.

Project 3A161101A91C IN HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 101 Thrombotic Mechanisms in Stress

[illegible] ted.

Publications:

1. Clagett, G.P., Russo, M., Hufnagel, H.: Platelet changes th in vivo exposure to prosthetic arterial surfaces. Surg Forum [illegible]26, 1978.

2. Clagett, G.P., Russo, M., Hufnagel, H.: Influence of prosthetic arterial surfaces on platelets. Circulation 58:II-225, 1978.

3. Clagett, G.P., Russo, M., Hufnagel, H.: Aspirin dosage and antithrombotic effect. Thromb Haemostas 42:60, 1979.

4. Clagett, G.P., Russo, M., Hufnagel, H.: Platelet-serotonin dynamics in animals with prosthetic arterial grafts. Thromb Haemostas 42:73, 1979.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6464	79 10 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	102
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Include with Security Classification Code)

(U) Chemotherapy and Chemoprophylaxis of African Trypanosomiasis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 10	Cont.	DA	C. In-House

17. CONTRACT GRANT

a. DATES/EFFECTIVE
b. NUMBER
c. TYPE
d. KIND OF AWARD
EXPIRATION
e. AMOUNT:
f. CUM. AMT

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 79	0.8	84
CURRENT FISCAL YEAR 80	0.8	95

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME: Childs, G.E., CPT
NAME: Mc Cormick, G.J.

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Trypanosomiasis; (U) Drug Development; (U) Biology; (U) Chemistry; (U) Toxicology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To find new drugs with chemoprophylactic or chemotherapeutic activity against African trypanosomiasis, a debilitating and frequently fatal disease which would pose a serious threat to military personnel operating in Central Africa. Currently available drugs are neither safe to use nor fully effective in therapy. There are no adequate drugs for prophylaxis.

24. (U) Laboratory models developed in this laboratory will be utilized to test selected chemical compounds from the malaria drug inventory for activity against Trypanosoma rhodesiense: (1) in vitro, (2) in laboratory mice, (3) in cynomolgus monkeys.

25. (U) 78 10 - 79 09. The development of the in vitro test system fo screening drugs has been completed and the system has been utilized for testing over 700 compounds. Correlation studies comparing in vitro results to those obtained in vivo are being conducted to ascertain which compounds or classes of compounds have activity per se or as metabolites. By contract, studies of in vivo activity have been done with 3,500 compounds and strains of T. rhodesiense have been developed which are resistant to several established drugs (berenil, suramin, melarsoprol, stilbamidine, and pentamidine) and are being used in studies of cross-resistance. In addition, a screen against T. cruzi has been established and is being evaluated with selected drugs. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. OD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 66 (FOR ARMY USE) ARE OBSOLETE

PROJECT 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 102 Chemotherapy and Chemoprophylaxis of African Trypanosomiasis

Investigators
Principal: COL David E. Davidson, Jr., VC
Associate: Gerald J. McCormick, Ph.D.
CPT George E. Childs, MSC

1. Description

African trypanosomiasis is a parasitic disease which poses a potentially serious threat to U.S. military personnel who may be required to operate in the large areas of Africa in which this disease is endemic. All drugs which are currently available for use against this disease are less than fully effective and/or have serious side effects. Efforts to develop drugs effective against this disease include screening and evaluation of candidate chemotherapeutic and chemoprophylactic agents by *in vivo* and *in vitro* techniques and elucidation of metabolic processes of the trypanosomes by laboratory investigation.

In-house research is supported by and coordinated with contract research monitored within the Department of Parasitology, Division of Experimental Therapeutics, WRAIR.

2. Progress

a. Primary Mouse Screen

The primary *in vivo* screen in mice utilizes the Wellcome CT strain of *Trypanosoma rhodesiense*. Compounds are administered subcutaneously to mice which have received a lethal dose of parasites (intraperitoneal injection of 0.5 ml of a 1:50,000 dilution of heparinized blood from heavily infected donor mice). Active compounds extend survival time beyond that of untreated infected control mice (4.5 $\pm$ 0.3 days). Treated mice surviving 30 days are considered to be cured.

Since 1972, approximately 17,000 compounds have been screened and of these approximately 500 have had reportable activity. In the past year more than 3000 compounds were tested and activity was exhibited by 105 compounds.

b. Drug Resistant Strains of Trypanosoma rhodesiense

Strains of T. rhodesiense which had been made resistant to berenil, suramin, melarsoprol, stilbamidine and pentamidine by increasing drug pressure during serial infections in mice were employed in studies of cross-resistance to these drugs and in studies of activities of various chemical classes of compounds. The relative resistances of the developed strains in comparison to the doses active against the sensitive strain (at least three cured mice of five infected) are:

berenil	>	260-fold resistance
suramin	>	64-fold resistance
melarsoprol	>	64-fold resistance
stilbamidine	>	16-fold resistance
pentamidine	>	128-fold resistance

Cross-resistance studies revealed that the berenil-resistant strain was >16-fold resistant to stilbamidine and >64-fold resistant to pentamidine. The stilbamidine-resistant strain was >64-fold resistant to pentamidine and >260-fold resistant to berenil. The pentamidine-resistant strain was >16-fold resistant to stilbamidine and >260-fold resistant to berenil. The suramin-resistant strain was 8-fold resistant to melarsoprol and the melarsoprol-resistant strain was 4-fold resistant to suramin.

Testing of selected compounds representative of various chemical classes of compounds for resistance of the melarsoprol- and suramin-resistant strains is in progress. Evidence of cross-resistance (at 4-fold or greater) of both strains has been obtained with several terephthalanilides and diamidines, a nitroimidazole and an arsenical compound. More precise determinations for these and other compounds are pending, as is testing in the other resistant strains.

c. In Vitro Antitrypanosomal Drug Screening System

The development of an in vitro system for the evaluation of potential antitrypanosomal drugs has been completed. The system is being used for routine screening and for investigations of activity of specific classes of compounds. Blood forms of T. rhodesiense, Wellcome strain, are isolated from rat blood by ion exchange chromatography and cultured in RPMI media supplemented with 30% horse serum. Parasites are incubated in

microtiter plates with serially diluted drugs and 3H-thymidine and 14C-leucine. After three hours, cells are collected on filter paper and washed. Residual activity of each radioisotope is assayed in a dual channel scintillation counter and the data analyzed using log-linear regression to obtain the ID-50 (the concentration corresponding to the 50% inhibition of the uptake of isotope). A compound is considered active if the ID-50 for either label is less than 12.5 µg/ml. A report describing the test system has been submitted for publication (1).

During the past year, approximately 500 compounds were tested and of these more than 275 were active. However, many were compounds which had been previously shown to be active in the in vivo mouse screen or were analogs of an active series of compounds.

Studies of several series of compounds have been completed or are currently being conducted. These compounds are standard antitrypanosomal drugs, aromatic diamidines, 2-acetylpyridine thiosemicarbazones, silver sulfonamides, and carbamodithioates.

A series of standard antitrypanosomal drugs (Table 1) were tested to relate the relative inhibition of the uptake of the radiolabelled precursors of nucleic acid and protein synthesis to probable modes of action of these compounds. Trivalent arsenicals are considered to react with sulfhydryl groups of proteins. The relatively greater inhibition of 14C-leucine uptake as compared to 3H-thymidine uptake is consistent with this hypothesis. The activity of tryparsamide in vitro supports evidence that pentavalent arsenicals exert their antitrypanosomal activity in vivo after reduction to a trivalent state. In contrast, the mode of action of the aromatic diamidines is related primarily to inhibition of nucleic acid synthesis and accordingly, stilbamidine, hydroxystilbamidine, and pentamidine showed greater inhibition of 3H-thymidine uptake than of 14C-leucine uptake. The inactivity of suramin may be due to either the relatively short incubation time of three hours or to the possibility that an in vivo metabolite is the active form.

In order to compare the relative activities in the in vitro screen to results of the in vivo mouse screen, the ID-50s of 25 aromatic diamidines as determined by both 3H-thymidine and 14C-leucine uptake inhibition, were correlated by rank order coefficients to the in vivo ED-50 as computed by log-probit analysis of results of tests of these drugs in the mouse in vivo screening system. There was a significantly high correlation

between in vivo activity and the inhibition of the in vitro uptake of 3H-thymidine ($0.02<\alpha<0.01$) and 14C-leucine ($0.01<\alpha<0.005$). These results indicate that the in vitro screen can rapidly and accurately predict relative in vivo activities of members of a closely related series of drugs.

Twenty-three analogs of 2-acetylpyridine thiocarbazone were assayed in the in vitro screen to relate variations in structural substituents to corresponding changes in antitrypanosomal activity. The analogs generally showed a greater inhibition of the uptake of 14C-leucine than the uptake of 3H-thymidine suggesting that the mode of active may be associated with protein synthesis.

Silver sulfadiazine, silver metachlorodine and silver sulfameter were found to have antitrypanosomal activity in the in vitro screening system. Silver sulfalene, silver sulfisamidine, silver sulfadoxine, zinc sulfadiazine, and aluminum sulfadiazine were inactive. Two additional polymeric forms of silver sulfadiazine were assayed; one showed antitrypanosomal activity. Disulfuram, a marketed drug used in the management of chronic alcoholism, was found to be highly antitrypanosomal in the in vitro screen.

The experiments conducted during the past year indicate that the in vitro antitrypanosomal drug screening system may be effectively employed for several areas of research. These include: 1) routine screening of drugs to identify new classes of compounds with antitrypanosomal activity; 2) providing evidence of possible modes of action of active compounds; 3) ranking in vivo activity in a series of closely related drugs based on results of in vitro testing; 4) providing a rapid assessment of the effects of structural changes on corresponding changes in activity; and 5) assaying metabolites of active parent compounds.

Table 1. In vitro activity of standard antitrypanosomal drugs as the concentration causing 50% suppression (ID-50) of the uptake of 3H-thymidine and 14C-leucine.

Drug	Isotope	
	3H-Thymidine (µg/ml)	14C-Leucine (µg/ml)
Arsenicals		
Melarsonyl (Mel W)	2.93	0.47
Melarsoprol (Mel B)	1.53	0.01
Tryparsamide	>12.5	>12.5
Diamidines		
Stilbamidine	2.27	2.97
Hydroxystilbamidine	1.62	2.59
Pentamidine	0.78	1.04
Complex polyanion		
Suramin	>12.5	>12.5

PROJECT 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 102 Chemotherapy and Chemoprophylaxis of African Trypanosomiasis

Literature Cited.

Publications

In Press:

1. Desjardins, R.E., Casero, R.A., Jr., Willet, G.P., Childs, G.E., and Canfield, C.J.: Trypanosoma rhodesiense: A Semiautomated Microtest System for Quantitative Assessment of Antitrypanosomal Activity In Vitro. Experimental Parasitology (submitted for publication).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
D OC 6466	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP R INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	103
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Infections of Cultured Intestines by Pathogenic Microbes

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 10	Cont	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION

b. NUMBER

c. TYPE NA

d. AMOUNT:

e. KIND OF AWARD

f. CUM AMT

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	1.5	30
FISCAL YEAR CURRENT 80	1.5	35

19. RESPONSIBLE DOD ORGANIZATION

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NAME

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U)Organ Culture;(U)Intestine;(U)Phase Microscopy;(U)Electron Microscopy

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To clarify interactions between cultured gut mucosa and pathogenic microbes including bacteria, viruses, parasites and microbial toxins. The results will provide new information which should clarify the pathogenesis of acute diarrheal diseases in military personnel.

24(U) Conventional morphologic techniques including phase contrast, light and electron microscopy, histochemistry, immunochemical, biochemical, and isotope tracer methods are being used. Methods such as interference microscopy and cinematography will also be utilized.

25(U) 78 10 - 79 09 Fetal mouse intestinal explants have been cultured in tricine buffered synthetic medium at 36 C in 5 percent carbon dioxide - 95 percent air. The function and structure of the mucosal epithelium was maintained for up to 20 days. The synthesis of protein, glycoprotein, and DNA were continuously observed in the intestinal explants in vitro. A steady level of cAMP concentration has been identified and activities of acid and alkaline phosphatases have been also observed in the cultured fetal intestine. The viability of the tissue was measured by light and electron microscopy. Another organ culture system for the adult intestine was developed. Utilizing a conditional medium and gas chamber, the adult mucosal explants were cultured for 20 days at 30 C. Current efforts are directed toward the replication of enteric viruses, and attachment and damage to intestinal mucosa by pathogenic bacteria in an organ culture system. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78 - 30 Sept 79.

*Available to contractors upon originator's approval

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Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 103 Infections of Cultured Intestines by Pathogenic Microbes

Investigators:
Principal: Akio Takeuchi, M. D.
Associate: Han Y. Cho, Ph.D.

Description

To develop a reliable and reproducible method for the organ culture of the small and large intestine of experimental animals and man. The cultured intestine will be employed in: (1) Studies of the response of the gut mucosa and submucosa to various enteric microbes and microbe-derived toxins; special attention will be paid to cinematographic recordings of as well as conventional static observations. (2) Studies of replication sites of certain enteroviruses in the cultured intestinal explants.

These studies should provide valuable new information which will clarify as yet unsolved problems in pathogenesis of acute infectious diarrheal diseases common in military personnel.

Problem and Progress

Utilizing a modified culture chamber and improved culture medium, we have succeeded in culturing fetal small and large intestine of mice for three weeks. We are completing manuscripts which describe (1) our method of the organ culture of intestines; (2) physico-chemical characteristics of growing fetal intestines. Recently we were able to culture the large intestine of adult mice for two weeks for the first time. Studies on the development of the immune system in growing fetal intestines have been initiated.

Results

The summary of current results is as follows:

1. Fetal mouse small and large intestines have been cultured in an improved culture medium in a new chamber for three weeks.

2. The viability of the intestinal explants was documented by physico-chemical and ultrastructural studies.
3. The phase-contrast microscope observation on growing intestines showed rhythmical movements and the formation of villi.
4. The development of the mid-gut to the well differentiated intestinal epithelium in the chamber has been determined by the transmission electron microscopy.
5. The intestinal explants synthesized and accumulated DNA, protein, and glycoprotein almost at a linear rate for at least 7 days.
6. De novo protein synthesis was inhibited by an inhibitor of protein synthesis, cycloheximide.
7. Cyclic AMP, acid and alkaline phosphastase have been identified in the cultured intestines.

Project 3A161101A91c IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 103 Infections of Cultured Intestines by Pathogenic Microbes

Presentations

1. Cho, H. Y. and Takeuchi, A.: Organ culture of the fetal mouse intestine. Presented at NATO International Congress, Monte Carlo, Monaco, Sept. 1979.

Publication

Cho, H. Y. and Takeuchi, A.: Organ culture of the fetal mouse intestine. In Vitro 15: 194, 1979.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6468	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DOD'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	106
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

mputer simulation of red blood cell metabolism

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE NA EXPIRATION

b. NUMBER

c. TYPE d. AMOUNT

e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	2	100
FISCAL YEAR FUNDING 80	2	100

19. RESPONSIBLE DOD ORGANIZATION

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NAME: LTC June M. Whaun, M.D. DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Malaria; (U) Metabolism; (U) Computer Simulation; (U) Blood Preservation

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) To develop a computer simulation model of intermediary metabolism in the erythrocyte, based on data both in existence and to be obtained in our laboratories. By constructing separate computer models of the metabolism of uninfected erythrocytes, malaria-infected erythrocytes, and free malaria parasites, we can gain a better understanding of how the parasite utilizes the metabolic machinery of the host erythrocyte. Malaria is a significant problem for soldiers stationed in many areas of the world.
24.(U) Laboratory work and computer model building will proceed simultaneously. Laboratory studies will include measurement of (1) intermediates and enzyme level of glycolysis, the pentose cycle, the tricarboxylic acid cycle, and fatty acid synthesis; and (2) intermediates and enzyme levels of the purine salvage pathways. Computer models will be developed using a simulation language which permits the investigator to describe his model as a series of biochemical reactions with specified rate constants and initial substrate concentrations. The simulation program then sets up and solves a series of simultaneous differential equations describing the rates of change of concentration of each substrate in terms of rate constants and of concentration of other substrates.
25.(U) 78 10 - 79 09. Laboratory procedures have been established for measurement of intermediates of purine metabolism and glycolysis, for assay of each of the enzymes involved in these two areas of metabolism, and for identification of host and parasite isotypes of specific enzymes. A collaborative arrangement has been initiated with Dr. M.J. Haut and Dr. David Garfinkel, an internationally recognized expert in simulation of intermediary metabolism. A data base has been accumulated for each of these two metabolic areas. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

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PROJECT 3A161101A91C IN HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 106 Computer Simulation of Red Cell Metabolism

Investigators

Principal: LTC Michael J. Haut, MC

Associate: Mr. John R. Prichard, GS-11; CPT H. Kyle Webster, MSC; LTC June Whaun, MC; David Garfinkel, Ph.D. (Professor of Computer Information Science, Moore School of Electrical Engineering, University of Pennsylvania); Michael Kohn, Ph.D. (Moore School of Electrical Engineering, University Pennsylvania); Jack London, Ph.D. (Assistant Professor of Radiology, University of Pennsylvania); Zelda B. Rose, Ph.D. (Associate Member, Institute for Cancer Research, Fox Chase); Irwin A. Rose, Ph.D. (Senior Member, Institute for Cancer Research, Fox Chase)

Description

The basic goal of this work unit is to develop a computer simulation model of intermediary metabolism in the erythrocyte, based on data both in existence and to be obtained in our laboratories. By constructing separate computer models of the metabolism of uninfected erythrocytes, malaria-infected erythrocytes and free malaria parasites, we can gain a better understanding of how the malaria parasite utilizes the metabolic machinery of the host erythrocyte. Two important practical goals this may help us attain are: (a) newer methods of metabolic attack on the malarial parasite, and (b) improved media for culture of the parasites in vitro.

A detailed description of the simultation methodology, as well as much of the experimental approach and laboratory methods, was given in last year's annual report. Therefore, the experimental approach and laboratory methodology will be discussed relatively briefly in this year's report.

A. Computer Simulation

The simulation language of Garfinkel (1,2) is the one used to formulate the models. This simulation language permits the investigator to describe his model as a series of biochemical reactions. Input for the model consists of: (1) a series of reactions which define the system being simulated (e.g., glycolysis in the Ehrlich ascites tumor cell; mitochondrial metabolism in the hypoxic heart); (2) rate constants for both the forward and (where applicable) for the reverse reaction; and (3) initial concentrations for each chemical compound in the system. The simulation program first sets up a series of simultaneous differential equations describing the rate of change of concentration of each substrate in terms of rate constants and of concentrations of other substrates. It then solves the series of simultaneous differential equations and plots the concentration of each substrate as a function of time. At selected times, the computer also prints out first and second derivatives of each concentration, to indicate the rate of change of that particular concentration.

The data base for initial construction of the computer model consists of: (1) literature and experimentally determined values for kinetic constants of each enzyme involved in red cell intermediary metabolism, and (2) literature and experimentally determined values for substrate concentrations in the erythrocyte, both in the steady state and after various types of perturbation. From this data, and from previous simulation studies by Garfinkel, et al., both of individual enzymes (3-5) and of entire metabolic pathways in specific tissues, such as Ehrlich ascites tumor cells (1,6), the perfused rat heart (7,8), the lactating rat mammary gland slice (9), and the perfused rat liver (10-12), a model in which each enzymatic reaction is described in detail will be constructed.

The model will then be refined by altering it so that it conforms to isotopic data as well. This will be done using the approach of Garfinkel in simulating use of radioactive tracers in brain metabolism (13,14). Matching models approximately describing the behavior of the biochemical system containing unlabeled compounds and the system with appropriate labeled inputs are constructed, and their behavior is compared with available experimental data. As the "unlabeled" model is usually much smaller than the "labeled" models, most of the manipulation is carried out with it, and the resulting kinetic parameters are repeatedly modified until the entire set of models closely approximates as much of the available experimental data as possible. Equations for the "labeled" models will be written by using a program that determines the labeling pattern of all metabolites after addition of a specifically labeled substrate to a specific unlabeled system (15).

Validity testing of the completed model will include examination of its ability (1) to conform to or explain data in the literature which were not used in construction of the model, and (2) to predict the results of experiments done in our laboratory or in other laboratories, in which the steady state metabolism of the erythrocyte is perturbed in a particular manner and metabolic changes are followed both with tracer studies and by measuring changes in levels of several key substrates.

B. Experimental Design and Laboratory Methodology

Malaria studies using the computer simulation model are focusing on two experimental systems - P. knowlesi infection in rhesus monkeys, and P. falciparum grown in vitro in human erythrocytes.

Our department is currently involved in a study of metabolic alterations in erythrocytes of rhesus monkeys during the course of a synchronous P. knowlesi infection. In this study (WRAIR protocol # 012-74), purine nucleotides, glycolytic intermediates, purine salvage pathway enzymes, and glycolytic enzymes are all measured in samples taken at 4 hour intervals during two or three 24 hour cycles of patent parasitemia.

Similarly, we are intensively investigating purine and pyrimidine metabolism in P. falciparum grown in vitro in human erythrocytes.

In both the in vivo and in vitro systems, our basic approach is to examine a specific aspect of metabolism in the uninfected erythrocyte, the infected erythrocyte, and the isolated parasite. Parasites are isolated by saponin lysis after the erythrocytes have been freed of contaminating leukocytes and platelets. As has been noted in a recent review (16), no current

method gives free parasites entirely uncontaminated by erythrocyte membrane fragments. Saponin lysis has the advantages of ease of performance, reproducibility of results, and contamination only by small pieces of attached red cell membrane. To be certain that our data for the free parasite are parasite-specific, we check the preparations for G6PD (which should be absent) and for pyruvate kinase (which should be present in high concentration as parasite-specific enzyme).

Purine and pyrimidine nucleotide, nucleoside, and nucleobase concentrations are determined using high performance liquid chromatography. Activities of enzymes of purine salvage and interconversion are measured as described by Snyder et. al (17); overall de novo purine synthesis is measured as described by Hershfield and Seegmiller (18).

Glycolytic enzyme activities will be determined spectrophotometrically as described by Beutler (19). Glycolytic intermediates will be determined fluorometrically as described by Minakami (20-22). $^{14}CO_2$ production will be determined by a modification of the method described by Yunis (23).

Progress

A. General

During the past year, work on this project became considerably more focused. Specifically, it was decided that this project's usefulness would be optimized by focusing on simulation of metabolic interrelationships between host erythrocytes and malaria parasites infecting these red cells. The major effort has therefore been directed toward examination of purine and pyrimidine metabolism in the uninfected erythrocyte, the malaria-infected erythrocyte, and the isolated parasite. A secondary effort was directed toward examination of glucose metabolism in the same three systems.

Because the quality of a computer model is highly dependent on the quality of the data on which it is based and the quality of the data used to test its predictive capacity, the major part of our effort this past year has been devoted to: (1) establishment of several systems for obtaining parsitized erythrocytes; (2) development of methods for biochemical examination of infected red cells and of isolated parasites, and (3) experimentally addressing specific questions of potential clinical importance or of importance to our understanding of the parasite's biology.

Work on construction of the computer models themselves has progressed concurrently. In particular, an extensive analysis of kinetic data available in the literature for each of the enzymes involved in red cell metabolism of glucose, purines, and pyrimidines has been performed. In this analysis, particular attention was paid to the proportion and kinetic properties of each of the isoenzyme forms of these enzymes, and to the occurrence and characteristics of parasite-specific isoenzymes. Our review of available data on glucose metabolizing enzymes in erthrocytes was particularly extensive, and indeed formed the basis for two major tables in recent textbook chapters written by the principal investigator.

Construction of the actual computer models is now beginning. Dr. Garfinkel has a grant pending with NIH for support of his part of the collaborative effort (a major part of the simulation), and plans to devote 30 per cent of his time for at least three years to this collaborative effort.

B. Specific Accomplishments

1) Methods development

With the help of LTC J.D. Haynes, LTC Whaun was able to establish an in vitro P. falciparum culture system in her laboratory. Using the methodology described by Trager and Jensen (24) and Haynes et al. (25), she was able to maintain the FCR_3 (Rockefeller drug-sensitive) strain of P. falciparum in continuous culture for more than 7 months. These cultures are now being used routinely by LTC Whaun and CPT Webster for examining uptake and interconversion of purines by infected erythrocytes.

Particular attention was paid during the past year to refining our procedures for obtaining parasitized erythrocytes free of contaminating leukocytes or platelets, and for isolating parasites from infected cells. Both of these problems were addressed in detail at a recent symposium (16, 26). We are currently able to obtain erythrocyte preparations virtually free of contaminating leukocytes or platelets; we have demonstrated the absence of the latter cells by both morphologic and biochemical means.

Considerable effort has been expended to establish methodology in our laboratory for separation of isoenzymes of the various enzymes of glucose and nucleotide metabolism. Electrophoretic separation of isoenzymes of most of the important glucose metabolizing enzymes and of some of the purine salvage enzymes have been established. We have found that addition of inhibitors to starch gel electrophoresis of lysates of infected red cells before staining permits us to demonstrate differential sensitivity of host and parasite iosenzymes of a given enzyme to various inhibitors.

We have extended our capability in enzyme activity determination, paying particular attention to nucleotide metabolism. We have developed and standardized methodology for examining the de novo pathway of purine biosynthesis. We now have the capability to examine both overall activity of the de novo pathway and the activity of particular enzymes, such as adenylosuccinate synthetase and adenylosuccinate lyase.

Methods have been established for high performance liquid chromatography (HPLC) analysis of purine and pyrimidine nucleotides, nucleosides, and nucleobases, using anion-exchange and reverse phase approaches. These methods are currently in very active use in our malaria studies (see below). Work is currently underway directed at development of a single-column method for separation of nucleotides, nucleosides, and nucleobases, employing a ternary gradient system and stop-scan spectroscopic analysis. Using a flow through radioactivity detector in addition to flow through UV-visible absorbance detectors, we have established methodology for examining in the same aliquot of extract from a tissue incubated with labeled substrate, both the concentration and the specific activity of all the nucleotides, nucleosides, and bases.

2) Examination of specific questions

As a result of our focused efforts on one area of metabolism, some significant information about purine metabolism in malaria-infected erythrocytes has been obtained. The two major efforts in this area have been (a) an investigation of blood purine and pyrimidine nucleotides during synchronous

malaria infection of rhesus monkeys, and (b) examination of the enzyme of the purine salvage pathway in malaria-infected erythrocytes.

a) Blood purine and pyrimidine nucleotides during synchronous malaria infection of rhesus monkeys

In order to examine all the major ribonucleotides during the course of the IE infection cycle in vivo, we employed rhesus monkeys infected with P. knowlesi, an intrinsically synchronous parasite in this host. By combining a rigidly controlled, sequentially sampled, animal model with anion-exchange high performance liquid chromatography (HPLC) of acid extracts of infected erythrocytes, it was possible to define characteristic blood nucleotide profiles over both the IE growth phase and successive IE cycles.

Adult male rhesus monkeys (6-7 kg) were surgically fitted with an indwelling venous catheter (femoral vein) kept patent by daily heparin flushes. Catheterized monkeys were placed in specially designed restraining chairs (27), housed individually in environmentally controlled modules, and permitted to adapt to these conditions over a 10-14 day period. Following adaptation, samples were collected to establish basal hematological and nucleotide metabolite values. Infections were produced by intravenous injection of cryopreserved parasitized erythrocytes (pool stabilates from a common donor monkey). A 3 day prepatent interval was observed followed by a patent infection with 2-3 synchronous quotidian cycles before the animal become terminal or was treated. Infections were monitored by microscopic examination of Leishman stained peripheral blood smears. Once a patent infection was observed sequential (serial) samples were obtained over 2-3 synchronous IE infection cycles. Blood samples for nucleotide analysis were processed immediately upon collection (1 ml whole blood in 2 ml 0.6N perchloric acid). Following acid extraction the neutralized supernatant was stored at -70^{o}C until analysis.

Nucleotide analysis was by anion-exchange HPLC using a modified technique (28) for gradient elution of purine and pyrimidine components. Chromatographic peaks were identified by retention time and absorbance ratio (280/254 nm) based on comparison to nucleotide standards of known purity. Quantitation was by an external standard method using a computing integrator on-line with the HPLC system.

Basal values for blood purine and pyrimidine nucleotides were obtained in a group of rhesus monkeys that were adapted to chair-restraint and catheterization, and had no previous history of malaria infection. Adenine nucleotides, particularly ATP, represented the predominant base class of nucleotides. Basal nucleotides were characterized in order of concentration from highest to lowest as follows: ATP>>ADP>GTP>UDP≈AMP> GDP≈CMP>IMP. Significant levels of GMP and UMP were not detected in basal blood samples. The basal composition of blood nucleotides in rhesus monkeys was noted to be similar to those reported in humans--AMP (0.02), ADP (0.22), ATP (1.35), GDP (0.02), GTP (0.06), IMP (0.03), values in μmoles/ml RBC (29).

Adenylate energy charge (AEC) values were calculated according to the relationship (ATP) + 0.5 (ADP)/{(AMP)+(ADP)+(ATP)} (30). The basal AEC for rhesus monkeys had a mean value of 0.90; an optimal AEC of about 0.85 has been found characteristic of a large number of organisms (31).

Content of major blood purine and pyrimidine nucleotides was determined on a group of rhesus monkeys infected with P. knowlesi. Sample points were selected to represent three phases of the parasites' growth during the IE infection cycle. Samples taken between hours 5-7 of the IE cycle represent what were termed mature "ring" forms (MR). Trophozoites (Tz) were obtained during hours 10-18 of the IE growth period. Samples referred to as post-lysis (P-L) were obtained during the two hours following rupture of schizont infected erythrocytes at the end of a given IE cycle.

The nucleotide profile of the mature "ring" (MR) phase (hours 5-7 of the intraerythrocytic cycle) is similar to that of the basal state, but differs from those of both the trophozoite and post-lysis phases. The basal and MR stages showed significantly higher ATP levels ($P<0.02$, Students "t" test) than the trophozoite or post-lysis phases of the intraerythrocytic cycle. Similar changes were observed for UDP ($P<0.05$) and GTP ($P<0.01$, in three of four monkeys). This trend--increased nucleotide levels associated with mature "ring" stage and reduced levels at trophozoite stage and post-lysis--was observed to repeat itself each day over successive IE cycles. This cycling over the IE growth period was shown most dramatically with ATP. The AEC paralleled the variation in ATP.

Nucleotide changes during the pre-patent interval were also observed. There was a moderate though significant decrease in ATP during the pre-patent period (1.21 ± 0.06 µmoles/ml RBC compared to 1.43 ± 0.03 µmoles/ml RBC, $P<0.05$ with $\bar{n} = 3$). GTP content was increased ($0.1\bar{1} \pm 0.05$ µmoles/ml RBC versus 0.07 ± 0.007 µmoles/ml RBC, basal) as was CMP ($\bar{0}.07 \pm 0.03$ µmoles/ml RBC compared to 0.03 ± 0.002 µmoles/ml RBC, basal).

At high parasitemias (>30% parasitized red blood cells PRBC often characterized by loss of parasite synchrony) the content of all nucleotides increased except for UDP. Only trace amounts of UDP were found at high parasitemias in monkeys suffering fatal infections. Chromatograms from these terminal animals with reduced UDP revealed a number of new peaks tentatively identified (using sample "spikes" with known standard) as UDP-sugars.

This study showed for the first time quantitative profiles for blood purine and pyrimidine ribonucleotides during the course of experimental malaria in the rhesus monkey infected by P. knowlesi. Changes in nucleotide profile were found to be characteristic for three phases of the IE infection cycle. During mature "ring" stage growth there was a build-up in nucleotide metabolites and energy rich compounds (high AEC) that were subsequently utilized over the ensuing IE growth period. The increased nucleotide content (especially ATP) was interpreted as critical for mature "ring" stage growth and the continued cycling of schizongonic growth stages. Studies of nucleic acid synthesis in P. knowlesi (32-35) suggests that the mature "ring" stage is associated with an intense utilization of nucleotide precursors which continues into the trophozoite growth phase. The parasite induced cycling of levels of purine nucleotides, specifically ATP, may account for loss of erythrocyte integrity within the infected host cell population, resulting in the generalized hemolysis which characterizes the end of a schizogonic cycle.

b) Examination of the enzymes of the purine salvage pathway in malaria-infected erythrocytes

Purine salvage enzyme levels were determined in lysates of erythrocytes infected with either the Camp strain or the FCR-3 strain of P. falciparum. In both strains, only adenosine deaminase levels were significantly increased in parasite cultures when compared to control cultures of uninfected erythrocytes. Levels of APRT, HGPRT, AK, and PNP did not rise significantly in infected erythrocytes. Since only 5-10% of the erythrocytes were parasitized, the 2-3 fold increase in ADA specific activity actually represents a 17-20 fold increase in the cellular ADA of parasitized erythrocytes. The ADA activity increased linearly with time in culture, which in turn was correlated with an increase in the number of parasitized cells. Other purine salvage enzymes measured did not increase significantly under the conditions employed.

When extracts of infected cultures were subjected to starch gel electrophoresis, an additional band of ADA activity with increased anodal mobility relative to the erythrocyte isozymes appeared. The new band showed an increase in staining intensity as parasite number increased, suggesting the additional band was due to production of a parasite adenosine deaminase. This hypothesis was confirmed by releasing the parasite from the host erythrocyte with saponin, and separating the resultant free parasites from the erythrocyte cytoplasm. Starch gel electrophoresis of extracts prepared from the free parasite fraction gave only the new enzyme, while the recovered erythrocyte cytoplasm gave predominantly the erythrocyte isozymes. Taken together, the data indicate that P. falciparum produces unusually high levels of a parasite specific adenosine deaminase.

Because P. falciparum contained such a high level of adenosine deaminase activity, it was of interest to examine the effect of ADA inhibition on parasite growth in culture. 2'-deoxycoformycin, a tight-binding ADA inhibitor, completely inhibited ADA activity in infected cultures, but did not affect parasite growth over a 48 hour period. However, our in vitro culture medium (RPMI 1640) does not contain purines, and thus does not expose the cultured erythrocytes to the continuous influx of nucleosides that occurs in vivo. Therefore, we investigated the effects of ADA substrates adenosine and deoxyadenosine on parasite growth when ADA is inhibited. Under these conditions, micromolar amounts of both compounds inhibited parasite growth, with deoxyadenosine more effective than adenosine. Neither compound inhibited parasite growth in the absence of the ADA inhibitor in this concentration range.

We tested two powerful ADA inhibitors, deoxycoformycin and erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) to determine if differential inhibition of host and parasite could be achieved. ADA from human erythrocytes and from P. falciparum obtained from infected chimpanzees was partially purified using modifications of the method of Osborne and Spencer(36), and then incubated in vitro with the inhibitors. The erythrocyte and parasite enzymes exhibited a remarkable difference with regard to inhibitor specificity. EHNA, which gives 95% inhibition of erythrocyte adenosine deaminase at a concentration of 3.6×10^{-6} M had no effect on the parasite enzyme at concentrations as high as 3.6×10^{-4} M. These results were confirmed for parasites grown in human erythrocytes in vitro by demonstrating that EHNA prevented staining of the host isozymes on starch gel but did not affect the parsite enzyme band (data not shown). Deoxycoformycin was effective against both enzymes, but was a slightly better inhibitor of the parasite enzyme. The difference in the interaction of the two enzymes with EHNA suggests a profound difference in the active sites of the two enzymes.

Work Unit 106 Computer Simulation of Red Cell Metabolism

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. Agency Accession	2. Date of Summary	Report Control Symbol
DA OC 6474	79 10 01	DD DR&E(AR)636

3. Date Prev Sum'ry	4. Kind of Summary	5. Summary Scty	6. Work Security	7. Regrading	8. Dissem Instr'n	9. Specific Data-Contractor Access	10. Level of Sum
78 10 01	D. Change	U	U	NA	NL	☒ Yes ☐ No	A. Work Unit

10. No./Codes	Program Element	Project Number	Task Area Number	Work Unit Number
a. Primary	61101A	BA161101A91C	00	107
b. Contributing				
c. Contributing				

11. TITLE (Precede with Security Classification Code)
(U) Neural and Behavioral Response to Sensory Stimulation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
013400 Psychology 012900 Physiology 016200 Stress Physiology

13. Start Date	14. Estimated Completion Date	15. Funding Agency	16. Performance Method
78 10	CONT	DA	C. In-House

17. CONTRACT/GRANT
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18. Resources Estimate		a. Professional Man Yrs	b. Funds (In Thousands)
Fiscal Year	Preceding 79	1.0	50
	Current 80	1.0	60

19. RESPONSIBLE DOD ORGANIZATION
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Foreign Intelligence Not Considered

22. KEYWORDS (Precede each with Security Classification Code) (U) Sensory Stimulation; (U) Nervous System; (U) Behavior; (U) Electrophysiology; (U) Attention; (U) Stress

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) This exploratory project will establish and evaluate an animal model to measure the interrelationships between sensory stimulation, nervous system responses (e.g., evoked potentials), and behavioral manifestations of stimulus control, attention and vigilance. Emphasis will be on methods requiring minimal physical and chemical restraint in order to permit realistic simulations of military environments demanding prolonged vigilance and cognitive performance, and of the disruptors (such as altered sleep and feeding patterns, and noxious stimuli) which impinge upon task performance in those situations. The data base generated will be part of our program to examine the maintenance and decrement of satisfactory military performance in stressful situations.

24. (U) Using the methods of animal psychophysics and electrophysiology, methods will be developed to permit measurement of neural electrical potentials from awake subjects attending and responding to environmental stimuli. Interrelationships between environmental signals and neural and behavioral responses will be established and then studied under conditions which interfere with optimal performance such as circadian desynchronization, sleep loss, increased noise or decreased signal resolution, or emotional disrupters.

25. It was shown that motor neurons in cerebral cortex are influenced by painful and by visceral stimuli and can be radically altered by the opiate antagonist naloxone. The initial set-up of an auditory psychophysics laboratory has been accomplished. For technical report see Walter Reed Army Institute of Research Annual Report 1 Oct 78-31 Sep 79.

*Available to contractors upon originator's approval

DD Form 1498, 1 Mar 68 — Previous editions of this form are obsolete. DD Forms 1498A, 1 Nov 65 and 1498-1, 1 Mar 68 (for Army use) are obsolete.

Project 3A161101A91C In-House Laboratory Independent Research

Work Unit 107 Neural and Behavioral Response to Sensory Stimulation

Investigators:
Principal: Tyner, LTC C.F.
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DESCRIPTION. This developmental project was initiated when it became clear that several areas of investigation were hampered by the absence of appropriate methodologies to measure behavioral and neural responses to sensory stimuli. Investigations of information processing, decision processes and attention under conditions of stress, fatigue and drug intoxication were impossible until a reliable and validated set of techniques were developed for measuring behavioral responses to sensory stimuli. An elementary component of information processing, decision making and attention is sensation. Evaluation of these "higher" processes must always include a rigorous specification of the sensory component. One portion of this ILIR project, then, focuses on the development of methods to analyze behavioral responses to sensory stimuli.

At the same time, it became clear that the understanding of neural responses to stress first requires a technique for measuring neural responses to sensory stimulation in general, both innocuous and noxious and an account of those pathways and central neuronal populations affected. An integral part of this endeavor involves using techniques for measuring neural responses to sensory stimulation in awake animals. This is important because the presence of an anesthesia may fundamentally alter the general pattern of neural response to stimulation. This last problem becomes particularly important when evaluating the influence of neurochemicals on neural response to sensory stimulation since many neurochemicals can interact with the anesthesia. Without an awake model for measuring neural activity the evaluation of neurochemical factors including chemical defense agents is impossible.

BEHAVIORAL RESPONSES TO SENSORY STIMULI

Introduction. The broad objective of research on this project is to study sensory information processing and its relation to attention under conditions of stress, fatigue and drug intoxication in animals. Although sensory information processing has been extensively studied in man, there is little information on that topic in animals because suitable behavioral techniques have not yet been developed. This ILIR project, therefore, is devoted to the development of such techniques.

The simplest form of sensory information processing which can be studied is concerned with the relationship between physical stimulus magnitudes and perceived or psychological magnitudes. It has long been recognized that psychological and physical magnitudes do not bear a one-to-one relationship to each other (Fechner, 1860), and that knowledge of the psychophysical relationship would hasten an understanding of brain mechanisms. Since brain mechanisms and the effects of altered physiological states are more easily and ethically studied in animals, the development of behavioral techniques to measure perceived magnitudes in animals would represent a major methodological advance in psychology and related fields. Hence, a focus of research on this project is to develop behavioral techniques for measuring perceived magnitudes in animals.

Work Accomplished To Date. In the five months that the project has been in effect, progress has been made in two areas necessary for the successful accomplishment of project goals. First, laboratory facilities to conduct auditory experiments with small animals have been partially established. The laboratory is expected to be fully operational within the next several months. Second, theoretical analyses have been made of two methods which can be used to measure perceived magnitudes in animals. The two methods employ behavioral tasks that may differ in their susceptibility to internal and external factors which modulate attention.

The bisection technique, which has been used by Boakes (1), Raslear (6) and Church & Deluty (2), employs a maintained generalization paradigm to measure perceived magnitude. In this technique animals are first trained in a two-choice discrimination between two stimuli, S_i and S_j. In the presence of S_i, responses on manipulandum A (R_A) are reinforced, while in the presence of S_j responses on R_B are reinforced. Once asymptotic performance has been achieved on this discrimination, several stimuli intermediate in magnitude to S_i and S_j are introduced. The original stimuli, S_i and S_j are still reinforced, but the new stimuli are not (maintained generalization). If percent responses to one of the manipulanda are examined as a function of stimulus magnitude, that stimulus magnitude which produces 50% R_A and 50% R_B can be determined. That stimulus is defined to be the mid-point (S_b) of the psychological distance defined by S_i and S_j. In other words, the perceived distance between S_i and S_b is equal to that between S_b and S_j. Fagot (1963) has shown that this information can be used to determine the relationship between perceived and physical magnitudes. For instance, if it is assumed that Fechner's Law ($P = k \log S$, where P is the perceived magnitude, S is the physical magnitude and k is a constant), applies, then S_b should fall at the

geometric mean of the interval defined by S_i and S_j:

$$S_b = (S_i S_j)^{1/2}, \quad (1)$$

If Stevens' Power Law ($P = kS^n$, where n is a constant pecular to each sense modality) applies, then the midpoint should fall at a point described by the following equation:

$$S_b = [1/2(S_i^n + S_j^n)]^{1/n} \quad (2)$$

Previous research (Raslear, 1975) has shown that the bisection technique is particularly sensitive to manipulations of the external environment (context effects) and a theoretical analysis of this technique (Raslear, 1979), suggests that this should be particularly so for large values of log (S_i/S_j). A protocol is in preparation to test this hypothesis, and should it be supported, the task would be an ideal means of examining the effects of a variety of environmental manipulations on perceptual processes in animals.

Because the bisection technique is very sensitive to environmental manipulations or context effects, any measurement made with that technique will be relative to the particular context in which it is made. Thus, it is desirable to also have an absolute measurement technique available for comparison. Pierrel-Sorrentino & Raslear (1979) have recently developed a method by which discrimination performances are used to measure perceived magnitudes. In this method, equal sensory distances are derived from pairs of stimuli which yield equivalent discrimination measures. It is assumed that differential responding within a discrimination procedure is chiefly controlled by differences in the experimenter-specified exteroceptive stimuli (e.g., sound pressure level). Other factors which influence differential responding are assumed to be constant. If these assumptions are correct, then the magnitude of the differential response performance should directly reflect the difference in perceived magnitude between the two stimuli (e.g., loudness). Equal magnitudes of differential responding (behavioral equivalents) are taken as defining equal sense distances between pairs of stimuli.

Once an equal sensory distance has been established, well-defined scales can be tested. For example, substituting the appropriate terms into Fechner's Law would yield:

$$\log I_A - \log I_B = \log I_C - \log I_D. \quad (3)$$

According to Fechner's Law, pairs of stimuli separated in sound

pressure by equal numbers of logarthmic units should produce equal differential response performances. A similar substitution can be made for Stevens' Law:

$$I_A^n - I_B^n = I_C^n - I_D^n. \qquad (4)$$

In this case, the exponent, n, can be determined by a process of iteration. CPT Raslear has conducted preliminary studies with this procedure elsewhere with rats and chinchillas and will further extend the method in new facilities prepared under this project. In particular, a study is in preparation to test the discrimination method in animals using procedures more comparable to those used in human studies. Due to the tighter behavioral constraints employed in discrimination techniques generally, the discrimination method will probably be less sensitive to manipulations of the external environment, but may serve as a useful tool to separate internal and external influences on sensory information processing when compared with the bisection technique.

NEURAL RESPONSES TO SENSORY STIMULI

During the past year we completed two projects directed at providing neurophysiologic descriptions of brain responses to sensory stimuli which may be generated when an organism is stressed. In the first, strong -- probably painful -- stimuli delivered to the viscera were shown to have a direct influence on the output neurons of motor cortex, the cortical neurons most closely concerned with control of skilled movement. In the second, we partly resolved a controversy about the influence of a newly-discovered spinal cord pathway -- the lateral cervical system -- on the motor cortex. We showed this ascending system -- argued by some to convey pain information -- to excite motor cortex output neurons and to inhibit local cortical interneurons. These results provide insight into possible mechanisms by which the ability to execute coordinated movements may be compromised if an organism is placed in stressful conditions.

In another project, performed both in awake and anesthetized animals, we have begun study of how the behavior of cerebral cortex neurons is changed following systemic injection of small amounts of chemicals. The initial experiments have used naloxone -- an opiate antagonist thought to reveal the brain's chemical systems for dealing with stress -- and picrotoxin, a more general excitatory drug. Thus far, all changes produced by naloxone, including motor cortex receptive field enlargement in the anesthetized state and increased spontaneous activity in the motor and sensory cortex in both the awake and anesthetized states, can be reproduced by small doses of picrotoxin. These results indicate the possibility of

selective pharmacologic modification of motor cortex neurons but raise doubts about the presumed specificity of naloxone for the study of stress.

Project 3A161101A91C In-House Laboratory Independent Research

Work Unit 107 Neural and Behavioral Response to Sensory Stimulation

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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3. DATE PREV SUM RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
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10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	108
b. CONTRIBUTING				
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003500 Clinical Medicine 012900 Physiology 002300 Biochemistry 012600 Pharmacology

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	CURRENT 80	1	60

19. RESPONSIBLE DOD ORGANIZATION

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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Post-traumatic epilepsy occurs in over 40 percent of soldiers subjected to dura-penetrating head injury. Neither improved neurosurgical care of head injuries nor prophylaxis with standard anticonvulsant medications has resulted in a decrease in the incidence of post-traumatic epilepsy. The onset of the seizure disorder usually occurs within two months of the injury but may not occur for up to 2 years. Understanding of the biochemical factors at work during this latent period could lead to effective preventive therapy that could be initiated immediately following the injury.

24. (U) Animal models such as the kindling procedure also provide a latent period between initial procedures and subsequent development of observable seizures. Other different animal models including rats given subpial injections of ferric chloride and rats sensitive to audiogenic seizures will be developed. Both in vivo and in vitro methods to investigate the role of cyclic nucleotides and neurotransmitters in specific brain regions on the development of epilepsy are planned in each of these models. This information will be used to design preventive measures.

25. (U) Preliminary studies in rats congenitally sensitive to audiogenic seizure show changes in cyclic nucleotides in several brain regions, including corpus striatum. These changes are particularly interesting by virtue of their occurrence in brain regions known to be associated with propagation of seizures. These studies are being extended to include the ferric chloride and kindling models. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

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Project 3A161101A91C In-House Laboratory Independent Research

Work Unit 108 Prevention of Post-traumatic Epilepsy

Investigators.
Principal: James L. Meyerhoff, M.D.

Associate: MAJ Vernice E. Bates, M.D., G. Jean Kant, Ph.D.; David R. Collins, B.S.

I. Neurochemical Mechanisms in the Development of Post-Traumatic Epilepsy

Medical management of the soldier following penetrating missile injuries of the brain must reflect the high probability of occurrance of post-traumatic epilepsy. More than 40% of men with penetrating missile injury have post-traumatic grand-mal seizures (1). Caviness, the leading clinical authority on this syndrome, has stressed the military-unique nature of the disorder (2). High risk of post-traumatic epilepsy has been associated with penetration of the dura mater, depth of injury, presence of infection, hypesthesia, coma, non-viable cerebral tissue and parietal location of the injury (3). Further study has shown that, despite significant reduction of incidence of infection over the period from WWI through the Korean War, the incidence of post-traumatic epilepsy was only slightly reduced (4). Moreover, during the Vietnam conflict attempts were made to reduce chronic seizure disorder by prophylactic administration of Dilantin following injury. These attempts were unsuccessful (5).

The onset of the seizure disorder usually occurs within two months of the injury but may not occur for up to 2 years. Understanding of the biochemical factors at work during this latent period could lead to effective preventive therapy that could be initiated immediately following the injury. Penfield noted that an "incubation" period of several months often exists between the causative brain trauma and the first epileptic attack (6). Seizures and spike foci on EEG may originate from an area of damaged cortex. Electrographically demonstrable "mirror foci" develop in homotopic cortex contralateral to a spike focus, presumably as a result of constant bombardment of this synaptically related contralateral cortex by the abnormally discharging focus. This suggests that the function of a previously normal area of brain has been altered by an abnormally discharging focus and may be related to the later development of seizures seen clinically (7-9). Because of the ineffectiveness of current neurosurgical and medical prophylaxis

of post-traumatic epilepsy, there is a clear requirement for new knowledge concerning the neurochemical events underlying such seizures. Such information could provide a rationale for new therapeutic measures to prevent chronic disability.

There are several experimental models for chronic seizure disorders, including injection of metals ($FeCl_3$, cobalt, aluminum, antimony, etc.) (8, 10-12), cortical freezing (13), kindling (14-20), rats congenitally sensitive to audiogenic seizures, as well as pharmacological techniques. As candidates for neurochemical models of post-traumatic epilepsy the ferric chloride and kindling models are particularly attractive because the experimental manipulation can be confined to one hemisphere, leaving the entire contralateral hemisphere accessible for regional neurochemical sampling, without the added complexity of drug interactions. The presence of a latent period between the initial event and development of a seizure disorder is an additional attraction. Kindling consists of repetitive, intermittent low intensity electrical stimulation of subcortical structures, especially the amygdala. This results in progressive changes in electrical activity and behavior and eventually culminates in a generalized seizure in response to an electrical stimulus which previously had produced no effect. This effect can be shown to be due to a relatively permanent transsynaptic change that results from electrical activation of neurons and not merely from tissue damage, toxicity, edema or gliosis (16). An orderly sequence of appearance of after-discharge (A.D.) and seizure discharges (I.I.D.) after amygdala stimulation has been described (16,19,20). These initially appeared only in the stimulated amygdala but successively appeared in the neostriatal system, then the midbrain reticular formation before finally causing bilateral neocortical and generalized convulsive activity. Subsequently Wada and Sato et al (20) studied the effect of the anticonvulsants phenytoin, phenobarbital and carbamazapine on this orderly sequence of development of A.D. and I.I.D., and found phenytoin to be relatively ineffective. Phenobarbital, which is known to exert a depressant action on multisynpatic reticular pathways, only partially prevented the development of distant I.I.D. and A.D. but effectively prevented the development of generalized seizures. Carbamazapine on the other hand, effectively prevented generalized seizures, but additionally markedly inhibited A.D. development and I.I.D. foci both at stimulated and distant sites (21). In the rat, diazepam or phenobarbital but not dilantin, prevented the spread of A.D. to contralateral amygdala and also prevented the development of motor seizures (22). Moreover, there is evidence that dilantin prolongs A.D. and facilitates spread to contralateral amygdala (23). When cortical sites are kindled, however, dilantin appears more effective than diazepam in blocking both A.D. and appearance of convulsions (23). We are developing the kindling model in our laboratory in order to study neurochemical mechanisms in the development of post-traumatic epilepsy.

Another experimental model useful in investigating post-traumatic epilepsy is the ferric chloride model. Biologic iron is normally protein bound in blood in transferrin and hemoglobin and isolated from the brain by vascular endothelium. Cortical contusion, such as occurs in blunt head injury or cortical laceration, such as occurs in penetrating missile injuries of the skull, result in a disruption of this physiologic barrier. This results in extravasation of blood and deposition of iron within the neuropil as hemosiderin (24). Conditions associated with hemosiderin deposition include intracerebral hematoma and cortical laceration (24), and are pathologic conditions highly associated with the development of post-traumatic epilepsy (25,26). In a recent study, the cortical injection of iron salts found to produce a chronic seizure focus in rats (27). Following subpial injection into rat cortex of 5 μl of $FeCl_2$ or $FeCl_3$, rats developed focal spiking on EEG followed by recurrent bilateral sustained bursts of epileptiform activity associated with behavioral convulsions. These seizures were spontaneous and recurred over a 12 wk observation period. Thus a metal that occurs naturally in the blood and that is deposited in the brain following trauma, has been shown to produce a seizure state that mimics post-traumatic seizures in several important respects: (1) a chronic seizure focus is established; (2) there is a variable latent period between the inciting event and the initial seizure; and (3) epileptiform discharges and behavioral seizures are spontaneous. We are developing the ferric chloride model as an additional means of studying neurochemical mechanisms in the development of post-traumatic epilepsy.

Although studies have attempted to examine the neurochemical mechanisms of seizures, few have utilized the focal models (i.e., $FeCl_3$, kindling) which appear to be most relevant to post-traumatic epilepsy. Some studies have used techniques that our laboratory and others have shown to be flawed by artifacts (28,29). Manipulations which decrease brain dopamine (DA), norepinephrine (NE) or serotonin (5HT) produced a lowered seizure threshold in an audiogenic seizure model (30-33), a metrazol model (34,35) and in a kindling model (36,37). Manipulations which potentiate monoaminergic function increase seizure threshold. Such manipulations include median raphe nucleus stimulation (38) and 5-hydroxy-tryptophan administration in a metrazol seizure model (39). In a kindling model, administration of L-DOPA, 5HT, the DA agonist bromocriptine (40), and the catecholaminergic agonists amphetamine (41) and cocaine (42) have resulted in an increased latency to kindled seizure. Other focal epilepsy models have shown a decrease in GABAergic terminals (43), gamma-aminobutyric acid (GABA) levels (44), as well as in levels of glutamic acid decarboxylase (the GABA-synthesizing enzyme) (45) in the seizure focus. Cyclic adenosine 3'5' monophosphate (cAMP) can be increased in brain tissue by NE

(46-48) and DA (49) and changes in cAMP may reflect post-synaptic changes in catecholaminergic stimulation. Cyclic GMP is increased in cerebellum (50) and substantia nigra (51) by pentylenetetrazol and GABA antagonists, which produce seizures, and decreased by GABA or Benzodiazepine (the latter being a potent inhibitor of kindled seizures (52). Using penicillin-induced seizure activity, Gottesfeld (44) found decreases of GABA and glutamate in the ictal and interictal stage and further decreases of glutamate alone during the ictal stage. Recent indications that GABA may be formed from putrescine, as well as preliminary data from our own laboratory, might point to mechanisms which act to limit decreases in GABA in the face of declining glutamate levels. This finding is made especially interesting in light of the observations that GABA may act to inhibit elevations in cGMP (53). In penicillin-induced seizures, an increase in cGMP is seen during the ictal, but not the inter-ictal phase (54). GABA (45) decreases during both phases. Thus it appears that the decrease in GABA may precede the increase in cGMP.

We are studying 3 animal models of epilepsy: rats congenitally sensitive to audiogenic seizure, and the $FeCl_3$ and kindling models. The latter 2 models are particularly attractive because of 3 advantages they offer: (1) they share with clinical post-traumatic epilepsy the phenomenon of a latent period between the insult and the ultimate development of seizure manifestations; and (2) the ability to study tissue from synaptically related areas (i.e., "secondary foci"), which develop independent seizure activity without direct proximity to the seizure-causing agent; (3) seizures in both models may occur spontaneously. These studies in turn could contribute to a new rationale for preventing chronic disability due to post-traumatic epilepsy.

During the first [illegible] ths program, we have studied neurochemical mechanisms in rats congenitally sensitive to audiogenic seizures. The subjects were seventh generation Wistar-derived male rats selectively bred for sensitivity to audiogenic seizure (AS). Groups of adult AS-strain rats and adult control (C) male Wistar rats of equal size and from identical maintenance conditions were subjected to audiogenic challenge. None had ever previously been exposed. Rats were placed singly in a metal grid chamber (size 33x24x13 cm) with a sound horn affixed to the top of the chamber. Auditory challenge was produced by an amplifier emitting white noise within 11.0 - 14.0 KHz range at an intensity of 105 $\pm$ 1 db at floor level. Each sound exposure was 1 min in duration. Of the 15 AS rats challenged, five (33%) responded with wild running followed by clonic-tonic seizures. None of the control strain responded. Under similar testing procedures at three weeks

of age, rats from the AS strain show a 95% incidence of clonic-tonic seizure. In different groups from the control strain, subjected to audiogenic challenge at 3 to 52 weeks of age, no clonic-tonic seizures have ever been observed. Separate groups of AS (N=6) and control rats (N=6) were sacrificed for neurochemical studies. Average body weight in each group was 321 grams. Neither group was ever subjected to audiogenic challenge. Sacrifice was accomplished by a 5 second exposure to 2.5 KW microwave irradiation at 2450 MHz. Brains were dissected into 25 regions and assayed for cAMP and cGMP by radioimmunoassay and for gamma-aminobutyric acid by enzymatic-fluorimetric assay. Brain regions assayed included lateral cerebellum and vermis, midbrain, brainstem, corpus striatum, substantia nigra, hypothalamus, thalamus, olfactory bulb, olfactory tubercle, nucleus accumbens, septal region, hippocampus, amygdala, inferior colliculus, superior colliculus, medial geniculate, lateral geniculate, frontal cortex, and cortical areas 2,17,24,29, 41, and 51. The pineal and pituitary were also assayed. In rats of the audiogenic seizure sensitive strain, cyclic GMP was elevated in the hippocampus, hypothalamus and vermis of the cerebellum.

Project 3A161101A91C IN HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 108 Prevention of Post-traumatic Epilepsy

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA0C6476	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISB'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	109
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Studies on Trypanosomiasis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 79	3	100
CURRENT FISCAL YEAR 80	4	110

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SOCIAL SECURITY ACCOUNT NUMBER

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NAME: GARSON, S., Ph.D

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Trypanosomes: (U) Antigenic Variants; (U) Coat Glycoprotein; (U) Protein Synthesis

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(V) The objective of this work unit is to develop immunological and chemotherapeutic protection for military personnel against parasitic and tropical diseases with emphasis on the isolation and characterization of variant-specific coat antigens from serodeme of salivarian trypanosomes.

24 (V) The synthesis of antigens on the cellular and molecular levels and the biochemical and genetic mechanisms controlling antigen synthesis will be studies as a rational approach to immunotherapy. Messenter RNA from enriched polysomes isolated from a specific variant strain will be purified. Using reverse transciptase and DNA-polymerase a specific genome unit will be synthesized and cloned. Genes for variant coat antigens will be mapped by hybridization competition technique.

25 (V) Surface coat glycoproteins were purified by Con A affinity chromatography from several different antigenic variants of a serodeme of Trypanosoma rhodesiense. SDS-polyacrylamide gel electrophoresis of each prep. gave a single band (MW 58,000-68,000. Isoelectric focusing resolved from one to four closely spaced components, the IEP of which were considerably different from variant to variant. Amino acid content appears to vary somewhat. Hyperimmune sera raised to purified glycoprotein produced a single precipitin line in immunoelectrophoretic or immunodiffusion tests with homologous glycoproteins. No interaction was detected in heterologous combinations. The antisera agglutinated only homologous trypanosomes. Immunization of mice with purified glycoprotein protected them from homologous but not heterologous variant trypanosome infection. These observations collectively attest to the immunogenic specificity of the glycoprotein preparations and to their identify as variant-specific surface coat antigens. For technical report see Walter Reed Army Institute of Research Annual Report 1 Oct 78 - 30 Sept 79.

*Available to contractors upon originator's approval

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 109 Biochemical Studies on Trypanosomiasis

Investigators:

Principal: Bhupendra P. Doctor, Ph.D
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Seymour Garson, Ph.D; John A. Kintzios, B.S.,
SP4 Brooks Montgomery

The objective of this work unit is the development of immunological and/or chemotherapeutic protection of military personnel against infection by parasitic protozoa. A detailed understanding of the biochemistry and molecular biology of parasitic protozoa and of host-parasite relationships is essential to a rational evaluation of the feasibility of immunotherapy, the application of chemotherapeutic measures, and the development of improved diagnostic procedures. The studies reported here are primarily concerned with the biochemistry and molecular biology of antigenic variation in salivarian trypanosomes. The present results are a delineation of the chemical and immunological properties of surface coat glycoproteins from antigenic variants of Trypanosoma rhodesiense. Future investigations are intended to furnish information on the biosynthesis and elaboration of surface coat glycoprotein and to elucidate the mechanism(s) of genetic expression of the antigenic variant surface coat structural genes.

Characterization of variant-specific surface coat glycoproteins.

Antigenic variants were derived from an isolate of the Wellcome strain of Trypanosoma rhodesiense. The isolate, termed CP3B4, was kindly supplied to us as a cloned stabilate by the Department of Immunology, Division of Communicable Diseases and Immunology. Trypanosomes from this stabilate were used as starting material and were injected intraperitoneally into a rat. After three days, infected rat blood was obtained and injected subcutaneously into a rabbit. Samples of blood were removed from the infected rabbit at sufficient intervals - days 15, 25, 35, and 45 - to maximize the probability of obtaining antigenically distinct variant types. Since the parasitemia in the infected rabbit was very low, the predominant antigenic variant in each sample was propagated by subinoculation into mice. Variants are listed according to their stabilate reference numbers and are designated 6, 10, 12, and 13. Stabilates were prepared by mixing infected heparinized blood with an equal volume of tissue culture medium 199 containing 20% glycerol. The mixtures were permitted to equilibrate for 30 min at 5 C and were

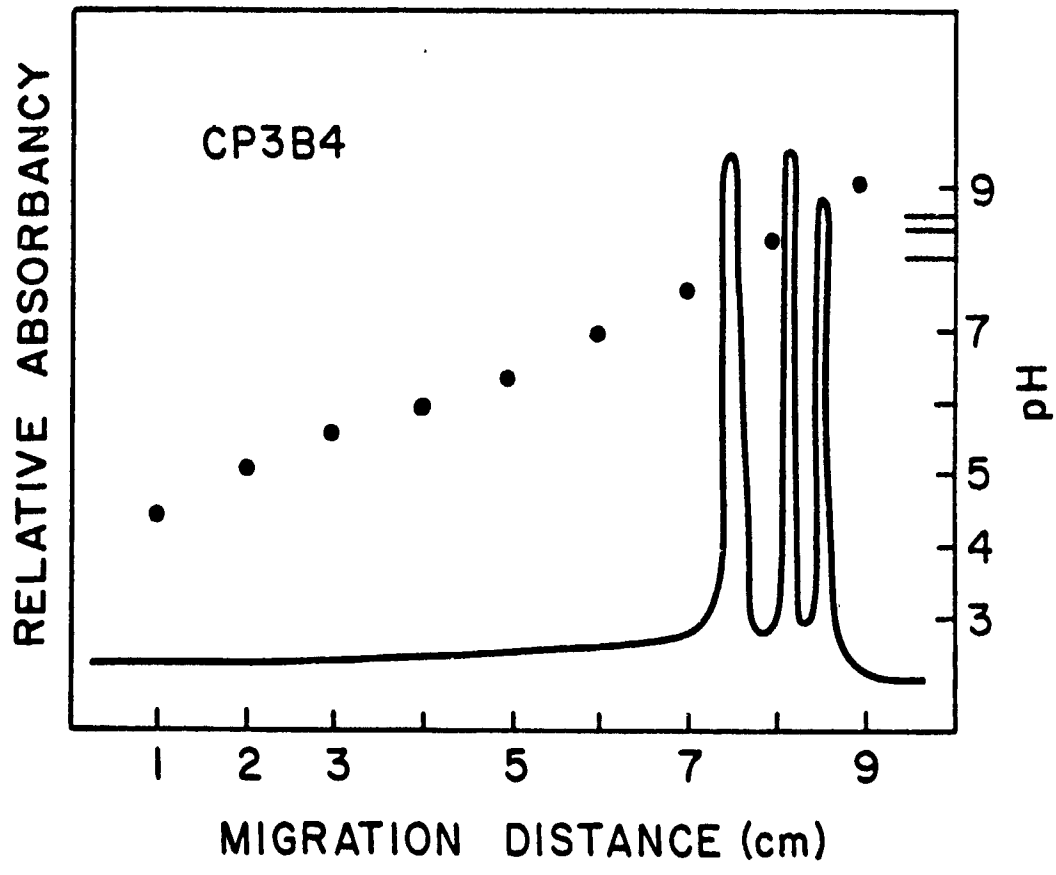

Fig. 1. Densitometric scan revealing stained protein banding pattern of gel isoelectric-focused surface coat glycoprotein from CP3B4 isolate of Trypanosoma rhodesiense.

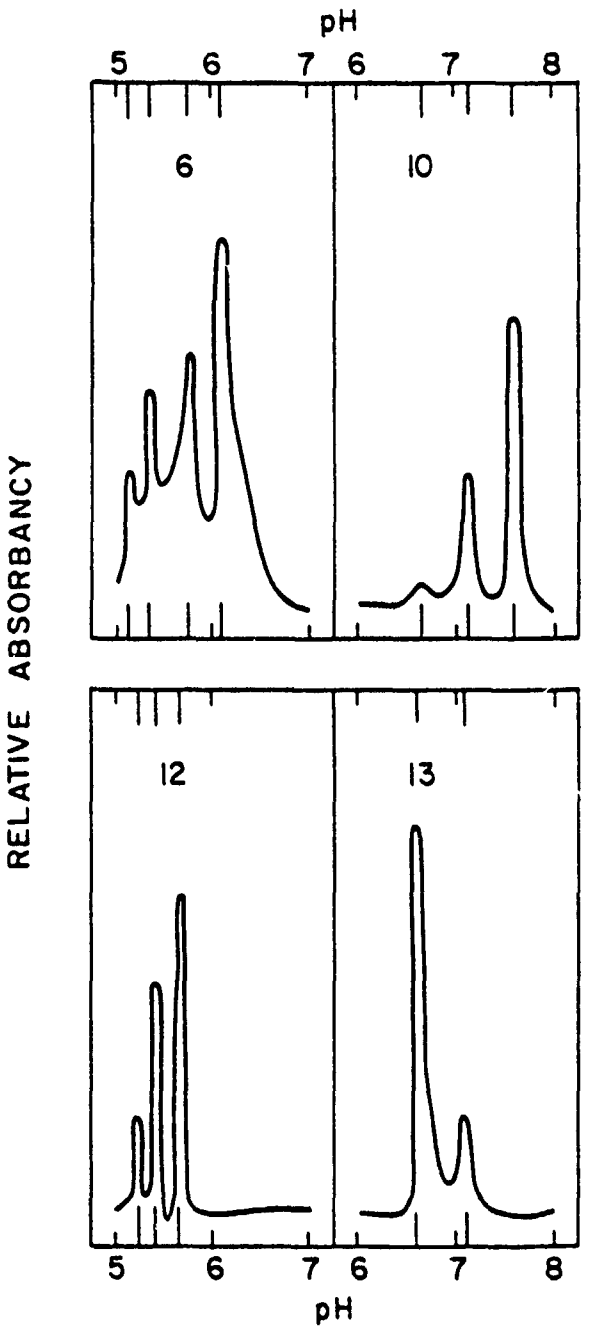

Fig. 2. Densitometric tracings of stained protein bands of gel isoelectric-focused surface coat glycoproteins from antigenic variants of CP3B4 isolate of Trypanosoma rhodesiense.

then stored at - 80 C.

Rats were injected intraperitoneally with inocula of trypanosomes obtained from thawed stabilates. After four days, rats were exsanguinated and trypanosomes were isolated from collected blood by differential centrifugation followed by selective passage through a column of DEAE-Sephacel. Surface coat glycoproteins were prepared and purified according to a procedure that was detailed in a previous annual report. The procedure consists essentially of an extraction of trypanosomes with phosphate buffer, pH 5.5, containing 1% glucose followed by affinity chromatography of the supernatant fraction on a column of Con A-Sepharose 4 B. Glycoprotein preparations were extensively dialyzed against distilled water at 4 C, concentrated to 1 mg protein per ml using immersible molecular separators, and stored at -80 C.

The molecular weight heterogeneity of surface coat glycoprotein preparations was analyzed by SDS-polyacrylamide tube gel electrophoresis. Following electrophoresis, gels were stained with a solution of Coomassie Brilliant Blue. The glycoprotein nature of the surface coat preparations was verified by staining duplicate gels with periodic acid-Schiff's reagent. Protein mobilities were expressed relative to the migration of the dye marker and molecular weights were calculated by reference to the mobilities of standard proteins. Standard protein molecular weight markers were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and trypsin inhibitor (20,000). Glycoprotein preparations from CP3B4 and the four derived variants - 6, 10, 12, and 13 - yielded only one band each, with apparent molecular weights of 65,000, 68,000, 67,000, 59,000, and 58,000. Electrophoresis under reducing conditions gave similar results indicating that the glycoproteins contained a single polypeptide chain.

The charge heterogeneity of surface coat glycoproteins was examined by isoelectric focusing in standard thin-layer polyacrylamide slab gels over the pH gradient 3.5 to 9.5. Focused gels were stained for protein with Coomassie Brilliant Blue. Inspection of the protein staining patterns of the surface coat glycoproteins from CP3B4 and the four variants revealed that, as a group, the glycoprotein preparations were resolved into two to four closely spaced bands within the pH range 5.1 to 8.6. Protein patterns of the focused stained gels were scanned using a Joyce-Loebl recording microdensitometer. Figure 1 shows a densitometer tracing of the protein banding pattern of isoelectric-focused surface coat glycoprotein from CP3B4. The pH gradient profile is also included in the figure and is depicted as a series of unconnected dots. Surface coat glycoprotein from CP3B4 was resolved into three peaks with isoelectric points of 8.0, 8.4, and 8.6. Figure 2 displays the densitometer tracings of protein banding

patterns obtained by isoelectric focusing of the four variant surface coat glycoproteins. Only part of the pH gradient is shown but in each case it embraces the region in which banding of protein was obtained. The surface coat glycoprotein of variant 6 (day 15), upper left panel, was found to separate into four components with isoelectric points of 5.2, 5.4, 5.8, and 6.1. Variant 10 (day 25), upper right panel, produced two bands with isoelectric points of 7.2 and 7.6. There is a suggestion of an additional band with an isoelectric point of 6.7. Three peaks with isoelectric points of 5.3, 5.4, and 5.7 were resolved for variant 12 (day 35), lower left panel. Variant 13 (day 45), lower right panel, revealed one major and one minor component with isoelectric points of 6.6 and 7.1. Without presently attributing any significance to the observation, it is of interest to note that, in the temporal succession of derivation of the variants, the isoelectric-focused bands alternate from one to the other end of the pH gradient.

The amino acid composition of surface coat glycoprotein form CP3B4 and each of the four derived variants was determined by automated analysis of samples hydrolyzed with 6 N HCl (Table 1). The amounts of tryptophan and proline were not measured. The values for each amino acid are expressed as residues per 100 amino acid residues. The surface coat glycoproteins were found to be rich in the couplets of aspartate-asparagine and glutamate-glutamine and in threonine, alanine, and lysine. Methionine was only detected in CP3B4. Examination of the amino acid analysis for each of the surface coat glycoproteins reveals that the overall amino composition for CP3B4 and for each of the derived variants is notably, although not markedly, different.

The antigenic activity of purified variant-specific surface coat glycoproteins was tested by determining if variant-specific immunoprotection against trypanosome infection could be produced in mice. One vol of Freund's complete adjuvant was emulsified with 3 vol of glycoprotein dissolved in distilled water. Each mouse in groups of 5 mice (Table 2) was injected intraperitoneally with 0.1 ml of the emulsion which contained 25 micrograms of glycoprotein. Mice were injected on days 0, 7, 14, and 21. Control mice received injections of an emulsion of Freund's complete adjuvant and distilled water. Fourteen days after the last injection, mice were individually challenged with an inoculum of about 2000 trypanosomes obtained from thawed stabilates. Excellent protection against homologous challenge was obtained for CP3B4, variant 6, and variant 10. Variant-specific protection was also afforded to variant 12 and variant 13 in that the lives of mice were extended as compared to nonimmunized control mice. No mice survived heterologous challenges.

Using hyperimmune sera raised in rabbits to purified glycoprotein preparation, it was shown that a single precipitin line was produced

Table 1. Amino acid compositions of variant specific surface coat glycoproteins

	Variant				
Amino acid	CP3B4	6	10	12	13
Asx	9	13	11	10	11
Thr	8	11	11	11	10
Ser	7	9	9	7	7
Glx	14	12	9	12	12
Gly	8	8	8	8	9
Ala	14	13	14	14	14
Cys	1	1	1	1	3
Val	3	2	3	3	4
Met	1	-	-	-	-
Ile	3	4	4	4	4
Leu	8	8	9	9	7
Tyr	4	2	2	2	3
Phe	3	4	4	2	4
Lys	11	10	11	11	11
His	3	2	2	4	2
Arg	3	3	3	3	3

Try and Pro content not measured.

Values expressed as residues per 100 amino acid residues.

Table 2. Variant-specific immunization of mice by purified surface coat glycoproteins

Variant		Number of mice dead by day							
Immunization	Challenge	5	6	7	8	9	10	15	30
CP3B4	CP3B4	.	.	1	.	.	.	.	1
	6	3	5	.	.	.	.	.	5
	10	4	5	.	.	.	.	.	5
	12	.	5	.	.	.	.	.	5
	13	2	5	.	.	.	.	.	5
6	CP3B4	1	2	5	.	.	.	.	5
	6	.	.	.	1	.	.	.	1
	10	1	4	.	5	.	.	.	5
	12	.	1	3	4	5	.	.	5
	13	.	3	5	.	.	.	.	5
10	CP3B4	.	5	.	.	.	.	.	5
	6	1	.	5	.	.	.	.	5
	10	.	.	.	1	2	.	.	2
	12	.	4	5	.	.	.	.	5
	13	3	4	5	.	.	.	.	5
12	CP3B4	5	.	.	.	.	.	.	5
	6	3	4	5	.	.	.	.	5
	10	3	5	.	.	.	.	.	5
	12	.	.	.	.	.	2	4	4
	13	4	5	.	.	.	.	.	5
13	CP3B4	1	4	5	.	.	.	.	5
	6	1	5	.	.	.	.	.	5
	10	.	2	4	.	5	.	.	5
	12	.	1	4	5	.	.	.	5
	13	.	.	.	.	4	5	.	5
Freund's	CP3B4	.	3	5	.	.	.	.	5
	6	.	2	4	5	.	.	.	5
	10	.	2	5	.	.	.	.	5
	12	.	1	3	5	.	.	.	5
	13	1	3	5	.	.	.	.	5

with homologous glycoproteins in immunoelectrophoretic or immunodiffusion tests and no interaction was detected in heterologous antiserum-glycoprotein combinations. Only homologous trypanosomes obtained from thawed stabilites were agglutinated with antisera and only erythrocytes coated with homologous glycoproteins were agglutinated with antisera in passive hemagglutination tests.

The presented results and the described data collectively attest to the immnogenic specificity and chemical uniqueness of the individual glycoprotein preparations, thereby identifying the variable surface coat antigenicity of Trypanosoma rhodesiense to reside in variant-specific surface coat glycoproteins.

Project 3A161101A91C IN-HOUSE LABORATORY INDEPENDENT RESEARCH

Work Unit 109 Biochemical Studies on Trypanosomiasis

Publications

1. Olenick, J.G., and P.E. Lorenz. 1979. A floating device to permit fractionation of density gradients from the top. Anal. Biochem. 97: 72-76.

2. Olenick, J.G. 1979. 2-Hydroxy-3-Alkyl-1,4-Naphthoquinones, p. 214-222. In F.E. Hahn (ed), Antibiotics, vol. 5, part 2, Springer, Verlag, Berlin-Heidelberg-New York.

3. Olenick, J.G., R.W. Travis, and S. Garson. Chemical and immunological characterization of surface coat glycoprotein antigens isolated from variants of Trypanosoma rhodesiense. Paper presented at the 54th Annual Meeting (29 July-3 August 1979) of the American Society of Parasitologists held in Minneapolis, Minnesota.

Project 3M161102BS01

BASIC RESEARCH ON MILITARY INJURY AND DISEASE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA 0A 6431	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8a. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	121
b. CONTRIBUTING				
c. [illegible]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Ecology and Control of Disease Vectors and Reservoirs

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 005900 Environmental Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
54 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION

b. NUMBER

c. TYPE d. AMOUNT

e. KIND OF AWARD f. CUM AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 79	6	318
FISCAL YEAR CURRENT 80	6	300

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Arboviruses; (U) Ecology; (U) Mosquitoes; (U) Disease Vectors; (U) Control; (U) Trombiculid mites

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Studies emphasize control of vectors of arbovirus, rickettsial and parasitic diseases of military significance. Objectives are incrimination of vectors and understanding of host-parasite relationships initially, understanding of vector biology and disease transmission mechanisms ultimately in order to develop more effective control procedures.

24. (U) Invertebrate vectors and vertebrate reservoirs and hosts are collected in areas of known disease activity. Seasonal changes in size of and infection rates in vector populations are determined; biological processes of vector species, such as pathogen transmission, diapause and reproductive physiology, are studied in the laboratory.

25. (U) 78 10 - 79 09 Six strains of St. Louis encephalitis virus have been isolated from overwintering Culex pipiens. To determine how the mosquitoes may have become infected, field studies on the winter survival of bloodfed mosquitoes are continuing. These studies have shown that non-diapausing bloodfed and non-bloodfed mosquitoes will not survive the winter, whereas from 15-25 percent of the diapausing bloodfed and wild caught mosquitoes will survive. It appears that populations of Cx. pipiens that have been conditioned to diapause become dissociated from the expected survivorship curve during hibernation. Mortality is no longer a function of age so that the probability of surviving from one age-interval to the next throughout hibernation remains more or less constant. The vector(s) responsible for transmission of Keystone and Jamestown Canyon viruses on Assateague Island Virginia have yet to be determined, however one isolation of Keystone virus from Aedes infirmatus had a temporal relationship to three Keystone seroconversions in sentinel hamsters. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

Project 3A161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 121 Ecology and Control of Disease Vectors and Reservoirs

Investigators

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Associate: CPT Michael E. Faran, MSC; CPT Thomas P. Gargan, MSC; CPT Michael W. Hastriter, MSC; CPT Alfred L. Hoch, MSC; Joel M. Dalrymple, Ph.D.; Douglas J. Gould, Ph.D.; COL Dan C. Cavanaugh, MSC; LTC David M. Robinson, VC; David E. Hayes, Ralph F. Tammariello and SP5 Ardith J. Regdon

Description

This task involves field and laboratory studies of the relationships between selected arthropods and various aspects of their natural environment, especially those aspects relating to certain pathogenic organisms, their hosts, and their reservoirs. Included are ecological and physiological studies on arthropods, studies of transmission mechanisms and the development of improved methods of control of arthropods of medical importance.

Progress

1. Overwintering Ecology of Culex pipiens and St. Louis Encephalitis Virus

One of the most important questions regarding the ecology of St. Louis encephalitis (SLE) and other North American mosquito-borne encephalitides is the mechanism by which these viruses survive the winter in temperate regions. In 1977 studies on overwintering females of Culex pipiens in Maryland, Pennsylvania and New Jersey resulted in the isolation of 2 strains of SLE from hibernating females of these mosquitoes (Bailey et al. 1978). In February, 1978, 4 more isolations of SLE came from this same region. Laboratory studies on Cx. pipiens have demonstrated that a proportion of prehibernating females, which have been conditioned with shortday photoperiods and cool temperatures, when warmed to 25°C take bloodmeals that do not result in the maturation of egg follicles. Those undergoing ovarian diapause use their bloodmeal for fat body development. It is believed that it is the quantity of fat carried into hibernation which is the important physiological factor determining the odds in favor of winter survival. In the 1978 annual report, results were presented on the overwintering survivorship of 3 groups of females of Cx. pipiens with different physiologic histories under field conditions. These data showed that a significant number of

preconditioned Cx. pipiens which had taken a prehibernation bloodmeal could survive the winter. However, no comparisons were made with survival rates of non-diapausing females nor was it possible to determine the stage of ovarian development in the mosquitoes that died.

It has been hypothesized that if females preconditioned to surviving the winter take a bloodmeal, this bloodmeal will not result in ovarian development but in hypertrophy of the fat body. In other words, if a mosquito after bloodfeeding becomes gravid it will not survive the winter. Likewise, if a female has not been conditioned for diapause, even though it is provided with a sugar source, it will not store this carbohydrate as fat and be incapable of surviving the winter.

To test these hypotheses concerning overwintering survival under field conditions, comparisons were made between 5 groups of Cx. pipiens with different physiologic histories.

The physiologic histories of each mosquito group were as follows:

1) "wild-caught", those which had entered the bunkers prior to the release date.

2) "lab-reared diapausing non-bloodfed" — Set I

3) "lab-reared diapausing blood"

4) "lab-reared non-diapausing blood" — a) non-gravid, b) gravid — Set II

5) "lab-reared non-diapausing non-bloodfed"

The mosquitoes in groups 2-5 were descendants of a colony established in 1975 from hibernating females collected at Ft. Mott, New Jersey. Larvae for the diapausing groups 2 and 3 were reared in climatic chambers at a temperature of 25°C and daily photoperiod of L:D 9:15 h. When the first pupae began to appear the temperature was lowered to 15°C. The pupae were randomly selected and placed into chambers programmed for 15°C, L:D 9:15 h and 80-90% RH. When the adults were 10-18 days old the sugar was removed and the mosquitoes were transferred to another climatic chamber at 25°C with the same photoperiod. After 24 hrs at 25°C these mosquitoes were offered 1-2 day old chicks for bloodmeals. Each morning for 3 successive days, all fully engorged females were removed and placed back into the 15°C environment. These mosquitoes were held for an additional 15-17 days under diapausing conditions to insure that the bloodmeal had been digested.

For the non-diapausing groups, 4 and 5, the larvae and adults were reared in the same type of climatic chamber at 25°C, 80-90% RH and a long photoperiod of L:D 15:9 h. When the adults of group 4 were 5-9 days old they were given bloodmeals on 1-2 day old chicks and then held for 8 days to allow digestion of the meal. The non-diapausing, non-bloodfed group was held in the laboratory for 8-11 days and fed on a 10% sucrose solution.

On 14 December, 1978, each group of mosquitoes was marked with a distinctive fluorescent dust and released inside an abandoned ammunition bunker at Fort Washington, Maryland. To insure that dead mosquitoes could be dissected and information obtained on their ovarian development, a sample of females from each physiologic group was also released into a plexiglass cage that was attached to an inside wall of the room. Nylon netting guarded the door to the room so that mosquitoes could neither enter nor leave throughout the study period. Temperature and relative humidity were monitored throughout the study by a recording hygrothermograph. Biweekly counts of the numbers of each group of mosquitoes were made with the aid of a portable black light.

Within the first week after release, the numbers of marked mosquitoes free in the room had decreased from 20-45% of there original numbers (Fig. 1). After the initial sharp decline, the numbers of mosquitoes in each group continued to decrease throughout the sampling period, but at a slower rate. The initial decline and later mortality in the non-diapausing and gravid groups (Set II) was greater than in the diapausing and wild groups (Set I) so that the curves diverged. At the termination of the experiment (8 March) all of the non-diapausing mosquitoes had died, whereas, 15-25% of the diapausing and-wild caught mosquitoes survived.

For the mosquitoes released in the cage, the survival curves of the groups in Set II strongly diverged from the curves of the groups in Set I (Fig. 2). Between 0-7% of the females in Set II were alive at the termination of the experiment versus 45.4% 58.0% of those in Set I. Mantel's (1966) chi square tests were performed comparing each curve with all the other curves. The three curves within each of the sets were not significantly different at the 0.05 level of significance. Whereas, the curves within each set compared with the curves of the other set were all statistically different even at the 0.005 level of significance.

Since within each set there was no statistical difference in the survivorship curves, a weighted average percent survival for each interval was calculated using the following formula:

$$\text{Weighted average \% survival} = \frac{S_{\blacksquare}t + S_{\blacktriangle}t + S_{\square}t}{N_{\blacksquare} + N_{\blacktriangle} + N_{\square}} \times 100 \text{ and } \frac{S_{O}t + S_{\bullet}t + S_{\Delta}t}{N_{O} + N_{\bullet} + N_{\Delta}} \times 100$$

Where S equals the actual number surviving at time interval t for each of the curves and N equals the females in the initial released population for each of the physiological groups (Tables 1 & 2). The resulting weight average percent survival for each set was plotted along the y-axis on semilog paper versus time in days along the x-axis (Fig. 3). The curve of Set I was approximately linear, indicating that the rate of mortality is constant and independent of the age of the individuals in the population. The mortality rate for these overwintering mosquitoes does not vary to any extent with age during their hibernation. The convex curve exhibited by Set II indicates that the mortality rate steadily increases with the age of the mosquitoes in the set; the rate of mortality for this set is considered to be age-dependent.

The full implication of the difference in the curves of Sets I and II has not been thoroughly investigated. It appears that populations of Cx. pipiens that have been conditioned to diapause become dissociated from the expected survivorship curve during hibernation. Mortality is no longer a function of the age of the females so that the probability of surviving from one age interval to the next throughout hibernation remains more or less constant.

2. Ecology of Eastern equine encephalitis (EEE) and Highlands J (HJ) in the Pocomoke Cypress Swamp (PCS)

During the years 1968-72, a great deal of research was done on the ecology of Eastern equine encephalitis (EEE) and Highlands J (HJ) viruses (previously reported as Western equine encephalitis) in the Pocomoke Cypress Swamp (PCS) in Worcester County, Maryland. Biological and distributional studies on Culiseta melanura (Coquillett), the enzootic vector of these viruses, have been reported by Joseph and Bickley (1969), Williams et al. (1971b) and Saugstad et al. (1972). Virological aspects of these studies were reported by Dalrymple et al. (1972) and Williams et al. (1972 and 1974). The main emphasis of the virological work was in defining virus amplification during the summer. In 1970 and 1971 these viruses were monitored by sentinel quail from early June through the end of November (WRAIR Annual Report, 1971-72). EEE first appeared in a single sentinel quail during 15-28 June; the next EEE virus infection was not detected until a month later with peak virus transmission occurring in August and September. EEE virus infections continued at a reduced rate through the last part of November. During the same period of time, HJ transmission began in mid-July and peak virus transmission

was recorded during July and August. HJ transmission gradually declined through September and October but abruptly increased during November. The onset and duration of quail infections with these two viruses during the summer corresponded closely to the density of Cs. melanura. However, the rise in transmission of both viruses in November occurred while the population of Cs. melanura was relatively low and declining. This observation raises the possibility that other mosquitoes may be involved in the late fall and early spring transmission of these viruses. A prime candidate for study is Culex restuans. This species is primarily an avian feeder, overwinters in the adult stage, and reaches its greatest abundance in the spring and fall (Barr 1958). EEE has been recovered from wild-caught Cx. restuans females (Hayes et al. 1960 and Morris et al. 1973, 1975). The objective of this research is to identify the overwintering mechanism(s) of EEE and HJ viruses in the PCS.

The seasonal transmission of both EEE and HJ viruses will be monitored by sentinel quail from early March 1979 through January 1980. Quail have been shown to be excellent sentinels for both viruses (Watts and Williams 1972 and Williams et al. 1971a) and are convenient to maintain.

Six sites were located in two different habitats (swamp and upland forest) in and around the study area in the PCS (Fig. 4). Each site contained the following: a CDC light trap augmented with dry ice, 3 resting boxes, a sentinel cage containing 5 quail, a hanging net trap containing 5 quail, and 20 one gal. oviposition pans. The hanging net traps were designed to allow mosquitoes to enter and take a bloodmeal but prevent their escape. A resting box and a light trap were placed in close proximity to each sentinel cage. Bloodfeeding insects were collected weekly from light traps and hanging net traps. Collections from the resting boxes were done biweekly. Oviposition pans were examined weekly and larval samples taken for identification. All bloodfeeding insects collected in the traps were stored at -70°C for future identification and virus assay.

Sentinels were replaced at each location every two weeks. The bleeding procedure was as follows: prebleed, expose sentinels for two weeks and bleed a second time, then hold sentinels in lab for two weeks and bleed for a third time. One ml of blood was taken from each quail at each bleeding, and blood samples were assayed by plaque reduction neutralization test (PRNT), for EEE and HJ antibodies.

To date, the number of mosquito collections from the various trapping methods in the PCS are as follows: resting boxes - 129,

net trap - 75, oviposition pans - 34, and light traps - 99 trap-night collections. Tabanids have also been collected from the net traps (22 collections) and light traps (64 trap-night collections). Identification of the mosquitoes and tabanids collected in these traps has not been completed.

Sera collected from quail through 25 July have been tested. The total serologic conversion of quail from the 6 sites (12 cages) are presented in Fig. 5. EEE virus transmission to sentinel quail was first detected in the last of May and beginning of June. A peak of EEE virus transmission was observed during late June when 100% of the birds exposed in the swamp were infected with virus. This year both the appearance and peak activity of EEE virus transmission in the PCS occurred much earlier than previously reported (Dalrymple et al. 1972 and Williams et al. 1972). In the past, EEE virus appeared in late July with peak transmission occurring in September. The habitat distribution of EEE antibody-positive quail is summarized in Tables 3 and 4. Neutralizing antibody to EEE virus has been found in quail housed in both the sentinel cages and the hanging net traps in all sites in the PCS. There does not appear to be a great difference in the percent of seroconversions within the birds exposed in sentinel cages (Table 3) nor within the birds in the net traps (Table 4) at the various upland and swamp sites. However, the railroad site, which is located deep in the swamp, does show a slightly higher percent of seroconversions than the other sites. The largest difference in the percent of seroconversions is observed between the birds housed in sentinel cages (48%, Table 3) vs. those in net traps (18%, Table 4). A probable explanation for this difference is because the quail in the net traps are at a lower risk of being bitten by mosquitoes than quail in the sentinel cages. Mosquitoes can enter the sentinel cages from five sides whereas they can only enter from the bottom in the net traps.

3. Ecology of Keystone and Jamestown Canyon Viruses in the Pocomoke Cypress Swamp

Two California encephalitis group viruses, Keystone (KEY) and Jamestown Canyon (JC), are endemic in the Del Mar VA peninsula. In the Pocomoke Cypress Swamp (PCS) the natural cycle of KEY virus transmission has been reported by LeDuc et al. (1975). This cycle involves Aedes atlanticus as the primary vector and gray squirrels and/or cottontail rabbits as the reservoirs. Transovarial transmission of KEY virus by Ae. atlanticus has also been demonstrated.

A serosurvey of a domestic goat herd in the vicinity of the Pocomoke Cypress Swamp yielded neutralizing antibody to California encephalitis group viruses. The antibody appeared to be directed

to Keystone (KEY) virus, however, significant cross reaction was observed to Jamestown Canyon (JC) virus. In an attempt to investigate the potential of using goats as sentinels for those two viruses an experimental infection experiment was conducted.

Goats approximately 1 year of age, which had been shown to be free of neutralizing antibody to KEY and JC viruses, were injected intravenously with either JC or KEY virus and maintained in mosquito proof field enclosures for the duration of the studies. The experimental groups are described in Table 5. Because of the requirement for a sensitive indicator of JC virus transmission in the study area, both high and low doses of JC virus were employed in this study. The JC virus used was a 1972 field isolate from Aedes canadensis which had been passed twice in suckling mice followed by two passages in BHK-21 cells. The KEY virus was received from American Type Culture Collection and had been passed four times in BHK-21 cells.

The goat bloods were assayed for viremia on BHK-21 cell monolayers for the first 6 days post inoculation (PI). Viremia was not detected in any of the 17 goats in this study. In order to determine whether the goats developed neutralizing antibody to these viruses, plaque reduction neutralization tests (PRNT) were performed on days 10, 20, 30, 45, 60, and 90 PI. Fifty percent plaque reduction endpoints were calculated where applicable. The results were presented in Tables 6, 7 and 8.

Responses to KEY virus infection appeared more specific than did those to JC virus, however, cross reactions were observed especially with high titered antibody containing samples. Antibody to JC virus was more cross reactive to KEY than KEY was to JC but never sufficient to preclude identification of the infective virus. No dose response was clearly evident but all of the JC high dose animals were high titered. It was interesting to note that all animals exhibited detectable antibody by day 10. The peak in antibody titer was attained at approximately day 20 PI, however detectable antibody persisted in all but one goat through day 90 PI.

The absence of viremia suggests that goats are not contributing to the KEY and JC virus cycle in the Pocomoke Cypress Swamp. However, this absence of viremia, when coupled with their relative sensitivity to KEY and JC viruses as measured by high titer antibody responses, makes goats an ideal sentinel animal to use for monitoring KEY and JC virus transmission.

During the summer of 1978, goats were used to monitor virus activity in the PCS. There were three seroconversions to KEY and one to JC during this time period. Collecting methods and site

locations were described in WRAIR Annual Report 1978. Neutralizing antibodies to KEY and JC viruses were detected by PRNT in BHK-21 cell monolayers. A sentinel goat housed in a Magoon trap at the Dorsey's Pasture site on 6 July seroconverted to JC. This represented the first JC seroconversion in a sentinel animal at the swamp. The KEY seroconversions were detected in a goat at the Slab Road site on 31 August and in two goats at the Atlanticus North site on 7 September.

Mosquitoes collected from the Magoon and light traps for the one month period prior to each of the seroconversions were processed for virus isolation (Table 9). All mosquito pools were assayed for virus on cell monolayers. Those pools associated with the JC seroconversion were also assayed for virus by inoculation into suckling mice. Over 12,000 mosquitoes, representing 15 species, were assayed for virus with no agents found.

4. Ecology of Keystone (KEY) and Jamestown Canyon (JC) virus on Assateague Island

Neutralizing antibody to both viruses has been reported from cottontail rabbits as well as seka and whitetail deer on the Chincoteague National Wildlife Refuge (CNWR) on Assateague Island, VA. However, neither Ae. atlanticus nor gray squirrels has previously been reported from the CNWR. The natural cycle of JC virus transmission is unknown in both the PCS and the CNWR.

For the past two summers sentinel hamsters, CDC light traps supplemented with dry ice, and bleeding of feral cottontail rabbits have been employed to determine KEY and JC virus activity in the CNWR. This year, four sites, two in a freshwater swamp and two in an upland pine forest, were used to monitor virus activity (Fig. 6). These sites in addition to six other sites were used in 1978. Each site had a cage containing two sentinel hamsters and a CO_2-baited CDC light trap. Sentinel hamsters were pre-bled, exposed in the field for one week and bled again, then held for an additional two weeks before being bled for the final time to determine presence or absence of neutralizing antibody to KEY and JC viruses. Bloodfeeding insects were removed from the light traps biweekly and stored at -70°C for future identification and virus assay. A trap, bleed, mark and release program was used to establish what time of year the cottontail rabbit population was seroconverting to either KEY and/or JC neutralizing antibody.

The results of the 1978 studies are presented in Tables 10 and 11 and Figs. 7 and 8. Twenty-three species of mosquitoes were collected, the majority of which were found in both habitats (Table 10). The CO_2-baited light traps collected a larger number

of specimens and also a greater species spectrum than did the hamster-baited net traps. Aedes atlanticus was collected (in low numbers) for the first time on Assateague Island.

Over 14,000 mosquitoes, representing 15 species, were processed for virus isolation from the CNWR (Table 11). The majority of mosquito pools were assayed for virus on both BHK-21 cell monolayers and by intracerebral inoculation of 3-5 day old mice. KEY virus was isolated in suckling mice from a pool of 36 Ae. infirmatus collected by light trap in the freshwater swamp on 8 August. The virus from the original supernate was not detected on BHK-21 monolayers. However, four of five suckling mice were found sick or dead on day 8 post inoculation of the original mosquito pool. The first 20% suckling mouse brain (smb) passage killed an additional litter of 14 suckling mice on days 3-4 post inoculation. The isolate was identified as KEY virus by PRNT.

Figs. 7 and 8 show the seasonal distribution of Ae. infirmatus collected by light and net traps respectively in the CNWR. Both trapping methods collected this mosquito species, however, light trapping collected the largest number of specimens. A total of 30 Ae. atlanticus females was collected between 23 July and 12 August, 21 of which were collected by light traps in the freshwater swamp. All specimens were assayed for virus in cell monolayers and suckling mice with no agents found. Considering the low levels of Ae. atlanticus collected, we do not believe this species could be totally responsible for KEY virus transmission on the CNWR.

Neutralizing antibody to KEY virus was detected in sera from two sentinel hamsters which had been exposed in the pine forest between 17-30 August. The virus isolation in Ae. infirmatus and the seroconversions in hamsters showed a temporal relationship to the second generation of Ae. infirmatus on the CNWR (Fig. 7). The minimum field infection rate for KEY virus in Ae. infirmatus was 1 per 6308 mosquitoes tested (Table 11).

In 1978, 5/9 (56%) of the feral cottontail rabbits that were trapped possessed antibody to JC virus, but none showed antibody to KEY virus. The percentage reductions to JC virus ranged from 60-100%. In 1979, 12 rabbits were trapped and 3 (25%) had antibody to JC virus and 2 (17%) had antibody to KEY virus. The ranges of percent reductions of KEY and JC viruses were 51-97% and 58-100%, respectively. Sera collected from sentinel hamsters through 13 August have been tested by PRNT with no evidence of seroconversion to either KEY or JC.

To date, 108 trap-night collections have been made from the four light trap sites (Fig. 6) in the CNWR. Identification of

mosquitoes and tabanids in these collections and sorting into pools for virus assay have not been completed.

5. Virus Transmission Study with Aedes infirmatus

In an attempt to demonstrate transmission of a naturally acquired infection of KEY virus approximately 1500 adult females of Ae. infirmatus were collected by light traps in the CNWR. These females were separated into two cages. Bloodmeals were offered by placing a KEY and JC antibody-free hamster in each cage for 24 hours. All bloodfed mosquitoes were removed daily and segregated into separate cages. This process was repeated for four consecutive days which resulted in eight hamsters that had been fed on by 1511 female Ae. infirmatus. All hamsters were held in a mosquito proof room for two weeks and then bled. Sera were assayed for antibodies to KEY and JC viruses. No evidence of neutralizing antibody was detected.

6. Laboratory Colonization of Aedes infirmatus

Approximately 6000 eggs were obtained from field collected adult Ae. infirmatus during the week of 10 June. Eggs were conditioned for two weeks and then approximately 2500 eggs were hatched for a colonization attempt. Larvae were reared in 300 cm. X 190 cm. X 50 cm. enamel pans. 100 larvae were added to each pan to which approximately 1500 ml of distilled water had been added. A rabbit pellet placed in the pans provided the infusion which served as food for the larvae. As the larvae matured more pellets were added to ensure the production of an abundant food supply. Pans were aerated to prevent the formation of scum. Pupae were removed from the pans daily by means of a pipette. The pupae were separated by sex by examining the terminal portion of the abdomen under a dissecting microscope. The pupae were then counted and placed in a cage. The larval and pupal rearing techniques resulted in minimal mortality. Over a period of 11 days approximately 1000 female and 1000 male pupae were placed in a 1^3 ft. wire emergence cage. Another 1^3 ft. cage was also set up containing 50 female and 50 male adults. Both cages were maintained in an incubator at a temperature of 25-26°C and a photoperiod of 15:8 L:D with a 30 min. dawn and dusk period. To increase the relative humidity each cage was covered with a wet towel and then a plastic bag was placed over the entire cage. There was minimal adult mortality throughout the experiment. Sucrose was usually present as food for the adults. Females readily fed on restrained hamsters placed inside the cages. Oviposition began approximately 10 days after engorgement. Five females were examined weekly for insemination with no sperm found in their spermathecae. Force mating attempts were unsuccessful in obtaining inseminated females. Approximately

12000 eggs were obtained throughout the experiment. All eggs were conditioned for 7-14 days and then hatching was attempted. None of the eggs had hatched after 48 hrs. The eggs were then cleared in a 5% chlorox solution and it was determined that none were fertile. The majority of the remaining females were then dissected and none were found to be inseminated.

7. Studies of Trombiculid Mite Vectors of Rickettsioses

Because of the absence of a principal investigator from June 1978 until January 1979, the infected and uninfected Leptotrombidium (Leptotrombidium) fletcheri and L. (L.) arenicola colonies were maintained at a minimum level. During this period the colonies were maintained under ambient conditions with extremes of temperature fluctuations (15-32°C). It became obvious that verification of the status of the chigger colonies was necessary to ensure that pure infected and uninfected colonies of L. fletcheri and L. arenicola were still available for future studies. To accomplish this, larval L. fletcheri and L. arenicola were fed on 5-6 week old female mice of the ICR and C3H strains, respectively. Infectivity was determined by mouse spleen passage and challenge with 1000 $MIPID_{50}$ of the Karp strain of Rickettsia tsutsugamushi. Few infected chiggers have been cultured from the existing colonies, consequently, new stock colonies of infected L. fletcheri and L. arenicola were recently obtained from the US Army Medical Research Unit (Malaysia) and are now being propagated.

8. Chigger Sex Ratio Conversion and Infectivity in Males.

Neal and Barnett (1961) found that of 13 presumably uninfected L. fletcheri (= Malaysian L. akamushi) females, 6 produced only female offspring, 3 only male, and 4 both sexes. Rapmund et al. (1969) reported that male progeny were not produced by R. tsutsugamushi infected L. fletcheri (=Malaysian L. L. akamushi) females over 5 generations. Roberts et al. (1977) demonstrated male/female sex ratios of 27/2938 and 1/906 for infected lines of L. fletcheri and L. arenicola, and males did not transmit R. tsutsugamushi to mice. Rapmund et al. (1972) reported rearing one infected male L. arenicola. The current laboratory colony has rarely produced male progeny; however, two infected L. arenicola females have been exceptions. The first female produced 15 male offspring and no females. None of these males transmitted R. tsutsugamushi during feeding. The second female produced one female and 14 male offspring. Each was fed singly on C3H mice. The female transmitted R. tsutsugamushi as demonstrated by survival of the host mouse to challenge with 1000 $MIPLD_{50}$ of the virulent Karp strain. The host mouse on which one of the 14 males was fed died on day 10 post-attachment. Because of the inability to

recover R. tsutsugamushi from dead mice, organs were not passed, but the mouse appeared sick several days before death and a greatly enlarged spleen was noted on necropsy. This is the second possible case of R. tsutsugamushi transmission by a male chigger of any species. Attempts are now being made to demonstrate venereal transmission via spermatophore uptake. The presumed infected male was placed in a sterile container, allowed to deposit 5-10 spermatophores, and then removed. A female from an established uninfected line (over ? generations) was placed in the container to collect the freshly laid spermatophores. F_1 progeny were fed on C3H mice to determine if venereally induced transovarial transmission had occurred. Results are pending. Additional tests for R. tsutsugamushi infection in this male will include fluorescent antibody techniques to detect rickettsial antigen in spermatophores and ultimately in specific tissues of the male.

9. Comparative Internal Morphology and Hisotology of Vector and Nonvector Trombiculidae

With the exception of work by Obata (1954) on Trombicula akamushi (Brumpt), detailed anatomical studies on known vector species are lacking. This laboratory has initiated morphological and histological studies on the known R. tsutsugamushi vector species L. arenicola and L. fletcheri.

Adult chiggers were fixed in Bouin's solution, embedded in wax, sectioned (6 microns) with a rotary microtome, stained with hematoxylin and eosin, and mounted on glass microscope slides with no. 1 microscope cover glasses and liquid mounting media. During preliminary studies several difficulties were encountered. The impermeable nature of chigger chitin does not permit Bouin's fixative to penetrate and adequately fix the internal tissue. Poor fixation was indicated by a lack of distinct cellular features and excessive compression of tissues during sectioning. Improved fixation might be attained by cutting the forelegs, palpi, and chelicerae close to the gnathasoma and placing the chigger in fixative in a vacuum. The optimum time interval required for proper fixation has not been determined. These techniques are being developed.

The minuteness of chiggers requires special handling techniques during wax embedding. Manipulation of chiggers is difficult to accomplish before the wax begins to harden. The most successful method for transferring and orientating specimens in wax is to pour a single specimen from a 10 ml beaker directly into an embedding mold under the dissecting microscope and quickly manipulate the specimen to the desired position with a hot stainless steel probe.

A differentiation of tissue types has not been detected in sectioned material because of fixation difficulties; however, some gross morphological features have been identified by microdissection techniques on adult L. fletcheri and L. arenicola.

Dissections are performed in a wax filled petri dish containing a shallow depression. The chigger is killed in 70% ethanol, the excess ethanol is removed, the chigger is positioned and immobilized for dissection by affixing it to the wax substrate with a hot stainless steel probe. The hydrophobic nature of chigger setae requires the application of 70% ethanol preceeding the application of French, Baker, and Kitzmiller's dissecting fluid (FBKDF). Once FBKDF has penetrated through an opening of the chitin, a few minutes are required to permit fixation of a mucilaginous substance that is so tenacious before fixation that dissection is impossible. When the chitinous exoskeleton is teased away, internal tissues are differentiated by staining. Lacto-orcein (2%) stains nervous, tracheal, gonadal, and glandular tissues a deep maroon. Only a few minutes are required for staining of these tissues, but repeated staining is necessary as solid digestive wastes and membranous structures which fill the abdomen interfere with the staining of deeper tissues.

Preliminary observations have revealed four paired salivary glands in both species which are similar in anatomical position and morphology. The salivary glands are located dorsal and anterior to the brain. The brain has large nerve trunks extending laterally and anteriorly to innervate the 8 legs, palpi, and chelicerae. The esophagus is encompassed by the brain and appears to bifurcate posteriorly as it exits the brain. The ventrally located testes are very large in L. arenicola in comparison to L. fletcheri. L. fletcheri males have a single pair of accessory glands which undoubtly exists but have not been found in L. (L.) arenicola. A pair of globular spermathecae were located in L. fletcheri. Ducts run from the spermathecae to the inner anterior portion of the genital opening. Several tubular accessory glands have been located in L. arenicola females. These observations are preliminary to further detailed investigations.

TABLE.1 (Set I) Weighted average and percent survival of wild caught, diapausing non-bleedfed and diapausing bloodfed, nongravid Culex pipiens at Ft. Washington, MD, during winter of 1978-9.

Date	t	Weighted Average % Survival: Wild $S_{\blacksquare}$	Lab-Reared Diap., Blood-Fed, Nongravid $S_{\blacktriangle}$	Lab-Reared Diap. Non-Bloodfed $S_{\square}$	Weighted % Survival
14 Dec	0	69*	44*	130*	100
15-18 Dec	4	68	42	127	97.5
12-25 Dec	11	65	42	124	95.1
26-Dec-1 Jan	18	65	38	116	90.1
2-8 Jan	25	63	37	110	86.4
9-15 Jan	32	61	37	108	84.8
16-27 Jan	39	60	37	104	82.7
28-29 Jan	46	57	35	97	77.8
30 Jan-5 Feb	53	56	34	93	75.3
6-12 Feb	60	55	34	88	72.8
13-26 Feb	74	45	28	69	58.0
27 Feb-8 Mar	84	40	25	59	51.0

* = N

TABLE 2. (Set II) Weighted average and pe cent survival of diapausing gravid, and non-diapausing, non-bloodfed and non-diapausing bloodfed C lex pipiens at Ft. Washington, Md, during winte. f 1978-9

Date	t	Weighted Average % Survival			
		Lab-Reared Diapausing Bloodfed Gravid S_A	Lab Reared Non-diap. Non-blood S_B	Lab Reared Non-diap. Bloodfed S_C	Weighted % Survival
14 Dec	0	43*	94*	86*	100
15-18 Dec	4	37	91	85	95.5
19-25 Dec	11	35	84	85	91.5
26 Dec-1 Jan	18	26	76	79	81.2
2-8 Jan	25	24	67	71	72.6
9-15 Jan	32	20	57	64	63.2
16-22 Jan	39	20	45	51	52.0
23-29 Jan	46	14	35	38	39.0
30 Jan-5 Feb	53	10	22	25	25.6
6-12 Feb	60	6	15	13	15.2
13-26 Feb	74	4	9	5	8.1
27 Feb-8 Mar	84	3	2	0	2.2

* = N

TABLE 3. EEE virus antibody conversions of quail in sentinel cages

Exposure Period	Habitat											
	Upland Sites						Swamp Sites					
	Bus	Cave	Fox	Tunnel	Total	%	Q	Railroad	Total	%	Grand Total	Over-all %
30 April-14 May	0/2[1,2]	0/1	0/1	0/2	0/6	0	0/2	0/2	0/4	0	0/10	0
14-28 May	0/3	0/3	0/3	0/3	0/12	0	0/2	0/3	0/5	0	0/17	0
28 May-12 June	0/3	0/3	0/3	0/3	0/12	0	0/3	1/2	1/5	20	1/17	6
12-26 June	2/2	2/2	0/0	3/3	7/7	100	3/3	2/2	5/5	100	12/12	100
26 June-10 July	3/3	1/2	2/3	0/3	6/11	54	1/3	3/3	4/6	67	10/17	59
10-25 July	4/5	5/5	4/5	3/4	16/19	84	5/5	4/4	9/9	100	25/28	89
TOTAL (%)	9/18(50)	8/16(50)	6/15(50)	6/18(50)	29/67	43	9/18(50)	10/16(62)	19/34	56	48/101	49

[1]Total number of quail exhibiting antibody following exposure/number of susceptible quail exposed.

[2]Positive antibody titer was determined by an $\geq$ 80% reduction at a 1:20 dilution of the sera assayed an BHK 21-15 monolayers

TABLE 4 EEE virus antibody conversions of quail in net traps

Exposure Period	Habitat											
	Upland Sites						Swamp Sites					
	Bus	Cave	Fox	Tunnel	Total	%	Q	Railroad	Total	%	Grand Total	Overall %
30 Apr-[illegible] May	0/1[1,2]	0/0	0/0	0/1	0/2	0	0/1	0/1	0/2	0	0/4	0
[illegible]-25 May	0/2	0/3	0/2	0/3	0/10	0	0/3	0/3	0/6	0	0/16	0
[illegible] May-12 Jun	0/2	0/1	0/2	0/2	0/7	0	0/2	0/2	0/4	0	0/11	0
12-26 Jun	1/2	1/1	1/2	1/2	4/7	57	2/2	2/2	4/4	100	8/11	73
26 Jun-10 Jul	0/2	1/2	0/2	0/1	1/7	14	0/2	0/2	0/4	0	1/11	9
10-25 Jul	0/5	0/5	3/5	0/5	3/20	15	0/5	3/6	3/11	27	6/31	19
TOTAL (%)	1/14(7)	2/12(17)	4/13(31)	1/14(7)	8/53	15	2/15(13)	5/16(31)	7/31	22	15/84	18

[1]Total number of quail exhibiting antibody following exposure/number of susceptible quail exposed.

[2]Positive antibody titer was determined by an $\geq$ 80% reduction at a 1:20 dilution of the sera assayed on BHK 21-15.

TABLE 5. Experimental Infection of Goats with KEY and JC Viruses

	Group Number		
	1	2	3
Virus	JC high	JC low	KEY
Dose	$2.9X10^4$ PFU+/ml	$1X10^2$ PFU/ml	$9.3X10^4$ PFU/ml
Number goats	6	4	6
Viremia	Neg	Neg	Neg

TABLE 6. JAMESTOWN CANYON (JC) HIGH DOSE INOCULATED GOATS

P.I. Serum Day	Goat Numbers											
	19		24		25		26		52		54	
	Virus											
	JC	KEY	JC	KEY	JC	KEY	JC	KEY	JC	KEY	JC	KEY
10	900*	160	1280	35	2000	200	2200	<10	2500	320	1900	200
20	1280	45	450	20	850	50	1280	40	1000	55	540	54
30	640	30	250	15	900	85	370	18	640	40	400	25
45	400	20	230	<10	200	35	220	15	500	40	220	<10
60	230	10	400	<10	160	10	250	10	1200	50	360	35
90	320	10	140	<10	100	10	130	10	540	40	230	24

*Reciprocal dilution exhibiting 50% reduction.

TABLE 7. JAMESTOWN CANYON (JC) LOW DOSE INOCULATED GOATS

P.I. Serum Day	Goat Numbers 22		27		33		51	
	Virus JC	KEY	JC	KEY	JC	KEY	JC	KEY
10	30*	<10	300	35	120	18	75	16
20	2100	130	280	200	290	54	390	75
30	1280	40	270	50	180	35	180	25
45	No sera		250	70	64	30	120	10
60	320	<10	350	56	100	85	160	<10
90	230	<10	160	22	90	56	100	<10

*Reciprocal dilution exhibiting 50% reduction.

TABLE 8. KEYSTONE (KEY) HIGH DOSE INOCULATED GOATS

P.I. Serum Day	Goat Numbers 13		18		23		28		31		53	
	Virus											
	JC	KEY	JC	KEY	JC	KEY	JC	KEY	JC	KEY	JC	KEY
10	<10*	10	20	420	10	640	<10	160	12	200	<10	10
20	<10	60	<10	160	20	1100	<10	210	<10	160	80	240
30	<10	15	<10	89	<10	500	<10	100	<10	95	10	90
45	<10	<10	<10	94	<10	220	<10	120	<10	82	<10	110
60	<10	<10	<10	90	<10	280	<10	120	<10	72	<10	40
90	<10	<10	<10	56	<10	55	<10	41	<10	45	<10	140

Reciprocal dilution exhibiting 50% reduction.

TABLE 9. Female mosquitoes tested for virus and comparison of species composition collected from light and Magoon traps in the Pocomoke Cypress Swamp, MD 1978

Species[1]	Light Trap	Magoon	Total
Aedes atlanticus	292	93	385
Ae. canadensis	66	2397	2463
Ae. cantator	27	6	33
Ae. triseriatus	89	129	218
Ae. sollicitans	36	142	178
Ae. vexans	0	2	2
Ae. spp.[2]	3	178	181
Anopheles bradleyi/crucians	29	157	186
An. punctipennis	1	1	2
Coquillettidia perturbans	2	32	34
Culiseta melanura	23	1	24
Culex salinarius	6854	1751	8605
Cx. spp.[2]	0	22	22
Psorophora confinnis	0	9	9
Ps. ferox	0	15	15
Ps. howardii	0	3	3
Orthopodomyia signifera	1	0	1
Total	7423	4938	12361

[1]species listed in alphabetical

[2]damaged specimens

TABLE 10. Mosquitoes collected in the Chincoteague National Wildlife Refuge, 1978[1].

Freshwater swamp	No. collected	Pine forest	No. collected
Aedes atlanticus	26	Aedes atlanticus	4
Ae. canadensis	2978	Ae. canadensis	1153
Ae. cantator	3	Ae. cantator	57
Ae. fitchii	19		
Ae. fulvus pallens	1		
Ae. grossbecki	7		
Ae. infirmatus	5249	Ae. infirmatus	1061
Ae. sollicitans	219	Ae. sollicitans	257
Ae. taeniorhynchus	5627	Ae. taeniorhynchus	1126
Ae. triseriatus	3	Ae. triseriatus	1
Ae. trivittatus	1		
Ae. vexans	11	Ae. vexans	19
Anopheles bradleyi/crucians	151	Anopheles bradleyi/crucians	124
An. punctipennis	6		
An. quadrimaculatus	1269	An. quadrimaculatus	174
Culex restuans	21		
Cx. salinarius	2702	Culex salinarius	6514
		Cx. territans	1
Culiseta inornata	3	Culiseta inornata	6
Psorophora ciliata	2		
Psorophora ferox	3450	Psorophora ferox	14
Ps. howardii	17		
Uranotaenia sapphirina	1	Uranotaenia sapphirina	6
TOTAL 22 species	21766	15 species	10517

[1]Species listed in alphabetical order

TABLE 11. Female mosquitoes tested for virus isolation from the Chincoteague National Wildlife Refuge, 1978

Species	No. tested	
	Pools	Specimens
Aedes infirmatus	235	6308*
Ae. atlanticus	15	30
Ae. canadensis	7	60
Ae. sollicitans	8	100
Ae. taeniorhynchus	52	1989
Ae. triseriatus	1	1
Ae. trivittatus	1	1
Ae. vexans	7	13
Ae. spp.[1]	8	63
Anopheles bradleyi/crucians	8	112
An. quadrimaculatus	15	238
An. spp.[1]	1	47
Culex salinarius	115	4571
Psorophora ciliata	1	1
Ps. ferox	15	485
Ps. howardii	1	13
Ps. spp.[1]	6	7
Uranotaenia sapphirina	1	4
	498	14043

[1]damaged specimens

*1 KEY virus isolation for a minimum field infection rate of 1/6308

Fig. 1

SURVIVAL OF CULEX PIPIENS IN DECATOR BUNKER AT FORT WASHINGTON, MARYLAND DURING WINTER OF 1978-79

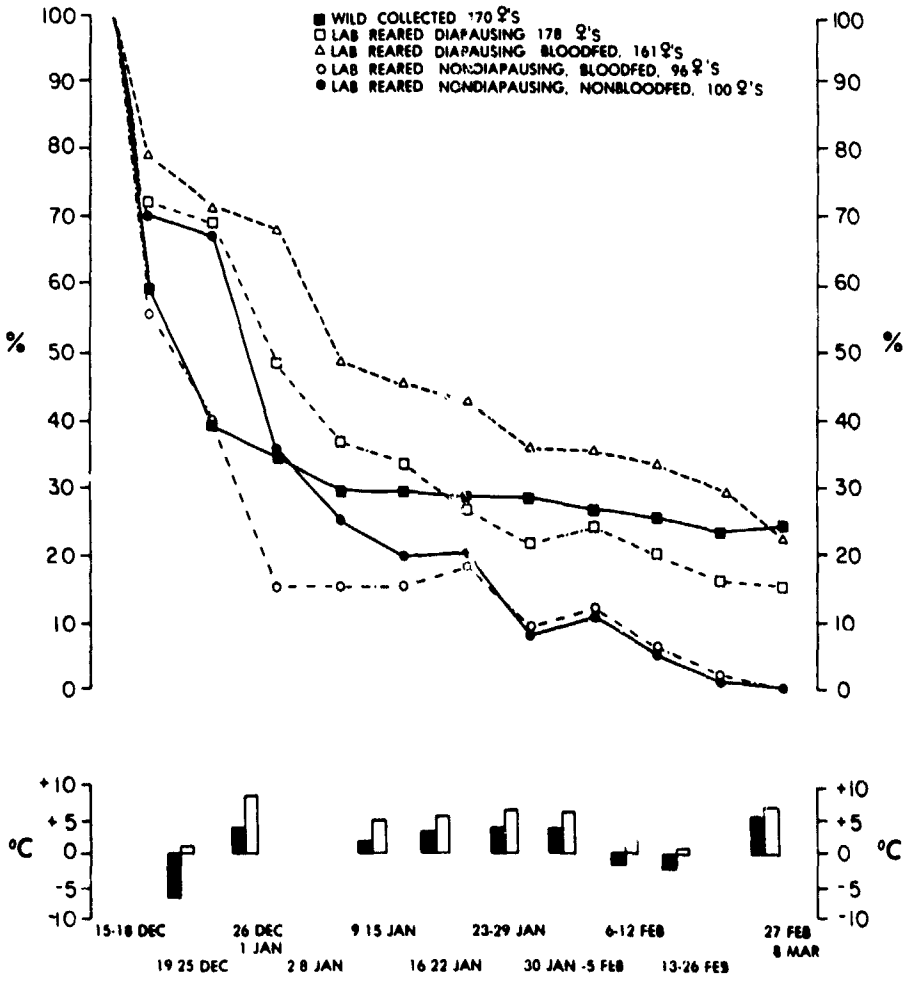


Fig. 2

SURVIVAL OF CULEX PIPIENS IN DECATOR CAGE AT FORT WASHINGTON, MARYLAND DURING WINTER OF 1978 - 79

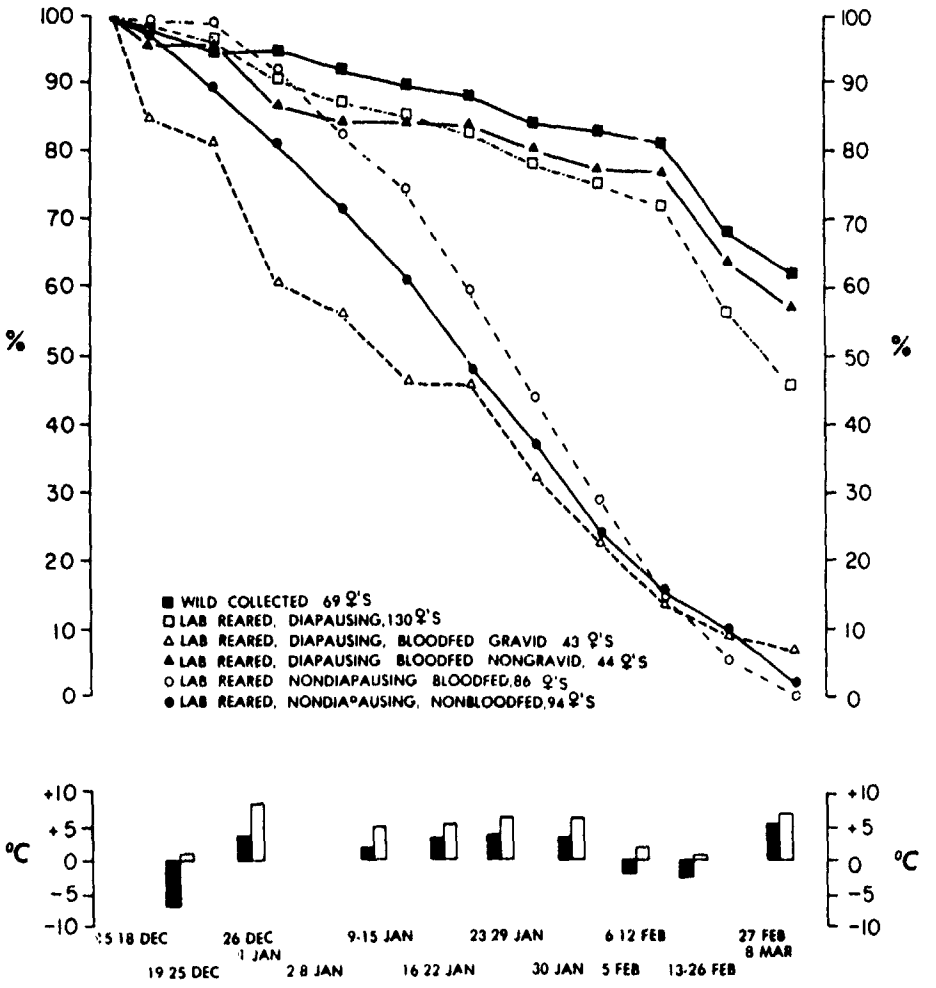


Fig. 3

SURVIVAL OF CULEX PIPIENS IN DECATOR CAGE AT FORT WASHINGTON, MARYLAND DURING WINTER OF 1978-79

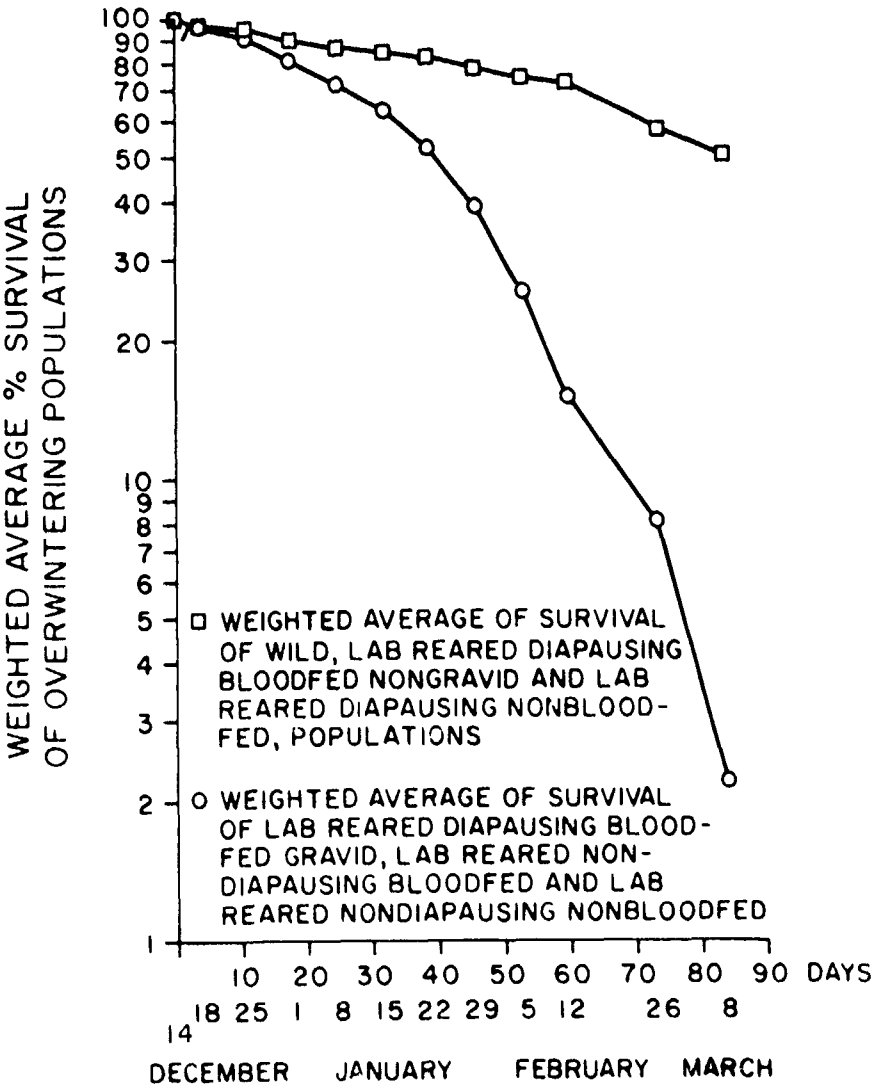


Fig. 4

MAP OF POCOMOKE CYPRESS SWAMP SHOWING LOCATION OF TRAPPING SITES

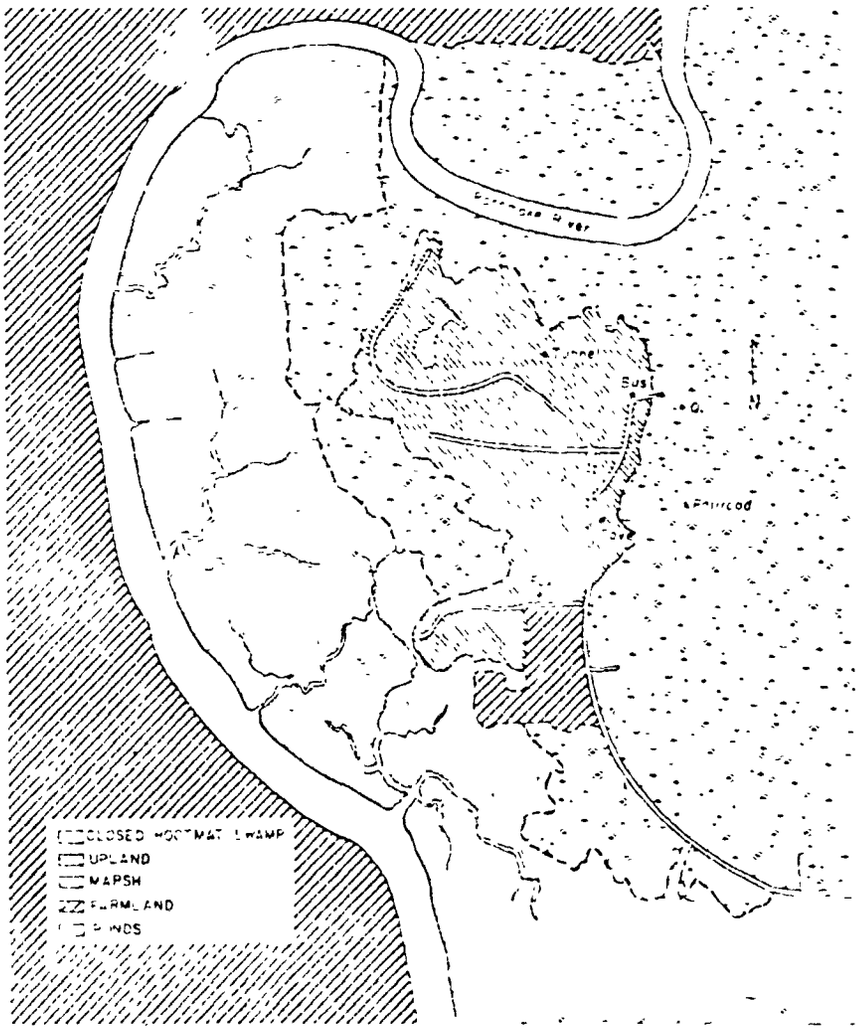


Fig. 5

SEASONAL DISTRIBUTION OF EEE NEUTRALIZING ANTIBODY IN QUAIL

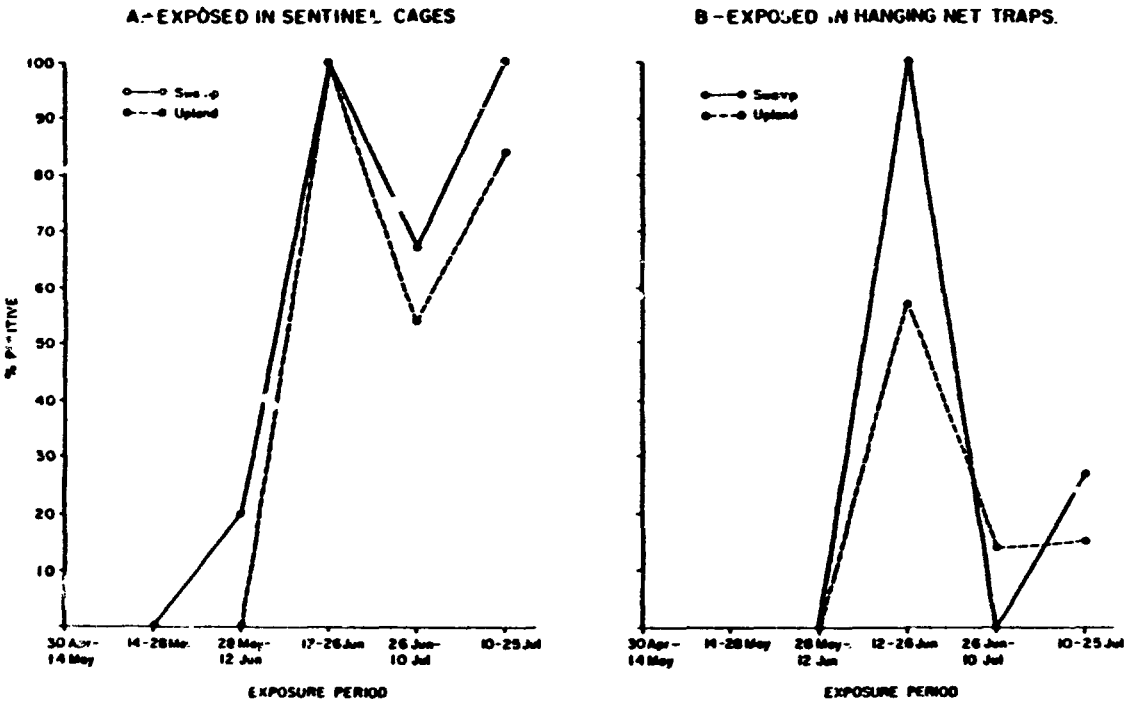


Fig. 6

MAP OF CHINCOTEAGUE NATIONAL WILDLIFE REFUGE SHOWING LOCATION OF SENTINEL CAGES AND LIGHT TRAPS

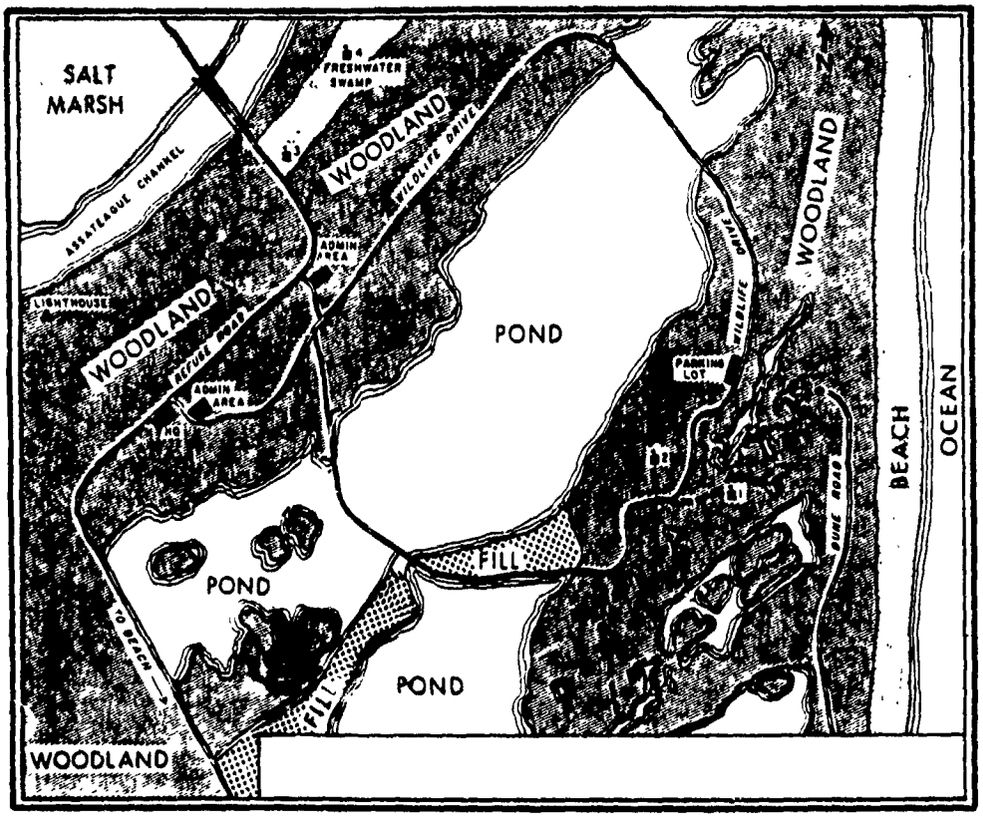


Fig. 7

ADULT FEMALE AEDES INFIRMATUS PER TRAP NIGHT AS SAMPLED BY CDC LIGHT TRAPS AND KEYSTONE VIRUS ISOLATIONS AND SEROCONVERSIONS, ASSATEAGUE ISLAND, VA 1978

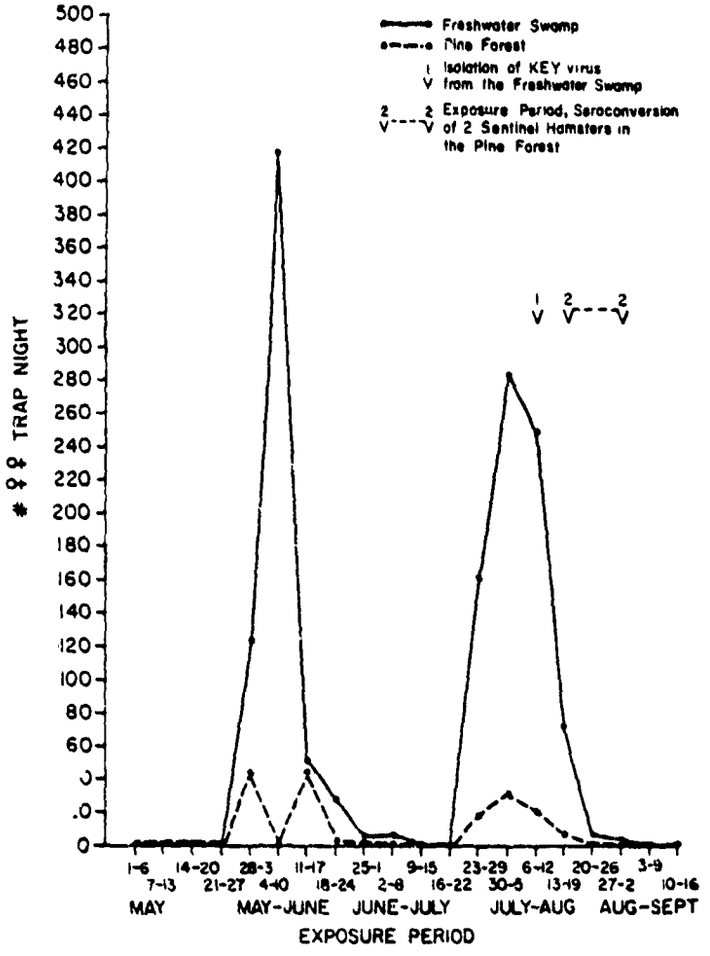
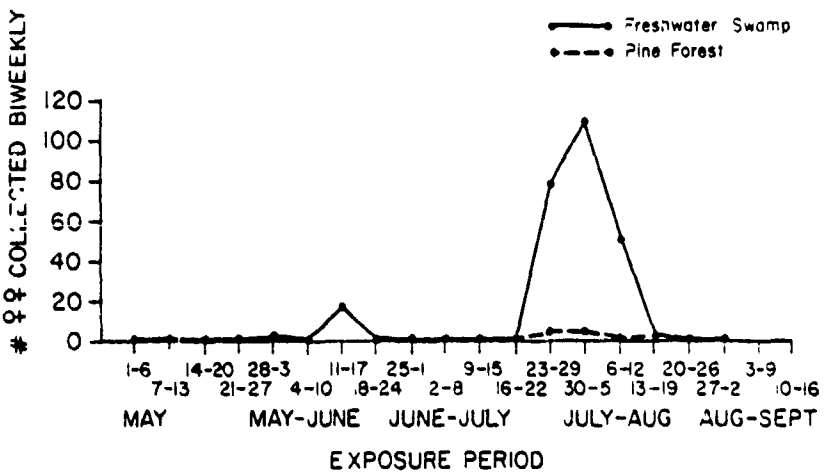


Fig. 8

ADULT FEMALE AEDES INFIRMATUS COLLECTED BIWEEKLY AS SAMPLED BY HAMSTER-BAITED NET TRAPS, ASSATEAGUE ISLAND, VA 1978



Captions

Fig. 1. Survival of Culex pipiens in Decator Bunker at Fort Washington, Maryland during winter of 1978-79.

Fig. 2. Survival of Culex pipiens in Decator Cage at Fort Washington, Maryland during winter of 1978-79.

Fig. 3. Survival of Culex pipiens in Decator Cage at Fort Washington, Maryland during winter of 1978-79.

Fig. 4. Map of Pocomoke Cypress Swamp showing location of trapping sites.

Fig. 5. Seasonal distribution of EEE neutralizing antibody in quail.

Fig. 6. Map of Chincoteague National Wildlife Refuge showing location of Sentinel cages and light traps.

Fig. 7. Adult female Aedes infirmatus per trap night as sampled by CDC light traps and Keystone virus isolations and seroconversions, Assateague Island, VA 1978.

Fig. 8. Adult female Aedes infirmatus collected biweekly as sampled by hamster-baited net traps, Assateague Island, VA 1978.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6448	79 10 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISB'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	122
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Basic Pharmacological Studies

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
68 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 79	3.0	137
CURRENT FISCAL YEAR 80	3.5	212

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME: KORTE, CPT D. W. Jr.

NAME: SCHUSTER, MAJ B.

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pharmacology; (U) Medicinals; (U) Drugs; (U) Shock; (U) Toxicity

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Research is directed toward investigating special areas of the pharmacology of potential drugs of military importance, their interactions, their mechanisms of action, and the development, characterization and improvement of animal models for defining specific applicable parameters.

24. (U) Drugs are tested in animal models specifically designed to pinpoint mechanisms of pharmacological effects, effects on physiological responses, and effects on protozoan systems. In vitro models are being used as well.

25. (U) 78 10 - 79 09 The acute cardiovascular and respiratory activity of intravenous Pentostam was compared in restrained versus unrestrained rabbits. Little difference was seen in responses between the two dosed groups in either heart rate or respiratory rate. The restrained rabbits showed more sensitivity to the drug in the amplitudes of the R and T waves and in the QTc interval. Conversely the mean arterial pressure was a more sensitive indicator in the unrestrained animals. A special laboratory has been set up to study the pharmacological effects of candidate antiparasitic drugs upon coronary blood flow and myocardial energetics. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 122 Basic pharmacological studies

Investigators:
Principal: Melvin H. Heiffer, Ph.D.
Associate: CPT D. Korte, MAJ B. Schuster, Dr. H. Lowensohn, Dr. R. Rozman, SP4 C. Basamania, SP4 J. Wilson

1. Description.

The basic research efforts of the department are directed towards several major areas. They are: the pharmacology of promising medicinal agents and of certain toxic substances; drug interactions with, and the nature of, adrenergic receptors; further characterization of new drug delivery techniques; and the development of new or modification of existing techniques to characterize drug effects.

Appropriate pharmacological, physiological, electrophysiological and biochemical studies are conducted both in vivo and in vitro. Many of these studies emphasize interactions of potential drugs with standard pharmacological agents. An important feature is ready access to the vast inventory of serially related and diverse chemicals which can be used in detailed studies of the nature of drug interactions with biological systems.

2. The cardiorespiratory effects of administering WR 229,870 (Pentostam®) to rabbits for ten consecutive days.

a. Background:

The Army is currently evaluating the efficacy of several dosing regimens of WR 229,870 (Pentostam®) for the treatment of leishmaniasis. One aspect of this evaluation involves defining the potential cardiovascular toxicity of the different dosing regimens of WR 229,870. Previous studies have shown that bolus injections of WR 229,870 equivalent to 40 mg of antimony (Sb) per kg produced hypotension and bradycardia plus increases in T and R wave amplitude and corrected QT interval (QTc) of the electrocardiogram when administered to pentobarbital-anesthetized rabbits (Korte, 1978). These effects were observed to be both transient and dependent upon the rate at which the WR 229,870 was administered.

One of the dosing regimens currently being evaluated by the Army is a 10 day continuous infusion of WR 229,870 versus the standard course of a daily injection of WR 229,870 for 10 consecutive days. To determine the potential cardiorespiratory toxicity of the two dosing regimens, a study has been designed in which rabbits will be given equivalent doses of WR 229,870 by either a daily injection or a continuous infusion for 10 consecutive days. On day 11 the rabbits will be anesthetized and challenged with a bolus injection of WR 229,870. The WR 229,870 will be injected or infused into the jugular vein via a cannula which is positioned subcutaneously so that it exits the body on the dorsal aspect of the neck. These rabbits will need to be restrained laterally (permitting only forward and backward movement along one axis) to prevent the cannula from being occluded or damaged by the rabbit's activity. This report presents the results of a preliminary study undertaken to determine whether lateral restraint of the rabbits for 10 days would affect the subsequent response of the rabbits to bolus injections of WR 229,870 on day 11. In addition, these studies will also permit evaluation of a ketamine/xylazine combination for induction of anesthesia and evaluation of a new lot of WR 229,870 which will be used for the 10 day study.

b. Methods and materials:

Ten male New Zealand white rabbits (2.6-3.3 kg) from the Walter Reed colony were randomly assigned to one of two groups each containing five rabbits. The rabbits in the first group (unrestrained) were placed in standard cages (22" x 14" x 9") and allowed food (Purina rabbit chow, Ralston Purina Co.) and water ad libitum. The rabbits in the second group (restrained) were placed in cages with adjustable baffles which were positioned to prevent lateral movement of the rabbit (22" x 4½" x 9"). These rabbits were also allowed food and water ad libitum. The rabbits were caged in this manner for 10 days.

On day 11, the rabbits were anesthetized with a combination of ketamine hydrochloride (Ketaset®, Bristol Laboratories), 35 mg/kg, and xylazine hydrochloride (Rompun®, Bayuet Division, Cutter Laboratories, Inc.), 5 mg/kg, given intravenously. The left jugular vein was cannulated for drug injection and the left common carotid artery was cannulated for recording arterial pressure. A tracheotomy was performed and respiratory rate monitored via a pneumotachometer. Leads I, II and III of the electrocardiogram were recorded and heart rate was derived from Lead III via an integrating cardiotachograph. The variables were monitored by and recorded on a Hewlett-Packard thermal recording system (Model 7758A). Measurement of drug effects

were made at a chart speed of 50 mm/sec. Temperature was monitored via a rectal thermistor probe and maintained at 37°C by use of thermostatically controlled heating pad. The above surgical procedures and electronic calibrations took approximately 30 min at which time a 12.5 mg/kg dose of sodium pentobarbital (V-Pento, A.J. Buck & Co.) was administered slowly over a one to 2 min period to supplement the ketamine/xylazine anesthesia.

The five rabbits in each group were then allowed to stabilize for a minimum of 15 min prior to the first bolus injection of WR 229,870 (Pentostam®, Lot 98440, The Wellcome Foundation, Ltd.). Each rabbit then received a bolus injection of WR 229,870 equivalent to 10 mg Sb/kg followed by 20 mg Sb/kg, 40 mg Sb/kg, 80 mg Sb/kg, 160 mg Sb/kg and then 320 mg Sb/kg at 10 min intervals. The maximum response obtained following each injection of WR 229,870 was compared with its respective preinjection baseline value using the paired t test of significance (Zar, 1974) with a p value of less than 0.05 considered significant.

c. Results:

Dose-response studies of WR 229,870 in the anesthetized rabbit indicate that doses of 320 mg Sb/kg produced a significant decrease in heart rate in both restrained and unrestrained rabbits (Table 1). WR 229,870 produced dose-related decreases in mean arterial pressure (MAP) beginning with the 20 mg Sb/kg dose in the unrestrained group and the 40 mg Sb/kg dose in the restrained group (Table 2). The maximum decrease in MAP observed with the 320 mg Sb/kg dose was approximately 56% of control in both the restrained and unrestrained groups. Although the 10 mg Sb/kg dose of WR 229,870 produced a significant increase in the respiratory rate in the unrestrained group, the overall effect of WR 229,870 on respiratory rate in both groups was negligible (Table 3). One of the unrestrained rabbits was deleted from these calculations due to difficulties in its evaluation because it exhibited a rhythmic pattern of respiration with approximately 50 sec of apnea followed by a 10 sec burst of respiration. This animal had a relatively low initial respiratory rate of 22/min suggesting a sensitivity to the respiratory depressant actions of the anesthesia.

WR 229,870 produced changes in the electrocardiogram which were especially prominent in Leads II and III. These changes included an increase in amplitude of the T and R waves plus an increase in the QTc interval. A quantitative description

of the changes observed in Lead II for both the restrained and unrestrained groups appears in Tables 4-6. The restrained rabbits were more sensitive than the unrestrained rabbits to WR 229,870-induced changes in the electrocardiogram. For example, the 20 mg Sb/kg dose of WR 229,870 produced a significant increase in the amplitude of the T wave, a response not observed in the unrestrained rabbits until the 80 mg Sb/kg dose of WR 229,870 had been administered (Table 4). However, 320 mg Sb/kg of WR 229,870 produced equivalent increases of 240% and 734% in T wave amplitude in the restrained and unrestrained rabbits respectively. A significant increase in the amplitude of the R wave was also recorded for the restrained group at a dose of 20 mg Sb/kg of WR 229,870 (Table 5). Even though a significant increase in amplitude of the R wave was first observed following the 40 mg Sb/kg dose in unrestrained rabbits, the maximum increase in amplitude produced by 320 mg Sb/kg of WR 229,870 was a 190% increase in unrestrained rabbits versus a 170% increase in restrained rabbits. The QTc interval was the most sensitive electrocardiographic index measured in the restrained rabbits since doses as low as 10 mg Sb/kg (the lowest dose given) produced a significant increase (Table 6). The QTc interval of the unrestrained rabbits was not significantly increased until administration of the 40 mg Sb/kg dose of WR 229,870. The enhanced responsiveness of the unrestrained rabbits was also observed at higher doses; 320 mg Sb/kg produced a 165% increase in the QTc interval in the restrained rabbits and a 143% increase in the unrestrained group.

d. Discussion:

The results of this study indicate that ten days of restraint prior to bolus injections of WR 229,870 on Day 11 caused no qualitative differences in the cardiorespiratory responses of the rabbit since the WR 229,870 injections produced decreases in heart rate and MAP, no consistent changes in the respiratory rate, and increases in the R and T wave amplitudes and QTc interval of the electrocardiogram in both the restrained and unrestrained rabbits. The quantitative differences observed in the responses of the restrained and unrestrained rabbits to injections of WR 229,870 (the WR 229,870 was more potent in increasing the electrocardiographic indices of the restrained group) might possibly be attributed to individual variation and the relative lack of resolution of the electrocardiographic measurements obtained at a 50 mm/sec recording speed. These differences are expected to be minimal in the ten day study comparing the constant infusion versus daily injection dosing regimens because both groups of rabbits will be restrained. Furthermore, the number of rabbits in each

group will be increased to eight which should reduce the effect of individual variation. In addition, the electrocardiographic measurements will be obtained from 200 mm/sec recordings.

A second function of the study was to evaluate a new lot of WR 229,870, which will be used in the 10 day comparison study. The new lot of WR 229,870 used in this study produced cardiorespiratory effects equivalent to those reported for previous lots of WR 229,870 (Korte, 1978). Quantitative differences in the responses are difficult to interpret because the two studies were not performed simultaneously and in the present study a ketamine/xylazine combination was used to anesthetize the rabbits, with a small supplemental dose of sodium pentobarbital, while previous work with WR 229,870 had employed sodium pentobarbital both to induce and maintain the level of anesthesia.

Previous studies of WR 229,870 in rabbits utilized sodium pentobarbital anesthesia (Korte, 1978). This method of anesthesia had proven to be unsatisfactory. The primary reason was the difficulty in titrating the dose of sodium pentobarbital required to produce the desired level of anesthesia. A second reason was the high baseline heart rates of the rabbits. The differences in individual sensitivity of the rabbits to sodium pentobarbital plus the relatively small range of anesthetic doses of sodium pentobarbital in the rabbit between the non-effect and lethal doses caused considerable difficulty in producing a consistent level of anesthesia. However, the ketamine/xylazine combination, which was administered intramuscularly, consistently produced a surgical level of anesthesia within 10 min of injection. This level of anesthesia persisted for 30-45 min at which time a small supplemental dose of sodium pentobarbital was administered.

Sodium pentobarbital significantly increased the heart rate of trained dogs due to vagolytic mechanisms (Cox, 1972). Correspondingly, the average heart rate recently reported for 15 rabbits anesthetized with sodium pentobarbital was 289.7 bpm (Korte, 1978). This relatively high heart rate makes interpretation of the electrocardiographic indices most difficult. The baseline heart rate for the unrestrained rabbits anesthetized with the ketamine/xylazine combination was 224.9 bpm (Table 1), a rate approximately that reported for unanesthetized rabbits.

3. <u>The direct effects of primaquine (WR 2975) upon cardiac function following infusion into the left circumflex artery of the dog: comparison to quinidine</u>.

a. Background:

Earlier this year the protocol for this study received approval. Motivation for this study stems from two basic questions. First, in screening candidate drugs for cardiovascular toxicity following intravenous injection, the hemodynamic events obtained present an equivocal picture. A clear-cut estimate of depressed myocardial function or pharmacological dilation of the peripheral vascular bed or a combination of responses has evaded cogent analysis to this point. Secondly, quinidine administration reduced myocardial efficiency (Moe and Abildskov, 1965; Rowe et al., 1957). Should primaquine demonstrate similar properties to quinidine, future consideration concerning changing of prophylactic doses to troops might follow. An abbreviated form of this protocol might evolve as a standard method for cardiac testing for future candidate antiparasitic drugs.

b. Discussion:

The major responsibility of the incumbent principal investigator involved accrual of appropriate laboratory equipment, standardization of technical instrumentation, development of procedures and design and manufacture of experimental devices not available from commercial sources. An entirely new laboratory has been devised to study the pharmacological effects of candidate antiparasitic drugs upon the coronary blood flow and myocardial energetics.

Part of this task has involved the modification in design and transvascular implantation of miniature catheters in the right coronary artery by the technique of Herd and Barger (1964). Catheter development consists of refinements made in designs previously used in coronary circulation studies (Elliot et al., 1968; Khouri et al., 1971). We plan to use these catheters in the left circumflex coronary artery to inject primaquine or quinidine directly into the left myocardium and in the coronary sinus to obtain left myocardial venous blood for oxygen determinations. Use of the catheters allows for selective blocking, via the S-A node artery, of the S-A node by acetylcholine without increasing total body responses to acetylcholine in chronic conscious animals. The overall studies permit pressure-flow hemodynamic studies of major organ systems during prolonged asystolic periods.

A prototype stainless steel ventricular cannula has been devised and tested. This cannula will facilitate monitoring

left ventricular pressures and the rate of contraction during drug testing. These cannulae are currently being manufactured for us.

Table 1

A Comparison of the Effect of 10 Days in Restraint Caging Versus 10 Days in Normal Caging on the Heart Rate of Anesthetized Rabbits Following Bolus Administration of WR 229,870 on Day 11

Dose of WR 229,870 (mg equiv Sb/kg)	Heart Rate (beats per minute)			
	Unrestrained		Restrained	
	Con[a]	Exp[b]	Con[a]	Exp[b]
10	224.9[c] ± 12.0	219.4 ± 13.7	241.1 ± 8.2	236.9 ± 6.6
20	220.7 ± 15.5	217.3 ± 15.0	242.9 ± 7.9	235.5 ± 8.1
40	221.0 ± 18.0	211.8 ± 20.9	244.9 ± 7.9	240.2 ± 11.3
80	221.7 ± 19.0	218.0 ± 21.6	239.4 ± 8.9	223.8 ± 9.9
160	235.5 ± 20.7	212.4 ± 23.7	245.4 ± 9.5	226.3 ± 12.0
320	236.2 ± 19.4	205.5[d] ± 22.4	243.2 ± 8.8	198.9[d] ± 12.1

[a]"Con" represents baseline control values immediately prior to WR 229,870 injection.

[b]"Exp" represents the maximal response of the variable to WR 229,870 injection.

[c]Values represent mean ± SEM for 5 rabbits.

[d]Significantly different from appropriate baseline value (paired t test, 5% level of significance).

Table 2

A Comparison of the Effect of 10 Days in Restraint Caging Versus 10 Days in Normal Caging on the Mean Arterial Pressure of Anesthetized Rabbits Following Bolus Administration of WR 229,870 on Day 11

Dose of WR 229,870 (mg equiv Sb/kg)	Mean Arterial Pressure (mmHg)			
	Unrestrained		Restrained	
	Con[a]	Exp[b]	Con[a]	Exp[b]
10	57.6[c] ± 3.4	56.0 ± 3.2	60.2 ± 4.2	59.2 ± 3.8
20	53.6 ± 2.7	49.0[d] ± 2.8	61.6 ± 5.1	62.4 ± 5.1
40	56.2 ± 3.4	47.6[d] ± 3.8	63.2 ± 6.3	56.2[d] ± 5.3
80	55.6 ± 4.4	43.2[d] ± 3.7	61.2 ± 4.4	50.2[d] ± 5.4
160	54.8 ± 3.2	34.0[d] ± 1.2	57.2 ± 5.0	39.2[d] ± 7.0
320	60.8 ± 3.4	27.0[d] ± 2.1	71.8 ± 6.4	31.6[d] ± 4.9

[a]"Con" represents baseline control values immediately prior to WR 229,870 injection.

[b]"Exp" represents the maximal response of the variable to WR 229,870 injection.

[c]Values represent mean ± SEM for 5 rabbits.

[d]Significantly different from baseline (paired t test, 5% level of significance).

Table 3

A Comparison of the Effect of Ten Days in Restraint Caging Versus Ten Days in Normal Caging on the Respiratory Rate of Anesthetized Rabbits Following Bolus Administration of WR 229,870 on Day 11

Dose of WR 229,870 (mg equiv Sb/kg)	Respiratory Rate (#/min)[a]			
	Unrestrained		Restrained	
	Con[b]	Exp[c]	Con[b]	Exp[c]
10	48.9 ± 3.3	56.2[d] ± 3.2	57.3 ± 6.4	58.0 ± 5.9
20	73.3 ± 12.4	71.4 ± 9.9	68.0 ± 10.4	71.1 ± 9.7
40	67.2 ± 5.3	71.4 ± 6.6	71.9 ± 10.9	76.5 ± 12.5
80	66.8 ± 7.6	68.0 ± 9.3	82.2 ± 13.4	83.7 ± 14.0
160	69.1 ± 7.9	69.1 ± 1.9	78.8 ± 12.8	67.9 ± 8.7
320	73.0 ± 9.9	76.6 ± 15.0	69.5 ± 13.8	73.5 ± 11.0

[a]Values in unrestrained group represent mean ± SEM of 4 rabbits while values in restrained group represent mean ± SEM of 5 rabbits.

[b]"Con" represents the baseline control values immediately prior to WR 229,870 injection.

[c]"Exp" represents the maximal response of the variable to WR 229,870 injection.

[d]Significantly different from baseline (paired t test, 5% level of significance).

Table 4

A Comparison of the Effect of Ten Days in Restraint Caging Versus Ten Days of Normal Caging on the T Wave Amplitude Following Bolus Injections of WR 229,870 in the Anesthetized Rabbit on Day 11

Dose of WR 229,870 (mg equiv Sb/kg)	T Wave Amplitude (mV)			
	Unrestrained		Restrained	
	Con[a]	Exp[b]	Con[a]	Exp[b]
10	0.29[c] ± .03	0.31 ± .02	0.28 ± .02	0.30 ± .02
20	0.29 ± .02	0.39 ± .05	0.29 ± .02	0.45[d] ± .04
40	0.31 ± .03	0.41 ± .04	0.29 ± .02	0.56[d] ± .07
80	0.28 ± .02	0.44[d] ± .04	0.29 ± .02	0.64[d] ± .03
160	0.29 ± .03	0.57[d] ± .05	0.30 ± .02	0.71[d] ± .02
320	0.29 ± .02	0.68[d] ± .08	0.32 ± .01	0.77[d] ± .04

[a]"Con" represents baseline control values immediately prior to WR 229,870 injection.

[b]"Exp" represents maximal response of variable to WR 229,870 injection.

[c]Values represent mean ± SEM for 5 rabbits.

[d]Significantly different from baseline (paired t test, 5% level of significance).

Table 5

A Comparison of the Effect of Ten Days in Restrained Caging Versus Ten Days of Normal Caging on the R Wave Amplitude Following Bolus Administration of WR 229,870 in the Anesthetized Rabbit on Day 11

Dose of WR 229,870 (mg equiv Sb/kg)	R Wave Amplitude (mV)			
	Unrestrained		Restrained	
	Con[a]	Exp[b]	Con[a]	Exp[b]
10	0.84[c] ± .09	0.86 ± .08	0.80 ± .06	0.83 ± .05
20	0.80 ± .07	0.84 ± .08	0.80 ± .05	1.00[d] ± .08
40	0.82 ± .08	0.98[d] ± .13	0.80 ± .05	1.05[d] ± .06
80	0.82 ± .08	1.16[d] ± .14	0.81 ± .06	1.21[d] ± .05
160	0.86 ± .09	1.33[d] ± .16	0.81 ± .06	1.24[d] ± .07
320	0.86 ± .09	1.64[d] ± .19	0.83 ± .05	1.41[d] ± .03

[a]"Con" represents baseline control values immediately prior to WR 229,870 injection.

[b]"Exp" represents maximal response of variable to WR 229,870 injection.

[c]Values represent mean ± SEM for 5 rabbits.

[d]Significantly different from baseline (paired t test, 5% level of significance).

Table 6

A Comparison of the Effect of Ten Days in Restraint Caging Versus Ten Days of Normal Caging on the QTc Interval Following Bolus Administration of WR 229,870 to the Anesthetized Rabbit on Day 11

Dose of WR 229,870 (mg equiv Sb/kg)	QTc Interval (msec)			
	Unrestrained		Restrained	
	Con[a]	Exp[b]	Con[a]	Exp[b]
10	0.282[c] ± .009	0.288 ± .010	0.266 ± .004	0.282[d] ± .006
20	0.285 ± .009	0.295 ± .009	0.267 ± .004	0.297[d] ± .009
40	0.284 ± .008	0.342[d] ± .018	0.266 ± .003	0.328[d] ± .010
80	0.288 ± .011	0.386[d] ± .014	0.273 ± .005	0.354[d] ± .013
160	0.308 ± .015	0.399[d] ± .014	0.277 ± .009	0.395[d] ± .007
320	0.312 ± .018	0.446[d] ± .024	0.284 ± .009	0.470[d] ± .017

[a]"Con" represents baseline control values immediately prior to WR 229,870 injection.

[b]"Exp" represents maximal response of variable to WR 229,870 injection.

[c]Values represent mean ± SEM for 5 rabbits.

[d]Significantly different from baseline (paired t test, 5% level of significance).

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 122 Basic pharmacological studies

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6445	79 10 01	DD-DRAE(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☐ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	123
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)*

(U) Biochemical Research on Cellular Injury

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

002300 Biochemistry 003000 Clinical Medicine 012900

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	6	379
FISCAL YEAR CURRENT 80	6	217

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: WOLFE, ALAN D., Ph.D.

NAME: HANSEN, BRIAN D., Ph.D.

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Cell Membranes (U) Parasites (U) Metabolism (U) Transport (U) Drugs

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The technical objectives of this work unit are (1) to define cellular processes and membranes transport systems as they apply to parasites, (2) to determine those mechanisms which are susceptible to pharmocological and immunological agents, (3) to ascertain fundamental difference in the metabolic pathways of insect and host stages of the parasite, (4) to study parasite resistance to therapy and (5) to study host-parasite interactions. This unit will support the Army's research on cellular injury.

24. (U) The approach will include the use of both animal and cell culture models. Radioactive compounds will be used to determine metabolic pathways, transport mechanisms, distribution and binding of drugs, and process inhibition. Products of interest will be isolated, identified and quantitated by chromatography, electrophoreses, gradient centrifugation and scintillation spectrometry. Enzyme and membrane structure analysis will be performed to support metabolic and drug resistance studies.

25. (U) The quinoline-4-methanols appear toxin to membranes. These drugs lyse bacteria and spheroplasts, and inhibit the cytoplasmic enzyme, NADH oxidase. The phenanthrene methanols possess bactericidal as well as antiplasmodial activity, and inhibit macromolecular synthesis in bacteria and plasmodia. The purine transport and the utilization and metabolism of amino acid have been investigated in leishmania. The conversion of amino acid to energy sources has been shown. For technical report, see WRAIR Annual Report, 1 Oct 78 to 30 Sept 79.

*Available to contractors upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498-1 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE.

Project 3 M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 123 Biochemical Research on Cellular Injury

Investigators
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SP4 Frank A. Stancato; Alan D. Wolfe, Ph.D.

The objective of this work unit is the definition of the biochemical parameters which may indicate or monitor cell injury, alteration, or proliferation. Cellular injury may result from combat wound trauma or infection, cellular alteration may occur upon antigen challenge or result in drug resistance, and proliferation of cells may be associated with wound healing, graft rejection, or pathological states. The following subjects were investigated:

A. The mechanisms of action of antimalarial drugs

B. The mechanisms of resistance to antimalarial drugs

C. DNA complementary among different plasmodial species

D. Correlation of a cellular enzyme with organ pathology and treatment

E. Metabolite changes as evidenced by alteration in their levels in body fluids and their secretion.

A. The Studies on The Mechanism of Action of Antimalarial Drugs.

It has been previously shown that the antimalarial drug mefloquine (WR 142,490) possesses antibacterial properties. Mefloquine causes a rapid loss in bacterial viability, induces lysis of bacteria, suppresses macromolecular synthesis, inhibits transport of macromolecular precursors and 2-methylalanine. It also increases cell permeability, and causes leakage of nucleic acids and proteins. The results suggest that mefloquine is probably a membrane active drug, exerting its effects through attachment to bacterial, and possibly to plasmodial membranes. Support for this hypothesis was obtained from the following investigations:

1. E. coli spheroplasts, prepared by the method of Zinder and Arndt (1956), were exposed to graded concentrations of mefloquine,

and the rate and extent of spheroplast lysis recorded turbidimetrically. Lysis of spheroplasts occurred in direct relation to the concentration of mefloquine, and a concentration of 1 x 10^{-4} M mefloquine caused 39% lysis in a twenty minute incubation period at 37 C. Polymyxin B, a membrane-active drug, induced 41% lysis during a similar incubation. Pyrimethamine, employed as a positive control, since it inhibits the soluble enzyme dihydrofolic acid reductase (Ferone et al, 1969), did not lyse spheroplasts.

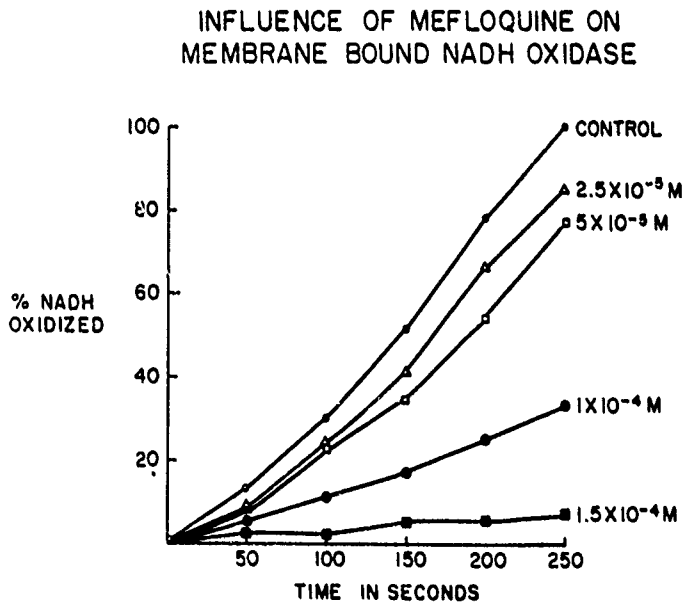

Figure 1. The oxidation of NADH by E. coli membrane fractions in the absence of mefloquine, and in the presence of the designated concentrations of drug. Reactions were assayed spectrophotometrically at 340 nm as a function of time. The volume of the reaction mixture was 2.0 ml, and the mixture contained 0.05 M Tris-HCl, pH 7.5, membrane fraction (protein content 66 µg/ml), and 1 x 10^{-4} M NADH. The order of addition was: buffer, membrane fraction, drug, and NADH. Permutation of the order of addition did not alter the extent of inhibition. Total disappearance of the NADH absorbance at 340 nm connoted 100% oxidation.

2. The membrane of E. coli is presently thought to contain in excess of eight enzymes, among them NADH oxidase (Osburn et al, 1972; Osborn and Munson, 1974). After isolation of the total E. coli membrane fraction (Osborn and Munson, 1974), the influence of mefloquine on the oxidation of NADH was determined. Figure 1 depicts the influence of graded concentrations of mefloquine on the membrane catalyzed oxidation of NADH, and the highest concentration of mefloquine employed, 1.5×10^{-4} M, decreased this rate by 93%. It is noteworthy that enzyme inhibition by mefloquine occurred in a concentration range identical to its antibacterial effects, and that NADH oxidase also occurs in the outer membrane of P. lophurae (Langreth, 1977).

3. The activity of membrane bound NADH oxidase was tested in the absence and the presence of a group of membrane active drugs, mefloquine, and drugs considered unrelated to membranes. Figure 2 shows that the membrane active drugs employed, polymyxin B and antimycin A, completely inhibited NADH oxidation at or below a concentration of 1×10^{-4} M, mefloquine caused almost complete inhibition, while chloroquine and the tetracycline achromycyin V, (not shown), exerted little or no inhibition.

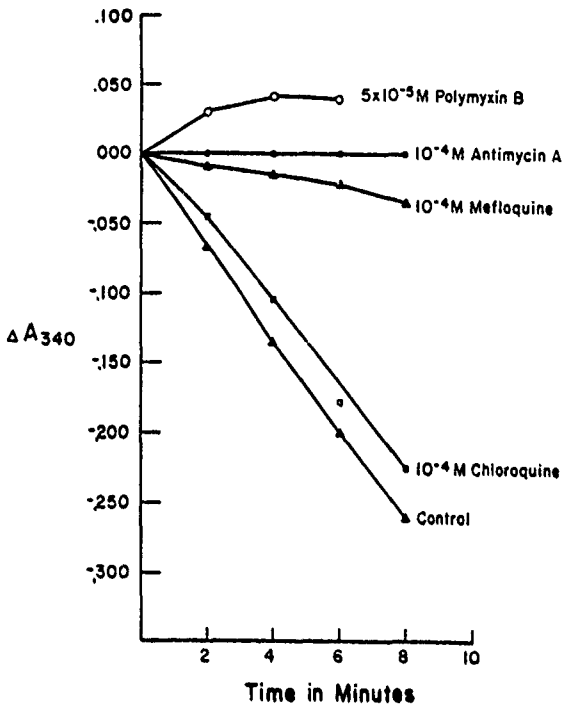

Figure 2. The oxidation of NADH by isolated E. coli membranes in the absence of drug, and in the presence of membrane-active drugs (polymyxin B and antimycin A), mefloquine, and chloroquine. Polymyxin B causes a conformational change in the membranes which is reflected by an increase in turbidity. The assay system is described in the legend to Figure 1.

4. The binding of (^{14}C)-mefloquine to membrane fractions isolated from both E. coli and P. berghei has also been under study, E. coli membrane (Osborn & Munson, 1974), were fractionated in a discontinuous sucrose gradient, a technique which yields the composite outer membrane of the organism as the heaviest fraction, a heterogeneous group of membrane components as an intermediate fraction, and the composite inner or cytoplasmic membrane as the lightest component. Figure 3 illustrates the results of such a fractionation using (^{3}H)-glycerol labelled membranes from E. coli AT-9.

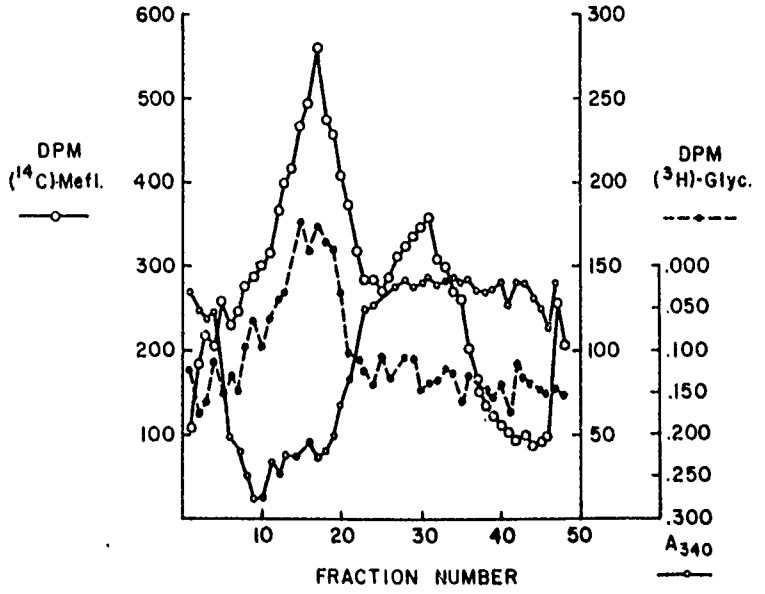

Figure 3. Discontinuous sucrose gradients demonstrating by (^{3}H)-glycerol (-o-) labelling, and analysis of the presence of NADH oxidase (-o-) activity,the location of the inner and outer membranes of E. coli. A large (^{14}C) mefloquine (-o-) peak coincides with the outer membrane, while a second (^{14}C)-mefloquine peak coincides with the heavier portion of the inner membrane, delineated by the onset of NADH oxidase activity. Approximately 1 mg of membrane protein is present per gradient.

Isolated membranes were incubated for 5 minutes at 37 C in the absence, or in the presence of 1 x 10^{-5} M (^{14}C)-mefloquine, centrifuged into the gradients, and fractionated. Determination of the distribution of (^{14}C)-mefloquine and (^{3}H)-glycerol was carried out on one gradient, while the distribution of NADH oxidase activity was assayed in the gradient which contained drug-free membrane. The heaviest fraction, consisting of the composite outer membrane, was heavily labelled with (^{3}H)-glycerol in comparison with the inner membrane, which was located in the gradients in proximity to NADH oxidase activity. The distribution of (^{14}C)-mefloquine coincided with the outer membrane peak, and also was centered about the leading edge of NADH oxidase activity, indicating mefloquine could bind to both the inner and outer membranes. Figure 4 presents the distribution in similar sucrose gradients of P. berghei membranes incubated in the presence or absence of 5 x 10^{-5} M (^{14}C)-mefloquine. The membranes were isolated as follows:

(a) Saponin lysis of parasitized rat reticulocytes (b) centrifugation at 1,086xg for 15 minutes (c) French pressure cell destruction of isolated plasmodia (d) centrifugation at 27,000xg for 30 minutes.

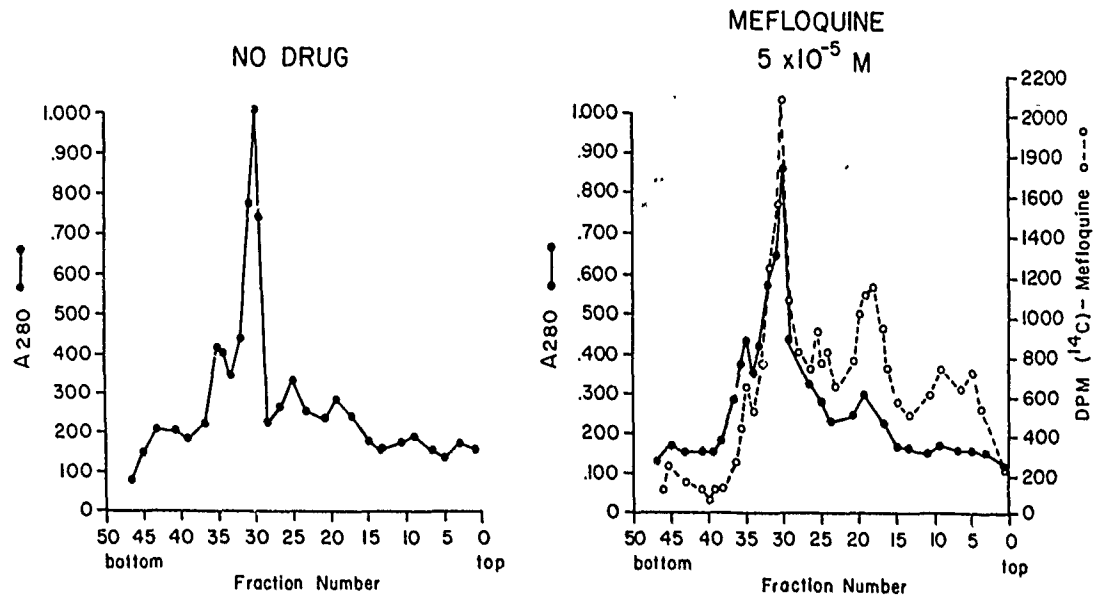

Figure 4. Discontinuous sucrose gradient fractionation of membranes of P. berghei incubated in the absence (left panel) or in the presence of (^{14}C)-mefloquine (right panel). Radioactive drug is distributed in a major peak in the center of the gradient, and also occurs in smaller peaks towards the upper region of the gradient. The gradient contained approximately 1 mg of membrane protein.

The A_{280nm} or turbidity profiles of discontinuous sucrose gradients containing plasmodial membranes incubated in (^{14}C)-mefloquine revealed a series of peaks of unknown content. The major portion of the drug itself was distributed in peaks from the center to the top of the gradient. Presence of mefloquine was also observed in a pellet below the gradient. Association of mefloquine with the different membrane or cellular components provides an important opportunity to identify the binding sites of the drug; the association of mefloquine with the pigment (pellet) is a interesting observation, since: (1) plasmodia produce haemozoin (pigment) as a specific protein end-product of hemoglobin digestion, and (2) chloroquine induced clumping of pigment is inhibited or reversed by many drugs, including mefloquine (Einheber et al, 1976; Peters et al, 1977) .

Studies on the uptake of (^{14}C)-mefloquine are being carried out with intact E. coli and parasitized mouse erythrocytes.

These studies indicate that both biological systems take mefloquine up rapidly but the bacteria appear to release a portion of the associated drug. Parasitized erythrocytes appear to take up appreciably more (^{14}C)-mefloquine than uninfected erythrocytes.

The antimalarials WR 184,806 and 226,253, also quinoline-methanols, are under study, and exhibit properties similar to but less intense than those possessed by mefloquine. The drugs inhibit the growth of E. coli, lyse cells and spheroplasts, and inhibit NADH oxidase. Major difference between studied quinoline-methanols have not been apparent. Phenanthrenemethanols: The mechanism of action of WR122,455 and WR 171,669 are also under investigation. DNA has been reported to alter the spectrum of WR 122,455 (Porter & Peters, 1976), while our studies have shown this drug to kill E. coli rapidly, to induce cell lysis, and to inhibit DNA, RNA, and protein synthesis in both E. coli and P. berghei. However, preferential inhibition of the synthesis of one class of macromolecule has not been observed in either organism. Thiosemicarbazone H: This drug possesses potent antibacterial and antiplasmodial properties, although screening tests indicate the drug is bacteriostatic rather that bacteriocidal. The observation suggests thiosemicarbazone H reversibly inhibits an enzyme or enzyme systems. A concentration of 1.1×10^{-5} M thiosemicarbzone H is sufficient to inhibit growth of E. coli AT-9 in medium M-9 (Osburn & Munson, 1974). The synthesis of RNA, DNA, and protein is blocked, although in E. coli RNA synthesis appears to be inhibited slightly more rapidly than other macromolecular synthesis. In P. berghei, drug H caused inhibition of all three tested classes of macromolecules. Inhibition of bacterial growth by drug H was reversed by a variety of unrelated compounds, yielding no single significant result, but suggesting that the minium inhibitory concentration of H for a biological species might well be a function of the growth medium. Drug H failed to inhibit L. casei dihydrofolic acid reductase.

B. Mechanisms of Resistance to Antimalarial Drugs: It was previously shown that bacteria can be adapted to grow in a normally lethal concentration of mefloquine. Since plasmodia may also be induced by serial transfer to grow in the presence of normally lethal concentrations of mefloquine, chloroquine, and other antimalarial drugs, studies on bacterial resistance mechanisms were continued in the hope that information might be obtained which would further the understanding of plasmodial drug resistance mechanisms.

Comparison of the uptake of (^{14}C)-mefloquine by mefloquine sensitive and mefloquine resistant E. coli suggest that less drug is bound to the resistant than to the sensitive variant.

Both variants appear to bind drug rapidly upon initial exposure, and to release a large proportion of the bound drug, but the resistant organisms appear to retain approximately one-half as much drug as do the sensitive cells.

Resistance to WR 184,806 has also been achieved through extended drug exposure of cultures of E. coli, and cross adaption of bacteria to mefloquine and to WR 184,806 has been demonstrated. In contrast, bacteria have been adapted to WR 122,455, but adapted cells become filamentous. Additionally, adaptation to mefloquine does not produce adaptation to WR 122,455, suggesting that the resistance & possibly actions modes differ between these drugs and their biological targets. It should be noted that mefloquine and WR 184,806 are quinolinemethanols, and apparently , do not bind to DNA, while WR 122,455 is a phenanthrenemethanol, and Porter and Peters (1976) have reported that DNA alters the spectrum of the latter drug.

Plasmodial resistance to Chloroquine: The plasmodial binding site associated with chloroquine sensitivity is under investigation. New Zealand rabbits have been immunized with: (1) mouse erythrocytes, (2) mouse erythrocytes parasitized with (a) chloroquine sensitive P. berghei, and (b) chloroquine resistant P. berghei, (3) membranes isolated from (a) chloroquine sensitive and (b) chloroquine resistant P. berghei, and other rabbits are being immunized directly with isolated plasmodia. Preliminary immunodiffusion analysis revealed rabbits immunized with parasitized and non-parasitized mouse erythrocytes produced an antibody to the erythrocyte itself rather than to the infecting plasmodium.

C. DNA complementarity among different plasmodial species: Attempts have been made to determine the degree of relatedness between the DNA of P. berghei and of P. falciparum. The underlying objective of these studies is the isolation of P. falciparum DNA with the expectation that a fraction encoded for surface determinants may be isolated, and the determinant replicated by cell free amino acid polymerization system and/or the encoded DNA inserted into E. coli by recombinant DNA technology. Results thus far have shown that DNA isolated from P. falciparum (infected erythrocytes provided by Dr. J. David Haynes, Dept. Imm., WRAIR) did not possess the expected, precise bands normally generated in CsCl gradients, and (2) the fragmented DNA had a molecular weight of 10-20 x 10^6 daltons. (courtesy of Dr. Jack Wohlheiter, Dept. Bact. Immun.). The molecular weight of fragmented P. berghei DNA was of a similar order of magnitude.

D. Correlation of a Cellular Enzyme with organ Pathology and Treatment.

The following two projects were carried out in collaboration with the Div of Surgery, WRAIR, Dept of Urology, WRAMC and funded by HSC.

Staging of Prostatic Carcinoma Using Radioimmune Assay for Prostatic Acid Phosphatase.

Enzymatic methods of analysis for prostatic acid phosphatase (PAP) have proven to be of limited value in staging of prostatic carcinoma. Recently radioimmune assay (RIA) has been employed for the analysis of PAP in an attempt to improve the sensitivity and specificity of this assay. Using RIA and enzymatic assay, the serum samples obtained from 56 controls, 164 patients with staged prostatic carcinoma, and patients with other disease were analyzed. In 30 patients was gastrointestinal cancer, no elevation of AP was found using RIA, whereas 30% of the samples had elevated values when assayed enzymatically using thymol-phthalein phosphate as substrate. In patients with bone metastases, 70% of samples have elevated values using thymol-phtalein phosphate, 67% elevation using β-glycerol phosphate, and 82% elevation was observed when RIA was employed. In contrast to the work of others, using the RIA procedure developed by us, we have not been able to demonstrate significant elevations of this tumor marker in patients with intracapsular (10%) and extracapsular (23%) disease. This discrepancy may be explained on the basis of a difference in (a) source and purity of PAP (b) specificity of antisera (c) radioimmune assay methodology and (d) staging procedure.

E. Determination of Metabolite Changes as Evidenced by Alteration in their Levels in Body Fluids and their Secretion

Determination of 5-Dimethylaminonaphthalene-1-Sulfonyl Derivatives of Urinary Polyamines by Ion-Pair High Performance Liquid Chromatography

A sensitive and specific method for the determination of diamines and polyamines by ion-pair high performance liquid chromatography has been developed. The 5-dimethylaminonaphthalene-1-sulfonyl derivations of putrescine, 1,6-diaminohexane, spermidine, and spermine are separated on a μ Bondapak C_{18} reversed-phase column with 1-heptane sulfonic acid and acetonitrile as the mobile phase. All compounds are eluted within 30 minutes using a programmed solvent gradient system. The method has a lower detection limit of 1 picomole (pM) on column.

Because of the simplicity of the method, its application provides a better means of closely monitoring patients undergoing treatment for various types of genito-urinary neoplastic diseases.

Dansylated polyamines were prepared as follows: Two hundred microliters of hydrolyzed urine, prepared as described below is pipetted into 13 mm x 100 mm silanized glass tubes. Twenty microliters of a 100 nM/ml solution of 1,6-diaminohexane (internal standard), 280 microliters of 0.5 M carbonate buffer (pH 9.2),

100 mg of anhydrous potassium carbonate, and 500 microliters of 10 mg/ml of dansyl chloride in acetone were added and throughly mixed. The tubes were sealed with parafilm and the samples were incubated in the dark at 54° C for 60 minutes. At the end of the incubation time, the reacted samples were allowed to cool to room temperature. The dansylated polyamines were extracted into 1 ml of ethyl acetate. The samples were throughly mixed. Five microliters of the ethyl acetate extract containing the dansylated polyamines were injected onto the column for analysis.

A prepacked 300 x 3.9 mm I.D. μ Bondapak C_{18} column was employed to chromatograph the dansylated polyamines. μBondapak C_{18} is a 10 μm particle size packing material, which is designed for both analytical and semi-preparative separations. The mobile phase consisted of 0.02 M solution of 1-heptane sulfonic acid combined with acetonitrile. The PIC B-7 reagent was prepared by mixing 40 ml of the pre-packaged reagent with 460 ml of glass distilled water. The pH of the solution was 3.40. A concave gradient (curv #8, solvent programmer) was used to elute the various dansylated polyamines from the column. Curve #8 may be produced in any dual pumping chromatographic system by using the following formulae.

a. % flow from pump B= $(FC-IC)(\ )^m+IC$
b. % flow from pump A= 100% - % flow from pump B

where:
FC = Final Concentration
IC = Initial Concentration
t = Time into the run
m = 3.00

Gradient parameters were 50% acetonitrile and 50% 1-heptane sulfonic acid at zero time. Upon injection, the acetonitrile was increased from 50% to 80% within a 20 minute period. Total analysis time was 30 minutes. Flow rate for the dual pumping system was 2 ml/minute. Column pressures ranged between 1200-1500 Psi. All separations were performed at ambient temperatures. 1,6-Diaminohexane was used as an internal standard. Each specimen was run in duplicate to insure reproducibility. Peak areas were measured by an on-line computing integrator. The detection limit of the method was 1 picomole on column with a signal-to noise ratio of 3 to 1.

Urine specimens collected from 20 normal subjects and 85 patients undergoing therapy for a variety of urologic malignancies were used for this study. One ml volumes of urine were mixed with an equal volume of concentrated HCl and incubated at 100° C for 14 hours. Analyses were performed immediately or several days later.

Early detection of various types of neoplasia is unusually important. The constant attempt by investigators to develop a simple and specific test to reveal the presence of neoplastic diseases, long before clinical symptoms have become apparent, has always been a major goal of the modern clinician. It is the purpose of this report to investigate the usefulness of the urinary polyamines in development of such a test.

An ion-pair reverse phase HPLC procedure was developed to separate polyamines in urine specimens from normal subjects and patients with known malignancies. From a series of standard solutions and experimental samples, the application of the new method is demonstrated by the chromatograms depicted below. Figures 1 and 2 represent the separation of a standard solution of standards and experimental samples for normalizing the values of solutions containing the dansylated derivatives of putrescine, spermidine, and spermine. 1,6-Diaminohexane was incorporated into both the standards and experimental samples for normalizing the values obtained for each separation. The chromatogram shown in Figure 5 represents the lower detection limit of the method (1.10 ρM). A higher concentration (100 ρM) of the standard solution was also applied to the column to determine the optimum operational range of the method (Figure 6). Linearity was observed for all concentrations of polyamines used in this study (25 ρM-1mM). The correlation coefficients for putrescine, spermidine, and spermine were 0.923, 0.961 and 0.942, respectively.

In the patients with known carcinomas of the kidney, prostate, bladder and testes, a two fold increase in the mean values of putrescine and spermidine were noted. At the same time, no appreciable differences were seen between the spermine values of the two groups. It has been suggested that a significant increase in the concentration of putrescine and spermidine in patients with genito-urinary diseases from those of normal subjects is a reliable indicator for use as a tumor marker.

Our HPLC technique is capable of delineating the parameters necessary to distinguish between normal subjects and patient with known genito-urinary carcinomas. Therefore the use of this new analytical procedure holds great promise as a tumor marker technique. It achieves the degree of sensitivity and specificity desired in polyamine profiling which has been unattainable in earlier methodologies.

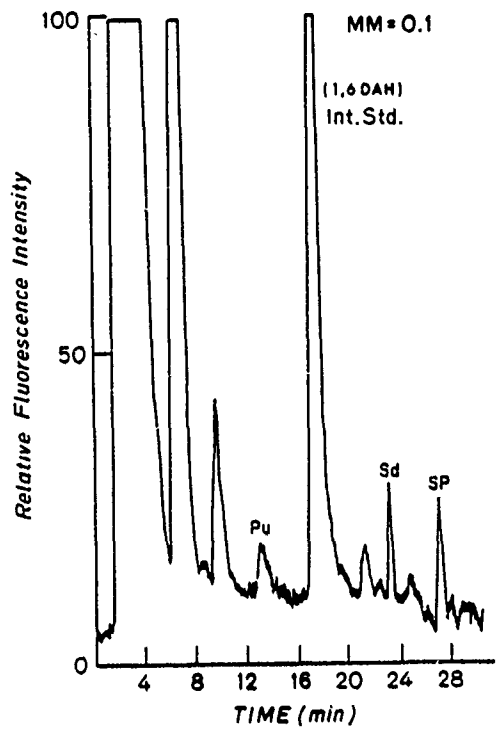

Figure 5 Separation of a standard solution containing 1.10 ρM of putrescine (pu), spermidine (sd), and spermine (sp) Column: 3.9mm x 30 cm μ Bondapak C_{18}. Mobile phase: Gradient mode 50% acetonitrile-50% 1-heptane sulfonic acid (zero time) 50%-80% acetonitrile (20 min.) 2 ml/min.

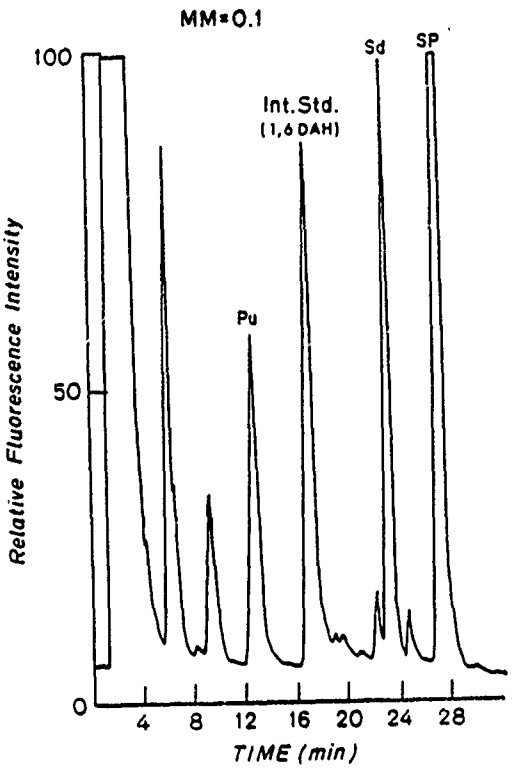

Figure 6 Chromatogram of a 100 pM sample of polyamine std. detected at 365 nm excitation and 510 nm emission. Meter Multiplier: 0.1.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 123 Biochemical Research on Cellular Injury

PUBLICATIONS

1. Brown, N.D., Sweet, R.B., Kintzios, J.A., Cox, H.D., and Doctor, B.P. Determination of 5-Dimethylaminonaphthalene-1-sulfonyl derivations of urinary polyamines by ion-pair high performance liquid chromatography. J. Chromat. 164: 35-40, 1979.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6444	79 10 01	DD-DR&E(AR)636

3. PRE-SUMPR	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	124
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Biochemical Research on Military Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

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b. NUMBER: NA
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
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FISCAL YEAR CURRENT 80	8	395

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Toxin (U) Immunoglobulin (U) Vaccine (U) DNA

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The objective of this work unit is the development of diagnostic procedures and of immunological and chemotherapeutic protection of military personnel by identification, purification, and characterization of molecules related to disease processes. These molecules may be products of the disease state, of infectious organisms, or of the immune system of the host. The classes of molecules under investigation include toxins and nucleic acids of bacterial origin, and enzymes, immunoglobulins, and amines from mammalian sources.

24. (U) Bacterial toxins to be investigated include those from the genera Shigella, Escherichia, and Clostridia. The relations between the structure and reactions of the toxin and pathogenicity of the bacterium will be determined. Antibodies and vaccines to the toxin will be induced and developed. Cloned lymphocytes will be fused with replicating cells to study antibody formation. DNA-DNA hybridization studies will be employed to relate newly found clinical isolates to known species of bacteria including pathogens.

25. (U) 78 10-79 09 Shiga toxin has been purified to apparent homogeniety. Attempts are being made to purify Clostridia sp. toxin. The role of shiga toxin in inhibitions of DNA, RNA and protein synthesis in intact mammalian cell is being elucidated. Role of C. deficile toxin in antibiotic associated colitis is elucidated. Monoclonal antibody against purified surface coat protein from variant strains of Trypanosomes has been successfully elicited. DNA:DNA hybritation studies with cholera sp. has been under taken and is nearing completion. For technical reports, See Annual Report, 1 Oct 78 to 30 Sept 79.

*Available to contractors upon originator's approval

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Project 3M161102 BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 124 Biochemical Research On Military Diseases.

Investigators:

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The objectives of this work unit are to conduct investigations related to causative effects of and biochemical alternations occurring during military relevant disease states. The ultimate aim is to develop diagnostic procedures for proper detection and afford immunological and chemotherapeutic protection of military personnel. This aim is pursued by studies on biochemical aspects of macromolecules related to disease processes.

1. Characterization of Shigella dysenteriae toxin.
2. Genetic and physiological aspects of shigella toxin production.
3. Characterization of Clostridium difficile toxin.
4. DNA relatedness among Enterobacteriaceae and Vibrionaceae.
5. Section of hybridomas producing monoclonal antibody to sindbis virus structural proteins.
6. Selection of hybridomas producing monoclonal antibody to surface coat proteins of Trypanosome rhodesiense.

1. Characterization of Shigella dysenteriae toxin.

The presence of a potent toxin is well-established in sterile filtered lysates from broth cultures of certain species of Shigella (1) Although the hallmark of bacillary dysentery is invasion of the colonic mucosa (2), the contribution of the toxin in the disease process is unknown. Several activities have been reported for shigella toxin: neurotoxicity in rabbits and mice (3), cytotoxicity (4), enterotoxicity (5) inhibition of cell-free polypeptide synthesis (6) and preferential inhibition of protein synthesis in intact HeLa cells (7).

Additionally a number of physiological reactions, unrelated to the

pathogenic invasion process are documented. For example, a live non-invasive strain S. dysenteriae 1 3818-0, causes fluid secretion in rabbit ileal loops (8) and produces altered myoelectric activity of the small intestine (9). Also the watery diarrhea from S. flexneri infection of monkeys was proposed as a jejunal secretion process (10). Moreover sterile filtrates produce an increase in adenylate cylase activity of intestinal mucosa (11). In order to understand the role of shigella toxin in bacillary dysentery, purification of toxin to homogeneity and biochemical characterization is essential.

A. Purification and Characterization of Toxin

Shigella toxin was purified on the basis of its cytoxicity to HeLa cell monolayers. A quantitative cytotoxin assay, developed by Gentry et al. (12), was used with several modifications. Crystal violet dye from the stained monolayers was dissolved in 50% ethanol containing 1% SDS. The absorbance was measured with a microtiter plate reading device using a 600 nm filter. The plate reader, built by the Division of Instrumentation at WRAIR, was designed by Dr. Robert Yolken at the NIH. Protein concentration was determined by the method of Lowry et al. (13).

Shigella dysenteriae 1 strain 3818-0 was provided by Dr. Sam Formal. Growth of bacteria and initial processing were performed at the New England Enzyme Center, Boston MA. Five hundred liters of modified syncase medium were inoculated with 5 liters of log-phase, rotary-shaken seed culture. Growth was continued for 18 hr with continuous aeration. The wet cell paste (4 kg) was resuspended in 6 liters of 0.05 M Tris-HCl, pH 8.0, containing 0.25 M KCl, 0.01 M Mg acetate, and 10 µg/ml phenylmethylsulfonyl fluoride. The suspension was passed three times through a Manton-Gaullin press and the supernate was collect after centrifugation in a Sharples continuous flow centrifuge. The effluent was subjected to ultracentrifugation in an Electronucleonics centrifuge using a zonal rotor at 40,000 rpm with a flow rate of 170 ml/min and again at 80 ml/min. The cleared lysate was frozen on dry ice and stored at -70° until use. Half of the prepared lysate was thawed and subjected to centrifugation at 20,000 rpm in a Beckman Type 21 rotor for 14 hr. After $(NH_4)_2 SO_4$ precipitation between 28% - 50% saturation, the toxin fraction was resuspended and dialysed against 0.020 M Tris-HCl pH 8.0 containing 0.10 M NaCl (20T8/100 buffer). Four DEAE-cellulose columns (15.5 x 5 cm) were equilibrated with 20T8/100 buffer. The dialysed toxin fraction was divided evenly and applied to the columns (7.4 g protein/column). Toxin was eluted with 20T8/100 buffer at 3.5ml/min. The toxin fraction was pooled and dialyzed. A CM-cellulose column (15.5 x 5 cm) was equilibrated with 0.010 M sodium phosphate, pH 6.0, 0.050 M NaCl (10P6/50). After dialysis against 10P6/50 buffer, the toxin fraction was applied to the column and eluted at a rate of 10 ml/min. After elution of the initial peak, a linear gradient is applied from

0.05 M NaCl to 0.35 M NaCl in 10P6/50 buffer. Fractions were adjusted to pH 8 with 1 M Tris and the toxin-containing fraction was brought to 70% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in 20T8/100 buffer and dialyzed. For gel filtration, Pharmacia S-200 was equilibrated with 0.13 M glycine-NaOH, pH 8.0 buffer (130G8). The column (3.3 x 9.5 cm) was eluted at a flow rate of 0.4 ml/min with 130G8 buffer. Preparative isoelectric focusing was performed on an LKB 110 ml electrofocusing column with 1% ampholytes, LKB pH 5 - pH 8 in a 0 - 40% sucrose gradient for 24 hrs (14). Initial voltage was 600V and was adjusted to 1000V while maintaining less than 10mA. Immediately after collecting the isofocused fractions, the pH was measured at 5°. The toxin-containing fractions were pooled and the pooled sample was applied to a G-25 column (3.3 x 40 cm) for removal of sucrose and ampholytes. The toxin was eluted with 20T8/100 buffer and was frozen at -70°. Discontinuous electrophoresis on 8% polyacrylamide gels (disc-PAGE) was performed by the method of Davis (15). Electrophoresis in the presence of SDS was performed on Pharmacia 4 - 28% gradient polyacrylamide slab gels (16). Analytical isoelectric focusing was performed with LKB PAG-Plates (17). For detection of cytotoxin activity, gels were sliced and eluted. Polyacrylamide rod gels was cut longitudinally. One side was stained; the other half was sliced with the Buchler electric gel slicer. Polyacrylamide slab gels were cut into lanes. Each lane was sliced manually into 1.4 mm slices. Toxin was eluted from the slices overnight with 0.2 ml 20T8/100 buffer containing 0.02% sodium azide.

The results in Table 1 show that shigella toxin is purified over 8000-fold by the procedure described. Several features of purification require special mention. Toxin activity is lost upon freezing/thawing so that aliquots for assay are frozen only once. The purification itself is carried out to the final stage without freezing. Concentration by ultrafiltration also inactivates toxin, therefore concentration is accomplished by $(NH_4)_2SO_4$ precipitation. During this process pH must be controlled carefully to prevent toxin inactivation. Likewise the toxin is unstable at pH 6 so that the pH is raised immediately as the fractions are collected from CM52 column.

The final product has been purified to apparent homogeneity. Discontinuous electrophoresis produces two closely migrating bands on 8% polyacrylamide rods gels (Figure 1). Both bands display cytotoxin activity after elution of the sliced gel. By densitometric analysis, these two toxin bands account for 96% of the protein stain, with the major (trailing) band containing 77%.

Several methods were used to estimate the molecular weight of the toxin. Gel permeation chromatography was performed with both BioGel P-100 and Pharmacia Sephacryl S-200. Purified toxin was applied to a

P-100 column equilibrated with 20T8/100 buffer. Cytotoxicity of the eluted fractions was assayed, indicating an M_r=46,000. Chromatography of partially purified material on the S-200 column (3.3 x 100 cm) gave a value of M_r=37,000. However, electrophoresis in polyacrylamide gradient gel slabs gave an estimated M_r value between 105,000 - 130,000. The discrepancy could result from a concentration-dependent dissociation during gel filtration or from an extremely low charge density during gradient gel electrophoresis.

Table 1. Purification of shigella toxin

Fractions	Total CD50's	CD50/mg Protein
Culture Broth	2.8×10^8	1.2×10^4
Cell Lysate	40×10^8	1.2×10^5
Supernate, 59,000 x g, 15hr	15×10^8	0.74×10^4
Pellet, 28-50% sat'd $(NH_4)_2SO_4$	13×10^8	1.1×10^5
DEAE - cellulose	11×10^8	4.7×10^6
CM - cellulose	3.0×10^8	2.8×10^7
Pellet, 0-70% sat'd $(NH_4)_2SO_4$	3.0×10^8	2.4×10^8
Sephacryl S - 200	0.42×10^8	0.88×10^8
Isoelectric Focusing, pH 5-8	0.098×10^8	4.8×10^8

The subunit structure of shigella toxin was examined by polyacrylamide gradient gel electrophoresis in the presence of SDS. The toxin was separated into at least two bands. Without 2-mercaptoethanol (2-ME), the bands have an apparent M_r = 29,000 and M_r = < 10,000. In the presence of 2-ME, the larger component migrates with an M_r = 31,000 and two other faint bands appear at M_r = 65,000 and at M_r = 27,000. Two dimentional PAGE revealed the relationship of the SDS-PAGE bands to the two components observed by disc PAGE. Both disc PAGE bands contain the 10,000 - dalton component. The major (trailing) band on the disc-PAGE rod contains a 31,000 dalton component in the presence of 2-ME. The major toxin component consists of subunits of M_r = 31,000 and M_r = < 10,000. The minor component contains subunits of M_r = 27,000 and M_r = < 10,000.

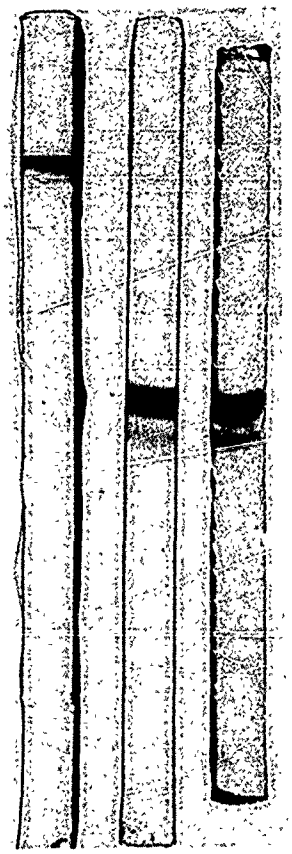

Figure 1. Discontinuous Buffer Polyacrylamide Gel Electrophoresis of Purified Toxin. a. Electrophoresis for 4 hours; b and c. Electrophoresis for 12 hours.

Analytical isoelectric focusing was employed to assess the homogeneity of the final preparation and to determine the isoelectric point. The toxin produced four bands on analytical polyacrylamide gel slabs. These components, in the range of pI 7.3, displayed cytotoxic activity. No cytotoxic activity was observed elsewhere on the gel slab.

The procedure described produced highly purified toxin from bacterial cell lysates. The methods used involved gentle biochemical techniques and minimized chemical or proteolytic artifacts. Large scale preparation allowed recovery of milligram quantities of purified toxin. Shigella toxin is a potent cytotoxic agent. In our assay system, complete cytotoxicity occurred at sub-femtomolar

quantities. Therefore HeLa cells appear to be killed at a ratio of about 250 - 1000 molecules per cell. Shigella toxin appears to contain two subunits. The larger subunit seemed to be susceptible to proteolytic cleavage, resulting in two bands by disc - PAGE analysis. The faster, minor band produced a larger subunit of M_r = 27,000 while the slower major band contained a larger subunit of M_r = 31,000. The isolated protein appears to account for all the classical exotoxigenic activities of Shigella dysenteriae. The toxin displays neurotoxin, enterotoxin and cytotoxin properties. In contrast to McIver et al. (13) no cytotoxin activity was detected at pH 6 after either preparative or analytical isoelectric focusing. Unlike the results of Thompson et al. (19) our toxin preparation consists of predominately one species when analyzed by disc - PAGE. Our use of PMSF-treated cell lysates may explain these discrepancies.

B. Use of Matrix Gel Blue A in Toxin Purification

Affinity chromatography is a widely-used technique in protein purification. Because minute amounts of purified shigella toxin are obtained by conventional procedures, purification by a fast, highly specific method remains desirable. A commerically available affinity chromatography material, Matrix Gel Blue A, has been tested for use in snigella toxin purification. A substantial purification was obtained.

Matrix Gel Blue A (Amicon #19011) consists of a 5% crosslinked agarose support matrix to which is coupled the dye Cibacron Blue F3GA. The gel has a high affinity for NAD^+-binding proteins. After washing and equilibration with 0.01 M Tris-HCl, pH 8, 0.05 M KCl, the gel was poured into a column (1.6cm x 3cm). Partially purified toxin (post-DEAE fraction) was applied. Elution at 0.1 ml/min was carried out first with 0.01 M Tris-HCl pH 8, 0.05 M NaCl. After elution of the initial, large absorbance peak the column was washed with 0.01 M Tris-HCl pH 8 containing 0.35 M NaCl. No toxin was detected in the initial material eluted from the column. Cytotoxic activity was detected in the fractions eluted with 10T8/350 buffer. The activity appeared approximately equal to that of the material applied, implying no loss of toxin. Other data indicated that toxin did not bind the agarose matrix, since toxin was not eluted with 0.01 M Tris-HCl pH 8, 0.05 M NaCl containing 0.2 M galactose. Further enhancement will probably be achieved by elution with a salt gradient. Application of this technique to the purification method will be tested after CM-cellulose chromatography. Advantages at that stage would be concentration as well as purification.

C. Mode of Action of Toxin

Using the purified toxin as described above, we have investigated its effects in intact HeLa cells on macromolecular synthesis, amino acid uptake and intracellular K^+ concentration. Shigella toxin appears to preferentially inhibit protein synthesis.

Detection of ^3H or ^{14}C in the TCA - precipitable fraction after incorporation was used to assay macromolecular synthesis in intact HeLa cells. Protein synthesis was assayed after incubation with ^{14}C-leucine (0.355 Ci/mmole). RNA or DNA synthesis were measured after incubation with ^3H-uridine (27.9 Ci/mmole) or ^3H-deoxythymidine (20 Ci/mmole). Cells were inoculated into 96 - well microtiter dishes (1.6×10^4 cells/well). Incubations overnight in complete medium (MEM containing 10% fetal calf serum, 0.18 mg/ml streptomycin, 180 units/ml penicillin) produced subconfluent monolayers. Medium was removed from all wells and replaced with 0.1 ml MEM minus leucine containing 1 mg/ml BSA. At the appropriate time interval, an additional aliquot (0.1 ml) was added containing toxin and the radioactive substrate. Radioactive incorporation was terminated by rapidly cooling the plate on a ice/water slurry, removal of medium and washing the cells with ice-cold complete medium.

For determination of ^{14}C-leucine and ^3H-deoxythymidine incorporation, cell monolayers were solubilized by addition to each well of 0.10 ml 1 M NaOH. After 30 minutes, 0.025 ml BSA solution (0.15%) was added as carrier protein and 0.10 ml aliquot of the mixture was withdrawn and dispersed into 0.9 ml cold 5% TCA. The precipitate was collected on glass fiber filters using the MASH II (Microbiological Associates) cell harvester. The filters were incubated overnight in 0.5 ml NCS and scintillation fluid was added for counting radioactivity. For measurement of ^3H-uridine and ^{14}C-leucine incorporation, the washed cells were detached by treatment with trypsin/ EDTA solution. After addition of 0.2 ml fetal calf serum, cells were transferred to glass fiber filters. The filters were washed extensively with cold 5% TCA, treated with NCS, and prepared for counting. Recovery of ^{14}C radioactivity was the same in both methods.

Uptake of amino acid was estimated by uptake of ^3H- α -aminoisobutyric acid (α -AIB) (10 Ci/mmol). Monolayers were established in 96-well microtiter dishes. Complete medium was replaced with MEM minus leucine containing 1 mg/ml BSA (0.10 ml/well). At appropriate intervals an additional aliquot (0.05 ml) containing toxin and ^{14}C-leucine (0.5 μCi/well) was added. Five minutes before the end of the experiment another aliquot (0.05 ml) containing ^3H-α-AIB was added (1 μCi/well). Uptake was linear for for 7 minutes. The experiment was terminated by placing the microtiter dish on a cold ice/water slurry. Medium was removed

and the wells were rinsed three times with ice-cold complete medium. Cells were dissolved with 0.10 ml 0.5 M KOH for 30 minutes at room temperature. After 0.025 ml 1% BSA was added, an aliquot (0.05 ml) was removed for counting, and another 0.05 ml aliquot was transferred to 0.90 ml cold 5% TCA. After 30 minutes the precipitate was collected on glass fiber filters in the cell harvester unit, dissolved in NCS, and counted for radioactivity.

Cellular potassium levels after washing in K^+ - free PBS were used as an indicator of intracellular [K^+]. Tissue culture dishes (60 x 15 mm, Costar) were inoculated with 1.4×10^6 HeLa cells. After overnight incubation, medium containing toxin was added to all plates. At different times the medium from three plates was transferred to a 50 ml centrifuge tube. The cell monolayers was rinsed three times with K^+-free PBS and the washes were combined with the medium. After draining the dishes, distilled H_2O (1.0 ml) was added, and the plates were frozen and thawed. DNA was hydrolyzed by addition of 1.0 ml buffer (0.20 M sodium acetate pH 5, 0.05 M NaCl, 8.2 mM $MgSO_4$) containing 160 units DNase I. After 1 hr incubation at 37°, the plates were dried overnight at 60°. To measure residual K^+ the plate contents were mixed with 2.0 ml 15 meg Li^+ solution and assayed by flame photometry.

The effect of shigella toxin on macromolecular synthesis is presented in Figures 2,3,4. Incorporation of ^{14}C-leucine, ^{3}H-uridine and ^{3}H-thymidine measured in the TCA-insoluble fraction was inhibited compared to the control wells. Inhibition by toxin is dose-dependent. Both the final incorporation levels and the time of onset of inhibition are decreased with larger amounts of toxin. In Figure 2 inhibition of ^{14}C-leucine incorporation is essentially complete at 45 minutes with 10 ng toxin (10^4 CD50). Inhibition of ^{3}H-thymidine incorporation and ^{3}H-uridine incorporation occur subsequent to inhibition of protein synthesis. No effect is observed on ^{3}H-uridine incorporation until at least 90 minutes. Inhibition of incorporation of ^{14}C-leucine might occur by failure of radioactive precursor to be transported into the cell. To examine this possibility the effect of shigella toxin on ^{3}H- α-AIB uptake was determined. Cells are exposed to ^{14}C-leucine and toxin for different time periods and then given a 5 minute pulse of ^{3}H-α-AIB. The results are shown in Table 2. No effect on uptake was observed during the course of the experiment. In contrast complete cessation of ^{14}C-leucine incorporation was observed by 90 minutes.

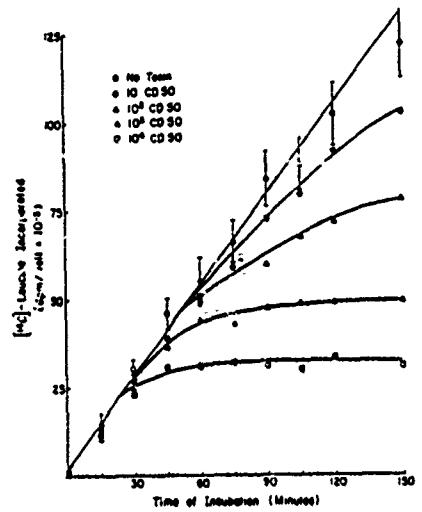

Figure 2.

Effect of Toxin on Intact Hela Cell Protein Synthesis.

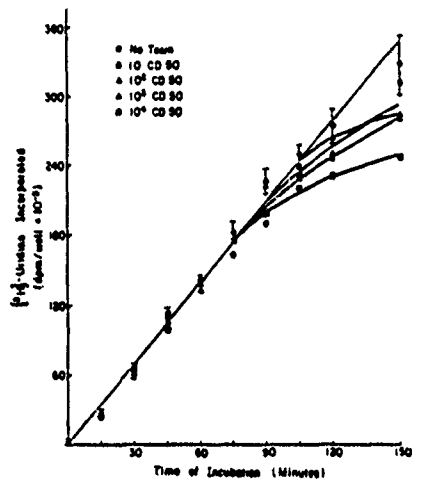

Figure 3.

Effect of Toxin on Intact HeLa Cell RNA Synthesis.

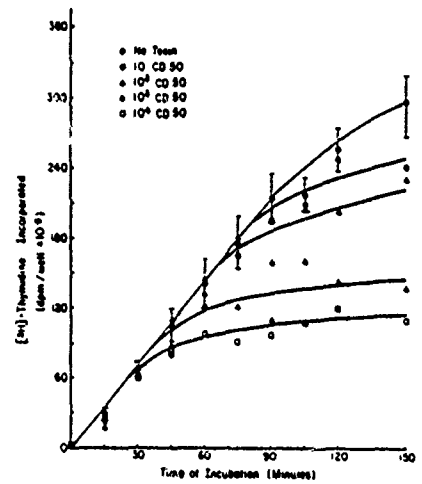

Figure 4.

Effect of Toxin on Intact HeLa Cell DNA Synthesis.

Table 2. Effect of Shigella Toxin on α-Amino-Isobutyric Acid Uptake

Time of Incubation (Minutes)	[^{3}H]-α-AIB Uptake (DPM/Well in 5 minutes)*	
	-Toxin	+Toxin
0	2200 ± 1200	2300 ± 270
15	2700 ± 190	2700 ± 180
30	3000 ± 260	2600 ± 340
45	2800 ± 420	2800 ± 240
60	3200 ± 160	2800 ± 160
75	3000 ± 360	2600 ± 350
90	2800 ± 410	2900 ± 290
105	3200 ± 650	2900 ± 220
120	2500 ± 340	2600 ± 410
150	2400 ± 580	1900 ± 190

*Values are the average of three determinations with error presented as the standard deviation.

Inhibition of macromolecular synthesis might also occur from depletion of cellular ATP due to gross membrane damage or inhibition of oxidative phosphorylation. Changes in cellular K^+ concentration would readily indicate this metabolic condition since high intracellular potassium concentration is maintained by an energy dependent process. Table 3 shows the effect of shigella toxin on intracellular K^+ concentration. Potassium levels remain constant for at least 2 hours after addition of toxin. Comparatively, ^{14}C-leucine incorporation was completely inhibited by 60 minutes at the same dose.

Table 3. Effect of Shigella toxin on Intracellular K^+ Concentration

Time Incubation (Hours)	Intracellular K^+ Content (μmoles K^+/60 mm Dish)*	
	-Toxin	+Toxin
0	1.16 $\pm$ 0.04	1.08 $\pm$ 0.07
1	1.07 $\pm$ 0.01	1.06 $\pm$ 0.03
2	1.17 $\pm$ 0.06	1.04 $\pm$ 0.05
3	1.13 $\pm$ 0.01	0.81 $\pm$ 0.03
4	1.18 $\pm$ 0.04	0.52 $\pm$ 0.02
8	1.31 $\pm$ 0.02	0.19 $\pm$ 0.02
16	1.92 $\pm$ 0.09	0.09 $\pm$ 0.01

*Values represent the average of three determinations with error presented as the standard deviation.

The inhibition of macromolecular synthesis can be abolished by inactivation of the toxin with heat or by antisera neutralization. In Table 4 the purified toxin was heated to various temperatures for 30 minutes, cooled and diluted with medium. Incorporation was allowed to proceed for 120 minutes. In agreement with Gentry et al. (12), toxin was fully active below 70°. The accumulated radioactivity represents incorporation during the lag period of toxin action. At 80° toxin was completely inactivated and DNA synthesis proceeds unimpaired.

Table 4. Inhibition of ^{3}H-Thymidine Incorporation after Heat Treatment of Toxin

Temperature of Toxin Pretreatment	^{3}H-Thymidine Incorporation
4°	32.1×10^3 dpm
60°	32.1×10^3
70°	33.7×10^3
80°	64.7×10^3
90°	64.7×10^3
100°	66.6×10^3
No Toxin	72.5×10^3

Inhibition of protein synthesis was completely blocked by neutralization with specific antiserum (Figure 5). The antisera, produced with toxin purified by Thompson et al.(19), was shown by A.D. O'Brien to be monospecific (20). Complete protection was provided against a 10^4 - fold excess of toxin in the experiment.

Inhibition of protein synthesis appears to be the initial effect of shigella toxin on cellular metabolism. Effects on DNA synthesis or RNA synthesis were less drastic and occur subsequently. Even at high toxin concentrations a lag period occurred before inhibition is observed. Osato et al. (21) have examined the effects on macromolecular synthesis of toxin parially purified from culture filtrates. An early effect on protein synthesis was observed, but in contrast to our studies, RNA synthesis was also inhibited.

Inhibition of protein synthesis was observed before any detectable effects on amino acid uptake, RNA synthesis, or cellular K^+ concentration. Therefore protein synthesis was not inhibited by depletion of amino acid pools, mRNA, or ATP. The site of action on intact cells appears to be translation.

McDonel et al. (22) proposed that Clostridium perfringens toxin induced membrane damage resulting in inhibition of protein synthesis. Our results with cellular K^+ concentration imply that shigella toxin does not act in a similar manner. Maintenance of high intracellular K^+ concentration would not continue if

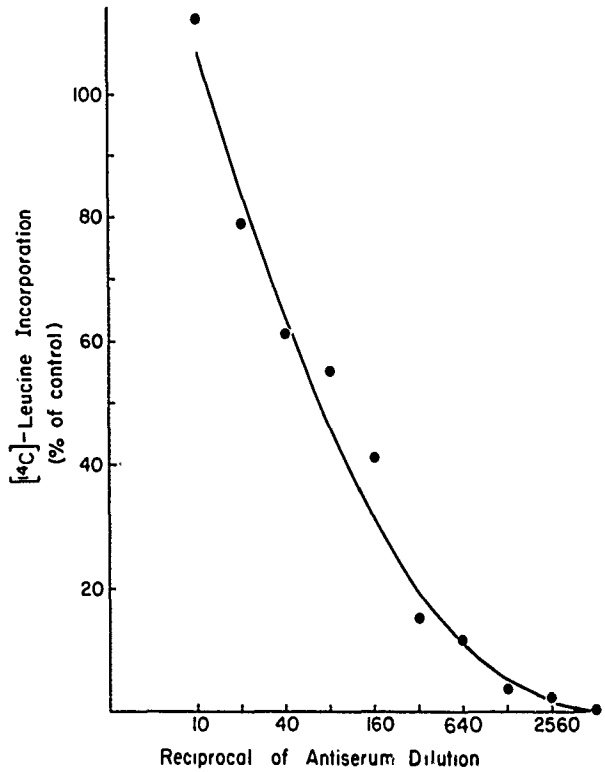

Figure 5. Protective Effect of Antiserum against Shigella Toxin.

toxin had induced gross permeability changes or was acting as a K^+ ionophore.

These experiments used toxin purified to apparent homogeneity, so that inhibition of protein synthesis appears to be a specific effect of the isolated toxin. Blocking of inhibition by heat inactivation and by antiserum neutralization confirm that observation.

Inhibition of protein synthesis has been observed with many protein toxins. One group, including diphtheria toxin and Pseudomonas aeruginosa toxin, produced inhibition by transfer of ADP-ribose from NAD to EF2 (23). Other toxins, such as abrin and ricin, do not appear to catalyze that reaction (24). Further work will elucidate the mechanism of shigella toxin action and its role in disease.

D. Inhibition of Protein Synthesis by Shigella Toxin in Sensitive and Resistant HeLa Cell line.

Gentry et al. (12) have observed that not all HeLa cell lines are sensitive to shigella toxin on the basis of a cell detachment assay. The refractory nature of the resistant HeLa cell line is unknown. Our observation of inhibition of protein

synthesis in intact cells within 60 minutes can be used to explore the problem by development of a short-term toxin assay procedure.

Initial data, shown Fig. 6, indicated that inhibition of protein synthesis does not occur in intact resistant cells at toxin levels producing complete inhibition of intact sensitive cells by 45 minutes. These results correlate closely with cytotoxicity as measured by the cell detachment assay. Subconfluent monolayers in 96-well microtiter plates were exposed to toxin (10^4 CD50/well) and ^{14}C-leucine for different time intervals. Incorporation into protein was measured by detection of ^{14}C in the TCA-insoluble fraction. Each data point is the result of three derminations.

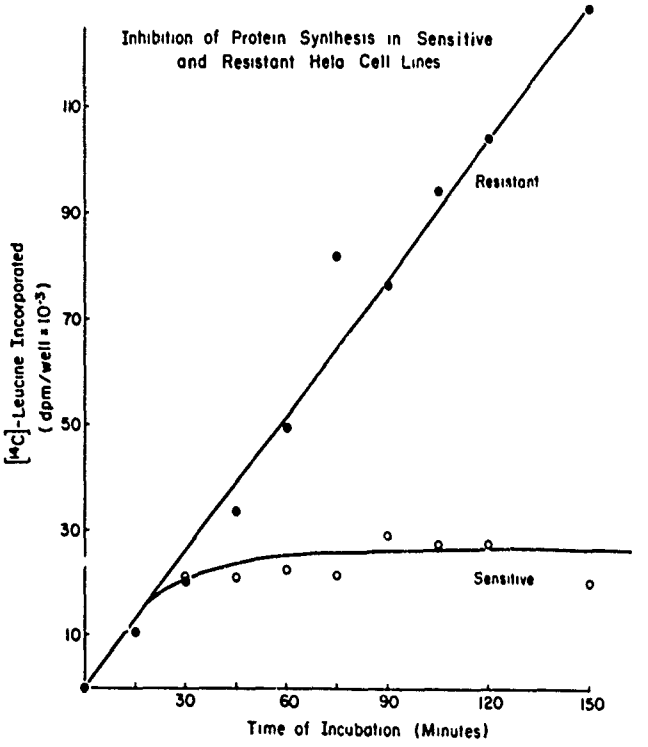

Figure 6. Inhibition of Protein Synthesis in Sensitive and Resistance Hela Cell lines.

Resistance to inhibition by toxin might result from several causes. Toxin may not bind to the cell membrane of resistant cells. It may not be internalized through the membrane. Resistant cells may contain refractory ribosomes. Each of these possibilities will be explored.

2. Genetic and Physiological Aspects of Shigella Toxin Production.

One approach to the study of the role of Shiga toxin in the pathogenesis of shigellosis involves the use of hyper-, hypo-, and nontoxigenic strains to study the effect of various toxin levels on the disease process. The usefulness of this approach has been illustrated by the use of chlorate resistant hypotoxigenic mutants [6,8]. Also recent studies with intergeneric hybrids of an E. coli donor and S. dysenteriae 1 recipient have yielded hybrid strains that have very low cytotoxic activity. Limited attempts isolate toxin producing E. coli hybrids and to mutagenize S. dysenteriae, S. flexneri, and E. coli into a hypertoxigenic phenotype have been unproductive, however.

In the past the isolation and characterization of such mutants was difficult due to the lack of a rapid, reliable screening procedure. Strains were screened for changes in toxin production using the following assays: mouse neurotoxicity, fluid secretion in ligated rabbit ileal loops, and detachment of HeLa cell monolayers. Moreover, the need for making a cell extract and the lack of a quick, sensitive, reproducible assay procedure made genetic studies cumbersome. Now, however, colonies can be screened using an extension of the microtiter HeLa cell cytotoxicity assay developed by Gentry et al. [12]. We have found that polymyxin B can induce the release of Shiga toxin from cells. This procedure also kills the bacteria thus obviating the need for a sterile filtration step for the cytotoxicity assay. As a consequence it is presently possible for one individual to screen more than 1000 clones per week.

The mutagenesis of Shiga toxin producing bacteria has been approached by two diverse methods. One approach utilized the alkylating agent, N-methyl-N'-nitro-N-nitrosoguanidine (NTG) which modifies DNA by causing nucleotide base substitutions. S. flexneri strain M4243 and an enteropathogenic E. coli strain SPSC (superpiliated South Carolinia) were mutagenized with NTG. 1000 randomly selected colonies of each strain were screened for increased Shiga toxin production. None of the colonies from either strain were found to have increased toxin levels in the microculture-polymyxin B screening method. The other approach to mutagenesis uses translocatable drug-resistance elements (transposons) which is able to insert themselves into a large number of different sites in prokaryote genomes [25], if the insertion occurs in the structural gene for Shiga toxin, one would get a nontoxigenic phenotype. If the transposition occurs in a regulatory gene, a change in the level of gene product will be obtained. Our present studies utilize the transposon Tn10 which carries genes conferring resistance to tetracycline (Tc). Strains of Salmonella typhimurium LT2 which carry Tn10 on a F'_{ts}

114 *lac*$^+$ plasmid were obtained from Dr. B.D. Stocker. Since many *S. dysenteriae* strains already carry Tc resistance genes, strains JVA70, 3818-0, and 1617 were screened for Tc resistance. Only *S. dysenteriae* strain 1617 was found to Tc sensitive. Initial attempts to transfer the F'$_{ts}$ *lac*$^+$::Tn10 plasmid from *S. typhimurium* strains TT627 and TT628 into *S. dysenteriae* strain 1617 were unsuccessful probably due to a restriction-modification difference between the two genera. In an attempt to alleviate the difference the F'$_{ts}$ *lac*$^+$::Tn10 plasmid was transfereed into a restriction-less, modification-less *E. coli* strain C600 Lac-. The *E. coli* C600 Lac- carrying the F'$_{ts}$ *lac*$^+$::Tn10 could then act as a donor for the transfer of Tn10 into *S. dysenteriae*. However, due to the lack of suitable counter-selection markers, additional markers were introduced in *S. dysenteriae* strain 1617. Spontaneously occurring mutants to streptomycin resistance and to nalidixic acid (Nal) resistance were isolated for use as recipients. Preliminary attempts to transfer Tc resistance into streptomycin resistant *S. dysenteriae* have been successful. However the sites of insertion of Tn10 and its effect on toxin level in these recent isolates have not been studied yet.

Genetic mapping of the toxin genes has been attempted using intergeneric conjugation and intragenetic transfer experiments. Intergeneric conjugation experiments were conducted with *S. dysenteriae* 1 strain 105-5 Hfr and *E. coli* K12 strain 395-1 or their hybrids as the recipients. The circular chromosomal map of *E. coli* K12 which is closely related to *Shigella* has been divided into 100 minutes and as of 1976 more than 650 loci have been reviewed and assigned distinct map position [26]. The ability to grow without an amino acid or the ability to use a sugar as the sole carbon source is denoted as a plus "+" phenotype. The requirement for an amino acid or the inability to ferment a sugar is denoted as a negative "-" phenotype.

In a conjugation experiment the integration of the donor DNA into the recipient DNA is sought. First the donor and the recipient must be characterized using biochemical markers as the use of a sugar as a sole carbon source and the ability to grow in the absence or the requirement for an amino acid. Next the biochemical markers of the donor and recipient are compared to see which markers differ. If they differ with respect to a biochemical marker, it may be useful as a marker to determine donor chormosome integration. In addition the recipient is usually resistant to an antibiotic to which the donor is sensitive. Consequently the donor is counterselected by the antibotic. If the donor's biochemical marker appears in the recipient, it is likely to be a consequence of the inheritance of appropriate genes by the recipient. Once hybrids are obtained for selected markers around the entire chromosome, they can be scored for inheritance of nonselected markers to determine how much of the

donor has been inherited. A map position for Shiga toxin genes can be determined in such a way. When F sex factor is integrated into the chromosome of a strain it is called a high frequency of recombination (Hfr) donor. A Hfr donor inserts its DNA starting at the end of the integrated F plasmid in a circular clockwise or counterclockwise manner away from the plasmid. The recipient or female strain is called F-.

After the mating of the donor and the recipient, hybrids are selected on minimal salts media containing the selective antibotic, tryptophan, nicotinic acid, and lack one of the following amino acids: proline (pro), leucine (leu), arginine (arg), and histidine (his); but contains all other amino acids necessary for growth. After selection each hybrid was purified by single colony isolation twice on the respective selective media. A master plate containing thirty hybrids was prepared and was then replica plated on media to determine the following unselected markers: ability to ferment maltose (mal), rhamnose (rham), fucose (fuc), galactose (gal), and lactose (lac), the ability to degrade tryptophan to indole (tna), and the ability to produce Shiga toxin (tox). Once the marker information is obtained for each hybrid, a determination can be made concerning which areas of the recipient DNA express donor phenotype.

Some of the representative classes of the hybrids are shown in Figure 7. The shaded areas represent integrated donor DNA. As shown hybrids have been found that contain one of the selected amino acid markers and none of the unselected markers. Additional hybrids have been found that contain various markers between Pro and His. One hybrid has been found that contains the entire Shiga chromosome from Pro to His which is 62% of the chromosome. Attempts to obtain hybrids with additional regions of the Shiga chromosome using hybrids containing only the his region have not been successful. None of the hybrids isolated produced Shiga toxin based on the results of the microculture polymyxin B HeLa cell cytotoxicity assay. Based on present data if the Shiga toxin genes are in close proximity, about 62% of the chromosome can be eliminated as the location of the toxin genes implying that the gene locus is in the chromosomal region between His and Lac. Another hypothesis would be that the genetic information could be on more than one locus which are widely separated. Once new Hfr strains are developed, this line of research will be continued.

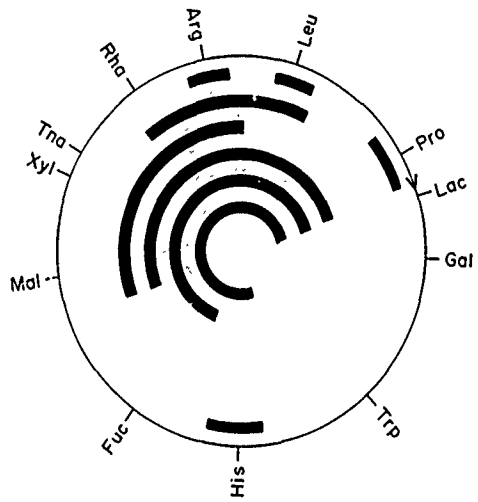

Fig 7. Chromosome map of S. dysenteriae x E. coli hybrid classes

Attempts to transfer toxinogenicity from S. dysenteriae to S. flexneri by plasmid recombination and transformation techniques are in the preliminary stages.

3. Characterization of Clostridial Toxin

Exotoxins of clostridia have recently been shown to cause antibiotic-associated colitis (AAC)--Clostridium perfringens E toxin in rabbits (27,28) and Clostridium difficile toxin in humans and hamsters (29-31). Clostridial toxin is also a factor in the fatal colitis in the guinea pig caused by single 50,000 unit doses of penicillin (32), or by clindamycin (33).

The syndrome in humans is usually characterized grossly by nodular inflammatory plaques and peudomembranes. AAC has been observed following treatment with tetracycline, chloramphenicol, clindamycin, penicillin and many other widely-used antibiotics (30,34). Any antimicrobial drug capable of changing the normal intestinal flora can presumably be responsible for precipitating pseudomembranous colitis. A similar disease was observed before the introduction of antibiotics, usually after gastrointestinal surgery (35), suggesting that other events which significantly alter the resident flora may cause pseudomembranous colitis due to clostridial toxins. For example, Clostridium perfringens E toxin was found in the cecal contents of rabbits who had died of "enteritis complex"; this disease is often seen experimentally

when rabbit chow is replaced by a low-fiber diet (36). Similarly, when brought into a conventional environment, formerly germ-free guinea pigs develop a fatal colitis; toxin-producing Clostridium perfringens E is found in the gut contents (37).

Many explanations for the development of AAC have been suggested. The essential etiologic mechanism in pseudomembranous colitis was seen to be intravascular coagulation (38), the coagulation being due to thrombogenic agents, possibly bacterial toxins, from the bowel lumen entering the blood stream. Recent studies have shown that bacterial toxins are indeed a most important cause of the disease--toxins from the genus Clostridium. When rabbits were given clindamycin orogastrically, they developed enterocolitis, and an enterotoxin was found in cell-free extracts of their cecal contents (27). The toxicity of these extracts was neutralized by incubating them with Clostridium perfringens E antitoxin (28). It has been previously shown that guinea pigs injected intramuscularly with single doses of 50,000 units of penicillin develop a fatal colitis, and that this colitis is associated with a gram-negative bacteremia (29). In addition, an enterotoxin can be found in cell-free extracts of their cecal contents (32). As in the clindamycin-associated colitis in hamsters (40,41), the toxicity of these extracts can be neutralized by incubating them with Clostridium sordellii antitoxin. In one study, both C. sordellii and C. difficile were recovered in large numbers from cecal contents of hamsters dying with colitis (31). However, injection of hamsters intracecally with either cell-free filtrates of C. difficile cultures or the organism itself caused enterocolitis (43), while injection of C. sordellii or its cell-free filtrates produced no intestinal lesions (41,44). C. sordellii antitoxin was found to neutralize the toxin derived from C. difficile cultures; C. difficile antitoxin is not yet available. Lastly, passive immunization of hamsters with C. sordellii antitoxin protects them from diarrhea and colitis after clindamycin treatment (45). The evidence shows that the disease in animals is mediated by clostridial toxins.

Study of humans with AAC after antibiotic treatment indicates that toxigenic C. difficile is involved. A toxin that has all of the characteristics of the toxin derived from C. difficile can be found in cell-free stool extracts from patients with AAC (29), and the organism can usually be found in the feces (30). C. difficile toxin has not been detected in stools from healthy adults, or patients with ulcerative colitis.

While it is presumed that antibiotics suppress the normal intestinal flora permitting resistant clostridia already present in the gut to over-grow and produce the toxin and the disease, this has not been proved. Clostridium difficile toxin, which is

presumed to caused AAC in humans, is detected by characteristic cytopathic effects in various cell culture monolayers (e.g., HeLa cells). The cytotoxicity is heat and acid-labile and can be neutralized by antitoxin to C. sordellii. The toxin is a high molecular weight protein, the molecular weight variously estimated as 107,000 to 110,000 daltons on Ultrogel AcA-44 (46,47), 240,000 on Sephadex G-200 (48), or 600,000 on agarose 6B and gradient PAGE (49).

A. Role of Clostridial toxin in penicillin-associated colitis in guinea pigs.

Guinea pigs given single injections of penicillin in the dose range of 50,000 units rapidly sicken and die on the third to sixth day after injection (50,51). The administration of penicillin is followed by a massive proliferation of gram-negative coliform bacteria in the guinea pig intestine. Farrar and Kent (39) found a high incidence of bacteremia in the penicillin-treated animals, as well as severe cecitis. They could prevent the syndrome by administration of antibiotics effective against coliform bacteria. They believed the disease due to the bacterial superinfection and in fact considered it a model for gram-negative septicemia. We reexamined the penicillin-treated guinea pig looking for toxin in the cecal contents, for bacteremia and for changes in gut flora. We looked for toxin in cecal contents. Table 5 shows that, in agreement with earlier observations (39), a single intramuscular dose of penicillin causes severe illness and death in guinea pigs within 3 to 6 days of the injection. All the guinea pigs found dead, and most of those who appeared ill, had toxin in their cecal contents; toxin was not found in healthy survivors. But half the healthy-appearing animals as well as half the moribund animals, had a gram-negative coliform bacteremia. Saline controls had no toxins; one of them had a bacteremia. We never observed toxin in blood or serum.

Again because of analogies with antibiotic-associated colitis, we chose to try neutralizing the toxin activity with clostridial antitoxins. The toxin was neutralized by Clostridium sordellii antitoxin but not by antitoxin to Clostridium perfringens types A and E, Clostridium histolyticum, or by normal horse serum. Because of the long association of this model with gram-negative septicemia, we also tried neutralizing the toxin with antisera prepared against E. coli. There was no neutralization by these sera.

We inoculated a small group of guinea pigs with clindamycin for comparison and found the same syndrome, with toxin in the stool. The toxin was neutralized by C. sordellii. Later a report appeared that clindamycin in guinea pigs caused appearance

of toxin neutralized by C. histolyticum (33). C. histolyticum antitoxin did not neutralize toxin from our guinea pigs given either penicillin or clindamycin.

Vancomycin prevents death in clostridial-toxin-associated colitis in rabbits, hamsters and man--caused by any number of antibiotics (27,53). When we administered vancomycin to guinea pigs which had been injected with penicillin, we prevented the high early mortality (Table 5), though a small percentage of animals died on the tenth day. None of the animals receiving vancomycin, with or without penicillin, had toxin in their cecal contents, though half the animals with both antibiotics had bacteremia. Vancomycin, then, both prevented toxin production and prevented the high, early mortality which is closely associated with the penicillin deaths. With vancomycin bacteremia was unchanged. Coliform organisms cultured from blood were Escherichia coli, Salmonella enteritidis, Klebsiella pneumoniae, and Citrobacter freundii. Farrar and Kent (39) had shown that the injection of penicillin caused proliferation of the gram-negative coliforms in the gut. These bacteria are present in low numbers in normal guinea pigs (54).

In the light of our finding a clostridial toxin in the cecum, we reasoned that there might be a rise in clostridia in the gut as well. In rabbits with clindamycin-associated colitis, in which Clostridium perfringens E toxin is found, there is a significant rise in clostridia in cecal contents (27,28). We found two distinct patterns (Table 6). The coliform bacteria and total aerobes were significantly higher in very sick animals and those given vancomycin. Survivors of penicillin and saline controls had similar coliform counts. There seems no relation between rise in coliform counts and survival (Table 5) nor between rise in coliform counts and bacteremia.

Clostridia and total anaerobes remained the same throughout. Toxin production cannot be ascribed to a rise in total clostridia, though it could be due to rise in numbers of one species.

To establish the role of the toxin in the disease, we wanted to make sure the toxin alone could kill guinea pigs with no help from bacteremia. Toxin injected directly into the cecum or ileum killed animals. The killing was dose dependent. The toxin was also lethal when injected intraperitoneally into guinea pigs. The toxin-treated cecum showed congestion and hemorrhage, with moderate thinning of epithelium. Goblet cells were discharged. No toxin was observed in the serum.

The question asked was whether toxin alone, toxin and bacteremia, or bacteremia alone are responsible for guinea pig

Table 5. Toxin, septicemia and death in antibiotic-treated guinea pigs

Treatment	No. of guinea pigs	Outcome (%)	No. of days after treatment	Toxin (No. positive/total)	Bacteremia (No. positive/total)
Saline	7	Survived (100)%	10	0/7[a]	1/7
Penicillin	35	Died (49%)	3-5	17/17[b]	ND[c]
		Moribund (31%)	3-6	8/11[b]	4/8
		Survived (20%)	10	0/7	2/4
Penicillin + vancomycin	12	Died (20)%	9-10	0/3	ND
		Survived (75%)	10	0/9	5/9
Vancomycin	5	Survived (100%)	10	0/5	2/5

[a]Negative toxin titer is failure to detect at $\leq$ 1/4 dilution.

[b]Toxin titer is defined as the last dilution causing cell detachment. Range of titers was from 1/64 to 1/262,144.

[c]Not determined.

deaths after penicillin administration. The case for toxin alone being responsible includes the observation that all the animals found dead at 3-5 days (and early death is a hallmark of the disease) had clostridial toxin in their cecal contents. Animals which survived, or who, when given vancomycin, died after an atypically long period (ten days), did not have toxin. Most animals which were considered moribund and were killed had toxin.

Table 6. Bacterial flora in cecal contents of antibiotic-treated guinea pigs

	Log_{10} of mean colony-forming units/ gram stool (wet weight)			
	Coliforms	Total aerobes	Clostridia	Total anaerobes
Class 1				
Penicillin (moribund) (n=23)	9	9	7	10
Penicillin + vancomycin (survived) (n=9)	9	9	7	9
Vancomycin (n=5)	8	8	5	8
Class 2				
Penicillin (Survived) (n=10)	4	6	7	9
Saline	3	7	8	10

The fact that bacteremia was found in animals of all groups at about the same level (except in the control in which only one animal had bacteremia) suggests that the bacteremia is not related to the disease. Then, too, no one organism was found--instead, we found a range of coliforms. While animals with bacteremia survived for the length of the experiment (10 days), when toxin alone was administered intracecally animals died. Toxin can kill without any bacteremia. The bacteremia may be incidental.

The mechanism of toxin production following antibiotic administration is unknown. The antibiotic may kill enough of the normal intestinal flora to allow C. difficile to increase in

number thereby increasing toxin which is otherwise present in small harmless amounts. There is no rise in total clostridia, but C. difficile alone could have increased in number without this being reflected in total clostridial counts. Or the change in composition of the flora could change the environment, causing C. difficile to produce toxin. Another possibility is that antibiotics induce higher toxin production. This last is not a likely mechanism because of the wide range of antibiotics with varying modes of action which can cause C. difficile colitis in animals and man.

The identity of the toxin in this case is inferred. The toxin is neutralized by a C. sordellii antitoxin preparation which has been shown to cross-react with C. difficile toxin (46), and the consensus has been that C. difficile is the causative organism. Very likely, in this model the lethal toxin is also from C. difficile. The toxin is more consistently associated with death after penicillin administration than is bacteremia, and the guinea pigs can be considered to have antibiotic-associated colitis caused by a clostridial toxin.

B. Purification of Clostridium difficile Toxin

Several laboratories have been attempting to purify Clostridium difficile toxin from supernatant fluids of long-term cultures, ranging from 3 to 10 days (47,49,50). Appearance of toxin in supernatant fluids after many days suggests that the toxin is intracellular, and released from cells upon autolysis. To avoid problems inherent in toxin extracted after autolysis, including partial digestion by cell proteases, we grew C. difficile for 18 hr in brain-heart infusion broth and extracted toxin from cells while they were still intact. Table 7 shows that after 18 hr toxin was almost all intracellular, while after several days supernatant fluids contained high levels of toxin.

C. Patient Diagnoses

The HeLa cell assay for C. difficile toxin is useful in patient diagnosis and can confirm sigmoidoscopic observations of pseudomembranes in the colon. The assay can provide a rapid diagnosis of cases of antibiotic-associated colitis where proctoscopy findings are negative (57). The extent of the patient population with this disease is unknown.

We used the assay to provide information on stool specimens from 4 patients at WRAMC and 1 patient whose specimen came to use through AFIP. Of the five specimens, three were negative, one was positive for another clostridial toxin and one was

positive for C. difficile toxin. The specimen during the patient's acute illness had a titer of $\geq$ 1:1024. After 2 days of oral vancomycin, the titer was reduced to 1:32 and the patient had improved markedly.

A preliminary comparison was made of different media--including chopped meat-glucose broth, yeast dialyzate diffusate medium (55) brain-heart infusion, and modified Lombard-Dowell broth (56). We had also compared toxin production by several strains of C. difficile. We are reasonably satified that the medium and strain we are using are optimal for toxin production. The toxin extracted from cells was partially purified by Amicon ultrafiltration and column chromatography. Attempts to concentrate the toxin by ammonium sulfate precipitation resulted in denaturation.

Table 7 Toxin in cells and supernatant fluids of *C. difficile* strain 106.

Growth Medium	Length of Incubation (days)	Toxin units [a] per liter	
		Supernatant	Cells
Brain-heart infusion broth	1	4×10^4	1×10^6
Yeast extract dialysis diffusate broth	6	1×10^6	ND[b]
Yeast extract dialysis diffusate broth	4	4×10^7	ND
Yeast extract dialysis diffusate broth	3	6×10^6	ND
Yeast extract dialysis diffusate broth	2	3×10^6	ND
Yeast extract dialysis diffusate broth	7	3×10^6	ND

[a]Toxin is assayed in HeLa cell microtiter assay. The number of units is defined as the reciprocal of the highest toxin dilution showing killing of HeLa cells.

[b]Not determined.

D. Production of C. difficile Antitoxin

We indicated above that C. difficile toxin is neutralized by C. sordellii antitoxin. C. difficile antitoxin is unavailable, with only an oblique reference by one laboratory to their having produced some in rabbits (50). We are attempting to produce antitoxin in rabbits and in a sheep.

1. Rabbits

Rabbits were inoculated IV by toxin of C. difficile treated with formalin to yield a toxoid (58). Inoculation of 2 different concentrations of toxoid followed by inoculation of increasing amounts of toxin did not yield neutralizing antibody.

Rabbits were inoculated with toxin and C. sordellii antitoxin in Freund's adjuvant, followed by inoculation s.c. of toxin and antitoxin in imcomplete Freund's adjuvant. This is being followed by s.c. injections of toxin with bleeding after 10 days to assay for neutralizing antibody.

2. Sheep

A pilot project is being undertaken in the attempt to produce C. difficile antitoxin in a sheep. The first day, blood was taken for a normal serum control. The animal has received 3 s.c. injections of partially purified toxin neutralized with C. sordellii antitoxin. Serum taken 10 days after the third injection yielded no neutralizing antibody. Inoculations are continuing. The toxin for inoculation of the sheep was supplied by Dr. N. Harvie of the University of Michigan.

E. Biological Characterization of C. difficile Toxin

1. Sensitivity of various tissue culture cell lines to C. difficile toxin.

Titers of C. difficile toxin were the same whether assayed on monolayers of WI-38 diploid human embryonic lung fibroblasts, Vero green monkey kidney, or HeLa human cervical carcinoma cells. Toxin titers are equivalent on our HeLa cell line, one grown at NIH, and on a HeLa line (CCL2) which is resistant to Shigella toxin.

2. Mode of Action in Cell Monolayers

Crude C. difficile toxin was added to HeLa cell monolayers together with [^{3}H]-thymidine and [^{14}C]-leucine to determine whether the toxin affected protein or DNA synthesis. The toxin caused

cells to round an hour after addition to the monolayers. Figure 8 shows that during the 6-hour incubation period [^{14}C]-leucine incorporation into TCA-precipitable material was unaffected. Figure 9 shows that [^{3}H]-thymidine incorporation was inhibited almost totally starting approximately 1 hour after toxin addition. When cells were incubated with toxin for 18 hr, the intracellular K^+ concentration as measured by flame photometry was 39% of the control.

F. HeLa Cell Assay for Toxins other than C. difficile

The sensitivity of the tissue culture cell assay was tested for toxins from other species. Besides C. perfringens E toxin, which has already been reported cytotoxic for HeLa cells (28), we found that the cells were sensitive to Clostridium perfringens type C (β toxin), but not to C. perfringens type C or to D botulinum type C toxins.

G. Search for Plasmids in a C. difficile Strain

The C. difficile strain we are using for toxin production is resistant to clindamycin, and can be grown in the presence of 200 µg/ml or more. In collaboration with J. Wohlheiter, we used the cleared lysate method (59) to determine whether the strain contained a plasmid which might be reponsible for its antibiotic resistance. No plasmids were detected.

This suggests the toxin causes disruption of the cell membrane and loss of cell integrity.

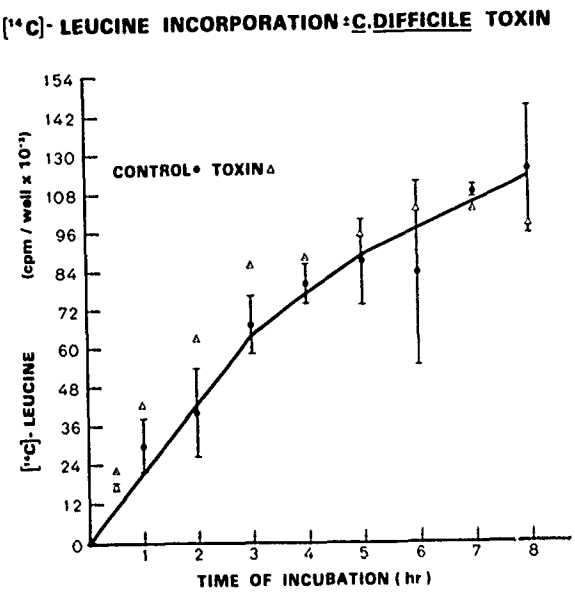

Fig 8. Incorporation of [^{14}C]-leucine into TCA-precipitable material by HeLa cells treated with Clostridium difficile toxin.

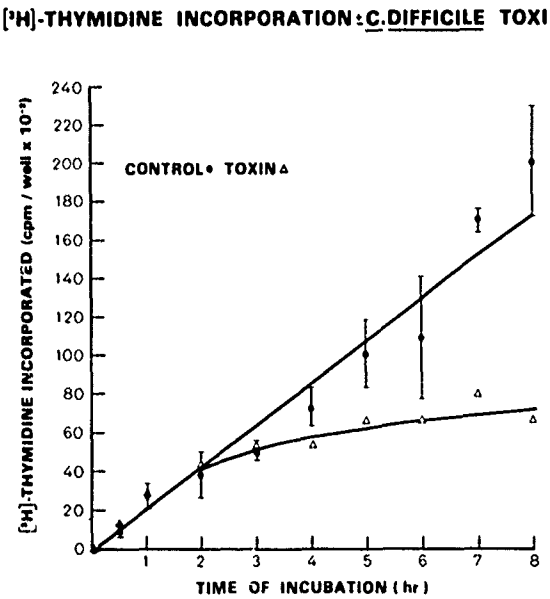

Fig 9. Incorporation of [^{3}H]-thymidine into TCA-precipitable material by HeLa cells treated with Clostridium difficile toxin.

4. DNA Studies with Enterobacteriacease and Vibrinonaceae

The family Enterobacteriaceae contains the bacteria causative agents of a variety of diseases including enteric fever, diarrhea, urinary tract infection, food poisoning and bacteremia. Additionally Enterobacteriaceae contains phytopathogenic bacteria. The isolation and identification of pathogenic bacteria and the determination of their frequency of occurence, especially for atypical organisms and/or organisms not usually associated with disease, is an extremely important aspect of medical microbiology. Our studies are designed to determine DNA relatedness in all organisms in this family of bacteria as well as other groups of bacteria, especically those associated with human disease. This data, as well as the phenotypic and serological data obtained at the Enteric Section, Center for Disease Control (CDC), Atlanta, Ga., are used for the following purposes: (a) To identify atypical clinical isolates for purposes of epidemiology and treatment, (b) To develop a classification based on genotypic relatedness, instead of a few phenotypic characteristics, (c) To develop a molecular definition of a bacterial species, (d) To accurately classify newly described bacteria, (e) To assess the lines of evolutionary divergence in pathogenic bacteria.

During the past year we have continued our work in assessing relatedness within specific genera, clarifying the status of recently described organisms, as well as atypical enteric organisms. Additionally we have begun work with the family Vibrinonaceae. Our research is in collaboration with Dr. Don J. Brenner, Chief Enteric Section, CDC, Atlanta, Ga.

Kluyvera: A New Genus in Enterobacteriaceae. Genotypic and Phenotypic Studies.

Kluyvera has been proposed for a group of gram-negative, polarly flagellated bacteria that produce large amounts of α-ketoglutaric acid during glucose fermentation. In 1962 Kluyvera was placed in the genus Escherichia because these organisms actually had peritrichous flagellae. Five culture collection strains and some 70 clinical isolates of Kluyvera and Kluyvera-like strains were characterized biochemically and with antimicrobial susceptibility profiles. About 50 strains were examined by deoxyribonucleic acid (DNA) hybridization. Two distinct DNA relatedness groups were detected. Relatedness between these groups was 60-65%. The type strains for K. citrophila and K. noncitrophila were in the same DNA relatedness group. The 10 strains in this group are all in the same species. 29 strains were in a second DNA relatedness group (K. no-name-yet). Both groups were 30-45% related to species of Escherichia, Salmonella, Citrobacter, Klebsiella, Enterobacter, and Serratia. The genus Kluyvera was

redefined to contain two species - K. citrophila (type = ATCC 14237) and a second, as yet, unnamed species. A third group of 5 strains were equally (65%) related to both K. citrophila and K. no-name-yet. These belong to the genus Kluyvera and may represent one or more additional species. Kluyvera strains have biochemical reactions distinct from those other species of Enterobacteriaceae. They resemble E. coli except they are citrate^{+}, KCN^{+}, and malonate^{+}. The two well defined Kluyvera species are almost identical biochemically, but can be differentiated with 95% accuracy because K. citrophila is more susceptible to the antibiotics cephalothin and carbenicillin. Human sputum is the most prevalent source of Kluyvera isolates, but strains have come from a wide variety of other clinical specimens. Pending better diagnostic tests and clinical revelance studies, all three groups should be reported as "Kluyvera species".

DNA Hybridization Studies in the Family Vibrionaceae

These organisms are generally classified as gram-negative, polarly flagellated, facultatively anaerobic rods. They are found in fresh and salt water, and in the alimentary canal of men and animals; some species are pathogenic for man and other vertebrates (fish).

There are five species of Vibrio recognized by the Subcommittee on Taxonomy of Vibrios: V. cholera, V. parahemolyticus, V. anguillarum, V. fischeri, and V. costicola. In addition there are at least 20 unspeciated strains that have been described. In addition to Vibrio there are four additional genera recognized in this family: Aeromonas, Plesiomonas, Photobacterium, and Lucibacterium.

In collaboration with the CDC, we are in the process of conducting a comprehensive program of study in order to properly identify and classify genotypically and phenotypically this large family of bacteria, many which are pathogenic to man. They are a diverse group of organisms having guanine-cytosine (GC) ratios ranging from 39-63%.

Preliminary hybridization studies in our laboratory have indicated wide genotypic diversity in this family. Using a V. cholerae reference strain only one other previously described sub-species, V. albensis shows a high degree of DNA relatedness.

Twelve other species of Vibrio and representatives of the other four genera of this family show only 2-15% relatedness to V. cholerae. This is in marked contrast to the enterics which have a core relatedness of approximately 20% with all genera.

Studies with genus Aeromonas show a higher degree of DNA

relatedness (45-75%) among species of this genus, thus indicating a closer degree of genetic relatedness in this genus. Work is being continued with this family of bacteria at the present time.

5. Selection of Hydridomas Producing Monoclonal Antibody to Sindbis Virus Structural Proteins.

This project is being conducted as a collaborative effort between the Dept. of Biological Chemistry and the Dept. of Virus Diseases (Dr. Joel M. Dalrymple). The detailed account of characterization of the antibodies may be found elsewhere in this annual report.

Significant progress has been made in the use of lymphocyte - hybridomas to produce monoclonal antibodies to virus proteins. Suspensions of spleen cells from BALB/C mice immunized with virus are fused with mouse myeloma line P3X63-Ag8 at a 10:1 ratio in the presence of polyethylene glycol 1000. Diluted suspensions of cells are dispensed into microtiter-plate wells. After the addition of a selective medium containing aminopterin, only fused cells continue to grow. After a period of 5-7 days, clumps of fused cells are visible microscopically in the wells. Supernatants from these wells are screened for antibody production by radioimmunoassay. Cells from those wells which are positive for antibody production are cloned on soft agarose, utilizing human fibroblasts as feeder cells either as monolayers or on microcarrier beads. Clumps of cells are picked from the agarose, returned to microtiter wells, and tested again for antibody production. Representative samples of these cells are frozen for long-term storage. Injection of these clones into pristane-primed mice results in the production of high levels of antibody in ascitic fluid.

Five fusions have been performed with spleen cells from mice immunized with either infectious suckling BALB/C mouse brain or purified CER cell-culture-propagated virions. Various combinations of material for initial immunizations and booster immunizations have been used. In the last fusion, parallel experiments were performed. A mouse initially immunized with purified virion and one immunized with mouse brain suspension were both boosted prior to fusion with injections of purified virion. Approximately equal numbers of growing fused cells resulted from both experiments, but the number of positive antibody-producing cells was increased two-fold in the latter fusion.

Immunogen		Microtiter Wells		
Initial	Booster	Screened	Containing Hybrids	Positive for Antibody
MBS	MBS	589	32	3
MBS	MBS	1,203	182	8
Virion	Virion	1,330	1,289	16 to date
Virion	Virion	444	348	64
MBS	Virion	444	345	129

Some losses have occurred, but to date a total of 130 anti-Sindbis antibody-producing cell lines have been cloned and stored in the frozen state. Preliminary characterization of 96 of the lines shows that 49 produce antibody directed against E_1 protein, 32 against E_2 protein, 8 against core protein, 1 of mixed specificity, and 6 with unknown specificity. Examination of lines from the first two fusions indicates that the cells retain their antibody-producing capability after freezing storage and subsequent thawing.

6. Selection of Hybridomas Producing Monoclonal Antibody to Surface Coat Proteins of Trypanosome Rhodesiense.

Efforts within the Division of Biochemistry at WRAIR to purifiy the coat protein from cloned and stabilated T. brucei rhodesiense (CP3B4) have consistently provided material which is homogeneous on SDS electrophoresis and heteogeneous on isolectric focusing on LKB-PAG plates. The observed charge heterogeneity is retained inspite of attempts to control for proteolytic digestion during coat protein purification (60).

In light of the effort which has been expended by investigators within the division in an effort to obtain charge homogeneous coat protein preparations it is felt that the charge heterogeneity observed may not be an artifact of the purification methodology employed. Possible alternative interpretations of coat protein charge heterogeneity include:

1. The protein may be homogeneous with respect to primary structure and charge heterogeneity may be reflective of differential post translational modifications.

2. The proteins may exhibit charge heterogeneity because they represent different primary amino acid sequences and every trypanosome in the cloned population elaborates a protein coat comprised of the three proteins observed on isoelecric focusing.

3. Either the stabilated trypanosomes used to infect rats may not have been homogeneous, or by the time the trypanosomes have been obtained from infected rats for coat protein isolation, sufficient antigenic differentiation may have occured so that the coat protein was actually derived from a serologically unresolvable heterogeneous population trypanosomes

In view of our orientation toward investigating the molecular biology of antigenic variation in trypanosomes, it is important that we obtain at least a qualitative understanding of the molecular basis of the charge heterogeneity of CP3B4 coat protein. To address this point through classical biochemical methodology requires obtaining substantial quantities of the three proteins demonstrated on isoelectric focusing in a purified form for amino acid, peptide, and sequence analysis. Alternatively, the question of charge heterogeneity can be defined qualitatively with unresolved coat protein preparations immunochemically using novel hybridoma technology described by Kohler and Milstein (61). The hybridoma methodology provides antibodies with a unique antigenic specificity by immortalizing the phenotype of a specific lymphocyte in a myeloma cell line. By cloning hybrids after fusion, one can obtain cells which generate monospecific immunochemcial reagents with specificity directed to a unique antigenic determinant in a complex antigenic mixture.

Using the hybridoma approach, we have obtained 13 cloned cell lines from fusion TRW1. These hybrids secrete antibodies which react specifically with coat protein from cloned CP3B4 organisms but not variants 6, 10, 12, or 13. The antibody secreted by each hybrid causes every CP3B4 organism to fluoresce in a indirect fluorescent antibody assay (IFA)(62). Furthermore, precipitation in gel of the three protein bands obained on isoelectric focusing of CP3B4 coat protein by rabbit antisera followed by IFA with the culture supernatant from hybrid TRW 1.201.4 causes fluorescense of each of the bands.
The results are taken to indicate that by the criteria applied:

1. The coat protein is being isolated from a homogeneous population of organisms.

2. Each of the proteins seen on isolectric focusing possess at least one identical antigen determinant. However, before the antigenic identity of the three proteins can be confirmed it is necessary that monoclonal antibodies with different anti-

genic specificities to the CP3B4 coat protein be identified and tested with protein resolved by isolectric focusing.

Work in this laboratory is continuing along the lines of defining the number of antigenic specificities which we have obtained in our hybridomas as well as preparing hybrids to the other variants from the CP3B4 serodeme in our hands.

Project: 3M161102 BSO1 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 124 Biochemical Research on Military Diseases

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION: DA OC 6451	2. DATE OF SUMMARY: 79 10 01		REPORT CONTROL SYMBOL: DD-DR&E(AR)636
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	CURRENT 80	7	619

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23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Provide text of each with Security Classification Code.)

23. (U) Investigations will seek to describe the means by which the nervous system effects bodily responses to stress and injury, and to discern those combinations of physiologic parameters which collectively define the optimal conditions for effective military performance.

24. (U) Animal models of performance will be created using the techniques of operant and respondent conditioning and the role of internal factors in performance variability assessed by neurophysiologic recording of intracellular and extracellular bioelectric potentials; the descriptive and experimental neuroanatomical techniques of light and electron microscopy and histochemistry; stimulation or lesioning of discrete brain areas; and experimental modifications of hormonal status by ablation and/or administration of exogenous hormones or other drugs.

25. (U) 78 10 - 79 09 Activities included: initiation and supervision of an extensive literature review in anticipation of renewed alcohol/drug abuse research; demonstration that in contrast to previous reports, BALB mice are relatively insensitive experimental subjects for the study of ethanol toxicity. Demonstration, in a new animal model of continuous performance, that the rhythmic fluctuations seen over time in the willingness to execute skilled behaviors can be controlled significantly by adjusting both task difficulty and the frequency/timing of rewards and check procedures; similar success was achieved in manipulating the rhythmicity in an individual's speed of attempts to solve new problems. Development of mathematical model of execution of skilled movement. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 127 Biological Modulation of Military Performance

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DESCRIPTION

Work in this unit directs the technologies of multiple disciplines toward the analysis of biological modulation of military performance. The interaction of biology and behavior can be conceptualized on several levels. At one extreme is the manner in which the biological endowment of the normal individual controls behavioral adjustment to the military environment. During fiscal year 1979, much attention has been given to the modulation of behavior by biologically based temporal rhythms, particularly the effect of time-of-day on work output and work quality. The principles derived from these analytic studies of behavioral rhythms will assist in the design and analysis of field studies of time-of-day effects and rapid time zone changes (jet lag) conducted elsewhere in this Division. In a related vein are economic variables that determine the equilibrium between behavior and the environment. Analysis continues in this area to develop principles that can guide the planning of animal models of natural behavioral systems and that can assist in the analysis of human behavioral systems. In particular, economic variables have a profound effect on the amplitude of behavioral rhythms.

At the other extreme are considerations of how alteration of the biological environment of the individual influences the normal structure and function of behavior. Work during this fiscal year has continued our interest in how the loss of normal limb afferents would alter motor functioning. In addition, preliminary work was initiated to pave the way for extensive studies of the effects of drugs of abuse and alcohol on performance and readiness.

Several new research endeavors related to this project, each dealing in broad terms with sensory systems and their interactions with behavior, have been supported this year under an ILIR project and progress is described elsewhere.

ANIMAL MODELS FOR THE STUDY OF BEHAVIORAL RHYTHMS

In support of the Division's efforts in the area of sustained operations, a series of studies is being conducted whose purpose is the development of general principles for the prediction of performance deficits as a result of time of day. It is well established that circadian rhythms (i.e., with a period of about 24 hours) occur in the behavior of virtually all species. Examples are rhythms in locomotor activity, feeding, and sleeping. Little is known, however, about rhythms in behavior patterns that are to a large extent acquired. In the development of animal models of rhythms in human behavior, it is these aspects of behavior with which we are most concerned. The questions that must be answered concern which behaviors are most likely to show decrements as a function of time of day, what variables are effective in modulating the amplitude of performance fluctuations across the day, and what can be done to minimize the effects of circadian rhythms in sustained operations. It would be expected that the results of these studies would be applicable as well to problems related to rapid time zone changes (i.e., jet lag).

The Rhesus monkeys used as subjects in these studies are trained to perform various tasks, then tested in their ability to perform these tasks at different times of day. The studies can be divided into two general categories, those concerned with quantitative aspects of behavior or behavioral output, and those concerned with qualitative aspects of behavior such as accuracy, consistency, or vigilance.

Quantitative behavioral rhythms

Given that rhythms in behavioral output occur, under what circumstances do they occur and what variables modulate their amplitude? We studied frequency of reinforcement, response cost, and reinforcement proximity.

Frequency of reinforcement. In an experiment that is now nearing completion, four Rhesus monkeys earned their total daily ration of food by pressing on a panel on the wall of their living/experimental cages (Elsmore and Conrad, 1979). During test sessions that were conducted every four hours around the clock, .75g food pellets were delivered at random intervals of time following presses on the panel. The average interval between food pellet availabilities was correlated with the color of the light illuminating the panel. Four different colors were used, correlating with different schedules of food availability. White, red, yellow, and green, respectively, signalled the availability of 3.6, 10.8, 32.4, and 97.2 pellets per hour. During test sessions, each color

and its associated schedule remained on for 7 min after which another color came on. Response rate (frequency of panel pressing) in the presence of all colors varied depending on time of day. A periodic regression analysis of a 12-day time series showed that, for all animals and all schedules, the best-fitting sine function had a period of 24 hours. In Figure 1, for each animal, the amplitudes of these 24-hour sine functions are plotted. Each data point was converted to % of the mean response rate for that animal at that reinforcement frequency. It is clear from this figure that the amplitude of the 24-hour fluctuation in behavior is strongly determined by the frequency with which that behavior is reinforced.

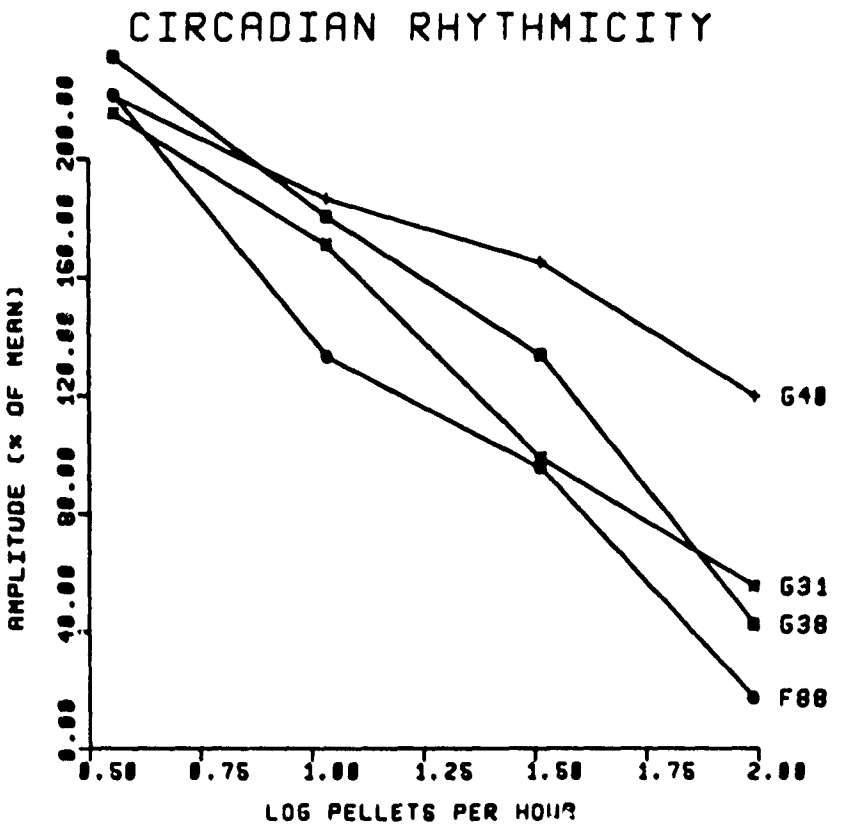

Figure 1

Current research involves maripulation of overall deprivation levels of the animals, since other work in this laboratory has shown that this variable is important in determining rhythm amplitudes (Elsmore, 1979).

Response cost or effort. In a study published this year (Elsmore, 1979), it was shown that the amount of effort required to obtain a food pellet also determines rhythm amplitude. Rhesus

monkeys earned their food by pressing on a panel which was one of four different colors, each associated with a different fixed-ratio reinforcement (FR) schedule. Each FR required a fixed number of presses for delivery of a food pellet, 1, 20, 60, or 180 presses. Sessions were run every other hour throughout the day, and there was constant illumination. In all cases, when rhythmicity did occur, it was greatest in those schedules requiring a larger number of responses. It is difficult, however, to separate the role of reinforcement frequency from that of FR in this experiment, since the two variables covary in this situation. Future research will attempt to separate the relative contributions of these variables.

Reinforcement proximity. Most human behavior outside the laboratory can be conceptualized as extended behavioral chains in which a directed sequence of behaviors results in some desired outcome. Writing a report, for example, involves sitting down at a desk, finding writing materials, organizing one's thoughts, and finally committing them to paper. The outcome is a completed report. Much animal research has demonstrated that the initial portions of behavioral chains are more susceptible than latter portions to disruption by a variety of events, including punishment, availability of alternative sources of reinforcement, and changes in the reinforcing efficacy of the outcome. A simple analog of a behavioral chain is the fixed ratio or FR schedule in which a fixed number of homogeneous responses produces reinforcement. In an experiment with Rhesus monkeys, 50 panel presses were required to obtain a food pellet. Test sessions were conducted at six different times of the day. Typical performance on FR schedules is characterized by a pause immediately following reinforcement followed by an abrupt transition to a more-or-less uniform rate of responding until the next reinforcement. In this experiment the variability in post-reinforcement pauses as a function of session time was shown to be considerably greater than the variability in interresponse times once responding commenced. In this continuing study, the effects of overall deprivation level and FR size will be explored.

Qualitative behavioral rhythms

Much of the concern with the possibility of human performance decrement in sustained operations lies with the quality of performance rather than its quantity. In attempting to construct animal models of qualitative tasks, two tasks were chosen, differing in the locus of the events controlling behavior: internal to the organism, or external, exteroceptive stimuli. A third task was studied which involved both internal and external control in the acquisition

of new behavior.

Behavior under the control of internal stimuli. Rhesus monkeys were trained in an analog of human counting. In this procedure, the animals had access to two panels. In order to obtain food, the animals were required to press on one panel at least ten, but less than fifteen times, then press on the other panel. Switches following fewer than 10 or more than 14 consecutive presses on the first panel produced a 30-second interruption of the experiment, and required the animals to start again. The only event cueing a correct switch, therefore, was internal, the completion of an appropriate number of first-panel presses. Sessions were conducted at six different times of day.

All animals showed both quantitative and qualitative circadian rhythms in performance. Overall rate of panel pressing was greatest during the light portion of the light-dark cycle and lowest during the early morning hours. Errors also showed a circadian pattern, with early switches being more probable at some times of the day than at others, and late switches showing a mirror-image pattern. The circadian pattern for errors, however, was different for different animals. A signal detection analysis of the data showed that the animals' sensitivity to number remained constant throughout the day, but that the bias towards making the switching response varied substantially. At night the subjects shifted the switching response toward the upper limit of the reinforced range of counting responses. Continuing research is investigating the role of problem difficulty upon the amplitude of the various rhythms in this situation, and is attempting to further characterize the signal detection characteristics of the results.

Behavior controlled by exteroceptive events. A complex task was designed in which animals are required to attend to the duration of an auditory stimulus and report it as being shorter or longer than a criterion duration by pressing on either a red or green panel. Delays ranging from 0 to 8 seconds were imposed between the offset of the tones and the opportunity to press on choice panels. Test sessions were run at six different times of the day. Again, rhythms were observed in both quantitative and qualitative aspects of performance. Greater rhythmicity was found with more difficult discriminations (i.e., those near the short-long cutoff point) than with easier discriminations. No differences, however, were found as a function of the delay between stimulus offset and opportunity to register a choice. Current research is investigating longer delays to determine if short-term memory is differentially susceptible to decrement as a function of time of day.

Acquisition of new behavior. Rhesus monkeys have been

trained to acquire a behavioral sequence requiring four responses on three push plates. For example, food may follow a sequence of presses such as key 1, key 2, key 3, key 1. The correct sequence of presses is changed at the start of each session so that the acquisition of the solution can be studied repeatedly. During the early part of each session the subjects typically attempt a variety of different solutions. Any correct response - such as key 1 in the last position of the example above - is signalled as correct regardless of the accuracy of the other three responses, but food only follows entirely correct sequences. The subjects achieve a high level of accuracy requiring about twenty attempts before solving the sequence problem consistently. A computer system of control and recording permits automatic programming of new problems without experimenter intervention and automatically records the details of each acquisition process. Last year we tested the subjects on two new problems each day occurring at any of six times-of-day. Across a broad range of food rations (motivation levels) we found only small time-of-day effects on the speed of pressing the keys and no time-of-day effects on accuracy of solving the problems. When switched to six problems a day around the clock providing a choice of work times and with a generous food ration, it was found that the subjects seldom worked at 0200 hrs and showed consistent decreases in speed and accuracy of working from a peak at 0600 or 1000 to a trough at 0200.

The primary focus of this year's work has been to study the susceptibility of this time-of-day effect to economic constraints on food supply. The number of food pellets available as a result of solving each problem was systematically reduced from 52 to 25 in five steps. It was found that for all subjects this economic constraint on food availability generated problem solving behavior at all test hours including 0200. For two of the four subjects this increased behavior at 0200 reflected a gradual reduction in overall rhythmicity as the food ration was reduced (see Figure 2a). For the other two subjects the increased behavior at 0200 reflected an overall increase in response rate at all times of day with no change in the range of the time-of-day rhythm (see Figure 2b). Despite these changes in the rhythmic patterns of response speed, no changes in the amplitude of the rhythmicity of problem solving accuracy was found even at a ration of only 25 pellets per problem. There may be some decrease in the day to day variability of problem solving behavior with reduced food rations, but the decreases in variability did not change the average accuracy with a given ration.

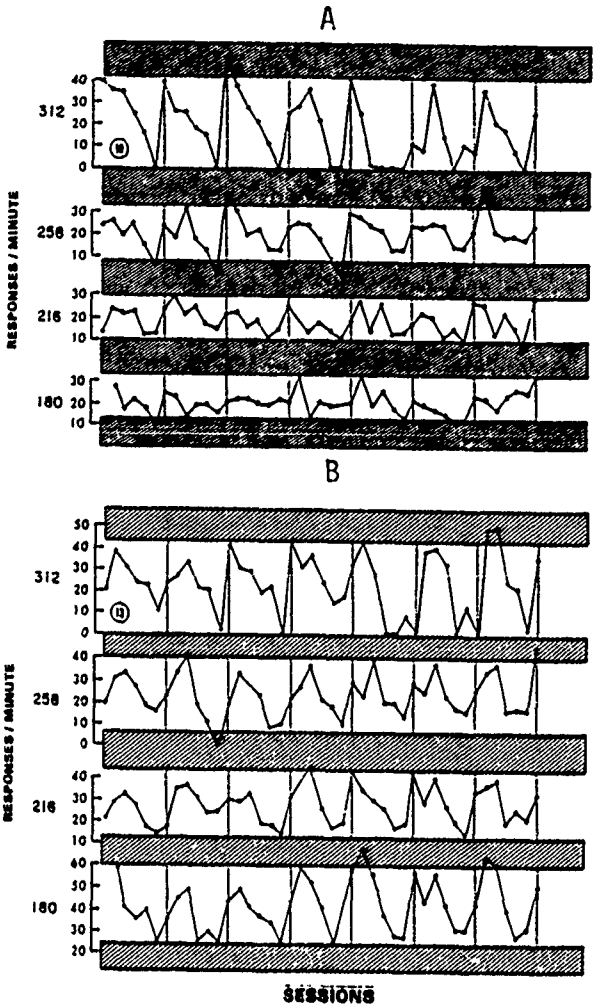

Figure 2

Summary of behavioral rhythm work

Animal models have been developed for the study of the role of various aspects of schedules of reinforcement in the determination of rhythms in learned behavior. To date the research suggests that:

1) There is an inverse relationship between frequency of reinforcement and amplitude of behavioral rhythms.

2) There is a direct relationship between effort required to obtain reinforcement and amplitude of behavioral rhythms.

3) Behaviors more proximal to reinforcement in a chain of behavior are less likely to show circadian rhythmicity than those distant from reinforcement.

4) There is little discernable difference between internally and externally controlled behavior in terms of behavioral rhythmicity.

5) More difficult discriminations are more subject to circadian variability than are easy discriminations.

6) In vigilance tasks, response bias (i.e., tendency to report a signal) may show considerable circadian variability, while sensitivity to the stimulus remains relatively constant.

7) Similarly, in a problem solving task, speed of working will be more rhythmic than accuracy.

8) Economic constraints that increase work output will alter the rhythm in speed more than the rhythm in accuracy.

PRELIMINARY STUDIES OF DRUG EFFECTS ON PERFORMANCE AND READINESS

During FY 79 two efforts in the area of drug abuse have been made. First, a contract was let to review the literature on drug effects and militarily relevant performance. This review is being performed by Associate Consultants Inc. of Washington, DC, and will be completed by the end of FY 79. Drugs being covered by the review include opiates, cannabinoids, and ethanol, as well as various other classes of abused drugs. Particular emphasis is being placed upon effects of withdrawal, interactions of drugs with stressors, drug-drug interactions, and chronobiological effects of drugs. Performance categories include sensory-motor function, memory, information processing, and complex simulation situations (e.g., driving). It is expected that this review will identify those areas in need of further research to be done both in-house and via extramural contract beginning in FY 80.

A second effort in the area of drug abuse, completed during FY 79, was a behavior-genetic analysis of the use of the conditioned flavor aversion paradigm in screening for behavioral toxicity of drugs (MacPhail and Elsmore, 1979). Briefly, in this experiment two inbred strains of mice (C57BL/6 and BALB/c) as well as their F1 cross were injected with various doses of ethanol immediately following their first exposure to the taste of saccharin. For one day on each of four subsequent weeks they were given saccharin solution as their only fluid. The C57BL/6 and F1 mice showed a dose-related reduction in their normal preference for saccharin, while the BALB/c mice showed no relationship between saccharin consumption and prior ethanol dose. These results are in direct contrast to other research showing that BALB mice are in general more sensitive to the effects of ethanol than are C57 mice. The

result suggest that the normal response of a strain to the flavor may be important, as the C57 control group increased fluid consumption by 100% on saccharin days, while the BALB control group's consumption of saccharin did not differ from water consumption. It was concluded that this particular flavor aversion paradigm was not well-suited for behavioral toxicity screening.

THE BIOLOGICAL ECONOMY CONTROLLING CHOICE BETWEEN DIFFERENT COMMODITIES

The concepts of micro-economics purport to describe the factors determining the equilibrium between a subject's demand for a commodity and the environment's supply. In this sense, microeconomics is a theory of steady-state behavior. The experimental analysis of behavior is another discipline that, among other things, has developed a theory of steady-state behavior. The economic model was originally developed from the observation of mass consumer behavior using money as a medium of exchange. The behavioral model was originally developed from the experimental analysis of individual test animals in rigidly controlled laboratory experiments using arbitrary discrete movements of levers or buttons as the behavioral measure. Each model can be extended to the domain of the other discipline with interesting results. An effort has been made this year to extend the economic model to the analysis of existing data from laboratory studies of animals. Four conclusions have been formulated that will guide future plans for behavioral experimentation with animals:

1. The economic system arranged in a behavioral experiment can determine the results.

2. Reinforcers (rewards or commodities) can be distinguished by a quality independent of relative value called elasticity of demand.

3. Reinforcers interact as complements, as well as substitutes.

4. Finally, because reinforcers differ in elasticity and because reinforcers can be complementary, no single choice rule can account for all choice behavior.

During the coming year, a battery of experiments are planned to test the limits of the economic model for the description of steady-state behavior in individual research subjects.

RECOVERY OF MOTOR FUNCTION AFTER LIMB DEAFFERENTATION (DORSAL RHIZOTOMY)

In previous reports results were presented showing that unilateral dorsal rhizotomy performed to abolish sensory inputs from the upper limb of a monkey does not interfere with the animal's ability to achieve a high density of reinforcement on a weight-lifting task. The paradigm required that the animal flex its arm at the elbow through at least a criterion arc in the vertical plane. Various loads were suspended from the arm restraint which limited movement to rotation in the vertical plane.

Comparison of the trajectories generated by normal and unilaterally deafferented monkeys on the first lift of an unknown load revealed that in the initial stages of testing the deafferented animals achieved the final position only after delayed error corrections which were apparent in the curves for acceleration as either decelerations exceeding that of gravity or increased accelerations which appeared as inflection or secondary maxima in the recordings of acceleration (Wylie and Tyner, 1978). It was inferred that the final peak positions achieved depended upon regulation of the muscle force during the rising phase of the movement. It was further inferred that the normal animals, to achieve about the same set of final peak heights, must also regulate the muscle force but that the short loop delays provided by proprioceptive feedback pathways (ca 20 msec.) allowed such rapid corrections of any errors consequent to the unknown loads that the corrections were not evident in the waveforms of acceleration.

Repeated testing of the deafferented animals has now revealed that the delayed error corrections declined in frequency and amplitude. The protocols for testing of the two deafferented animals were sufficiently different that I can not extract any common variable which contributes to the increasing smoothness of the trajectories other than repeated testing.

The observation that with repeated practice the deafferented animals were able to generate lifts which at least approximated the smoothness of those generated by normal animals leads to several alternatives. One possibility is that the disappearance of delayed error corrections might be correlated with the acquisition of a visual tracking skill. An attempt at directly testing this hypothesis by requiring the animal to perform in complete darkness was unsuccessful because the animal, in darkness, did not generate sufficient lifts to yield analyzable data. The visual tracking hypothesis, however, is not fully satisfactory

on the grounds that the final position achieved is still steeply weight dependent even after extensive practice. If visual tracking underlies the behavior, the error signal is evidently insufficient to fully compensate for the external load.

A second possibility is that the behavior observed (both that of the normal and of the deafferented monkeys) does not depend upon regulation of the ongoing movement. That is, given the mechanical properties of the system, a constant driving function applied through the motor nerves is sufficient to move the system through the observed trajectories given the range of external loads applied. That is, the presence of neither a light load nor of a heavy load requires modulation of the neural signals applied to the muscles. Three approaches have been used to address this question; recording of the electrical activity of the muscles, development of a mathematical model, and analysis of the forces applied to the external loads during movements.

Electromyograms

The electromyograms clearly indicate that the signals to the muscles are modulated during the course of a lift of an unknown load. Inspection of the raw EMG's obtained over a range of 0 to 608 g with 3 normal animals and one deafferented animal clearly show that lightest loads are accompanied by the least EMG activity and the heaviest loads by the most EMG activity. The extent of overlap in the intermediate range of loads is considerable and explains why the effects are not obvious in the recordings from the second deafferented animal who was tested only over the range of 114 to 414 g. Furthermore, comparison of rectified and integrated EMG's from one normal and one deafferented animal indicate that whereas the normal animal generates a peak EMG early in the lift, the deafferented animal, when confronted with the heaviest loads, reaches a peak in the EMG only relatively late in the trajectory. The differences in the phase relations of the peak EMG and the peaks of the variables of motion (acceleration, velocity and position) between the animals presumably reflects the differences in the loop delays earlier inferred from recordings of acceleration. The rectified and integrated EMG from the deafferented animal also indicates that the signals to the muscles increase monotonically until some 200 msec after the start of the movement. The implication is that the driving function is invariant with respect to load until well after the onset of movement. This agrees well with the results to be discussed below.

Mathematical model of limb trajectory

The mathematical model was developed to predict the trajectories across a range of loads given a constant driving function.

The model is a mechanistic model in that it contains terms for each of the major physical forces in the paradigm. These include the gravitational forces on the load, arm restraint and monkey's arm, the rotational inertia of the arm restraint and monkey's arm, the apparent viscosity inherent in muscle and the springlike properties of muscle. The model allows the experimenter to calculate a time-dependent driving function from the observed trajectory of a lift generated by a monkey and some assumed values for parameters reflecting the velocity and position dependent properties of muscle. Having obtained numerical values for a driving function from a real lift of the lightest load in the range to be explored, the experimenter can then calculate the trajectories that would be expected if a system described by the model were required to lift each of the heavier weights. Preliminary results indicate that if the parameter for viscosity is sufficiently high, then such a system can move all of the weights but that the final peak position tends to show a stronger inverse relationship to load than that generated by either normal or deafferented monkeys. The model at present has some weaknesses, principally the assumption that viscosity should be represented as a constant rather than as a time-dependent variable. It does serve to focus on the force-velocity properties of muscle and agrees with the results suggested by the EMG's that the onset of movement generated by deafferented monkeys across loads is the consequence of an invariant driving force.

Torque calculations

The third approach to the problem has been to calculate the torques exerted on the apparatus by the muscle-limb system to see if the peak accelerations achieved early in a lift are the consequence of a load dependent torque. That is, does the torque exerted on the experimental apparatus at the time of the peak acceleration, to overcome the inertial resistance of the system, increase as a function of the test load? The results indicate that at the time of peak acceleration, which falls early in the rising phase of a lift, the torque applied to the inertial resistances of the system by the deafferented animals is not an increasing function of the test load. On the other hand, the performance of normal animals ranges from that of the deafferented monkeys to a "best" performance by one normal monkey in which the torque increases as a linear function of the load. All of the normal animals tend to approach this "best" performance with repeated testing.

In conclusion, the results suggest that performance of skilled motor tasks in which unexpected external loads may be encountered depends on the interaction of the mechanical properties of the

muscle-limb system and active regulation of the ongoing movement. The visco-elastic properties of muscle provide some stabilization in the face of external perturbations and may be effective over a considerable range of loads but active regulation is required for the achievement of large displacements occurring over time.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 127 Biological Modulation of Military Performance

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Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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78 10 01	D. Change	U	U	NA	NA	☒ YES ☐ NO	A. WORK UNIT

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(U) stress (U) cyclic nucleotides (U) neurotransmitters (U) neurochemistry (U) microwave inactivation (U) lateralization of cerebral function

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number preceds test of each with Security Classification Code)

23. (U) To examine neurochemical mechanisms regulating neuroendocrine responses involved in adaptation to stress, providing database for interpretation of military field studies and recommendations for prevention and/or treatment of breakdown in soldiers. To examine neurochemical mechanisms mediating lateralization of function, spatial abilities and recovery from cerebral injury.

24. (U) Analysis of role of neurotransmitter pathways in regulation of hormonal response to stress. Effect of stimulation or lesion of specific pathway (i.e., noradrenergic dopaminerg ., serotonergic). Effect of stress or centrally-acting hormones on cyclic nucleotides and neurotransmitters in specific brain regions. In-vivo determination permitted by use of microwave enzyme inactivation system designed in this laboratory. Role of dopamine in lateralization of cerebral function.

25. (U) 77 10 - 79 09 We have found that muscarinic cholinergic agonists markedly elevate cyclic AMP in the hypothalamus interpeduncular region and the pituitary. Cyclic GMP was also increased in several brain regions including the cerebellum, brainstem, midbrain, hippocampus and septal region. Drug-induced changes in brain regional cyclic GMP are frequently, but not always correlated with changes in motor activity, which we have shown can increase cyclic GMP within 30 seconds. We have shown that dopamine in substantia nigra is released by amphetamine but not by depolarization, possibly examining the unique behavioral effects of amphetamine. We have found that chronic amphetamine administration increases the sensitivity of the hormonal response to dopaminergic stimulation. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

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Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 128 Mechanism of Response to Military Stress

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I. Mechanism of Neuroendocrine Response to Stress

In order to study neurochemical mechanisms determining adaptive hormonal response to environmental stress, we have successfully developed new models. It is well established that stress causes elevations in plasma corticosterone (1-5) and prolactin (5) while lowering plasma growth hormone (3,4). Corticosterone levels following stress vary with time of day, reflecting the circadian rhythm in testing levels (2). It has been demonstrated that habituation occurs to the corticosterone response induced by handling (3,4). Stress is reported to activate central noradrenergic (NE), dopaminergic (DA), and serotonergic (5HT) neurons (6-12). Increases in cAMP levels in brain tissues have been demonstrated following exposure to NE, DA or 5HT in vitro (16,17) and to NE (but only minimally to DA) in vivo (18). It has been recently reported that cold stress elevates cerebellar cyclic GMP (13,14). Cyclic nucleotides are known to activate protein kinases. Rodknight (15) notes a possible stress component in brain protein kinase activity. The foregoing led us to predict that psychological stressors will elevate cGMP and/or cAMP. An ACTH fragment reportedly lowers brain GABA (19). As ACTH is released in stress and GABA administration lowers cGMP (20), it would seem highly desirable to study the effects of stress (as well as ACTH and other peptides) on brain cGMP and GABA, as well as norepinephrine and cAMP. A method has been established in our laboratory which permits assay of gamma-aminobutyric acid (GABA), glutamic acid (GLU), cyclic adenosine 3'5' monophosphate (cAMP), cyclic guanosine 3'5' monophosphate (cGMP) and norepinephrine in the same sample of brain tissue after microwave inactivation of enzymes, thereby increasing the amount of information obtainable from a single experiment. The assays employed are the radioimmunoassay of Steiner for cyclic nucleotides (21,22) and the enzymatic method of Graham and Aprison for GABA and GLU (23). To ensure optimal support of the stress studies, we have modified assay procedures to increase the sensitivity of our cyclic nucleotide assay system by acetylating our samples

as described by Harper and Brooker (J. of Cyclic Nucleotide Research 1:207-218, 1975). This has increased our sensitivity into the femtomole range. It is thought that cGMP is responsive to cholinergic transmission (24,27) and under various conditions, brain tissue cAMP is stimulated by norepinephrine, dopamine, serotonin and histamine (25-27). Further studies in our laboratory have demonstrated that the technique of using high-intensity microwave irradiation for enzyme inactivation is indispensable for determining levels of cAMP, cGMP and GABA in brain regions (28,29). The elimination of artifact has permitted accurate assessment of levels of these substances in the regions studied (30-38). In addition, for many of the regions, the work is unique in that levels have never previously been reported. Brief exposure to certain environmental stressors has been reported to elevate cyclic guanosine 3'5'-monophosphate (cGMP) in cerebellum of rats and mice (39,40). We were surprised to find that forced immobilization failed to produce elevations in cerebellar cGMP (41) in rats even though the plasma hormone profile was typical of the response to stress in the rat (1-5) with marked elevations in plasma corticosterone and prolactin with reductions in plasma growth hormone. This finding led us to examine the effect of locomotor activity on cGMP in rat brain regions. We found that 5 minutes of running in an activity wheel produced marked elevations in cGMP in a number of brain regions (42). The regional pattern of cGMP increase was similar to that produced by cold exposure (41) in that the elevations were most marked in the cerebellum and midbrain. These findings led us to speculate that locomotor activity might be an intervening variable in producing some of the changes in cerebellar cGMP reported to occur following drugs or exposure to environmental stressors (42). Accordingly we decided to examine the interaction of immobilization with the robust elevations of cerebellar cGMP produced by either cold exposure or injection of apomorphine.

A 5 min. exposure to cold ($4^{o}C$) produced a robust elevation in cerebellar cGMP compared to controls sacrificed immediately upon removal from their home cages (4.63 ± 1.48 vs 0.74 ± 0.18 picomoles per mg. wet wt). Animals moved from their home cages to a novel cage at $22^{o}C$ for 5 minutes also had elevated cerebellar cGMP compared to controls (2.00 ± 0.34 vs 0.737 ± 0.18 picomoles per mg. wet wt). Superimposition of immobilization on the cold exposure condition produced a marked attenuation of the cold exposure effect. In fact cerebellar cGMP levels in the immobilized/cold-exposed group were slightly lower than levels in the freely moving group that explored a novel cage for 5 minutes at room temperature. Levels in the immobilized/cold-exposed group were slightly elevated compared to the group immobilized for 5 minutes at room temperature. Apomorphine produced a marked increase in cerebellar cGMP ten minutes

after injection in freely moving animals, compared to freely moving saline-injected controls (5.26 ± 0.63 vs 1.72 ± 0.24 picomoles per mg wet wt). The immobilized/apomorphine group had cerebellar cGMP levels slightly lower than the freely moving/saline group. The animals immobilized for 10 minutes following saline injection had very markedly reduced levels compared to the freely moving/saline group (0.24 ± 0.06 vs 1.72 ± 0.24 picomoles per mg wet wt). Although immobilization seems to have markedly attenuated the apomorphine effect on cerebellar cGMP compared to the effect in freely moving animals, the immobilized/apomorphine group had significantly elevated levels compared to the immobilized/saline group. In addition to previously demonstrating that locomotor activity elevates cGMP in the cerebellum in rats (42), while forced immobilization for 5 minutes has no effect on cerebellar cGMP (41), we have also shown that prolonging immobilization to 15 minutes reduces levels to 40% of control levels (42). The present study demonstrates that immobilization markedly attenuates the elevation in cerebellar cGMP following either cold exposure or injection of apomorphine. These data also indicate that immobilization for as brief a period as 10 minutes significantly reduces cerebellar cGMP. It is important to note that the immobilization technique did not prevent all motor activity. The movements associated with respiration were observable as were some head movements. It is possible that cold exposure and apomorphine increase the rates of these movements. Numerous drugs are reported to elevate cerebellar cGMP. Of these, the locomotor effects of amphetamine are well known; apomorphine induces stereotypic movement (73); oxotremorine and harmaline produce tremors (43); pentylenetetrazol produces seizures and subconvulsive doses produce irregular, jerking movements; and morphine withdrawal produces "wet-dog shakes". Numerous environmental conditions have also been reported to elevate brain cGMP, including forced swimming, fighting, hot plate exposure and cold exposure. Although the effects were ascribed to "stress", the locomotor aspects of swimming or fighting are obvious; cold exposure has been shown to increase locomotor activity in rats (44,45); and the hot plate exposure cited was for a duration of 20 seconds at 56°C. Under the latter conditions Eddy (46) describes mice "dancing", jumping and shaking their paws after only 8-10 seconds. Many drugs are reported to depress cerebellar cGMP. Most of these have sedative properties and some have been specifically shown to depress locomotor activity. Although locomotor activity or its suppression might contribute to the changes in cerebellar cGMP in many of the examples cited, it cannot be assumed that locomotor activity is a sole or consistent factor. Any number of the drugs cited might produce their effects on cGMP independent of any effects on activity. For example RO 20-1724, a phosphodiesterase inhibitor we found elevates cGMP, has marked sedative properties (47).

Since increased activity might be an unavoidable and confounding variable following administration of various drugs or exposure to stressful stimuli, we have conducted further experiments designed to allow the estimation of the activity related component of observed cyclic GMP changes. Rats were habituated to motor-driven activity wheels which rotated at a rate of 5 rpm, a rate that most of the rats appeared to comfortably tolerate. On the day of the experiment rats were subjected to 0, 15 seconds, 30 seconds, 1 minute, 2 minutes or 5 minutes of motorized running at a constant rate. The rats were then quickly sacrificed by microwave irradiation and the lateral and medial portions of the cerebellum were dissected. These tissue samples were weighed, sonicated, and centrifuged. Supernatants were frozen and later analyzed for cyclic GMP. Cyclic GMP levels were doubled after 30 seconds of running and quadrupled at 5 minutes. This experiment both verified our earlier work regarding the relationship between cyclic GMP and activity and also demonstrated the rapidity of the response and the small amount of activity required to generate cyclic GMP increases. A second experiment was designed to determine the length of time required for cyclic GMP to return to baseline levels after activity. Rats were subjected to 5 minutes of moderate activity in the motorized running wheels and then sacrificed immediately, 5, 10, 15, 20, or 25 minutes after the activity stopped. Cyclic GMP levels were highest (2.3 pmoles/mg wet weight) in the cerebellum of rats sacrificed immediately after the 5 min activity period. Levels had decreased to 1.5 (pmoles/mg wet weight) in rats sacrificed after a 5 min rest period following the activity. Rats given 10 or 15 minutes of rest prior to sacrifice had only slightly elevated cyclic GMP levels. Rats given more than 15 minutes of rest had cyclic GMP levels similar to those of rats who had not had running exposure (0.8 pmoles/mg wet weight). As a result of these studies, measurements of locomotor activity prior to sacrifice after various types of treatments is routinely accomplished in the following manner. Rats are habituated to open-field activity cages which are circular arenas which contain photocells. Electronic equipment connected to the photocells record the number of beam breaks in each cage. On the day of an experiment, rats are placed in these cages and their activity recorded at 5 minute intervals before and after drug or stress manipulations until the time of sacrifice. In this way changes in activity levels among treatment groups can be quantitated and the activity contributions to cyclic GMP increases can be estimated.

Centrally-acting antimuscarinic agents play an important part in the therapy of poisoning due to organophosphates, but unfortunately at the dosage required, they severely disrupt behavior. Atropine, a centrally active muscarinic cholinergic receptor blocker, has been shown to markedly affect the level of cyclic 3'5' Guanosine

Monophosphate (cGMP) in mouse brain (48). Levels in cerebral cortex were trebled while cerebellar levels were halved. Oxotremorine, a centrally active cholinergic receptor agonist, caused increases in cerebellar cGMP, an effect reversible by atropine. An understanding of the neurochemical mechanism by which anticholinergic agents such as atropine and benactyzine disrupt behavior requires assessment of effects in many more brain regions than those cited. Cholinergic systems in brain have been mapped using cholinesterase activity, choline acetyltransferase activity, choline uptake, acetylcholine receptor activity as well as acetylcholine levels. Pathways described include the septohippocampal pathway--thought to be involved in memory, the cholinergic neurons intrinsic to the striatum, the habenulo-interpeduncular tract, and the olivocochlear pathway. In addition, the amacrine cells of the retina are thought to be cholinergic (49,50) and cholinesterase staining suggests that the superior colliculi may have cholinergic input as well (51). The technique employed in the referenced study (1) is unreliable because the slowness of freezing permits biochemical artifacts to occur. It is also limited because of the difficulty of performing rapid reliable regional brain dissection on frozen tissue. Our laboratory, as noted, has developed a technique for simulataneous sacrifice and rapid inactivation of brain enzymes in the rat which leaves the brain in a suitable condition for regional dissection (28-38). We have examined in detail the regional cyclic nucleotide response following central cholinergic stimulation in the brain of the rat. Male albino rats WRC strain, weighing between 250-300 grams were maintained in a light cycled chamber and all experiments took place at the same time of day. Animals received an intraperitoneal injection of either oxotremorine (2 mg/kg), physostigmine (0.5 mg/kg), or saline 10 minutes prior to sacrifice by exposure to high power microwave irradiation. We used a 2.5 kilowatt, 2450 MHz, microwave inactivation system as modified in our laboratory. Animals receiving the cholinergic agonists were pretreated (30 min.) with methylatropine (0.5 mg/kg) to avoid excess peripheral cholinergic stimulation. Following sacrifice and decapitation, trunk blood was collected for radioimmunoassay for corticosterone (CS) prolactin (Prl) and growth hormone (GH), and 18 brain regions were dissected for radioimmunoassay for cyclic AMP and cyclic GMP. Levels of cyclic GMP in the cerebellum of animals receiving oxotremorine increased significantly (0.98 $\pm$ .11 to 2.03 $\pm$ .18 pmoles/mg tissue) as did levels in several other regions, i.e., brainstem, midbrain, hippocampus and thalamus. Physostigmine only increased levels significantly in the midbrain and hippocampus. Oxotremorine increased the levels of cyclic AMP in several regions, e.g., the hypothalamus, substantia nigra and interpeduncular region, with the most dramatic increase in the pituitary (1.15 + .13 to 14.63 + 2.74 pmoles/mg tissue). There were no significant differences in CS or Prl levels among the groups. GH however, was significantly reduced following oxotremorine.

The regional pattern of increase in cyclic GMP levels in the animals receiving oxotremorine is similar to the response following locomotor activity (5) consistent with the behavioral observation of tremor. The findings of increased regional levels of cyclic AMP, on the other hand, are quite unique and open up an entirely new area for study. Inquiry into the neurochemical basis of the action of general anesthetics and other central nervous system depressant drugs has led a number of investigators to examine the effects of these drugs on levels of the cyclic nucleotides adenosine 3',5'-monophosphate (cyclic AMP) and guanosine 3',5'-monophosphate (cyclic GMP). Both cyclic AMP and cyclic GMP are found in the CNS in relatively high concentrations, and both compounds have been suggested as mediators of synaptic transmission (52,53). At present, a large body of evidence points to alterations in synaptic transmission as being responsible for much of the phenomena of general anesthesia (54,55). Biebuyck et al. (56) found that whole brain levels of cAMP were increased approximately 2-fold after one hour of anesthesia with either halothane, ketamine, or morphine in rats ventilated to control pCO_2 at normal values. Nahrwald et al. (57), reported no significant change in cAMP in mouse cortex, cerebellum, or spinal cord during halothane anesthesia, except for a decrease in cAMP in the cortex at concentrations of halothane of 2.4% or greater. These higher doses of halothane were associated with hypoxemia and attendant acidosis. Barbiturates produce centrally mediated behavioral effects but their mode of action remains unknown. Neurochemical studies in rats during acute barbiturate administration have shown changes in the turnover of the catecholamines, serotonin, and acetylcholine (58). These changes might cause alterations in the post-synaptic activities of the cyclic AMP and cyclic GMP systems. Cyclic GMP levels in the cerebellum have been shown to decrease with acute administration of pentobarbital (43) and our laboratory has reported cyclic GMP decreases in many brain regions in rats maintained chronically on barbiturates (59). Mueller et al. (60) have demonstrated an inverse correlation between cerebellar cGMP and pCO_2 as ventilation was varied. Their studies illustrate the need to verify that arterial blood gas values are in the normal range before the effects of a drug on brain cGMP levels can be reliably assessed. The effects of two general anesthetics, pentobarbital and halothane, on in vivo levels of cyclic AMP and cyclic GMP were examined in 17 brain regions and the pituitary in the rat. Ventillation was controlled to produce normal values of arterial pH, pCO_2, and pO_2, to eliminate changes in cerebral perfusion and oxygen delivery which occur as a result of the respiratory depressant effect of these drugs. Arterial pressure was monitored, and colonic temperature was maintained within normal limits. Experimental and control animals were sacrificed by microwave irradiation one hour after the start of anesthesia.

All animals receiving pentobarbital or halothane were found to have pO_2, pCO_2, pH and mean arterial pressure within the physiologic range. Pentobarbital significantly decreased levels of cyclic GMP in 14 of the 17 brain regions examined as well as in the pituitary. In most of these regions the cyclic GMP levels were decreased by over 50%. The most dramatic decreases were seen in the cerebellum (to 7% of control levels), the pineal (to 13% of control) and in the cortical areas (24-33% of control). A striking increase of more than seven-fold in pituitary cyclic AMP levels was found after one hour of pentobarbital anesthesia. Other statistically significant changes occurred in the frontal cortex where cyclic AMP levels dropped by 28% and in the amygdala where cyclic AMP levels were increased by 27%. All other regions showed small and statistically insignificant changes with pentobarbital. Halothane significantly decreased levels of cyclic GMP in 15 of the 17 brain regions measured and in the pituitary. Again the decreases in the cerebellum and pineal were most marked, to 10% and 12% of control levels respectively. Halothane elevated cyclic AMP levels in the pituitary to 270% of control levels after one hour of anesthesia. Significant increases (with levels reported as percentage of control) were also observed in the cerebellum (158%), brainstem (149%), and hypothalamus (165%). In the striatum levels were significantly decreased to 75% of control levels. Both pentobarbital and halothane anesthesia greatly decreased levels of cyclic GMP in most of the brain regions examined especially in the cerebellum and in the pineal. We have previously reported similar decreases in cyclic GMP after chronic intake of sodium barbital or 15 minutes of forced immobilization (41,59). Others have reported decreases of cyclic GMP in cerebellum after sedative or depressant drugs (43,61-64). Interestingly, we have reported <u>increases</u> in cyclic GMP in many rat brain regions after 5 minutes of activity in a running wheel especially in the cerebellum where there was a 2 fold elevation of cyclic GMP (42). One logical interpretation of these findings would be that drugs or behavior which decrease activity also decrease firing rates in neuronal pathways linked to post-synaptic cyclic GMP generating systems, while drugs or activities that increase cyclic GMP such as apomorphine, cold stress or running (42,47,61) do so via increased neuronal firing in these pathways. The changes in cyclic AMP observed were less widespread throughout the brain and specific to a few regions especially the pituitary. We did not observe changes in cyclic AMP levels in a previously reported chronic sodium barbital model (in which pituitary cyclic AMP was not measured). The lack of significant alterations in cyclic AMP levels after pentobarbital in any regions except the amygdala (27% increase) and the pituitary (680% increase) are thus consistent with the chronic data (59). The pituitary cyclic AMP system appears to be remarkably responsive. We have recently reported large pituitary cyclic AMP increases after phosphodiesterase inhibition with

RO 20-1724 or after the dopamine agonist, apomorphine (47). Catecholamine-sensitive adenylate cyclase have been found in the anterior and posterior pituitary (65,66). Reserpine has also been found to increase pituitary cyclic AMP in vivo (67). Possibly, the pentobarbital induced cyclic AMP rise reported here is mediated via alterations in activity in the tubero-infundibular dopamine system, or other neuropeptide-neurotransmitter systems linked to the pituitary. Alternatively, pentobarbital might increase pituitary cyclic AMP via increased release of adenosine as recently reported by Cohn et al (68) for whole brain. The pattern of cyclic AMP response after halothane was qualitatively different than that after pentobarbital. Again pituitary cyclic AMP was increased although to a lesser extent than seen after pentobarbital. Possibly changes in transmitter turnover in the hypothalmo-pituitary region are responsible. Halothane has also been reported to directly increase adenylate cyclase activity in the uterus (69) and this anesthetic might affect adenylate cyclase activity directly in the pituitary as well as in the other brain regions (hypothalamus, cerebellum and brainstem) where cyclic AMP increases were observed. Although cyclic AMP in the pituitary was less increased after halothane than after pentobarbital, cyclic AMP levels in several brain regions were increased after halothane but not after pentobarbital. These findings are consistent with the reported increases in whole brain cyclic AMP after halothane (56). However, as this regional study demonstrates, the increases in cyclic AMP after halothane are not uniform but restricted to specific brain regions. These results show that two anesthetics which modify synaptic transmission selectively alter cyclic GMP and cyclic AMP levels in specific brain regions and the pituitary. The neuronal pathways involved in each instance and the exact molecular mechanisms remain to be defined.

Monoamine neurotransmitter systems in the CNS have been implicated in numerous studies with behavioral and physiological changes occurring during a variety of stimuli including psychotropic agents and stress (6-10). One such monoamine system is the dopaminergic (8,10), whose cell bodies lying in both brainstem and hypothalamus, project to such discrete areas as the neostriatum, limbic structures and infundibular stalk (70). Because of the location of these dopamine pathways and significant pharmacological evidence, dopamine has been associated as one of the primary neurotransmitters in a variety of disorders including: amphetamine-induced psychosis (71), schizophrenia (71,72), dyskinesias associated with long term administration of neuroleptics or anti-parkinsonian agents, neuroendocrine manifestations secondary to conditions such as stress as well as Parkinsonism and Huntington's Chorea. Additionally dopaminergic neurotransmission has been strongly implicated in the stereotypy response of experimental animals following administration of dopamine (DA) agonists such as apomorphine (APO) (73). The discovery of a dopamine sensitive adenylate cyclase

in the central nervous system (27) offered a biochemically quantitative method of measuring an indicator of dopamine response at the post-synaptic membrane. Thus a change in dopamine activity in a specific pathway might be reflected by an alteration in cyclic AMP levels in a specific region of the brain to which that pathway projects. We studied cAMP response to APO in 3 models previously described as producing behavioral sensitivity to APO. These included (a) chronic administration of the DA antagonist haloperidol (74), (b) chronic administration of the DA agonist amphetamine (75), and (c) chronic administration of thyroxine (76). Dopaminergic stimulation inhibits the secretion of prolactin and may be "prolactin inhibitory factor" (77). In each of our studies blood samples were taken from each rat at time of sacrifice and prolactin and GH levels were determined. It was hypothesized that increased sensitivity of the DA receptor would be reflected in increased responses of prolactin and GH to APO challenge. An increased stereotypic response to APO challenge was seen only in the chronic haloperidol model ($p < .05$). Neither cAMP response nor cGMP response to APO challenge was increased following any of the chronic treatments. Significant increases in cGMP were observed in several regions following apomorphine 10 mg/Kg (possibly due to increases in motor activity). Serum prolactin suppression was significantly greater in response to APO challenge in both the chronic haloperidol ($p < .02$) and chronic amphetamine ($p < .05$) models. Serum growth hormone levels were not significantly changed by APO challenge in any of these models. We conclude that behavioral supersensitivity to APO can occur without demonstratable changes in the in vivo response of cAMP at the times and in the regions tested. However, receptor supersensitivity to APO challenge can be inferred in both the haloperidol and the amphetamine models as measured by serum prolactin response. These findings suggest that cAMP response may not reflect dopaminergic activity in the neostriatal and mesolimbic DA pathways. However, serum prolactin may be a useful measure of dopaminergic supersensitivity in the tubero-infundibular DA pathway. Moreover, a DA sensitive adenyl cyclase has been demonstrated in the pituitary (78) and studies in our laboratory demonstrate a pronounced rise in cAMP in rat pituitary to APO challenge (47). Clearly future studies should investigate the use of pituitary cAMP response as a measure of altered DA receptor sensitivity in the tubero-infundibular pathway using the above described models.

Goddard et al. (79) reported that brief bursts of subconvulsive non-polarizing electrical brain stimulation presented chronically to certain brain structures resulted eventually in a permanent change in the response of the animal to include localized seizure discharge, behavioral automatisms and generalized convulsions. This "kindling effect" has been shown to be trans-synaptic in nature, but the mechanism and specific neurons involved have not been identified.

Considerable data indicate that brain catecholamine systems suppress various types of seizure susceptibility. It has been shown that drugs that potentiate norepinephrine and dopamine function suppress seizures while drugs that interfere with these monoamines can increase seizure susceptibility. In our laboratory we have developed a sensitive model technique to measure small absolute amounts of endogenous norepinephrine and dopamine released in vitro (80-82). We decided to use this method to determine whether amygdaloid kindling involves permanent changes in the releasability of NE and/or DA from various brain regions. There is ample evidence that stress releases catecholamines from the adrenal medulla and by some indications, from brain neurons as well. Although neuronal firing rates are one determinant of release local presynaptic receptors are reported to influence releasability of brain NE. The latter work was done with exogenous, labelled NE. Such a model could prove misleading in that numerous storage compartments may be labelled, and therefore, specificity limited. Our laboratory has developed the first in vitro model of release of endogenous NE from brain tissue (80). This advance permits us to examine the effects of acute and chronic stress or injury on presynaptic modulation of transmitter release. Such studies would not have been feasible without the development of our in vitro model. We have previously measured the release of NE in vitro from hypothalamus, a region with a high NE concentration, in response to depolarizing concentrations of KCl (80). We also reported that DA was released from striatum, a region rich in DA, after incubation with KCl (81). We have recently shown that a portion of the DA release in corpus striatum may be calcium-independent (81) in marked contrast to NE release from hypothalamus. We have demonstrated (82) that release of endogenous catecholamines might not always parallel the release observed during previously taken up radiolabeled amine. The present data with regard to s. nigra DA release demonstrate the utility of the endogenous release model. d-Amphetamine stimulated both NE and DA release from regions throughout the rat brain. This widespread release of both transmitters should be considered in attributing the behavioral effects of amphetamine to specific neuronal systems.

Thirty-four male Long-Evans rats were stereotaxically implanted with bipolar electrodes into the basolateral amygdala. Seventeen animals were kindled beginning 10 days after surgery. Each day the rats received 1 sec of constant current 60 Hz square wave stimulation. The remaining rats served as yoked controls, receiving electrical stimulation at 3 Hz (a non-kindling frequency) for 1 sec. daily. Three weeks after the last seizure, the animals were sacrificed by decapitation; the brain was removed and dissected into: amygdala-pyriform, frontal cortex, parietal cortex, caudate, accumbens-tubercle, thalamus, hippocampus, hypothalamus, and septal region. The brain tissue pieces were weighed, chopped, and washed twice with cold Krebs-Ringer bicarbonate buffer. Each sample was then resuspended in 1.0 ml of 37^{0}C buffer and incubated at 37^{0}C for 10 minutes with

or without added KCl to bring the final KCl concentratior to 5,15 or 45 mM. Release was terminated by centrifugation and the supernatants were analyzed for NE and DA by radioenzymatic assay. Spontaneous release of NE and DA was measurable in all regions studied. Added Potassium stimulated an increase in release of both transmitters with 45mM KCl having a greater effect than 15mM KCl. Both spontaneous and KCL-stimulated release of NE and DA were similar for both kindled and control tissue in all tested regions. This suggests that the finding by Engel of lowered DA in kindled amygdalar tissue is not attributable to a lowered threshold for release.

We have studied the possibility that electroconvulsive shock (ECS), a technique which is efficacious in treating severe depression, may function in part through an activation of endorphin systems. In addition to their application to the understanding of mental disorders, these studies provide an additional model of neuroendocrine activation through the application of a stressful stimulus. Moreover, since ECS produces a generalized seizure, studies on endorphin activation by ECS also relate to seizure disorders such as may occur in post-traumatic epilepsy. We found that transauricular ECS in rats induced a state of catalepsy characterized by a loss of righting reflex. This treatment also resulted in an increase in nociceptive latencies. In fact, ECS treated rats closely resembled rats which we have observed following injections of morphine or β endorphin. These opiate-like effects of ECS appeared to be reduced when naloxone was injected prior to ECS. In an extension of these studies, we specifically focused upon respiratory rates and blood pressure changes following ECS. We found respiratory rates post-ECS to be higher in those rats preinjected with naloxone, suggesting that endorphin release during ECS may serve to maintain normal respiratory rates following this procedure. Since ECT in humans is also known to produce transient hypertension, we characterized the ECS-induced changes in blood pressure in saline and naloxone-preinjected rats. Peak elevations in mean arterial pressure were the same in both groups of rats, however, naloxone-injected rats sustained a higher blood pressure for the first 20 seconds. Subsequently, these rats became significantly more hypotensive until 30 sec. following ECS. Since ECS in rats produced a spectrum of opiate-like effects which were, to varying degrees, attenuated or modified by naloxone pretreatment, we suggest that ECS stimulates the functional release of endorphins. It is possible that such an endorphin release also occurs during ECT in humans and may contribute to the established therapeutic efficacy of that procedure. More conservatively, however, our data suggest that respiratory and cardiovascular changes following ECS may be related to endorphin release. Our findings of naloxone-reversible opiate-like effects following a single ECS led us to study the effects of repeated ECS on subsequent morphine tolerance, and to study the effects of previously induced morphine tolerance upon the

acute opiate-like effects of ECS. In a complementary set of studies we found that repeated ECS sensitives opiate-naive rats to the acute effects of a single morphine injection, and conversely, the induction of morphine tolerance sensitized ECS-naive rats to the acute opiate-like effects of a single ECS. Body temperatures were significantly elevated in the repeated ECS groups, and there was a trend toward elevated temperatures in the morphine tolerant group. The cross-sensitivity between repeated ECS and morphine tolerance coupled with the temperature data suggest that the physiological changes following repeated ECS and the induction of morphine tolerance share common neurobiological mechanisms, mechanisms which may be involved in the regulation of body temperature. The question arises, is the sensitivity to morphine seen following repeated ECS unique to ECS or does it occur with other repeated stressors? If other repeated stressors sensitize to the effects of opiates then perhaps such sensitization is part of adaptation to chronic stress. Studies are underway to investigate this possibility. Since our evidence indicates that electroconvulsive shock (ECS) functionally activates endorphin systems, we were interested in determining whether pituitary β endorphin was specifically involved. Ideally, an assay of plasma extracts from ECS and sham ECS treated rats for β endorphin content would provide information in that regard. However, owing to the minute (sub-picogram/ml) concentrations found in plasma as well as the lack of highly sensitive and specific antisera for β endorphin, plasma β endorphin assays are at present unreliable at best. By contrast, concentrations of β endorphin in the anterior and intermediate lobes of the rat pituitary gland are sufficiently high to allow for column chromatographic purification of β endorphin from pituitary extracts. Thus, a more specific evaluation of β endorphin changes in pituitary glands is feasible with present technology. Rats were subjected to repeated ECS (1/day for 9 days) and rapidly decapitated for removal of the pituitary glands. These glands were microdissected into anterior and intermediate-posterior lobe components to evaluate changes in β endorphin content following ECS. Preliminary data indicate a lack of evidence for a depletion of pituitary βb endorphin by repeated ECS, thus suggesting the possibility that other endogenous opiate systems (such as brain enkephalins) may be involved in the effects of ECS that are naloxone sensitive.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 128 Mechanism of Response to Military Stress

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Publications:

1. Kant, G.J., and Meyerhoff, J.L. Release of endogenous norepinephrine and dopamine from rat brain regions in vitro. Life Sci. 23:2111-2118, 1978.

2. Meyerhoff, J.L., Lenox, R.H., Kant, G.J., Mougey, E.H., Pennington, L.L., and Sessions, G.R. The effects of locomotor activity on cerebellar cGMP. Neuroscience Abstracts, 4:1111, 1978.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA OA 6440	2. DATE OF SUMMARY* 79 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 78 10 01	4. KIND OF SUMMARY D. Change	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8. DISP'N INSTR'N NL	8b. SPECIFIC DATA-CONTRACTOR ACCESS ☒ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT
10. NO./CODES.*	PROGRAM ELEMENT	PROJECT NUMBER		TASK AREA NUMBER	WORK UNIT NUMBER		
a. PRIMARY	61102A	3M161102BS01		00	129		
b. CONTRIBUTING							
c. CONTRIBUTING	CARDS 114F						

11. TITLE (Precede with Security Classification Code)*

(U) Parasitic Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
54 09	CONT	DA			C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 79	3.0	237
	CURRENT 80	3.0	140

19. RESPONSIBLE DOD ORGANIZATION

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NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Parasite; (U) Schistosomiasis; (U) Malaria; (U) Primate; (U) Trypanosomiasis; (U) Leishmaniasis

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To study physiological, biochemical, pathological and epidemiological aspects of parasitic diseases of military importance. To evaluate existing techniques and to develop new techniques for diagnosis, prevention, treatment and control.

24. (U) Culture systems and animal models of parasitic diseases will be developed and used to study the parasites of interest, the parasitic disease process, and the effectiveness of new diagnostic, preventive and therapeutic measures. Studies will emphasize but will not be restricted to malaria, leishmaniasis, trypanosomiasis, and schistosomiasis.

25. (U) 78 10-79 09 Studies of the metabolism of promastigotes of Leishmania braziliensis panamanensis have determined that purine uptake occurs by both diffusion and mediated systems and 3 transport loci were identified. Glucose, glutamine and glutamate are rapidly converted to alanine, while proline and alanine remain largely unmetabolized for up to 10 minutes. In attempts to develop a useful mouse model of cutaneous leishmaniasis, 3 cutaneous strains of Leishmania were inoculated into 13 inbred strains of mice. L. mexicana in C57L/J mice and L. braziliensis panamanensis in A/J and SWR/J mice produced persistent cutaneous lesions which may be useful for laboratory investigations. Progressive increases in serum antibody were observed in Mystromys infected with L. mexicana. Metal sulfonamides, especially silver sulfadiazine, exhibited activity against trypanosome, malaria and leishmania parasites in culture and low toxicity for laboratory rodents. Attempts to cure infections in rodents by development of optimal regimens of administration are in progress. Disulfiram and several of its metabolites are highly active against trypanosomes and plasmodia in vitro. For Technical Report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78-30 Sep 79.

*Available to contractors upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 129 Parasitic Diseases of Military Importance

Investigators:

Principal: COL David E. Davidson, MAJ Larry D. Hendricks, CPT George Childs, CPT Larry Lightner, CPT Michael Wyson

Associate: LT CDR Erich Stafford, CPT LuAnn McKinney, CPT Charlotte Keenan, Brian Hansen, Ph.D., J.E. Decker Jackson, Ph.D.

1. Description.

Basic Research Studies on Parasitic Diseases of Military Importance are being conducted with the goal of developing improved techniques and new knowledge to assist in diagnosis, prevention, treatment and control. Parasite culture systems and animal models of the parasitic diseases of interest are being developed to study the physiology and biochemistry of the parasite, and to study the host-parasite relationship and the pathogenesis of these diseases. Emphasis is placed upon, but is not restricted to malaria, leishmaniasis, schistosomiasis and trypanosomiasis.

2. Progress.

a. Studies of parasite biochemistry:

(1) Leishmanial metabolism studies:

Knowledge of the metabolic pathways of various leishmanial organisms could assist in rational development of new drugs, more rapid means of parasite identification, and information useful in control of this militarily important disease. With these ideas in mind, a collaborative research effort between this Division and Dr. Brian Hansen, Division of Biochemistry, has been initiated.

We are studying purine base and nucleoside uptake by promastigotes of L. b. panamensis (WR-008), the parasite with which most of this laboratory's in vitro and in vivo investigations to date have been conducted. The rates of absorption of ^{3}H-adenine, ^{3}H-adenosine, ^{3}H-hypoxanthine and ^{14}C-inosine by promastigotes of Leishmania b. panamensis have been determined. The uptakes of these labeled substrates, (1 mM) measured as a

function of time, were linear during the first two to three minutes of a 15-minute incubation period. Following two-minute incubations, 90% of the radioactivity in perchloric acid extracts of the promastigotes was recovered in the unchanged form of the added substrates. These data suggest minimal efflux or metabolism of the labeled substrates during the initial two minutes of incubation. The uptake of these labeled substrates by promastigotes occurred by both mediated and diffusion systems. Inhibition studies using purine base and nucleoside analogues indicated that there are three transport loci. Locus 1 transported only adenosine, with adenine, hypoxanthine and inosine binding nonproductively. Locus 2 transported the purine bases hypoxanthine and adenine, with inosine binding nonproductively. Locus 3 transported inosine.

Investigations into the short-term metabolism of radiolabeled glucose and amino acids have also been initiated. The uptake and metabolism of ^{14}C-alanine, ^{14}C-proline, ^{14}C-glutamate, ^{14}C-glutamine and ^{14}C-glucose were determined in promastigotes of L. b. panamensis (WR-008) following a 10-minute incubation period at 27°C. The promastigotes were extracted overnight in 70% ethanol at room temperature to remove free amino acids. The resulting pellets were hydrolyzed under nitrogen in sealed ampules with 4 ml of 6 N HCl for 18 hr at 100°C. In all cases, no radiolabel was found in amino acids from the protein hydrolysates, and >90% of the ^{14}C-label was recoverable in the free amino acid (ethanol extract) fraction. 48% of the absorbed label from ^{14}C-glucose was recovered in free alanine. Moreover, significant amounts (>30%) of the ^{14}C-label from added glutamate or glutamine were also recoverable as alanine. More than 70% of ^{14}C-alanine was recovered as free alanine. These data suggest that significant amounts of glucose, glutamine, and glutamate are initially converted to alanine by promastigotes of Leishmania braziliensis panamensis. ^{14}C-proline was largely unmetabolized in 10 minutes and 98% of the label remained in the free proline pool. Similar investigations of the metabolism of the amastigote stage of this parasite are planned.

(2) Rapid biochemical identification of leishmanial strains and species by radiorespirometry:

A contract between the WRAIR and the World Health Organization was recently made to provide support for Dr. J.E. Decker Jackson to develop and enlarge a data base using radiorespirometry as a means of rapidly identifying various isolates of Leishmania. This work is to be done in association with the

Leishmania Section of our Division. The inability to specifically identify organisms of the Leishmania complex has been a major obstacle in all areas of Leishmania research, in attempts to study the epidemiology of the disease, in the clinical management of military patients, and in the design of therapeutic treatment regimens.

b. Development of animal models of militarily important parasitic diseases:

(1) Cutaneous leishmaniasis in inbred mouse strains:

Susceptibilities of mice to various species of leishmania have not been extensively studied heretofore. Thirteen inbred mouse strains representing a broad genetic spectrum were surveyed for the development and resolution of cutaneous lesions in response to supranasal inoculation with human strains of Leishmania braziliensis panamensis, L. mexicana mexicana and L. aethopica.

Infections of L. mexicana mexicana on BALB/cJ and RF/J mice appeared to be similar. Cutaneous swelling and nodules at the site of inoculation appeared relatively late, at 50 to 60 days, and grew progressively larger with no evidence of resolution. Lesions on the C57L/J mice appeared much earlier. By 28 to 39 days, three of four mice developed distinct nodules; one of these lesions resolved and two progressed to ulcers. Histologically, the C57L/J mice with ulcers showed a moderate host response with local infiltration of lymphocytes and plasma cells which followed planes of connective tissue. Massive numbers of amastigotes were observed in vacuolated macrophages.

Lesions at the site of inoculation were observed in 11 of the 13 strains of mice infected with L. braziliensis panamensis. Lesions ranged from swelling in only one or two animals per group to 100% swelling and scabbing in other groups. None of the infections produced the chronic, progressive nodules that were observed in several of the L. mexicana infections. Almost all lesions eventually showed signs of resolution.

The infections of L. b. panamensis in the A/J mice produced local swelling as early as two weeks, and by four weeks 100% of the mice had swelling and crusty scabs. Lesions developed until 42-56 days and then most resolved. At 50 days, histologically, focal areas of eosinophilic cell infiltration with few lymphocytes and plasma cells were observed. Many of these infiltrating cells were heavily infected with amastigotes.

The gross development of the lesions of L. braziliensis panamensis infection in SWR/J mice was similar to that in A/J mice with a progression of early swelling, scabbing, and eventual healing at about 56 to 70 days. However, the histologic character of the lesions suggests that the SWR/J mouse was more reactive in responding to the infection. In contrast to the infection on the A/J mouse, there were many lymphocytes and plasma cells in the SWR/J mice. Only a few amastigotes were found focally in the papillary dermis.

Only two strains developed consistent lesions with L. aethiopica: RF/J and CBA/J. The RF/J mice exhibited swelling and early loss of vibrissae. The swelling persisted for the course of the experiment. Although the cutaneous lesions were culture-negative, the spleens were culture-positive at day 55. Histologic examination of the lesions showed no alterations which might be associated with an active leishmanial infection.

One of the most unusual lesions was seen on the CBA/J mice infected with L. aethiopica. Three of four mice showed a slight cutaneous swelling at the site of inoculation with loss of vibrissae at three to four weeks which progressed to an extensive hair loss over a large area of the face. Otherwise, there was no other evidence of infection either grossly or histologically and the nature of the hair loss could not be determined.

Results of these studies are summarized in Table 1. Several of the cutaneous leishmanial infections in inbred mice appear well suited for use as potential models; in particular, the infection of L. mexicana mexicana in the C57L/J mice and L. braziliensis panamensis in the A/J and SWR/J mice.

(2) Visceral leishmaniasis in dogs:

To date, no attempts to induce visceral leishmaniasis in canines have yielded sufficiently high infection rates to establish dose-response relationships in chemotherapeutic drug trials. Thus, a project to study visceral leishmaniasis in German Shepherd dogs was initiated to determine if these animals might be susceptible to the infection to the extent that chemotherapeutic studies would be feasible. Experimental groups consisted of: (1) three three-month old puppies inoculated IV with 1.7×10^8 amastigotes of L. chagasi per kg body weight, (2) three three-month old puppies inoculated IV with 2.8×10^8 amastigotes of L. donovani (Khartoum) per kg body weight and (3) two three-month old control dogs, one of which was inoculated

IV with a 20 ml suspension (in medium 199) of uninfected hamster spleens. All animals are currently being monitored daily for changes in rectal temperature, pulse, respiration rate and general physical condition. Bone marrow and blood samples are being examined bi-weekly. Cultures of bone marrow taken at two weeks post-inoculation were positive for amastigotes in all six of the experimental dogs. This project is continuing in collaboration with CPT C. Keenan, Division of Pathology, and will be completed as per the protocol schedule.

(3) Visceral leishmaniasis in Aotus monkeys:

Several discoveries have recently been made in this laboratory which show excellent promise for possible use in the chemotherapy of leishmaniasis, i.e., liposome-encapsulated drugs, and the lepidine compounds, particularly WR 6026. These new developments have been studied extensively in the L. donovani/ hamster model. Additional evaluation of efficacy in other mammalian model systems would be highly desirable prior to possible clinical trial.

Previous pilot experiments with leishmanial infections in rhesus and cynomolgus monkeys as well as canines, while initially showing promise, have yet to produce a consistently heavy enough infection to evaluate promising new drugs.

As the Aotus monkey has proven to be a susceptible and useful host in studies with human malaria, a pilot project to evaluate the susceptibility of this primate to visceral leishmaniasis has been initiated. Two Aotus monkeys were infected with L. donovani (Khartoum) amastigotes derived from infected hamster spleens, and the progress of the infection is being monitored, in collaboration with Dr. William Hanson, University of Georgia, Athens, GA.

c. Studies of the immunology of leishmaniasis:

Sera collected from African white tailed rats (Mystromys albicaudatus) during the course of leishmanial infections induced by inoculation of promastigotes of L. mexicana mexicana have been used to characterize the development of the immune response in studies being conducted collaboratively with the Immunology Section of the Uniformed Services University of the Health Sciences (USUHS).

A radioimmunoassay was used to detect specific anti-leishmanial antibody in this experimental model of cutaneous

leishmaniasis. Promastigote and amastigote forms of the parasite grown in vitro were used to prepare soluble antigens for a solid phase, double antibody, radioimmunoassay. Antisera to Mystromys immunoglobulin were prepared by immunization of rabbits with the 50% saturated $(NH_4)_2SO_4$ cut of Mystromys serum in Freund's complete adjuvant. Adult male and female Mystromys were infected by subcutaneous inoculation of 5×10^6 promastigotes. All animals with proven infections (visible cutaneous lesions and positive skin cultures) had serum antibody to leishmanial antigens, and antibody titers tended to increase with the duration of infection. Although promastigote and amastigote antigens seemed to be cross-reactive, antibodies to the promastigote antigens generally appeared earlier. Moreover, while anti-promastigote antibody levels remained elevated, anti-amastigote titers became negative following regression of cutaneous lesions. The involvement of cell-mediated immunity in resistance to leishmanial infections is well documented, but our data also demonstrate that African white tailed rats infected with L. mexicana produce specific antileishmanial serum antibody. Although it is not known whether serum antibody plays a protective role in immunity against cutaneous leishmaniasis, the progressive increase in serum antibody with time may be useful in the diagnosis and prognosis of leishmaniasis.

d. Pathology of Leishmania braziliensis panamanensis infection in Mystromys albicaudatus:

The description of the gross and histological pathology of Leishmania b. panamanensis infections in Mystromys albicaudatus from the time of inoculation through 12 wks has been completed by CPT L.A. McKinney of the Division of Pathology. A paper describing this work has been accepted for publication by the American Journal of Tropical Medicine and Hygiene (1).

e. Studies of antiparasitic drugs directed toward specific biochemical targets:

We have attempted to explore potential new classes of drugs based upon a theoretical construct rather than a straight empirical approach. Exploitation of "parasitic weaknesses" has stemmed from an examination of the pertinent literature and from previous experimental results of studies conducted at WRAIR.

(1) Silver sulfadiazine:

Silver sulfadiazine was originally developed as a topical agent effective against bacterial and fungal colonizations of the surface of third degree burns. This highly insoluble material (0.009 μM/ml water) was absorbed sytemically after oral or subcutaneous administration and was effective in multiple doses against Plasmodium berghei infections in mice. The compound was also effective against systemic infections of Pseudomonas aeruginosa. The drug produces minimal toxicity in rodents even at doses of 1,050 mg/kg/day for thirty days. The pharmacokinetics of silver sulfadiazine resemble sulfadiazine itself with significant quantities of the drug localizing in the liver.

We have demonstrated that silver sulfadiazine has potent anti-trypanosomal activity in vitro against T. rhodesiense. Other silver and metal sulfonamides were synthesized. Silver metachloridine and silver sulfameter also showed in vitro activity, while silver sulfalene, silver sulfisomidine, silver sulfamethazine, silver sulfapyrazine, silver sulfadoxine, zinc sulfadiazine and aluminum sulfadiazine were inactive.

Silver sulfadiazine also had activity against T. rhodesiense infections in mice. The drug must be administered in water as a suspension orally q.i.d. for 5 days; this drug regimen matches that of sulfadiazine itself (which is ineffective against trypanosomes).

Silver sulfadiazine was discovered to be effective in killing amastigotes of Leishmania braziliensis in vitro. Activity was also observed in healing cutaneous lesions of L. braziliensis in Mystromys when the drug was suspended in DMSO and applied directly to the lesion topically. Limited testing in the prophylactic mouse malaria screen indicates activity which may possibly be explained by the high levels of drug that localize in liver. The drug is effective in vitro against P. falciparum.

Because of silver sulfadiazine's in vitro activity against trypanosomes and leishmanial organisms, the drug will be tested against T. cruzi in the near future.

Experiments with this agent yielded results that seem to indicate the existence of two separate polymeric forms of the drug in solution. Collaborative studies with Professor

Minoru Tsutsui of Texas A&M University are being performed in the hopes of identifying and synthesizing each of the two forms; one form is active and the other is inactive against T. rhodesiense. (Both forms are active against bacteria.)

(2) Disulfiram congeners:

Disulfiram was active in vitro against Trypanosoma rhodesiense. It is the most active anti-trypanosomal agent ever tested by this laboratory, its ED_{50} being on the order of 100 nanagrams/ml. Its analogues, diethyldithiocarbamate, tetramethyldithiocarbamate and dithiocarbamate, were also active in vitro, possessing nearly identical ED_{50} values, but different inhibition slopes.

Disulfiram has also demonstrated anti-trypanosomal activity in infected rats, but the compound is not active in mice. Disulfiram extended the life of the test rats, but did not cure the animals. The major metabolite of disulfiram is diethyldithiocarbamate (also active in vitro) which is metabolized to methylDDC, which is inactive in vitro. The lack of activity of methylDDC may explain the disappointing in vivo results. Superoxide dismutase, an enzyme which blocks formation of hydroxyl radicals which may be the target of the drug in the trypanosomes is also inhibited by DDC but not by methylDDC.

Disulfiram was also active in vitro against P. falciparum malaria.

Testing of metallic analogues of disulfiram (e.g., zinc and iron derivatives) revealed that these compounds were also active in vitro. Analogues with bulky aliphatic groups substituted on the tetraethyl positions were also active. These compounds will be tested in vivo with the idea that they are less likely to be rendered inactive by methylation.

(3) Immunopotentiating agents:

Agents that affect cell-mediated immunity are being investigated as possible adjuncts to the treatment of leishmaniasis. These agents include dextran derivatives, drugs that affect the susceptibility of the parasite to the immune system (enhanced antigenicity) and drugs that modulate the immune response, such as BCG and Levamisole.

(4) Antitrypanosomal agents utilizing free radical kill mechanisms:

Several prophyrins and quinone derivatives have been shown to kill trypanosomes by exploiting their large pool of intracellular hydrogen peroxide. In the presence of these antitrypanosomal compounds, hydroxyl radicals and singlet molecular oxygen are produced and these components produce extensive parasite damage by lipid peroxidation. Quinone compounds are not effective *in vivo* but are highly effective *in vitro*. Several porphyrins have been reported to possess potent antitrypanosomal activity. Donor-recipient complexes of porphyrins with quinones, arsenicals and other free radical reactive compounds are presently being synthesized. Such compounds would exploit the free radical sensitivity weakness of the parasite.

f. Chemotherapy of filariasis:

Chemical compounds selected from the drug inventory of the Division of Experimental Therapeutics are being provided to Dr. John McCall of the University of Georgia under a special USAMRDC agreement with the World Health Organization and the University. These compounds are being screened by Dr. McCall in jirds infected with both *Brugia pahangi* and *Litomosoides carinii*. Of approximately 100 compounds submitted for screening this fiscal year, approximately 20 compounds had activity against one or both parasites. Analogs of these active compounds have been submitted and are currently under test.

Table 1. Infection of inbred mice with cutaneous strains of leishmania.

Strain of Mouse	L. b. panamanensis	L. mexicana mexicana	L. aethiopica
A/J	+++	-	-
A/HeJ	++	-	-
AKR/J	-	-	-
BALB/cJ	++	+++	-
C3H/HeJ	+	-	-
C57Bl/6J	+	-	-
C57L/J	+	++	-
CBA/J	-	-	++
CBA/CaJ	+	-	+
DBA/1J	+	-	-
DBA/2J	+	-	-
RF/J	++	++	++
SWR/J	++	-	-

- No evidence of infection
+ Swelling in 25-50% of mice
++ Small lesions in 75-100% of mice
+++ Large lesions in 100% of mice

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASES

Work Unit 129 Parasitic Diseases of Military Importance

Literature Cited:

1. McKinney, L.A. and Hendricks, L.D.: Experimental infection of Mystromys albicaudatus with Leishmania braziliensis: Pathology. Am. J. Trop. Med. Hyg. (accepted for publication).

Papers Presented:

1. Sales, P., Hunter, K., Stafford, E., Hendricks, L., Strickland, G. and Kinnamon, K.: Antibody response to New World cutaneous leishmaniasis (Leishmania mexicana) in African white tailed rats (Mystromys albicaudatus). To be presented at the 75th Annual Meeting of the American Society of Tropical Medicine and Hygiene, 14-16 Nov 1979, Tucson, Arizona.

2. Hansen, B.D., Brown, N.D. and Hendricks, L.D.: Short-interval metabolism of radiolabeled amino acids in Leishmania braziliensis panamensis (WR 008). To be presented at the 75th Annual Meeting of the ASTMH, 14-16 Nov 1979, Tucson, Arizona.

3. Hansen, B.D. and Hendricks, L.D.: Purine base and nucleoside uptake by promastigotes of Leishmania braziliensis panamensis (WR 008). Presented at the 54th Annual Meeting of the American Society of Parasitologists, 29 July - 3 August 1979, Minneapolis, Minnesota.

4. Childs, G.E., Groves, M.G., Hendricks, L.D., Price, E.E. and McKinney, L.A.: Inbred mice as model hosts for cutaneous leishmaniasis. Presented at the 54th Annual Meeting of the ASP, 29 July - 3 August 1979, Minneapolis, Minnesota.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6441	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	II	II	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	661102A	3M161102BS01	00	130
b. CONTRIBUTING				
c. XXXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Viral Infections of Man

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 79	4.5	479
FISCAL YEAR CURRENT 80	4.5	459

19. RESPONSIBLE DOD ORGANIZATION

NAME: Walter Reed Army Institute of Research

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: DALRYMPLE, Joel M.

NAME: BRANDT, Walter E.

21. GENERAL USE

Foreign intelligence not considered.

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Virology; (U) Immunology; (U) Arbovirus Infections; (U) Adenovirus Respiratory Diseases; (U) Influenza; (U) Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Provide Individual paragraphs identified by number, precede text of each with Security Classification Code)

23 (U) To define etiology of acute infectious diseases of special hazard to military personnel, to determine and evaluate factors influencing occurrence, distribution, severity and medical result of human virus infections, and to develop means for reducing disability due to virus diseases.

24 (U) Contemporary virological and immunological methods are applied to disease problems occurring in troops or in susceptible civilian populations in strategically important areas. New conceptual approaches and methods are developed as needed for specific problems.

25 (U) 78 10 - 79 09 Adenovirus. Acute respiratory disease rates remained low on all basic training posts except for brief period of influenza transmission in January and February. Adenovirus type 21 was the predominant virus isolated but types 4 and 7 occurred on most posts and type 3 occurred briefly at Ft Knox. Eleven lots of Adenovirus vaccines were titered and shown to slowly lose potency on storage beyond one year. A type 21 vaccine used in a field trial at Ft Dix in 1977 lost 1.7 logs of virus 2 years after manufacture. Neutralizing antibody assays on vaccine recipients were completed. The frequency of type 21 antibody seroconversion was the same (9 percent) for vaccinees as it was for controls (11 percent). Measles. Studies following outbreaks of measles indicate that 12.2 percent of troops at Ft Lewis and 9.9 percent of special forces personnel at Ft Bragg did not have hemagglutination inhibiting antibody to rubeola virus. An epidemic of rubeola in silvered leaf monkeys at USAMRU Kuala Lumpur was confirmed in a collaborative study. Interferon. A virulent and avirulent strain of Semliki Forest virus were shown to differ in the sensitivities to and induction of interferon in cell culture. Interferon induction was found to be temperature dependent. For technical report, see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78 to 30 Sep 79.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

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3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	130
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

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0026 Biology 010100 Microbiology 003500 Clinical Medicine

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63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING		
FISCAL YEAR CURRENT		

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21. GENERAL USE

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22. KEYWORDS (Precede EACH with Security Classification Code) (U) Viruses; (U) Immunology; (U) Arbovirus Infections; (U) Adenovirus Respiratory Diseases; (U) Influenza; (U) Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code)

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24 (U) Contemporary virological and immunological methods are applied to disease problems occurring in troops or in susceptible civilian populations in strategically important areas. New conceptual approaches and methods are developed as needed for specific problems.

25 (U) 78 10 - 79 09 Arboviruses. Nineteen (19) volunteers without flavivirus antibody were immunized with graded doses of an attenuated live dengue virus vaccine. Five volunteers developed viremia and 3 others seroconverted. The immune response was not solely dependent on vaccine dose. Few clinical symptoms occurred. Transmission of vaccine virus to A. aegypti mosquito vectors was demonstrated. Antibody to dengue vaccine persisted for 12 months in earlier vaccinees. Antibody enhancement of dengue infection of human monocytes is modulated by a trypsin resistant Fc receptor. A method for oligonucleotide mapping of dengue virus RNA was introduced to this program and confirmed the serotype specificity of the dengues. Monospecific Sindbis virus antibody was produced in hybrid mouse cells. The feasibility of making monospecific antibody to individual viral proteins was shown and will be used to evaluate the strain variations. Dengue virus replication in vitro was shown to be sensitive to an antiviral drug, Ribavirin. For technical report, see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78 to 30 Sep 79.

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Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 130, Viral Infections of Man

Investigators:

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Description

To define the etiology and ecology of human virus infections particularly those of military medical significance; to devise and evaluate means for precise diagnosis, control and/or prevention of disease. Studies have applied virological, immunological, epidemiological and physiological approaches to understanding disease caused by respiratory, arthropod-borne and other viruses, the factors influencing transmission to men, other vertebrates and invertebrates, and their survival in nature.

Progress

I The Arthropod-Borne Viruses

A. Immunization with Live Attenuated Dengue Virus Vaccines Study #1. Safety and Immunogenicity of DEN-2 (PR-159/S-1) in Adult Volunteers.

1. Background

The WRAIR has developed an attenuated dengue type 2

virus, PR-159 (S-1) as a candidate live virus vaccine. The selection, characterization and testing of the S-1 virus strain has been described in previous annual progress reports. The vaccine virus is temperature sensitive and less virulent for mice by intracerebral inoculation than the parent (PR-159) virus. The S-1 virus also has markedly reduced ability to produce viremia in rhesus monkeys and chimpanzees compared to the parent virus.

The first human trial of the DEN-2 PR 159 (S-1) virus involved six adult male volunteers who received one dose of undiluted vaccine ($2.5\text{-}4.5 \times 10^5$ pfu/0.5ml) in February or April 1978. All volunteers were heterologous flavivirus (yellow fever) immunes. Details on volunteer selection, study design and results of clinical, viremia and serological responses are described in the WRAIR annual progress report, 1978. This report includes additional serological results through 12 months after immunization.

2. Results

Serum antibody responses to immunization were measured by complement fixation (CF), hemagglutination inhibition (HI) and plaque reduction neutralization tests (PRNT) using routine procedures. The strain of DEN-2 virus used in the CF and HI tests was New Guinea C (NG-C) and in the PRNT, PR-159 parent virus.

Four volunteers had no detectable DEN-2 antibody at the time of vaccination (Day 0). Volunteer DDB had a 1:8 CF titer on Day 0 which was not present on Day 14 and may have been a nonspecific reaction. Volunteer G.R. had PRNT antibody titer of 1:20 on Day 0 and past history of a dengue type 4 infection. Peak geometric mean antibody titers by all tests were obtained on Day 30 (Table 1). All six volunteers demonstrated a four-fold or greater rise in HI antibody titer, but GR had the least response. CF antibody was of short duration but HI and PRNT antibodies remained detectable for 12 months.

3. Discussion

The serologic responses of six heterologous flavivirus

Table 1. Dengue Type 2 Antibody Titers*
in Six Yellow Fever Immune Volunteers

	FHT	WLO	GR	WHB	DDB	MAU	GMT**
Complement Fixation (NG-C)							
Day 0	<4	<4	<8	<8	8	<8	1.4
Day 14	<8	<8	<8	<8	<8	<8	1.0
Day 21	64	512	<8	<8	64	<8	11
Day 30	64	512	8	256	64	<8	40
Day 60	16	32	<4	16	8	<4	6.3
6 Mon	4	4	16	32	8	16	10
12 Mon	<4	4	8	16	8	8	5.7
Hemagglutination Inhibition (NG-C)							
Day 0	<10	<10	<10	<10	<10	<10	1.0
Day 14	<10	10	<10	<10	<10	<10	1.5
Day 21	640	2560	40	80	640	80	254
Day 30	640	2560	40	2560	160	80	359
Day 60	160	640	20	320	80	<10	61
6 Mon	80	40	20	320	80	40	63
12 Mon	40	20	10	80	20	10	22
Plaque Reduction Neutralization Test (PR-159)							
Day 0	<10	<10	20	<10	<10	<10	1.6
Day 30	140	400	20	350	160	60	125
Day 60	140	230	20	500	100	60	112
6 Mon	100	90	20	620	80	80	95
12 Mon	100	70	20	220	100	80	79

*Reciprocal serum dilutions

**Geometric mean titer. Antibody titers of <4, <8 and <10 were assumed to equal 1.0.

immune volunteers showed the DEN-2 vaccine to be immunogenic. The low level antibody response of GR may indicate that heterologous dengue antibody may suppress the immune response to the DEN-2 vaccine.

B. Immunization with Live Attenuated Dengue Virus Vaccines Study No. 2. Response to Varied Doses of DEN-2 (PR-159/S-1) Vaccine in Adult Volunteers.

1. Background

This study was designed to determine the response of people who had no flavivirus antibody to various doses of the DEN-2 PR-159/S1 vaccine. Vaccine effect was evaluated in terms of clinical, virological and serological responses. In addition, an effort was made to determine if persons with vaccine viremia were capable of infecting vector mosquitoes. Permission for this human volunteer studied was granted by the OTSG on 30 Jan 1979.

2. Methods

a. Volunteers

Twenty-one (21) male and female active duty military personnel assigned to Ft Detrick, MD volunteered for the study. Most of them were designated Medical Research Volunteers (MVRs). The others were members of the staff who were interested in participation in a research program.

All of the volunteers were thoroughly briefed on the dengue vaccine development program, the potential risks and benefits to vaccinees, the results of Study No. 1 and the procedure for Study No. 2. Each volunteer signed a consent form and passed complete physical examination prior to the initiation of the study.

Although the protocol called for 36 people divided into 6 study groups, there were insufficient volunteers to fill each group. The 21 volunteers were utilized so that 19 people with no detectable flavivirus antibody by HI tests received one of four different doses of vaccine; two other volunteers who had pre-existing flavivirus antibody were

designated to receive a placebo (Table 1). The placebo recipients served as controls for the clinical response to vaccination.

b. Place and Time

The volunteers were vaccinated in three groups on 16 Oct 78, 21 Feb 79 and 14 Mar 79 (Table 2). They were permitted to live at home and carry out their routine professional duties, but were restricted from leaving the Frederick Valley for 21 days after vaccination. Each volunteer was required to report in person to Ward 200, USAMRIID, daily for examination and laboratory tests.

c. Clinical Evaluation

Each volunteer had a complete physical examination including a chest x-ray, electrocardiogram, and urinalysis. Preliminary blood studies included CBC, platelet count, partial thromboplastin time, prothrombin time, BUN, creatinine, SGOT, SGPT, and fibrin split products. Female volunteers were tested for pregnancy with an assay for human chorionic gonadotropic hormone in the urine.

Following vaccination on Day 0, through Day 21, oral temperatures were recorded four times a day, and each volunteer was interviewed for symptoms and signs from a standard check list. Serum samples for viremia studies were collected daily. Serology sera were collected twice a week to Day 21, then Day 30 and 60. Clinical Laboratory studies included daily CBC and platelet counts. On Days 0, 7, 14 and 21 blood was drawn for serum SGOT, SGPT, BUN, C_3, and fibrin split products and a urinalysis was done.

d. Vaccine Administration

The vaccine used in this study was a live, attenuated dengue type 2 virus designated DEN-2 (PR-159/S-1) Lot No. 1 (Mfg date Jan 76) prepared by the Department of Biologics Research. On the days of vaccination, vials of vaccine were resuspended with sterile water for injection and diluted by Dr. Eckels to obtain the desired dosages. Vaccine vials were later coded and transported to USAMRIID on

Table 2. Volunteers for DEN-2 Vaccine, Study #2

Vaccine Dilution	Volunteer	Sex	Age	Date of Vaccination (Day 0)	Previous Flavivirus Experience
undilute	RKB	F	24	21 Feb 79	none
	JDH	M	24	"	"
	NLH	F	22	"	"
	KMM	F	23	"	"
	RAU	M	27	"	"
10^{-1}	SDF	M	20	16 Oct 78	"
	SEG	F	19	"	"
	JSR	M	22	"	"
	RLS	M	19	"	"
	KLT	F	21	"	"
10^{-2}	PCA	M	21	"	"
	BMB	F	23	"	"
	KMJ	F	18	"	"
	GFM	M	34	"	"
	JFS	M	17	"	"
10^{-3}	JFG	M	24	"	"
	JLB	F	24	14 Mar 79	"
	CAJ	F	20	"	"
	TLK	F	20	"	"
Placebo	SCC	M	30	21 Feb 79	natural infection
	RLW	F	19	14 Mar 79	YF vaccine

wet ice. Each volunteer received 0.5 ml subcutaneously in the upper arm on the morning of Day 0.

The unused portions of vaccine were immediately placed in wet ice and transported back to WRAIR for virus titration. Each dilution of vaccine was titrated by plaque assay in LLC-MK2 cells at 35 C. The observed titers are shown in Table 3.

e. Detection of Viremia

Daily serum samples were assayed directly on LLC-MK2 cell monolayers in 25 cm^2 plastic flasks. One flask was inoculated with 0.2 ml of serum and incubated at 35 C for 1-2 hours to allow virus adsorption; then an agar overlay medium was added for direct plaque assay. Another flask inoculated with 0.2 ml serum was incubated with liquid nutrient medium at 35 C for 14 days before the supernate was tested for virus by direct plaque assay at 35 C and 39.3 C.

f. Detection of Antibody

Antibody responses to immunization were measured by hemagglutination inhibition (HI), complement fixation (CF) and plaque reduction neutralization tests (PRNT). The antigen used in the HI and CF tests was dengue type 2, New Guinea-C. The PRNT was done with the vaccine parent virus, PR-159. Methodologies for the tests were the same as in Study No. 1. Volunteers were considered to be infected if they developed viremia and/or seroconverted to titers of $\geq$ 1:16 by CF and $\geq$1:20 by HI or PRNT.

g. Virus Transmission to Vector Mosquitoes

Aedes aegypti mosquito eggs collected in Thailand in 1978 were raised to adulthood in the Department of Entomology, WRAIR. Mosquito rearing was timed so that adults emerged approximately four days prior to feeding. Adult, female mosquitoes were held in groups of 30-40 each in mesh-covered cartons. One carton was used for each 30-minute feeding on each volunteer. After feeding, the mosquitoes were maintained at 28 C until tested for virus.

Table 3. Dengue 2 Virus Vaccine Doses

Vaccine Dilution	Date Used	Virus Titer/ 0.5 ml dose
Undiluted	21 Feb 79	3.0×10^5
10^{-1}	16 Oct 78	2.8×10^4
10^{-2}	16 Oct 78	3.0×10^3
10^{-3}	16 Oct 78	3.3×10^2
	14 Mar 79	3.3×10^2
Placebo	21 Feb 79	no growth
	14 Mar 79	no growth

Virus was assayed by direct fluorescent antibody (DFA) and direct plaque assay on days 14 and 19. Sixteen (16) mosquitoes (3 from RLS and 13 from KLT) were held for 28 days to attempt a probing experiment. These mosquitoes were allowed to feed on a nutrient droplet which was later assayed for virus.

3. Results

a. Virus Isolation

Viremia was detected in 5 of 9 vaccinees from 5 to 7 days. The onset of viremia ranged from Day 9 to Day 13 and persisted from 5 to 7 days. The latest virus isolate was obtained from the Day 17 serum of JSR.

Seventeen (17) isolates obtained from 14-day supernate cultures were studied for growth characteristics; all formed small plaques and remained temperature sensitive (Table 4). Some isolates from KLT obtained by direct plaque assays formed mixed small-to-medium sized plaque.

b. Antibody Response

Eight of 19 vaccinees demonstrated seroconversion including all five viremic people (Table 5). The response to immunization was incomplete at all dosage levels, which made it impossible to estimate an optimal dose (Table 6).

c. Clinical Response to Vaccination

The volunteers experienced a wide variety of incidental injuries and infections unrelated to the dengue vaccine during the period following vaccination (Table 7). Of the eight people who seroconverted by PRNT, KMM developed a dengue-like illness with rash following the onset of viremia, RAU developed an asymptomatic leukopenia, CAJ developed an acute low back strain and four (JSR, RLS, GFM, and JFS) had no illness. The frequency of dengue-like symptoms (2/8) was similar to that observed in Study No. 1 (1/6). The relationships of temperature and rash to viremia are illustrated in Figure 1. Of the 11 vaccine

Table 4. Characteristics of Virus Isolates from Serum

Volunteer	Day Post Vaccination	Plaque Size	Virus titer at 35°	Virus titer at 39.3°	Identity by PRNT
KMM	10	s	1.2×10^4	no growth	
	12	s	3.2×10^4	" "	
	13	s	1.3×10^5	" "	
	14	s	1.4×10^4	" "	DEN-2
JSR	13	s	3.1×10^4	" "	
	14	s	1.3×10^4	" "	DEN-2
RLS	10	s	3.2×10^2	" "	
	11	s	5.6×10^4	" "	
	12	s	1.3×10^3	" "	DEN-2
	13	s	6.0×10^4	" "	
	14	s	6.4×10^3	" "	
JFS	12	s	1.1×10^3	" "	
	13	s	1.9×10^4	" "	
	14	s	7.5×10^3	" "	DEN-2
KLT	10	s	1.0×10^2	" "	
	11	s	1.1×10^4	" "	
	12	s	1.2×10^3	" "	DEN-2
	13	s	9.3×10^3	" "	
	14	s	3.8×10^3	" "	
	15	s	4.0×10^2	" "	
Mosquito 2	13	s	7.0×10^3	" "	DEN-2
Mosquito 22	13	s	1.6×10^1	not done	not done

Table 5. Dengue Type 2 Antibody Response in Flavivirus Nonimmunes

Dose	Volunteer	Reciprocal Antibody Titer											
		Day 0			Day 30			Day 60			6 MON		
		HI	CF	N	HI	CF	N	HI	CF	N	HI	CF	N
$3.0x10^5$	RKB	0*	0*	0*	10	0	0	0		0**			
	JDH	0	0	0	0	0	0	0	0	0			
	NLH	0	0	0	0	0	0	0	0	0			
	KMM	0	0	0	320	8 (D21)	700	40		260**			
	RAU	0	0	0	0	0	0	0	0	20			
$2.8x10^4$	SDF	0	0	0	0	0	0	0	0	0	0	0	0
	SEG	0	0	0	0	0	0	0	0	0	no serum		
	JSR	0	0	0	40	0	380	no serum			0	0	0
	RLS	0	0	0	40	8	100	0	8	90	0	8	50
	KLT	0	0	0	0	0	90	0	16	20	0	0	30
$3.0x10^3$	PCA	0	0	0	0	0	0	0	0	0	no serum		
	BMB	0	0	0	0	0	0	0	0	0	0	0	0
	KMJ	0	0	0	0	0	10	0	0	0	no serum		
	GFM**	0	0	0	10	0	40	0	0	90	0	0	40
	JFS	0	0	0	0	8	140	20	8	30	0	0	0
$3.3x10^2$	JFG	0	0	0	0	0	0	0	0	0	no serum		
	JLB	0	4	0	0	4	0	0	0	0			
	CAJ	0	0	0	40	4	130	20	8	50			
	TLK	0	0	0	0	0	0	0	0	0			
Placebo	SCC	10	0	240	20	8	280	0	0	320			
	RLW	0	0	0	0	0	0	0	0	0			

*0 = <10 for HI and N; <4 for CF
** 4 month
***YF vaccine on Day 42

Table 6. Dose Response of Flavivirus Nonimmunes

Dose	Volunteer Recipients	Viremic Recipients	Seroconversion No.	(%)
$3.0x10^5$	5	1	2	(40)
$2.8x10^4$	5	3	3	(60)
$3.0x10^3$	5	1	2	(40)
$3.3x10^2$	4	0	1	(25)

Table 7. Clinical Status Following Dengue-2 Vaccination

Dose	Volunteer	Den-2 Seroconversion	Clinical Status
$3.0x10^5$	RKB	No	No illness. leukopenia Days 14, 19-21
	JDH	No	URI with fever, leukocytosis Days 19-21
	NLH	No	Fever, Headache, back pain and abdominal pain Days 11-14
	KMM	Yes	Headache, mylagia, eye pain, rash Days 15-17
	RAU	Yes	No illness. Leukopenia Days 12-18
$2.8x10^4$	SDF	No	No illness
	SEG	No	Group G Streptoccocal pharyngitis with fever Days 10-12
	JSR	Yes	No illness
	RLS	Yes	No illness. Leukopenia Days 13-18
	KLT	Yes	Asymptomatic rash Days 14-18
$3.0x10^3$	PCA	No	Leg injury from auto accident Days 4-11
	BMB	No	No illness
	KMJ	No	Acute pharyntitis Days 1-8
	GFM	Yes	No illness
	JFS	Yes	No illness. Fever Day 3
$3.3x10^2$	JFG	No	URI with fever Days 1-2
	JLB	No	Pelvic Inflammatory Disease Day 14
	CAJ	Yes	Acute low back strain Days 1-8
	TLK	No	No illness
Placebo	SCC	-	Headache, myalgia, malar flush Days 6-10
	RLW	-	Persistent headache and sorethroat

recipients who did not seroconvert, 4 had upper respiratory infections and one had pelvic inflammatory disease. Others including the placebo recipients frequently reported symptoms on direct questioning. RKB had an asymptomatic leukopenia between Days 14-21. There were no changes in hematocrit, serum biochemistries, serum complement levels or urinalyses that could be attributed to dengue infection. Weekly determinations of fibrin split products were uniformly negative.

d. Virus Transmission to Vector Mosquitoes

Seven (7) people who received DEN-2 vaccine on 16 October 1978 volunteered to permit mosquitoes to feed on them on Days 9, 11 and 13. Among the group were three who had documented viremia and one other who seroconverted.

Only two volunteers were definitely viremic at the time of mosquito feeding (Table 8). Two mosquitoes that fed on KLT on Day 13 yielded virus isolates by plaque assay. One isolate came from the body of a mosquito held 19 days whose head was negative by DFA. The other isolate came from a mosquito used in a probing experiment after 28 days. No virus was detected in droplets tested in the probing experiment. Therefore, although infection of mosquitoes by feeding on vaccine recipients was demonstrated, evidence was not obtained that the mosquito could transmit the virus.

The virus isolated from mosquitoes retained the same temperature sensitivity and small plaque growth characteristics as the vaccine virus (Table 4).

4. Discussion

Infection with the DEN-2 vaccine did not occur uniformly at any dosage level, but some recipients were infected at every dosage. The response to this vaccine by this group of volunteers was clearly dependent on some factor other than total dose. The possibility exists that some of the non-responders developed levels of antibody which are undetectable by these techniques.

Table 8. Transmission of DEN-2 Vaccine Virus to Aedes aegypti Mosquitoes

Volunteer	Days of Viremia	Mosquito Feeding			
		Pre-viremia		During Viremia	
		No. Fed	No. Infected	No. Fed	No. Infected
JFS	12-14, 16	40	0	0	-
RLS	10-14	20	0	42	0
KLT	9-15	0	-	72	2*
PCA	none	80	0	-	-
SDF	"	83	0	-	-
KMJ	"	64	0	-	-
GFM	"	60	0	-	-

*Virus isolates came from mosquitoes that fed on Day 13.

The vaccine virus showed no evidence of reversion to the growth characteristics of the parent virus either after passage through humans or mosquitoes. Although mosquito vectors can become infected by feeding on vaccinees, it remains to be determined if the virus can be transmitted to other people.

C. Differential Effect of Trypsin on Dengue Virus Receptors and Fc Receptors on Human Monocytes.

1. Background

Dengue virus replicates in adherent human monocytes from normal donors, and this replication is enhanced when very dilute (non-neutralizing) antibody is added to the virus inoculum and to the medium in which the infected monocytes are cultured (Annual Report 1977, 1978). The concept of immune enhancement of dengue virus replication was suggested previously by work in Halstead's laboratory where it was observed that peripheral blood leukocyte suspensions from dengue immune donors replicated more virus than leukocyte suspensions from normal donors. Immune enhancement of dengue virus replication may be one of the factors contributing to dengue hemorrhagic fever, since most cases occur in individuals with prior antibody to other dengue serotypes.

Studies of immune enhancement in our laboratory in a characterized human cell population (adherent phagocytic monocytes) were complicated by variation in virus yield. Specifically, the quantity of virus produced by monocytes cultured in normal medium ranged from 2 to 6 logs. To demonstrate immune enhancement, very dilute antibody was mixed with the inoculum as well as the culture medium, and the virus yield was increased several hundred fold in those cultures that normally yield 2 logs of virus, but only increased 10-fold in those cultures that yield 4 to 5 logs of virus. Immune enhancement could not be demonstrated at all in those cultures that yield 6 logs of virus in normal medium.

In order for immune enhancement to be demonstrated in a predictable manner, the interaction of virus with monocytes cultured in normal medium had to be blocked so that infection and replication would occur to a more measurable extent in the presence of dilute antibody. When immune enhancement of virus replication does occur, it is probably mediated by an Fc receptor on the monocyte surface; virus-antibody complexes infect more monocytes by the binding of the antibody Fc piece to the monocyte Fc receptor. Since Fc receptors are relatively resistant to trypsin digestion, monocytes were treated with trypsin in an attempt to remove other cell surface receptors that bound virus in normal medium. This would reduce the amount of virus replication in monocytes cultured in normal medium, and thus allow us to detect a greater increase in virus yield from monocytes cultured in dilute antibody. The following report describes the experiments with trypsin-treated monocytes, and the resulting discrimination between viral receptors on the surface of monocytes.

2. Methods

The procedures for isolating adherent phagocytic monocytes were described in detail in the 1977 WRAIR Annual Report. Briefly, blood was drawn into 50 ml syringes containing heparin, and then diluted in Ca++ and Mg++ free Hanks balanced salt solution containing EDTA and Hepes buffer (diluent). The diluted blood was centrifuged in ficoll hypaque gradients, and the white cell layer was washed and plated in 25 cm^2 plastic cell culture flasks or 9 cm^2 dishes molded into 6-well trays. Following incubation for 3 hours, the nonadherent cells (lymphocytes) were removed and the adherent cells were washed, incubated overnight, washed again and then infected. We originally scraped off the adherent cells and replated them to obtain a population that was essentially 100% phagocytic (secondary adherent cells that injested latex beads) but 90% of the cells were lost by this procedure. The present experiments employ primary adherent cells with a variable number of sticky lymphocytes. We showed previously that the Puerto Rico 159 strain of dengue-2 virus used in these

experiments does not replicate in lymphocytes cultured with or without mitogens (1977 WRAIR Annual Report). RPMI 1640 medium containing 10% fetal bovine serum, Hepes buffer and antibiotics was used as the monocyte culture medium.

3. Results

Trypsin-treated human monocytes were evaluated first for their ability to remain attached to the plastic culture vessel surface. The adherent cells were washed with diluent prior to trypsin treatment. The concentration of trypsin used ordinarily to passage cell culture monolayers (0.25%) removed 84% of the adherent monocytes after 30 minutes incubation at 35 C; 0.125% trypsin appeared also to remove most of the adherent cells, while 0.075% trypsin had little, if any, effect on the attached monocytes.

Trypsin-treated adherent monocytes were evaluated next for their ability to replicate dengue-2 virus. One flask of 5.5×10^5 cells received 5 ml of 0.075% trypsin in diluent; another flask of 5.6×10^5 cells received diluent only. Both flasks were incubated for 30 minutes at 35 C, washed, and infected with dengue-2 virus. Procedures for infection and sampling were described previously (1977 WRAIR Annual Report). It can be seen in Figure 1 that dengue-2 virus replicated to levels in excess of 5 logs in the untreated cells, but only 2.5 logs (several hundred fold less virus) in the trypsin-treated cells. By the fifth day after infection, the number of adherent cells decreased to 1.3×10^5 cells in the trypsin-treated flask and 1.5×10^5 cells in the untreated control flask (Table 9). It appears that trypsin treatment reduced markedly the ability of monocytes to support dengue virus replication without affecting significantly the number of adherent cells during the culture period.

We determined next if dilute antibody mixed with the virus inoculum (as well as the culture medium) would enhance viral replication in trypsin-treated cells. Cells from the donor shown in Figure 1 as well as another donor

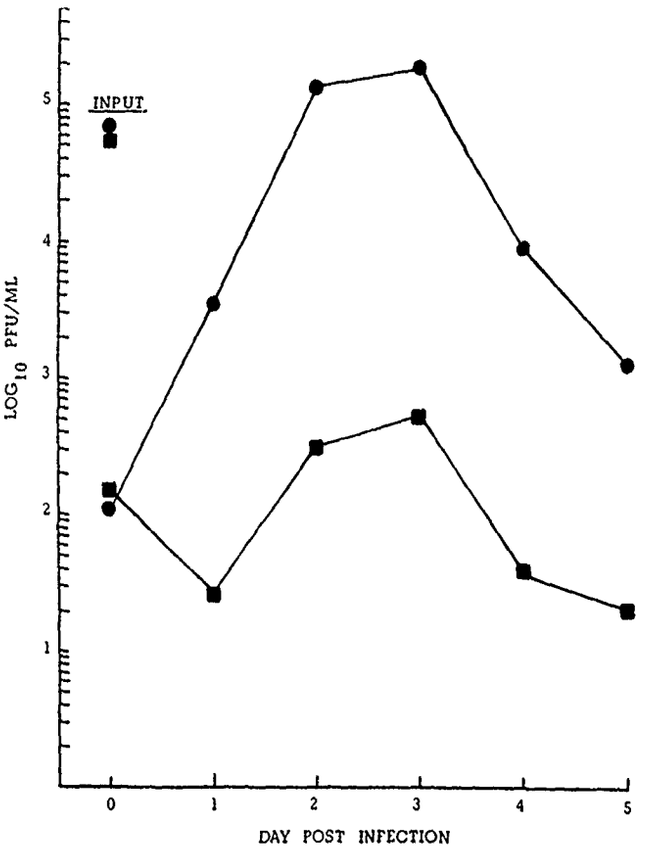


Figure 1. Dengue-2 virus replication in trypsin-treated (■) and untreated (●) human monocytes. The number of cells in each flask (25 cm^2) for each day post infection is given in Table 9 ; $1.6x10^5$ trypsin-treated cells and $1.9x10^5$ untreated cells were present the third day post infection.

Table 9. Number of Trypsin-Treated and Untreated Adherent Human Monocytes before and after Infection with Dengue-2 Virus.[a]

	Before Treatment	Day Post Infection 1	2	3	4	5
Flask 1 (trypsin)	5.5	2.4	1.6	1.6	1.3	1.3
Flask 2 (control)	5.6	3.0	2.9	1.9	1.8	1.5

[a]All numbers x 10^5. Cells treated with 0.075% trypsin or diluent for 30 min at 35 C prior to infection with PR-159 dengue virus (GMK6/LLC-1).

were tested at the same time. Duplicate cultures of monocytes from each donor were infected with and without the presence of dilute antibody. DEN-2 immune human serum that exhibited a neutralization titer of 1:80 was diluted to 1:500 (final concentration). The growth curves presented in Figure 2 show again that dengue did not replicate very well in trypsin-treated cells cultured in normal medium, but in the presence of dilute antibody, virus titers exceeded 5 logs. In another experiment, we measured immune enhancement in both untreated and trypsin-treated cells simultaneously in cells from the same donor (Figure 3). Immune enhancement of viral replication was several thousand fold in the trypin-treated cells as compared to several hundred fold in the untreated cells. These experiments show that trypsin-treated monocytes can support dengue virus replication very well once the virus gets inside the monocyte. The capacity of dilute antibody to facilitate infection of trypsin-treated monocytes suggests that the mechanism of immune enhancement is active at the cell surface, and is compatible with the hypotheses that a non-neutralizing antigen-antibody complex binds to the monocyte Fc receptor.

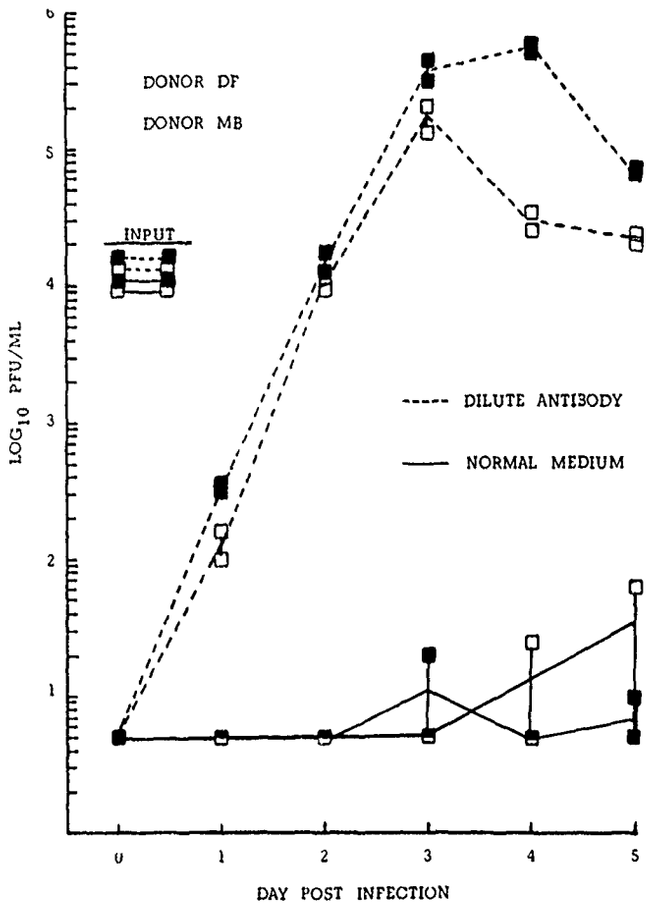


Figure 2. Antibody enhancement of dengue-2 virus replication in duplicate cultures of trypsin-treated human monocytes. Untreated monocytes from both donors previously replicated 4 to 5 logs of virus in normal medium. Donor DF ■ Donor MB ▭. The average numbers of monocytes in 9 cm^2 wells the third day post infection from donors DF and MB were: $4.5x10^4$ and $5.0x10^4$ in normal medium; $3.4x10^4$ and $4.5x10^4$ in medium containing dilute antibody, respectively.

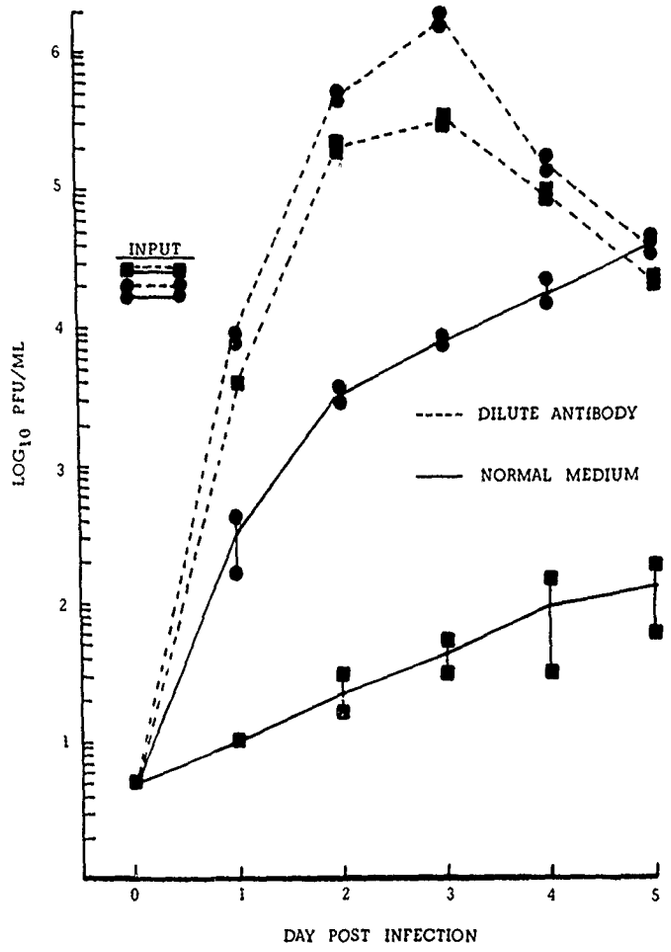


Figure 3. Antibody enhancement of dengue virus replication in duplicate cultures of trypsin-treated (■) and untreated (●) human monocytes. On the third day post infection, there were an average of 4.0×10^4 and 1.9×10^4 trypsin-treated monocytes cultured in normal medium and dilute antibody medium, respectively; 6.8×10^4 and 3.8×10^4 untreated monocytes in normal medium and antibody medium, respectively (9 cm^2 wells).

Complement is not required for immune enhancement, since virus replication occurred in the presence of heat-inactivated dengue immune serum as well as fresh serum (Figure 4). this experiment was done as part of the work shown in Figure 3. Slightly lower yields with heat-inactivated serum were observed in another experiment (Figure 5) and may represent either less available immune globulin due to partial aggregation caused by heat, or, that complement may be an additive factor. It can be seen also in Figure 5 that immune enhancement could not be demonstrated readily in untreated (non-trypsinized) cells. On the other hand, immune enhancement was demonstrated very well in trypsin-treated cells (Figure 5); the yield was about 7000 fold greater from monocytes cultured in dilute antibody. We attempted to standardize the immune enhancement system in media free of fetal bovine serum, since different lots of this serum may have variable effects of leukocytes. Neither untreated, nor trypsin-treated monocytes cultured in Neuman-Tytell serumless medium without dilute antibody supported dengue virus replication. Antibody enhancement of viral replication using the serumless medium was unpredictable.

D. Monocyte Fc Receptors in Immune Enhancement of Dengue Infections.

1. Background

We have shown above that antibody enhancement of viral replication could be demonstrated regularly in trypsin-treated monocytes cultured in RPMI 1640 and fetal bovine serum. This parameter was used to examine the mechanism of infection of monocytes, the most plausible explanation being the attachment of the virus-antibody complex to the monocyte Fc receptor. Halstead observed that Fab fragments did not effect immune enhancement of viral replication, suggesting that the missing Fc piece was required to react with the monocyte Fc receptor. We reasoned that the hypothesis could be tested by adding back an Fc piece in the form of a complete rabbit IgG molecule directed against human Fab. Human monocytes recognize the Fc piece on

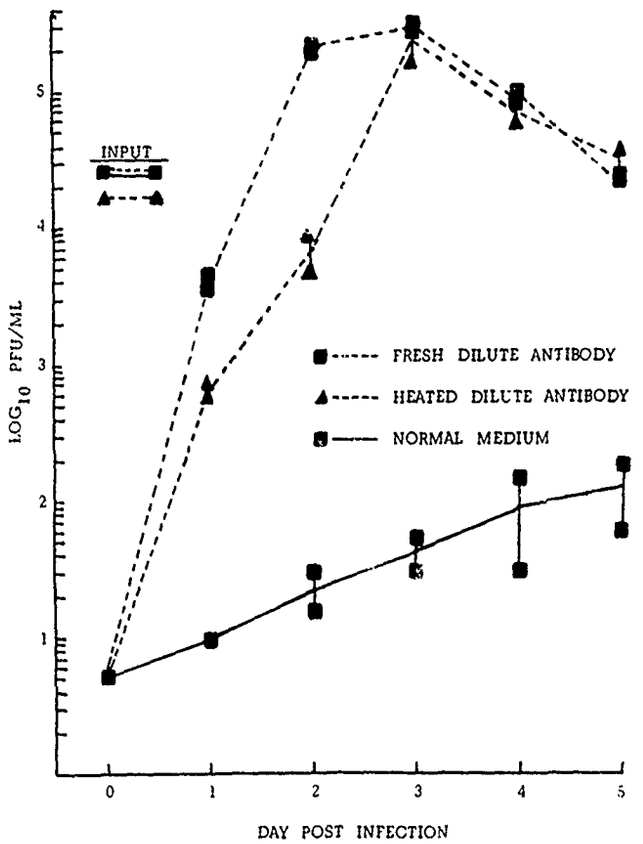


Figure 4. Antibody enhancement of dengue virus replication in trypsin-treated monocytes. Duplicate cultures were tested with heat-inactivated human dengue antiserum as well as unheated serum as part of the experiment shown in Figure 3. There was an average of 2.3×10^4 monocytes infected and cultured with heat-inactivated antiserum.

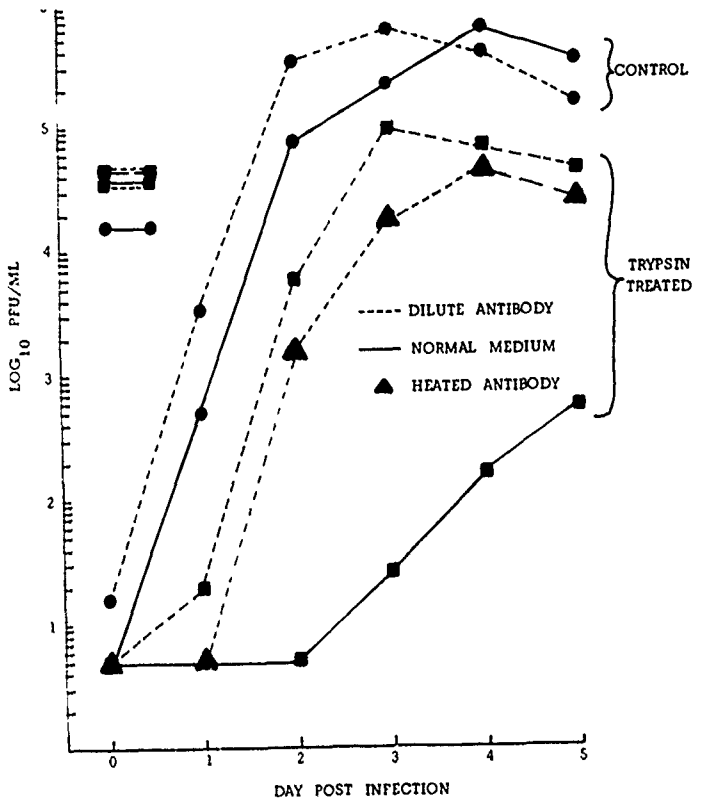


Figure 5. Antibody enhancement of dengue virus replication in untreated (control) and trypsin-treated human monocytes. Heat-inactivated serum (▲) does not enhance virus replication quite as well as unheated serum. On the third day post infection, there were 1.3×10^5 cells in the 25 cm^2 flasks containing untreated monocytes and 5.0×10^4 cells in the flasks containing trypsin-treated monocytes.

rabbit IgG, but not ruminant IgG (fetal bovine serum does not block the reaction).

2. Methods

Human $F(ab)_2$ prepared from dengue-2 immune human serum exhibited a plaque reduction neutralization titer of 1:15. This material was used at a final concentration of 1:100 to approximate the dilution of intact antibodies that permit immune enhancement without neutralizing the virus. Large volumes of blood were not drawn from the donors for dose response at this time. It was empirically determined that Rabbit anti-Fab serum should not be used at a final concentration of greater than 5% in the culture system. Therefore, a final dilution of rabbit anti-Fab of 1:20 was included in the monocyte culture medium and in the viral inoculum. Preliminary experiments showed that no enhancement of viral replication in the trypsin-treated monocytes occurred. The reason for this lack of enhancement may be that the $F(ab)_2$ and rabbit anti-Fab formed immune complexes prior to reacting with the virus, and then the Fab portion was sterically blocked from reacting with the virus. This could be resolved in the viral inoculum by first reacting the virus with the $F(ab)_2$ and then adding the anti-Fab to form an immune complex involving the virus. However, the experiments involve a 5-day growth curve in order to amplify the viral yield to detectable levels since dengue is such a poor replicating virus. The use of $F(ab)_2$ and rabbit anti-Fab in the culture medium would not enhance this yield, as shown above, so intact dengue-2 IgG would have to be used in its place (in the culture medium only) to amplify the yield after the first cycle of replication.

3. Results

The results are shown in Figure 6, carried out as part of a larger experiment with other controls (Fab alone, Rabbit anti-Fab, etc.). Essentially, no virus was produced by monocytes infected and cultured with $F(ab)_2$-Anti-Fab mixed together before reacting with the virus (open hexagons). When the monocytes were infected with

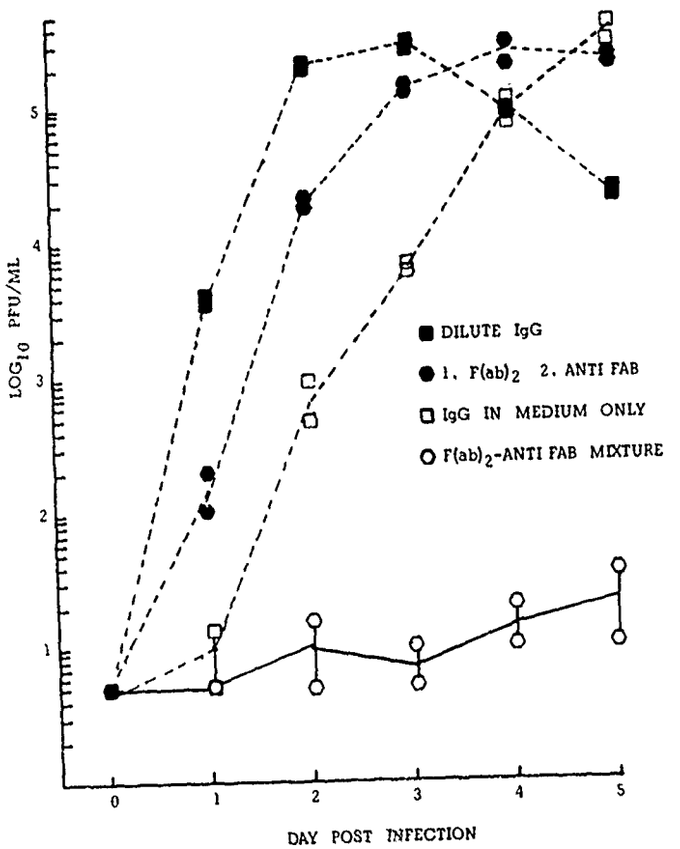


Figure 6. Dengue F(ab)2-anti Fab effect on dengue replication in trypsin-treated monocytes. The F(ab)2 was incubated with the virus prior to addition of the anti-Fab (●). IgG was added to the medium to amplify the yield after one 12-hour cycle of replication since a previously formed complex of F(ab)2-anti Fab did not enhance replication (⬡). Average number of monocytes in duplicate cultures the third day after infection: ■ $1.9x10^4$; ● $2.6x10^4$; □ $3.5x10^4$; ⬡ $1.6x10^4$.

virus alone, washed, and then cultured in dilute intact D-2 IgG antibody as a control, the progeny virus was first detected on the second day post-infection (open squares). When the virus inoculum was first reacted with dengue $F(ab)_2$ and then with rabbit anti-Fab, the progeny virus was detected the first day of infection (closed hexagons). The use of dilute intact dengue IgG with the inoculum and in the culture medium was judged to be most efficient for enhancing infection, since most virus was recovered the first day post-infection from the monocytes infected with the virus-IgG mixture (closed squares). Fab fragments alone or anti-Fab alone did not effect immune enhancement. Thus, an immune complex requiring an Fc piece is necessary to increase the yield of dengue virus. These results suggest that dengue virus can infect a cultured monocyte in 2 ways: a) through a viral receptor that is trypsin sensitive; b) through an Fc receptor that is not trypsin sensitive.

E. Characterization of the Flavivirus Genome and Replication Characteristics In Vivo

1. Objectives

The primary objectives are to ascertain 1) the occurrence and prevalence of defective interfering (DI) particles in both cytolytic and persistent flavivirus infections in vertebrate vs invertebrate cells, as well as the synthesis of predominant viral RNAs in these cells; 2) genome homologies between the dengue serotypes, and characterization of dengue DI particles in relation to their parental viral cistrons; and 3) the replication characteristics of a temperature-sensitive (ts) mutant of dengue type 2 virus, PR 159 (S1), designated as an experimental human vaccine candidate.

2. Optimization of Dengue Virus Detection and Replication

Before engaging in research on the above objectives, it was necessary to optimize dengue virus replication

in preliminary experiments. Since much of this work depends on plaque assays to titer virus samples, we thought it desirable to use the most sensitive assay available. The original system in use at WRAIR consists of incubating the virus for 6-7 days on LLC-MK2 (Rhesus monkey kidney) cell monolayers under a highly nutrient overlay medium in 1% agar. This is followed by a second agar overlay containing neutral red stain and plaques are counted 24 hours later. We were successful in shortening the assay time from 7-8 days to 4-5 days using a modification of a system published previously (5). In the improved assay, virus is incubated at 35°C in an atmosphere of 5% CO_2 on BHK (baby hamster kidney) cell monolayers in E-MEM containing 0.4% agarose and 10% fetal bovine serum. After 4-5 days the overlays are removed and the monolayers stained instantaneously with crystal violet using the procedure of Holland and McLaren, 1959 (2). Plaques can be counted immediately and the stained monolayers stored indefinitely as a permanent record. The BHK assay system appears to be more sensitive in that 0.5 - 1.0 log more prototype virus can be detected.

For our purposes, dengue virus does not grow to sufficient titer in LLC-MK2 cells and does not incorporate adequate amounts of radioisotopes into the virion or viral RNA (Table 10).Subsequently, by growth curves and by radioisotope incorporation studies, we have determined that the C6/36 clone of Aedes albopictus mosquito cells and BHK cells yield the greatest amount of highly radioactive dengue type 1 (DEN-1) virus. Both the A. albopictus and the Panama strain of VERO (African green monkey kidney) cells have proven to be the best cell types for optimal yields of dengue type 2 (DEN-2) virus--New Guinea C strain, as well as the ts mutant PR 159 (S1) (Figure 7). Experiments are in progress to determine which cell types will yield optimal amounts of dengue types 3 and 4 viruses.

Dengue virus stocks at WRAIR are normally prepared from suckling mouse brains and are not cloned. Because pure virus stocks are required for molecular hybridization and RNA analyses, inoculum of DEN-1 virus, free of DI particles, was prepared by a 5-fold clonal purification on BHK cell

Table 10. Comparison of DEN-2 (NGC) Replication and Radiolabeling in Three Cell Lines

	DEN-2 (NGC) Grown In:		
	$LLC\text{-}MK_2$	Panama-Vero	*A. albopictus*
Released Virus (PFU from $5x10^6$ cells)	$1.5x10^6$	$1.2x10^7$	$1.2x10^7$
Purified Virus (PFU from $5x10^7$ cells)	$6.5x10^5$	$1.5x10^8$	$1.0x10^9$
HA Units (Purified virus from $5x10^7$ cells)	≤ 128	> 4096	> 4096
^3H-Uridine-CPM Incorporated (Purified virus from $5x10^7$ cells)	$3.5x10^3$	N.T.*	$4.7x10^5$
^{32}P-CPM Incorporated (Purified virus from $5x10^7$ cells)	$6.5x10^5$	$2.5x10^6$	$2.4x10^6$

*not tested

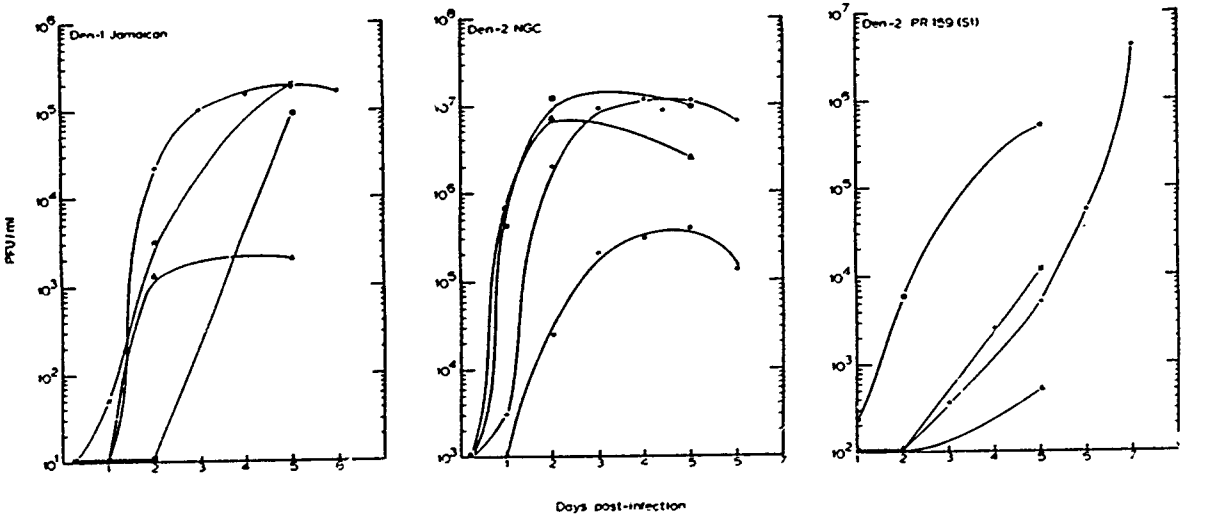


Figure 7. Growth of DEN-1 Jamaican, DEN-2 (NGC), and DEN-2 PR 159 (S1) Vaccine Strain in Vertebrate and Invertebrate Cells.

●—● A. albopictus

0—0 A. pseudoscutellaris

□—□ Panama-VERO

Δ—Δ mouse neuroblastoma

X—X BHK

monolayers followed by a single propagation step in both A. albopictus and BHK cells. DEN-2 inoculum was similarly prepared but was propagated in A. albopictus and VERO cells.

3. Results

a. DI Particles and Viral RNA

During clonal purification of dengue types 1 and 2, both viruses generated large and small plaque variants. Both variants were clonally purified and subsequently propagated in both vertebrate and invertebrate cells. The cloned inocula are currently undergoing successive undiluted passage in both vertebrate (35°C) and invertebrate (28°C) cells in order to generate DI particles and to evaluate their capability to establish persistent infections. There is a definite difference in the infection process between DEN-2-New Guinea C (NGC) strain large (LP) and small (SP) plaque infections in mosquito cells. Although the LP infection released a greater amount of infectious virus, there was noticeably less cytopathic effect (60%) by Day 21 and no surviving cells by 60 days post-infection. In contract, the SP infection yielded less infectious virus while displaying a much greater CPE (95%) by Day 21. At 60 days post-infection, numerous rapidly-dividing cells were observed which may indicate the establishment of a persistent infection.

We consistently find in our laboratory that virions released from DEN-2 infected mosquito and VERO cells separate into 3 optically defined bands upon potassium tartrate (KT)--glycerol density gradient centrifugation (Figure 8, left). Following subsequent rate-zonal purification on sucrose gradients (Figure 8, right), these infectious bards were treated with proteinase K to remove nucleases, lysed with SDS, and the RNA extracted with a phenol: cresol: hydroxyquinoline mixture as described by Repik and Bishop (4). The RNA pellet was resuspended in a low salt buffer containing 0,1% SDS and the RNA species resolved in a linear gradient of 30 to 15% sucrose in the same buffer (1). Essentially, two species of RNAs were resolved (Figure 9): 4s RNA from the "A" band, 42s RNA from the "C" band (viral) along with a small amount of aggregated RNA, and a mixture of both 42s and 4s RNA from the "B" band plus aggregated RNA. To determine both size and

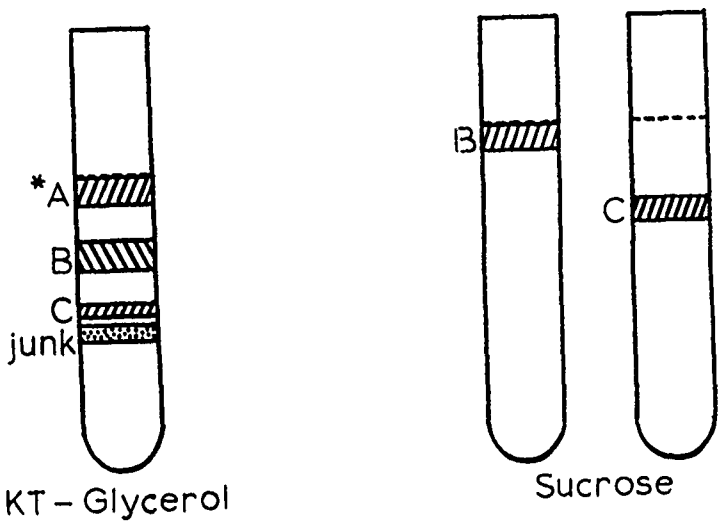


Figure 8. Purification of DEN-2 (NGC)
Left) 50% potassium tartrate - 30% glycerol gradient density centrifugation. Beckman SW 41 rotor, 35 K for 18 hours.
Infectivities: "A" band, 3.8×10^5 PFU/ml
"B" band, 3.1×10^6 PFU/ml
"C" band, 3.8×10^7 PFU/ml

Right) 70 to 20% sucrose gradient (rate zonal) centrifugation in 1M NaCl, 0.01 M Tris-HCl, pH 8.5. Beckman SW 41 rotor, 40 K for 90 min.

*Note: The "A" band from the KT-glycerol gradient remains in the load zone on sucrose gradients. Therefore this band was not normally put onto the sucrose gradient.

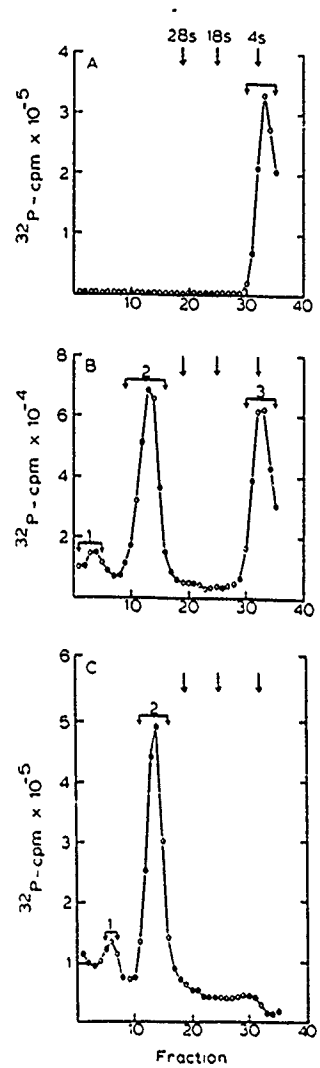


Figure 9. Resolution of DEN-2 (NGC) RNA by centrifugation in 30 to 15% sucrose containing 0.1% SDS, 0.1 M NaCl, 0.002 M EDTA, 0.02 M Tris-HCl, pH 7.4. Beckman SW 41 rotor, 40 K for 4 hours at 20°C.

A) RNA from "A" band
B) RNA from "B" band
C) RNA from "C" band

purity, an aliquot of each RNA species was subjected to electrophoresis in 2.2% aqueous polyacrymamide gels. As shown in Figure 10, both the 42s and the 4s RNAs migrated as homogeneous species with a molecular weight of 3.8×10^6 and 0.35×10^6 daltons, respectively. The RNAs from each virus band are currently being subjected to fingerprint analysis to determine if their oligonucleotides are of viral or cellular origin. A curious finding has been a 2-fold increase in the 4s ribosomal RNA extracted from DEN-2-infected cells as compared to that from uninfected cells (Figure 11).

b. Genome Homologies

In collaboration with others, we have determined by oligonucleotide fingerprinting that virion RNAs of all four dengue virus types grown in A. albopictus cells are distinct from one another (Figure 12) (6). In addition, DEN-2 NGC RNA isolated from Panama-Vero (Figure 13) and LLC-MK_2 propagated virus display essentially identical oligonucleotide patterns as compared to that from A. albopictus, meaning that there is little or no cellular influence on the viral RNA. A detailed description of the RNA fingerprinting technique has been published elsewhere (1). Basically, it consists of treating phenol-extracted and gradient-purified ^{32}P-RNA species with ribonuclease T_1 and then subjecting the digested sample to two-dimensional polyacrylamide gel electrophoresis to separate the oligonucleotides.

Because the fingerprint technique allows one to detect only 15% of the genome, RNA-RNA hybridization will also be employed in the future to determine the extent of homology (in percent) between different strains of the same virus, as well as between DI particles that we may generate..

c. DEN-2 ts Mutant PR 159 (S1)

We are planning to look at the RNA profiles of PR 159 (S1)—infected cells as well as that of released virus to determine if the RNA is in any way altered. In addition, fingerprint analyses are also planned to determine if the ts lesion can be detected by oligonucleotide pattern differences.

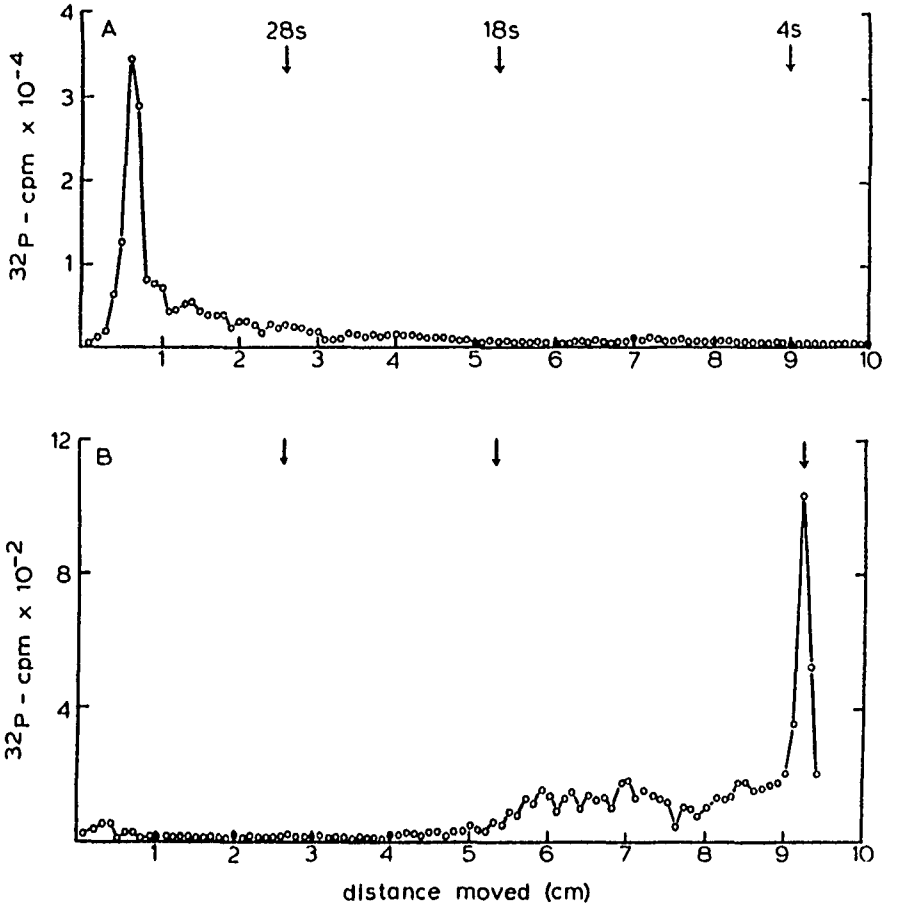


Figure 10. Polyacrylamide (2.2%) gel electrophoresis of purified RNA. 60V, 10 mA/gel for 4 hr.

A) 42 s RNA species (viral RNA) from "C" band (pool 2). Identical results were obtained from "C" band (pool 1) and "B" band (pools 1 and 2).

B) 4 s RNA species from "A" band. Identical results were obtained from "B" band (pool 3).

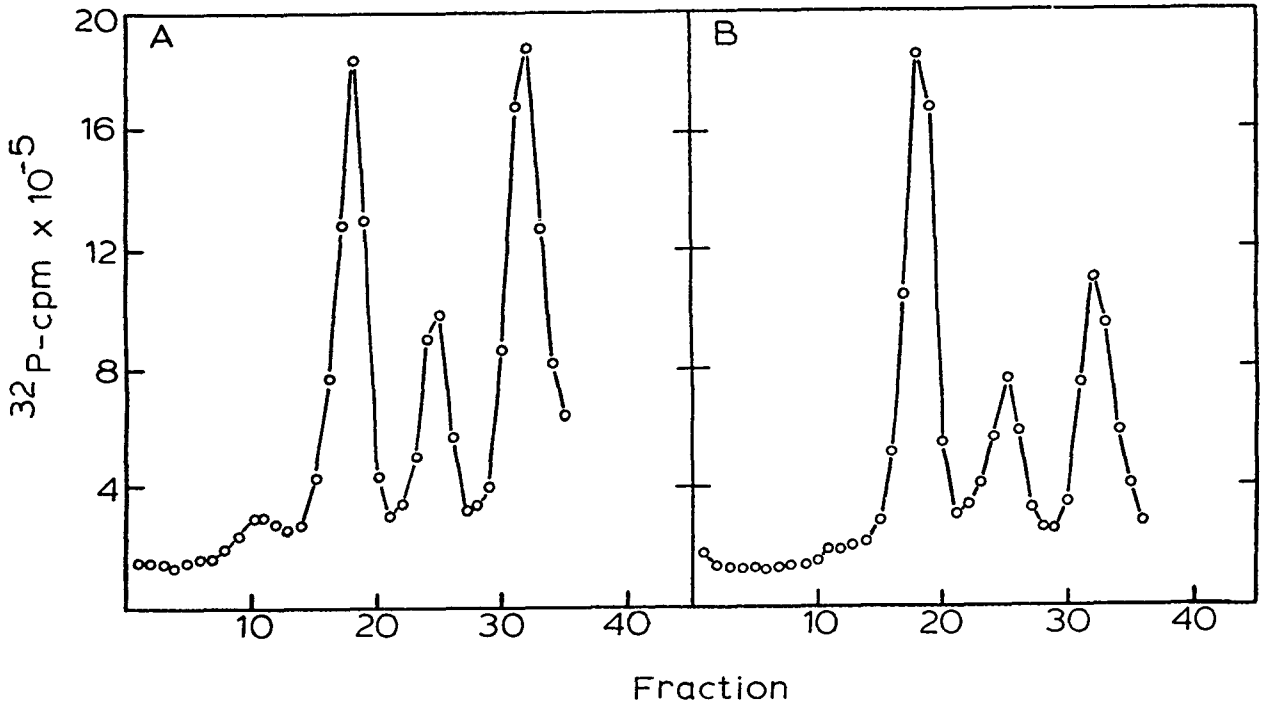


Figure 11. Resolution of ribosomal RNA by sucrose gradient centrifugation. (Conditions as in Figure 3).

A) rRNA extracted from DEN-2 (NGC)-infected Panama-VERO cells.

B) rRNA extracted from uninfected Panama-VERO cells.

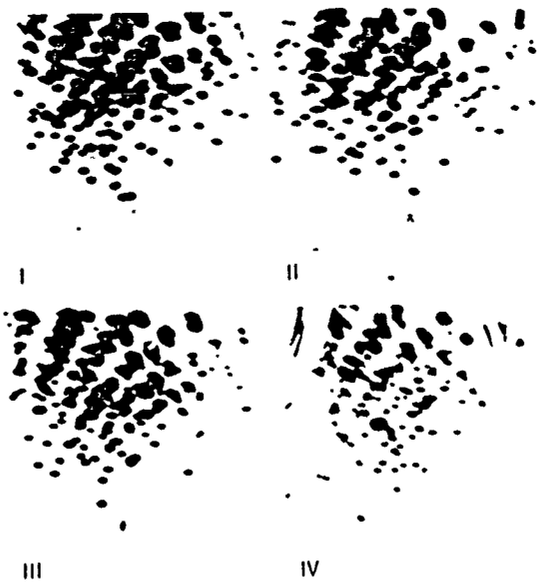


Figure 12. Two-dimensional gel electrophoresis of oligonucleotides from prototype dengue viruses grown in A. albopictus cells.

I) DEN-1 Hawaiian
II) DEN-2 (NGC)
III) DEN-3 H-87
IV) DEN-4 H-241

Figure 13. Two-dimensional gel electrophoresis of oligonucleotides from DEN-2 (NGC) grown in Panama-VERO cells.

F. Analysis of Monoclonal Antibody to Sindbis Virus Antigens Produced by Lymphocyte Hybridoma Cell Cultures

1. Background

Recent advances in the production of monoclonal antibody by lymphocyte hybridoma cell cultures have made this procedure applicable to the detailed analysis of the antigenic structure of viruses. The antigenic analysis of alphaviruses (Sindbis virus in particular) has been a continuing research effort directed at a better understanding of the virus antigens which elicit protection or immunity to disease. Efforts involving a direct biochemical analysis of chemically degraded virus have been frustrated by the fact that most virion components isolated by these procedures have been denatured in the process and most probably no longer resemble their antigenic and immunogenic structure in vivo. Furthermore, the small size of certain antigenic components restricts the immunogenic potential of antigens prepared by virion degradation. For all of these reasons, a monoclonal antibody approach to interpreting virus antigen architecture was pursued.

From a research standpoint, this approach to the problems associated with non-infectious arbovirus structural protein immunizations offers many advantages. 1) Animals used to generate "immune" lymphocytes can be either infected or immunized with intact, infectious virus. 2) Animal immunization can be selected that should yield either predominantly IgM or IgG type antibody. 3) Various myeloma cell lines can be selected that have either a known class of immunoglobulin or are "non-secreting" with respect to immunoglobulin to influence the predominant antibody class produced. 4) Fusing cells of different species is possible, allowing an examination of species influence on immunological reactivity. 5) Radioactive precursors can be added to lymphocyte hybridoma cell cultures to produce intrinsically radiolabeled antibody. 6) Lymphocyte hybridoma clones can be injected into animals to produce high-titered immune ascitic fluids with titers similar to those obtained by conventional immunization of these animals.

2. Materials and Methods

A detailed description is included elsewhere in this report by the Division of Biochemistry. Briefly, young adult female BALB/c mice from Jackson Laboratories were immunized with an initial dose of virus antigen given both IP and IV. A second antigen dose was given IP and IV 3-8 months later and spleens were harvested 3 days following this second injection. A mono-dispersed suspension of mouse spleen cells was mixed 10:1 with a myeloma cell line (P3x63-Ag8), cells fused with polyethylene glycol 1000 and inoculated into microtiter culture dishes. All wells exhibiting growth in aminopterin containing medium (designed to kill all residual non-fused myeloma cells) were subjected to three media changes at daily intervals and the final media supernatant was examined for anti-virus antibody.

All supernatants and subsequent immune ascitic fluids were tested using a solid-phase radioimmune assay (RIA). Procedures have been described in detail in previous reports and employ virus antigen (mixtures, purified or specific components) electrostatically adsorbed to a microtiter plate, a protein "filler" or blocking protein solution, incubation with the suspect antibody preparation and a final incubation with extrinsically iodinated anti-mouse immunoglobulins. Recent modifications of this assay involve the substitution of iodinated protein A or anti-mouse Fab '2 as the secondary antibody in the test.

Antibody-positive cell cultures were cloned to preclude the possibility of mixed cell populations producing different monoclonal antibodies of differing specificities. The original cultures were inoculated at a cell density generally yielding less than 50% growing wells of which less than 30% were positive for virus antibody. Cells were plated in semi-solid agar plates overlaying a human foreskin fibroblast (HR) "feeder" layer at concentrations resulting in individual, well-separated cell colonies. Cell clones were transferred to liquid media, supernatants were again tested by RIA and cells from selected antibody positive cultures were frozen in liquid nitrogen for future reference. Hybridoma-injected mouse ascitic fluid (HIMAF) was prepared to selected clones

by injecting BALB/c mice IP with 10^7 cloned hybridoma cells. Mice used for HIMAF production were "primed" by injecting 0.5 ml Pristane IP at least 2 weeks prior to the injection of cells; ascites generally developed between 8 and 16 days post-injection of the tumor cells.

3. Results

Five separate fusion experiments have been attempted and data describing their relative success is shown in Table 11. The antigens employed in mouse immunizations are detailed in the table; however, it may be premature to suggest that purified Sindbis virus is a superior immunogen to infected suckling mouse brain suspensions simply because technical proficiency with these procedures increased greatly over the course of these experiments. A higher percentage of antibody-positive clones was observed in experiments 4 and 5 in which purified Sindbis virus was used for the booster immunogen. Experiment 3 is somewhat misleading because it also utilized a purified Sindbis virus booster. In this experiment, virtually all of the inoculated wells yielded growing cells, many of which were initially antibody positive, but they subsequently turned negative upon passage suggesting mixed cell populations were present. Of the antibody-positive cell clones described, considerably less than half were positive using iodinated protein-A as a secondary antibody. These data would suggest that most hybridoma cell cultures produce IgM antibody. Similarly, most, but not all, of these cell lines have been successfully frozen for future reference.

An important aspect of the characterization of these monoclonal antibody preparations was to examine their behavior in established arbovirus serological procedures. Many, but not all, of the RIA-positive supernatants were tested by complement-fixation (CF), hemagglutination inhibition (HAI), neutralization (NT) and the indirect fluorescent antibody test (FA). As expected, most titers were relatively low on supernatant fluids using these serological procedures, which are known to be considerably less sensitive than RIA. Serological testing with SIN virus antigens is in progress, and CF, HAI, NT and FAT reactive supernatants have been observed.

Table 11. Characteristics of Lymphocyte-Hybridoma Fusion Experiments

Fusion Experiment	Primary Immunization	Secondary Immunization	Cultures Inoculated	Hybrid Growth	Antibody Positive Clones
1	SIN-smb susp*	SIN-smb susp	589	-	3
2	SIN-smb susp	SIN-smb susp	1203	182	8 (4%)
3	Purified SIN+	Purified SIN	98	96	16 (17%)
4	SIN-smb susp	Purified SIN	345	129	51 (40%)
5	Purified SIN	Purified SIN	348	64	22 (34%)

* - Sindbis virus infected suckling BALB/c mouse brain suspension - 20% w/v

+ - Cell culture propagated Sindbis virus concentrated by polyethylene glycol precipitation and purified by density and rate-zonal centrifugation.

The specificity of these antibody-secreting clones was tested by RIA in plates containing isolated structural components of Sindbis virion (Table 12). Purified Sindbis virus was disrupted with Triton X-100 and the nucleocapsid separated by rate-zonal sucrose centrifugation used as the nucleocapsid antigen even though it still contained the virus RNA. The envelope glycoproteins were separated by isoelectric focusing, fractions pooled and used directly as antigens in the RIA. Only 9 of the 89 positive reactors appeared specific for nucleocapsid; only one of the 9 positives was derived from the last two fusion experiemnts which yielded the great majority of the antibody-positive clones. This interesting observation may suggest that infected mouse brain is a better immunogen than purified virus to produce nucleocapsid reactive clones. Antibodies to E_1 and E_2 were produced with almost equal frequency. The unknown category represents cell clones that either did not react in any of the 3 viral component assays (but were positive to Sindbis virion) or exhibited low level reactivity to more than one component.

The cross-reactivity was similarly measured by RIA using infected suckling mouse brain suspensions of Sindbis (SIN), western and eastern equine encephalitis (WEE and EEE) viruses as antigens (Table 12). Although most clones were SIN specific, all possible cross reactivities were observed. Detailed examination of these cross-reactions is awaiting the preparation of immune ascitic fluids to the clones in question.

Table 12. Reactivity of Monoclonal Antibody Preparations to Sindbis Virus Structural Antigens and Cross Reactivity with WEE and EEE Viruses

Antigens	Frequency of Reaction
Sindbis Structural Proteins	
E1	43/89 (48%)
E2	29/89 (33%)
Nucleocapsid	9/89 (10%)
Unknown	8/89 (9%)
Alphavirus Cross Reactivity	
SIN only	30/63 (41%)
SIN and WEE	15/63 (23%)
SIN and EEE	6/63 (10%)
SIN,WEE and EEE	12/63 (19%)

II Respiratory Viruses

A. Acute Respiratory Disease (ARD) in Military Personnel

1. Adenovirus and Influenza

ARD on basic combat training posts was not a major problem during FY 79. Adenovirus (ADV) disease caused unexpectedly little disease. However, an epidemic of influenza occurred on most post during January and February 1979 with ARD rates greater than 2.5/100/week for periods of up to two weeks.

Isolations of ADV types 4 and 7 were uncommon in basic combat trainees (BCT) and were distributed throughout the year. ADV type 4 vaccine (Lot 7901) titering 5.0 $\log_{10}$ $TCID_{50}$ and type 7 vaccine (Lot 8001) titering 6.2 $\log_{10}$ $TCID_{50}$, administered on all basic combat training posts from 1 October 1978 thru 31 March 1979, appeared to effectively suppress ADV types 4 and 7 ARD.

As has occurred in previous years, ADV type 21 was prevalent at many basic combat training posts. Particularly affected during FY 79 were Forts Benning, Dix, Knox and Wood. This virus was isolated throughout the year at these posts, but the virus was not associated with sustained ARD rates greater than 2.0/per 100 men/week in the absence of influenza. The proportion of trainees hospitalized with ARD who yielded isolates of ADV type 21 from the nasal pharynx during the winter season rarely exceeded 25%. This proportion was considerably less than that seen during previous outbreaks of type 4 and type 7 ARD when isolation rates of 40 to 60 percent were observed.

There was one outbreak of ARD at Fort Knox in May and June which was associated with the isolation of ADV type 3 as well as type 21. This outbreak lasted for approximately 2 months and during this period ARD rates peaked at 2.3/per 100 men per week (Table 13). This is the first time in recent years that ADV type 3 was associated with a sustained outbreak of ARD in BCTs. Nonetheless, ADV type 21 was responsible for a larger percentage of virus isolates. ADV types 4 and 7 vaccines were administered to male BCTs only.

Table 13. Acute Respiratory Disease Due to Adenovirus at Fort Knox, KY 1979

Week Ending	ARD Rate/100/Week	No Sampled	% With Isolation of	
			ADV 3	ADV 21
21 April	0.9	19	0	11
28 April	1.3	18	0	17
5 May	1.4	22	0	40
12 May	1.5	20	0	15
19 May	1.4	20	0	10
26 May	1.6	17	5	17
2 June	2.3	14	14	42
9 June	1.3	18	11	55
16 June	1.8	25	12	32
23 June	1.2	19	21	5
30 June	0.9	25	16	25

Females were trained at Fort McClellan early in FY 79; females' training began at Fort Jackson, Fort Dix and Fort Wood during the spring of 1979.

In the spring of 1979, there was uncertainty when additional supplies of types 4 and 7 vaccines would be delivered to the WRAIR. At the recommendation of WRAIR, OTSG notified each BCT post to cease immunization on 1 April 1979 instead of 1 May as is usual and to delay initiation of immunization from 1 October 1979 to 15 October. The purpose of modifying the vaccination period was to conserve vaccine during months of low risk of ADV infection in order to assure adequate supplies for the 1979-1980 respiratory season.

Throughout FY 79, all BCTs were immunized against Influenza A and B by parenteral injection of killed subunit vaccines. On arrival at the reception center, each trainee received a monovalent vaccine containing 24 ug of HA of A/USSR/77 (H_1N_1). This was followed 4 weeks later by trivalent vaccine containing 20 ug of HA each of A/USSR/77 (H_1N_1), A/Texas/77 (H_3N_2) and B/Hong Kong/72. Despite immunization, an influenza epidemic occurred during the winter of 1979 on all BCT posts. During this period, complement-fixation conversions for influenza were seen and isolations of influenza virus were made. The epidemic was largely due to influenza type A (H_1N_1) but influenza type B was also seen on some posts. The disease was self limited and less extensive than that which occurred with the same serotype virus during the same period one year previously.

The following is a summary of respiratory disease patterns in the nine basic combat training posts:

a. Fort Benning. Basic combat training resumed at Fort Benning during the first week of December. Trainees were immunized with types 4 and 7 vaccines from 27 November - 31 March 1979. ADV isolates included two type 4 strains and 28 type 21 strains, no type 7 strains were obtained. The type 21 strains were isolated intermittently throughout the year and were not associated with any ARD rates greater than 1.0/100 men/week. For eight of

the ten months of training, ARD rates ranged from 0.2 to 1.0/100 man/week. In late December and early January, however, rates peaked to 2.1/100 men/week. During this period serological conversions to influenza type A began to appear and continued through February.

b. Fort Bliss. Adenovirus vaccines were administered from 1 Oct 78 through 31 Mar 79. Only 5 isolations of adenoviruses were made, two of type 4, one of type 7 and two of type 21. In no case were adenovirus isolations associated with ARD rates greater than 0.7/100 men/week. ARD rates never exceeded 1.0/100 men/week through the 12 months of survey; however, seroconversions to Influenza A were noted from January through March.

c. Fort Dix. Trainees received ADV types 4 and 7 vaccines from 1 Oct 78 through 31 Mar 79. From 831 trainees sampled from 1 Oct 78 through 31 Aug 79 there were 77 ADV isolates; 31 type 4, 7 type 7, 53 type 21 and two other adenovirus serotypes. ADV was isolated from 11% of the ARD patients sampled; ADV type 21 accounted for 6.3%. The ARD rates were less than 1.0/100 men/week, through the first week in December. They peaked at 2.3/100 men/week during the week of 24 Feb 79 after which they returned to less than 1.0/100 men/week by the week of 12 May. Adenoviruses were isolated throughout the year. During the week of Aug 25, ADV type 4 was associated with 32% of ARD samples submitted. At that time, the ARD ratio had climbed to 1.5/100 men/week. ADV vaccines were started on 1 Sep at this post. Serological conversions to Influenza A and B were noted from December through March while ARD rates were high. Two isolates of Influenza type A (H_1N_1) were made in March 1979. Serological conversions between 2 Dec 78 and 31 Mar 79 were Influenza A, 20%; Influenza B, 4.7% and adenovirus, 11.4%. These were interpreted as being due to naturally acquired infections.

d. Fort Gordon. ADV types 4 and 7 were used from 1 Oct 78 through 31 Mar 79. From 275 trainees admitted to hospitals for ARD during this period, 13 (4.7%) ADVs were isolated; 9 strains of type 7, 3 strains of type 21 and 1 strain of type 4. Four (3.4%) strains of ADV type 7 were isolated from 116 samples submitted after 31 March. Median ARD rates never exceeded 1.0/100 men/week

during the 12 months of surveillance. Fort Gordon did experience a mild epidemic of Influenza A from Dec 78 through Mar 79 when 60 (41%) of 145 patients studied showed significant antibody rises to Influenza A. During this same period only 10 (6%) patients seroconverted to ADV.

e. Fort Jackson. BCTs received ADV types 4 and 7 vaccines at the reception centers at Fort Jackson from 1 Oct 78 through 31 Mar 79. Only three ADV isolations were made during this period; two type 4 and one type 7. There were no ADV type 21 isolates made during FY 79. Median weekly ARD rates for February and March were 1.9 and 1.2, respectively, in association with the Influenza A epidemic. The ARD admission rates for the remainder of the year were less then 1.0/100 men/week.

f. Fort Knox. Vaccines for ADV types 4 and 7 were administered from 1 Oct through 31 Mar 79. During this period ADV was isolated from 39 (8%) of 496 samples submitted: there were four type 3, 10 type 4, one type 7 and 24 type 21 isolates. The ARD rate rose to a median level of greater than 1.0/100 men/week in December 1978, peaked in January at 2.2 during the Influenza A outbreak and remained high until April when it dropped to 1.0/100 men/week. There was a minor increase of ARD in May and June 1979 during which time ARD rates peaked at 2.3/100 men/week in the first week of June. This increased ARD was due to adenovirus infections following the discontinuation of routine ADV immunization. ADV seroconversion was found in 42% (35/83) and ADV isolates obtained from 35% (63/179) of which 9% were type 3 and 26% were type 21 (Table 13). After June ARD rates fell to 1.0/100 men/week or lower and remained so for the duration of the fiscal year.

g. Fort McClellan. ADV types 4 and 7 vaccines were administered to males throughout the fiscal year. During this period, ADV was not isolated from 52 specimens submitted. The combined male and female ARD admission rates did not rise above 0.2/100 men/week except for the weeks ending 13 and 20 January when they rose preciptiously to 1.7 and 2.0/100 men/week, respectively. This acute outbreak of ARD was shown by serology to be due to Influenza A, and

the majority of cases occurred in one training company.

h. Fort Sill. BCTs received ADV types 4 and 7 vaccines from 1 Oct 78 through 31 May 79. During this time there was only one isolate of ADV type 21 from specimen submitted. The ARD admission rate remained below 1.0/100 men/week with the exception of January and February when it rose to a peak level of 5.2/100 men/week during the Influenza A epidemic.

i. Fort Wood. ADV types 4 and 7 vaccines have been administered year round in recent years due to sustained ARD rates greater than 1.0/100 men/week. In FY 79 vaccines were administered from 1 Oct 78 through 31 May 79 when they were stopped to conserve vaccine supplies. The frequency of ADV isolation during the period of immunization was 42/484 (9%) ARD cases sampled. Six ADV isolates were type 4, one was type 7 and 24 were type 21. During the spring and summer following the withdrawal of immunization an additional 10 (6.4%) ADVs were isolated from 156 specimens submitted, including two ADV type 4 and 8 ADV type 21. Fort Wood also underwent an Influenza A epidemic, with seroconversion to Influenza A in over 25% of samples submitted between the first week in January and the second week in March. During this time the ARD rates peaked at 5.8/100 men/week. During the remainder of the year, Fort Wood continued to have a high ARD admission rate which rarely dropped below 1.5/100 men/week.

2. Titration of Adenovirus Vaccines

a. Purpose

This study was performed in order to establish the ability of HEK-T cells to titrate ADV vaccines and to determine if there was a loss in the titer of these vaccine with time in storage.

b. Method

Samples were taken from currently and previously used lots of vaccines. Five lots each of ADV 4 and 7

and one lot of ADV 21 vaccine were sampled. In addition, a placebo lot, prepared for the ADV 21 vaccine trial, was also tested as a control. Two tablets of each lot of vaccine were tituated together in 10 ml of growth media (Eagles minimal essential medium, Earles base). Twenty-five lambda samples of the resulting vaccine suspension were diluted in 25λ of growth media and 10 to $20x10^3$ HEK-T cells in 100λ of growth media were added. Plates were incubated at 35° in 5% CO_2. Six replicate titrations were performed for each sample. Plates were examined daily for CPE. When CPE stabilized, the plates were fixed and stained by immersion in a solution of 5.6% formaldahyde and 0.7% crystal violet in water for two hours. Following this, the plates were repeatedly washed by gentle immersion in tap water and dried. The titers were analyzed using the Reed-Munch equation.

c. Results

The results may be seen in Table 14. That HEK-T cells appear to be capable of accurate titration of adenovirus vaccine is seen by the similarity of the titrations obtained by this technique and those provided by the manufacturer for lots of ADV 4 and 7 vaccines currently in use. Older lots of vaccine tablets showed some loss of potency after storage for more than one year.

B. Respiratory Viruses

Use of transformed human embryonic kidney cells 293 (HEK-T) in adenovirus (ADV) neutralizing antibody assays.

1. Background

In response to dwindling supplies of primary human embryonic kidney cells (HEK-P), an alternative method was devised to detect ADV neutralizing antibody. This method, a microtiter technique using HEK-T cells (Annual Report 1978), was found to be a useful test for ADV antibodies and to compare favorably with the HEK-P tube neutralization assay. Further studies into the sensitivity and specificity of the HEK-T technique for ADV type 21

Table 14. Titration of Adenovirus Vaccines

ADV Type	Lot No.	Production Date	Production Log $TCID_{50}$	WRAIR Date	WRAIR Storage Temp.	WRAIR Log $TCID_{50}$	WRAIR Log titer Loss
4	5601	10 May 74	5.5	9 Nov 78	-20°C	3.5	2.0
	7101	17 May 76	4.9	9 Nov 78	-20°C	5.0	0
	7401	3 Dec 76	5.1	9 Nov 78	-20°C	4.0	1.1
	7701	16 May 77	5.0	26 Oct 78	+ 4°C	5.0	0
	7901	26 May 78	5.0	26 Oct 78	+ 4°C	5.2	0
7	5701	17 May 74	6.5	9 Nov 78	-20°C	4.2	2.3
	7201	24 May 76	6.2	9 Nov 78	-20°C	4.2	2.0
	7501	10 Dec 76	6.1	9 Nov 78	-20°C	4.2	1.9
	7801	23 May 77	6.0	26 Oct 78	+ 4°C	5.4	0.6
	8001	9 Jun 78	6.2	26 Oct 78	+ 4°C	6.2	0
21	00101	14 Jun 76	5.7	26 Oct 78	+ 4°C	4.0	1.7
Placebo	00200	14 Jun 76	Neg	28 Oct 77	+ 4°C	Neg	-

antibody were carried out in FY 79. Sensitivity and specificity are determined by comparison of methods used to determine the occurrence of an event with the actual occurrence of that event. In the case of ADV antibody, the event is the actual development of antibody and the standard way of determining this was the HEK-P test tube neutralization test.

2. Methods

In order to determine the sensitivity and specificity of the HEK-T microneutralization test for ADV type 21 antibody, we re-examined a group of sera previously studied with the HEK-P tube neutralization test. These sera were acute and convalescent serum samples collected from 38 men who were hospitalized for ARD in 1976 at Fort Wood. ADV type 21 had been isolated from 17 of these men.

3. Results

The results of re-examination of the sera by HEK-T are seen in Figure 14. A four-fold or greater rise in ADV type 21 antibody was seen in all 17 men from whom ADV type 21 was isolated.

The sensitivity and specificity of the HEK-T test was determined by arraying the results in a decision matrix and calculating the "conditional probability" of true positive results (sensitivity) and true negative results (specificity). The decision matrix was constructed by comparing the HEK-T results with the standard HEK-P results as follows.

		HEK-P +	HEK-P -
HEK-T	+	24	17
	-	3	32

$$\text{Sensitivity (true positive)} = \frac{24}{24 + 3} = \frac{24}{27} = 88\%$$

$$\text{Specificity (true negative)} = \frac{32}{32+17} = \frac{32}{49} = 65\%$$

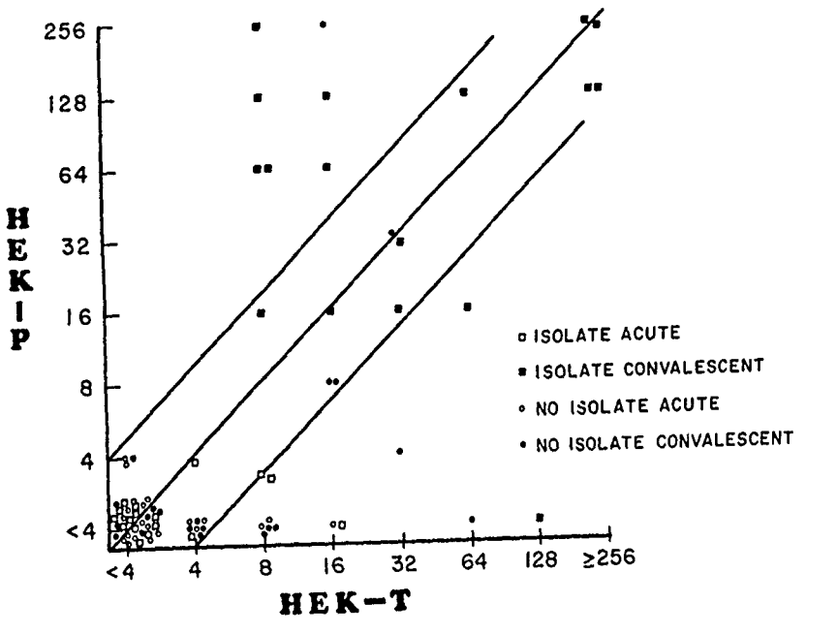


Figure 14. Adenovirus type 21 neutralizing antibody titers in persons with and without an adenovirus type 21 isolate. Titers determined by the transformed human embryonic kidney (HEK-T) microtiter method are compared to titers determined by the primary human embryonic kidney (HEK-P) tube method.

This analysis indicated that the HEK-T test for ADV type 21 antibody, was highly sensitive, but lacked specificity. The reduced specificity was largely due to the inability of the HEK-T cells to maintain confluency for the duration of the test. This clouded the interpretation of CPE and led to the low titered false positive results seen in the absence of antibody.

Application of this analysis to the data on the HEK-T test previously presented for ADV 4 and 7 antibody (Annual Report 1978) showed sensitivities similar to that seen for ADV type 21 antibody. Specificity, however, was variable ranging from 91% to 58% respectively for the 68 ADV type 7 and 90 ADV type 4 determinations reported. Despite the relatively low specificity, the HEK-T test was considered satisfactory for analysis of ADV antibody.

C. ADV Type 21 Vaccine

1. Background

In November 1976, an ADV type 21 vaccine efficacy trial was performed at Fort Dix, New Jersey. The study was timed to correspond with the expected autumn rise in ADV disease. Two groups of 300 volunteers were studied; one group received ADV types 4, 7, and placebo (4/7/P) vaccines. The vaccines were administered within 72 hours of arrival at Fort Dix and recipients were followed throughout the basic combat training period. Vaccine safety and efficacy were to be estimated by monitoring admissions to the hospital. Paired sera were collected from all volunteers at the time of admission (Day 0) and three weeks later (Day 21).

Three events, previously described, prevented the successful completion of this study. First, ARD rates did not increase as expected, precluding a valid study of vaccine efficacy. Second, differences between the study groups in hospital admission rates early in the course of training could not be explained by the viruses isolated.

Third, the commercial sources of HEK-P cells could no longer supply sufficient cell cultures to test serum pairs for evaluation of the immunogenicity of the three vaccines in immunized troops.

The development of a satisfactory test for the determination of ADV antibody (HEK-T micro-neutralization) allowed for completion of the serological studies for the ADV vaccine study.

2. Evaluation of Immunogenicity of ADV Types 4, 7 and 21 Vaccines.

To determine the immune response of vaccine, 76 subjects in each group were randomly selected for study. Day 0 and Day 21 sera were tested in duplicate for ADV types 4, 7 and 21 antibodies using the micro-neutralization technique with HEK-T cells (Table 15). Preliminary data, resulting from initial studies for ADV type 21 antibody have been previously presented (Annual Report 1978). Serological studies on the initial sera of the 152 volunteers indicated 68% of the men lacked antibody at a 1:14 dilution for ADV type 4, 61% for ADV type 7 and 85% for ADV type 21. Seroconversion rates to 1:4 or greater were 87.5% for ADV type 4 and 62.1% for ADV type 7. Seroconversion frequency for ADV type 21 were low (10%) in agreement with the preliminary data. There was no difference in the frequency of seroconversion between the immunized and nonimmunized groups.

The good antibody responses to immunization seen with ADV types 4 and 7 contrasted sharply with those seen with ADV type 21. Since the sensitivity of the HEK-T micro-neutralization test for ADV type 21 antibody has been established, the lack of seroconversion seen in the ADV type 21 vaccinees may have been due to interference with the immune response by the concomitant administration of ADV types 4 and 7 vaccine as suggested in an earlier study at Lackland AFB. However, the most likely explanation is that ADV type 21 vaccine lost potency prior to its administration to the volunteers (Table 14).

Table 15. Type-Specific Neutralizing Antibody Responses of Adenovirus Vaccine Recipients (Fort Dix, NJ 1976)

Vaccine Study Group*	Total No.	Adenovirus Type 4		Adenovirus Type 7		Adenovirus Type 21	
		Initial	Three Week	Initial	Three Week	Initial	Three Week
		<1:4 No (%)**	>1:4 No (%)***	<1:4 No (%)**	>1:4 No (%)***	<1:4 No (%)*	>1.4 No (%)***
4/7/21	76	52 (68)	45 (87)	41 (53)	30 (73)	66 (86)	6 (9)
4/7/P	76	52 (68)	46 (88)	56 (73)	31 (55)	64 (84)	7 (11)
TOTAL	152	104 (68)	91 (88)	97 (63)	61 (62)	130 (85)	13 (10)

* Adenovi. s vaccines are indicated by number, placebo by P.

** Percent of total = susceptible

*** Percent of number having initial titer of <1:4.

D. Measles (Rubeola) Antibody in Active Duty Military Personnel

1. Background

Between December 1978 and early February 1979, an outbreak of measles (rubeola) occurred at Ft. Lewis, Washington, and the surrounding area. At least 90 cases were reported in the military community alone, including 13 active duty personnel. Many people were hospitalized, and several pneumonias, 2 cases of congestive heart failure (CHF) and 1 death were reported. The adults with disease were incapicitated for at least a week with fever, cough, myalgias, conjunctivitis, and the classic rash. One adult had myocarditis, subaortic stenosis, and CHF. The active duty personnel with measles came from different units, but is unknown whether or not they were married or had children. Because of the severity of the disease and the possibility of a large epidemic affecting combat troops, sera from soldiers of 2 units at Ft. Lewis were surveyed for the presence of measles antibody. In addition, sera taken from Special Forces Troops during Operation Jack Frost, January 1977, was also tested for measles antibody. High susceptibility rates would suggest the need for immunization of troops.

2. Methods

The standard CDC microhemagglutination inhibition (HI) test was selected for antibody determination. This test is both sensitive and specific. Results were compared using RBC from both Rhesus monkeys (RM) and African Green monkeys (AGM).

3. Results

a. Ft. Lewis

Sera from 180 soldiers were evaluated for antibody using both RM and AGM cells. No demographic data were available on these soldiers except that they were from two units which had no acute measles infections. The distribution of HI Ab titers with the two types of cells is seen in Table 16. RM cells were more sensitive--only 7%

of troops had titers <1:10, whereas 12% were <1:10 by AGM cells. Most soldiers had titers in the range of 1:20 and 1:40.

b. Ft. Bragg

Sera from 274 special forces soldiers deployed to Alaska for Operation Jack Frost in January 1977 were tested for measles HI antibody. All troops had received gamma globulin approximately two weeks prior to serum collection. Age, rank, and sex were known on all troops. The distribution of antibody titers is seen in Table 16. Again, most sera titered at 1:20 and 1:40. AGM cells proved to be more sensitive with these samples giving only 10% <1:10 vs 16% with RM cells.

There was some variability in the frequency of antibody by age (Table 17). Of the troops between 31-35 years old, 17.3% had titers less than 1:10, suggesting they have the most susceptibility to infection. The absence of antibody was not related to rank (Table 18). Of 14 women tested, only one had an antibody titer of less than 1:10.

4. Discussion

Both Ft. Bragg and Ft. Lewis troops had a 90% or greater prevalence of measles antibody yet a higher percentage of troops might be protected since the titer of measles Ab that is protective is unknown. Recently, data show that 15/19 people with measles HI of <1:5 had a secondary IgG response to measles vaccination (3). Whether or not these people would have been protected against natural measles is unknown, but it has been suggested that a measles titer of 1:2 or even less is protective. Thus, the 7-10% of troops who had titers of <1:10 would represent the maximum of measles susceptibility in our population. Nonetheless, more troops are susceptible now than in 1966 (1% susceptible) and epidemics have occurred in populations with only 2% susceptibility (3).

Table 16. Comparison of Measles HI Ab Titers by Rhesus and African Green Monkey Cells

Study Group (No. tested)	Indicator Cells	Frequency (%) of HI Titer				
		<1:10	1:10	1:20	1:40	≥1:80
Ft Lewis (180)	RM	6.7%	3.3%	33.9%	38.3%	17.8%
	AGM	12.2%	9.4%	28.9%	29.4%	20%
Ft Bragg (274)	RM	15.7%	9.5%	35%	31.9%	7.7%
	AGM	9.9%	5.9%	33%	33.3%	17.9%

Table 17. Age Distribution of Measles HI Antibody* in Special Forces Troops

Age	Number	Titers Less Than 1:10	
		Number	%
17-20	30	3	10.0
21-25	89	9	10.1
26-30	58	4	6.9
31-35	52	9	17.3
36 +	45	2	4.4
Total	274	27	9.8

*African Green Monkey Cells

Table 18. Measles HI Antibody Prevalence by Rank (Special Forces Troops, Jan. 77)*

Rank	Number of Sera		%
	Ab ≥1:10	Ab <1:10	AB <1:10
Officers (including W.O.)	41	6	12.8
Enlisted	206	21	9.3

* AGM cells

E. Measles in Silver Leaf (S.L.) Monkeys

1. Background

In December 1976 in the USAMRU, Kuala Lumpur, an outbreak of suspected measles occurred in 31 leaf leaf monkeys that had been in captivity for 4 to 12 months. While three monkeys were being closely monitored for baseline data, they developed a maculopapulor rash, distributed primarily on the ventral body surface. One to three days after the rash started, leukopenia developed which lasted one-two days. One monkey had fever. Following recognition of the rash on these monkeys, the other animals were examined. Twenty-four of the 31 monkeys developed a rash which persisted six to nine days and subsequently resulted in desquamation of the involved skin. Nasal discharge and conjunctivitis were occasionally noted. Eight monkeys dies--six of whom had a rash--but none had postmortem lesions pathognomonic for expected measles. Although the death rate of these monkeys in captivity is high, it appeared to increase during this epidemic. Following this epidemic, all silver-leafed monkeys were given measles vaccine during the early days of captivity.

Paired sera taken one to two months prior to and approximately five weeks after the midpoint of the epidemic were sent to WRAIR for HI Ab titers against measles as were pre- and post-vaccination sera from nine other monkeys.

2. Methods

The anti-measles HI test described above was used to determine antibody titers.

3. Results

a. Epidemic sera

Paired sera of the 23 surviving monkeys had previously been tested for measles antibody by complement fixation. All sera initially had titers less than 1:4. Following the epidemic, 21 monkeys had developed CF antibody (Table 19a).

Table 19. Anti-Measles Antibody in Silver Leaf Monkeys

a)

	Number with CF Titer of:					
	<1:4	1:8	1:16	1:32	1:64	1:128
Pre-Epidemic	23	0	0	0	0	0
Post-Epidemic	2	2	2	9	6	2

b)

	Number with HI Titer of:			
	<1:10	1:20	1:40	≥1:80
Pre-Epidemic	23	0	0	0
Post-Epidemic	1	0	4	18
Pre-Vaccination	9	0	0	0
Post-Vaccination	0	2	5	2

By the HI test, all pre-epidemic sera were less than 1:10 and 22 of the 23 post epidemic sera had at least a four-fold titer rise. Four sera had titers of 1:40; the other 18 sera had titers of 1:80 or greater (Table 19b). The monkey who failed to have an antibody rise evidently did have a rash but it is not known how long after the rash the convalescent serum was drawn. No postmortem blood was obtained from any of eight dead monkeys.

b. Vaccinee sera

Sera from nine monkeys vaccinated after capture were tested by HI. Prior to vaccination, all nine animals had measles antibody titers of less than 1:10. All convalescent sera were drawn 6-8 months later and all had demonstrable antibody titers (Table 19b). No rashes or illnesses were seen in these animals.

4. Discussion

Rhesus, cynomoulogous, gibbon and vervet monkeys as well as baboons, chimpanzees and orangutans are apparently free of measles antibody in their natural forest habitat. This is evidently true also for silver leaf monkeys--all 33 monkeys were initially negative by HI for measles antibody. Following capture, either by natural exposure or vaccination, at least 31 monkeys developed HI antibody. This demonstrates that silver leaf monkeys are susceptible to measles virus, transmitted both by close contact with humans and by vaccination, and develop measles HI and CF antibodies as well as probably clinical symptoms.

III Anti-Viral Substances

A. Ribavirin

1. Background

Ribavirin (virazole: 1-β-D ribofuranosyl - 1,2,4 - triazole -3 - carboxamide) has been previously shown to be an effective antiviral agent against a number of infectious

diseases caused by both DNA and RNA viruses. The objectives of the following studies are 1) determination of the sensitivity of certain flaviviruses to treatment with ribavirin and 2) investigation of the antiviral action of the drug.

2. Effect of Ribavirin on Dengue Virus Production

To determine the effect of ribavirin on dengue virus replication, LLC-MK_2 cell monolayers were treated with various concentrations of ribavirin at different time points in the virus growth cycle. In all the experiments described in Figures 15-18, the cells were exposed to the drug continuously from the time of addition until the experiment was terminated at seven days post-infection. Figure 15 shows the effect of ribavirin on the replication of dengue type 1 virus when the drug was added 24 hr before virus adsorption. Virus growth characteristics in the presence of 0.1, 1.0 or 5.0 mcg/ml were indistinguishable from untreated control cultures over the seven-day course of infection, reaching an average maximal titer of 1.6×10^6 pfu/ml. The presence of 10 mcg/ml caused a delay of approximately 24 hr in the appearance of logarithmic virus release and also caused a reduction in maximal titer at seven days of approximately 50%. By contrast, no replication was detected with 30 mcg/ml ribavirin until six days post infection when a maximal titer of 5×10^2 pfu/ml was achieved. No replication was detected when cells were treated with 100 mcg/ml ribavirin.

When addition of ribavirin was delayed until 1 hr post infection (i.e. immediately after adsorption) similar patterns of inhibition were obtained (Figure 16); however, concentrations as high as 10 mcg/ml did not cause significant virus inhibition. Virus replication at 100 mcg/ml was still below the detection limits of the plaque assay.

When ribavirin addition was delayed until 24 hr post infection, less drug effect was observed (Figure 17). Concentrations of 5 mcg/ml or less caused no discernible inhibition. Greater concentrations caused some delay in the release of infectious virus. Maximal virus titers achieved on Day 7 were 10 mcg/ml, 1.1×10^6 pfu/ml; 30 mcg/ml, 6×10^4 pfu/ml; 7.1×10^6 pfu/ml in the absence of ribavirin and 100 mcg/ml, 7×10^2 pfu/ml.

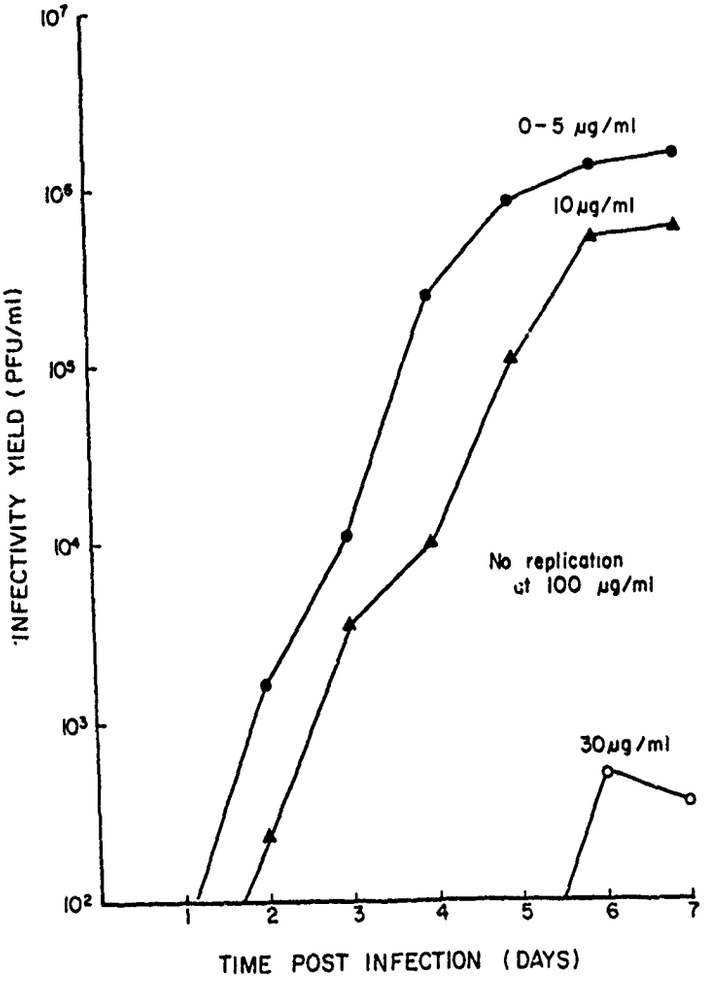


Figure 15. Effect of Various Ribavirin Concentrations Added 1 Day Before Dengue Virus Infection. Infected LLC-MK_2 cells were treated with ribavirin continuously during the course of infection beginning 24 hr before infection.

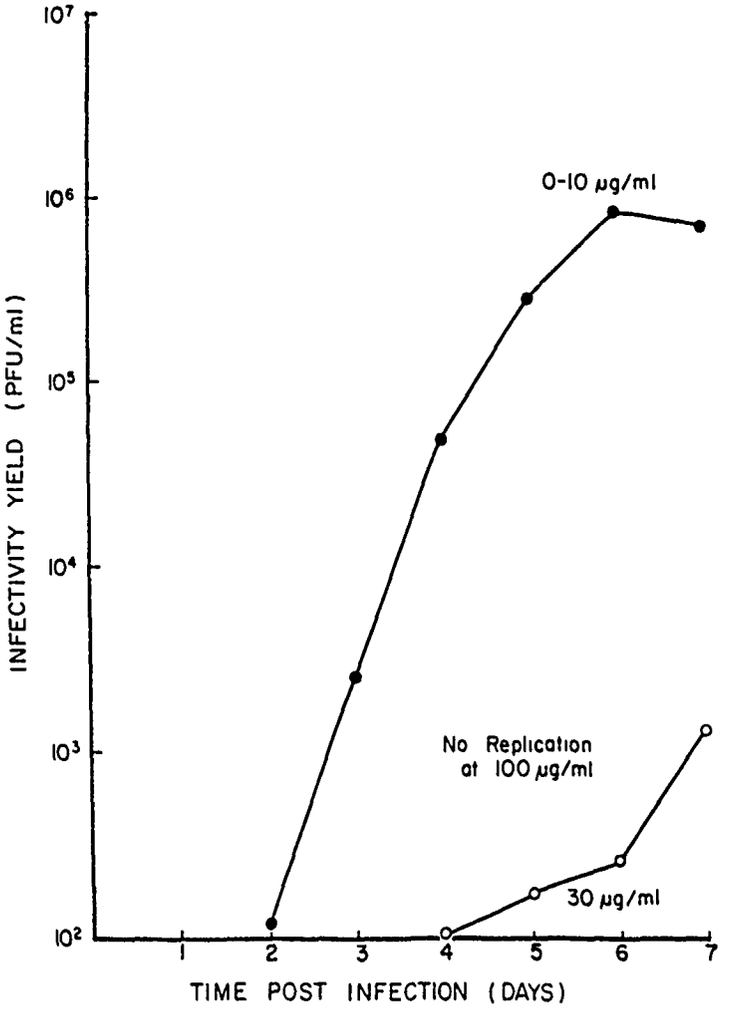


Figure 16. Effect of Various Ribavirin Concentrations Added Immediately After Dengue Virus Infection. Infected LLC-MK_2 cells were treated with ribavirin continuously during the course of infection beginning immediately after virus adsorption.

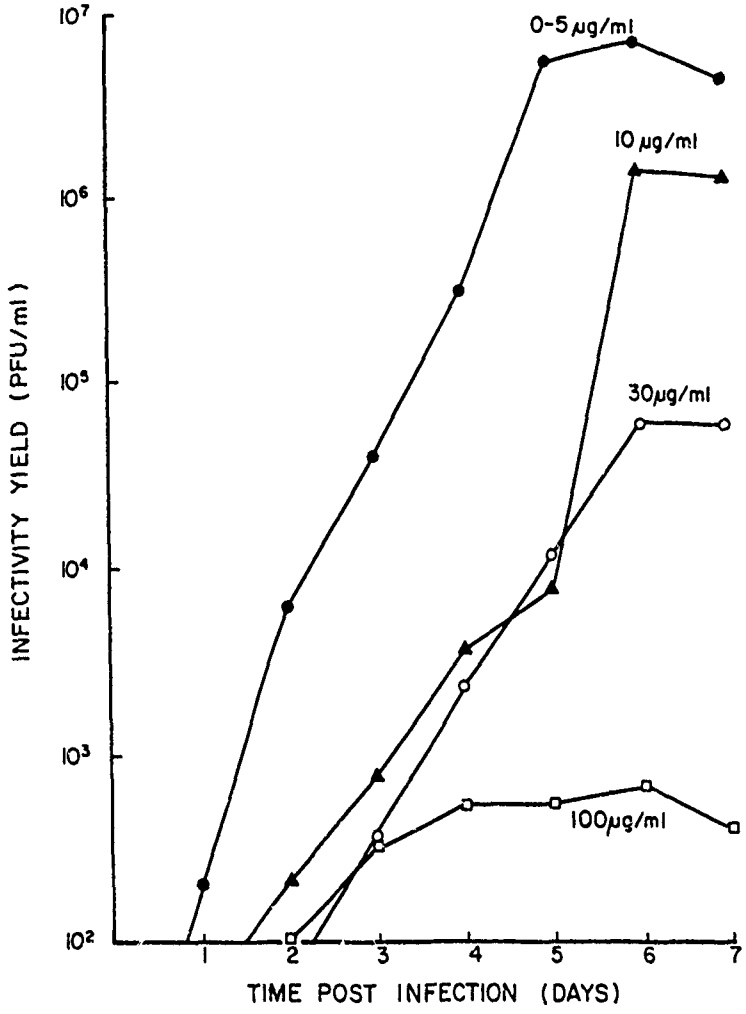


Figure 17. Effect of Various Ribavirin Concentrations Added 1 Day After Dengue Virus Infection. Infected LLC-MK_2 cells were treated with ribavirin continuously beginning 24 hr post-infection.

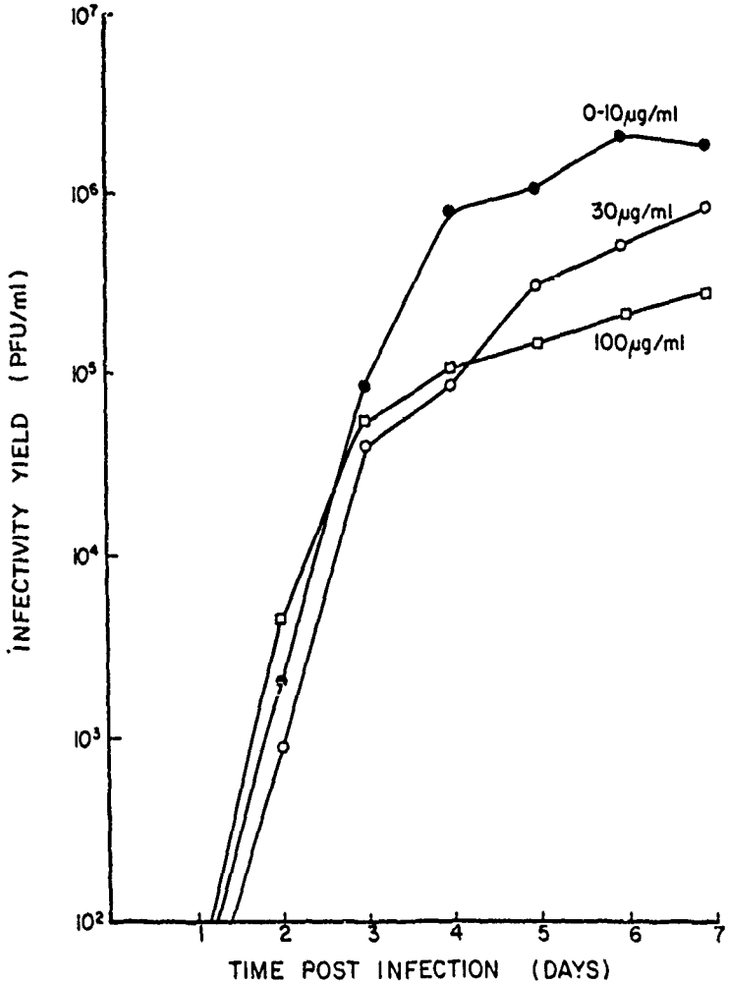


Figure 18. Effect of Various Ribavirin Concentrations Added 3 Days After Dengue Virus Infection. Infected LLC-MK_2 cells were treated with ribavirin continuously beginning 3 days post-infection.

When ribavirin was added 72 hr post infection, an immediate reduction in the kinetics of release was noticeable with concentrations greater than 10 mcg/ml. However, maximal titers were $8x10^5$ pfu/ml in the presence of 30 mcg/ml and $2.6x10^5$ pfu/ml in the presence of 100 mcg/ml.

From the experiments described above, it was impossible to determine whether the inhibition observed reflected a decrease in the total number of virus particles released or a decrease in the proportion of infectious virus particles (i.e. pfu to particle ratio). Dengue type 1 virus was grown in the presence of various concentrations of ribavirin beginning 24 hr before infection and harvested seven days post infection. The virus samples were concentrated by polyethylene glycol precipitation and centrifuged on a preformed 20-70% (w/v) sucrose gradient, in a SW-41 rotor at 40,000 rpm for 90 min. Fractions of 1.0 ml were collected and assayed for infectious particles (pfu) and total particles estimated by hemagglutination (HA titer). Figure 19 shows the suppression of infectious virus release was directly proportional to the ribavirin concentration. HA titers were determined on the gradient fraction 8 shown in Figure 19; the results are listed in Table 20. The pfu/HA ratio indicated some preferential loss of infectious particles at high dosage, with an overall decrease in both.

3. Macromolecular Syntheses

Microscopic examination of LLC-MK_2 cell monolayers showed little if any visible evidence of drug toxicity through eight days of treatment. DNA synthesis was measured by the incorporation of ^{3}H-thymidine into trichloroacetic acid (TCA) precipitable counts (Figure 20). Very little effect was noticeable, although higher concentrations of drug caused a transient stimulation of about 200% of control values after 2-3 days of drug treatment. RNA synthesis was dramatically stimulated by higher concentrations of ribavirin (Figure 21). The incorporation of leucine into protein was only affected by high concentrations of ribavirin (30 and 100 mcg/ml - Figure 22). This inhibition to 60% of control levels (30% after five days of treatment) is not sufficient to attribute the

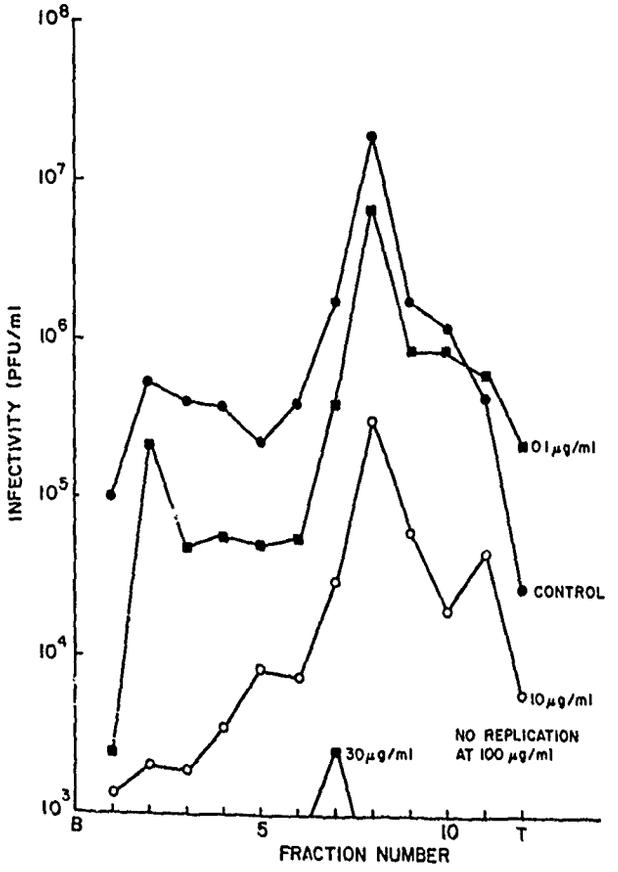


Figure 19. Inhibition of dengue virus release by ribavirin as measured by rate-zonal centrifugation. Infected LLC-MK_2 cells were treated with various concentrations of ribavirin continuously for 8 days beginning 24 hr before infection. Released virus was concentrated by polyethylene glycol precipitation and centrifuged on a preformed 20-70% (w/v)sucrose gradient in a SW 41 rotor at 40,000rpm for 90 min.

Table 20. Effect of Ribavirin Treatment on the Production of Virus Hemagglutinin

Ribavirin Concentration (mcg/ml)	pfu/ml	Virion HA	pfu/HA ratio
0	$2x10^7$	128	$1.6x10^5$
0.1	$6.8x10^6$	64	$1.0x10^5$
1	$6.6x10^6$	64	$1.0x10^5$
10	$3.4x10^5$	8	$4.2x10^4$
30	$2.5x10^3$	4	$6.2x10^2$
100	$<2.5x10^1$	<2	-

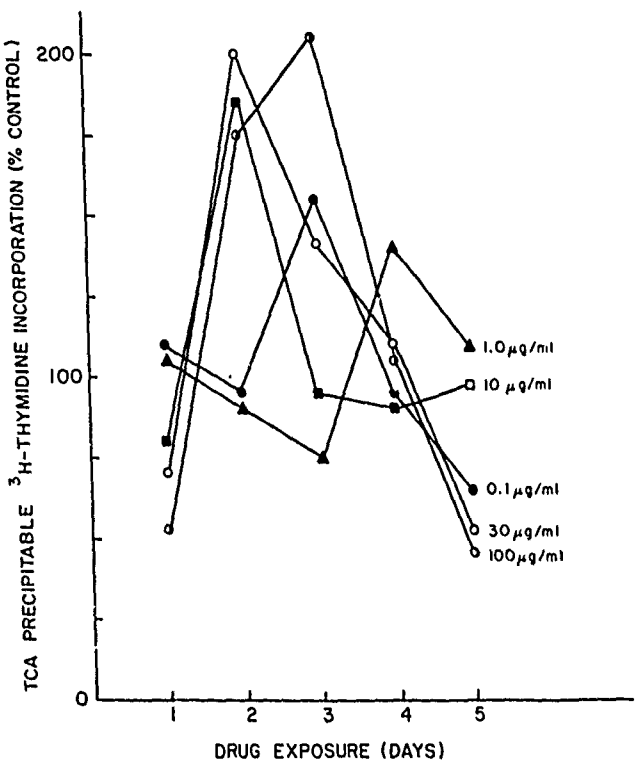


Figure 20. Effect of ribavirin on cellular DNA synthesis. Uninfected LLC-MK_2 were treated with various concentrations of ribavirin. The uptake of ^{3}H-thymidine (2 micro Curies/ml) during a 3-hour incubation period was measured daily by TCA precipitation.

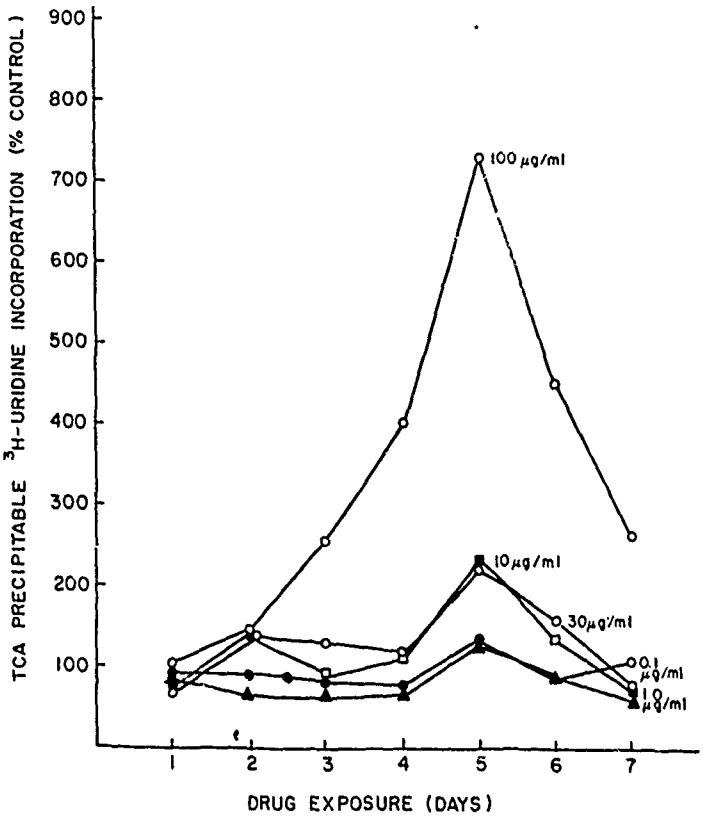


Figure 21. Effect of ribavirin on cellular RNA synthesis. Uninfected LLC-MK_2 cells were treated with various concentrations of ribavirin. The uptake of ^{3}H-uridine (2 micro Curies/ml) during a 3-hour incubation period was measured daily by cold TCA precipitation.

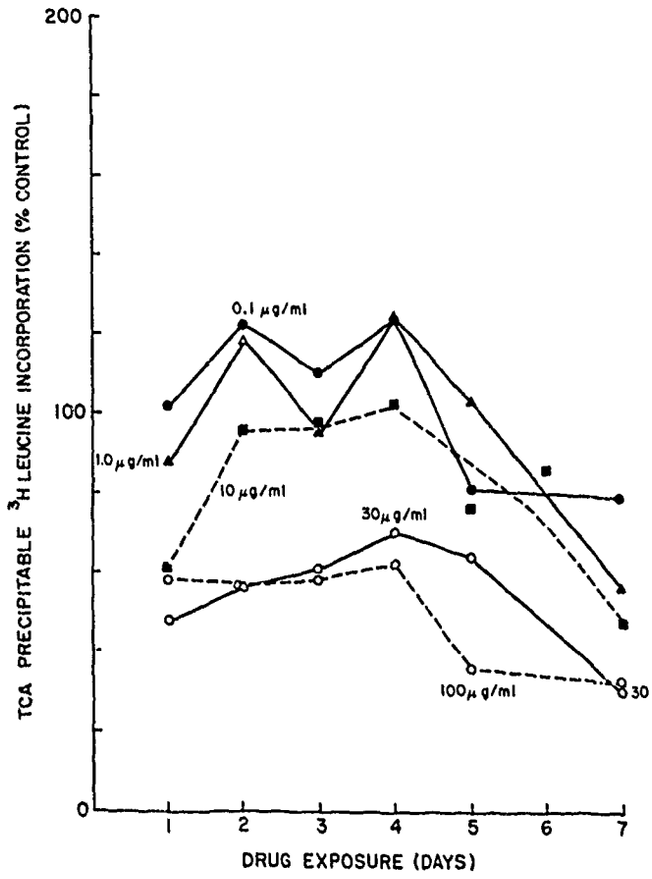


Figure 22. Effect of ribavirin on cellular protein synthesis. Uninfected LLC-MK_2 cells were treated with various concentrations of ribavirin. The uptake of ^{3}H-leucine (2 micro Curies/ml) during a 3-hour incubation period was measured daily by TCA precipitation.

dramatic decreases in virus production to nonselective inhibition of total protein synthesis.

4. Effect of Ribavirin Treatment on the Production of Virus-Specific Intracellular Proteins

The production of virus-specific proteins was examined by indirect immunofluorescence using ribavirin-treated, dengue type 1 infected LLC-MK_2 cells and dengue non-type specific Thai human antiserum. The percentage of cells showing dengue-specific perinuclear fluorescence during the course of infection shown in Figure 23. Compared to control-infected cells, very little antigen was detected in the presence of 30 mcg/ml ribavirin and even less in the presence of 100 mcg/ml. These data suggest that ribavirin blocks the production of dengue antigens.

5. Reversibility of Ribavirin Inhibition

The reversibility of the inhibition of dengue virus production by ribavirin was measured by treating infected cells with drug for various intervals of time rather than over the entire course of infection, as described in Figures 15-18. Cells were treated with 30 or 100 mcg/ml for 1, 2, 3 or 5 days beginning one day before infection. The removal of the 30 mcg/ml ribavirin dose resulted in a recovery of virus release with relatively normal kinetics after a delay of 24-36 hr (Figure 24a). Cells treated with 100 mcg/ml also released virus after ribavirin was removed but only after a delay of about 72 hr (Figure 24b).

6. Conclusions

The replication of dengue type 1 virus in LLC-MK_2 cells has been shown to be relatively sensitive to moderate doses of ribavirin. This drug effect cannot be explained by nonspecific inhibition of the synthesis of cellular macromolecules. This inhibition is reversible after the drug is removed from the culture medium. Although the mechanism of action is unknown, the striking inhibition of the production of virus-specific antigens suggests that

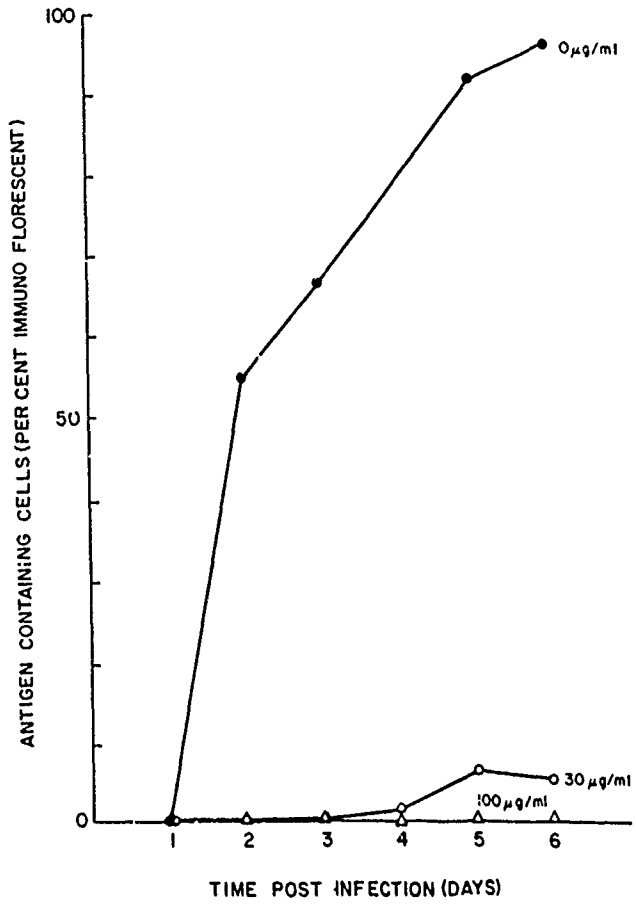
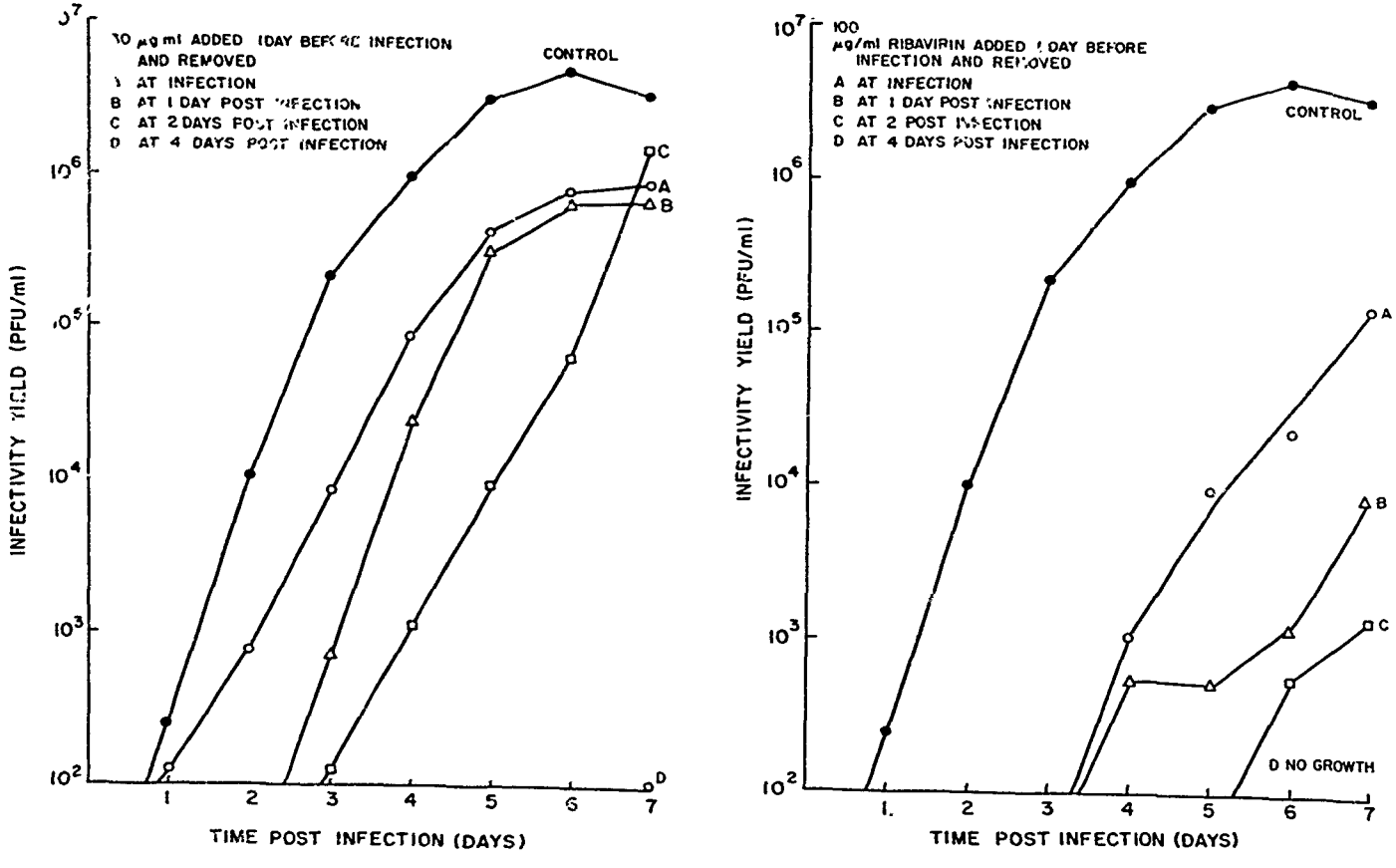


Figure 23. Percentage of infected LLC-MK_2 cells showing dengue-specific perinuclear fluorescence by indirect immunofluorescence. Cells were incubated in the presence of 0,30 or 100 mcg/ml ribavirin beginning 24 hours before infection.



(a) (b)

re 24. Reversibility of the ribavirin-dependent inhibition of dengue virus production removal of drug. Infected LLC-MK_2 cells were treated with 30 mcg/ml (a) or 100 mc bavirin beginni before infection. Drug was removed
vario

ribavirin acts on or before the synthesis of virus-specific proteins. Investigations are continuing to determine the mode of action of ribavirin at the molecular level.

B. Interferon

1. Background

The A774 and L10 strains of Semliki Forest virus (SFV) differ markedly in virulence for adult mice as well as rabbits and guinea pigs. L10 has been shown in this laboratory to have an LD_{50} of <2 pfu/ml in mice; conversely, high concentrations of A774 fail to cause death. The pathophysiology of the A774 and L10 seemingly differ only in that L10 virus reaches higher titers in brain on Day 4 whereas A774 titers are higher in lymph nodes and spleen. To further characterize the virulence patterns of these two strains of SFV, and to determine the possible role of interferon (IF), we previously examined the ability of L10 and A774 to induce interferon. L10 consistently induced more IF in L929 cells than did A774 regardless of MOI or time of IF harvest (Annual Report 1978). Further experiments have confirmed and extended the previous results.

2. Methods

Details of IF induction and the IF assay are in the previous annual report (1978).

3. Results

a. Effect of Viral Replication on IF Induction

Both induction of high amounts and low amounts of interferon has been associated with either poor or good viral replication. One possible explanation for the differences in IF induction by L10 and A774 is variation in virus replication in L929 cells--conceivably L10 would grow to higher titers inducing more interferon. Multiple experiments have now indicated that this is not the case. In a typical experiment L10 and A774 were each adsorbed

to confluent monolayers of L929 cells in 25 cm^2 plastic flasks at a MOI of 1.0. After a one-hour adsorption period at 36°C, the virus was aspirated, the monolayers were washed, and the cells fed with EMEM media with 2% fetal bovine serum. At each time point, 0.5 ml of media was removed from triplicate flasks and replaced with fresh media. The samples from each virus were initially pooled, then divided for either IF assay or virus titration and finally frozen at -70°C until tested. The peak A774 titer was 3.3×10^7 pfu/ml which was almost one log higher than the peak L10 titer (Table 21). Generally, A774 replicates to a titer of 10^7 to 10^8 which is usually higher than peak L10 titers. Peak viral titers occur at 24 or 48 hours and there is a tendency for L10 to reach a peak earlier than A774, as was seen in this experiment. Despite the slightly better viral replication of A774, L10 induced the production of much more IF. It may be that the higher amounts of IF induced by L10 results in early suppression of viral growth.

b. Effect of Temperature on IF Induction

Temperature sensitive mutants of SFV can vary in their ability to induce IF depending on the temperature. Since L10 and A774 were selected on the basis of virulence and since virulence and temperature sensitivity are frequently inversely correlated, it seemed mandatory to test the effect of temperature on growth of and IF induction by these two strains. Monolayers of L929 cells were infected with L10 or A774 at a MOI of 1.0 and treated as above except that triplicate flasks of each strain were placed at both 35°C and 39°C. Neither virus was particularly temperature sensitive (Table 22). L10 grew to a slightly lower titer at 39°C compared to 35°C but in a second experiment, this small difference was not noted. There was no noticeable difference in growth of A774 at the two temperatures. L10 induced more IF than A774 at both temperatures.

In spite of the similar growth curves at 35°C and 39°C, L10 induced significantly less IF at the higher temperature. This suggests that the IF induction or production pathways of the cells are temperature sensitive. That this is the

Table 21. Virus Replication and Interferon Induction at Different Times After Infection by L10 and A774

Hours Post-Inoculation	L10		A774	
	Virus Titer*	IF Titer**	Virus Titer	IF Titer
¼	2.5×10^1	<10	1.7×10^4	<10
2	2.5×10^3	<10	2.3×10^4	<10
4	2.5×10^3	<10	---	<10
8	3.7×10^5	<10	5.5×10^5	<10
24	3.1×10^6	70	3.3×10^7	<30
48	2.0×10^6	2300	3.3×10^7	310
72	2.5×10^5	1850	1.1×10^7	210
120	2.7×10^5	1500	3.3×10^6	105

* PFU/ml

** Inverse of dilution protecting 50% of cells after viral challenge.

Table 22. Viral Growth of L10 and A774 at 35°C and 39°C

Hours Post-Inoculation	Virus Titer (PFU/ml)			
	L10		A774	
	35°	39°	35°	39°
¼	8.3×10^4	2.4×10^5	1.9×10^4	1.4×10^4
2	3.8×10^5	6.1×10^5	2.5×10^5	1.6×10^5
4	5.1×10^5	8.1×10^5	5.1×10^5	1.0×10^6
8	2.7×10^7	1.1×10^7	3.1×10^7	8.3×10^7
24	8.8×10^7	1.2×10^7	1.4×10^8	1.7×10^8
48	1.5×10^8	2.7×10^7	5.3×10^7	7.9×10^7
72	5.8×10^7	8.0×10^5	1.3×10^7	3.8×10^6
96	2.5×10^7	5.5×10^4	3.7×10^6	3.0×10^5

case was demonstrated by inducing IF by both rIn:rCn and Newcastle Disease virus (NDV). NDV does not grow in mouse cells yet is a good inducer. It induced IF at 35°C but not at 39°C. rIn:rCn also induced IF at 35°C but less at 39°C (Table 23).

c. Sensitivity of L10 and A774 to Exogenous IF

Multiple experiments have now indicated that L10 in addition to inducing more IF is also more sensitive to the effects of IF than is A774. Serial two-fold dilutions of stock solution of IF (containing 12,000 units of IF) were added to duplicate wells of confluent L929 cells. After a incubation period of 24 hr, the interferon containing media was removed, the cells washed, and a constant amount of either L10 or A774 added. After a one-hour adsorption period, the cells were overlayed with agar. At two days, neutral red was added and the number of plaques counted (Table 24). Approximately 30 units of IF were required to decrease by 50% the number of A774 plaques but only 7 units to decrease L10 plaques by an equivalent amount.

d. Summary

The virulent and avirulent strains of SFV, L10 and A774, differ markedly in both ability to induce interferon and to respond to interferon in mouse L929 cells. The mechanism of enhanced IF induction by L10 is not clear but is not due to increased viral growth or temperature sensitivity of A774. Differences in intracellular RNA or in formations of DI particles are yet to be examined. Plaque purification of stock L10 and subsequent serial MOI passage of the samples have led to loss of virulence. Whether this is a result of a mixed population of viruses in L10 is unclear.

Table 23. Interferon Induction by rIn:rCn-Dextran and NDV at 35°C and 39°C

Hours Post-Inoculation	IF Titer			
	rIn:rCn		NDV	
	35°	39°	35°	39°
24	100	15	<20	<20
48	20	<10	65	<20

Table 24. Effect of Exogenous Interferon on the Plaquing of L10 and A774 Viruses

Interferon (International Units)	# of Plaques	
	L10	A774
0	97	30
0.5	79	33
1.0	73	35
1.9	87	32
3.7	81	32
7.5	47	27
15	42	31
30	20	18
60	1.5	6.5
120	0	0
240	0	0
480	0	0

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Work Unit 130, Viral Infections of Man

Publications.

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Work Unit 130, Viral Infections of Man

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA OA 6443	2. DATE OF SUMMARY* 79 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
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b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

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(U) Bacterial Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
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58 05	CONT	DA	C. In-House

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e. KIND OF AWARD: f. CUM. AMT.

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21. GENERAL USE
Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Pseudomonas aeruginosa; (U) Neisseria meningitidis; (U) Gonococcus; (U) Immunology; (U) Antibiotics; (U) Infectious Diseases

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

25 (U) Studies on the etiology, ecology, epidemiology, pathogenesis, physiological, immunological and diagnostic aspects of diseases of microbial origin which are current or potential problems to military forces. Current emphasis on control of meningococcal, gonococcal and pseudomonas infections in military forces.

24 (U) Development of bacteriologic techniques for study of various infectious diseases. Field studies on prophylactic regimens, spread and persistence of organisms in various military populations.

25 (U) 78 10 - 79 09 A prototype parenteral gonococcal pilus vaccine was tested in 71 laboratory and troop volunteers, and found to be safe and immunogenic. N. meningitidis polysaccharide vaccines for serogroup 29E and a combined Y-W135 were titrated in 200 recruit volunteers at Ft. Dix, NJ. The vaccines were found safe. The Y-W135 vaccine was immunogenic at all doses tested. A case strain of N. meningitidis was isolated that elaborated both the serogroup Y and W135 capsular determinates. This organism is a potential vaccine strain. A new meningococcal group B vaccine was evaluated in 80 Army recruits and found to be safe and immunogenic. Six new lots of group B vaccine have been prepared for human use for the purpose of determining optimal vaccine composition. (For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.)

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 132 Bacterial Diseases of Military Importance

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1. A prototype parenteral gonococcal pilus vaccine was tested in 72 laboratory and troop volunteers, and found to be safe and immunogenic.

2. Gonococcal pili were found to be broadly cross reactive when examined with regard to their ability to block attachment of gonococci to epithelial cells.

3. Several mammalian cell lines are being examined for the ability of N. gonorrhoeae to attach to them. Nine cell lines have been examined and the percentage of attachment has ranged from 1.5% to 35%.

4. Meaningful epidemiology requires a mechanism for distinguishing strains. The three typing systems we used measured independent variables in N. gonorrhoeae and when used in concert should prove useful in epidemiologic studies.

5. The inhibition of attachment assay measures the functional ability of antipilus antibody. The test which is done by direct microscopic examination is simple, accurate and reproducible but is tedious and time consuming. The need for a new and equally sensitive assay is paramount. Three approaches have been taken; 1) radiolabelling organisms 2) determining limulus lysate activity and 3) radiolabelling high titered specific antiserum.

6. A mouse lung model has been developed to study the

pathogenesis of gonococcal infections and as a possible model to assay potential vaccines.

7. A high molecular weight substance probably representing the gonococcal capsule has been isolated from the supernatant of organisms that have previously been passed through guinea pig chambers.

8. A direct microtiter test has been developed to assay the bactericidal activity of sera against gonococci.

9. WRAIR volunteers (10 for each vaccine) were injected with N. meningitidis vaccines for serogroups 29E, Y, W135 and a combined Y-W135. These vaccines were found safe, and immuno genic by a group specific hemagglutination assay, a primary antigen binding assay and a bactericidal antibody assay.

10. N. meningitidis polysaccharide vaccines for serogroup 29E and a combined Y-W135 were titrated in 200 recruit volunteers at Ft. Dix, NJ. The vaccines were found safe. The Y-W135 vaccine was immunogenic in all doses tested; data is not available at this time for the 29E vaccine.

11. A case strain of N. meningitidis was isolated that elaborated both the serogroup Y and W135 capsular determinates. This organism is a potential vaccine strain.

12. A new meningococcal group B vaccine was evaluated in 80 Army recruits and found to be safe and immunogenic. Six new lots of group B vaccine have been prepared for human use for the purpose of determining optimal vaccine composition.

13. Studies of the quality of human antibodies to meningococcal group B polysaccharide have shown them to be of relatively low avidity.

14. The protein responsible for serotype specificity among group A strains has been partially purified and shown to represent a new class of meningococcal serotype proteins.

15. Collaborative studies on the role of toxin A and S and the development of toxoids has continued. High molecular weight polysaccharides isolated from P. aeruginosa do not contain the antigenic determinant found in lipopolysaccharide against which human opsonic antibody is directed. Removal of ester linked fatty acids from the lipid A of P. aeruginosa by sodium methoxy treatment does not destroy the opsonic determinant but does remove a majority of the toxicity.

16. Pyocin receptors were demonstrated in the core sugar areas of the lipopolysaccharides of Ra, R1, and R3 core types of Salmonella, Shigella and E. coli. These receptors could be blocked by O side chain on the molecule or by the K1 surface antigen. Meningococcal polysaccharide capsules were also able to block pyocin killing of meningococci.

17. Gamma globulin has opsonic antibody against six of the seven immunotypes of P. aeruginosa, Shigella and some E. coli. Modification of gamma globulin by alkylation destroys opsonic but not binding antibody. Binding antibody against the core glycolipids J-5 and Re and Toxin A are also found in gamma globulin.

18. K1 capsular polysaccharide is most commonly associated with E. coli that have incomplete LPS. It protects these otherwise serum-sensitive organisms from the bactericidal activity of human serum and from the killing by polymorphonuclear leukocytes.

19. Mononuclear cells, upon exposure to the specific antigens SKSD or the LPS of Shigella flexneri 2A, produces a factor released into the supernatant that is able to favorably influence bacterial kill by polymorphonuclear leukocytes.

20. Tetracycline, ampicillin and carbenicillin-resistance are the most common transmissible plasmids that confer antibiotic resistance among clinical isolates of gram-negative bacteria at WRAMC. Cross-resistance to aminoglycosides can develop in response to a high level of usage of gentamicin.

21. An 11 month prospective survey of patients hospitalized at WRAMC revealed 107 patients with nosocomial pneumonia (incidence 0,82%). Contaminated respirators are an unusual cause of endemic nosocomial pneumonia (6/107 cases).

22. In vitro secretion of anti-meningococcal, anti-gonococcal and anti-tetanus toxoid IgG, IgM and IgA by human peripheral blood B cells can be amplified and suppressed by autologous T cells. Since these T cell influences have exhibited antigen and immunoglobulin class specificity, this newly developed assay system can now be used to identify those immunopotentiating agents which can specifically amplify human antibody responses to antigenically relevant bacterial antigens.

23. Thymosin (Fraction V) can stimulate the secretion of anti-meningococcal, antigonococcal and anti-tetanus toxoid IgG, IgM and IgA by human peripheral blood lymphocytes.

24. Serum from adults immunized with pneumococcal vaccine is opsonic in vitro and protective in vivo for group B type III streptococci.

1. The incidence of gonorrhea has reached epidemic proportions in the United States (approximately 3-4 million cases per year). The greatest proportion of these cases occur in individuals less than 30 years of age, the age group which makes up the greatest proportion of individuals serving in the Armed Forces. Clearly, the present methods employed for controlling this disease has been a failure. Furthermore, American military personnel are frequently deployed in foreign countries where the indiscriminate use of antibiotics is widespread. As a result the gonococcus has steadily developed increased resistance to antibiotics which has led to an increased morbidity due to gonorrhea and threatens to overwhelm our medical resources for care for these patients. Thus the need for a gonococcal vaccine is acute.

The general approach to the development of a gonococcal vaccine has been to examine the human immune response to this infection, to determine the important GC antigens eliciting this response, and finally to purify and characterize the antigens as potential vaccine candidates.

Previous studies have demonstrated the development of local genital antibodies to gonococci (Annual Report 1975, 1976); that these antibodies function to inhibit the attachment of gonococci to epithelial cells (Annual Report 1975, 1976), and that the gonococcal antigen principally responsible for eliciting this response is pili (Annual Report 1977).

A prototype gonococcal pilus vaccine (gonococcal pilus vaccine P-3-2 IND #1351) was prepared by Charles C. Brinton, Ph.D., University of Pittsburgh, Pittsburgh, PA. This vaccine was shown to meet all FDA safety testing by independent testing at the University of Pittsburgh, and the Department of Biologics Research, Division of Communicable Diseases and Immunology, WRAIR. Initial studies for safety and immunogenicity were conducted on laboratory and troop volunteers at the WRAIR and Fort Bragg, North Carolina. Two laboratory volunteers received 2 mg. doses and 7 laboratory volunteers received 1 mg. doses 4 weeks apart. Immunogenicity was determined by the SPRIA using the pilus (vaccine) as the antigen (Tables 1, 2, 3). All 9 volunteers developed an immunogenic response to pili. None of the volunteers developed an antibody response to LPS purified from the prototype strain as the antigen. Sixty three troop volunteers received either a placebo, 0.1 mg, 0,2 mg, .5 mg, or 1.0 mg. vaccine doses. The analysis of the immune response is incomplete in the male volunteers from this group, All 14 female volunteers who had received the vaccine

developed serum antibodies but only 3 developed local antibodies. The sera of eight volunteers were tested for rises of opsonic antibody. The pre and post bleed sera were tested on the same day at various dilutions for the ability to kill gonococci in the presence of human white blood cell (WBC) and complement from an aggamaglobulonemic donor. The highest dilution of serum able to kill more than 50% of the organisms in 45 minutes has been arbitrarily defined as the titer. There are 10^6 organisms in the 100 ml test. Controls in each test include: serum plus WBC, serum plus complement, WBC plus complement and complement alone. None of these combinations kill the test organism GC P-3-2 in a valid test. The GCP-3-2 strain is the one from which the pili vaccine was produced. The pre and post bleed opsonic titers are shown in table 4. Since only 10µl of sera are used in a final volume of 100µl the final dilution is 10 times that listed in the table. Seven of the eight vaccinated volunteers demonstrated rises in opsonic antibody against the homologous vaccine strain. Inhibition experiments are currently being performed to prove that the opsonic antibody that was induced by pili vaccination is directed against pili. Except for arm soreness which lasted in some cases up to 48 hours, the vaccine was well tolerated by all of the volunteers. Some volunteers also developed arm redness. No volunteers developed a fever, or lost time from work. None of the 9 volunteers who had a SMAC-20, CBC or urinalysis performed every week for 8 weeks developed any abnormalities.

2. In order for a gonococcal pilus vaccine to be protective, it must stimulate cross reactive antibodies. Gonococcal pili have previously been demonstrated to have considerable antigenic heterogenicity. However, by using the assay which measures inhibition of attachment of whole gonococci to human epithelial cells (see Annual Report 1975), gonococcal pili were demonstrated to be broadly cross reactive. This was demonstrated by competitive inhibition and by absorption of vaginal antibody. Gonococcal pili were capable of competitively inhibiting the attachment of heterologous strains of gonococci to human epithelial cells, although not as efficiently as they inhibited the homologous strain. When genital antibody was absorbed with the homologous pili, this blocking activity was completely removed; while absorption with the heterologous pili removed all of the inhibiting activity against the heterologous strains, but only partially reduced the titer against the infecting strain (Table 5). Thus there appears to be a common set of antigenic determinants on gonococcal pili which mediate attachment and the human local antibody response is directed at least in part against this common set of determinants.

3. The day to day variance in buccal epithelial cells obtained

from a pool of human volunteers and its influence on the ability of N. gonorrhoeae to attach is a continuing problem. An approach to solving this problem is to propagate stable cell lines using tissue culture techniques. The ability of two different strains of N. gonorrhoeae to adhere to nine mammalian cell lines has been examined. The percentage of bacteria adhering to a specific number of tissue culture cells was determined using both a viable bacteria count and radiolabelled N. gonorrhoeae. The percentage of gonococci attaching to each cell line varied considerably and was different for each cell line (Table 6, Table 7).

4. Meaningful epidemiology requires a mechanism for distinguishing strains. Many different typing systems for N. gonorrhoeae have been previously developed by many different investigators, but have in general not helped much in unraveling important immuno-epidiologic questions.

A study was undertaken at Fort Bragg, North Carolina to obtain topographical, epidemiologic and strain identity in anticipation for a possible future field trial of a gonococcal vaccine at Fort Bragg. 527 patients over a 6-month period (Dec 1978-May 1979) with probable gonorrhea (gram stain, and/or positive culture) were enrolled.

One hundred sixty strains of N. gonorrhoeae from Fort Bragg, North Carolina were examined utilizing auxotyping, pyocin typing, and a lipopolysaccharide (LPS) radioimmune inhibition typing system (RI-LPS). 18 distinct nutritional classes were found by auxotyping. The major auxotype required proline (17%). One arginine, uracil, hypoxanthine (AUH) requiring strain was found; 68% of the strains had no nutritional markers in our system and were classified as wild types. The sensitivity of the GC strains to 20 pyocins from 6 pyocin groups was tested. The majority of strains (63%) were killed by pyocins in Groups 1 and 5, while 14% were killed only by group 5 pyocins and 9% by all 6 groups. The remaining GC strains were classified into 7 different pyocin types. The RI-LPS system based on 4 GC LPS serotypes typed 29% of the GC strains as type 2, 25% as type 4; types 1 and 3 each contained 4% of the strains. Eleven percent of the strains had 2 types and 27% were nontypable. The single AUH auxotype was a pyocin type 1, 5 and was nontypable by the RI-LPS system. There was no correlation between auxotype, pyocin type and RI-LPS type. Therefore, these three typing systems measure independent variables in GC and thus when used in concert should prove useful in epidemiologic studies. The lack of correlation between pyocin and RI-LPS types also implies that the LPS immunologic marker is distinct from the pyocin receptor area in GC LPS.

5. The inhibition of attachment assay measures the functional ability of antipilus antibody. The test which is done by direct

microscopic examination is simple, accurate and reproducible but is tedious and time consuming. The need for a new and equally sensitive assay is paramount. Three approaches have been taken, 1) radiolabelling organisms, 2) determining limulus lysate activity and 3) radiolabelling high titered specific antiserum.

N. gonorrhoeae has been labelled with ^{3}H adenine. The surface of a GC agar plate is coated with 500 µl of a balanced buffered salt solution containing 50 µl of ^{3}H adenine (1mCi/ml). This solution is allowed to dry on the plate surface. The GC plate is then streaked with bacteria, and incubated for 18 hrs. The labelled bacteria can now be used in the standard IEA assay. The percentage of bacteria attached to the cells is determined by the amount of radioactivity associated with the cells. This method is being developed in conjunction with the tissue culture project because the sensitivity of this test is related to the ability of N. gonorrhoeae to attach to the cells. Our results have confirmed the feasibility of this approach but have also emphasized the need for using a cell line to which the bacteria adhere at a high percentage. The limulus lysate activity of N. gonorrhoeae attached to tissue culture cells can be determined. To do this requires that the IEA test be performed in a well in which a tissue culture cell line is growing. At the completion of the IEA test the mixture in the well is removed and the cells washed with sterile distilled water. The limulus reagents are then added directly to the well. Those wells in which sufficient bacteria are attached to the cell will cause the limulus test to gel and will be related to the titre of the sample being tested. This system has proven to be very sensitive. The usefulness of it is limited by the requirement that the sample being tested be free of bacterial, LPS and proteolytic enzyme contamination.

High titered GC specific antiserum has been raised in rabbits, fractionated with ammonium sulfate, absorbed with the tissue culture cells with which it will be used and labelled with ^{125}I. This labelled antiserum is used to enumerate the bacteria adhering to the TC cells. The results of these studies are incomplete.

6. Animal models for gonococcal infections have proven elusive; their lack has frustrated studies of immunity and of the pathogenesis of gonorrhoeae. Others have used the mouse lung infection model to study host response to infection with pathogenic and non-pathogenic enteric bacilli. In collaboration with the Department of Pathology, we have adapted this model to study the response to infection with gonococci. Results from preliminary experiments indicated that organisms from laboratory maintained strains, even though they were of the virulent colony types, were not suitable. They appeared to provoke little host response and were very rapidly cleared; they were considered avirulent

in this model. Use of such strains was discontinued; however, in retrospect, the failure of these organisms in this model could to some extent be ascribed to our initial failure to master and standardize the challenge procedure. To better insure virulent challenge strains we obtained and used strains of gonococci from primary isolation cultures of male patients attending a local VD clinic, isolated on GC medium with defined supplement (GCD) and BYE medium, both containing the VCN inhibitory antibiotic mixture. Four such strains were tested with essentially the same results.

Mice were infected intranasally by passing a nasal catheter into the nasopharynal and instilling 0.05 ml of a suspension of freshly isolated gonococci in Mueller-Hunter broth containing 1×10^8 organisms. They were sacrificed at intervals after challenge and their left lungs preserved for pathological examination and the right lungs used to assess the numbers of infecting organisms. Results of challenge with four different strains were essentially the same. Organisms were recovered in larger numbers, 2×10^6 to 2×10^7, immediately after (0hr) and at 3 and 6 hr after challenge. However, at 12 hr, organisms were detected in only small numbers or not at all, and could not be detected at 24 hr after challenge. Histologically at 0 and 3 hr after infection, many organisms were found in the bronchial lumen and attached to the bronchial epithelium and were also frequently seen in the alocoli. At 6 hr larger numbers of leukocytes and macrophages were evident in the bronchial lumen and alvcoli, as were large numbers of intracellular and extracellular diplococci. Further, capillaries and larger vessels were congested. Twelve hours after challenge the numbers of leukocytes and macrophages containing phagocytosed gonococci increased considerably while there was a concomitant decrease in the intracellular organisms. At 24 hr the infection started to subside; fewer leukocytes were seen and edema and congestion were less severe. The recovery of organisms through 6 hr post challenge correlates with the large number of extracellular organisms seen in sections of infectioned lung tissue. Our inability to recover gonococci 12 hr at post-infection correlates with the absence of extracellular organisms in infected tissue and suggests that the large numbers of intracellular gonococci seen in infected tissue have been killed by the leukocytes and macrophages.

We have also tested virulent colony type 1 organisms of strain 22, along with its phenotype "avirulent" colony type 3 variants in this model after 3 and 6 in vitro passages on GC agar. The patterns seen in mice sacrificed at 6 hr after infection with organisms of either of two colony types after 3 passages are quite similar. We have also investigated the effect on virulence of passage of a freshly isolated G.C. strains on artificial media as expressed in the mouse lung model.

Colony type 1 (T_1) organisms and variant colony type 3 (T_3) organisms were used to infect mice intranasally after 3 and 6 passages on GCD agar and the resultant infections compared with the infection produced by the first passage, primary isolation culture. The numbers of virulent phenotype T_1, organisms recovered with mice infected with 3rd and 6th passage cultures were the same as those from mice infected with the primary isolation culture. Histology of the lungs of these mice was also similar; large numbers of PMN and intracellular and extracellular gonococci and macrophages were present in the bronchi and alvoli. The picture resulting from infection with avirulent phenotype T_3 organisms obtained colonially pure on the 3rd passage was the same as that resulting from the T_1 infections. However, the infection with 6th passage T_3 organisms was markedly different. Compared with T_1 infected mice, not only were fewer organisms recovered from lung cultures and fewer organisms, either extracellular or intracellular, seen on histological examination, there appeared to be fewer PMN and macrophages. These data suggest that the 6th passage T_3 organisms had suffered a loss in virulence after 6 passages; they were cleared more rapidly than the T_1 or 3rd passage T_3 organism and the cellular response was less intense.

This lung infection model could provide a system to study the cellular response of animals to the presence of N. gonorrhoeae; to define some of the host and parasite factors which play a role in the intense response of the male urethra to gonococci. The system can be manipulated immunologically and could provide the means to define virulence factors of the parasite and antigens with vaccine potential.

7. Attempts to isolate, purify and chemically characterize the gonococcal capsule are still in progress. Guinea pigs (CG-P) have been implanted subcutaneously with plastic chambers and allowed to epithelialyze for at least one month. The chambers have then been injected with 10^5 live gonococci. Three days later these organisms were collected from the chamber and plated on GC-agar. By india ink techniques the majority of the organisms are encapsulated. These GP- passed organism were then grown in medium 199 (Microbiologic Assoc). The pH of the media after addition of 1% isovitalex was maintained at 7.2 by addition of 7.5% HaBicarbonate. The organisms were grown 24 hr, harvested and the supernatant is filter-sterilized and lyophilized. The lyophilized supernatant was then dialyzed extensively and relyophilized. The material was treated with Pronase and Nuclease at room temperature for 2 hrs, placed into final concentration of 2% deoxycholate and run over a G1-0 deoxycholate, glycine buffered column, pH 7.8 with 0.0 1M FDTD to remove lipopolysaccharide contamination. The pooled, dialyzed, and alcohol-washed

void volume contained a high molecular weight polysaccharide which is probably the gonococcal capsule. Immunologic and chemical studies are underway to confirm its structure and relation if any to gonococcal lipopolysaccharide.

Gonococcal serum bactericidal tests (BCT) have become standard procedures to assay for antibody in hyperimmune rabbit sera, and human sera from normal uninfected individuals, gonorrhea patients and individuals immunized with gonococcal antigens. Serum bactericidal tests provide reliable data; however they are both cumbersome and time consuming. Recently, a microtiter bactericidal test procedure was described to assay antibodies in the sera of individuals immunized with meningococcal polysaccharide. This test is contained entirely within a microtiter plate, and relies on the dye triphenyltetrazolium (TTC) to visualize growth of colonies within each well. We have used this procedure to test assay for gonococcal antibodies in normal human serum (NHS) bactericidal activity. The test sera, NHS or rabbit antisera, are first diluted in Geys' BSS containing 0.007% TTC in the microtiter plate. The test sera, NHS, using its intrinsic complement, or rabbit antisera, are serially diluted in the microtiter plate in 25 mcl volumes of Geys BSS (G-BSSS) containing 0.0075% TTC (GBSS/TTC). A similar volume of complement in the rabbit antiserum test, or G-BSS in NHS is added next followed by 25 µl of G-BSS containing 400-500 test strain organism. After gentle agitation the plate is covered and incubated for 45 min at 37°C. Finally, 0.1 ml of molten GCD medium at 48-50°C is added to each well and held at room temperature for 5 min to permit the agar to gel. The microtiter plate is then covered, sealed with tape and incubated overnight at 37°C. To test the inhibitory activity of GC lipopolysaccharides (LPS) for NHS bactericidal activity, the LPS is first serially diluted in the microtiter plate in 75 µl GBSS/TTC. A standard dilution of NHS which kills approximately 90-95% of test organisms is added to the LPS dilutions and the mixtures are reacted for 30 min at 37°C. Test organisms are then added and after an additional 45 min incubation 0.1 ml GCD agar is added and the plates are sealed and incubated as above. The results of the test bactericidal endpoints, are readily apparent by visual inspection. GC-colonies when present are colored by the TTC dye, and wells which contain large numbers of colonies indicating no bactericidal activity are visibly red. One can make relatively accurate estimates of 80%-90% bactericidal endpoints if one enumerates the numbers of organisms added to each well. As with most microtiter tests, this test is readily assembled and conserves reagents. It eliminates the considerable time and effort expended in acquiring results of classical bactericidal tests by colony counts, the results of the microtiter bactericidal test can be read as easily as any microtiter serological test.

One of our prime objectives is the definition of the LPS antigens

of serum sensitive GC strains responsible for serum sensitivity. The most direct route is to test the LPS' and their isolated oligosaccharides in a bactericidal inhibition test. The microtiter bactericidal test will not only serve admirably for this purpose but will provide the opportunity to test extracted antigens which will be available only in small quantities.

9. Serogroup 29E, Y and W135 Neisseria meningitidis capsular polysaccharide vaccines as well as a divalent Y-W135 polysaccharide vaccine were tested in 10 adult human volunteers each at WRAIR. A dose of 50 mcgm (monovalent) or 100 mcgm (divalent) was injected subcutaneously in the deltoid region.

The mean age of volunteers was 31.4 years for 29E, 33.1 years for Y, 38.5 years for W135 and 32.1 years for Y-W135 combined. All three of the oldest volunteers received W135, resulting in a greater range, but a similar mean for this preparation.

Reactogenicity was evaluated in each recipient by questioning him/her about local pain and tenderness at the vaccination site, systemic symptoms (headache, chills, fever, malaise, etc.) and by noting and measuring erythema and/or induration at the vaccination site. Each recipient's evaluation of the local reaction was given a numerical score, according to the following criteria.

0 - no noticeable pain or tenderness

1 - slight pain and tenderness, not noticeable at rest. Easily ignored and considered minimal.

2 - slight to moderate pain and tenderness, intermittently noticeable at rest, without interference with activities. Ignorable and considered "par" for a "mild" vaccine (less than influenza or typhoid).

3 - moderate pain and tenderness intermittently bothersome at rest and requiring slight adjustment in activity. Considered cause for complaining, but acceptable.

4 - moderate to severe pain and tenderness which interferred with activities, was noticeable during sleep, or led to taking of analgesics. Considered a painful vaccine on a par with influenza or typhoid.

Chills without fever, mild malaise and/or headache were considered evidence of minor systemic reactions. Fever >101°F, chills, malaise, coryza and myalgia, with or without headache, were considered evidence for a major systemic reaction.

Table 8 presents results of reactogenicity evaluation. Groups 29E, Y and Y-W135 combined gave acceptable levels. Group Y was clearly the least reactive (it also passed pyrogenicity testing at the 2.5 mcgm level, the highest of the four). Groups 29E and Y-W135 combined were intermediate. Only one minor systemic reaction was observed (headache alone in one recipient of 29E).

Group W135 produced greater side effects, including one major systemic reaction consisting of fever to 102.6°F, myalgia, moderately severe malaise, coryza and chills, lasting 24 hours. Two other minor systemic reactions (one, headache, alone; one, headache, mild malaise, chills without fever and restlessness during sleep) also were noted. In addition, four volunteers developed erythema with induration. Overall, group W135 was judged borderline acceptable and will not be tested further.

Preliminary immunogenicity data was obtained by indirect hemagglutination assay (IHA). Final judgement of immunogenicity will be based on Primary Antigen Binding Assay and Bactericidal Assay data, which provide better quantification and an estimate of functional efficacy, however, this data is complete only for the Y and W135 vaccines. A $\log_2$ rise in IHA titre between pre-vaccination and two-week post-vaccination sera was considered positive. Each set of sera was assayed against the homologous polysaccharide or polysaccharides. In addition, sera from volunteers vaccinated with 29E polysaccharide were also assayed against Z polysaccharide which is similar in chemical composition and sera from volunteers vaccinated with Y and W135 polysaccharides were assayed against W135 and Y polysaccharides, respectively.

Ten of 10 volunteers responded in the homologous hemagglutination assay to 29E and W135 vaccines. Eight of ten volunteers responded to Y. One of the non-responders had a high and unchanged titre (8 $\log_2$), and was a nasopharyngeal carrier of group Y Neisseria meningitidis. The other non-responder had only a $\log_2$ rise in titre. For the Y-W135 combined vaccine, 8/8 responded to Y polysaccharide, 7/8 to W135.

Two 29E vaccines also responded to Z polysaccharide. Eight of ten Y vaccines also responded to W135 polysaccharide, while 5/10 W135 vaccine responded to Y polysaccharide. For the most part, the heterologous response was muted compared to the homologous response, although exceptions were noted.

The range and geometric mean of binding and bactericidal antibody levels for the Y, W135 and Y-W135 vaccines at 0 and 4 wks, for all 30 volunteers, are displayed in figures 1 and 2, respectively. Values for the group Y carrier are boxed. Immunologic response, for both assays, and conversion of "susceptibles" are summarized in table 9. A binding antibody response to the homologous polysaccharide(s) was induced in 100% of the volunteers by 2 wks and persisted through six months. In addition, a heterologous response to the opposite polysaccharide was induced in 60-80% of volunteers at 4 weeks. Lytic antibody response was somewhat less - 85% at 4 weeks for those vaccinated with W135 polysaccharide, either alone or in combination with Y polysaccharide, and 100% at 4 wks for Y polysaccharide, either alone or in combination. A heterologous response was induced in 30-70% of

volunteers. The geometric mean homologous response was considerably greater than the heterologous response for both assays. Differences in mean homologous response between the mono and divalent preparations did not consistently favor either formulation: response to group Y was greater when administered in combination with group W135; response to group W135 was greater when administered alone.

Using the absence of bactericidal antibody as the determinant of susceptibility, 11 of 29 volunteers (38%) were susceptible to the standard group Y strain prior to vaccination. All 11 developed bactericidal antibody which persisted through 6 months, regardless of which of the three vaccines they received. For the W135 strain, 15/29 volunteers (52%) were susceptible prior to vaccination, and three remained susceptible after vaccination (1 vaccinated with W135 polysaccharide; 2 with Y polysaccharide).

Persistence of antibody is depicted in figure 3. Binding antibody remained constant over 6 months, while bactericidal antibody diminished by 1-2 $\log_2$ over the same period.

The effect of pre-vaccination antibody levels on response is shown in figure 4. For each assay, pre-vaccination values for each of the 20 volunteers receiving group Y or group W135 vaccine, alone or in combination, are arrayed in order of increasing values. The corresponding post-vaccination value (at 2 wks) is displayed above each pre-vaccination value. For binding antibody values the correlative line of best fit is displayed. For bactericidal antibody, equal pre-vaccination values (e.g. 0 $\log_2$) are arrayed according to increasing corresponding post-vaccination values. Pre-vaccination levels tended to affect post-vaccination binding antibody levels, with volunteers with higher pre-existing antibody responding to correspondingly higher post-immunization levels. For bactericidal antibody, however, no consistent relationship was seen. Individuals developed a titre of about 10 $\log_2$ regardless of pre-existing antibody. The net change in bactericidal titre was, therefore, much less for individuals with high pre-existing levels, than those without.

We conclude that 1) both the group Y and group W135 capsular polysaccharide are safe and effective immunogens for adult humans when administered alone or in combination at a dose of 50 mcgm; 2) that the immunologic cross reaction between these structurally similar polysaccharides can be maximized by combined administration; 3) that induced antibody persists for over six months at presumably effective levels; 4) that bactericidal antibody response is greatest in individuals without pre-existing bactericidal antibody, and, hence, 5) >90% of susceptibles can be converted to immunes by a single dose of combined vaccine.

10. Two hundred and fifty volunteers were solicited from basic

training recruits in their first week of training at Ft. Dix, NJ. Approximately 25 men at each antigen concentration were injected subcutaneously in the deltoid region with 10, 25, 50 or 100 µg of 29E or 10, 25, 50, or 100 µg or 25 µg in a constant volume (cv) of 0.5 ml of Y-W135 vaccine to determine which antigen concentration will induce optimal antibody response.

Nasopharyngeal carrier survey and serum specimens were obtained prior to and at four and six weeks post vaccination. Recruits carrying the same serogroup to which he/she was immunized was excluded from the immunological analysis.

The dose-response curve for the Y-W135 vaccine (Figure 5) was biphasic for binding antibody, with the higher two doses inducing a greater response than the lower two. Interestingly, the constant volume dose was intermediate between these two phases. The dose-response curve for bactericidal antibody was, in contrast, flat. Since immunity to meningococcal dissemination correlates with bactericidal, rather than binding antibody, the higher doses would appear to offer no advantages.

This is more clearly seen in the table 10 where the volunteers are broken down into two overlapping subcategories; those without pre-existing bactericidal antibody and those with ≤ 4 $\log_2$ of bactericidal antibody, pre-vaccination. No consistent differences in the percent of total volunteers who responded with a rise of ≥ 2 $\log_2$ were seen among the doses, and 100% of susceptibles and those with low pre-existing antibody levels responded to all doses.

No data is available at this time for the 29E vaccine titration.

11. A single strain (8021) of N. meningitidis, isolated from a child with disseminated meningococcal disease, was found to elaborate two serogroup-specific capsular polysaccharides - Y and W135. The original isolate as well as the progeny of 10 single colony sub-isolates each agglutinated with both group Y and group W135 serogrouping antisera. The capsular polysaccharide of strain 8021 contained the chemical constituents of both the W135 (sialic acid-galactose) and Y (sialic acid-glucose) capsular polysaccharides in a ratio of 2.5:1 (table 11). The patient responded immunologically to both capsular polysaccharides with hemagglutinating antibodies (group W135 1:16 → 1:64, group Y 1:2 → 1:16). Analysis by double diffusion in agar revealed that the capsular polysaccharide of strain 8021 contained individual molecules of group W135 and group Y capsular polysaccharides as well as a mosaic molecule containing both antigenic determinants. The testing of a strain 8021 capsular polysaccharide vaccine is currently underway to determine its antigenicity. Utilization of the naturally occurring polyvalent capsular polysaccharide would permit combination of fewer individual lots in the production

of a tetra-valent vaccine (groups A, C, Y and W135), and, therefore, less cumulative endotoxin.

An immunological cross-reaction between agarose, a naturally occurring galactan, and an antigenic determinant which is a locus for human bactericidal antibody within the LPS of a group Y, type IX strain of N. meningitidis was investigated. Bactericidal antibody in the convalescent serum of the child from whom the strain was isolated could be absorbed by highly purified agarose in bead form (Sepharose), but not by a dextran gel (Sephadex). It was inhibited by agarose as a linear polymer and by the strain's LPS, but not by a heterologous LPS from a group B, type II strain of N. meningitidis, nor by the homologous capsular polysaccharide. Both LPS contained galactose; neither was anti-complementary. Agarose antiserum, raised in a rabbit, was bactericidal for the group Y strain, but not for the group B strain. Bactericidal antibody in the agarose antiserum could be inhibited by agarose. Immunization with agarose induced hemagglutination antibody against the group Y strain's LPS which could be absorbed by the group Y, but not the group B strain, and could be reduced by absorption with Sepharose. We conclude that the cross-reaction resides in the fine structure of the galactose constituent of the groups Y strain's LPS.

12. Group B Neisseria meningitidis continues to be responsible for a major proportion of meningococcal disease in the United States, Europe, England, and South Africa. Since the development of successful capsular polysaccharide vaccines for groups A and C in the late 1960's there has been a continuing effort at the WRAIR as well as in several other laboratories to develop a suitable group B vaccine. Several products including purified high molecular weight group B polysaccharide, and isolated serotype outer membrane protein have been tested in human volunteers with disappointing results. We recently demonstrated in a phase I study that a non-covalent complex of group B polysaccharide and outer membrane protein prepared under mild conditions was safe and immunogenic in eight laboratory volunteers. The present report describes the results of phase IIa testing of the vaccine in Army recruits.

In the phase IIa study, conducted at Ft. Dix, New Jersey, two lots of vaccine (BPS-WZ-2 and BP2-4) were each tested in about 40 recruits. Seventy volunteers were obtained from among recruits during their first week of basic training. Forty of the volunteers were given a single 120 µg dose of vaccine subcutaneously in the upper arm. The remaining 30 weregiven a saline placebo in the same manner. The volunteers did not know which they received. Throat cultures and blood samples were obtained prior to vaccination and at either 2, 5 and 7 weeks or at 4 and 6 weeks.

Reactogenicity of the vaccine is summarized in table 12. No immediate reactions to the vaccine were detected. At 16-24 hours,

local reactions, consisting mainly of erythema and slight tenderness at the vaccination site, were observed in 53% of those who received one of the vaccines and 17% of those who received the placebo (recruits received several other immunizations, including typhoid and tetanus toxoid, during the same two day period). Four volunteers who received vaccine and five who received the placebo reported mild systemic effects principally consisting of headache and or fever. Four of the five systemic complaints were from among 10 females who received the placebo (no females received vaccine).

The two lots of vaccine were tested separately about 3 months apart. During testing of lot BP2-WZ-2 the initial nasopharyngeal carrier rate for Neisseria meningitidis was 28% and increased to 34% by the seventh week of training. Initially, there were no group B carriers, but two in the vaccine group and one in the placebo group acquired a group B strain during the 7 wk period. During testing of lot BP2-4 the initial carrier rate was 14.3% and increased to 23% by the seventh week. There were two in the vaccine group and three in the placebo group that were carriers of a group B strain at some point during the seven weeks.

The pre and post vaccination sera were assayed for total bactericidal antibody (by using the vaccine strain), for bactericidal antibodies to the group B polysaccharide (by using a group B strain with different serotype antigens), and for bactericidal antibodies to the serotype protein antigens (by absorbing out the group B antibodies with purified capsular polysaccharide and testing for residual killing of the vaccine strain). These results which are summarized in table 13 demonstrate that the vaccine induces bactericidal antibodies against both the group B polysaccharide and the serotype protein(s). Since serum bactericidal antibodies have been correlated with protection against meningococcal disease, the induction of bactericidal antibodies by a candidate vaccine is of prime importance. Most of the bactericidal antibodies induced appear to be directed against the group B polysaccharide. This is good since one might expect such antibodies to protect against all group B strains. A disappointing aspect of these results is that the antibody titers did not remain high, but instead decreased markedly between 2 and 6 weeks.

In the case of lot BP2-WZ-2 all 14 recruits who initially lacked bactericidal antibodies to the vaccine strain had acquired bactericidal antibodies at 2 wk, and among those immunized with lot BP2-4 6 of 7 who were seronegative converted (table 14). Overall the seroconversion rate was 95%. By 6 to 7 weeks post-vaccination, however, the seroconversion rate was down to 71% due to the lack of persistence of the antibody that was induced.

The antibody response to the outer membrane proteins was also measured by solid phase radioimmunoassay using outer membrane

complex from the vaccine strain as the antigen. The mean antibody concentration changed very little in the placebo group but increased from 24 µg/ml to 65 µg/ml in those who received vaccine lot BP2-WZ-2. At 6-7 weeks the mean level had decreased to 50 µg/ml. The pre-vaccination antibody level for those who received lot BP2-4 was 7.6 µg/ml. This level rose to 19 µg/ml at two weeks and decreased somewhat to 16 µg/ml at 6-7 weeks. A two-fold or greater increase in antibodies to the outer membrane proteins was induced in 73% of those immunized with lot BP2-WZ-2 and in 59% of those immunized with lot BP2-4.

Thus, both the polysaccharide and protein components of the vaccine were found to be immunogenic in recruits, and although the bactericidal antibody response was quite transient in some individuals, immunity may well be of sufficient duration to protect recruits during basic training. The results obtained in phase I testing of the vaccine in laboratory volunteers suggest that a booster dose of vaccine given at 4 wk is able to significantly increase the duration of the antibody response. The effects of a booster dose in the recruit setting needs to be studied. Additional studies are needed and are underway to determine optimum dose, optimum composition with respect to the relative amounts of each component present in the vaccine, and the basis for the increased reactogenicity as compared to the meningococcal purified polysaccharide vaccines.

Preparation of New Vaccine Lots.

To approach the question of standardization of vaccine composition, five new lots of vaccine were prepared from the same cultures and adjusted to differ systematically in the relative proportions of outer membrane protein and polysaccharide. The proportions of polysaccharide to protein in these lots are: 1:0, 4:1, 1:1, 1:2.5, and 1:9. They were prepared by first isolating the polysaccharide and protein components separately and then re-complexing them in the desire proportions. Testing of these lots will demonstrate the extent to which the complexing of the polysaccharide and protein increases their immunogenicity, and will establish the best composition for the complex.

The basis for the complexing of the polysaccharide and protein appears to be a hydrophobic moiety (probably a fatty acid or containing a fatty acid) attached to one end of the polysaccharide which binds to hydrophoic sites on the outer membrane proteins. We have found that the polysaccharide with the hydrophobic moiety intact binds spontaneously to bovine serum albumin, while polysaccharide which has lost the hydrophobic moiety does not. The linkage of the hydrophobic moiety to the polysaccharide appears to be quite labile, and in most meningococcal cultures a mixture of "binding" and "nonbinding" polysaccharide is present.

A procedure was developed to separate the "binding" and "non-binding" polysaccharide. This procedure, which involves chromatography of the crude polysaccharide on a column of Sepharose CL-2B, was used in the preparation of the polysaccharide for the five new lots that were made. Thus, in these lots all the polysaccharide present has the capacity to bind to proteins. In the lots previously tested (BP2-WZ-2 and BP2-4) only about 60-70% of the polysaccharide was bound to protein. This new procedure should result in a better and more standardizable vaccine.

13. Suggestive evidence has accumulated indicating that human antibodies to group B polysaccharide, although present in over 90% of normal adult sera, may be of poor quality, i.e. low avidity. The following studies were undertaken to develop an improved primary binding assay for antibodies to group B polysaccharide and to compare the avidity of human anti-B polysaccharide antibodies with human anti-C polysaccharide antibodies which are known to be protective.

A modification of the Farr assay was devised using intrinsically labelled, high specific activity capsular polysaccharide from group B or C meningococci. The high specific activity capsular polysaccharide was prepared from small liquid cultures. Briefly, 0.5ml of a 6 hr culture of meningococci in modified Franz medium containing 0.1% glucose was inoculated into 20 ml of β-glycerol-phosphate medium containing 1% casamino acids and 3mCi of ^{3}H-N-Acetylmannoseamine, but no glucose. After overnight growth at 37°C in a shaking water bath the culture was precipitated with 0.1% hexadecyltrimethylammonium bromide and the crude polysaccharide prepared by the usual method of $CaCl_2$ extraction and ethanol fractionation. The crude polysaccharide was separated into "binding" and "non-binding" fractions by chromatography on a column of Sepharose CL-2B equilibrated in 0.02 M Tris-Cl, 0.15M NaCl, pH 7.4. The "binding" polysaccharide elutes first as a polysaccharide-protein complex followed by a second peak of free or "non-binding" polysaccharide. These two peaks were collected separately and subjected to cold phenol-water extraction to remove the protein. The aqueous phase was dialyzed against distilled water, and the specific activity of the polysaccharide determined by dividing the total radioactivity by the total polysaccharide as measured by the thiobarbituric acid assay for sialic acid.

This procedure for intrinsically labelling capsular polysaccharide resulted in specific activities in the range of 400-3000 cpm/ng.

Pool 2 polysaccharide which lacks the hydrophobic moiety which mediates binding to protein has been used in most of our studies with the Farr assay since there is less non-specific binding than with pool 1 or "binding polysaccharide."

These tritium labelled polysaccharide antigens from group B and C meningococci were used in a dissociation rate assay to compare the avidity of antibodies in different human sera.

Tritium labelled group B and C polysaccharides containing an equivalent number of cpm of ^{36}Cl as a volume marker, were incubated with sufficient human antibody to give 40-60% binding of 2 to 10 mg of the ^{3}H-polysaccharide. After incubation overnight at 4°C, the mixture was aliquoted into replicate tubes. A 15-fold excess of unlabelled homologous polysaccharide was added to the appropriate tubes and incubated in an ice bath. At time intervals between 1 and 60 min. saturated ammonium sulfate was added and the tubes spun in a microfuge. Approximately one third of the supernatant was counted for both ^{3}H and ^{36}Cl, and the ^{3}H cpm adjusted to identical volume based on the ^{36}Cl volume marker. The per cent antigen bound was calculated for controls and samples and the per cent dissociation of bound labelled antigen as a function of incubation time with excess unlabelled antigen determined.

A significant difference in avidity between the anti-B antibodies and the anti-C antibodies can be seen in table 14A After 30 min with competitor the anti-group B antibodies are 82% dissociated by the unlabelled polysaccharide as compared to 34% in the group C system. The dissociation is faster and more complete in the B system. The same group B and group C systems were also run in parallel at room temperature (23°C) with similar results. At competitor incubation times less than 60 min the temperature was an important factor in both the group B and group C systems. The amount of labelled group B antigen bound after overnight incubation at 4°C (before addition of unlabelled antigen) was reduced by 31% when subsequently equilibrated to room temperature. By comparison, the binding of anti-C antibody to C polysaccharide was only reduced by 4% when the temperature was raised. This result also indicates that a higher proportion of the binding by anti-group B polysaccharide antibodies is of low avidity.

14. Identification of distinct protein and lipopolysaccharide serotype antigens on group B and C meningococci has allowed serotyping of strains within these serogroups. Such serotyping has provided useful epidemiological information. For example, the protein serotype 2 determinant (which is associated with the 41,000 dalton principal outer membrane protein) has been correlated with epidemic potential of group B and C strains. Group A strains, however, have been reported to be uniform with respect to protein serotype. The pattern of protein bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is identical for all group A strains, whereas on group B and C strains the pattern varies with serotype. We have

recently applied the solid phase radioimmunoassay (SPRIA) inhibition method of serotyping to group A strains and were able to clearly distinguish among group A strains on the basis of both outer membrane protein and lipopolysaccharide antigens. Since group A strains all appear to have the same principal outer membrane protein, the determinant responsible for the observed protein serotype specificity was most likely associated with a different outer membrane protein.

In order to identify the protein(s) with which the serotype determinants were associated, outer membrane complex (OMC) isolated from prototype group A strains was fractionated by dissolving the complex in buffer containing 3% sodium deoxycholate at pH 8.8 and passing the solution over a column of Sephadex G-150 in the presence of 2% sodium deoxycholate. The sample eluted as two protein peaks followed by a peak containing LPS and lipids. The protein peaks were pooled, concentrated by ultrafiltration, and separately rechromatographed on a smaller column of the same kind. The isolated peaks termed fractions I and II were recovered by precipitation and washing with ethanol, assayed, and dissolved in distilled water. Analysis of these two fractions by SDS-PAGE revealed that fraction I consisted mainly of the 45,500 and 37,500 dalton major outer membrane proteins while fraction II consisted mainly of a 31,000 dalton protein. These two fractions along with the intact OMC were analyzed for the presence of the serotype determinant by testing 2 fold dilutions of each for the capacity to inhibit the specific SPRIA serotype reaction. The concentration of each required to inhibit the appropriate reaction 50% (MIC_{50}) was determined and used as a measure of antigenic activity. The results as summarized in table 14B indicate that for all three group A-associated serotypes investigated (P6, P22, and P23) fraction II was between 40 and 330 fold more active than fraction I and several fold more active than the intact OMC. The principal outer membrane protein serotype determinant P2, however, was clearly different in that fraction I was much more active than fraction II. Since fraction II consists principally of a single protein of about 31,000 daltons, it is probable that the serotype determinants are on this protein. The group A serotype proteins appear to represent a second general class of meningococcal serotype proteins. Since P6 is not only found on group A strains but is also present on some group B and C strains, it is clear that this class of serotype proteins is not unique to group A. The epidemiological usefulness of these group A protein serotypes is presently under investigation.

15. The goal of this program has been to develop safe and effective vaccines against Pseudomonas aeruginosa. We have examined the role of extracellular virulence factors such as toxin A and the

newly discovered toxin S in terms of their production by disease producing organisms and their role in human infection. Evidence has accumulated that these two toxins are playing a part in human infection. Toxoids of these two toxins are being developed under collaborative arrangements with Dr. Barbara Iglewski of the University of Oregon.

We have investigated the surface antigens of P. aeruginosa in an attempt to discover which of these antigenic determinants were important vaccine candidates. Our underlying assumption has been that of human opsonic antibody has an important function in defense against these organisms. A surface antigen that could induce opsonic antibody would, therefore, be a good vaccine candidate. The majority of the opsonic antibody found in convalescent sera of patients infected with P. aeruginosa or in pooled gammma globulin of normal people is directed against determinants found in the lipopolysaccharide (LPS) of the infecting strain. These determinants are also found in the LPS of other P. aeruginosa of the same immunotype. Evidence for this idea can be seen in experiments utilizing inhibition of microphagocytosis, a technique we developed specifically for this purpose. Human polymorphonuclear leukocytes (PMN), baby rabbit complement (10 µl), human convalescent sera or gamma globulin (10 µl), and LPS (10 µl) are incubated with P. aeruginosa in a total volume of 100 µl with vigorous shaking for 90 minutes and sampled for bacterial colony forming units at time 0 and 50 minutes. The ratio of PMN to organisms is approximately 1:1 each being 10^7/ml. Data from a representative experiment is shown in table 15. Serum from a patient infected with P. aeruginosa immunotype 5 was used at a dilution of 1:4 (final dilution 1:40) and the γ-globulin at a dilution of 1:16 (final 1:160). These dilutions were determined in previous phagocytosis experiments to be the break point in the titer curves for these sera. This is done so that the inhibition is not overwhelmed by excess antibody. The LPS used as the inhibitor in these experiments was purified by hot phenol-water and shown to have less than 1% protein by the Lowry technique and less than 1% nucleic acid by absorption at OD_{260} in the ultraviolet spectrophotometer. The organism used was the Fisher immunotype 5 prototype. No killing was seen when WBC alone, WBC plus complement, WBC plus serum, or serum plus complement were used. Only WBC plus complement plus serum were able to kill this strain of P. aeruginosa. As can be seen from the table 15 LPS at a final concentration of 50 µgm/ml was able to inhibit all of the opsonic antibody in the patient's serum. At 50 µgm/ml, LPS was able to inhibit 51% of the antibody in the pooled gamma globulin. Those concentrations of LPS are neither anticomplementary nor inhibit non-specifically as demonstrated by experiments using a totally heterologous complement dependent group B streptococcal phagocytosis system. In this system no inhibition was demonstrated

when concentrations of up to 100 µgm/ml final concentration of LPS were used. Thus it appeared that convalescent opsonic antibody is directed against antigenic determinants found in the LPS molecules. Opsonic antibody may also be directed against other non-LPS determinants as suggested by the inability of LPS to inhibit more than 50% of the opsonic antibody in pooled γ-globulin.

We have previously reported the isolation of a non-toxic high molecular weight polysaccharide from P. aeruginosa which induced protective immunity in mice and showed major antigenic determinants with LPS. In order to remove contaminating LPS from this preparation it had been necessary to treat the product with mild acetic acid thereby cleaning the lipid A from the contaminating LPS. The lipid A was removed by chloroform extraction and the low molecular weight O-side separated from the polysaccharide and high molecular weight O-side chain by Sephadex chromatography. High molecular weight, non-toxic, polysaccharides were prepared from Fischer prototype immunotypes 1, 5 and from a patient strain of immunotype 6. These polysaccharides were immunologically active by ouchterlony analysis. None of them were able to inhibit phagocytosis of P. aeruginosa by human convalescent sera, WBC and complement. Concentrations as high as 1000 µgm/ml were attempted. In all of these trials LPS of the proper immunotype was able to inhibit the greater part of the antibody. Thus it appeared that the LPS determinant which humans normally respond to are either not shared with the polysaccharide or are acid labile.

Removal of contaminating LPS from polysaccharide preparations was, therefore, attempted using non-acidic and gentler means. An alcohol precipitated crude preparation of T-5 polysaccharide which had previously been treated with cetavalon to remove nucleic acid was dissolved in 2% deoxycholate, 0.15% $NaPO_4$, 0.5% glycine buffer at pH 8.8 and spun at 20,000 RPM's in a Sorvall centrifuge for 1 hr. There was very little pellet. The supernatant was applied to a Sephadex G100 column equilibrated in the same buffer. The fractions were analyzed for protein and the void volume was pooled. This preparation and the crude alcohol precipitated polysaccharide were able to inhibit 100% of the opsonic antibody in convalescent sera at final concentrations of 50 µgm/ml. Cold phenol extraction of deoxycholate-column purified material removed 95% of the protein but did not alter its ability to inhibit opsonization. However, despite removal of most of the LPS, endotoxin activity by limulus lysate gelatin, KDO analysis and rabbit pyrogenicity (0.75-.5 µgm/kilo) remained. Suspension of the material in 75% alcohol with spinning at 60,000 xg for 1 hr is a technique used for removal of pyrogenicity from meningococcal vaccines. This was attempted and a large pellet was obtained. The supernatant was alcohol precipitated and found to be low in

rabbit pyrogenicity (10-20 µgm/kilo). This product, however, had lost its ability to inhibit opsonization. Thus it appeared that gentle techniques that were successful in removing LPS contamination also removed inhibiting activity in opsonization experiments. It appears likely that the isolated high molecular polysaccharides of P. aeruginosa free of lipid A do not contain the antigenic determinants humans respond to with opsonic antibody when infected with P. aeruginosa. This does not mean that these polysaccharides would not be able to induce opsonic responses to their own dominant determinants and therefore a limited safety and immunogenicity trial with these high molecular weight polysaccharides is planned.

The lipopolysaccharide molecule itself, therefore, appeared to be the leading vaccine candidate. Detoxification of the molecule while maintaining its antigenicity and immunogenicity was the problem. Toxicity of the molecule resides in the lipid A. Most of the lipids are hooked to the glucosamine backbone by alkaline labile ester linkages and one by a very chemically stable amide linkage. We attempted to remove the ester linked fatty acids in as gentle a manner as possible. By using very dry methanol in which 0.25% sodium methoxy was generated by the addition of weighed dry metallic sodium, treatment of LPS at 62°C for 18 hrs accomplished complete removal of the ester linked fatty acids. The alkali lable determinants in the polysaccharide portion of the molecule are probably not subject to nucleophilic attack because they are not in water solution. Inhibition experiments utilizing the sodium methoxy treated LPS are shown in table 16. LPS was immunotype 5 as was the patients infecting strain. The immunotype 5 prototype organism was used in the assay. A group B streptococcal phagocytosis control test was run in the same experiment to demonstrate the specificty of the inhibition. It appeared that sodium methoxy treatment destroyed at most 50% of the active immunologic determinants involved in phagocytosis. This is based on the data that 25 µgm/ml of NaMeOH treated LPS inhibited about the same as 12.5 µgm/ml untreated. Neither NaMeOH treated LPS or untreated LPS were anticomplementery as no inhibition at 50 µgm/ml occurred with either in the heterologous streptococcal system. Removal of the ester linked fatty acids could, therefore, be accomplished wihout destruction of the important immunologic determinants, and at the same time reduced toxicity as measured by limulus lysate gelatin (table 17). Because any vaccine candidate for P. aeruginosa should be tested in the best animal models available that mimic P. aeruginosa disease in man the burned rat model has been set up. This model developed and used extensively at the USAISR has pathologic and clinical correlates of burn wound sepsis. We have been able to duplicate the Fort Sam work demonstrating that live vaccination induces protection against death from P. aeruginosa burn wound sepsis. Thirteen rats were

vaccinated with 10^5 organism I.P. followed by 10^4 7 days later followed by 10^3, 21 days later. Sixteen controls were simultaneously vaccinated with tryptical soy broth - the media the vaccine strains were grown in. The animals were burned 6 weeks following the last vaccination with a 25% boiling water burn and the burn was swabbed with P. aeruginosa (strain #1477). Moribund animals were sacrificed and pure cultures of P. aeruginosa were obtained from their blood and organs. As shown in table 18 all of the controls died while only (8/14) immunized animals died. Immunization prolonged the survival time. The controls all died by the 12th post burn day. Seven of the 8 animals, that died in the vaccinated group, did so on the 12th post burn day or after.

16. The discovery that receptors for pyocins were located in the core structures of lipopolysaccharide (LPS) of core defective mutants of Salmonella minnesota was extended to the other core types found in enterobacteriaceae. The importance of this work is that is further documents the common structures found in heterogeneous LPS's and provides a model for accessibility and inaccessibility of structures on the gram negative cell surface. The core types tested for sensitivity are shown in table 19.

E. coli C (complete LPS core of R1 type), (table 20), E. coli J-5 (defective core R-3 type) (table 22), were sensitive while E. coli K12C600 was resistant, indicating accessible pyocin receptors in some but not all E. coli LPS cores. Several isogenic R mutants derived from wild-type smooth (ie containing complete O-side chains), pyocin resistant E. coli became sensitive to pyocins. This can be seen in Table 21 where the smooth parent E-308 which is totally resistant to pyocins become sensitive to pyocins of group 5 when O-side chain was removed (daughter E-308-9, E308-17 and E308-6 are sensitive to pyocins). The suggestion that O-side chain can block pyocin sensitivity is further supported by the finding that E. coli O111 is resistant to pyocins while its well defined J-5 daughter mutant (Rc) is partially sensitive to pyocins of groups 2 and 5. Likewise, the smooth Shigella flexneri 4 (FH 6) and the Shigella flexneri Ra core mutant FH 17 were resistant to pyocins while the Sh-flexneri 4 (FH 15) Rb core mutant was sensitive to pyocins of groups 2,4,5. (Table 22) This is a pattern similar to the sensitivity of Salmonella core defective mutants SF 1119 (Rc) and SF 1177 (Rb). Cross inhibition with purified core LPS from these strains confirmed the sharing of pyocin receptor areas in these strains.

Four wild type E. coli were determined to have rough LPS structure by sensitivity to core specific phages, and by sizing of their LPS after 1% acetic acid treatment over Sephadex

G-50 columns. These wild type rough E. coli were also shown to contain the K1 antigen by their sensitivity to K1 specific phages and by halo-precipitation with specific goat anti-K1 antibody. These strains were found to be pyocin resistant. Isogenic mutants from two of these, lacking K1 antigen, became sensitive to pyocins. One of these E412 is shown in Table 23. The K1 containing parent is resistant and each of 5 independent isogenic daughter mutants lacking K1 is sensitive to pyocins of group 5. This suggested that K1 could block pyocin accessibility to the underlying core receptors in the LPS of these strains. The K1 antigen is not universally able to block pyocins as is demonstrated by numerous naturally occurring and constructed mutants which contain K1 and are sensitive to pyocins. It may be a quantitative phenomenon as suggested by E182, a rough K1 containing E. coli which was partially sensitive to pyocins as indicated by the very weak zones of killing and low titers of pyocin activity against it. Four of five isogenic daughters lacking K1 became more sensitive to the same pyocins which they only showed weak reactions with before.

Blocking by capsular polysaccharide antigens were further demonstrated by the finding that all meningococci trested thus far have been pyocin resistant. Isogenic mutants lacking capsule and non-typable meningococci (also presumably lacking capsule) were sensitive to pyocins.

17. Fisher and Manning showed that human gamma globulin contains specific antibody to P. aeruginosa. Mouse models have demonstrated the efficacy of gamma globulin against P. aeruginosa and other gram negative organisms. The clinical efficacy of gamma globulins against septicemia in man has not been confirmed. No one has administered gamma globulin in high doses intravenously in a controlled trial because of dangers associated with its I.V. administration. Several new preparations of γ globulin suitable for intravenous use will shortly be available. We, therefore, looked at several of these preparations for primary binding antibody and opsonic antibody against P. aeruginosa. These experiments were performed to lay the foundations for a clinical trial of the new gamma globulins and to determine if such trials was warranted. The solid phase radioimmunoassay was utilized to measure primary binding antibody against P. aeruginosa LPS and a complement dependent microphagocytic assay was utilized to measure opsonic antibody. Killing of P. aeruginosa required the presence of human polymorphonuclear leukocytes (PMN), complement and gamma globulin. Gamma globulin alone, gamma globulin plus complement, gamma globulin plus PMN plus complement did not kill the organism. This is shown in table 24. The gamma globulins

were tested at a series of dilutions for their ability to aid in opsonic killing of P. aeruginosa. The titer was defined as that point on the killing curve where the percent kill of organisms began to sharply decrease. A standard lot of Armour gamma globulin which had previously been shown to be low in activatable enzymes of the kallikrein system and possibly suitable for I.V. use without further modification, was tested against each of the seven immunotypes of P. aeruginosa. As can be seen in table 25 the gamma globulin was opsonic for six of the seven immunotypes. The titers varied from undilute to 1:32 (final dilution in the test 1:320). There was not a good correlation between solid phase binding antibody against lipopolysaccharide and opsonic titer. In the case of immunotype T-3 there was no killing in spite of high levels of antibody (86 micrograms/ml). The levels of antibody by both assays varied depending on immunotypes.

Cutter has manufactured a modified gamma globulin which is in the process of being licensed for intravenous use. This modified product was compared to the unmodified gamma globulin from which it was produced. The normal gamma globulin comes as a 16% solution while the modified is a 5% protein solution. By comparing adjusted titers and binding antibody levels in table 26 it can be seen that modification reduces or eliminates the opsonic titer while having little if any effect on the primary binding antibody against lipopolysaccharide. Armour gamma globulin was tested for opsonic antibody against other gram negative organisms including Shigella, Salmonella, E. coli, Klebsiella and Serratia. The results are shown in table 27. Opsonic antibody was found against Shigella and on one E. coli that did not have K1 capsular antigen. Opsonic levels were undetectable in this assay for Klebsiella, Serratia and the other E. coli thus far tested. Gamma globulin has good levels of opsonic antibody against most P. aeruginosa but not against other common gram negative disease producing organisms.

Gamma globulin does contain binding antibody against lipopolysaccharide from each of the seven immunotypes of P. aeruginosa. It also has antibody against common core determinants found in all gram negative LPS, for example, 3.36 µgm of antibody/ml against the J-5 determinant, 7.84 µgm/ml against Re determinant in Armour lot #S19710. It also contains IgG against toxin A of P. aeruginosa, 8.7 µgm/ml in this Armour lot.

18. A survey of 161 consecutive blood isolates of E. coli revealed that 25% of strains had rough LPS and 20% had K1 capsular polysaccharide; however, 69% of these K1 positive strains occurred on rough organisms. Only 8% of smooth E. coli had K1 polysaccharide versus 56% of smooth strains. All isolates were resistant to serum bactericidal activity (SBA). Isogenic mutants of rough, K-1 positive strains that were selected for loss of K-1 polysaccharide became

sensitive to the SBA of both normal human serum and C-4 deficient guinea pig serum. This implies that K1 capsular polysaccharide protects rough, otherwise serum-sensitive E. coli from SBA. Other capsular polysaccharide besides K1 may be important in this protection. Heating abolishes the SBA of both normal and C-4 deficient guinea pig sera for the rough, K-1 negative mutants. Preliminary data also suggest that antibody is important to this reaction. Rough, K1-negative organisms are readily phagocytized by PMN, whereas smooth LPS and K1 polysaccharide results in no phagocytic kill of E. coli. Addition of antisera raised against Group B meningococcal polysaccharide does not alter these data. Thus lipopolysaccharide and K1 antigen may independently determine serological activity of E. coli.

19. In our study of the role of opsonic antibody in host protection against gram-negative bacteria, we have previously shown that human mononuclear cells exposed to phytohemagglutinin and cultured lymphoid cell lines each release into the supernatant a factor that enhances bacterial killing by polymorphonuclear leukocytes. Preliminary data now suggest that exposure of human mononuclear cells to either SKSD or lipopolysaccharide of Shigella flexneri 2a results in the release of a factor into the supernatant that enhances the killing of a heterologous organism, Serratia marcescens, by polymorphonucear leukocytes. We are now attempting to study the requirements for the induction of this stimulating factor.

20. Contaminated respiratory assistance devices have repeatedly been complicated in epidemic nosocomial pneumonia. A prospective surveillance of nosocomial pneumonia was done to assess the role of contamined respirators in endemic nosocomial pneumonia. Diagnosis of pneumonia was made by established criteria. One hundred seven (0.82%) patients in an eleven-month period had nosocomial pneumonia. Pseudomonas aeruginosa was the most common etiologic agent; surprisingly S. pneumoniae occurred in 10 cases. Contaminated respirators accounted for 6 cases, contaminated tracheal suction solutions for 2 cases and respirometers, 1 case. The mortality from nosocomial pneumonia was 39%. All patients who died of nosocomial pneumonia had serious underlying disease. Contaminated respiratory case devices are a minor cause of endemic nosocomial pneumonia at WRAMC.

21. Isolates of resistant organisms from the clinical microbiology laboratory at WRAMC were obtained since 1975 and saved

in skim milk at -20°C. These isolates were mated with an E. coli K12 recipient in an effort to examine the relative incidence of transmissible plasmids among clinical isolates. Tetracycline, ampicillin and carbenicillin were the most common resistance markers transferred. Despite increasing resistance of these isolates to gentamicin, and despite increasing use of this agent, gentamicin resistance was rarely transferred. Amikacin resistance was never found to be transmissible.

The incidence of resistance to antimicrobial agents of E. coli, Pseudomonas aeruginosa, Proteus mirabilis & Klebsiella pneumonia was compared to the amount of antibiotics used at WRAMC. This was an attempt to determine whether levels of antibiotic usage affects the incidence of resistance. One striking finding was that the incidence of tobramycin resistance increased with gentamicin, but not tobramycin, use. A policy of antibiotics held "in reserve" may be ineffective if all drugs of that class are not included.

22. Specific antibacterial antibody secretion by human lymphocytes. Human peripheral blood lymphocytes (PBL) isolated from normal adults who have been recently immunized with both tetanus toxoid (TT) and meningococcal (Mgc) group C polysaccharide can secrete anti-TT and anti-Mgc IgG, IgM & IgA when cultured in vitro with pokeweed mitogen (PWM). In vitro secretion of these specific antibodies also can occur in the absence of PWM when PBL are obtained 5-9 days following in vivo immunization. We have adapted Zollinger et al's solid phase radioimmunoassay (SPRIA) to investigate the ability of T-cells (isolated using neuraminidase-treated sheep RBC rosettes) to influence anti-TT and anti-Mgc IgG, IgM & IgA secretion by autologous (T-cell depleted) B-cells (see protocol, Fig. 6). Helper and suppressor T-cell influences were differentiated by varying the number of T-cells added and by comparing the effects of irradiated T-cells to those of non-irradiated T-cells. By examining each supernatant for anti-TT and anti-Mgc IgG, IgM & IgA, antigen and Ig class specificity of helper and suppressor T-cell influences could be determined. In the presence of pokeweed mitogen (PWM), antigen-specific IgG, IgM & IgA production by unseparated mononuclear cells (U) was substantially greater than that secreted by either non-adherent mononuclear cells (NA) or T-cell-depleted B-cells (B) -- (representative expts. shown in Fig. 7, graphs A&B). T-cells alone secreted no Ig (not shown).

When non-irradiated T-cells were added to a T:B ratio of 1:2, specific Ig secretion increased only slightly. Irradiated T-cells, however, caused a marked increase in specific Ig secretion when added at this low T:B ratio (Fig. 7, graphs A&B). These data suggest that radiosensitive suppressor T-cells exert a greater influence on specific Ig secretion than radioresistant helper T-cells when both

are present among non-irradiated T-cells. When T-cells were added at a higher T:B ratio (2:1), however, non-irradiated T-cells were more effective than irradiated T-cells in enhancing specific Ig secretion. Moreover, less specific Ig was secreted in the presence of irradiated T-cells when this higher T:B ratio was used as compared to when the T:B ratio was 1:2 (Fig. 2, graphs A&B). These data suggest that there may exist A) radioresistant suppressor T-cells which can exert a greater influence than radioresistant helper T-cells and B) radiosensitive helper T-cells which, when present among non-irradiated T-cells, exert a greater influence on Ig secretion than either suppressor T-cell. The fact that radiosensitive helper T-cell influence and radioresistant suppressor T-cell influence can be demonstrated only when greater numbers of T-cells are used implies that these two cell types are either fewer in number or that they require closer cell-to-cell association in order to be effective. These helper and suppressor T-cell influences were also demonstrable when B-cell populations were able to secrete specific Ig in the absence of PWM (see representative Expt. shown in Fig. 7, graph C).

When cells were obtained one year post-in vivo immunization with TT & Mgc Group C polysaccharide, A) Radiosensitive helper T-cells (as determined using the higher T:B ratio of 2:1) influenced in vitro anti-TT IgG, IgM & IgA secretion but did not influence the secretion of anti-Mgc, IgG, IgM & IgA (Fig. 8 A B & B) radiosensitive suppressor T-cells (as determined using the lower T:B ratio of 1:2) influenced antigen-specific IgG & IgA secretion but did not influence antigen-specific IgM secretion (Fig. 8 C) These data suggested that radiosensitive helper T-cells are antigen specific and that radiosensitive suppressor T-cells are Ig class-specific. In contrast, there was no evidence for antigen of Ig class specificity of radioresistant helper T-cells since this activity was invariably present. 2) Our data suggest that there exist among PBL T-cells which can provide A) radioresistant help, B) radiosensitive suppression, C) radiosensitive help, and D) radioresistant suppression of anti-TT and anti-Mgc Ig secretion. The latter two T-cell influences can be demonstrated only when relatively higher T-cell concentrations are used suggesting that they are either present in fewer numbers or that they require closer cell-to-cell association to be effective. 3) While radioresistant helper T-cell influence is invariably evident, one year post-immunization A) radiosensitive helper T-cells can influence anti-TT Ig secretion but not anti-Mgc Ig secretion suggesting that these helper T-cells are antigen-specific and B) radiosensitive suppressor T-cells can influence anti-TT or anti-Mgc IgG and IgA secretion but not anti-TT or anti-Mgc IgM secretion suggesting that these suppressor T-cells are Ig class specific. 4) Since these T-cell influences have

exhibited antigen and immunoglobulin class specificity, this newly developed assay system ca be used to identify those immunopotentiating agents which can specifically amplify human antibody responses to antigenically relevant bacterial antigens.

23. Identification of immunopotentiating agents which can amplify the production of specific and relevant antibacterial antibodies in man has in the past been hampered by the lack of in vitro methodology in which the effects of promising immunopotentiating agents could be tested on human peripheral blood antibody producing lymphocytes (PBAL). We have shown (1) that following in vivo immunization, the solid phase radioimmunoassay developed by Zollinger et al can be used to measure in vitro secretion by human PBAL of anti-meningococcal (Mgc) & anti-tetanus toxoid (TT) IgG, IgM and IgA. We have also modified this assay in order to demonstrate secretion by PBAL of polyclonal and anti-gonococcal pili (GCP) IgG, IgM and IgA. Our purpose has been to determine whether or not immunopotentiating agents can be identified which can enhance the production of specific antibacterial antibodies in man. To this end, we have tested thymosin (Fraction V) which has been shown to increase the production of anti-sheep erythrocyte antibodies in animals. Thymosin (Fraction V) consists of a family of polypeptides some of which are postulated to act by maturing precursors of T helper (or T suppressor) lymphocytes into actively functioning T cells.

When thymosin (Fraction V) was added in vitro to pokeweed mitogen stimulated cultures of human PBAL obtained from adults immunized with tetanus toxoid, meningococcal group C polysaccharide and/or gonococcal pili vaccines, secretion of IgG, IgM and IgA specifically directed against these antigens was significantly increased (Table 28). That thymosin (Fraction V) is not a B cell mitogen was evidenced by the lack of effect on cultures lacking pokeweed mitogen (data not shown). In contrast to this stimulating effect on antigen-specific antibody production, thymosin (Fraction V) often inhibited secretion of polyclonal antibodies (Table 29).

This contrasting effect (increased specific antibody production accompanied by decreased polyclonal secretion) suggests that 1) the T-cells which govern antigen-specific antibody production are different from those which control total antibody production and 2) different polypeptide components of thymosin (Fraction V) may have opposing effects on human cells depending on which precursor T cells are present. The clinical implication of these results is that administration of thymosin (Fraction V)

in vivo can enhance specific antibody secretion against relevant bacterial antigens while not stimulating polyclonal antibody production. This would have in vivo significance since stimulation of polyclonal antibody production might result in potentiation of auto-antibody formation with accompanying immune complex-generated side effects which could be disadvantageous to thymosin's clinical use in vivo. Since thymosin (Fraction V) is currently being given to certain patients, this new use suggested by our data (viz. enhancement of specific antibacterial antibody production) can be tested. Military personnel may benefit from this type of immune enhancement by having their antibody production against various bacteria (or, perhaps, viruses or parasites) boosted by administration of one immunopotentiating agent prior to their deployment in endemic areas of infection.

24. Pneumococcal Vaccination Against Group B Streptococci.

Over the past several years, group B type III streptococci (GBS III) disease, has emerged as a major (and, in some areas, the major) cause of neonatal sepsis and meningitis and has been associated with a high degree of morbidity and mortality. It has been demonstrated that a deficiency of type-specific antibody in maternal and cord sera is a major factor predisposing infants to invasive group B streptococcal disease. Based on the fortuitous observation that antisera directed against type 14 pneumococci immunoprecipitates with the hot HCl-extracted polysaccharide antigen of group B type III streptococci, we have recently reported that rabbit antisera directed against type 14 pneumococci (P14) not only promotes in vitro opsonophagocytic bactericidal activity against GBS III but also protects suckling rats against GBS III sepsis and death.

In the present study we prospectively immunized adult human volunteers with the commercially available polyvalent pneumococcal vaccine (Pneumovax) which contains the capsular polysaccharide of P14. We investigated whether or not this pneumococcal immunization caused 1) a rise in titer of serum antibodies which are opsonic for GBS III in vitro and 2) significantly enhanced in vivo protection against GBS III sepsis in the suckling rat model of neonatal streptococcal disease.

In this study, we used the previously reported microtiter adaptation of a neutrophile-mediated opsonophagocytic bactericidal assay which we modified to increase the sensitivity of the assay by decreasing the number of target bacteria and increasing the concentration of serum in the assay. We used this modified assay in order to identify those individuals which were most deficient in anti-GBS III antibodies (and, therefore, most

susceptible). We screened sera of 25 healthy adults and found that 32% had titres of <1/2.5 while 68% had titres equal to or greater than 1/5 against GBS III.

We immunized five individuals with anti-GBSS III serum titres less than 1/2.5, and three individuals whose titres were 1/2.5, 1/5 & 1/10, respectively. When sera from these individuals were tested for anti-GBSS III opsonic activity 6-8 weeks post-immunization with pneumovax, it was found that their titres had risen to between 1/40 and 1/150. (Figure 9). As expected, when these pre & post sera were tested against type 14 pnuemococci, titres rose between 4 and 6 two-fold dilutions thus demonstrating that each of the individuals had adequately responded to the pneumococcal vaccine (not shown). Figure 10 shows growth curves of two strains of GBS III and one strain of P14 using pre-immunization sera and two dilutions of six-eight week post-immunization sera in the neutrophile-mediated opsonophagocytic bactericidal assay. This confirms our previous findings (using rabbit antisera) that anti-P14 antibodies are opsonic for many strains of GBC III (6) and demonstrated that anti-GBS III and anti-P14 opsonic antibodies could persist in the sera of Pneumovax-immunized individuals for nine months (Fig 11A). We further demonstrated that the anti-GBS III opsonic activity in human post immunization sera was inhibited by small amounts (0.03 mcg/ml) of purified P14 polysaccharide (Fig 11B). Polysaccharides purified from type 5 pneumococci, group C meningococci or pseudomonas did not significantly inhibit even when using one hundred fold more antigen in the assay.

In vivo studies using a suckling rat model of neonatal GBS III disease demonstrated that human post-immunization sera afford significantly enhanced protection compared to pre-immunization sera (Fig. 12).

Our results show that immunization of susceptible adults with Pneumovax would generate antibodies which are opsonophagocytic in vitro and protective in vivo against GBS III. Our data strongly suggest that Pneumovax immunization of women who are deficient in anti-GBS III opsonic antibodies may help protect their infants against GBS III sepsis.

Table 1

Serum Antibody Response (SPRIA) to P-3-2 Vaccine

Anti Pili Antibody (IgG) µg/ml

Vol	PRE	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
TRA	.54	15.4	133.	55.0	62.0	50.2	51.2
FOR	.23	.8	3.9	4.8	4.8	12.0	12.2
BOS	.38	2.9	5.4			31.3	26.6
LEM	.60	1.5		2.4	4.5	13.6	11.0
GEM	.33	1.5	11.8	12.6	9.6	18.5	16.0
OAK	.33	10.2	26.2	24.5	20.6	23.2	
GRO	.40	7.4	11.3	15.6	16.8	20.0	24.5
SAD	.49	10.9	26.0	19.5	18.7	18.2	18.2
CRO	.73	34.7	37.0	29.0	23.0	22.8	40.0

Table 2

Serum Antibody Response (SPRIA) to P-3-2 Pilus Vaccine

Anti Pili Antibody (IgA) µg/ml

Vol	PRE	1 wk	2 wk	3 wk	4 wk	wk	6 wk
TRA	.19	6.6	37.9	12.5	9.8	8.1	8.1
FOR	1.0	5.1	13.0	9.3	6.0	6.5	7.4
BOS	.27	1.2	7.5			10.8	5.7
LEM	.19	2.2		7.5	4.2	12.1	7.8
GEM	.6	2.5	13.3	9.7	4.2	5.4	4.0
OAK	.29	19.3	56.0	37.7	25.3	22.6	
GRO	.39	37.1	23.2	22.3	16.1	12.9	12.5
SAD	.28	2.6	4.9	3.6	2.8	2.9	2.2
CRO	.37	13.5	12.4	8.0	4.9	6.4	6.1

1 Volunteers were vaccinated at 0 week and boosted at 4 weeks.

2 TRA & FOR received 2 mg vaccine. All others 1 mg vaccine.

Table 3

Serum Antibody Response (SPRIA) to P-3-2 Pili Vaccine

Anti Pili Antibody (IgM) µg/ml

Vol	PRE	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk
TRA	1.8	25.6	59.0	25.6	21.8	13.0	11.9
FOR	1.1	16.3	33.1	26.1	17.3	14.1	13.7
BOS	2.1	28.9	20.1			13.6	11.4
LEM	1.2	11.5		10.2	12.8	10.6	9.6
GEM	1.8	5.7	18.1	18.0	13.0	12.3	12.7
OAK	1.5	10.9	12.1	13.2	7.5	8.6	
GRO	2.6	193.	84.2	88.4	62.6	64.5	500
SAD	2.1	20.8	23.7	178	17.6	16.1	17.9
CRO	5.0	149.	78.1	53.6	33.1	32.3	33.8

Table 4

Opsonic Titers of Sera from Volunteers Vaccinated with Pili

Serum	Titer	% organisms killed at end point
pre 1011	64	73
post 1056	1024	67
pre 1010	8	50
post 1070	64	67
pre 1026	32	51.2
post 1036	128	85.3
pre 1013	32	58.6
post 1057	32	64.8
pre 1012	32	88.6
post 1055	128	70.9
pre 1015	4	84.1
post 1039	256	79.8
pre 1006	16	62
post 1051	256	69
pre 1001	2	70.6
post 1034	64	58.3

Table 5

Inhibition of Attachment by Vaginal Secretions

Absorbed with Homologous and Heterologous Pili

	Strains		
Vag. sec. 418	418	135	149
Unabsorbed	1:32	1:4	1:32
abs. with 418 pili	0	0	0
abs. with 135 pili	1:8	0	0
abs. with 149 pili	1:1	0	0

Table 6

Comparison of Mean Percentages of

Attachment and Number of Bacteria Attached/Cell

Cell Line	Percentage of Bacterial Attachment	Bacteria Attached per Cell
	P3-2	
BHK-21	1.47[a]	9.2[a]
Hep-2	2.18[b]	9.2[b]
Vero	13.6 [b]	9.7[b]
Henle	2.27[b]	7.8[b]
HeLa	5.39[c]	17.3[c]
Foreskin	1.26[c]	2.6[c]
Tonsil	2.78[c]	7.9[c]
	CG418	
BHK-21	3.54[d]	6.7[d]
Hep-2	3.70[b]	6.6[b]
Vero	1.89[e]	4.4[e]
Henle	2.19[b]	2.8[b]
HeLa	1.28[c]	9.8[c]
Foreskin	7.86[c]	34.5[c]
Tonsil	14.58[c]	15.8[c]

Table 6. Continued Comparison of Mean Percentages of Attachment and Number of Bacteria Attached/Cell

[a] The mean is derived from 7 individual tests run at two separate times.

[b] The mean is derived from 10 individual tests run at two separate times.

[c] The mean is derived from 5 individual tests run at one time.

[d] The mean is derived from 8 individual tests run at two separate times.

[e] The mean is derived from 9 individual tests run at two separate times.

All cell lines were counted using a hemocytometer and a bacterial suspension was made in M199 + 2% w/v BSA. The bacteria and cells were combined in a 50:1 ratio and incubated for 30 minutes at 37°C. Dilutions were made of the mixture of cells and bacteria and were then counted by the bacterial plate counting method.

Table 7

Mean Percentages of Attachment

and Number of Bacteria Attached per Cell

	Cell Line	Percentage of Bacterial Attachment	Bacteria Attached per Cell
		P3-2	
	Flow 1000	35.5[a,b]	103[a,b]
	HeLa	19.7	18
	BHK	3.7	3
P3-2	Hep-2	13.3	11
	Vero	6.5	6
	Henle	6.4	5
	Flow 2000	ND	ND
	Tonsil	ND	ND
	Foreskin	ND	ND
		418	
	Flow 1000	19.2[a]	18[a]
	HeLa	17.1	5
	BHK	3.5	1
	Hep-2	10.5	2.9
	Vero	8.3	2

Table 7 (cont)

Henle	6	1
Flow 2000	13.7	3.1
Tonsil	ND	ND
Foreskin	8	2

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a
The mean is derived from 5 individual tests

b
All cell lines were counted using a hemocytometer. A bacterial cell suspension was made in M199 + 2% BSA. The percentage of attachment and the bacteria attached per cell were determined from the amount of radioactivity associated with each cell line.

Table 8

Reactogenicity of Minor Serogroup Polysaccharide Vaccines

Vaccine	Nr	$\bar{X}$ score	Score of 4	Eryth.	Indur.	Minor Sys.	Major Sys.
Y	10	0.33	0	0	0	0	0
29E	10	1.9	1	2	0	1	0
Y-W135	10	2.1	0	4	1	0	0
W135	10	2.0	2	4	4	2	1

Table 9

Immunologic Response of Volunteers Vaccinated with Group Y Group W135 or a Combined Y-W135 Vaccine

Antigen:	Y					W135				
Assay:	Binding Ab		Lytic Ab			Binding Ab		Lytic Ab		
Week:	4	26	0	4	26	4	26	0	4	26
Y Vaccine (N:9)										
% Responding*	100	100	-	100	89	78	67	-	71	57
% Susceptible+	-	-	67	0	0	-	-	67	22	22
W135 Vaccine (N:10)										
% Responding	60	43	-	30	25	100	100	-	90	80
% Susceptible	-	-	30	0	0	-	-	40	0	0
Y-W135 Vaccine (N:10)										
% Responding	100	100	-	100	90	100	-		80	80
% Susceptible	-	-	20	0	0	-	-	50	10	10

* Percent with $\geq$ 10 ngm rise in ABC between pre-vaccination sera and indicated post-vaccination sera, for binding Ab, and percent with $\geq$ 2 $\log_2$ increase in serum bactericidal activity, for lytic Ab.

+ Percent with a titre of bactericidal activity < 1 $\log_2$.

Table 10

Bactericidal Antibody Response, at 4 wks, to Varying Doses of Divalent Group Y-W135 Capsular Polysaccharide Vaccine. For Each Category, the Per Cent of Volunteers who Responded with $\geq$ 2 $\log_2$ Dilutions of Bactericidal Antibody Against Each Group, at Each Dose, are Given. Number in Parentheses is the Number of Volunteers in Each Category for Each Serogroup.

Category:		Total		Susceptibles*		Low pre-existing Ab*	
Serogroup:		Y	W135	Y	W135	Y	W135
Dose:	10 mcgm	76.9 (26)	96.2 (26)	100 (10)	100 (16)	100 (12)	100 (20)
	25 mcgm	84.0 (25)	83.3 (24)	100 (8)	100 (13)	100 (10)	100 (16)
	25 mcgm CV	88.5 (26)	88.0 (25)	100 (8)	100 (10)	100 (11)	100 (12)
	50 mcgm	84.4 (32)	90.3 (31)	100 (5)	100 (10)	100 (8)	100 (18)
	100 mcgm	87.5 (24)	87.5 (24)	100 (6)	100 (10)	100 (11)	100 (13)

* Susceptible: titre $\leq$ 1 $\log_2$ in pre-vaccination serum; Low existing Ab: titre $\leq$ 4 $\log_2$ in pre-vaccination serum.

Table 11

Chemical Characterization of Capsular Polysaccharide of Strain 8021

% "Dry" Weight[1]	Lot Nr 1	Lot Nr 2
Galactose	N.D.	19.0
Glucose	N.D.	7.1
Sialic Acid	N.D.	47.7
Total Carbohydrate		73.9
Moles % of total carbohydrate residues detected[2]		
Hexose	49.8	48.5
Sialic Acid	50.2	51.5
Galactose	35.1	35.3
Glucose	19.7	13.2
Galactose/glucose	2.38	2.65

[1]Determined by quantitative gas-liquid chromatography, using mannitol as the internal standard, for Lot 2, only. ND: not determined.

[2]Determined by qualitative gas-liquid chromatography.

Table 12

Reactogenicity of Meningococcal Group B Vaccine Lots BP2-WZ-2 and BP2-4

Vaccine lot	Number vaccinated	Number with			
		Erythema (mean diameter)	Induration (mean diameter)	Sore Arm	Mild systemic effects*
BP2-WZ-2	40	18 (15 mm)	0	3	2
BP2-4	41	25 (17.8 mm)	8 (19.4 mm)	13	2
Placebo	60	10 (13.7 mm)	4 (12.7 mm)	1	5‡

* Headache and/or fever

‡ Ten of those who received the placebo and none who received the vaccine were females. Four of the systemic complaints were from females.

Table 13

Bactericidal Antibody Response of Army Recruits to Meningococcal Group B Vaccine

Vaccine Lot (recruits)	Specificity of Bactericidal antibody	Geometric mean reciprocal bactericidal titer at indicated time			
		0 wk	2 wk	4-5 wk	6-7 wk
BP-WZ-2	Total	13	220	64	45
(35)	B polysaccharide	20	194	64	52
	Serotype protein	<2	4.4	3.3	2.7
BP2-4	Total	21	126	71	44
(41)	B polysaccharide	12	101	57	37
	Serotype protein	ND*	ND	ND	ND

* Not determined.

Table 14

Bactericidal Antibody Response of Recruits who were Initially Seronegative

Vaccine Lot	Specificity of bactericidal assay	Number sero-negative* at indicated time			
		0 wk	2 wk	4-5 wk	6-7 wk
BP-WZ-2	Total	14	0**	1	3
	B polysaccharide	11	0**	3	4
	Serotype protein	28	7**	9	14
BP-2-4	Total	7	1	2	3
	B polysaccharide	9	1	1	3
	Serotype protein	ND	ND	ND	ND

* Bactericidal titer less than 1:2.
** Two-week sera were not available from about 25% of the individuals.

Table 14A

Inhibition of specific SPRIA serotype reactions by two protein-subfractions of OMC

SPRIA serotype	Prototype strain	MIC_{50} of indicated homologous antigen			
		OMC	Fraction I	Fraction II	I/II
P23	106(A)	5*	40	0.75	67
P22	7889(A)	1	5	0.12	42
P6	32I(C)	1.5	100	0.3	330
P2	99M(B)	1.5	5	>100	<0.05

* µg protein/ml required for 50% inhibition.

Table 14B

Dissociation rate of human anti-meningococcal polysaccharide antibodies at 4°C

Antigen-antibody system	Percentage of bound antigen dissociated after indicated number of minutes*				
	0	1	2	10	30
Group B	0	24	28	53	82
Group C	0	4	4	13	34

* A 150-fold excess of unlabelled homologous polysaccharide was added at time 0.

Table 15

Inhibition of Opsonic Antibody in Human Convalescent Sera and ɣ-globulin by LPS.

Sera	Inhibitor	Concentration µgm/ml	% kill at 90 min	% inhibition
Convalescent	None		99.999	
"	LPS	50	0	100
"	"	25	0	100
"	"	12.5	40.96	40.9
"	"	6.25	94.57	5.4
"	"	3.00	99.999	0
ɣ-globulin	None		97.26	
"	LPS	50	47.4	51.2
"	"	25	66.3	31.8
"	"	12.5	70.2	27.8
"	"	6.25	78.3	19.4
"	"	3.1	83.8	13.8

* $$\% \text{ inhibition} = 100 - \frac{\% \text{ kill of inhibited reaction}}{\% \text{ kill of uninhibited reaction}}$$

Table 16

Inhibition of Opsonic Antibody in Human Convalescent Sera by Sodium Methoxy Treated Lipopolysaccharide+

Sera	Organism	Inhibitor	Concentration µgm/ml (final)	% kill	% Inhibition
Convalescent	Ps-T5	None		99.5	0
"	"	MeOH LPS+	50	0	100
"	"	"	25	30	69.7
"	"	"	12.5	92	6.8
"	"	"	6.2	96.8	2.6
"	"	"	3.1	99.5	0
"	"	LPS	25	0	100
"	"	"	12.5	41	41
Rabbit	Group B strep	None		100	0
"	"	MeOH LPS	50	99.8	.20
"	"	LPS	50	99.4	.6

MeOH LPS - Immunotype 5 LPS treated with 0.25 sodium methoxy for 18 hr at 62°C.

Table 17

Limulus Gelation by Pseudomonas Lipopolysaccharide

Preparation	Concentration in ng/ml								
	10^5	10^4	10^3	10^2	10	1	0.5	0.75	0.175
T-5 LPS	+[6]	+	+	+	‡	-	-	-	-
NaMeoH[+] T-5 LPS	+	‡	-	-	-	-	-	-	-
Water (Control)	-	-	-						
E. coli Reference							+	+	‡

[+] Mean gelatin of limulus lysate

NaMeoH - Sodium methoxy treated LPS

Table 18

Protection of Burned Rats from Death Due to P. aeruginosa Burn Wound Sepsis

	Animals	Days Following Burn								Total Dead
		5	7	8	9	12	14	15	20	
Vaccinated	14			1*		2	1	2	2	8/14
Controls	16	2	2	4	6	2				16/16

*Number of animals dead from P. aeruginosa on given post burn day.

Table 19

Core Types in Enterobacteriaceae Tested for Pyocin Activity

Ra	R1	R2	R3	R4
Salmonella minnesota core mutant	*E. coli* C	Not Tested	*E. coli* 0111(J-5)	*E. coli* K12*
	S. sonnei phase II		*Sh.* flexneri 4 core (Ra) core mutant (Rb) core mutant	

*Resistant to all pyocins tested

Table 20

Pyocin Sensitivity of R1 Core Type Organisms

Strain	Pyocin Type KR-1 K L M N O P R S T	KR-2 D F J	KR-2 I	KR-4 H	A B C E G	KR-6 W	Pyocin Group
E. coli C	0 0 0 0 0 0 0 0 0	± ±)	0	0	+ + + + 0	0	2,5
S. sonnei Phase II	0 0 0 0 0 0 0 0 0	0 + 0	0	±	+ + + + ±	0	2,5
S. sonnei Phase I	0 0 0 0 0 0 0 0 0	0 0 0	0	0	0 0 0 0 0	0	resistant

Table 21

Pyocin Sensitivity of Rough Mutants from a Smooth $K1^+$ E. coli

Strain	LPS	K^+	Pyocin Type: KR-1 K	L	M	N	O	P	R	S	T	KR-2 D	F	J	KR-3 I	KR-4 H	KR-5 A	B	C	E	G	KR-6 W	Pyocin Group
Parent E-308	Sm	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	resistant
E-308-9	Rf	+	0	0	0	0	0	0	0	0	0	0	±	0	0	0	±	+	+	+	±	0	5
E-308-17	Rf	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+	+	+	±	0	5
E-308-6	Rf	+	0	0	0	0	0	0	0	0	0	0	±	0	0	0	±	+	+	±	0	0	5
E-308-7	Rf	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	resistant
E-308-11	Rf	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	resistant

Table 22

Pyocin Sensitivity of R3 Core Types

Strain	Pyocin Type: KR-1 K	L	M	N	O	P	R	S	T	KR-2 D	F	J	KR-3 I	KR-4 H	KR-5 A	B	C	E	G	KR-6 W	Pyocin Group
E. coli 0111 parent	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	resistant
E. coli 0111 (J-5) Rc mutant	0	0	0	0	0	0	0	0	0	±	0	±	0	0	±	±	±	±	0	0	2,5
Sh. flexneri 4 (FH6) Smooth	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	resistant
Sh. flexneri 4 (FH17) (Ra) core mutant	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	resistant
Sh. flexneri 4 (FH15) (Rb) core mutant	0	0	0	0	0	0	0	0	0	+	+	+	0	+	+	+	+	+	0	0	2,4,5

Table 23 Masking of Pyocin Sensitivity by K1 Antigen in Rough E. coli

Strain	LPS*	K1**	KR-1 K L M N O P R S T	KR-2 D F J	KR-3 I	KR-4 H	KR-5 A B C E G	KR-6 W	Pyocin Group
E412 parent	R	+	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	0 0 0 0 0	0	resistant
E412^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	+ + + + 0	0	5
E412B^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	+ + + + 0	0	5
E412C^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	+ + + + 0	0	5
E412D^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	0 ± ± ± 0	0	5
E412E^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	± + + ± 0	0	5
E182 parent	R	+	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	± ± ± ± 0	0	5
E182A^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	± + + + 0	0	5
E182B^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 ± 0	0	0	+ + + + ±	0	5
E182C^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	± ± ± + 0	0	5
E182D^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	+ + + + ±	0	5
E182E^{R}	R	-	0 0 0 0 0 0 0 0 0 0	0 0 0	0	0	+ + + + ±	0	5

* R - Rough LPS as shown by sensitivity of strain to rough specific phages.
**Presence of K1 shown by halo precipitation plates and sensitivity to K1 specific phages.

Table 24

Phagocytosis utilizing gamma globulin

Contents of well	CFU at 0 minutes	CFU 90 min	% kill or growth
gamma globulin***, C'	2.38×10^6	7.14×10^6	200% growth
PMN, C'	3.50×10^6	3.74×10^6	6.8% growth
PMN, ** gamma glob	3.88×10^6	3.18×10^6	18% kill
PMN, glob, C'	3.64×10^6	1.48×10^5	95.9% kill

*C' is complement (10 µl) from infant bunnies

**PMN - isolated Polymorphonuclear leukocytes

***Cutter 3-2163-35 non-modified gamma globulin

Table 25

ɣ-globulin against 7 immunotypes of P. aeruginosa

	T-1	T-2	T-3	T-4	T-5	T-6	T-7
Titer	Un	1:4	NK*	1:16	1:32	1:4	1:4
% kill at titer	94.8	90.7	-	93.6	91.1	91.1	89.6
Anti LPS IgG µgm/ml**	13.58	89.1	86.8	199	30.4	63.5	33

*NK, no kill

**Antibody measured using anti IgG in a solid phase radioimmunoassay

Table 26

Cutter normal and modified gamma globulin against the seven immunotypes of Pseudomonas aeruginosa

	T-1	T-2	T-3	T-4	T-5	T-6	T-5
				Opsonic Titer			
Normal gamma globulin	1:2	1:8	NK*	1:32	1:64	1:4	1:8
Corrected to 5%	Un	1:2	NK	1:10	1:30	Un	1:2
Modified gamma globulin	NIC	1:4	NIC	1:4	NIC	NIC	NK
				**Anti Lipopolysaccharide (IgG)			
Normal gamma globulin	76.3	82.3	96.7	232	34	78	84
Corrected to 5%	23.8	25.8	30.2	72.5	10.6	24	26
Modified gamma globulin	19.8	23.2	26.2	30.5	8.1	10.3	19

*NK, no kill
**Ig against LPS measured in a solid phase radioimmunoassay in micrograms/ml

Table 27

Opsonic antibody in gamma globulin against gram negative organism

	Characteristics of Strain	Titer
Shigella M4243		1:2
Shigella 2457T	Rf344	1:2
Salmonella T1627		NK*
E. coli 537	$K1^{+**}$ Smooth LPS***	NK
E. coli 646	$K1^{+}$ Rough LPS	NK
E. coli 540	K1 - Smooth LPS	1:2
Serratia E311		NK
Klebsiella 659		NK

* NK; no kill

** $K1^{+}$,K1 capsular antigen or not

*** Smooth LPS; contains complete O side chains in the lipopolysaccharide

Table 28

Stimulation of Specific Antibody Secretion Using Thymosin Fr V

Exp #	Ig	Antigen*	Thymosin (mcg/ml)	Ab secreted (ng/ml) Thymosin present	Thymosin absent
1	IgG	TT	0.1	16.0 ± 0.8	2.9 ± 0.2
2	"	"	100	5.5 ± 0.1	3.8 ± 1.0
3	"	Mgc	0.1	4.2 ± 0.3	1.2 ± 0.4
4	"	"	100	14.2 ± 1.3	2.5 ± 0.6
5	IgM	TT	1	21.6 ± 6.9	5.1 ± 0.2
6	"	"	0.1	1.2 ± 0.2	0
7	"	Mgc	100	8.7 ± 2.0	2.9 ± 1.3
8	"	"	100	14.2 ± 1.3	2.5 ± 0.6
9	"	GCP	100	179.7 ± 4.5	95.9 ± 22.3
10	"	"	0.1	164.9 ± 18.7	112.3 ± 8.4
11	IgA	TT	100	3.4 ± 0.5	0
12	"	"	100	2.9 ± 1.1	0
13	"	"	0.01	1.2 ± 0.2	0
14	"	Mgc	0.1	1.9 ± 0.7	0
15	"	"	0.1	3.9 ± 0.3	1.4 ± 0.2
16	IgA	GCP	1	82.0 ± 3.8	54.4 ± 7.1
17	"	"	100	235.2 ± 12.5	180.5 ± 19.7

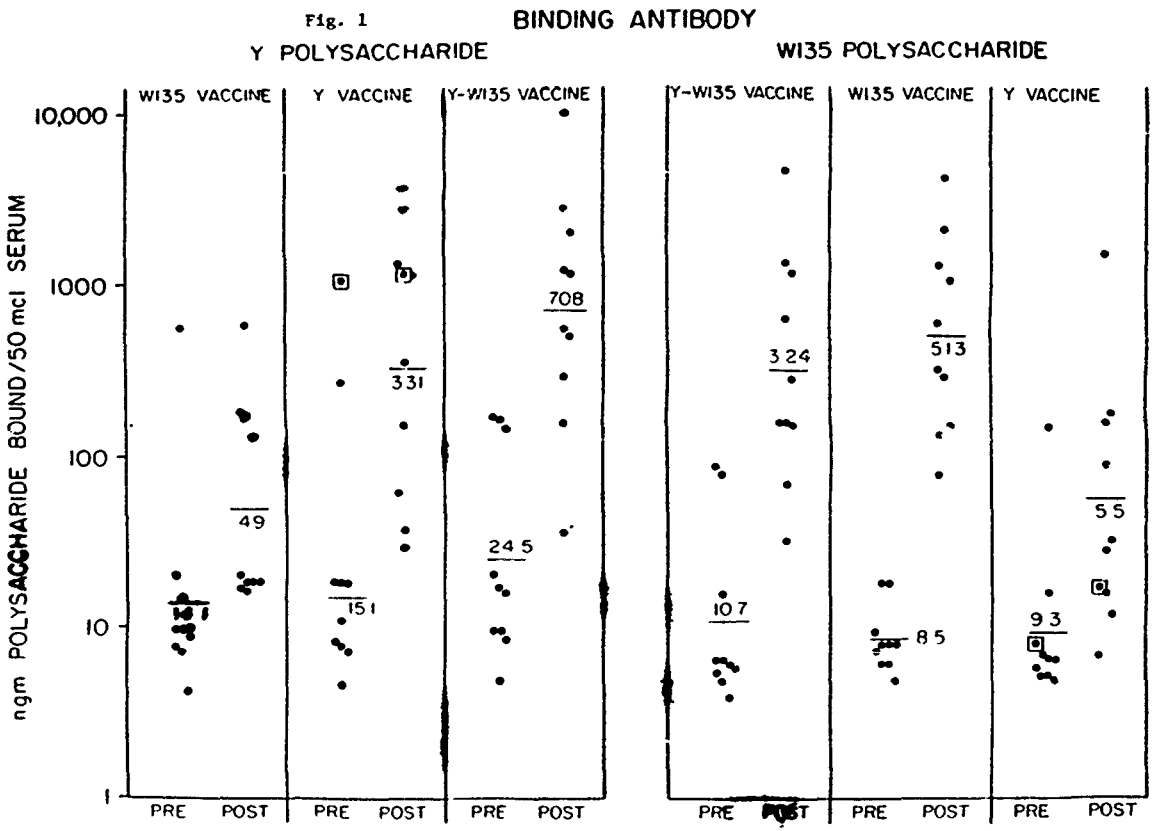
* Antigens against which specific antibodies were directed are abbreviated as follows: TT, tetanus toxoid; Mgc, meningococcal group C polysaccharide (partially purified); Gcp, gonococcal pili.

Table 29

Suppression of Polyclonal Antibody Secretion by Thymosin Fr V

Exp. #	Ig	Fr V (mcg/ml)	Ab secreted (ng/ml)
1	IgG	-	799 ± 14
		0.01	0
2	"	-	436 ± 139
		10.0	79 ± 92
3	"	-	794 ± 11
		100.0	618 ± 63
4	IgA	-	116 ± 6
		0.1	75 ± 9
5	"	-	1165 ± 25
		10.0	890 ± 43
6	IgM	-	389 ± 71
		0.01	0
7	"	-	1738 ± 274
		1.0	1157 ± 23
8	"	-	433 ± 4
		100.0	79 ± 1
9	"	10.0	2951 ± 338
10	"	-	3859 ± 157
		100.0	3456 ± 71

Fig. 1


BINDING ANTIBODY
Y POLYSACCHARIDE
WI35 POLYSACCHARIDE
WI35 VACCINE
Y VACCINE
Y-WI35 VACCINE
Y-WI35 VACCINE
WI35 VACCINE
Y VACCINE
ngm POLYSACCHARIDE BOUND/50 mcl SERUM
10,000
1000
100
10
1
PRE
POST
49
151
331
245
708
107
324
85
513
93
55

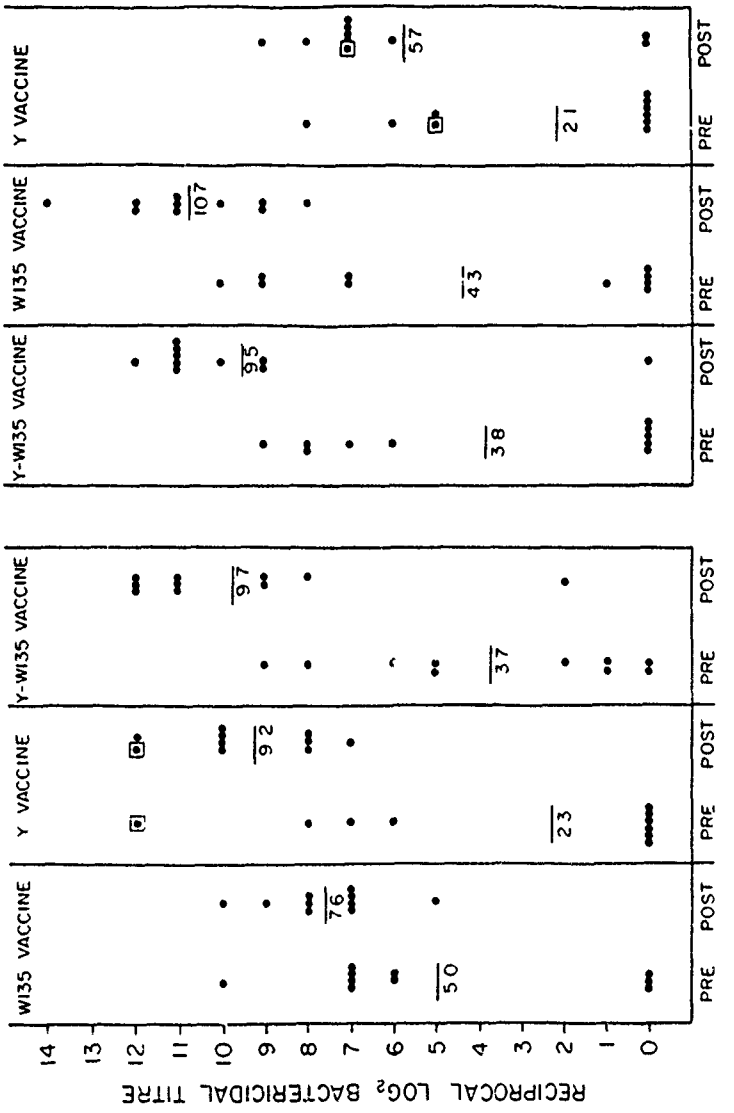

BACTERICIDAL ANTIBODY
Y STRAIN
W135 STRAIN
W135 VACCINE
Y VACCINE
Y-W135 VACCINE
PRE
POST
RECIPROCAL LOG2 BACTERICIDAL TITRE
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
50
76
23
92
37
97
38
95
43
107
21
57

Fig. 2

Fig. 3

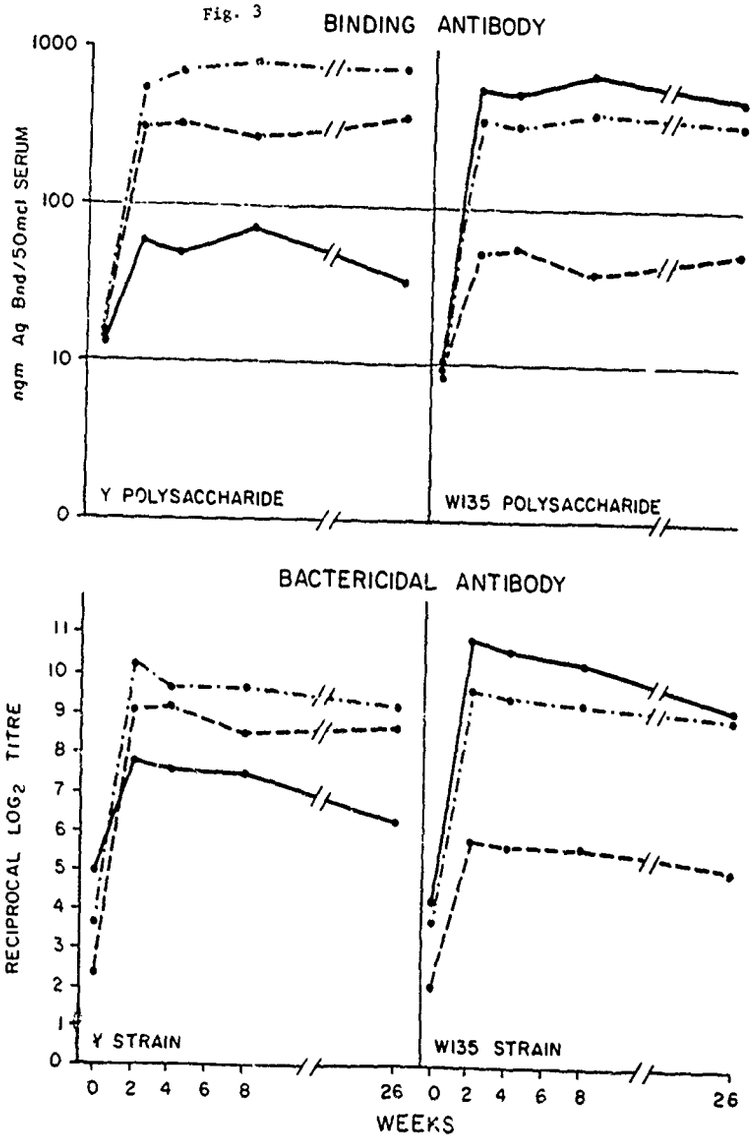

BINDING ANTIBODY
1000
100
10
0
ngm Ag Bnd/50mcl SERUM
Y POLYSACCHARIDE
W135 POLYSACCHARIDE
BACTERICIDAL ANTIBODY
RECIPROCAL LOG2 TITRE
Y STRAIN
W135 STRAIN
0 2 4 6 8 26 0 2 4 6 8 26
WEEKS

Fig. 4

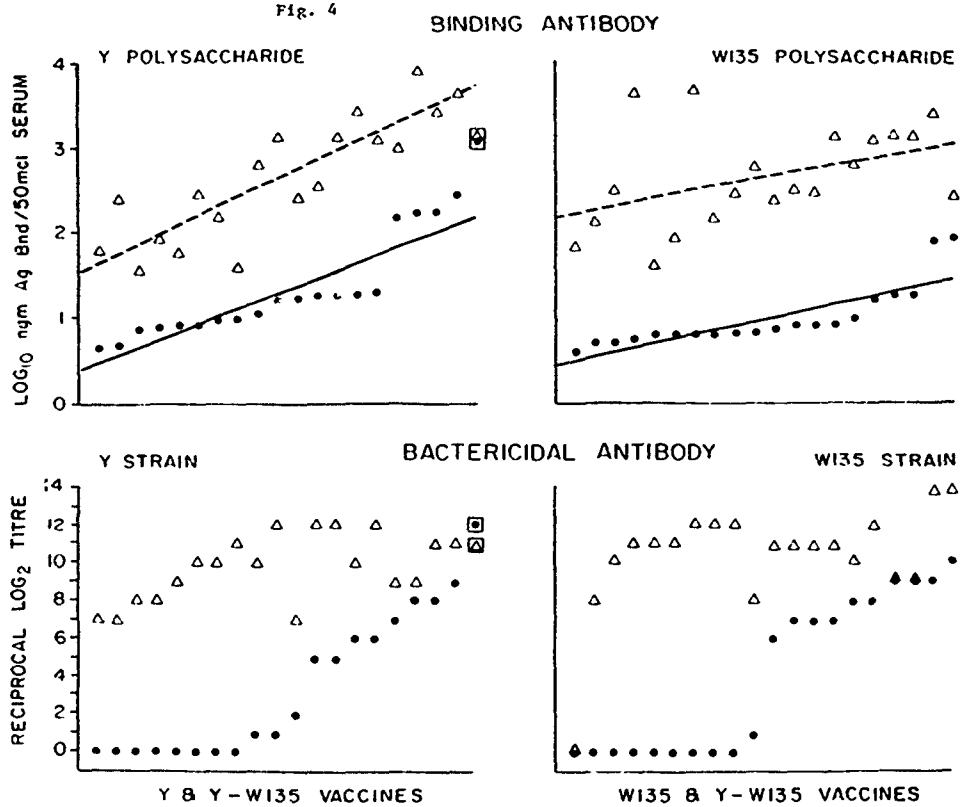

BINDING ANTIBODY
Y POLYSACCHARIDE
W135 POLYSACCHARIDE
LOG10 ngm Ag Bnd/50mcl SERUM
0
1
2
3
4
BACTERICIDAL ANTIBODY
Y STRAIN
W135 STRAIN
RECIPROCAL LOG2 TITRE
0
2
4
6
8
10
12
14
Y & Y-W135 VACCINES
W135 & Y-W135 VACCINES

Fig. 5

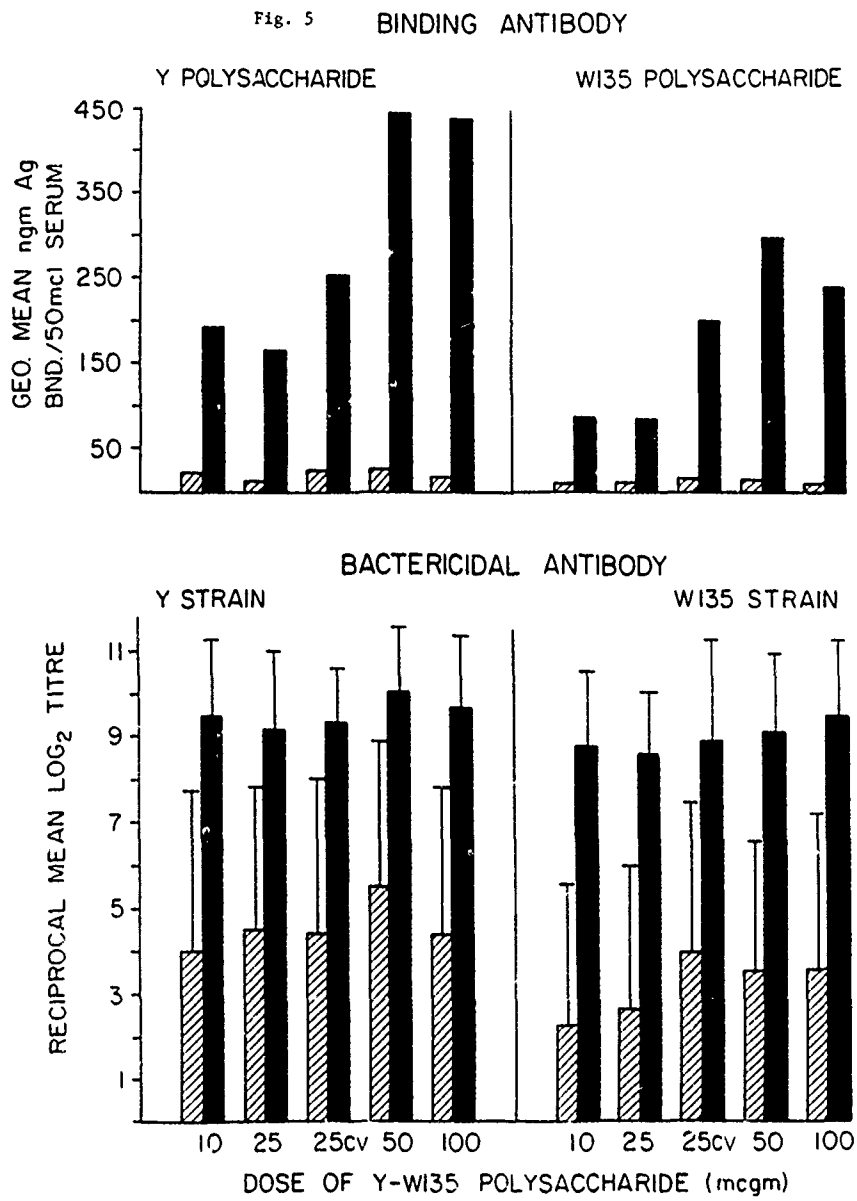

BINDING ANTIBODY
Y POLYSACCHARIDE
W135 POLYSACCHARIDE
GEO. MEAN ngm Ag BND./50mcl SERUM
450
350
250
150
50
BACTERICIDAL ANTIBODY
Y STRAIN
W135 STRAIN
RECIPROCAL MEAN LOG2 TITRE
11
9
7
5
3
1
10 25 25cv 50 100
10 25 25cv 50 100
DOSE OF Y-W135 POLYSACCHARIDE (mcgm)

Fig. 6

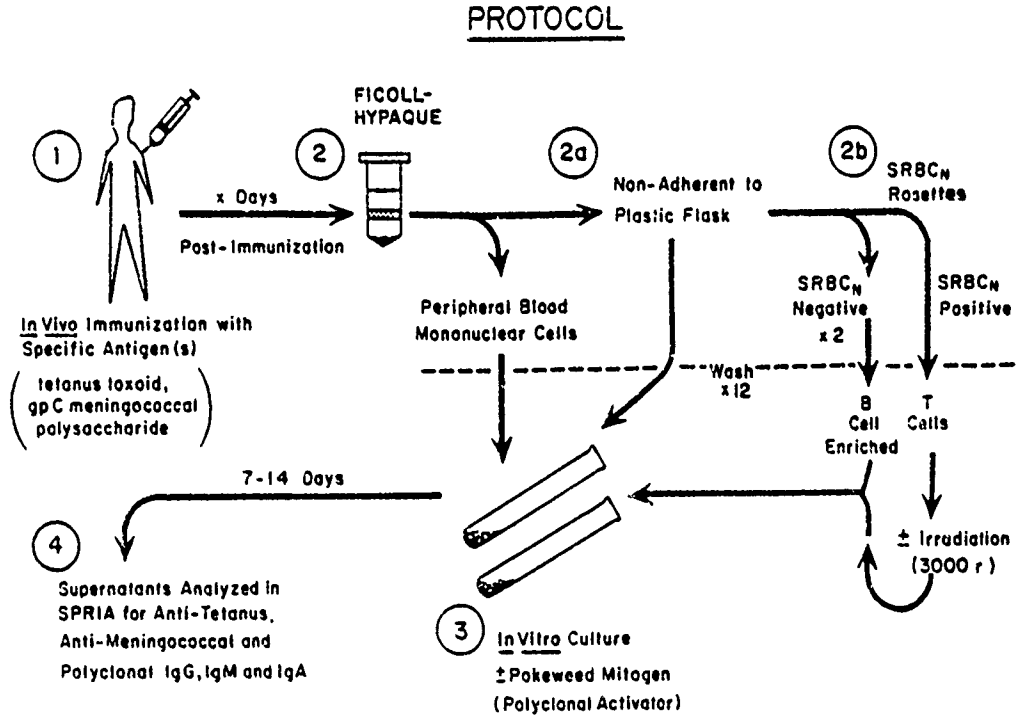
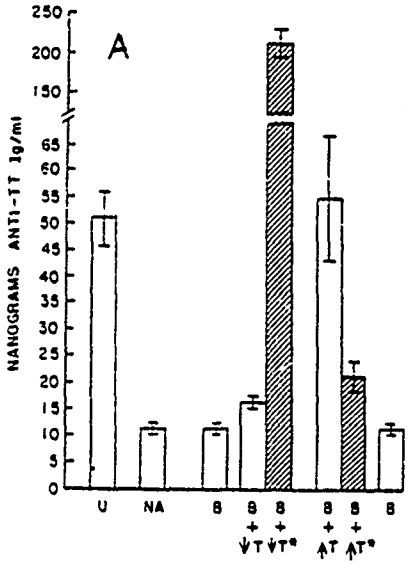

PROTOCOL
FICOLL-HYPAQUE
1
2
2a
2b
x Days
Post-Immunization
Non-Adherent to Plastic Flask
SRBC$_N$ Rosettes
SRBC$_N$ Negative x 2
SRBC$_N$ Positive
In Vivo Immunization with Specific Antigen(s)
(tetanus toxoid, gpC meningococcal polysaccharide)
Peripheral Blood Mononuclear Cells
Wash x12
B Cell Enriched
T Cells
7-14 Days
± Irradiation (3000 r)
4
Supernatants Analyzed in SPRIA for Anti-Tetanus, Anti-Meningococcal and Polyclonal IgG, IgM and IgA
3
In Vitro Culture
±Pokeweed Mitogen
(Polyclonal Activator)

Fig. 7

RADIOSENSITIVITY OF T-HELPER AND
T-SUPPRESSOR ACTIVITY IN ANTI-TT
IgG PRODUCTION 6 DAYS POST IMMUNIZATION



KEY

U = UNSEPARATED MONONUCLEAR CELLS

NA = NON-ADHERENT MONONUCLEAR CELLS

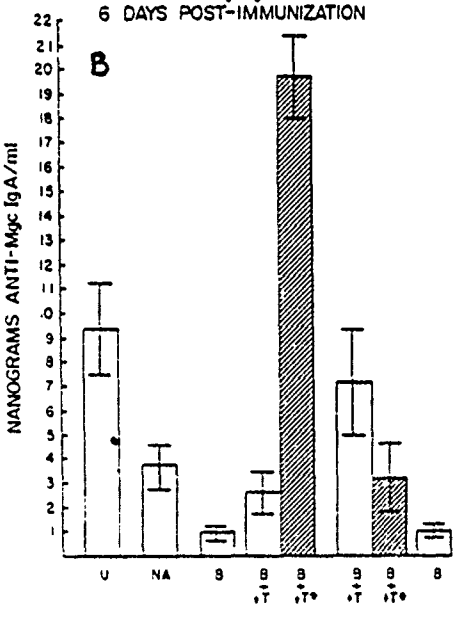
B = B-CELLS & MONOCYTES (T-CELLS DEPLETED)

↓T = T-CELLS ADDED AT T : B RATIO OF 1 : 2

↑T = T-CELLS ADDED AT T : B RATIO OF 2 : 1

* = T-CELLS IRRADIATED PRIOR TO ADDITION

RADIOSENSITIVITY OF T-HELPER & T-SUPPRESSOR
ACTIVITY IN ANTI-Mgc IgA PRODUCTION BY PBL
6 DAYS POST-IMMUNIZATION



RADIOSENSITIVITY OF T-HELPER AND
T-SUPPRESSOR ACTIVITY IN ANTI-TT
IgG PRODUCTION BY PBL 6 DAYS POST-IMMUNIZATION
(POKEWEED MITOGEN ABSENT)

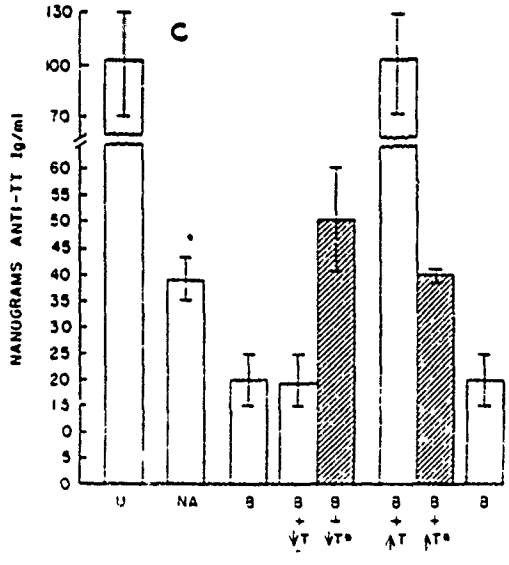


Fig. 8

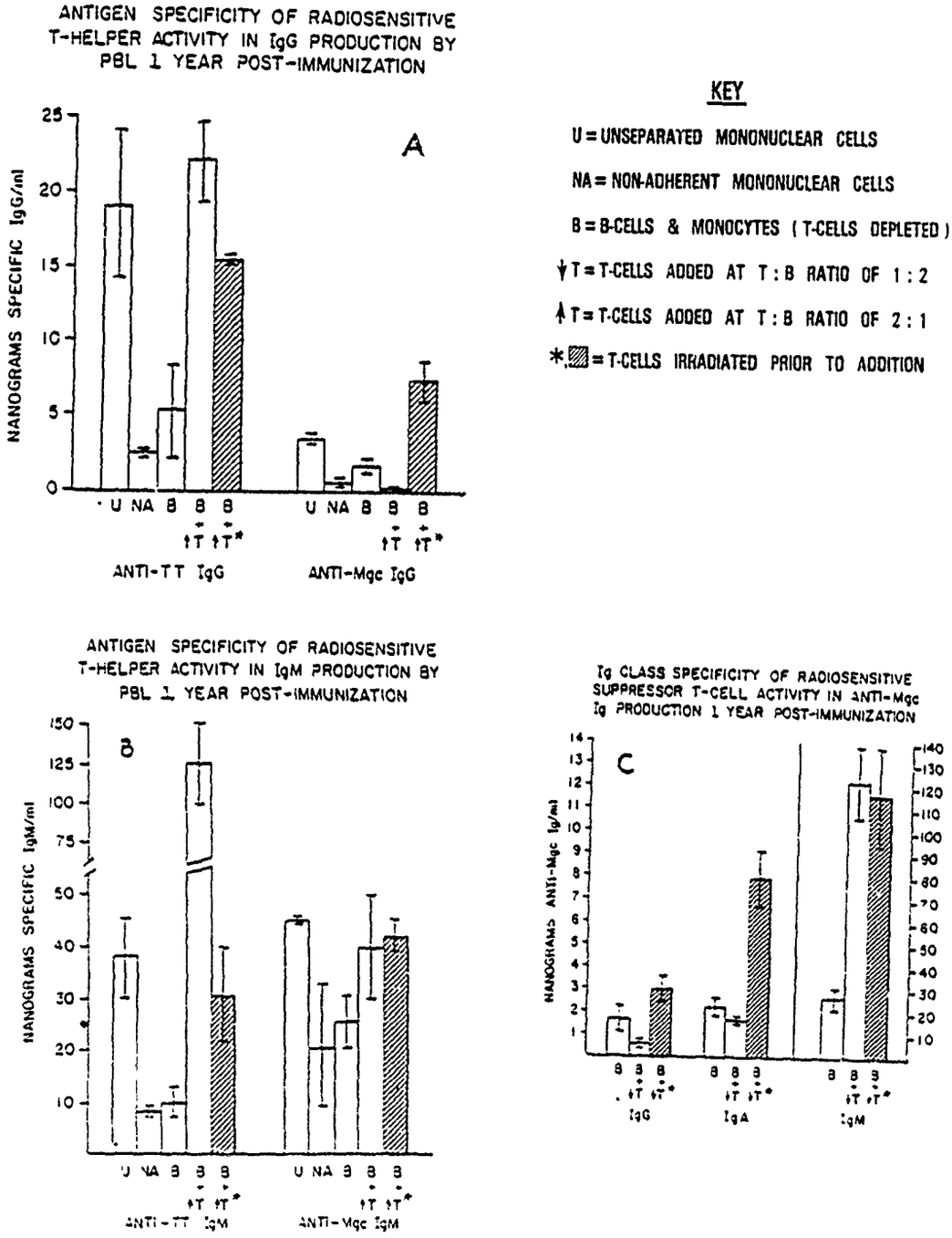

ANTIGEN SPECIFICITY OF RADIOSENSITIVE T-HELPER ACTIVITY IN IgG PRODUCTION BY PBL 1 YEAR POST-IMMUNIZATION
A
NANOGRAMS SPECIFIC IgG/ml
25
20
15
10
5
0
U NA B B B
U NA B B B
ANTI-TT IgG
ANTI-Mgc IgG
KEY
U = UNSEPARATED MONONUCLEAR CELLS
NA = NON-ADHERENT MONONUCLEAR CELLS
B = B-CELLS & MONOCYTES (T-CELLS DEPLETED)
T = T-CELLS ADDED AT T : B RATIO OF 1 : 2
T = T-CELLS ADDED AT T : B RATIO OF 2 : 1
* = T-CELLS IRRADIATED PRIOR TO ADDITION
ANTIGEN SPECIFICITY OF RADIOSENSITIVE T-HELPER ACTIVITY IN IgM PRODUCTION BY PBL 1 YEAR POST-IMMUNIZATION
B
NANOGRAMS SPECIFIC IgM/ml
150
125
100
75
50
40
30
20
10
U NA B B B
U NA B B B
ANTI-TT IgM
ANTI-Mgc IgM
Ig CLASS SPECIFICITY OF RADIOSENSITIVE SUPPRESSOR T-CELL ACTIVITY IN ANTI-Mgc Ig PRODUCTION 1 YEAR POST-IMMUNIZATION
C
NANOGRAMS ANTI-Mgc Ig/ml
IgG
IgA
IgM

Fig. 9

PMN BACTERICIDAL ACTIVITY OF HUMAN SERA AGAINST GBS III PRIOR TO AND FOLLOWING IMMUNIZATION OF ADULTS WITH POLYVALENT PNEUMOCOCCAL VACCINE

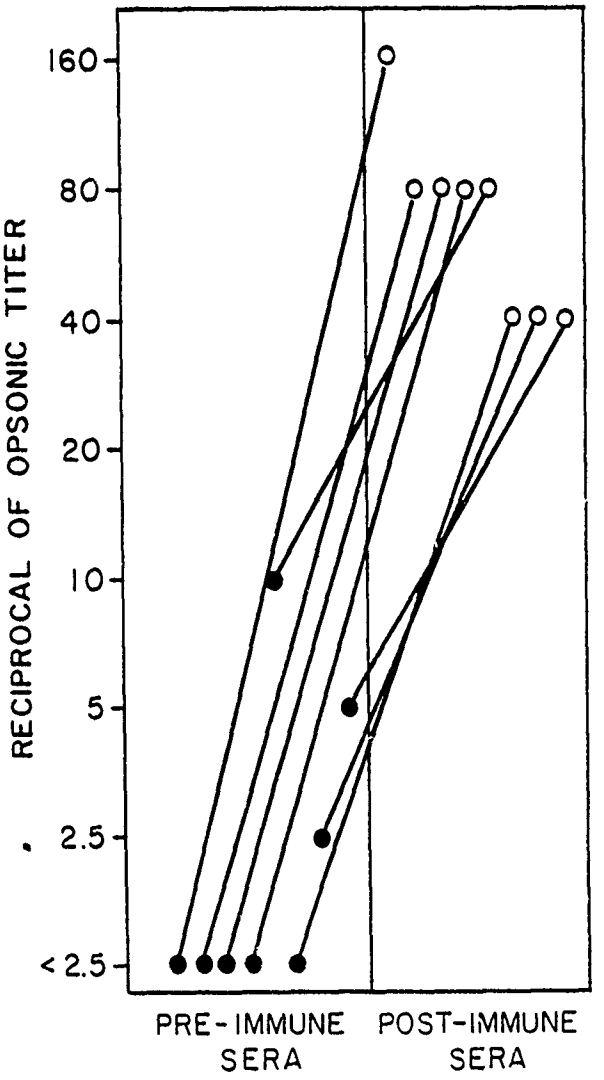


Fig. 10

PMN BACTERICIDAL ACTIVITY AGAINST GBS III AND P14 USING HUMAN SERA OBTAINED PRIOR TO AND FOLLOWING IMMUNIZATION WITH "PNEUMOVAX"

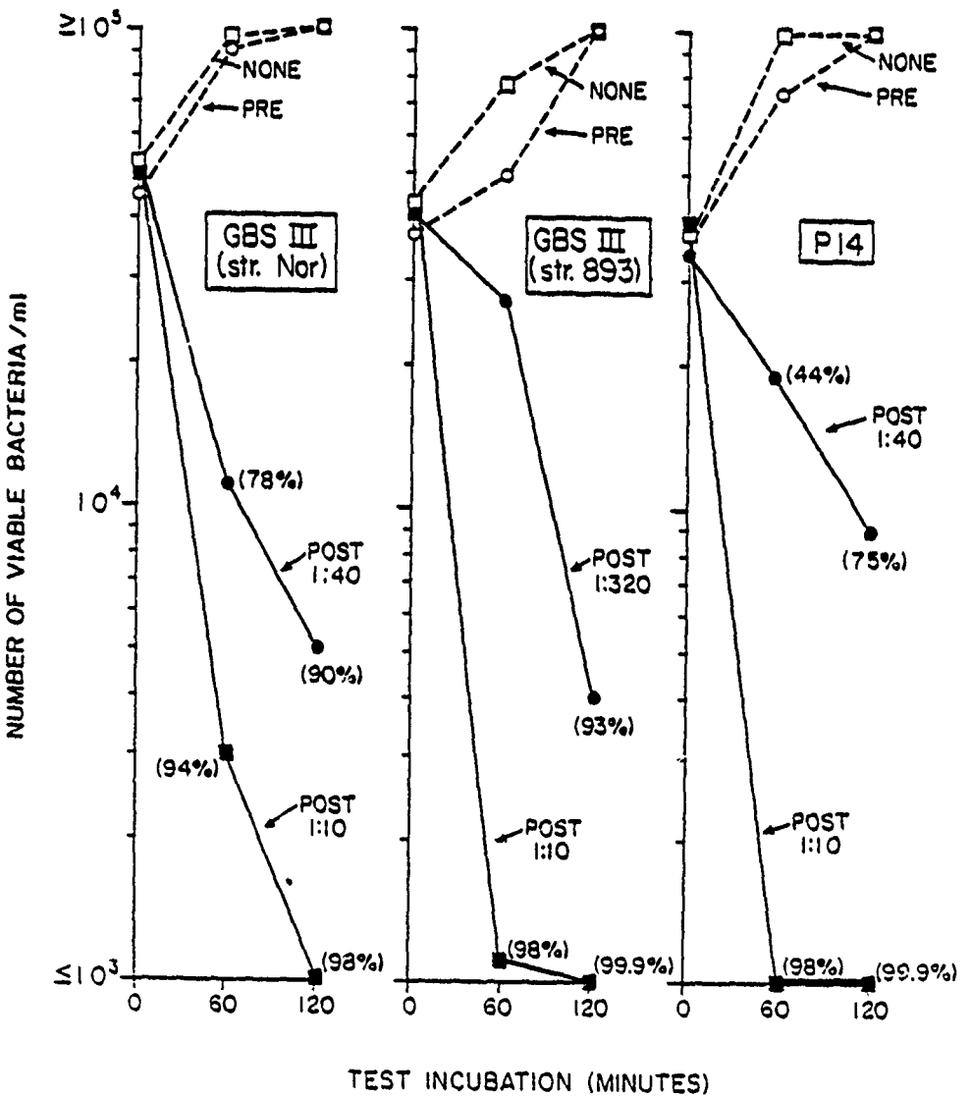
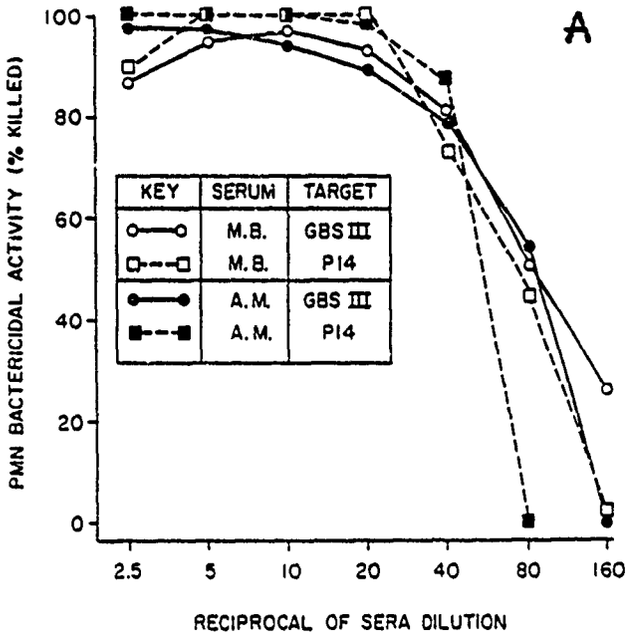


Fig. 11 OPSONIZATION OF GBS III AND PI4 BY HUMAN SERA OBTAINED NINE MONTHS POST-IMMUNIZATION WITH "PNEUMOVAX"



INHIBITION OF PMN BACTERICIDAL ACTIVITY AGAINST GBS III BY PI4

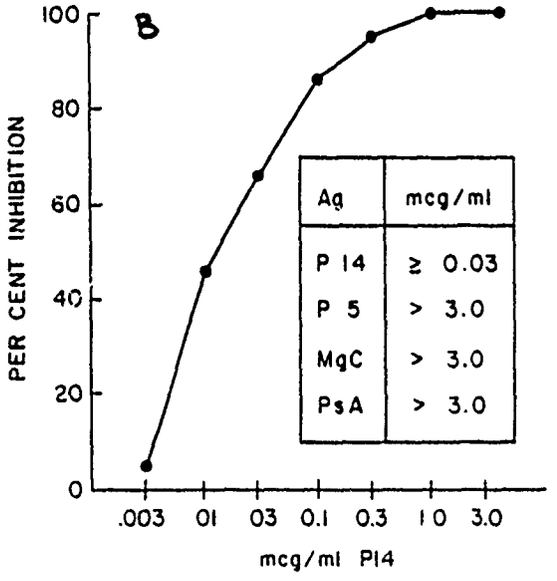
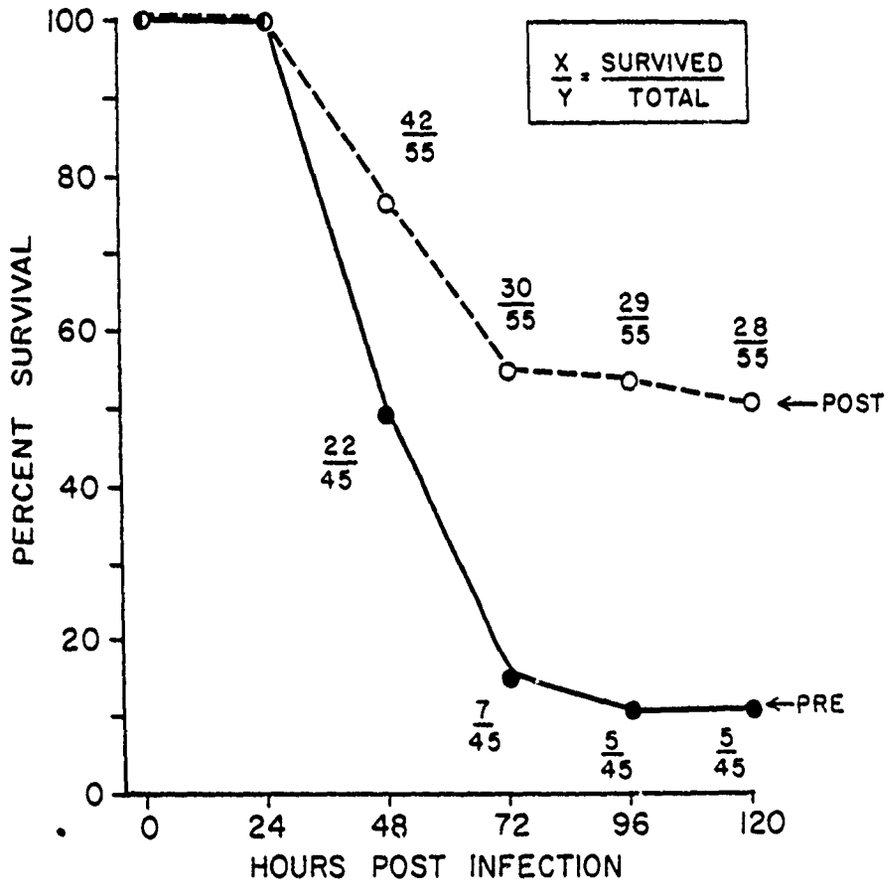


Fig. 12

IN VIVO PROTECTION OF SUCKLING RATS AGAINST GBS III SEPSIS USING SERA OBTAINED FROM FIVE INDIVIDUALS PRIOR TO AND FOLLOWING IMMUNIZATION WITH POLYVALENT PNEUMOCOCCAL VACCINE



Legends

Figure 1. Range and mean binding antibody values, pre and 4 weeks post-vaccination, in ngn of antigen bound by 50 µl of serum.

Figure 2. Range and mean bactericidal antibody values, pre and 4 weeks post-vaccination values are expressed as the reciprocal at the highest $\log_2$ dilution which lysed at least 50% of 2×10^3 organisms of each serogroup.

Figure 3. Geometric mean binding and bactericidal antibody levels, by week following vaccination, for each vaccine: group W135.__., group Y.__., group Y-W135 combined .__.__.

Figure 4. Effect of pre vaccination and body levels on binding and bactericidal antibody responses.

Figure 5. Geometric mean binding and bactericidal antibody response to five doses of divalent Y-W135 polysaccharide vaccine.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 132 Bacterial Diseases of Military Importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B 6531	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	133
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Ecology of Plague

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
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FISCAL YEAR PRECEDING 79	2	198
FISCAL YEAR CURRENT 80	2	275

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21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Yersinia pestis; (U) Plague; (U) Vaccines; (U) Immunization; (U) Serological tests; (U) Genetics

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Determine the factors influencing outbreaks of plague and the most appropriate methods to prevent the infection of troops engaged in field operation.

24. (U) Specimens and sera from humans and animals are tested for the presence of Y. pestis and antibody to Y. pestis. Strains of Y. pestis are characterized for determinants of virulence and antibiotic susceptibilities.

25. (U) 78 10 - 79 09 Strains isolated from chronic rodent infections contain virulent organisms which do not produce F-1 antigen. Examination of saline extracts of acetone killed and dried Y. pestis identified at least 17 antigens, including murine toxin, in addition to F-1. This multiplicity of antigens in simple extracts emphasizes the importance of precise characterization and standardization of diagnostic antigens. An international committee has been organized to direct this work. ELISA tests have been developed using both F-1 and other antigens purified by isoelectric focusing to detect antibody following infection with aberrant strains. The ability of these antigens to specifically detect Y. pestis infections will be determined. An ELISA inhibition test has been developed to confirm the specificity of the reaction as well as measure antigen content. Utilizing a series of isogenic pairs of strains, diagnostic antigens are being prepared from cultures genetically deficient in the ability to produce unwanted antigens. Y. enterocolitica strains which are virulent for mice are sensitive to the action of P-I pesticin and spontaneously agglutinate during growth in cell culture media. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 133 Ecology of Plague

Investigators.

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Description.

To determine the factors influencing outbreaks of plague infection and the most appropriate methods to prevent the infection of troops engaged in field operations.

Progress.

Plague has continued to be a sporatic problem in the U.S. with 9 cases reported this year. Outbreaks have occurred in several countries of the world in areas of former plague activity which have been thought to be free of the disease.

Research during FY79 has involved the following main areas: 1) continuation of the development and testing of methodologies directly applicable to the rapid differentiation of yersiniae phenotypes; 2) further development of the enzyme-linked immunoassay for diagnosis of plague and surveillance of animal populations under field conditions; 3) an intensive program for the standardization of diagnostic tests and reagents to reflect a growing requirement for these materials throughout the world; and 4) transmission of Yersinia pestis by rodents and fleas.

1. Differentiation of Yersiniae phenotypes.

A. Relationship of autoagglutinability to virulence.

In the course of studies of virulence factors of Yersinia species we have made the fortuitous discovery that strains which are virulent when fed to adult mice invariably share the ability to autoagglutinate when grown at 36 C in tissue culture media. Strains which failed to autoagglutinate were found to be avirulent. This observation has enabled us to develop a simple test for distinguishing virulent and avirulent strains of yersiniae. In

addition to its value in preparing isogenic derivatives needed for studies of pathogenesis, we have found this test to be reliable for rapid screening of large numbers of yersiniae from clinical and food sources for their pathogenic potential. Thus it is now possible to avoid the labor and expense of the animal and tissue culture models currently being used to screen such diverse isolates.

Many of the strains employed in this study are part of the stock culture collection of the Department of Hazardous Microorganisms, Walter Reed Army Institute of Research, Washington, D.C. Some strains were also provided by Dr. Lee Wei Hwa, USDA-FSQS, Beltsville, Md., Dr. Ira J. Mehlman, FDA, Washington, D.C., and Dr. Jean M. Alonso, Institut Pasteur, Paris, France. All strains were maintained at room temperature in screw-capped tubes containing 10.0 ml of semi-solid medium (Bacto-Tryptose 10.0 g, sodium chloride 5.0 g, and 5.0 g Bacto-Agar per liter H_2O). Organisms were routinely cultivated on trypticase soy agar medium at 26 C.

The autoagglutination test was performed in sterile 13 x 100 mm disposable glass test tubes containing 2.0 ml of RPMI-1640 medium with 25 mM Hepes (Microbiological Associates, Walkersville, Md.). Strains were streaked on trypticase soy agar plates and grown for two to three days at 26 C. Cells from an isolated colony were inoculated into a pair of tubes. One tube was incubated for 18 hours at 36 C while the other was incubated for the same time period at 26 C. Routinely ten colonies of each strain were tested to insure an adequate sample size. The growth in each tube was examined for evidence of bacterial agglutination. The growth of autoagglutinating (Ag+) strains consisted of an irregular edged layer of agglutinated bacteria which formed a flocculate covering the bottom of the tube. Usually, the medium in such tubes was clear. The growth of non-agglutinating (Ag-) strains was distinctly different. Although some bacteria had settled out to form a smooth round pellet in the center of the bottom of the tube, the majority of the bacteria remained in suspension therefore creating a turbid medium. When the various tubes were gently shaken the agglutinated bacteria remained clumped whereas the non-agglutinated bacteria in the pellet formed a smooth turbid suspension.

This agglutination phenomenon was found to be dependent on temperature of growth. Strains were scored as Ag+ if the 36 C tube was positive and the 24 C tube was negative. Bacteria which agglutinated at both temperatures were considered to be false positives. In most cases these false reactions were produced by bacteria which formed colonies of a rough morphology.

We selected colonies from representative wild-type virulent strains of Y. pestis, Y. pseudotuberculosis and Y. enterocolitica and tested them for their ability to autoagglutinate. These cultures were found to contain both Ag+ and Ag- bacteria. Therefore, it was possible to select isogenic pairs of each of these strains differing in their ability to autoagglutinate. These isogenic pairs were then tested for virulence by oral feeding of adult mice. Groups of 5 Swiss albino mice (Walter Reed ICR) weighing 15-20 g were deprived of water for 18 hours and then allowed to drink ad libitum from a 50 ml water suspension of each strain containing about 1×10^9 bacteria per ml. Mice were examined twice a day for evidence of diarrhea and sepsis. Liquid feces as well as the spleens of dead mice were cultured using standard bacteriological techniques to detect the presence of the challenge organism.

As shown in Table 1, all Ag+ strains proved to be virulent whereas their isogenic Ag- derivitives were avirulent. Virulence was defined as the ability of a strain to induce diarrhea and/or produce a fatal systemic infection following oral infection.

These results suggested that the autoagglutination test might be useful as a presumptive test of the pathogenic potential of yersiniae isolates. Over the last year we have tested 220 different strains for their ability to autoagglutinate. Four out of 10 Y. pestis, 6 out of 30 Y. pseudotuberculosis and 25 out of 180 Y. enterocolitica strains were found to be Ag+. All proved to be virulent to mice by the oral route. None of 185 Ag- strains proved to be virulent. Nine strains of Y. enterocolitica autoagglutinated at both 36 C and 24 C but were avirulent for mice. One strain of Y. pseudotuberculosis produced a positive reaction at both 36 C and 24 C. This strain, however, did prove to be virulent. Therefore, out of the 220 strains tested by our autoagglutination method only one strain was presumptively falsely characterized as avirulent.

The biochemical basis of this autoagglutination phenomenon is obscure. Nonetheless, there is without question a correlation between the autoagglutination and virulence properties of yersiniae. As a consequence, we are now examining the possible relationship of this phenomenon to known virulence determinants of yersiniae. At least 5 such determinants have been defined in Y. pestis; V and W antigen (VW), purine synthesis (pur), fraction 1 (F1), pesticin-T-coagulase-fibrinolysin complex (PCF) and the ability to absorb certain dyes (P) when grown on solid media. VW, pur and possibly the P determinants are also shared by Y. pseudotuberculosis. Recent evidence (unpublished observations)

Table 1. Comparison of virulence properties of autoagglutinating and non-agglutinating isogenic derivitives of Yersinia species.

Species	Strain	Auto-agglutinating	Virulence to mice	
			Diarrhea[a]	Death[b]
Y. pestis	195(Ag+)	+	-	5/5
	195(Ag-)	-	-	0/5
	MP6(Ag+)	+	-	5/5
	MP6(Ag-)	-	-	0/5
Y. pseudo-tuberculosis	13-13(Ag+)	+	+	5/5
	13-13(Ag-)	-	-	0/5
	13-14(Ag+)	+	+	5/5
	13-14(Ag-)	-	-	0/5
	13-10(Ag+)	+	+	5/5
	13-10(Ag-)	-	-	0/5
Y. enterocolitica	Y7(Ag+)	+	+	5/5
	Y7(Ag-)	-	-	0/5
	4052(Ag+)	+	+	0/5
	4052(Ag-)	-	-	0/5

[a] Mice which were scored as diarrhea positive produced multiple soft to liquid stools over a three to five day period usually starting on the third day after bacterial challenge.

[b] Death occurred within 14 days.

suggest that some virulent strains of Y. enterocolitica also are VW positive. At least two biological roles have been associated with the V and W antigens: resistance to phagocytosis by neutrophils, and the survival and multiplication within free or fixed macrophages. We are currently investigating the possibility that the autoagglutination property and the VW antigen are indicators of the same virulence determinant. The fact that the production of the VW antigens as well as the ability to autoagglutinate are temperature dependent suggests that this is a reasonable possibility.

B. Association of PI sensitivity to virulence in strains of Yersinia enterocolitica.

Outbreaks of Yersinia enterocolitica infections in Japan, Canada, the United States, and in Europe, demonstrate the wide distribution and the pathogenic nature of the newest member of the genus yersiniae. Y. enterocolitica has been isolated from a variety of sources including wild and domestic animals, meats, fruits, dairy foods, and non-chlorinated drinking water. Its' common occurrence in foods and its ability to grow at refrigerator temperatures pose significant public health problems.

Pesticin I, a bacteriocin produced by wild type cells of Y. pestis, is active against not only serotype 1 of Y. pseudotuberculosis and E. coli but also against certain strains of Y. pestis and Y. enterocolitica. In our studies of plague bacteriocins, we noted that Pesticin I sensitivity in Y. enterocolitica strains appeared to be related to the ability of the particular strain to kill mice. This study examined the possible application of Pesticin I as a diagnostic tool to rapidly detect fully virulent isolates of Y. enterocolitica.

All Y. enterocolitica cultures were obtained from Dr. W.J. Laird. The plague strains were from the stocks of this Department. The bacteria were routinely streaked from the stock slants onto Brain Heart Infusion Agar (BHIA, Difco) and were transferred into Brain Heart Infusion Broth (BHIB, Difco) after growth became visible (6-24 hrs).

Pesticin I was produced by adding Mitomycin C to a 6 hour broth culture of Y. pestis strain M23 to give a final concentration of 0.5 μg/ml. The cultures were shaken overnight at 37 C, sterilized by filtration and stored at -20 C until use. To detect PI sensitive strains, 0.01 ml of a BHIB culture containing approximately 10^6 organisms per ml was added to

5 ml of soft agar, mixed and poured onto sterile BHIA plates and allowed to harden. The pesticin preparation was dropped onto each strain and incubated at 37 C overnight.

We have examined over 200 strains and showed that Y. enterocolitica can be divided into 3 or more classes according to the degree of virulence for mice. In Table 2, Pesticin I shows a clear cut distinction between Classes I and II. The 7 avirulent, but Pesticin I sensitive strains in the unclassified group are now being re-examined for virulence. Other studies will be conducted to determine whether they comprise yet other classes of Y. enterocolitica. This is a reasonable alternative since the avirulent, isogenic pairs to Class I strains are also sensitive to the action of PI.

Classes I and II, whose only apparent difference is the killing of mice,are completely separated by PI sensitivity. This may indicate the cells of Class II have undergone a mutation from full virulence that has a relationship to the cell wall sites of PI attachment.

2. Enzyme Linked Immunosorbent Assay.

Serological techniques appear to be far more successful in detecting plague infection than are the more classical bacteriological methods. Studies on the ELISA technique, recommended by the World Health Organization for other diagnostic purposes have been undertaken at WRAIR. The ELISA is a simple and highly reproducible technique for demonstrating the presence of F-1 antibody in sera collected from plague infected R. norvegicus or human beings. It has particular value in that both IgG and IgM antibodies to F-1 can be detected and measured directly. Data from a titration of convalescent sera collected from 4 of 38 plague infected R. norvegicus (Fig. 1) demonstrate that F-1 antibody (IgG) can be easily detected in the sera of this important plague reservoir. The ELISA tests appear to be equally valuable in the examination of sera collected from bacteriologically confirmed plague patients (Table 3). A classical serological response to infection with Y. pestis is present in confirmed plague cases with both IgG and IgM antibody to F-1 in the sera of convalescent patients.

Experiments with the ELISA for detecting plague antibodies have revealed that a critical consideration for standardizing the procedure to achieve reproducible results is consistent volumes of reactant fluid from well-to-well and test-to-test. Inactivation of sera is unnecessary, and serum dilutions made directly in the ELISA plate exhibit the same titers as dilutions made otherwise and transferred to the ELISA plate, so

Table 2. Strains of Y. enterocolitica classed according to phenotype.

	Plasmid	Diarrhea	Colonization	Virulence in mice	P-1 Sensitive
Unclassified	0/101	0/101	0/101	0/101	7/101
Class I	27/27	27/27	27/27	27/27	27/27
Class II	12/12	12/12	12/12	0/12	0/12

* Isogenic pairs to Class I strains have been isolated which are P-1 sensitive but do not colonize or kill mice.

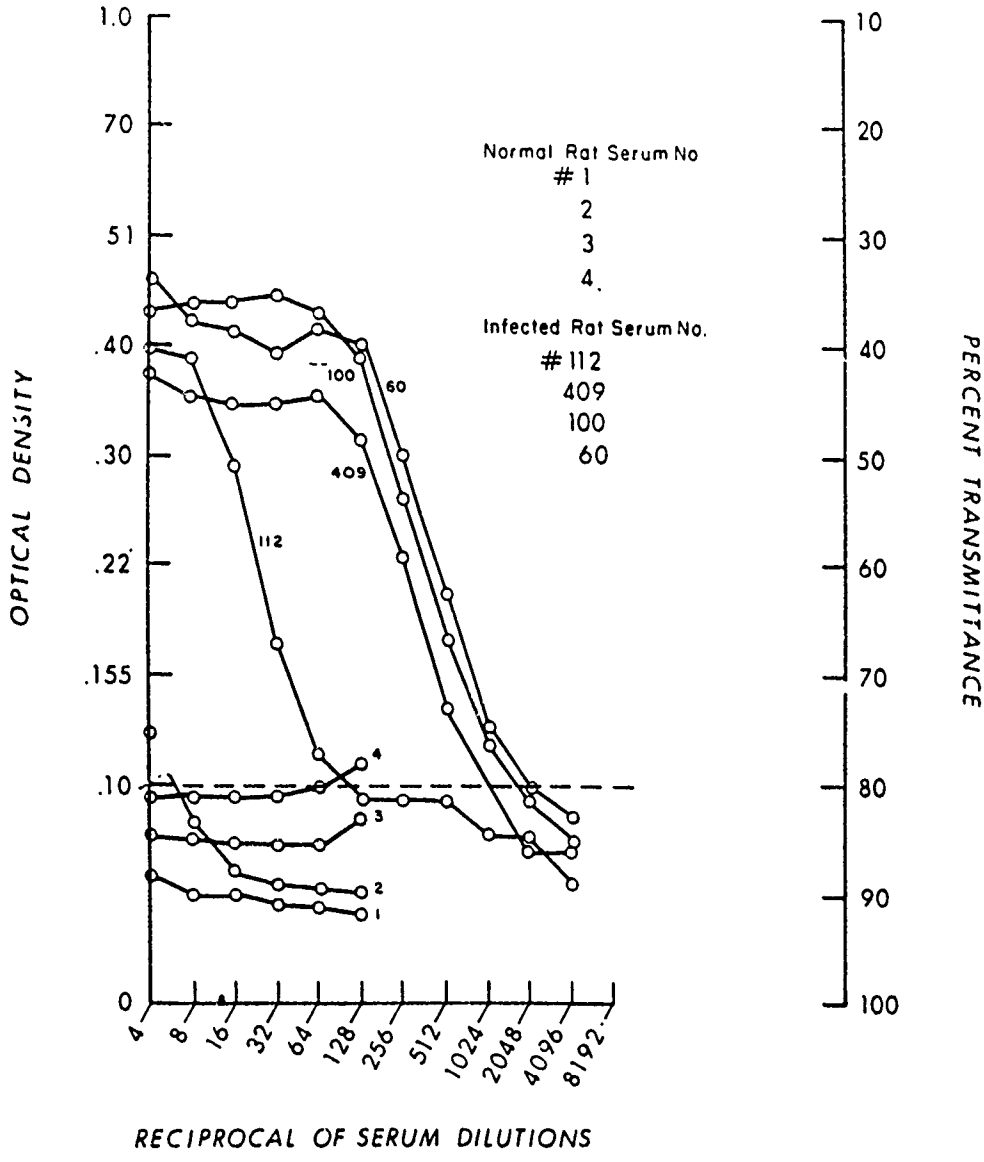

Fig. 1 ELISA tests for F-1 antibody. Results compare the antibody response of infected R. norvegicus with values obtained with normal rat sera.

Table 3. Serological response to infection or presumed infection observed in bacteriologically confirmed plague patients or contacts measured by the ELISA technique.

Patients	Acute		Convalescent	
	IgG	IgM	IgG	IgM
1	1:32	-*	1:16,384	1:2,048
2	-	-	1:512	1:128
4	1:64	-	1:4,096	1:128
5	1:128	1:128	1:16,384	1:2,048
6	1:32	-	1:2,048	1:2,048
7	-	-	1:1,024	1:1,024
8	1:64	-	1:256	1:256
9	1:4,096	1:256	1:32,768	1:4,096
10	1:128	1:256	1:2,048	1:2,048
Contacts**				
1	N.D.***	N.D.	1:4,096	1:1,024
2	N.D.	N.D.	1:256	1:256

* Less than 1:4.
** Sera from 2/26 contacts exposed to plague in nature. Acute sera and specimens for bacteriological examination were not collected.
*** Not done.

long as reactant volumes are equal. ELISA plates sensitized with the F-1 antigen of Y. pestis can be stored in the refrigerator for at least 3 months prior to use without any deterioration in test performance.

An opportunity to investigate the applicability of the ELISA test to field situations was afforded the laboratory when Major Williams served as a PAHO consultant in Peru. All reagents were shipped by air, non-refrigerated in a dry form. Results of this first field trial indicated that the reagents are stable during shipment and short term storage at ambient temperatures. Further studies aimed at developing a temperature stable, complete, compact, and light antibody/antigen detection kit are planned.

In an effort to quantify specific antigens of plague and corraborate the results of the regular ELISA test, we have developed an ELISA inhibition procedure which relies on either reacting a known antigen with the test serum or a known serum with the test antigen prior to the exposure of these reactants to the microtiter plate coated with a known antigen. Briefly, serial two-fold dilutions of the test reagent are prepared in phosphate buffer saline containing 0.05% Tween 20 and 1% BSA (PBSTB) in a microtiter plate. A standard amount of serum or antigen depending on the experimental design is added to these dilutions. The plates are placed at 37 C for 1 hr. The mixture is then transferred to the wells of a micro-ELISA plate that has been pre-coated with a second antigen and washed 3 times with PBSTB. Most of the experiments conducted to date have used the capsular antigen, F-1, of Y. pestis as the coating antigen. The mixture containing the unknown and the serum or antigen is incubated in the coated wells for 30 min at 37 C and aspirated. The wells are then washed with PBSTB. Anti-immunoglobulins conjugated with horseradish peroxidase against the appropriate species are added and the plate incubated at 37 C for 30 min. The contents are aspirated, the plate washed and substrate is added. The plate is incubated at 37 C for 1 hr and the OD's of the samples are read at 455 nm on a spectrophotometer.

Experiments with F-1 as both the coating antigen and the test antigen indicated that the test is capable of routinely quantifying nanogram amounts of F-1. When murine toxin was reacted with serum containing F-1 antibody, the resultant mixture had a titer to F-1 identical to unreacted serum. Thus, the inhibition test is a sensitive and specific measure of the F-1 antibody content of sera or the F-1 antigen content of various preparations.

3. Standardization of diagnostic tests and reagents.

A. Murine toxin and the V-W complex.

Recently, observations on cryptic plague (Annual Report, 1977) have indicated a requirement for serological procedures that employ specific antigens, other than F-1, of the plague bacillus. Strains of this phenotype have been isolated in nature: the Bryant strain isolated in the USA and the Nairobi strain isolated in Kenya. We have now completed a re-examination of serological data collected from 18 confirmed plague cases (by isolation of Y. pestis, Fig. 2) that occurred during the 1950's. The sera were tested for antibody to the F-1 and murine toxin antigens of Y. pestis in HA tests and in mouse protection tests. The results in Fig. 2 compare the geometric mean titers for the group with the titers measured in the serum of one patient. In these tests, an anticipated serological response to F-1 was not observed in the sera of this one particular patient although a satisfactory response was observed to murine toxin and in the mouse protection test. F-1 antibody response appears to resemble that observed in R. norvegicus infected with non-encapsulated F-1⁻ Y. pestis. Two Y. pestis antigens that may prove valuable to detect aberrant F-1⁻ Y. pestis infections are murine toxin and the V-W complex.

Isolation of murine toxin and V-W was achieved by growing M23P (an aberrant although virulent strain completely devoid of F-1) on TSA agar. The cells were harvested in saline and precipitated with -20 C acetone. Precipitated cells were then centrifuged at 2,000 rpm (1,060 xg) and dried under vacuum. A crude extract was obtained by absorbing these dried cells with a 2% saline solution and precipitating the soluble proteins with saturated ammonium sulfate. Centrifugation of the floating precipitate at 20,000 rpm (34,880 xg) in Spinco centrifuge was required to pellet the proteins. Dialysis against distilled water was utilized to remove ammonium sulfate from the partially purified extracts. These extracts were then isoelectric-focused in a 110 ml LKB column to separate the proteins which were present. Five to fifty percent sucrose was used to stabilize the pH gradient obtained by adding 3.25 ml of pH 3.5-5 ampholyte and .75 ml of pH 4-6 ampholyte to the dense gradient solution and 1.07 ml of pH 3.5-5 ampholyte and .33 ml of pH 4-6 ampholyte to the light gradient solution. Triethanolamine (1.6%)was used as the cathode electrolyte and (.15 M) phosphoric acid as the anode electrolyte. Samples were then run at 5 watts constant power for 72 hrs. Fractions (2 ml) taken from the column were analyzed for pH, protein content, mouse IV LD_{50}, and assayed for the presence of

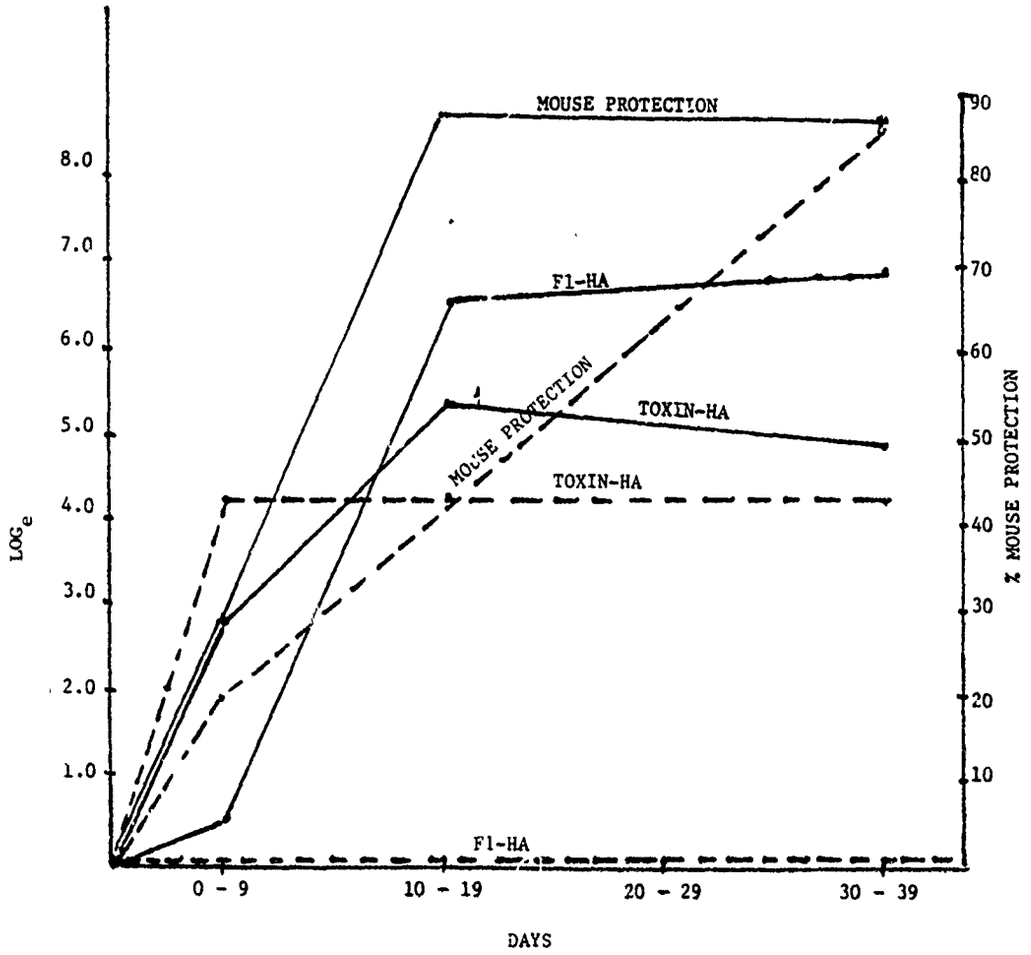

Figure 2.

Serological response of 18 plague patients to pulmonary plague measured by three techniques: the IHA test employing either the F-1 or murine toxin antigens of Y. pestis and the mouse protection test of K.F. Meyer. Solid lines are the normal curves for the group. Dashed lines are the responses measured in a single patient. Note the absence of F-1 antibody.
Source: official records, WRAIR.

specific antigens using monovalent and polyvalent antisera. Preliminary protein content of each fraction was determined with a Fracto-Scan Mini-monitor attached to a LKB Fraction Collector. More precise protein determinations were later made using the protein dye method.

Figures 3 and 4 show the linear pH gradients and protein concentrations obtained for saline extracts of organisms grown at 28 C and 37 C. Five peaks were identified in the extract of cells incubated at 28 C and four in the extract of cells incubated at 37 C. For convenience, peaks have been numbered (Tables 4 and 5) to facilitate subsequent identification.

Pooled fractions comprising each peak were assayed for murine toxin by intravenous injection of 0.5 ml of graded decimal dilutions of test samples in the lateral tail veins of 4-5 week old male mice to determine the LD_{50} of each protein peak. Two highly toxic peaks were identified in cells grown at 28 C (Table 4), one occurring at pH 6.1 with an LD_{50} of 1.2 μg/ml and the other at pH 5.50 with an LD_{50} of 2.0 μg/ml. Two toxic peaks were also identified in cells incubated at 37 C, one occurring at pH 6.62 with an LD_{50} of 2.5 μg/ml and the other occurring at pH 5.58 with an LD_{50} of .94 μg/ml. Two species of murine toxin, toxin A and toxin B, are produced at 28 C, but only toxin B at 37 C. Isoelectric focusing, however, resolved two distinct murine toxin peaks at 37 C (Fig. 4). Other significant differences in both production and relative toxicity can be noted between the 28 C and 37 C antigens (Tables 4 and 5). Although the production of pH 6.10 toxin at 28 C is relatively small compared to pH 5.50 toxin production, the pH 6.10 pool is more toxic having a lower LD_{50}. Curiously, the entire picture is reversed when cells are grown at 37 C. Production of the pH 6.62 toxin is both more abundant than that of the pH 5.58 toxin, and the lethality is now less than that of the pH 5.58 toxin. Preliminary experiments using the purified toxin (pooled fractions 40-50) have been done. Fig. 5 shows results of initial checkerboard titrations using murine toxin. The optimal concentration of the antigen can be seen to be 10 μg/ml or less. Presently, sera from laboratory induced infections with aberrant strains are being tested to analyze the feasibility and reliability of the system.

V and W antigens were identified by rocket immunoelectrophoresis using monovalent antiserum prepared by Lawton. In the focused extracts of cells incubated at 37 C, peaks 1 and 2 were both strongly positive for the presence of both V and W antigens

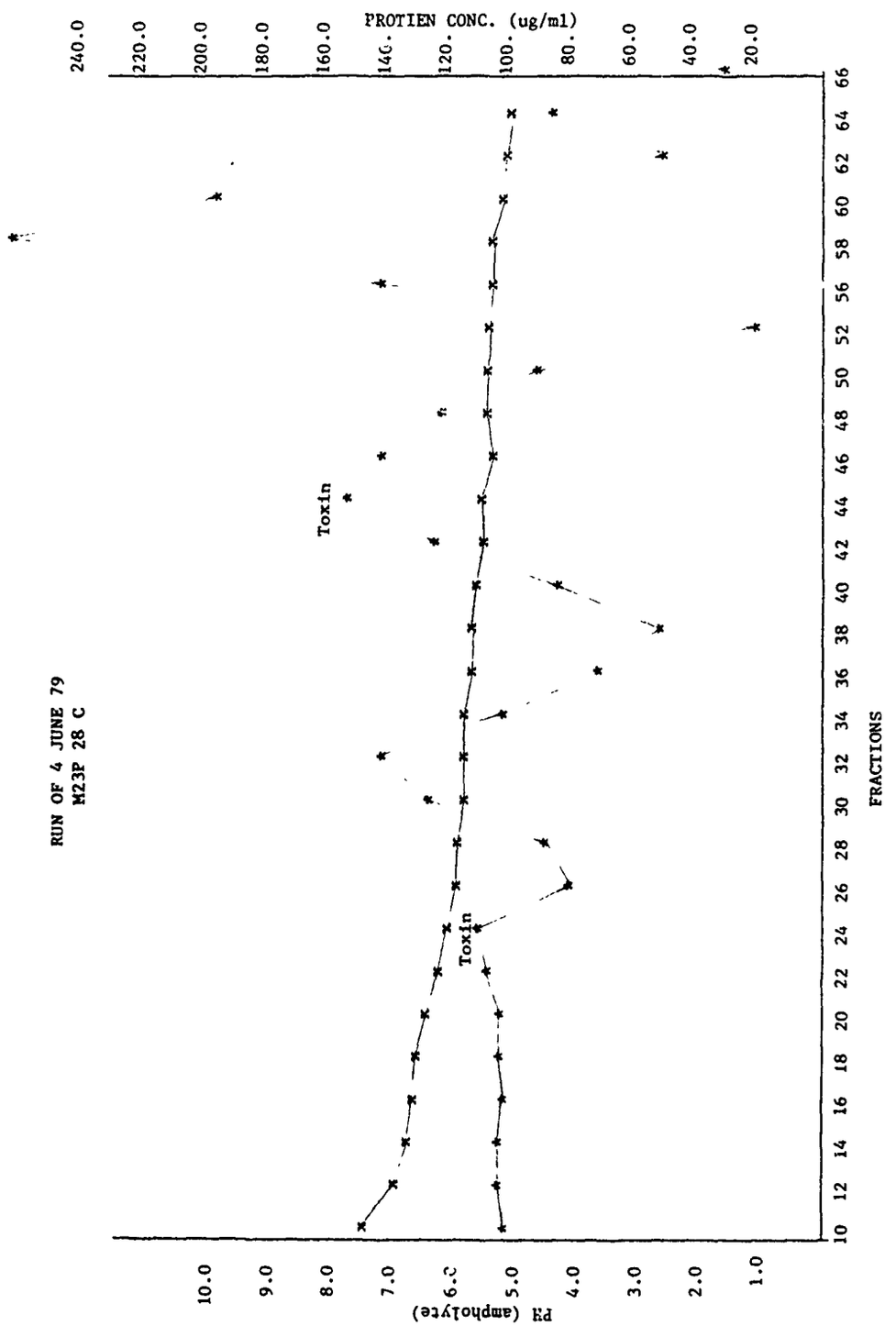

Fig. 3 Graph of protein concentration and pH plotted against fractions of isoelectric-focused M23P grown at 28°C.

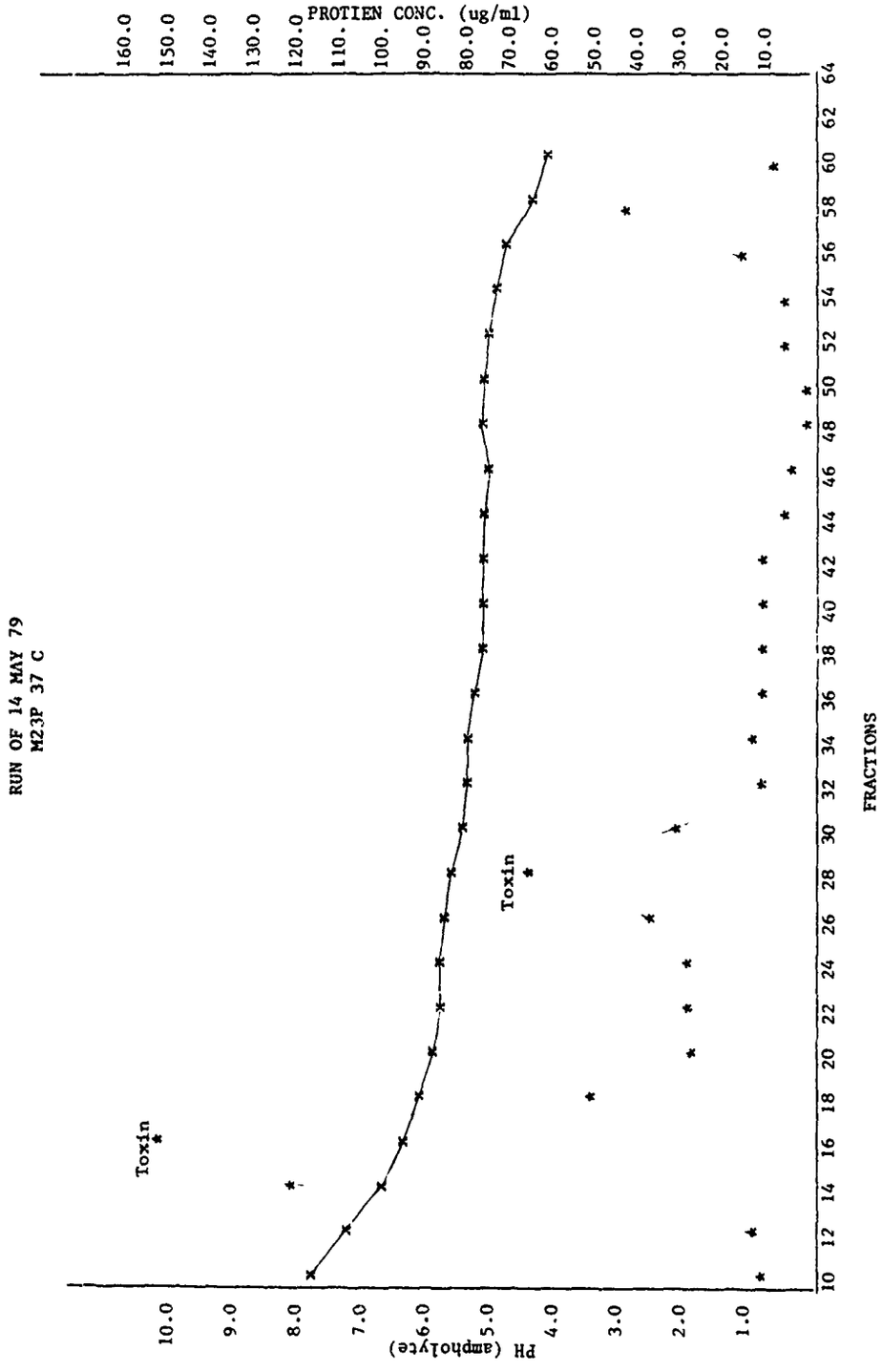

Fig. 4 Graph of protein concentration and pH plotted against fractions of isoelectric-focused M23P grown at 37°C.

Table 4. Fractionation by isoelectric focusing of saline extracts of the M23P strain of Y. pestis grown at 28 C.

Temp 28 C							
Peak	Fractions Pooled	pH	Antisera 1122	111	V	W	LD_{50} *
1	23-24	6.10	+	+	+	-	1.2
2	**						
3	40-50	5.50	+	+	±	-	2.0
4	53-62	5.31	+		±	-	22.0
5	67-69	***	+		-	-	>17.0

* Mouse IV LD_{50} µcg protein/ml.
** As yet uncharacterized protein component.
*** Accurate pH is not available because the protein migrated to the end of the pH gradient.

Table 5. Fractionation by isoelectric focusing of saline extracts of the M23P strain of Y. pestis grown at 37 C.

Temp 37 C							
Peak	Fractions Pooled	pH	Antisera 1122	111	V	W	LD_{50} *
1	14-18	6.62	+		+	+	2.5
2	26-31	5.58	+	+	+	+	.94
3	33-35	5.39	+		+	-	>6.0
4	54-59	**	+		-	-	>9.5

* Mouse IV LD_{50} µcg protein/ml.
** Accurate pH is not available because the protein migrated to the endo of the pH gradient.

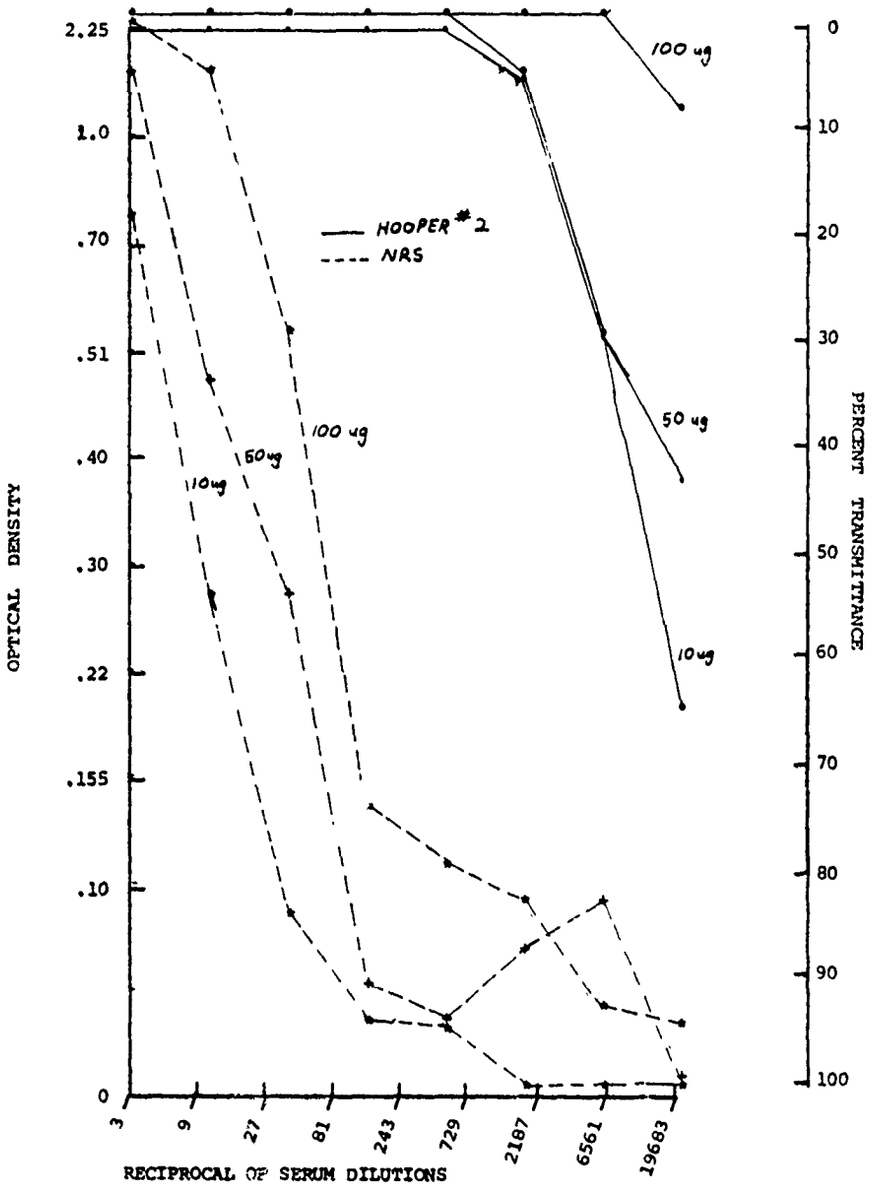

Fig. 5 ELISA tests for murine toxin antibody. Results compare the antibody response of an infected rabbit with values obtained with normal rabbit sera.

(Table 5). Traces of V antigen were also present in extracts of Y. pestis incubated at 28 C (peaks 1, 3, and 4), although W antigen was not detected (Table 4). High concentrations of murine toxin were also present in the same peaks. Murine toxin and the V and W antigens have similar isoelectric points and thus are difficult to separate by this technique.

B. Standardization of the F-1 Diagnostic Antigen.

The serological diagnosis of Yersinia pestis infection is based on the detection of antibody to the F-1 antigen. Since the plague organism shares antigens with other members of the genera and with members of the Enterobacteriacea the quality of the data is related to the purity of the F-1 preparation used as antigen. The present production procedure calls for a series of ammonium sulfate precipitations of acetone killed and dried microorganisms. Studies conducted in this laboratory of the F-1 lot presently being used for detection of antibody indicates that it contains significant amounts of contaminating material. As an approach to preparing a better diagnostic antigen we have fractionated the present Lot 4, F-1 antigen on an isoelectric focusing column. Preliminary experiments with pH 3.5-10 columns indicated that all of the antigenic material, as measured by an ELISA test, was concentrated near pH 4. Although several peaks appeared to be present they were poorly separated. Further studies were conducted with an LKB 110 column using a pH gradient produced by adding 2.2 ml of pH 3.5-5.0 ampholyte, 2.2 ml of pH 4-6 ampholyte, and 1 ml of pH 6-8 ampholyte to a 5-55% linear sucrose gradient to stabilize the pH gradient produced during electrophoresis. Either 10 or 50 mg of F-1 was added to the 55% sucrose prior to the pouring of the gradient. Following 72 hr electrophoresis at 1,000 volts the column was fractioned into 30 drop, approximately 2 ml, fractions. The pH of each fraction was determined, the fractions were dialyzed against distilled water to remove the sucrose and ampholytes, and protein concentrations were determined by the method of Lowry. The fractions were titered in the ELISA using an antiserum raised against whole organisms. The ability of these fractions to inhibit the standard test antigen/standard serum ELISA inhibition was quantified by dividing the µg of protein in each fraction which reduced the OD to 0.25 by the µg of F-1 diagnostic antigen which gave the same result. This was expressed as an ELISA inhibition index.

There was a wide variation in the ability of the fractions to interfere with the ELISA (Table 6). Fraction 51, which titered 1/1280, required 62 times more protein than F-1 to inhibit the

Table 6. Comparison of the properties of antigenic peaks isolated following isoelectric focusing of Lot 4 F-1 diagnostic antigen.

Fraction number	ELISA titer[a]	pH	ELISA Inhibition index[b]
44	1/320	4.3	15
51	1/1280	3.9	62
60	1/1280	3.6	0.2
66	1/1280	1.8	28

[a] Last dilution of the fraction producing a visible color change when tested with a 1/100 dilution of serum produced against whole Y. pestis organisms, Antigua strain.

[b] µg of protein in fraction required to reduce the OD to 0.25 divided by the µg of Lot F-1 protein required to achieve the same results.

ELISA to the same extent. Conversely, fraction 60, with an equivalent ELISA titer, required less protein than the F-1. Intervening fractions gave a logical progression of intermediate values. These data indicate that the present F-1 diagnostic antigen contains several microbial antigens with differing isoelectric points and abilities to interfere with standard serological assays, but with equivalent antigenic contents.

4. Transmission of Y. pestis by rodents and fleas.

A. Influence of plague on rodent population sex ratio.

Rodents trapped in endemic plague foci have occasionally demonstrated sex ratios favoring females. Rattus rattus trapped in the Cumbun Valley of India from 1931-1933 were 63% females. R. rattus captured in a rural plague focus in Kenya from 1934-1937 were 58% females. R. norvegicus collected at Nha Trang, Vietnam in 1966-1967 were 57-62% females during epidemic plague seasons and 62-73% females in non-epidemic seasons. Such ratios may have resulted from influences unrelated to plague per se, as numerous observations of 52-71% females have been reported for

rat populations in plague-free areas. But where rodent plague exists, resistance to disease or exposure to infection might also influence sex ratio. To date, different susceptibilities in male and female rats have not been demonstrated, and in Kenya, at least, male and female rats exhibited identical infestation rates for vector fleas, so exposure to infection via fleabite appeared similar for the sexes. Nevertheless, plague transmission among rodents also may occur by the consumption of infected carcasses, and the influence of this process on population sex ratios has not been evaluated.

Here we report on the effects of oral transmission of plague in populations of laboratory mice with a high and uniform susceptibility to the disease. The conditions necessary to induce cannibalistic behavior were determined in groups of 20 mice (10 males and 10 females) given various rations of baked biscuits. Cannibalism was not observed among mice fed $\geq$ 2 g/day, although feeding behavior and body weights indicated that stress was experienced by mice given less than 4 g/day (Table 7). Starved mice cannibalized, and a greater mortality in males than females resulted.

Experiments then were conducted to measure the effects of plague on sex ratios of laboratory mouse populations fed 1.5 g/day per mouse. Our first experiment included two cages of 30 mice, 50% each sex. In one cage (i.e., infected), 3 male and 3 female mice were inoculated subcutaneously (sc) with 31,500 Yersinia pestis of the virulent Indian strain 195/P cultured at 25 C. Mice from the other cage (i.e., noninfected) were not inoculated. A second experiment employed five sets of cages: 20 males; 15 males plus 5 females; 10 mice of each sex; 5 males plus 15 females; 20 females. Two mice from the infected cage in each set were inoculated sc with 3,000 Y. pestis strain 195/P cultured at 25 C. In both experiments, a census of mice was conducted three times a week for four weeks after inoculation of Y. pestis, carcasses found in cages were collected and used to confirm infection by isolation of plague bacilli from tissues, and normal mice were added to replace those that had died.

Deaths occurred in noninfected and infected cages, carcasses were cannibalized readily by cagemates, and mortality was rather constant over time. The mice inoculated with Y. pestis died within five days, but Y. pestis was isolated from carcasses taken from infected cages over the course of the experiments. Similar isolation attempts with carcasses collected from noninfected cages

Table 7. The effects of limited food supplies on mice.

Food provided (g/day per mouse)	Mortality after 10 days		Weight (g) after 10 days		Observations
	Males	Females	$\bar{X}$ (range) males	$\bar{X}$ (range) females	
0	100%	60%	-	16.5 (15.6-18.7)	Cannibalism frequently commenced immediately after death or even during agonal stage.
2	0	0	20.9 (12.5-27.6)	18.4 (15.6-21.9)	Food consumed quickly when placed in cage.
4	0	0	30.8 (25.7-35.8)	25.8 (17.7-35.2)	All food consumed in 24 hours.
8	0	0	33.0 (28.8-37.1)	28.6 (25.0-32.7)	Food always present in cage.

were always negative. Adequately fed normal mice added to dirty cages that had been replaced by clean cages for experimental groups never experienced plague in our experiments. Other workers also have shown that laboratory mice do not derive plague infections from soiled litter.

When food was scarce, laboratory mouse populations experienced significantly higher mortalities for males than females ($p < .005$ by Chi-square), irregardless of the presence or absence of plague within the group (Tables 7 and 8). Cages that had initial sex ratios of 1:1 usually exhibited ratios that favored females when subsequently censused. When plague was present, the disproportionate death-rate for males was not as evident, although mortality for both sexes increased significantly ($p < .005$ by Chi-square). However, total mortality increased as males comprised a greater percentage of the mice in infected cages. This observation prompted us to undertake a third experiment to compare mortalities in populations of males held at various densities (Table 8). Three males from each infected cage were inoculated sc with 31,500 Y. pestis 195/P cultured at 25 C. Surprisingly, in the absence of females, male population density had no demonstrable influence on rates of mortality in either noninfected or infected cages. It may be that aggression among males, manifested only when females are present and more severe when females are few, influences the mortality of males, irrespective of plague in the population. In our experiments, some transmission of disease by fighting cannot be ruled out, but the equalization of male and female mortalities when plague was present suggests that transmission occurred predominantly via the cannibalism of infected cagemates.

These experiments illustrate two effects of plague on murine populations: an increase in mortality overall, as observed often in wild rodents, and an equalization of the percentages of males and females in the population, as seen for R. norvegicus caught during epidemic periods in Vietnam compared with captures during non-epidemic seasons.

B. Transmission studies of aberrant (Fraction 1 negative) strains of Yersinia pestis by fleas (Siphonaptera).

1) Background.

Recently, aberrant strains of Y. pestis have been recovered from natural infections (Annual Progress Reports, WRAIR). The efficiency with which fleas can transmit these aberrant plague

Table 8. Mortality in laboratory mouse populations.

Initial population sex ratio (male:female)	Mice per cage	Floorspace per mouse (cm^2)	Number dead over 28 days		Deaths/day per 1,000 mice		Males per female dead	
			Noninfected cage	"Infected" cage	Noninfected cage	"Infected" cage	Noninfected cage	"Infected" cage
Experiment No. 1								
1:1	30	95	33	83	39	99	15.5	1.7
Experiment No. 2								
all males	20	55	24	90	43	161	-	-
3:1	20	55	35	89	63	159	4.8	4.6
1:1	20	55	35	81	63	145	7.8	1.5
1:3	20	55	25	81	45	145	0.4	0.6
all females	20	55	17	72	30	129	-	-
Experiment No. 3								
all males	20	64	49	91	44	81	-	-
	40	32	102	177	46	79	-	-
	80	16	196	338	44	75	-	-

strains is unknown. A collaborative study has been established with the Department of Microbiology, School of Medicine, University of Maryland, Baltimore, Md., to investigate the ability of the Oriental rat flea, Xenopsylla cheopis, to transmit aberrant strains of Y. pestis. This flea is the classical vector of plague.

Eskey and Haas (1), Burroughs (2, 3), Cavanaugh (4), Holdenreid (5), and Wheeler and Douglas (6, 7) have proposed various techniques for safely handling plague infected fleas in the laboratory. Although these workers have made significant advances toward understanding the epidemiology of urban and sylvatic plague, we felt their methods of handling and feeding fleas during Y. pestis transmission studies were not adequate for our requirements. Studies with aberrant strains require absolute accountability for every flea. These strains produce fulminating, lethal infections when inoculated into vaccinated rodents and have produced frank disease in man. Therefore, the development of suitable techniques was mandatory.

2) Development of a flea feeding apparatus.

A colonizing and feeding apparatus has been designed that satisfies the stringent safety requirements (Figs. 6 and 7). The apparatus, incorporating some of the Berlese principles, is constructed of a clear, acrylic tube (outside diameter, 2.6 cm; inside diameter; 1.9 cm) divided into 3 easily connectable sections. The bottom (Section 1), 3.0 cm long, is filled with a substrate composed of a charcoal/Plaster-of-Paris mixture (1:9), which provides a continuous source of humidity in the flea holding chamber (Section 2). The substrate is moistened periodically by adding a few drops of distilled water. Section 2 is long (21 cm) to prevent fleas from jumping out while connecting and disconnecting the mouse-holding chamber (Section 3). A stainless steel grid (2.2 cm in diameter with 0.25 cm holes) separates Sections 2 and 3, and confines the rodent to Section 3. Section 3 is 14.8 cm in length.

During operation, Sections 1 and 2 are connected and the desired number of fleas are placed in Section 2. The fleas are contained in this Section by a nylon mesh cover over the free end except during feeding.

During feeding, a 3-7 day old suckling mouse is placed in Section 3. The open end of Section 3 is securely covered with nylon mesh to prevent the escape of fleas. The nylon mesh is removed from Section 2 and it is connected to Section 3. The entire apparatus is inverted allowing the fleas to pass into Section 3. The apparatus is laid on its side while the fleas are feeding on the mouse (Fig. 7). When feeding is complete, the apparatus is placed upright with Section 1 and 2 down until all fleas have returned to Section 2. Section 3 is removed, and Section 2 is covered with nylon mesh. The apparatus provides a closed system except during connection and disconnection of Sections 2 and 3. Each apparatus is stored and operated in a 60 X 60 X 18 cm stainless steel pan. Preliminary testing of the apparatus with noninfected fleas has proven its effectiveness in support of flea maintenance and safety.

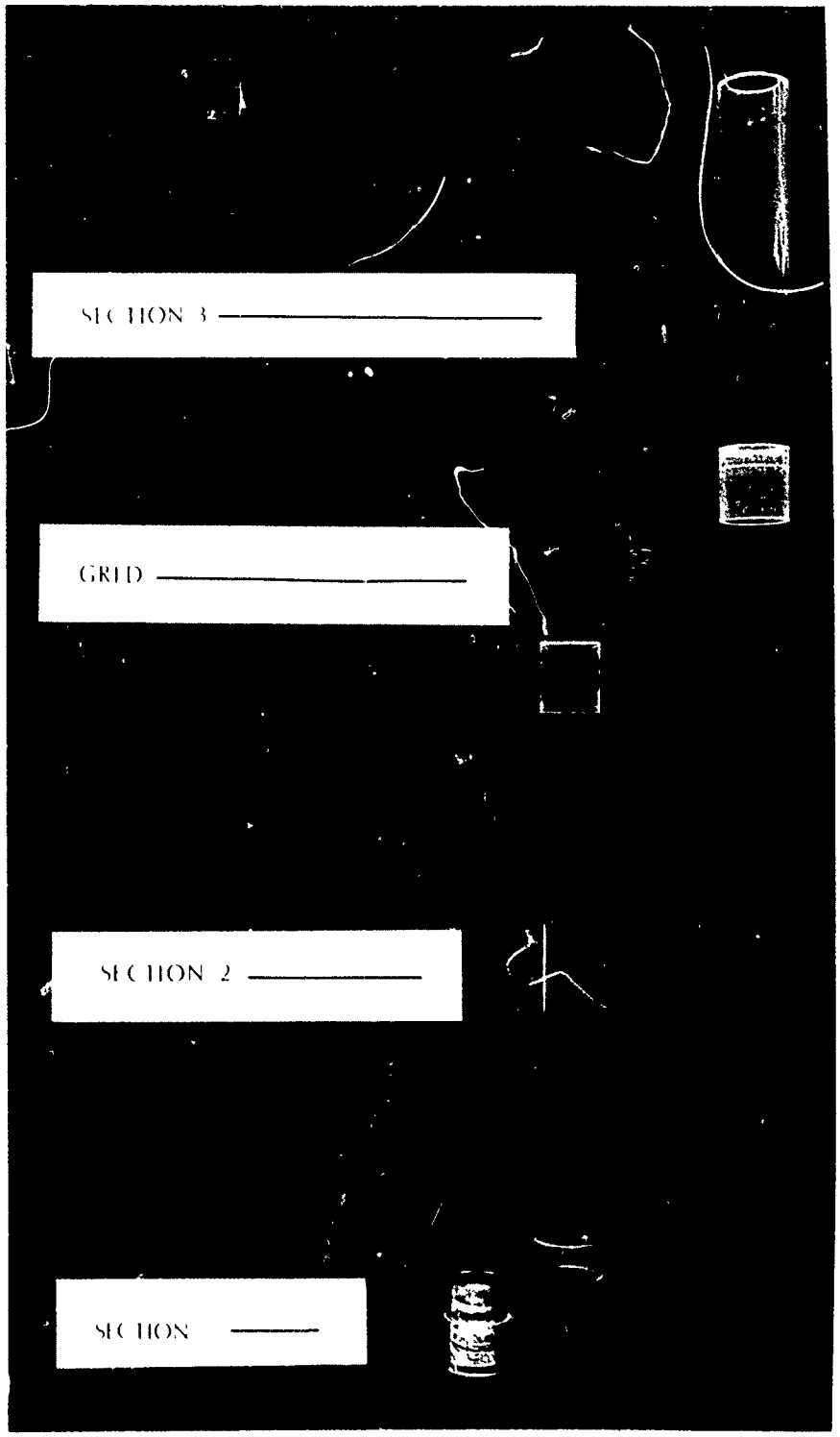

Figure 6. Disassembled flea feeding apparatus illustrating components.

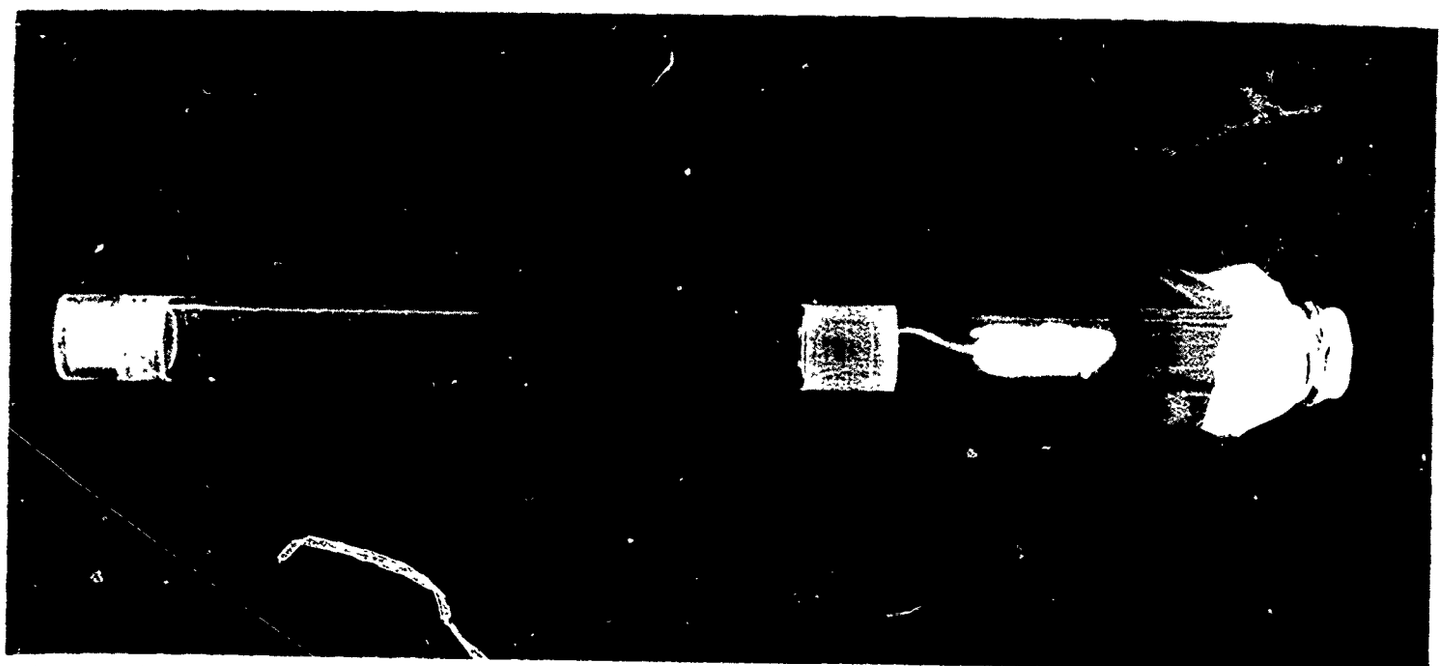

Figure 7. Assembled flea feeding apparatus illustrating horizontal position during flea feeding.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 133 Ecology of Plague

Literature Cited.

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7. Wheeler, C.M. and Douglas, J.R. Sylvatic plague studies. V. The determination of vector efficiency. J. Infect. Dis. 77: 1-12, 1945.

Publications:

1. Williams, James E. and Cavanaugh, Dan C. Measuring the efficacy of vaccination in affording protection against plague. Bull. World Health Org. 57(2): 309-313, 1979.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6449	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	134
b. CONTRIBUTING				
c. XXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Immunological Mechanisms in Microbial Infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology 003400 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION

b. NUMBER

c. TYPE d. AMOUNT

e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	2.0	186
FISCAL YEAR CURRENT 80	2.0	163

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Immunity; (U) Antibodies; (U) Infectious Diseases; (U) Complement Fixation; (U) Radioimmunoassay; (U) Serodiagnosis

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) The objective of this work unit is to elucidate the mechanisms operative in the natural and artificial induction of immunity to a variety of microbial infections of military importance. This includes the study of infections in model systems and the development of methodologies for the study of the immune reaction in humans for research as well as diagnostic evaluations.

24 (U) The approaches used for these studies involve the measurement of various parameters of disease and of the immune response to disease in both in vivo and in vitro experiments. A variety of diseases are attacked. Immunological phenomena common to a variety of different diseases are also studied.

25 (U) 78 10-79 09 Murine peritoneal macrophages exposed to amastigote form of Leishmania tropica showed an increase both in the percent infected macrophages and the number of intracellular survival of amastigotes in cultures containing lymphokines. Pretreatment of macrophages with lymphokine reduced the number of infected macrophages when exposed to amastigotes. Lymphokines added to infected macrophages induced intracellular killing of amastigotes. All patients with active atopic dermatitis had markedly reduced number of TG cells, while the total T cell population and TM cells were within normal limits. There was an inverse relationship between TG cells and serum IgE levels. Guinea pig IgG1 and IgG2 immunoglobulins can be effectively separated on protein A-Sepharose as a function of pH. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 1979.

*Available to contractors upon originator's approval

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Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 134 Immunological Mechanisms in Microbial Infections

Investigators:

John Barbaro, Ph.D.; Barry Ellis; Carol Nacy, Ph.D.; Donald Wong, Ph.D.

I. Isolation of immunoglobulins from sera using protein A-Sepharose.

Objective: This study was undertaken to determine if protein A -Sepharose offered a simple, rapid and better method for the isolation and purification of immunoglobulin subclasses.

Description: The major feature of protein A is its affinity for immunoglobulins of a wide variety of species. Although protein A's binding is notably with IgG immunoglobulins, IgM and IgA bind from certain species (1). Ey et al described the fractionation of mouse serum on protein A-Sepharose as a function of pH. Essentially, all the mouse immunoglobulin IgG subclasses were bound at pH 8.0 and the various IgGs could be sequentially eluted with buffers of decreasing pH(2). The presence of immunoglobulins with overlapping biologic, electrophoretic and immunologic properties often makes it difficult to isolate immunoglobulins of suitable purity in adequate quantities for analysis or testing in various assay systems. The procedure described for separation of mouse immunoglobulin may be most useful for the fractionation of IgG subclasses from other species. In order to determine the suitability of this method, immune guinea pig serum was fractionated on a protein A-Sepharose column. Immune guinea pig serum was selected because it contains two types of IgG immunoglobulin, IgG1 and IgG2, distinguishable by their electrophoretic, biologic and antigenic properties (3,4,5).

Guinea pig anti ovalbumin serum pool was fractionated on a protein A-Sepharose column using phosphate-citrate buffer (pH 3.0 - pH 6.0) for elution of the various immunoglobulins. The fractions were pooled and assayed for IgG2 (complement fixation) and IgG1 (passive cutaneous anaphylaxis (PCA)).

Progress: Figure 1 depicts both the elution pattern of guinea pig immunoglobulins from protein A-Sepharose and the biologic activity of each fraction. The results show that 48.6 percent of the complement activity (IgG2) is located in fraction V and that fraction V and VI accounts for 62.9 percent immunoglobulin IgG2 activity. On the other hand, fraction III possesses 52.8 percent of the PCA activity (IgG1) and fraction III and IV have 69.9 percent of immunoglobulin IgG1 activity. Figure 2 shows the different electrophoretic mobility between fraction III (IgG1) and fraction V (IgG2).

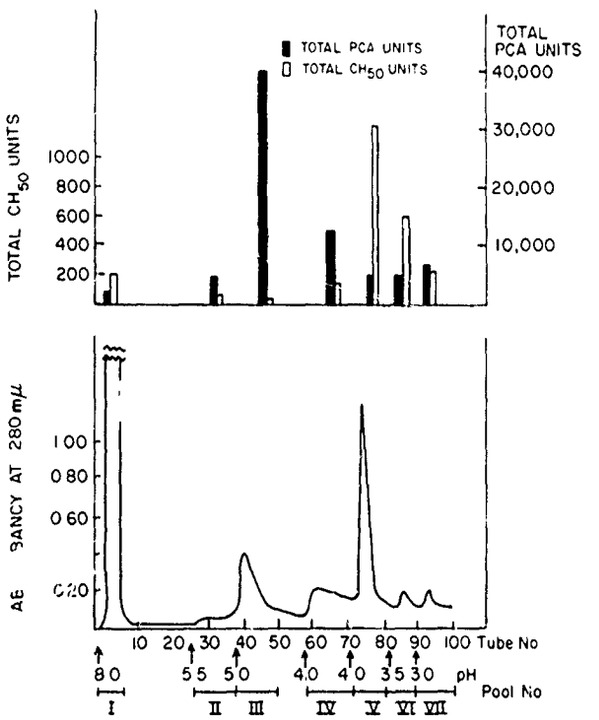

Fig. 1. Elution pattern and biologic activity of guinea pig antiserum.

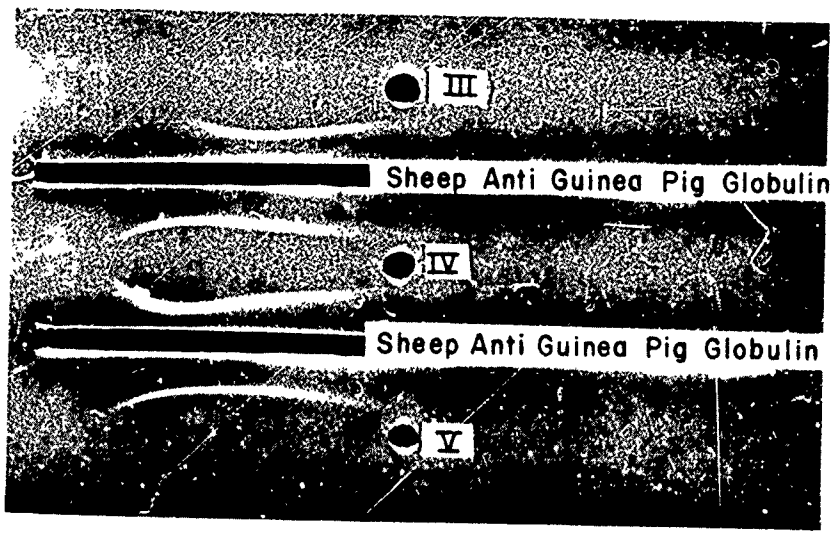

Fig. 2. Immunoelectrophoresis of guinea pig fractions isolated on protein A-Sepharose.

Discussion: Although the results clearly demonstrate the suitability of separating guinea pig immunoglobulins as a function of pH, the optimal conditions for the complete separation of IgG1 and IgG2 need to be determined. Parish has reported that guinea pig serum has two IgG1s, distinguishable by their latencies and persistence in skin (6). Future work will attempt to isolate these two immunoglobulins and to extend the work to include sera from other species.

II. Interaction of Leishmania with macrophages.

Objective: To understand the growth of leishmania in macrophages and the killing of leishmania by macrophages activated with soluble T lymphocyte products. This background information will be useful for the development of an effective vaccine for leishmaniasis.

Description: Different antigenic species of Leishmania cause cutaneous, mucocutaneous, or visceral infections in man.

These diseases are vector transmitted. Infected sand flies contain the flagellated promastigote form of the parasite which transforms into the obligate intracellular amastigote form when introduced into the host. Observation of lesions developing from any of several leishmanial species suggests that the only form present is the amastigote, and the organisms are confined to macrophages (L. McKinney, personal communication).

Immunity to leishmaniasis has been correlated with the development of cellular immunity, and delayed hypersentitivity can be demonstrated to leishmanial antigens. A useful in vitro correlate of cellular immunity is the activation of macrophages by soluble products (lymphokines) of sensitized T lymphocytes for the cytolysis or cytostasis of neoplastic cells and bacteria (8, 9). Since the leishmania are obligate intracellular parasites of macrophages, we were critically interested in the ability of macrophages to be activated by lymphokines for leishmanial killing. We chose a mouse model for our initial studies, since a great deal is known about mouse macrophages, and macrophages are readily available in large numbers in the peritoneal cavity.

Progress: Peritoneal cells (PC) were harvested from C3H/He mice by peritoneal lavage, adjusted to $1x10^6$ macrophages/ml, and dispensed in 0.5 ml quantities in polypropylene tubes. Amastigotes, harvested from foot pad lesions of BALB/c mice, were added to PC cultures at a ratio of 7 amastigotes/macrophage for 1 hour. Samples were removed 1, 24, 48, 96, and 192 hours after infection: growth of amastigotes in macrophages was monitored by examination of Giemsa-stained cell smears (Table 1) for percent of infected macrophages and number of intracellular amastigotes infected macrophages.

Table 1
Infection of resident peritoneal macrophages by Leishmania tropica.

Time (Hour)	Percent Macrophages Infected	Mean Amastigote/ infected macrophage
1	41	2.7
24	90	9.9

Time (Hour)	Percent Macrophages Infected	Mean Amastigote/ infected macrophage
48	89	9.1
96	97	10.3
192	97	26.0

Both percent infected macrophages and numbers of intracellular amastigotes increased with time in culture; however, it was difficult to know if the dramatic increase observed at 24 hours was due to rapid multiplication of the amastigote or increased uptake of remaining amastigote inoculum. Several efforts were then made to remove excess amastigotes: 1) By washing PC after infection, 70% of the original amastigote inoculum could be accounted for in the discarded supernatant and intracellularly in macrophages. The remainder of the amastigotes were pelleted with PC as amastigote clumps or attached to lymphocytes. 2) We then attempted to remove the inoculum by Ficol-Hypaque sedimentation. Surprisingly, the amastigotes banded in exactly the same area as the macrophages. Separation was not achieved by this technique. For the remainder of the studies, amastigote inoculum was reduced (1-3 amastigotes/ macrophages) and macrophage cultures were washed after the infection period. A persistant increase in infected cells occurred at 24 hours, but the level of increase was predictable using the treatments described above.

In an effort to understand the nature of the increase at 24 hours, macrophage cultures were exposed to latex beads alone, amastigotes alone, or latex beads and amastigotes combined (Table 2).

Although the number of macrophages ingesting latex beads was the same as the number ingesting amastigotes (34%), it is clear that nonspecific phagocytosis is not the only way in which amastigotes entered cells. In experiments where macrophages were exposed to latex beads and amasti-

Table 2

Exposure of resident peritoneal macrophages to latex beads or amastigotes of *Leishmania tropica*.

Time (hrs)	Percent Infected Macrophages				
	Latex Beads	Amastigotes	Latex Beads and Amastigotes		
			Latex beads	Amastigotes	both
1	34 ± 2.9	34.5 ± 1.4	18.6 ± .5	33.5 ± .8	6.5 ± .6
24	32 ± 2.1	55 ± 1.6	23 ± 1.3	35.5 ± 1.2	17 ± 1.2

gotes, the number of cells ingesting both was minimal (6%). The number of cells ingesting amastigotes alone remained the same as control cultures (34%). The 24 hour increase in percent of infected macrophages (55%) can be accounted for by an increased ingestion of amastigotes by cells which are non-specifically phagocytic (contain latex beads), while those cells containing amastigotes alone (35%) remain the same as control cells at 1 hour.

We produced lymphokines from spleen cells of Mycobacterium bovis strain BCG infected C3H/He mice stimulated by PPD in vitro (8). The direct effect of lymphokine on amastigote viability was tested by adding lymphokines to several samples during the infection period. No difference was detected in infectivity or intracellular survival of amastigotes in control culture containing lymphokines (Table 3). Pretreatment of macrophage cultures for 4 hours with lymphokine significantly reduced the number of infected macrophages after 1 hour exposure to amastigotes. Lymphokines added after infection also induced intracellular killing of amastigotes. The numbers of infected macrophages at 24 hours in cultures treated with lymphokines after infection were significantly reduced compared to control cultures.

Discussion. Although considerable headway has been made in the understanding of leishmanial infection and growth in macrophages, results for killing of leishmania by activated macrophages are in the preliminary stage. Further studies are planned to analyze the population of macrophages capable of killing leishmania, the signals necessary to activate the killing of leishmania, and the mechanism(s) of killing.

III Investigation of immunologic imbalance in atopic dermatitis.

Objective: Continue the collaborative study with the Allergy-Clinical Immunology Service, WRAMC, to determine if an immunologic imbalance exists in atopic dermatitis, particularly in regard to suppressor T-cell function.

Table 3

Exposure of amastigotes of L. tropica to lymphokines (LK): effect on infectivity and intracellular survival.

Time (Hours)	Percent Infected Macrophages					
	Control Macrophages:		LK Pretreatment of MØ: 4 hr		Treatment of MØ with LK after infection	
	No LK at infection	LK at 1 hr infection	No LK at infection	LK at 1 hr infection	No LK at infection	LK at infection
1	34.5 ± 1.4	35 ± 1.5	23.8 ± .8	23.8 ± 1.4	ND	ND
24	55 ± 1.6	54.4 + 2.9	34.2 ± 1.1	36.5 ± 0.9	42.2 ± 1.3	44.5 ± 0.6

Description: The previous annual report described in detail the preparation of reagents and the procedures used in this study. Briefly, the IgE levels, total lymphocyte, and the absolute and relative number of TG and TM cells were compared in four groups consisting of: 1) 12 patients with active atopic dermatitis, with mean IgE concentration of 5678±1382 IU/ml with a range of 350 to 14,800 IU/ml. 2) 5 patients with inactive atopic dermatitis with mean IgE level of 291±141 with a range of 22 to 500 IU/ml. 3) 4 patients with allergic rhinitis and/or asthma without atopic dermatitis with mean IgE level of 1263±974 IU/ml with a range of 110 to 3200 IU/ml and 4) 12 healthy individuals with mean IgE concentration of 33±17 IU/ml with a range of 0.5 to 121 IU/ml.

Progress: Absolute numbers of lymphocytes were comparable in the patient and normal control group. Both groups also had comparable relative and absolute numbers of T-lymphocytes. Analysis of T-cell subpopulations revealed similar values for relative and absolute numbers of TM cells. However, atopic dermatitis patients had markedly reduced relative TG levels, 1.79±0.37% compared to normals 10.5± 0.67% ($p < 0.001$). The patients also had reduced absolute TG levels, 29±7% compared to 181 ±36% ($p < 0.0005$). There was no significant decrease in the relative or absolute numbers of TG cells in either inactive atopic dermatitis patients or in patients with allergic rhinitis and/or asthma when compared to normals. Sequential studies of one patient with active atopic dermatitis showed normalization of TG levels from 3 to 9% together with clinical improvement.

Discussion: Results of this study are highlighted by the finding of a selective deficiency of TG cells in patients with active atopic dermatitis. All patients with active atopic dermatitis had markedly reduced values of TG cells; whereas, numbers of total T-cells as well as TM cells were normal. There was a striking inverse relationship between low TG cells and serum levels of IgE. The basis of the relationship of decreased TG cells to the increased levels of IgE found in active atopic dermatitis is not known at this time.

IV. Characterization of virulent (Vi) antigen variation.

Objective: A collaborative study with the Department of Bacterial Immunology has started to investigate the virulent (Vi) antigen variation. Various immunological procedures will be used to determine chemical differences among Vi antigen containing hybrids.

Description: Understanding the genetic and structural changes that occur during Vi antigen variation will result in better immunoprophylactic methods for the prevention and control of typhoid fever. There have been conflicting reports concerning the value of Vi antigen in human immunity to typhoid. The difficulty in resolving the problem is an incomplete understanding of the chemical nature of Vi antigen, resulting in studies done with contaminated or chemically modified antigen.

The genetic determinants of the Vi antigen of Citrobacter ballerup were conjugally transferred to a Salmonella typhi recipient whose own Vi antigen determining genes had first been removed. Many of the hybrids generated expressed an incomplete (intermediate) form of the Vi antigen while others expressed an additional Vi antigen component (designated orange) that is found in the complete antigen of the C. ballerup parent. Synthesis of the orange hybrids were subject to the same rapid reversible variation seen in the parent donor strain; whereas, synthesis of antigen from intermediate hybrids appeared to be continuous and not subject to regulatory control (7).

Progress: Progress has been limited by the lack of suitable antisera to distinguish different Vi antigens. A "purified" Vi antigen was obtained from Dr. Robbins, NIH. On immunoelectrophoresis the "purified" Vi antigen give a single line when tested with antiserum prepared against C. ballerup (parent donor strain) and multiple bands when tested against Burro anti S. typhi. Although only a single precipitin band developed with anti-C. ballerup there is an indication of considerable charge heterogeneity with the "purified" Vi antigen. Presently, our efforts have been directed towards preparing various antigen extracts

from different Vi bearing hybrids. The extracts will be analyzed with the available antisera and then will be used to prepare antisera.

Discussion: The preliminary results with the "purified" Vi antigen reinforces the need for monospecific antisera and antigens. Functional monospecific antisera will be prepared using affinity chromatography to remove unwanted antibodies. This will enable the isolation, purification and characterization of the Vi antigen. The preliminary results also indicate that simple immunoelectrophoresis will be of limited value and that such procedures as tandem crossed and crossed-line immunoelectrophoresis will be needed to analyze various preparations.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 134 Immunological mechanisms in microbial infections

Literature Cited:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OR 5513	79 10 01	DD DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	135
b. CONTRIBUTING				
c. CONXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Mechanisms of Transmission of Hepatitis Viruses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology 003500 Clinical Medicine

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
72 07	CONT	DA			C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA — EXPIRATION:
b. NUMBER:
c. TYPE: — d. AMOUNT:
e. KIND OF AWARD: — f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	2.5	87
	CURRENT 80	2.0	133

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
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21. GENERAL USE

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22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Viruses; (U) Hepatitis; (U) Antigen; (U) Immunology

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific methods for detection of hepatitis viruses, antigens and antibodies to determine factors important in resistance to disease and infection in military personnel.

24 (U) New methods for identification and antigenic analysis of hepatitis viruses are under development. The immune response of patients infected with hepatitis viruses is studied to define sensitive parameters of infection and to define factors critical in immunity. The epidemiology of hepatitis B in military populations is defined.

25 (U) 78 10 - 79 09 A sensitive and specific assay for IgM antibody to hepatitis A virus was used to study an outbreak of hepatitis at SHAPE, headquarters in Belgium. It was found that the asymptomatic to symptomatic ratio of hepatitis A infection in children at SHAPE was much lower that in children studied at Ft Richardson in 1977. Peak IgM antibody responses occurred within 30 days after the onset of symptoms. IgM antibody was detectable as long as 120 days after the onset. Preliminary analysis of hepatitis patients admitted to hospitals in Germany, Korea and Ft Hood indicate hepatitis B virus causes 70 percent of hospitalized cases OCONUS and at least 40 percent at Ft Hood. This study permits the first assessment of the frequency of non-A, non-B hepatitis in active duty military. This information and the diagnostic assays will aid in the evaluation of immunoprophylactic measures in the future. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 135 Mechanisms of Transmission of Hepatitis Viruses

Investigators:

Principal: COL W.H. Bancroft, MC; MAJ S.M. Lemon, MC

Associate: LTC M. Benenson; SFC T.E. Simms; SSG D.S. Brooks; SSG C.D. Brown; SP4 J.R. Payne; H.G. Cannon

Description

To define the epidemiology of hepatitis in military populations in order to establish methods for reducing disability from hepatitis. Emphasis is on developing and applying sensitive and specific methods for detection of hepatitis viruses/antigens and antibody to determine host factors important in resistance to disease and infection.

Progress

I Hepatitis A Virus (HAV)

A. Determination of anti-HAV antibody titer in provisional reference "Antibody to Hepatitis A Antigen" lot #1.

1. Background

Despite the widespread use of immune serum globulin (ISG) for the prophylaxis of HAV infections, commercial lots of ISG do not contain standard concentrations of anti-HAV, and the anti-HAV titer of available commercial lots remains poorly defined. The Hepatitis Branch, Division of Blood and Blood Products, Bureau of Biologics therefore initiated a collaborative international evaluation of six samples (code letters A through F) of a provisional reference antibody lot.

2. Methods

Antibody titer was determined in three replicate specimens

of each provisional reference sample utilizing the solid phase radioimmunoassay (SPRIA) for anti-HAV described in the FY 78 Annual Report. The first specimen of each sample was tested twice in replicate microtiter plates. In addition, 2 lots of Hyland Laboratories ISG, recently purchased by the Department of Defense, were tested as well. Prior to testing, each specimen was diluted to 10^{-2} in phosphate buffered saline; subsequent dilutions were made in 1% normal human serum (anti-HAV negative) so that the absolute protein concentrations would be roughly equal at each dilution. IgG and IgM concentrations were determined by radial immunodiffusion.

3. Results

Results are shown in Table 1. For each specimen tested, the $-\log_{10}$ 50% blocking dilution was determined by interpolation. The final titer of antibody was derived from the mean of the replicate samples tested. Anti-HAV titer varied from 1:1300 (Sample C) to 1:6900 (Sample A), and correlated roughly with total IgG. Anti-HAV titers of the two commercial lots were 1:2500 and 1:4300, respectively. These results were forwarded to the Bureau of Biologics.

B. Comparison of commercial radioimmunoassay (HAVAB (R)) with WRAIR SPRIA.

1. Background

During this fiscal year, a commercial assay for determination of anti-HAV antibody, termed HAVAB, was marketed by Abbott Laboratory, Chicago. The HAVAB test is conceptually similar to the WRAIR SPRIA but uses plastic beads rather than microtiter plates as a solid phase support. Use of the commercial assay is advantageous in terms of technician time spent on the procedure, and the conservation of hepatitis A antigen. Therefore, an effort was made to compare the specificity of HAVAB with the WRAIR SPRIA.

2. Methods

Sera collected during the Fort Richardson hepatitis A

Table 1. Anti-HAV Reference Lots: WRAIR SPRIA

Sample	SPRIA RESULTS: Replicate Determinations $-\log_{10}$ 50% Blocking Dilution				mean	estimated titer	RADIAL IMMUNODIFFUSION: IgG (mgm/ml)	IgM (mgm/dl)
A	>4.00	3.84	3.50	3.71	3.8	1:6900	140	146
B	3.77	3.54	3.22	3.51	3.5	1:3200	102	60
C	3.66	2.92	2.66	2.96	3.1	1:1300	33	<10
D	----	3.48	2.92	3.33	3.2	1:1600	112	146
E	3.68	3.70	3.66	3.48	3.6	1:4000	162	60
F	3.57	3.59	3.34	3.61	3.5	1:3200	126	146
Hyland 1AA*	3.46	3.54	3.29	3.33	3.4	1:2500	---	146
Hyland 2AA*	3.68	3.78	3.53	3.53	3.6	1:4300	---	146

*Hyland ISG lots 0632C001AA and 0632C002AA

Table 2. Comparison of HAVAB Test with Anti-HAV SPRIA Developed at WRAIR and at Baylor University*

Reference Test		HAVAB Result	
		negative	positive
WRAIR	negative	11	0
	positive	0	28
BAYLOR	negative	8	0
	positive	2	30

*Total of 49 sera tested by HAVAB, 39 by WRAIR and 40 by Baylor.

outbreak (see Annual Report FY 77) and previously tested by SPRIA at WRAIR and at Baylor University (see Annual Report FY 78) were tested by HAVAB according to instructions included with the kit.

3. Results

Table 2 demonstrates 100% concordance between results of the HAVAB and WRAIR test on 39 sera. The Baylor test also correlated well with the HAVAB assay (40 sera), although two specimens that were positive in the Baylor assay were negative in both HAVAB and WRAIR tests. The HAVAB test was subsequently adopted for routine anti-HAV determinations in this laboratory.

C. Development of a solid phase radioimmunoassay specific for IgM class antibody to hepatitis A virus.

1. Background

Currently, available assays for detection of antibody to hepatitis A virus include the SPRIA, ELISA, immune adherence hemagglutination and immune electron microscopy techniques (see Annual Report FY 78). The SPRIA, ELISA and immune electron microscopy techniques detect IgM class antibody to hepatitis A virus and are usually positive at the time of onset of symptoms. Because IgG antibody to hepatitis A virus persists for the life of the individual, the testing of a single serum sample by SPRIA or ELISA is of limited diagnostic value. Techniques to determine subsequent increases in antibody titer are laborious, expensive and possibly misleading. The immune adherence test detects predominantly IgG antibody, and therefore can frequently demonstrate a seroconversion during acute hepatitis A virus infection. However, this requires paired serum samples which necessarily results in a delayed diagnosis. The ideal assay would be a test capable of detecting IgM antibody to HAV specifically. Such antibody has been shown in at least a small number of patients to be short lived, and therefore such a test would provide a rapid and specific method for identifying acute hepatitis A infection.

2. Methods

Initial attempts utilizing rate-zonal centrifugation of serum in a 10%-40% sucrose gradient demonstrated the presence of IgM antibody in acute phase specimens (see Annual Report FY 78). Subsequently, a solid phase radioimmunoassay for IgM anti-HAV (IgM-SPRIA) was developed based on the brief description of a technique by Duermeyer and vander Veen (Lancet ii, 684-685, 1978). This technique is based on the binding of heterologous anti-human IgM antibody to a solid phase support, followed by incubation with suspect acute hepatitis A serum. IgM anti-HAV bound to the solid phase is subsequently detected by sequential incubation with hepatitis A virus antigen and radiolabelled purified human IgG anti-HAV. Because 1) the antigen employed is not highly purified and 2) the radiolabelled IgG is derived from a convalescent patient and is not hepatitis A specific, the solid phase is flooded with an excess of normal human serum (anti-HAV negative) before the addition of the radiolabel. This assay is thus a 4-layer "sandwich" (anti-IgM--IgM antibody--HAV--IgG antibody-^{125}I) and as a direct technique provides significant advantages over existing "blocking" SPRIA methods (see Annual Report, FY 78).

a. Reagent Preparation and Materials

(1) Goat anti-human IgM (t=3.5) was purchased from Antibodies, Inc., Davis, California. This material was shown to produce optimal binding of IgM when used at a dilution of 1:1000 in a pH 9.0 carbonate buffer (1.59 gm Na_2CO_3, 2.93 gm $NaHCO_3$ and NaN_3 0.2 gm per L).

(2) Purified IgG anti-HAV was prepared as follows: 5cc AK003 ("Smith") plasma was precipitated sequentially by 45% and 35% $NaHSO_4$, the final precipitate resuspended in 1 ml 0.25 M phosphate buffer, pH 7.4, and dialyzed extensively against the same buffer. This

material was then applied to a 107 cm x 1 cm G200 Sephadex column equilibrated with 0.25 M phosphate buffer and 3 ml fractions were collected. Two major protein peaks were identified: fractions comprising the second peak were pooled, concentrated against an XM50 Diaflo filter (Amicon), and then pressure dialyzed against 0.01 M phosphate buffer, pH 6.8. The sample was then applied to a 10 ml DEAE-Sephadex A25 column equilibrated with 0.01 M phosphate buffer, pH 6.8, and eluted with the same buffer. A single protein peak was obtained, and the pooled fractions contained IgG at a concentration of approximately 2 mgm/ml. The material was aliquoted and stored at -20° until iodinated by the chloramine-T method.

(3) Hepatitis A antigen. Chimpanzee stool extracts were prepared as described in FY 78 Annual Report. HAV-positive stool samples used were CH 173 3/6 and CH 173 3/7 (see Annual Report FY 78).

(4) Anti-HAV-negative blocking serum. Serum from 4 HAVAB-negative individuals was pooled and diluted 10^{-2} in phosphate buffered saline, pH 7.2 with sodium azide 0.2% (PBS).

(5) Test sera. Sera collected during the Ft. Richardson, Schofield Barracks (Annual Report FY 78), and SHAPE (Belgium) outbreaks of hepatitis A (see below) were tested. Control sera from cases of type B hepatitis were collected as part of the study of etiology of hepatitis in active duty personnel (see below). Rheumatoid factor-positive sera were obtained from the Immunology Service, WRAMC.

b. Procedure

(1) Ninety-six well polyvinyl microtiter plates were washed with tap water and shaken dry. Only the central 60 wells were used. All subsequent manipulations of the plates were performed on damp towels.

(2) 100 µl goat anti-human IgM, diluted 1:1000 in pH 9 carbonate buffer, was added to each well. The plate was incubated for 4 hrs at 30°C.

(3) The plates were washed 5x with PBS containing 0.05% Tween 20.

(4) Wells were filled with 1% Bovine serum albumin in Dulbecco's PBS with 0.2% sodium azide and 0.5% phenol red, and the plates were placed at 4°C overnight in a humidified chamber.

(5) The plates were washed 7x with PBS containing 0.05% Tween 20.

(6) 50 µl of test serum was added to each of two replicate wells. Sera were diluted 1:10 in PBS for screening purposes. Further dilutions were carried out in PBS. The plates were incubated at 30°C for 4 hours.

(7) Plates were washed as in step 5.

(8) 20 µl of of a HAV-positive 10% chimpanzee stool extract was placed into each well, and plates were placed at 4°C overnight.

(9) Plates were washed as in step 5.

(10) 20 µl of the anti-HAV-negative serum pool was added to each well, and the plates were held at room temperature for 15 minutes.

(11) Without washing, 30 µl of the ^{125}I-IgG anti-HAV (250,000 cpm/30 µl) was added to each well, and the plates gently agitated and placed at 4°C for 2.5 hours.

(12) Plates were washed as in step 5.

(13) The plates were tapped dry, and individual wells were cut out and bound ^{125}I was measured.

c. Interpretation of Test

On each plate, 4 sera were included as IgM anti-HAV negative controls (2 sera were HAVAB positive, 2 were HAVAB negative). From the 8 wells filled with these 4 sera, a "negative mean" cpm value was calculated. Subsequently, for each serum tested, a mean cpm value for the replicate wells was determined, and a P/N value calculated by dividing this value by the "negative mean." P/N values greater than or equal to 2.2 were considered positive (see below).

A series of dilutions of an IgM anti-HAV-positive control serum was included on each plate. The titer of anti-HAV was established as the highest serum dilution yielding a P/N value $\geq$ 2.2.

3. Results

The P/N values obtained with the anti-HAV positive serum control (SHAPE B021) ranged from 28.6 (1:100 dilution) to 1.8 (1:1000,000) when a 10% fecal extract was employed as antigen (Figure 1). Reducing the concentration of the antigen to 6% fecal extract resulted in no reduction in the observed P/N values. However, further reduction in the antigen concentration did result in a general reduction of P/N values, especially at lower dilutions of test sera (also Figure 1). Ten percent extract was routinely used in further studies.

One hundred sera collected during the studies listed above and lacking detectable anti-HAV activity by HAVAB were tested at a 1:10 dilution. The mean P/N value obtained was 1.12 (S.D.=0.359). In subsequent testing, all P/N values greater than three standard deviations from this value (i.e. $\geq$ 2.2) were considered to be positive. Twenty-two sera taken from individuals without a recent history of hepatitis but containing anti-HAV detectable by HAVAB were also tested. The mean P/N of these specimens was 1.14 (S.D.=.303). None of these sera gave a P/N which fell in the positive range.

Seventy sera collected from patients within 30 days of onset of symptoms during three epidemic outbreaks

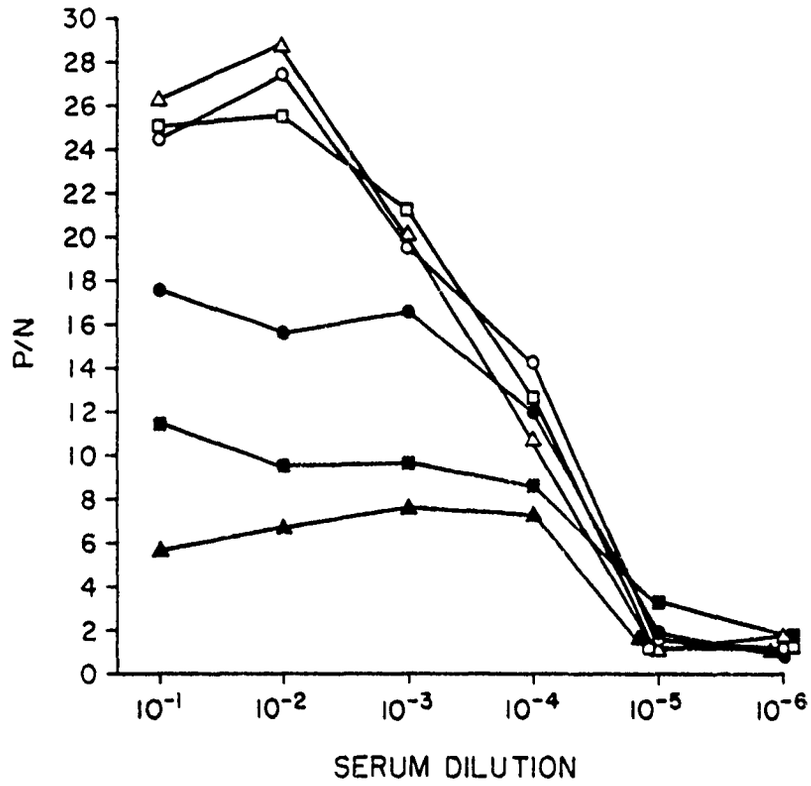

Figure 1. SPRIA for IgM anti-HAV employing varying concentrations of HAV antigen at different dilutions of an IgM anti-HAV positive serum. HAV antigen preparations were 10% (O), 8% (□), 6% (Δ), 5% (●), 2.5% (■) and 1% (▲) chimpanzee stool extracts. There is a progressive reduction in the positive/negative ratio (P/N) with stool extracts of less than 6%.

of hepatitis A were tested for IgM antibody to HAV (Figure 2). All of these sera had anti-HAV detectable by HAVAB, and all gave a P/N value greater than 2.2 in the assay for IgM antibody. Twenty-five sera collected during the SHAPE outbreak gave a mean P/N of 6.49 (range 2.46-11.23, S.D.=2.16); 18 sera from the Schofield Barracks outbreak gave a mean P/N of 8.83 (range 2.83-13.50, S.D.=2.65); and 27 sera from the Ft. Richardson epidemic yielded a mean P/N of 7.43 (range 3.10-11.44, S.D.=2.02). The titer of IgM anti-HAV in acute sera ranged from 1:6400 to $\geq$ 1:51,200. Thus, the IgM-SPRIA had excellent specificity for acute or recent hepatitis A virus infection.

The specificity of the assay was further assessed by testing sera obtained within 30 days of onset of symptoms of hepatitis B and non-A non-B hepatitis (Figure 2). Forty-five sera obtained from cases of hepatitis B yielded a mean P/N of 1.08 (range 0.40-1.91, S.D.=.346), and 5 non-A non-B sera mean P/N of 1.14 (range 0.61-1.62, S.D.= .370).

IgM rheumatoid factor (RF) could theoretically cause a false elevation of the P/N in the IgM anti-HAV assay by binding radiolabelled IgG directly to the goat anti-IgM precoat. Therefore, 10 RF-positive sera from patients with rheumatoid arthritis were tested; 6 of these were HAVAB positive. The mean P/N value obtained in the IgM anti-HAV test was 1.65 (range 1.01-3.2). Test values from two sera (20%) fell in the low positive range (Figure 2) indicating that RF could interfere with the specificity of the assay. Nonetheless, the highest P/N obtained with RF-positive rheumatoid arthritis sera was only 3.2, a value lower than that obtained with 95% of the acute phase hepatitis A sera.

Eleven of 107 patients (10%) with recent hepatitis type A possessed detectable RF-activity when sera collected within 90 days of onset of symptoms was tested by latex agglutination. RF invariably occurred during the first 30 days after the onset of symptoms (Table 3), and in one patient was found to persist for over 3 weeks. RF

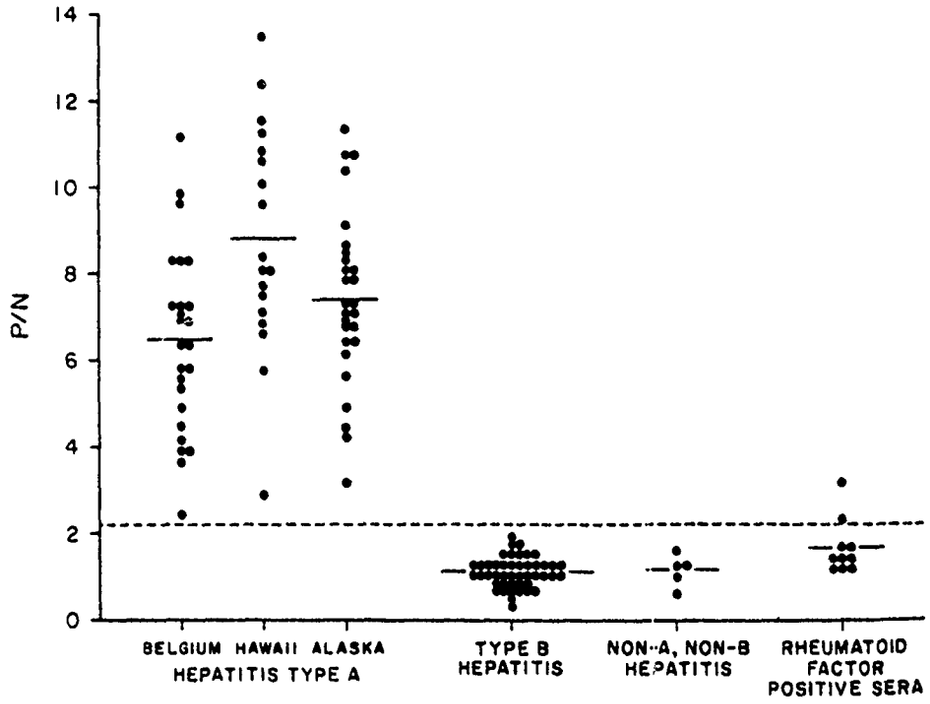

Figure 2. Specificity of the IgM-SPRIA for anti-HAV using human sera at a 1:10 dilution.

Table 3. Presence of Rheumatoid Factor (RF) in HAV Infection.* (SHAPE, Schofield Barracks, Ft. Richardson Outbreaks)

Days After Symptom Onset	Positive Sera/ Total Tested	% Positive
0-10	5/38	13%
11-20	6/36	17%
20-30	1/19	5%
31-40	0/15	0%
41-50	0/8	0%
51-90	0/15	0%

*RF detected by latex agglutination.

occurring during acute hepatitis A infection did not appear to exert a significant influence on the result of the IgM anti-HAV assay. The mean P/N value obtained with RF-positive sera was 8.04 (range 2.46-11.6, S.D.=3.07) whereas with RF-negative sera it was 7.41 (range 2.83-13.5, S.D.= 2.32).

Paired acute and convalescent sera, drawn approximately 100 days apart, afforded an opportunity to study sequential changes in IgM anti-HAV during the SHAPE outbreak (Figure 3). A return of the P/N value towards normal was seen in all 16 patients with available sera, with convalescent results falling within the normal range in 10. On the other hand, three sera fortuitously collected before the onset of type A hepatitis lacked IgM anti-HAV whereas late convalescent sera from all three cases were positive by the IgM-SPRIA.

Plotting the results from all sera collected from cases during the SHAPE outbreak as a function of the time of collection relative to onset of symptoms allowed an estimate of the time course of the IgM antibody response to hepatitis A infection (Figure 4). IgM antibody could be detected as early as two days after the onset of symptoms, but was absent in one patient who was bled within hours of her first symptoms. P/N values generally increased during the first 30 days and subsequently declined over the next three months. Two of four sera collected over 120 days after the onset of symptoms still yielded P/N values slightly greater than 2.2. Similar results were obtained when sera from the Alaska outbreak were analyzed in a similar fashion (Figure 5), although fewer late convalescent sera were collected during this outbreak.

There was absolute concordance between the IgM-SPRIA assay and the conventional radioimmunoassay for anti-HAV (HAVAB) inasmuch as all sera that were positive for IgM anti-HAV resulted in greater than 50% blocking in the latter test.

Total IgM concentrations were greater than 280 mgm/dl in 11 of 20 sera collected (55%) during the first 30 days after the onset of symptoms during the Belgium outbreak.

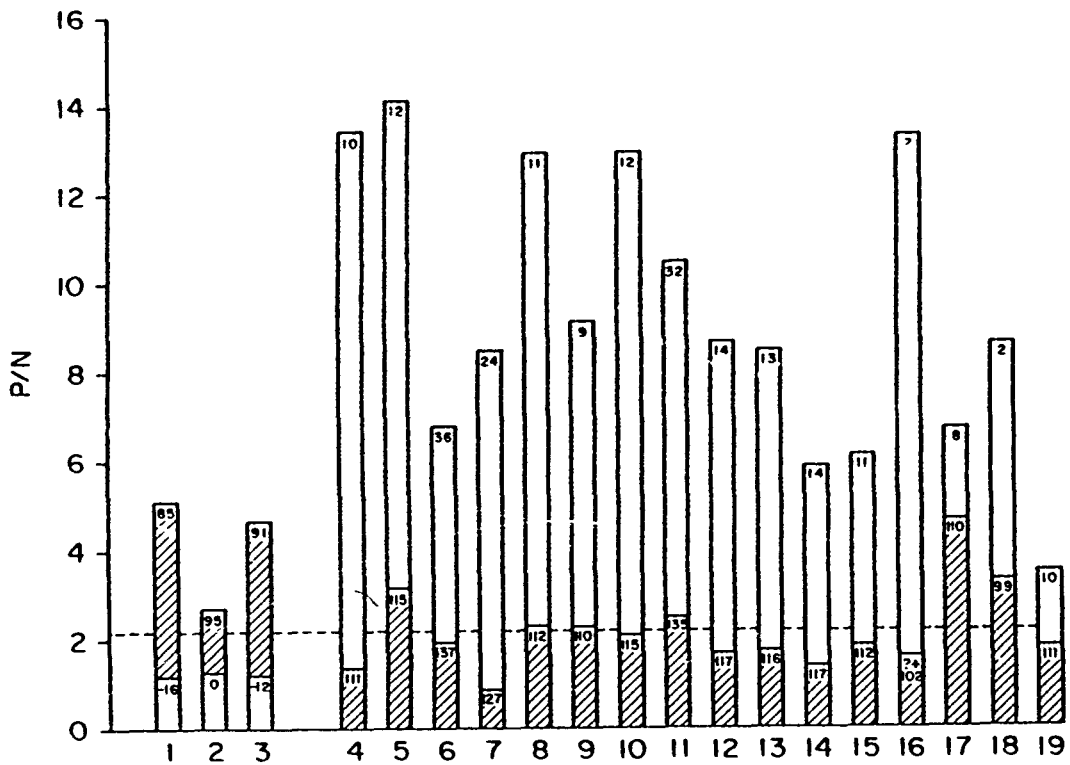

Figure 3. Sequential changes in the IgM-SPRIA P/N value following acute hepatitis A infection in patients. The days elapsed since the onset of symptoms are shown for each serum specimen tested; results obtained with the first serum sample appear in unshaded columns, whereas results of testing a second serum sample appear in the shaded columns. In cases 1-3, sero-conversion occurred following acute infection. Cases 4-19, however, were all bled for the first time following the onset of disease and show in each case a fall in the P/N value to or toward normal (P/N < 2.2).

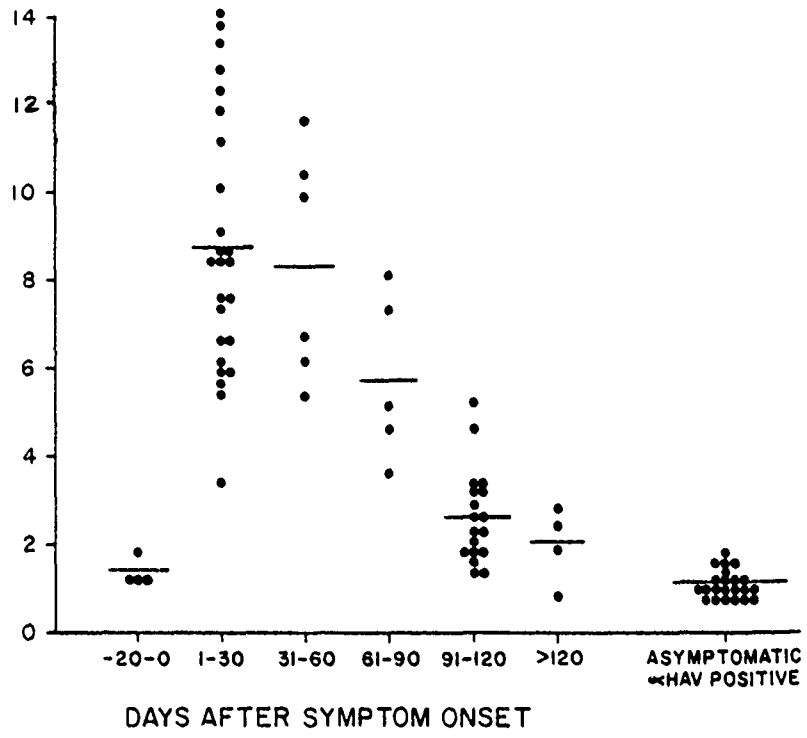

Figure 4. Relationship of IgM anti-HAV reactivity (P/N) to time after onset of symptoms in sera collected during SHAPE outbreak.

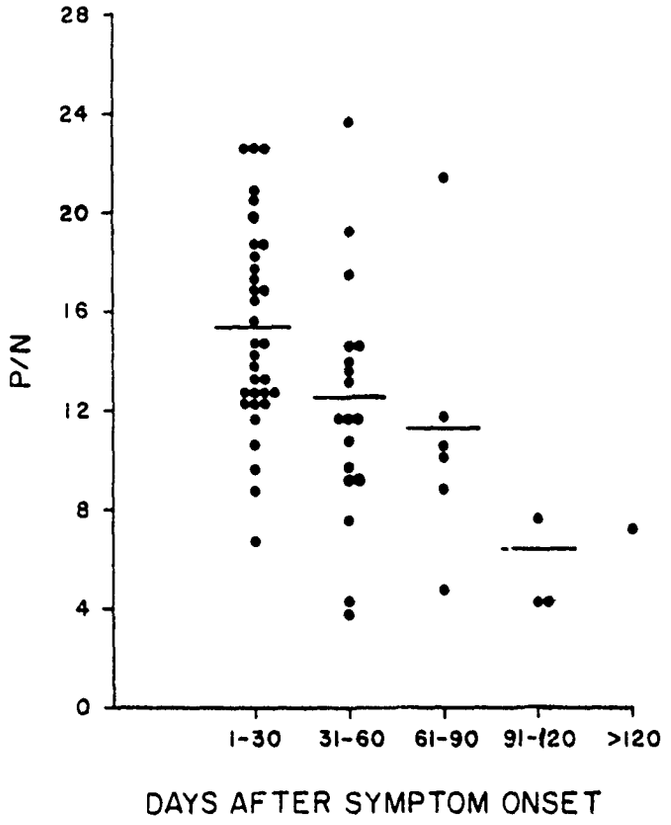

Figure 5. Relationship of IgM anti-HAV reactivity (P/N) to time after onset of symptoms in sera collected during the Ft. Richardson outbreak.

Slightly higher P/N values were found with sera containing a greater than normal concentration of IgM.

When questionnaire data from the SHAPE outbreak was reviewed, no correlation was evident between the P/N value and the 1) age, 2) sex, 3) maximum observed SGOT, 4) serum bilirubin value or 5) the presence or absence of any of the following signs and symptoms: fever, nausea, vomiting, diarrhea, myalgia or arthralgia. High P/N values were found with sera collected from totally asymptomatic children who were epidemiologically implicated in transmitting the virus to their parents and older siblings. Thus, the only factor influencing the P/N value appeared to be the duration of the interval between onset of symptoms and the collection of sera. Such analyses are however of necessity quite crude because the P/N value is not linearly related to the titer of antibody present. IgM antibody titers are now being determined in the laboratory and will be correlated with these variables.

D. Anti-HAV prevalence among special forces troops.

1. Background

The antibody prevalence, and hence susceptibility to hepatitis A virus, of American soldiers is poorly defined. Current civilian studies would suggest that the vast majority of young Americans are susceptible to this virus however. To better establish hepatitis A immunity among today's soldiers, sera collected from special forces troops in 1977 and 1978 were studied.

2. Method

Sera from 275 individuals participating in the Alaskan "Jack Frost" exercise in 1977 were studied (see Annual Report FY 77). Both pre-deployment and post-deployment sera were available. A second collection of sera, taken from 106 special forces troops after their return from the Iranian "ARESH" exercise of 1978, was also studied.

Each serum sample was tested for HBsAg (Ausria II),

Table 4. Hepatitis Antibody Prevalence in Special Forces Troops

	Soldiers Tested	Anti-HAV Positive
Operation "Jack Frost" - 1977	271	61 (23%)
HBV serum markers*	52 (19%)	17 (33%)‡
No HBV markers	219 (81%)	45 (20%)‡
Operation "ARESH" - 1978	104	22 (21%)
HBV serum markers	23 (22%)	7 (30%)**
No HBV markers	81 (78%)	15 (19%)**

*Includes any combination HBsAg, anti-HBs, or anti-HBc.
‡Chi Square = 2.86
**Chi Square = 0.84

anti-HBs (AUSAB), anti-HBc (CORAB) and anti-HAV (HAVAB) by commercially available radioimmunoassay. Questionnaires administered during the time of blood collection are currently being analyzed and correlated with serological results by the Division of Preventive Medicine (see Work Unit 001, Epidemiologic Studies of Military Diseases).

3. Results and Conclusions

Overall anti-HAV prevalence in both groups of soldiers was 22.8% and 21.2%, respectively (Table 4). The total prevalence of occurrence of any marker of hepatitis B infection was 19% and 22% (including 3 of 104 ARESH participants who were HBsAg positive). A positive relationship between past exposure to hepatitis B virus and immunity to hepatitis A virus appears to be present, for in both groups anti-HAV frequences were somewhat higher in individuals with HBV markers. Although Chi square values were low, a similar relationhsip has been demonstrated in civilian studies. No seroconversion to hepatitis A virus occurred during operation "Jack Frost." The vast majority of seasoned troops are susceptible to both viruses.

E. Purification of hepatitis A virus from chimpanzee stool.

1. Background

Preliminary attempts to purify virus (see Annual Report FY 78) have been continued in the hope of obtaining a purified product suitable for viral polypeptide analyses. Analysis of viral proteins may assist in proper classification of the virus and will be essential to the final understanding of virus replication and assembly. Purified virus may also serve as an antigen for antibody production, either by hybridoma cultures or in intact animals.

2. Methods and Results

A 20% w/v suspension of CH 173 3/13 stool (see Annual Report 78) was prepared in 50 mM TRIS buffer pH 7.4, shaken with glass beads, and centrifuged at 1500 g x 15 minutes. The supernatant fluids were decanted, and the

stool was re-extracted 5x with an equal volume of buffer. Supernatant fluids were then pooled, centrifuged at 17,000 x g for 1 hr.; the supernatant fluids decanted again and recentrifuged under identical conditions. Supernatant fluids from the last centrifugation were spun at 100,000 x g for 3 hrs. The pellet from this centrifugation step was resuspended in 50 mM TRIS, pH 7.4.

The pelleted virus was extracted with an equal volume of chloroform. The aqueous phase was centrifuged at 100,000 x g for 3 hrs. and the pellet was resuspended in TRIS buffer. This material was applied to a 93 x 2.5 cm Sepharose 2BCL column equilibrated with the TRIS buffer. Fractions (3.5 ml each) were collected into siliconized tubes, and absorbancy at 280 nM was monitored continuously. Fractions were tested for HAV antigen by a modification of the IgM-SPRIA (see above) using a high-titered IgM anti-HAV serum. Results appear in Figure 6. A broad band of anti-HAV reactive material appeared in fractions 70-100, well separated from two major peaks of material absorbing light at 280 nM. Fractions 76 through 91 were pooled and filter-concentrated against an XM300 Amicon filter to a volume of approximately 10 ml.

The concentrated virus was layered into 10 ml preformed cesium chloride step gradients of 1.16 to 1.38 density. Gradients were spun at 246,000 x g for 24 hours, and 0.5 ml fractions were collected from the bottom. Fractions were tested by SPRIA; results are shown in Figure 7. A single peak of HAV appeared at an average density of 1.31 (range 1.30-1.32).

Efforts are currently in progress to evaluate the material in this peak by electron microscopy.

F. Outbreak of hepatitis A infection at SHAPE.

An outbreak of HAV disease occurred in February 1979 among active duty personnel and dependents at SHAPE near Mons, Belgium. An EPICON investigation (see Work Unit 001, Epidemiologic Studies of Military Diseases) was supported by this laboratory both in terms of manpower and specific

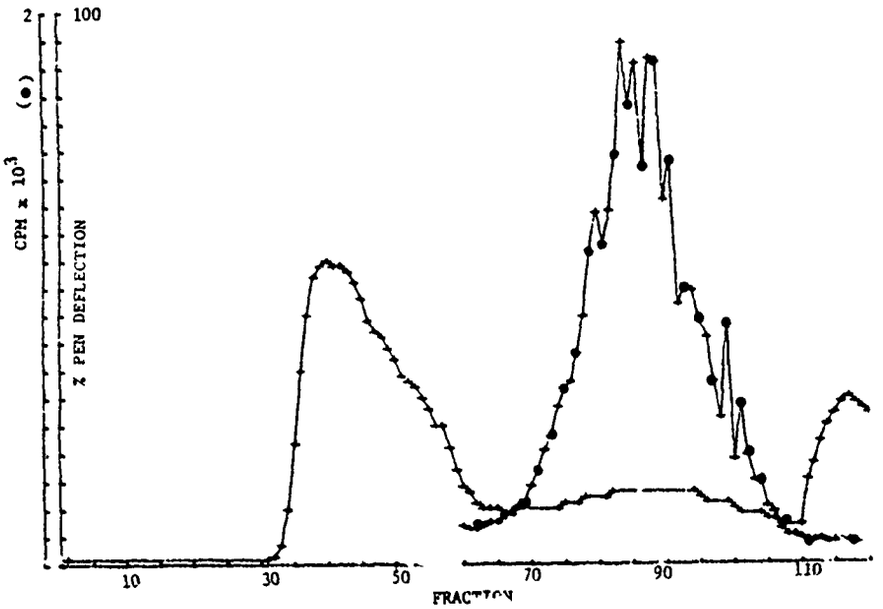

Figure 6. Sepharose 2BCL chromatography of chloroform-extracted chimpanzee stool containing HAV. Relative absorbancy at 280 nm (+——+) is represented by % pen deflection (LKB 8300 Uvicord II), with two major protein peaks occurring at fraction 40 and fraction 117. Anti-HAV reactive material (•——•) detectable by SPRIA appeared in a broad band centered at fraction 85.

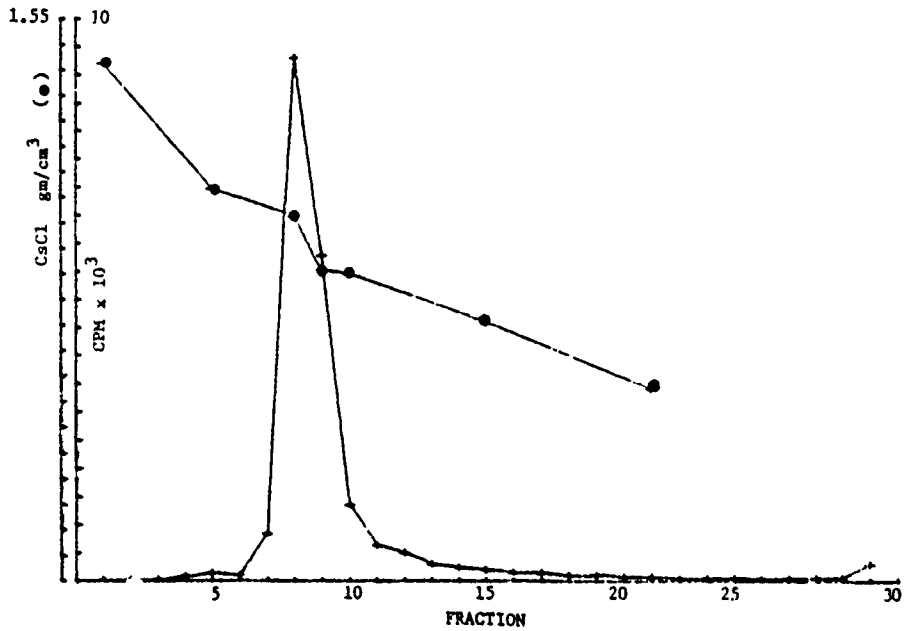

Figure 7. CsCl equilibrium gradient centrifugation of partially purified chimpanzee stool-derived HAV. A single sharp peak of anti-HAV reactive material (+——+) was found at a density of 1.31 gms/cm^3. Density of individual fractions (●——●) was estimated from the refractive index.

virus serology. The outbreak was similar to that which occurred in 1976 at Ft. Richardson, Alaska, inasmuch as the primary areas of transmission were a preschool nursery and a child care facility. This outbreak differed from the Ft. Richardson experience, however, for a substantially higher symptomatic/asymptomatic infection ratio existed among the involved children. Secondary spread to older siblings and parents was common at SHAPE, as it was at Ft. Richardson.

Sera collected during this outbreak, and during a second visit to SHAPE in May 1979 provided critical material for the development of the IgM SPRIA described above.

II Etiology of viral hepatitis among active duty personnel.

A. Background

Endemic viral hepatitis has been a longstanding problem for U.S. Army personnel stationed overseas. Annual incidence rates range between 5-10 cases per thousand per year among troops in both Europe and Korea. These cases of hepatitis represent a considerable cost to the Army, especially in time lost from duty. Hepatitis control measures are difficult to develop without a firm understanding of the exact virological cause of hepatitis and the mode of virus transmission in each theatre. Therefore, a preliminary study was designed to determine 1) the viral etiology of cases of hepatitis requiring hospitalization among U.S. Army personnel within CONUS, the Federal Republic of Germany and the Republic of Korea; 2) the severity of the disease; and 3) the basic demographic characteristics of the population of soldiers developing hepatitis, including such data as age, sex, rank, length of time in country of assignment and possible exposures. To date, sera and questionnaires have been collected from over 400 hospitalized patients.

B. Methods

1. Study Design

The study is currently being carried out at five major U.S. Army general hospitals located at Fort Hood, TX; Seoul, Republic of Korea; and Landstuhl, Frankfurt and Bad Canstatt, Federal Republic of Germany. Since 1 October 1978, active duty personnel hospitalized with hepatitis have been identified and a serum sample obtained as soon as possible after admission. A brief questionnaire history concerning the patient and his illness were completed. A second serum sample and second questionnaire were obtained 4-8 weeks after hospitalization. Both sera and questionnaires were forwarded to WRAIR for evaluation.

Criteria for inclusion of a case in this study are: 1) hospitalized active duty personnel 2) a single serum SGOT level greater than 3x normal, 3) no evidence of extrahepatic obstruction, congestive heart failure, malignancy or liver disease attributable directly to drugs (medications), chemicals or alcohol.

2. Serological Methods

HBsAg, anti-HBs, anti-HBc and anti-HAV are determined by commercial radioimmunoassay (Abbott Laboratories). IgM class antibody to HAV is being detected by SPRIA as described above. In addition, in cases classified as due to neither type A nor type B virus, heterophile antibodies and complement-fixing antibodies to cytomegalovirus are being measured. Heterophile-positive cases are confirmed as having current Epstein-Barr virus (EBV) infection by testing for EBV-viral capsid antibody by immunofluorescent techniques.

C. Results

Preliminary results appear in Table 5. Hepatitis B virus is the predominant cause of viral hepatitis in all three locations. However, it is striking to note that non-A non-B cases comprise approximately 17% of cases in Germany, and 27% of cases in CONUS, but 4% of cases in Korea. Also notable is the virtual absence of type A hepatitis in Germany. Further testing of collected sera is currently in progress, as is an evaluation of questionnaire data.

Table 5. Etiology of Viral Hepatitis in Hospitalized Active Duty Personnel

	Study Area		
	Korea	Ft Hood TX	Germany
Number of patients†	76	29	190
Virus Etiology:			
Current Hepatitis B virus infection	57 (75%)	14 (48%)	134 (71%)
Prior Hepatitis B infection‡	2 (3%)	3 (10%)	17 (9%)
Current Hepatitis A infection	11 (15%)	4 (14%)	5 (2.5%)
Non A-Non B virus(es)	3 4%)	8*(27%)	33**(17%)
Cytomegalovirus (CMV)	0	-	1 (.5%)
Epstein-Barr virus (EBV)	3*** (4%)	-	-

†SGOT ⩾ 3x normal
‡possess anti-HBs (± anti-HBc) without HBsAg, thus time of infection (recent vs. longpast) remains uncertain
*EBV and CMV testing incomplete
**EBV testing incomplete
***includes one patient with concurrent CMV infection.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 135 Mechanisms of Transmission of Hepatitis Viruses

Publications.

1. Lemon, S.M.; Hutt, L.M.; Huang, Y.; Blum, J.; Pagano, J.S. Simultaneous Infection with Multiple Herpesviruses. Am. J. Med. 66:270-276, 1979.

2. Lemon, S.M.; Pagano, J.S.; Utsinger, P.D.; and Sinkovics, J.G. Cultured "Hairy Cells" Infected with Epstein-Barr Virus. Evidence for B-Lymphocyte Origin. Ann. Int. Med. 90:54-55, 1979.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6448	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	136
b. CONTRIBUTING				
c. ~~XXXXXXX~~	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Development of Biological Products

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
58 05	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION

b. NUMBER

c. TYPE d. AMOUNT:

e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	4	439
	CURRENT 80	4	423

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Berman, S.L., Ph.D.

NAME: Altieri, P.L.

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Bioassays; (U) Biological Products; (U) Dengue Virus Vaccine; (U) Febrile Antigens; (U) Meningococcal Vaccines; (U) Pseudomonas Vaccine; (U) Vaccines

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) This work unit is concerned with the development of manufacturing methods and the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

24. (U) Increased effectiveness and reduced reactivity are pursued by use of new physical and chemical methods for processing. Improvement in stability and reduction of logistic requirements are achieved by application of modern freeze-drying and packaging techniques.

25. (U) 78 10 - 79 09 Investigations on the development of new and improved biological products for military use have continued. 1. Studies were carried out on the preparation of purified polysaccharides from meningococcal groups A, C, 29E, 8021, and protein-polysaccharide complex antigens from group B for use in a combined vaccine for human use. 2. Two polysaccharide vaccines derived from cultures of Pseudomonas aeruginosa, type 5, were produced. 3. Studies were initiated on the preparation of Yersinia pestis Fraction 1 antigen for use as a standard preparation for worldwide testing. 4. Investigations were continued on the development of a more attenuated dengue virus type 2 vaccine. 5. The evaluation of the stability of freeze-dried diagnostic antisera was continued. 6. Additional studies on mouse protection tests for the evaluation of typhoid vaccines and meningococcal vaccines were carried out. For technical report, see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

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Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 136 Development of Biological Products

Investigators.
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Description.

This work unit is concerned with the development of manufacturing methods for the production of new effective vaccines for military use, and with the modification of existing biological products to increase effectiveness and reduce reactivity, to afford greater stability, and to minimize logistic requirements.

Progress.

1. Meningococcal Vaccines.

During this period laboratory studies were continued on Neisseria meningitidis groups A, C, and 29E for the purpose of preparing purified polysaccharides to be used in a combined vaccine and tested as an immunogen in man. In addition, studies were initiated on the production of a purified polysaccharide from the group 8021 N. meningitidis. This polysaccharide, used as an antigen, cross-reacts in serological tests with group Y and group 135 sera, and will be tested in man to see if antibodies against the groups Y and 135 organisms will be elicited. If this is the case, the 8021 polysaccharide will be incorporated into the proposed polyvalent vaccine in place of the polysaccharide derived from the Y and 135 organisms. Investigations also continued on processing methods for the production of purified protein-polysaccharide complex antigens derived from group B meningococcus for use as immunogens.

a. A new production lot of polysaccharide derived from the A-4 (M1027) strain of N. meningitidis was prepared from a 12 hour culture grown in a modified Franz medium

containing 0.2% yeast extract dialysate. The crude Sharples sediment was processed to the purified group A meningococcal polysaccharide by previously determined optimum methods (Annual Report, 1976). Results of in vitro assays of the final product are shown in Table 1.

Table 1. Assays of Group A meningococcal polysaccharide.

Yield (mg/L culture)	70.4
Protein (%)	0.47
Nucleic Acid (%)	0.20
Phosphorus (%)	8.28
Molecular Weight	> 100,000
Pyrogenicity	Non-Pyrogenic at 2.5 μg/Kg

By the criteria set up by the Bureau of Biologics, FDA, for a group A meningococcal vaccine, this purified polysaccharide is suitable for use in humans.

b. A new production lot of polysaccharide derived from the C-11 strain of N. meningitidis was prepared on a 16 hr culture grown in modified Franz medium. The crude polysaccharide was purified using 75% phenol extractions. Previous lots of the C-11 crude polysaccharide had been purified by means of chloroform extractions. In both, the final step was ethanol opalescence. A comparison of the final purified polysaccharides obtained by both purification procedures is shown in Table 2.

Table 2. Comparison of Group C meningococcal polysaccharides obtained by chloroform and phenol extractions.

In vitro assay	Chloroform	Phenol
Yield (mg/L culture)	16.0	35.8
Protein (%)	0.16	0.42
Nucleic Acid (%)	0.31	0.35
Sialic Acid (%)	85.0	85.0
Molecular Weight	> 100,000	> 100,000
Pyrogenicity	Non-Pyrogenic at 2.5 μg/Kg	Pyrogenic at 2.5 and 0.5 μg/Kg Non-Pyrogenic at 0.25 μg/Kg

It can be seen that the phenol extracted product is more reactive in rabbits than the chloroform extracted polysaccharide. However, the phenol extracted product still meets the Bureau of Biologics requirement that the lowest acceptable limit for pyrogenicity is 0.25 μg/Kg rabbit. An advantage of the phenol procedure is that the yield of polysaccharide was doubled. This group C polysaccharide will also be included in a proposed polyvalent vaccine.

c. Three production lots of N. meningitidis strain 29E-106 were grown and processed in order to obtain sufficient purified polysaccharide for inclusion in a polyvalent vaccine. These were processed in the same manner as Lot 1 of this strain (Annual Report, 1978). After phenol extraction, the three lots were combined for ethanol opalescence and high speed centrifugation. In vitro assays of the final purified polysaccharide are shown in the following table (Table 3).

Table 3. In vitro assays of N. meningitidis strain 29E-106 polysaccharide

Yield (mg/L culture)	18.85
Protein (%)	0.62
Nucleic Acid (%)	0.60
Keto-Deoxyoctonic acid (%)	71.8
Carbohydrate	12.2
Molecular Weight	≥ 100,000
Pyrogenicity	Non-pyrogenic at 0.25 μg/Kg

The results of the in vitro assays of this product were similar to those obtained with the first lot of this polysaccharide (Annual Report, 1977), which was found to be immunogenic in man.

d. At the request of personnel of the Department of Bacterial Diseases, WRAIR, studies were initiated on production and processing methods to obtain a purified polysaccharide from the Group 8021 strain of N. meningitidis. This polysaccharide is of interest because it reacts serologically with both group Y and group 135, and could possibly serve as a substitute for these two polysaccharides.

Sixteen hour cultures were grown in modified Franz medium and in modified Franz medium containing 4 times the normal phosphate concentration. The Sharples sediments were processed to the crude polysaccharides in the usual manner (calcium chloride extraction, nucleic acid removal, ethanol precipitation and drying of precipitate). A comparison of the two products obtained is given in Table 4.

Table 4. Comparison of the crude polysaccharides obtained with N. meningitidis Group 8021 in different growth media.

In vitro assay	Modified Franz Medium	Modified Franz with 4 X PO_4
Yield (mg/L culture)	65.9	17.5
Protein (%)	37.8	57.8
Nucleic Acid (%)	14.3	13.4
Sialic Acid (%)	19.5	2.5

The results indicate that the modified Franz medium was superior to the 4 X PO_4 medium in yield of polysaccharide. This is shown not only by mg/L culture obtained, but also by the sialic acid content. Therefore, no further work was done with the 4 X PO_4 crude polysaccharide.

Difficulty was experienced in obtaining a polysaccharide from this strain with a protein and nucleic acid content of less than 1%. With the polysaccharide at a concentration of 5 mg/ml, repeated ethanol opalescence steps followed by high speed centrifugations were carried out. The results are shown in the table below (Table 5).

Table 5. Effect of ethanol opalescence and high speed centrifugation (HSC) on protein and nucleic acid content of Group 8021 polysaccharide.

Treatment	Yield mg/L culture	Protein (%)	Nucleic Acid (%)
Post phenol extraction	21.7	6.58	4.56
25% ETOH + 3 hr HSC	13.7	1.34	1.04
25% ETOH + 3 hr HSC	11.3	1.76	ND
6 hr HSC	10.1	1.58	1.15

The only significant change by the above treatments was a continued loss of polysaccharide. Concentrations of ethanol greater than 25% caused precipitation of the polysaccharide.

Another lot of this polysaccharide was prepared in order to determine what changes could be made in the processing procedures to improve the purity of the product. To start, the results obtained with three phenol extractions were compared to those obtained with five phenol extractions. The results are shown in Table 6.

Table 6. Comparison of results obtained with three versus five phenol extractions of N. meningitidis Group 8021 polysaccharide.

In vitro assay	Phenol Extractions	
	three	five
Yield (mg/L culture)	15.9	13.1
Protein (%)	1.77	1.29
Nucleic Acid (%)	1.56	1.74
Sialic Acid (%)	58.9	58.1

The data indicate that there was no significant increase in purity as the result of the two additional phenol extractions.

Both products were then dissolved in 0.02 M $CaCl_2$ at a concentration of 1 mg/ml instead of 5 mg/ml. Ethanol was added to a final concentration of 25% alcohol. The solutions were chilled to 5°C, centrifuged at 100,000 xg for 3 hours, and the polysaccharides were ethanol precipitated from the supernatants. In vitro assays of these products are given in the table below (Table 7).

Table 7. Effect of ethanol treatment and high speed centrifugation on a dilute (1 mg/ml) solution of N. meningitidis Group 8021 polysaccharide.

In vitro assay	25% Ethanol + 3 hr HSC	
	Phenol Ext. 3	Phenol Ext. 5
Yield (mg/L culture)	7.1	8.2
Protein (%)	0.79	0.83
Nucleic acid (%)	1.19	1.17
Sialic acid (%)	53.5	57.3
Molecular weight	> 100,000	> 100,000

Since the above treatment reduced the protein content to less than 1% and brought the nucleic acid to approximately a 1% level, another production lot was grown and processed to a purified polysaccharide suitable for use as an immunogen in man. In vitro assays of the intermediate products are given in Table 8 (on the following page).

The purified polysaccharide (Column 4 in Table 8) was sterilized by filtration through a 0.2 μm membrane filter, filled at 2.5 mg polysaccharide per vial and freeze-dried. This material will be tested in man by the Department of Bacterial Diseases, WRAIR.

e. The investigations on the production of a protein-polysaccharide antigen prepared from the B-11 strain of N. meningitidis grown in CAG medium (Annual Report, 1978) continued.

A 16 hour culture (30 L) was inactivated with Cetavlon and the precipitate collected by Sharples centrifugation. The Sharples sediment was divided into two parts in order to compare the usual calcium chloride extraction procedure with a 1.0 M sodium chloride extraction to obtain the crude protein-polysaccharide complex. The divided sediment was processed with both extractants in the same manner as previously described for Lot PA-3 (Annual Report, 1977). A comparison of the crude protein-polysaccharide complexes is given in Table 9.

Table 9. Comparison of crude polysaccharide yields obtained with sodium chloride and calcium chloride extraction of sedimented N. meningitidis strain B-11 from CAG medium.

In vitro assay	1.0 M NaCl extract	1.0 M $CaCl_2.2H_2O$ extract
Yield (mg/L culture)	72	60
Protein (%)	32.8	73.1
Nucleic acid (%)	21.9	5.7
Sialic acid (%)	38.6	38.7
Molecular weight	75% of product $\leq$ 100,000	$>$100,000

The sodium chloride-extracted polysaccharide is unsatisfactory because of the high nucleic acid content and low molecular weight. This indicates that the 1.0 M $CaCl_2.2H_2O$ is the extractant of choice at the present time.

Table 8. *In vitro* assays of the intermediate products in processing of *N. meningitidis* Group 8021.

In vitro assay	Crude Extract	Phenol Extract	25% Alcohol + HSC	25% Alcohol 6 hr. HSC
Yield (mg/L culture)	49.5	20.4	10.7	8.3
Sialic acid (%)	31.1	49.4	50.3	52.4
Protein (%)	33.3	0.67	0.60	0.56
Nucleic acid (%)	7.93	1.71	1.41	1.61
Carbohydrate (%)	22.4	36.5	35.4	34.8
Molecular weight (G-200 Gel)	ND	> 100,000	> 100,000	> 100,000
Pyrogenicity	ND	ND	ND	Non-pyrogenic at 2.5 µg/Kg

f. At the request of personnel of the Department of Bacterial Diseases, WRAIR, the Department of Biologics Research, prepared 14 and 16 hour cultures of the B-11 strain of N. meningitidis for processing to purified protein, polysaccharide, and protein-polysaccharide antigens, using the methods developed by W. Zollinger.

Two 15 L cultures were grown in CAG medium. The 14 hour cultures were inactivated with phenol at a final concentration of 0.5% phenol. The 30 L of phenol-inactivated suspension were passed through the B-16 continuous-flow rotor of the Spinco L4 centrifuge operating at 73,340 xg at a flow rate of approximately 9.6 L/hr. The yield of pelleted organisms was 119 gm (wet weight). The outer-membrane protein (614 mg) was obtained from this material and was turned over to W. Zollinger for further processing.

Another three 15 L cultures were grown in the same medium with a total incubation time of 16 hours. These were inactivated with Cetavlon and processed to a protein-polysaccharide complex in the same manner as Lot PA-3 (Annual Report, 1977). The material obtained was then chromatographed on a Sepharose CL-2B column by W. Zollinger. The protein-polysaccharide complex from the column was extracted three times with 75% phenol in order to obtain a relatively pure polysaccharide. This preparation was then given to W. Zollinger for further processing.

Seven lots of vaccine were prepared by W. Zollinger using various combinations of the purified protein and polysaccharide preparations to make protein-polysaccharide complexes. One of the seven lots consisted of the protein alone, and one of the polysaccharide alone. These were filled into vials at 3 mg/vial and freeze-dried. Tests of the final products are in progress at the present time. When all the tests are completed, these vaccines will be tested in man.

2. Pseudomonas Vaccines.

During the past year, in collaboration with the Department of Bacterial Diseases, WRAIR, two polysaccharide vaccines derived from cultures of Pseudomonas aeruginosa, type 5, were produced from the supernatant growth fluid. The sediment (inactivated microorganisms) was also extracted to see if additional polysaccharide could be obtained.

a. Growth of the type 5 strain of P. aeruginosa was harvested from Tryptic Soy Agar (TSA) plates and transferred to two 1.5 L of Tryptic Soy Broth (containing 3% glycerol). These were incubated at 36°C on a reciprocal shaker operating at 110-120 cycles/minute. After 5 1/2 hrs, the 1.5 L cultures were transferred to two 13.5 L of Tryptic Soy Broth (containing 3% glycerol and 25 ml of UCON antifoam) in 20 L carboys. These were incubated at 36°C with aeration (2-3 liters/minute). After 48 hrs, the cultures were removed from the incubator and to each was added 83.5 ml of a 90% phenol solution. The inactivating cultures were thoroughly mixed and held at room temperature for 18 hours. Then, to each of the two bottles, 150 ml of a 10% solution of Cetavlon were added and the cultures were thoroughly mixed and held at room temperature for another 24 hours. Samples of the inactivating cultures were removed at timed intervals and distributed on TSA plates which were incubated at 36°C. No apparent growth in either bottle was observed after 24 hour post phenol plus 6 hour post Cetavlon.

The 30 liters of phenol-Cetavlon-inactivated culture were passed through the Sharples centrifuge and the supernatant was given to personnel of the Department of Bacterial Diseases, WRAIR, for processing. The Sharples sediment was stored at -60°C.

The final purified polysaccharide was sterilized by filtration through a 0.2 μm membrane filter, filled at 2.5 mg polysaccharide per vial and freeze-dried. In vitro assays of the final product are shown in Table 10.

Table 10. In vitro assays of P. aeruginosa, type 5, Lot 1 vaccine.

Carbohydrate (%)	75.4
Protein (%)	5.1
Nucleic acid (%)	0.6
Keto-Deoxyoctonic acid (%)	0.3
Total Solids (mg/vial)	2.5
Pyrogenicity	Non-pyrogenic at 25 μg/kg

b. Cultures of P. aeruginosa, type 5 strain, were grown and inactivated as in Section a. The Sharples supernatant was processed by personnel of the Department of Bacterial Diseases, WRAIR. Their procedure consisted of an ethanol

precipitation, Sephadex G-100 chromatography, phenol extraction, and a final ethanol precipitation. This polysaccharide was sterilized by passage through a 0.2 μm membrane filter. Rabbit pyrogenicity tests showed the vaccine to be pyrogenic at 1.0 μg/Kg and borderline at 0.25 ug/Kg. Therefore, the bulk filtrate was thawed, 1 M $CaCl_2.2H_2O$ added to a final concentration of 0.02 M, and ethanol added to a final concentration of 25% alcohol. This solution was chilled to 5°C, then centrifuged at 100,000 xg for 4 hours, and the polysaccharide precipitated from the supernatant fluid. The result of this treatment are shown in the table below (Table 11).

Table 11. Effect of ethanol treatment and high speed centrifugation on P. aeruginosa, type 5 polysaccharide.

Carbohydrate (%)	98.0
Protein (%)	2.5
Nucleic acid (%)	0.6
Keto-Deoxyoctonic acid (%)	0.3
Pyrogenicity	Non-pyrogenic at 2.5 μg/Kg - Pyrogenic at 5.0 μg/Kg

It was decided by personnel of the Department of Bacterial Diseases that this polysaccharide would not be tested in man because of the results obtained in serological tests.

c. The Sharples sediment from Section a was extracted with 1 M $CaCl_2.2H_2O$ and processed to a crude preparation in the same manner as used for processing meningococcal Sharples sediment. The crude material was then extracted with a 75% phenol solution. The results of this treatment are shown in Table 12.

Table 12. Effect of meningococcal processing procedures on Sharples sediment of P. aeruginosa, type 5.

In vitro assay	Crude	Phenol Extracted
Carbohydrate (%)	1.84	1.27
Protein (%)	41.8	35.2
Nucleic acid (%)	11.3	10.4

It can be seen that the percentage of carbohydrate is extremely low in both preparations. This indicates that polysaccharide either is not present in the Sharples sediment or that the methods used failed to extract it.

3. Plague antigen.

In collaboration with the Department of Hazardous Microorganisms, WRAIR, work has continued on the study of the F-1 antigen. Fraction 1, the specific Yersinia pestis envelope antigen used in diagnostic plague serology, was previously prepared from the Y. pestis virulent strain 195/P and the attenuated strain ATTC 11953.

The antigens from both strains had been prepared a number of years ago for CF and HA tests by fractionating with saturated ammonium sulfate (pH 7.0) at 0.4 saturation. The freeze-dried antigens had been stored at -20°C. Lot 3 (ATTC 11953) had been prepared in July 1966 and Lot 4 (195/P) in July 1971.

Some of the dried antigen from each lot was taken and dissolved in pH 8.0 water. Both were then fractionated with saturated ammonium sulfate, first at 0.33 saturation and then at 0.4 saturation. These fractions and the supernatant from the 0.4 saturation were dialyzed and freeze-dried. The yields of antigens from each fraction of both strains are shown in Table 13.

Table 13. Yields obtained in fractions from 100 mg of Y. pestis Fraction 1 antigens, Lot 3 and Lot 4.

Sat. $(NH_4)_2SO_4$ Fraction	Lot 3	Lot 4
0.33	85.2 mg	79.4 mg
0.4	4.0 mg	10.0 mg
0.4 supernatant	2.6 mg	13.7 mg

Some differences appeared between the two lots. The initial protein content of the starting solutions was essentially the same, 1.50 mg/ml versus 1.44 mg/ml. The surprising factor was that the 0.33 fraction of Lot 4 gave an LD_{50} of 500 µg/mouse in mouse toxin tests. Lot 3 (0.33 and 0.4 fractions) and Lot 4 (0.4 fraction) did not kill at 1 mg/mouse, the highest concentration tested.

The production of another lot of F-1 antigen from the ATTC 11953 is planned for the near future to be used as a standard preparation for worldwide testing.

4. Dengue Virus Vaccine.

During this period studies were continued on the development of a more attenuated dengue virus type 2 (DEN-2) vaccine for human use. In addition, attempts were made to increase the sensitivity of a mouse potency assay for the evaluation of dengue virus vaccines.

a. The S-1 DEN-2 virus from the lot #2 live, attenuated dengue virus vaccine (Eckels, K.H. et al., 1976) was passaged further in certified fetal rhesus monkey lung (DBS-FRhL-2) cells at 31°C in order to determine if passage of the virus at a lower temperature would increase temperature sensitivity and promote further attenuation in terms of pathogenicity for suckling mice.

The results of the passages of DEN-2 in DBS-FRhL-2 monolayers at 31°C, as well as the temperature sensitivities of the virus from selected passages, were presented last year (Annual Report, 1978). As reported previously, the passage of the S-1 virus at 31°C not only changed the plaquing characteristic of the S-1 virus from small plaques into a mixed population of large and small plaques, but also lessened the sensitivity of the virus to temperature.

The pathogenicities of the same selected passages of the S-1 virus were then investigated. Suckling mice (1-3 days old) were inoculated intracerebrally (IC) with 0.02 ml of the selected S-1 virus passages. As a control, the S-1 vaccine was also included in the titration. At the same time, the titer of each sample, in terms of plaque-forming units (PFU) per 0.2 ml, was determined in LLC-MK_2 monolayers. The results are presented in Table 14.

At the passage 10 level, the LD_{50} could not be calculated because the virus was more virulent than expected and the sample was not diluted sufficiently. Therefore, half a log beyond the last dilution was conservatively selected as the endpoint, so that the ratio between LD_{50} and plaque forming units per 0.2 ml could be estimated. It is obvious from Table 14 that the virulence of the S-1 virus at the 10th passage increased significantly (18-fold) when compared to the control (S-1 vaccine).

It appears, therefore, that as the virus becomes adapted to replication at 31°C, instead of further attenuation, there is a concomitant increase in virulence for suckling mice. This is not

Table 14. Inoculation of suckling mice with selected passages of DEN-2 S-1 virus.

Passage	Dilution	Survivors/Total Inoculated (0.02 ml)	LD_{50}/0.2 ml	Titer (PFU/0.2 ml)	Ratio (LD_{50}/PFU)
1	Undil	11/27	$10^{-1.9}$	4.6×10^4	0.0017
	10^{-1}	14/27			
	10^{-2}	17/26			
2	Undil	15/27	$10^{-1.6}$	9.7×10^3	0.0041
	10^{-1}	12/27			
	10^{-2}	17/20			
4	Undil	8/27	$10^{-2.5}$	1.1×10^5	0.0029
	10^{-1}	7/27			
	10^{-2}	15/27			
6	Undil	9/26	$10^{-2.5}$	9.8×10^5	0.00032
	10^{-1}	3/27			
	10^{-2}	18/27			
8	Undil	2/26	$10^{-2.3}$	3.1×10^5	0.00064
	10^{-1}	11/27			
	10^{-2}	13/18			
10	Undil	0/27	$>10^{-3.5}$	1.1×10^6	0.0029
	10^{-1}	2/27			
	10^{-2}	3/27			
S-1 vaccine	Undil	9/27	$10^{-1.7}$	3.1×10^5	0.00016
	10^{-1}	19/27			
	10^{-2}	18/27			

surprising since previous studies have shown an increase in the number of large plaques and a drop in temperature sensitivity, both indicators of enhanced virulence, with further passage of the S-1 virus.

b. The following study was initiated in an attempt to produce a heat resistant DEN-2 virus by passaging at 39°C. Confluent monolayers of certified DBS-FRhL-2 cells in 25 cm^2 flasks were inoculated with 0.5 ml of a 1:4 dilution of the infected human serum (PR 159, Natal Ortiz, 8/18/69) in maintenance medium (MM). After an adsorption period of 90 min at 35°C, 5 ml of MM was added (without removing the inoculum) and the inoculated flasks were then incubated at 39°C. One flask was reincubated at 35°C to serve as a control. On days 4, 7, and 10 post-infection, the culture fluids were harvested and the cell sheets were re-fed with fresh MM. The final harvests were made on day 14 post-infection and all samples were assayed by plaque titration in monolayers of LLC-MK_2 cells in 25 cm^2 plastic flasks. A summary of the results of these passages is presented in Table 15.

After the first passage, the fluid from the control flask at 35°C clearly showed DEN-2 replication. However, there was very little virus present in the harvested fluids from the flask at 39°C, as indicated in Table 15. In fact, there is a very good probability that the low titer observed was due solely to residual virus from the inoculum. It was thus feared that upon a second passage at 39°C, the virus would be lost. For this reason, three flasks containing monolayers of DBS-FRhL-2 cells were inoculated for the second passage. Two of these flasks received the 39°C (day 4) harvest from passage 1. After an adsorption period of 90 min at 35°C, one of these flasks was incubated at 39°C, whereas the other flask was incubated at 35°C. The third flask, the control, received the 35°C (day 14) harvest from passage 1 and was incubated at 35°C.

Surprisingly, the results of the second passage definitely demonstrated the replication of DEN-2 virus in all three situations. The final harvest of the control flask at 35°C was done on day 11 instead of day 14 because of extensive cytopathogenic effect (CPE) at that time.

For the third passage, the day 11 harvest from the previous passage was used in all three categories. Thereafter, the harvest that gave the highest titer was used as the inoculum for the next passage.

Table 15. Passages of Dengue 2 virus (PR159) in FRhL cells at various temperatures.

Passage	Day of Harvest	Incubation temperatures		
		39°C	35°C	39°C→35°C
1	4	2.5×10^{0}*	1.5×10^{1}	
	7	0	2.0×10^{1}	
	10	0	3.2×10^{2}	
	14	0	9.4×10^{2}	
2	3	0	1.1×10^{3}	1.5×10^{0}
	7	1.1×10^{1}	2.0×10^{5}	4.0×10^{1}
	11	1.3×10^{1}	1.1×10^{6}	5.0×10^{1}
	14	2.1×10^{2}	ND	2.2×10^{2}
3	4	5.5×10^{0}	4.5×10^{4}	4×10^{0}
	7	4.5×10^{1}	2.1×10^{6}	3.5×10^{1}
	10	2.8×10^{2}	1.5×10^{6}	1.7×10^{2}
	14	3.5×10^{1}	ND	6.1×10^{3}
4	4	4.0×10^{0}	1.3×10^{6}	2.9×10^{1}
	7	7.0×10^{2}	6.6×10^{5}	9.4×10^{2}
	10	4.0×10^{2}		9.8×10^{3}
	14	1.6×10^{2}		6.1×10^{3}
5	4	1.8×10^{3}	1.1×10^{6}	4.3×10^{3}
	7	1.6×10^{3}	3.4×10^{5}	1.1×10^{4}
	10	2.0×10^{2}	ND	3.2×10^{3}
	14	6.0×10^{1}	ND	8.0×10^{2}
6	4	1.6×10^{3}	1.3×10^{6}	1.2×10^{3}
	7	1.5×10^{2}	3.2×10^{5}	1.3×10^{3}
	11	5.5×10^{1}	ND	4.0×10^{2}
	14	5.5×10^{1}	ND	1.0×10^{2}

* Titer (PFU/0.2 ml)

After six passages, the DEN-2 virus appeared to have adapted fairly rapidly to the conditions of each passage, as evidenced by the fact that the highest titers of the virus were attained in shorter time intervals, as indicated in the table.

Interestingly, the virus that was initially passaged at 39°C and then transferred to 35°C for the second passage appears to be defective. After five passages at 35°C, this virus has been unable to equal the titer of the virus passaged only at 35°C. The selection of this defective virus appears to be due entirely to chance.

Currently, the viruses from the three passage series are being investigated by means of growth curves at various temperatures.

c. A mouse potency assay was previously described for evaluation of the immunogenicity of dengue virus vaccines (Annual Reports 1977 and 1978). In the development of the assay, the mice were challenged 1 week after the last of three inoculations of formalin inactivated vaccine. Recently Chaturvedi et al. demonstrated by passive transfer that serum obtained from mice 3 to 5 weeks after three IP doses of live virulent dengue-2 virus, protected recipient mice against intracerebral challenge with DEN-2 virus, whereas the serum obtained 1 and 2 weeks post-immunization provided minimum protection.

The following experiment was done to determine if a later challenge would be more sensitive in the evaluation of vaccines by the mouse potency assay. A comparison was made between challenging at 1 week versus 3 weeks post-immunization. The results are presented in Table 16.

Table 16. Comparison of one week versus three weeks interval between immunization and challenge on the mouse potency assay.

	Interval between immunization & challenge	
Three doses of vaccine	one week	three weeks
undiluted	22%*	44%
1/5	22%	30%
1/25	10%	20%

* Percent of survival. All mice challenged IC with 450 LD_{50} doses of WP130 strain of DEN-2 virus.

The formalin inactivated DEN-2 vaccine used to immunize the mice was low in antigenic content (in terms of HA and infectious titer in LLC-MK_2 monolayers) and this could account for the low protection of the mice. Even so, the results indicate that challenging the mice three weeks post-immunization would be a better time interval for the evaluation of dengue vaccines by the mouse potency assay.

d. Passage of DEN-2 virus in chick fibroblasts was previously described (Annual Reports 1977 and 1978). During this year further passages were done in chick fibroblast cultures in an attempt to increase the yield of infectious virus. After numerous trials little or no virus in the fluids from the infected cultures was detected by plaquing in LLC-MK_2 monolayers. As a result, the attempt to adapt the DEN-2 virus to chick fibroblast cell cultures was abandoned.

5. Diagnostic reagents.

As part of a continuing effort to improve the stability of diagnostic reagents required by military laboratories, a study was initiated in 1977 on the feasibility of preparing stable, freeze-dried diagnostic antisera (for slide agglutination tests) in pre-packaged single-test units. These would be rehydrated and used in situ only as required for the number of tests actually to be accomplished in a laboratory in place of the present method which involves the withdrawing of the volumes needed from a multi-test container, and distribution to a series of slides for use.

The six diagnostic antisera, prepared and freeze-dried in the wells of Linbro Dispo Trays, were stored at $4^{o}C$. One set of trays was placed under vacuum in a vacuum jar and the second set was sealed with "Scotch" tape and samples of each were periodically tested to determine the stability of the freeze-dried diagnostic antisera. The results of the continuing tests performed after 9, 12, 15, and 22 months are recorded in Table 17.

After 12 months' storage under vacuum, the freeze-dried samples in the wells exhibited physical changes in the character of the dried product and did not go into solution, so testing of 4 of the 6 antisera stored this way was discontinued. The other two antisera, S. typhosa "H" and S. schottmuelleri "H", showed a loss in ability to agglutinate at 12 months and were

Table 17. Stability of freeze-dried antisera in Disposo-Tray wells.

Antiserum	Months stored @ 4°C			
	9	12	15	22
Salmonella typhosa "H"				
Freeze-dried - under vacuum	4+	2	±	ND
Freeze-dried - no vacuum	4+	4+	4+	4+
Salmonella schottmuelleri "H"				
Freeze-dried - under vacuum	4+	2+	±	ND
Freeze-dried - no vacuum	4+	4+	4+	4+
Shigella boydii 5				
Freeze-dried - under vacuum	4+	ND*	ND	ND
Freeze-dried - no vacuum	4+	4+	2+	+
Vibrio cholerae "O"				
Freeze-dried - under vacuum	2+	ND*	ND	ND
Freeze-dried - no vacuum	2+	2+	2+	±
Escherichia coli				
Freeze-dried - under vacuum	+	ND*	ND	ND
Freeze-dried - no vacuum	+	4+	4+	+
Shigella flexneri 1				
Freeze-dried - under vacuum	4	ND*	ND	
Freeze-dried - no vacuum	4	+	±	-

ND* = Samples in the wells did not go into solution - testing discontinued.

completely inactive at 15 months. However, these latter two antisera, when stored without vacuum, retained their agglutinability through the 22 months of storage at 4°C. The S. boydii 5, V. cholerae "O", and E. coli antisera appeared stable through 15 months of storage but all three were unsatisfactory after 22 months. The S. flexneri 1 antiserum was the least stable sample, retaining agglutinability only through 12 months of storage. Studies on preparing diagnostic reagents in single-test units will continue.

6. Vaccine potency assays.

a. Typhoid vaccine potency assay.

Studies have continued during the past year to determine whether iron-supplemented mucin preparations can be employed as virulence enhancers in the mouse protection test to evaluate typhoid vaccines.

Previous investigations in this laboratory have shown that the addition of iron, in the form of ferric ammonium citrate, to sub-standard lots of 5% gastric mucin used in challenge suspensions, resulted in a further enhancement of virulence for mice of typhoid strain Ty2 (Annual Reports, 1975, 1976, 1977). In addition, mouse protection test assays were performed and preliminary results indicated that the addition of iron to sub-standard lots of mucin did not appear to interfere with the test for determining the potency of typhoid vaccines (Annual Report, 1976).

Additional mouse potency studies have been performed utilizing challenge suspensions containing mucin lots which require the addition of iron to obtain the virulence desired, along with mucin lots proven to be effective without iron supplement. In each of these experiments, commercial typhoid vaccines were compared with the U.S. standard vaccine Lot 6A. The object of these experiments was to determine whether sub-standard lots of mucin, by the addition of iron, could be used as effectively in evaluating typhoid vaccines as were other mucin lots which did not require additional iron to be satisfactory.

The results of some of these experiments, all of which conform to the FDA requirements for the evaluation of typhoid vaccines, are given in Tables 18 and 19.

Table 18. The mean values for the dose-response slopes (b) obtained in tests using different vaccines and different mucin preparations.

Vaccine	Mucins with no added iron		Mucins with added iron			
	A	B	C	D	E	F
6A	0.59(1)	0.69(4)	0.55(5)	0.93(2)	0.39(1)	0.41(1)
W-1	0.48(1)	0.57(1)	0.47(2)	0.62(2)	0.45(1)	0.76(1)
W-2		0.29(1)			0.29(1)	0.76(1)
W-3		0.47(3)	0.46(3)			
W-4		0.64(3)	0.59(3)			
All tests:	0.53(14)		0.56(23)			

(number of tests in parenthesis)

Table 19. Mean values for relative potencies of typhoid vaccines using different mucin preparations.

Vaccine	Mucins with no added iron		Mucins with added iron			
	A	B	C	D	E	F
6A	1.0(1)	1.0(4)	1.0(5)	1.0(2)	1.0(1)	1.0(1)
W-1	0.9(1)	0.5(1)	0.9(2)	0.5(2)	0.8(1)	0.4(1)
W-2		0.4(1)			0.4(1)	0.2(1)
W-3		0.4(3)	0.4(3)			
W-4		0.5(3)	0.4(3)			
All tests	0.67		0.67			

(number of tests in parenthesis)

Table 18 records the slopes (b-values) of the dose-response lines for the various experiments. Although there is considerable variability in the individual slope values, no significant differences were found between tests performed with typhoid challenges enhanced with mucin lots with no added iron and with challenges using mucin lots which required added iron, indicating that similar mechanisms are involved.

Table 19 demonstrates the relative potencies of the various vaccines employed in these experiments. These data also show that there are no significant differences between the values obtained with mucin lots with no added iron and with mucin lots which required added iron.

Thus it appears that sub-standard lots of gastric mucin, with the addition of added concentrations of iron, may sufficiently enhance the virulence of the challenge organism to permit their use in mouse potency assays for the evaluation of typhoid vaccines.

b. Meningococcal vaccine potency assays.

During the past year, studies have continued using the mouse protection assay previously described (Annual Report, 1974), on an investigation of the protective responses of mice to experimental Group B meningococcal protein-polysaccharide complex vaccines.

Previous studies (Annual Report, 1974) have demonstrated that excellent protection was afforded mice immunized with miningococcal protein vaccines and later challenged with virulent Neisseria meningitidis, Strain B-11, suspended in 5% hog gastric mucin.

Walter Reed ICR mice weighing 13-16 gms, were immunized with a single IP dose of one of five Group B protein-polysaccharide vaccines prepared jointly in the Department of Biologics Research and in the Department of Bacterial Diseases, WRAIR. The mice were subsequently challenged one week later with meningococcal strain B-11(#1). The results of these experiments are given in Table 20. Low levels of protection were demonstrated with all of the vaccines as evidenced by the high ED_{50} values. Additional experiments were carried out to determine whether protective responses in mice could be improved with a longer time interval between immunization and challenge.

Table 20. Mouse protection assays with meningococcal protein-polysaccharide complex vaccines (one week interval between immunization and challenge).

Vaccine	ED_{50} (ml)	(1) Standard Deviation
WZ-1	0.1000	63-159%
WZ-2	0.2083	78-128%
PA-1	0.1701	66-151%
PA-3	0.0492	66-151%
BP2-4	0.0525	66-151%

Since vaccines PA-3 and WZ-2 contain equal proportions of protein and polysaccharide and have been reported to provide some degree of immunogenicity in humans (Zollinger et al., 1979), these two vaccines were selected for use in subsequent mouse potency assays.

Mice were immunized with the two vaccines and were challenged one and three weeks later. An additional group of mice were immunized with vaccine PA-3 so that the challenge dose could be administered at intervals of one, two, or three weeks post-immunization. The results of these experiments are summarized in Table 21. Mice challenged two or three weeks post-immunization showed significantly increased protective levels when compared to the protection exhibited by mice challenged after only one week post-immunization.

Additional mouse potency assays were performed with three vaccines to determine if any large differences could be detected using two and three week intervals between immunization and challenge. These results are recorded in Table 22. The ED_{50} values obtained indicate higher protective responses with a three week interval between immunization and challenge, with differences of 2, 6, and 22-fold exhibited for the three vaccines, WZ-2, PA-3 and BP2-4, respectively. It should be noted that the 6-fold difference in ED_{50} value with vaccine PA-3 was not evidenced in an earlier experiment (Table 21).

To determine the reproducibility of the mouse potency assay additional mouse protection experiments were performed utilizing two and three week intervals between immunization and challenge. The same vaccines were employed in these assays. The results demonstrated that the protection afforded mice with the two

week immunization-challenge interval was consistent with that achieved in earlier experiments. Although somewhat greater protection was afforded mice challenged three weeks post-immunization, this advantage was offset by the extra time required to obtain results and the additional cost of housing and caring for the animals.

On the basis of the above results, the use of the mouse potency assay as an in vivo assay for the evaluation of protein-polysaccharide complex vaccines prepared from N. meningitidis, strain B-11 appears feasible. Correlation of the protection afforded mice immunized with protein-polysaccharide complex vaccines and the results obtained from human immunizations will be made. The usefulness of the mouse protection test for the evaluation of Groups "A" and "C" meningococcal polysaccharide vaccines will also be evaluated.

Table 21. Effect of 1, 2 and 3 week intervals between immunization and challenge on mouse protection potency of meningococcal vaccines.

Vaccine	1 Week		2 Weeks		3 Weeks	
	ED_{50}(ml)	(1) Standard Deviation	ED_{50}(ml)	(1) Standard Deviation	ED_{50}(ml)	(1) Standard Deviation
WZ-2	0.0231	37 - 268%	----------		<0.00080	----
PA-3	0.0200	59-170%	----------		0.00086	35 - 288%
PA-3	0.0123	70-144%	0.00036	66-151%	0.00032	80 - 125%

Table 22. Comparison of 2 and 3 week intervals between immunization and challenge on mouse protection potency of meningogocccal vaccines.

Vaccine	2 Weeks		3 Weeks	
	ED_{50} (ml)	(1) Standard Deviation	ED_{50} (ml)	(1) Standard Deviation
WZ-2	0.00119	66-151%	0.00042	66-151%
PA-3	0.00276	70-144%	0.0042	57-174%
BP2-4	0.00473	50-199%	0.00012	47-213%

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 136 Development of Biological Products

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6537	79 10 01	DD DR&I (AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BSU1	00	137
b. CONTRIBUTING				
c. XXXXXXXX	Cards 114F			

11. TITLE (Precede with Security Classification Code)

(U) Pathologic Manifestations of Zoonotic Diseases of Military Importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
74 02	Cont	DA		C. In House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE — EXPIRATION

b. NUMBER

c. TYPE NA — d. AMOUNT

e. KIND OF AWARD — f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING FISCAL YEAR 79	8	16
CURRENT FISCAL YEAR 80	8	126

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATOR

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NAME

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Morphologic pathology
(U) Animal model; (U) Wildlife; (U) Trypanosomiasis; (U) Leishmaniasis; (U) Pathogenesis

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23(U) To study and define the pathogenesis of experimental trypanosomiasis, leishmaniasis, and sarcocystosis and the effects of other infectious, toxic, and environmental biohazards in a variety of animal hosts. Provide morphologic pathology support for wildlife expidemiologic surveys in the TransAmazon and other military installations. Provide diagnostic pathology for animals acquiring spontaneous diseases and deaths during quarantine or colonization at the Walter Reed Army Institute of Research. Provide the pathology parameter for quality assurance of laboratory animals reared in the animal facility of the WRAIR and those acquired on contract. Provide morphologic pathology, ultrastructural microscopy, and immunopathology in an investigative study of spontaneous, severe renal disease and deaths in colonized aotus monkeys. All projects are generated from approved protocols and are related to military medical problems.
24(U) Studies utilize conventional gross and histopathology, clinical pathology, histochemistry, immunohistochemistry and electron microscopic techniques.
25(U) 78 10-79 09 The role of disseminated intravascular coagulation in the pathogenesis of Trypanosoma rhodesiense infection in rats will be continued as an interdepartmental study with the Department of Immunology. The pathology of chronic Trypanosoma rhodesiense infection in mice is being prepared for manuscript draft. The pathology and pathogenesis of experimental leishmaniasis in mice and dogs are being studied. The study to define the pathogenesis of glomerulonephritis induced in bovines by Sarcocystis fusiformis is being completed for manuscript draft. The pathology and pathogenesis of spontaneous disease with progressive renal injury in the aotus monkey is being continued. As a part of an epidemiologic survey, the morphologic pathology os wildlife captured in the areas of the TransAmazon Highway is being continued into a second phase. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

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*Available in controlled form upon originator's approval

DD FORM 1 MAR 68 1498 — PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498 1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 137 Pathologic Manifestations of Zoonotic Diseases of Military Importance

Investigators
Principal: Anthony J. Johnson, MAJ, VC
Associate: Michael J. Reardon, MAJ, VC

Background:

To diagnose, define, investigate and compare known and potential diseases common to man and animals. Special emphasis will be focused on control and protective methods against the inflammation and tissue injury induced by these diseases. A major effort will be directed toward defining the mechanistic events and reactions at the cellular and subcellular levels occurring during the development of these diseases. Gross pathology, histopathology, clinical pathology, ultrastructural pathology, histochemistry, and immunohistochemistry will be used.

During the reporting period, research activities have included: 1) Pathology of chronic Trypanosoma rhodesiense infection in mice (strain C57BL/6J; 2) The role of disseminated intravascular coagulation in the pathogenesis of Trypanosoma rhodesiense infection in rats; 3) Pathology and pathogenesis of leishmaniasis in dogs; 4) Inbred mice as model hosts for cutaneous leishmaniasis; 5) Mortality of aotus monkeys in the WRAIR colony; 6) Diagnoses and morphologic pathology of wildlife from the Trans Amazon and CONUS military installations; 7) Clinical Pathology laboratory: Support and Collaborative Studies; 8) Pathology of spontaneous coronavirus infection in dogs; 9) Experimental investigation of nephrotoxic effects of gentamicin in the rat; and 10) Pathogenesis of glomerulonephritis induced in bovines by Sarcocystis fusiformis.

1. Pathology Of Chronic Trypanosoma rhodesiense Infection In Mice (strain C57BL/6J).

Interdepartmental study with the Department of Immunology, WRAIR.

The early symptoms of fever, general malaise, lymphadenopathy and splenomegaly are considered to be the direct consequences of invasion and multiplication of trypanosomes in the general circulation in Trypanosoma rhodesiense infections. This

acute infection in man is comparable to those observed in experimental infections in rodents in which death can occur within 5 days with terminal parasitemias of up to 10^9 trypanosomes/ml blood. The more well-known and specific symptoms such as meningoencephalitis, myocarditis, edema and anemia develop slowly and are considered of immunopathologic origin in man. The pathogenesis of these chronic symptoms are complex and the cause of death is still somewhat obscure. Thus, the need for a laboratory rodent model to study chronic Trypanosoma rhodesiense is well-recognized. Chronic Trypanosoma rhodesiense infection in mice strain C57BL/6J is a recent finding by the Department of Immunology, WRAIR. Meningoencephalitis, myocarditis, splenomegaly, lymphadenopathy and a marked erythrocytic response are some of the host's changes. Also, deposits of IgM, IgG and C3 are demonstrated in the kidneys. The severity of these changes are clearly related to the duration of the infection. These chronic pathologic changes are being characterized and assembled for publication.

2. The Role Of Disseminated Intravascular Coagulation In The Pathogenesis Of Trypanosoma rhodesiense Infection In The Rat.

   Interdepartmental study with the Department of Immunology, WRAIR.

The pathogenesis of anemia in Trypanosoma rhodesiense infection is complex and somewhat obscure. Damage to the tissue is mainly the results of the metabolic activities of the trypanosomes, especially through the repeated insults offered by the emergence of successive trypanosome variants and attempts made to control the infection by the host's defense mechanisms. Antigen-antibody reactions are accompanied by activation of biologic proteins, release of pharmacologic active substances, changes in the state of the blood-clotting mechanisms and deposition of immune complexes in the kidneys and other organs. The altered clotting mechanism is accompanied by thrombocytopenia and disseminated intravascular coagulation in some human patients.

Rats developing acute Trypanosoma rhodesiense infection from experimental intraperitoneal injections are thrombocytopenic but without significant changes in RBC counts and plasma fibrinogen and fibrin split products levels. The complement system is activated and deposits of immune complexes are demonstrated in the kidneys. A second study is in progress to analyze the blood coagulopathy in a chronic infection.

3. Preliminary Studies On The Use Of The German Shepherd Dog As An Experimental Model For Visceral Leishmaniasis.

Interdepartmental study with the Department of Parasitic Diseases, WRAIR.

The need to control or prevent fatal visceral leishmaniasis is obvious. Methods currently available to prevent or treat infections are inadequate. Drugs currently available for prophylactic or therapeutic treatment are neither entirely safe nor entirely effective. Most test drugs are being evaluated in rodent models because the susceptibility of nonrodent species to visceral leishmaniasis is not well-characterized. The German shepherd has shown a unique breed susceptibility to certain tropical infections (e.g., Ehrlichia canis) as compared to beagles or mixed breed dogs. Because of this susceptibility and the special advantage of acquiring direct evaluation of drugs for possible use in military working dogs, this species was selected.

Hematological, biochemical, serological and clinical changes will be evaluated and characterized in German shepherd dogs exposed to three strains of Leishmania that induce visceral leishmaniasis. The use of this animal for drug testing procedures will be determined.

4. Inbred Mice As Model Hosts For Cutaneous Leishmaniasis.

Interdepartmental study with the Department of Parasitic Diseases, WRAIR.

Susceptibility of mice to various human strains of Leishmania are being studied. Thirteen inbred mouse strains from a broad genetic background are variable in their development and resolution of nasal lesions in response to infections with human strains of Leishmania braziliensis, L. mexicana and L. aethiopica. Efforts are in progress to evaluate and characterize these infections as models of human disease. Studies of the pathogenesis, immunity and chemotherapy of cutaneous leishmaniasis are needed.

5. Mortality Of Aotus Monkeys In The WRAIR Colony.

Interdivisional study with the Division of Veterinary Medicine, WRAIR.

During the period of this annual report, juvenile, adolescent, and adult aotus monkeys continue to die unexpectedly of

natural diseases and/or conditions. To better define and categorize the pathology, and to identify the casual factors, complete case reports including thorough clinical histories, clinical pathology, gross examinations, histopathology, bacteriology and virology are prepared for each death. While the complications of renal disease, anemia and septicemia are the primary causes of death, agents and/or conditions which precipitate these morbid morphologic and functional changes are, for the majority of deaths, unknown. Strains of Klebsiella pneumoniae are frequently isolated from septicemic deaths but a majority of the monkeys dying of renal failure and anemia go unresolved for etiologic cause(s). The latter deaths constitute the majority.

These coordinated efforts to determine the origin, nature, cause(s) and development of disease and/or condition in the aotus colony at the WRAIR have provided information which resulted in improved methods of husbandry and in a decrease in aotus deaths. These procedures are being continued.

6. Diagnosis And Morphologic Pathology Of Wildlife From The TransAmazon Epidemiological Survey.

In collaboration with the USAMRU-Belem.

Reports of the histopathology and diagnoses of disease in rodents, marsupials, bats, and other miscellaneous mammals are being furnished the WRAIR Team-Belem. A total of 4100 cases have been submitted for histopathologic evaluation.

7. Clinical Pathology Laboratory Support And Collaborative Studies.

The clinical pathology laboratory handled approximately 21,000 requests for hematology and 35,000 determinations for serum or plasma biochemistry during the reporting period. This laboratory supports research and diagnostic pathology at the WRAIR. The clinical pathology may be a part of an interdepartmental study in which a written report is prepared or the raw test results are returned to the investigator. Consultation as to needs and types of clinical tests, and interpretation of test results are available.

a. Clinical Pathology Of The Aotus Monkey.

Interdivisional study with the Division of Veterinary Medicine, WRAIR.

To define the clinical histories as well as provide data to determine the cause(s) of natural illnesses and deaths of colonized aotus monkeys, a systematic clinical pathology reporting procedure was established. Using this systematic criterion, ill monkeys without obvious clinical signs as well as the degree of illness could be made with a good measure of accuracy. This reporting procedure was designed for computer storage.

Serum electrophoretic patterns of karyotypically defined aotus monkeys have been defined and a paper has been accepted for publication (Lab. An. Sci. Oct. 1979).

b. Serum Isoenzymes of Creatine Phosphokinase and Lactic Dehydrogenase as Aids in the Diagnosis of Intestinal Infarction.

Interdivisional study with the Division of Surgery, WRAIR.

The serum isoenzyme patterns of creatine phosphokinase (CK) and lactic dehydrogenase (LDH) of experimentally manipulated dogs and volunteer human patients with abdominal disorders, including intestinal infarction, are being studied. Preliminary data indicate: 1) that the dog is an acceptable laboratory model and has tissue isoenzyme distribution patterns similar to man; 2) the serum concentration of the isoenzymes change with intestinal infarction; 3) the pattern of change is different from myocardial infarction and peritonitis and is of both diagnostic and prognostic value.

8. Pathology Of Spontaneous Coronavirus Infection In Dogs.

Interdivisional study with the Division of Veterinary Medicine, WRAIR.

Canine coronavirus was recovered and characterized first from fecal specimens of an epizootic of diarrheal disease in USAF military dogs in Germany (Binn, et al, 1974). The pathogenesis of this isolate was investigated and determined to be mildly pathogenic (Keenan, et al, 1976). Recently, a new strain of coronavirus was isolated from the small intestine of two dogs which died with diarrhea and enteritis in the WRAIR animal colony. The pathology and pathogenesis of coronavirus infection are being studied.

9. Experimental Investigation Of Nephrotoxic Effects Of Gentamicin In The Rat.

Interdepartmental study with the Department of Nephrology, WRAIR.

Aminoglycoside antibiotics are known to be nephrotoxic in animals and man. Gentamicin, a member of the aminoglycoside group, renal toxicity has been reported. However, estimation of the clinical incidence has been obscured by the setting in which this antibiotic is given, i.e., serious sepsis and by the insensitivity of parameters to monitor toxicity in patients. This pilot study in rats is designed to acquire dose-effect data.

A histopathologic description of the renal pathology in rats induced by variable doses of Gentamicin is provided to the Department of Nephrology.

10. Pathogenesis Of Glomerulonephritis Induced In Bovine By Sarcocystis fusiformis.

In collaboration with the Animal Parasite Institute, USDA, Beltsville, MD.

The nature and cause of the glomerulonephritis have been studied. A rough draft manuscript is being prepared.

Project: 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit: 137 Pathologic Manifestations of Zoonotic Diseases of Military Importance

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Project: 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit: 137 Pathologic Manifestations of Zoonotic Diseases of Military Importance

Manuscripts

Published:

1. Binn, L. N., Alford, J. P., Marchwicki, R. H., Keefe, T. J., Battie, R. J., and Wall, H. G.: Studies of Respiratory Disease in Random-Source Dogs: Viral Infection in Unconditioned Dogs. Lab. Ani. Sci., 29: 46-50, 1979.

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3. Jacobson, E. R., Seely, J. C., Novilla, M. N. and Davidson, J. P.: Heart Failure Associated with Unusual Hepatic Inclusions in a Deckert's Rat Snake. J. Wildlife Dis., 15: 75-81, 1979.

4. Keenan, K. P., Binn, L. N., and Takeuchi, A.: Animal Model of Human Disease, Acute Nonbacterial Gastroenteritis. Am. J. Path., 94(2): 439-442, 1979.

In Press:

1. Jacobson, E. R., and Seely, J. C.: Lymphosarcoma Associated with Viral-Like Intranuclear Inclusions in a California King Snake (Colubridae: Lampropeltis). J. Nat. Cancer Inst., 1979.

2. McKinney, L. A., and Hendricks, L. D.: Experimental Infection of Mystromys albicaudatus with Leishmania braziliensis. Am. Soc. Top. Med. & Hyg., 1979.

3. Seely, J. C.: Electron Microscopy as a Technique in Diagnostic Pathology. J. Wildlife Dis., 1979.

4. Reardon, M. J., Hall, R. D., and Davidson, C. E.: Serum Electrophoretic Patterns of Karyotypically Defined Owl Monkeys (Aotus trivirgatus). Lab. Ani. Sci., 29(5), Oct. 1979.

Project: 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit: 137 Pathologic Manifestations of Zoonotic Diseases of Military Importance

Abstracts

1. Childs, G.E., Groves, M.G., Hendricks, L.D., Price, E.E., and McKinney, L.A.: Inbred Mice as Model Hosts for Cutaneous Leishmaniasis. Presented at annual meeting of American Society of Parasitologists, Minneapolis, MN, August 1978.

2. Bunte, R.M., Beattie, R.J., Marchwicki, R.H., Sims, R.E., and Binn, L.N.: Fatal Enteric Disease in Foxhounds with Coronavirus Infection. Presented at annual meeting of Conference of Research Workers in Animal Diseases, Oct. 1978.

3. Bunte, R.M., Marchwicki, R.H., Beattie, R.J., and Binn, L.N.: Coronavirus Infection in Laboratory Foxhounds. Presented at annual meeting of American Association for Laboratory Animal Science, Atlanta, GA, Sept. 1979.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 6530	79 10 01	DD DR&I (AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	9. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102 BS01	00	138
b. CONTRIBUTING				
c. XXXXXXXXX	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Vaccine Development in Trypanosomiasis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 09	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION

b. NUMBER

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 79	3	85
	CURRENT 80	4	100

19. RESPONSIBLE DOD ORGANIZATION

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NAME: WELLDE, B.T.

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Kenya; (U) Trypanosomiasis; (U) Vaccine; (U) Africa; (U) Cattle; (U) Immunity

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) The objective of this program is to develop an effective, practical vaccine against African trypanosomiasis, useful to both military and civilian agencies. Related benefits include acquisition of knowledge pertaining to trypanosome immunity, host response and pathology of infection. There is a requirement for these studies which should provide a basis for rational development of a vaccine for this disease which would constitute a serious hazard for military personnel operating in the endemic area.

24. (U) Experiments conducted at WRAIR and in Kenya have demonstrated that experimental animals can be successfully immunized with irradiated trypanosomes. Rodents, cattle, and monkeys can be rendered completely resistant to a challenging infection of T. rhodesiense. Complete immunity has been achieved against T. congolense.

25. (U) 77 10 - 78 09 During this period the investigators demonstrated that the antigenic character of the parasite population of T. rhodesiense from an endemic area was composed of perhaps as few as one serodeme which was antigenically stable over an 8-year period. They also found that immunity could be induced to blood and tsetse fly (metacyclic) forms by exposure of experimental animals to a broad spectrum of antigenic variants of the same serodeme. The sterile immunity was long lasting. (U) 78 10 - 79 09 Cross serodeme challenge with both blood and metacyclic forms did not result in protection and indicates that any vaccine would have to be developed for a specific area of which the antigenic composition of the trypanosome population were known. Metacyclic trypanosome may be more homogeneous antigenically than blood forms. Techniques have been developed to isolate metacyclics from tsetse flies and immunization trials with metacyclics are under way. It is believed that these findings enhance the likelihood of immunologic control of trypanosomiasis. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 October 1978-30 September 1979.

*Available to contractors upon request before approval

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (* IN ARMY USE) ARE OBSOLETE

PROJECT 3M161102 BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

WORK UNIT 138 VACCINE DEVELOPMENT IN TRYPANOSOMIASIS

INVESTIGATORS:
PRINCIPAL: MAJ W.T. HOCKMEYER, MSC
ASSOCIATES: MAJ L.W. ROBERTS, MSC; B.T. WELLDE, GS13

PART I: TRYPANOSOMA CONGOLENSE: NATURAL AND ACQUIRED RESISTANCE IN THE BOVINE

INTRODUCTION

In nature, there is some evidence of natural and acquired immunity in cattle to trypanosomiasis. It has been postulated that young animals are more resistant to trypanosomiasis than adults (Reviewed by Fiennes, 1970), possibly through transmission of an immune factor to calves born of immune or partially immune dams (Whiteside, 1962). Certain breeds of cattle also appear to be naturally resistant to trypanosome infections (Stephen, 1970). Attempts to induce immunity to trypanosomiasis under field conditions, however, have produced contradictory results. Reports of several investigators have shown no evidence of immunity in cattle maintained under therapy in endemic areas over long periods of time (Hornby, 1941; Wilson, Paris and Dar, 1975). Other workers, however, claim that drug therapy induced a degree of protective immunity in treated animals (Bevan, 1928; van Saceghem, 1938; Fiennes, 1953; Soltys, 1955; Smith, 1958; Wilson, et al. 1976). Many of these field observations, however, are difficult to interpret because of the use of small numbers of animals of unknown age and condition, the question of persisting trypanocides, and the meager information concerning the antigenic nature of the trypanosome complex in given endemic areas.

In the laboratory on the other hand, a variety of immunization procedures have been used which produce a strong resistance in animals to a challenging trypanosome infection (Dodin and Fromentin, 1962; Johnson, et al. 1963; Seed and Weinman, 1963; Duxbury and Sadun, 1969; Welde, et al. 1979). None of these procedures, however, has been shown to be effective against the disease in nature. This has been due, in part, to the variant specific nature of the protective immune response and to the relatively obscure antigenic structure of the naturally transmitted metacyclic trypanosome. The early literature on the subject of immunity to African trypanosomiasis has been amply reviewed by Taliaferro (1929), and the more recent literature by Clarkson (1976) and Murray and Urquhart, (1977).

The lack of substantial laboratory investigations regarding immunity in the bovine to Trypanosoma congolense led us to examine the questions of immunity in reference to the following: age resistance, self-cure, chemotherapeutic cure and the relationship between blood and tsetse fly induced infections.

MATERIALS AND METHODS

Animals

Cattle of a predominantly Hereford breed were obtained from the veterinary department farm at Kabete or from other trypanosomiasis-free areas in Kenya. Upon their arrival at our laboratory, all animals were routinely treated before experimentation with recommended levels of Terramycin (Pfizer International - New York, New York), Phenamidine (May and Baker, Dagenham, England) and Ranizole (Merck Sharpe and Dohme, B.V., Haarlem, Netherlands). Ranizole treatment was continued on a periodic basis. All animals also received foot and mouth vaccine (Wellcome-Kenya). In general, the experimental animals were kept outside and supplemental food was provided during periods of poor pasture conditions. All experimental animals were dipped or sprayed in an acaracide weekly, with the exception of animals undergoing tsetse fly challenge.

Trypanosomes

The Trans-Mara I strain of Trypanosoma congolense which was isolated from an infected cow in the Trans-Mara area near the Kenya-Tanzania border in 1966 was the primary parasite used in these studies. A stabilate was made from a pool of blood collected from 3 infected steers in 1971. Other stabilates were prepared in 1973 and 1975 and all animals in this study were infected with trypanosomes originating from one of these three stabilates. Usually, infected mice were used as donors after being infected with stabilate trypanosomes. Sometimes, however, animals were infected or challenged with blood obtained from infected cattle.

For blood induced infection or challenge, trypanosomes in heparinized blood were enumerated in a hemocytometer and diluted with phosphate buffered saline (pH 7.8) containing 5% glucose and 10% fetal calf serum and injected into the jugular vein. Cattle were infected and challenged with 10,000 Trypanosoma congolense per 500 Lbs. body weight unless otherwise noted.

For tsetse fly infection or challenge, newly emerged flies (Glossina morsitans) were fed on an infected bovine donor for 14 consecutive days. Thereafter, the flies were fed for 5-day intervals on non-infected bovines until needed to induce a challenging infection.

A second strain of T. congolense was used for testing immunity to a heterologous strain. This parasite (designated Yoani I strain) was isolated in 1977 from an infected dairy cow at Yoani, Kenya, about 40 miles south of Nairobi.

Detection of Parasites

All animals were tested for the presence of trypanosomes by injecting their blood (0.5 ml) into mice intraperitoneally before the initiation of experiments. Subinoculations of blood were also done in some experimental animals in an effort to detect subpatent infections.

Parasitemias in experimental animals were estimated by counting the numbers of trypanosomes per 100 leucocytes on thick blood smears and relating these values to the total leucocyte counts per cubic millimeter.

Chemotherapy

Curative chemotherapy was initiated with Berenil (Farbwerke Hoechst, Frankfurt (M) Germany) at a level of 1.05 g of active ingredient per 660 Lbs. of body weight. Generally, animals which were treated were severely anemic, extremely weak and occasionally prostrate. These animals appeared to be near death at the time of treatment.

Assessment of Immunity

Immunity in experimental animals was assessed by comparing prepatent periods, levels and frequency of parasitemia, hematologic parameters, general clinical signs, and the ability to survive a challenging infection with those of controls.

Hematology

Packed cell volumes (PCV) were done by the microhematocrit method and leucocytes were counted using an electronic cell counter (Coulter Electronic, Harpenden, England). Methods used in collecting samples and counting thrombocytes have been published previously (Wellde, et al. 1978). Reference in the text to experimental values are given plus or minus one standard deviation (±SD) unless otherwise noted.

RESULTS

Effect of dose of trypanosomes and sex of host

Within the range of numbers of trypanosomes injected into cattle, no relationship between dose and the survival time of animals was observed. The dose of trypanosomes was, however, related inversely to the prepatent period (Fig. 1). Both male and female animals developed similar infections and there was no apparent difference in survival times between animals of different sexes.

Age Resistance

Table 1 depicts the results of infections in animals of different age groups. It can readily be seen that most animals under one year of age survived the infection without treatment. Some animals between 1 and 2 years of age also were able to survive the infection, whereas all animals over 2 years of age either died or required treatment to survive.

Infections in Young Animals

Eventhough young animals were able to survive the infection without treatment, they underwent a severe disease. Clinical parameters were compared in 11 young animals who survived and 6 uninfected controls over a 31-week period. Fig. 2 shows the average level of trypanosomes in the peripheral blood of the survivors over a 31-week period. Average levels of parasitemia were gradually reduced as the disease progressed. While animals had patent infections throughout the first 8 weeks, after this time an increasing number of animals became apatent for periods which became greater with time. An average preinfection packed cell volume of 34.0($\pm$4.0) was reduced to a level of 17.9($\pm$3.2) at 8 weeks after infection (Fig. 2). Packed cell volumes gradually increased after this time and by 31 weeks after infection had risen to 24.1($\pm$4.8). Packed cell volumes did not appear to reach preinfection levels in individual animals for long periods even though trypanosomes were only infrequently found in the blood. Thrombocytopenia and leucopenia were also prominent manifestations of the disease (Fig. 2). Intermittent fever was accompanied by an early weight loss after which a minimal weight gain was apparent. Controls of the same age, however, had gained an average of 116 Lbs. while the infected animals gained only an average of 9 Lbs. during the 31-week period (Fig. 2). Many of these young infected animals remained small in stature throughout their adult life (Fig. 3).

Immunity in Self-Cured Calves

Animals which had apparently self-cured the primary infection and whose blood was negative when sub-inoculated into mice were challenged with the same strain of Trypanosoma congolense up to a year after their last patent parasitemia. No detectable infections developed in the self-cured animals whereas the controls developed typical infections and required treatment to survive (Table 2).

Infections in Adult Animals

Animals over one year of age developed an acute or chronic course of disease that was usually considered fatal. Clinical parameters were compared in 11 adults and 11 uninfected controls (Fig. 4). The average parasitemia in adult animals was twice that of the surviving young animals, however, the anemia which developed was similar in degree. A leucopenia which was comparable to that found in young animals was also present (Fig. 4). Average thrombocyte levels were lower in infected adult animals although younger animals naturally have a higher level of thrombocytes. Weight loss was marked in adult animals with up to a 34% decrease in preinfection values. Ten of the 11 infected animals died or required treatment to survive by the 15th week of infection. The remaining animal developed a protracted chronic course of disease and died during the 32nd week of infection. This chronic disease state was characterized by a low level relapsing parasitemia accompanying a continued low PCV.

Immunity in Treated Adults

Adult animals which required therapy to survive were challenged along with controls at a later time. Table 3 shows that an appreciable immunity had developed in these animals and many self-cured the challenge infection. Even when the challenging infection was given about two years after treatment there was evidence of persisting immunity. Although most of these animals which were challenged at this time needed treatment to survive, their infections were of a chronic nature and less severe than those of control animals. Treatment was required in these animals at a later time than in their primary infections or in the controls. Figure 5 illustrates the pattern of parasitemia and level of packed cell volume in an animal undergoing infection, treatment and challenge. The challenging infection was much less severe than the primary infection; the animal had limited periods of parasitemia which were similar to that of a chronically infected animal and a minimal decrease in packed cell volume and other hematologic parameters.

When animals described in Table 3 were challenged a second time, no detectable infections or clinical signs of disease were observed while all controls developed parasitemia and required treatment to survive (Table 4). Animals were also strongly resistant to challenge with relapse parasites obtained from chronically infected bovines. Figure 6 illustrates the patterns of parasitemia and levels of packed cell volumes in an animal immunized by infection and cure and a control animal challenged with parasites isolated from a bovine undergoing a relapsing infection of 250 days duration. When compared to the control animal, the infection in the immunized animal was brief and much less severe. The control required treatment while the immunized animal self-cured.

Tsetse Fly Challenge

Animals presumed to be immune to challenge with blood forms were subjected to tsetse fly challenge with the homologous strain of Trypanosoma congolense. Each of twelve immune, three partially immune and nine control animals received an average of 428 fly bites from a pool of flies having a 32% infection rate of metacyclic trypanosomes. Of the twelve immune bovines challenged by fly bite, five did not develop parasitemia or clinical evidence of disease (Table 5). The other seven had limited periods of patent parasitemia (Fig. 7), and only one animal developed signs of clinical disease. All twelve immune animals survived infections and eight required treatment to survive.

Average parasitemias were greatly reduced in immune animals and followed a relapsing pattern somewhat similar to that of chronic infections or that of immune animals challenged with blood forms. Prepatent periods were not always increased in immune animals, however, and three immune animals had prepatent periods similar or shorter than controls. Although parasites appeared in the blood of these animals early after challenge they were suppressed quickly (Fig. 8). Clinical parameters such as PCV (Fig. 9) thrombocyte levels (Fig. 10) and leucocyte levels (Fig. 11) remained within normal limits in immunized animals while the values in controls were severely affected.

One year after cyclical transmission was initiated, experimental animals remained highly resistant to challenge with the Trans Mara strain by fly bite whereas control animals required treatment to survive.

One animal, which had undergone primary infection and challenge in 1970 and was rechallenged periodically during the subsequent 6 years with syringe induced infections, was challenged by tsetse fly bite in 1977. Table 6 summarizes the results over the 8-year period. Control animals injected at each challenge either required treatment to survive or died.

Heterologous Challenge

To determine whether or not cross strain immunity was present in animals immune to the Trans Mara I strain of T. congolense, 3 immune and 3 control animals were challenged with the Yoani strain of T. congolense by blood induced or tsetse fly induced infections (Table 7). No immunity was observed in any of the animals whether challenged by either method. All animals were treated during the fifth week of infection when packed cell volumes had decreased to below 20%.

DISCUSSION

Our studies demonstrate that under certain conditions an appreciable immunity to T. congolense can develop in bovines. We found a substantial age resistance to Trypanosoma congolense and although young animals underwent a relatively severe disease process, almost all survived while animals over two years of age invariably succumbed to infection. Although the mechanism(s) for such resistance is not clear, in our experiments it did not involve specific maternal antibody since the dams of our calves had never been infected and the calves had been weaned at least 1 month before infection. These studies confirm and extend the observations of others (reviewed by Fiennes, 1970). Although a more virulent strain of T. congolense might kill young animals, we believe a relative resistance would be found in them when compared to adults.

Although Weitz (1970) suggested there was no evidence for an acquired protection in animals after recovery from the disease, we have shown that young surviving animals are resistant for extended periods of time to a challenge infection of the same strain by either syringe inoculation of blood forms or by tsetse fly bite (metacyclic forms). Many of these immune animals, however, are stunted and are relatively non-productive. As well as being a poor source of meat, the small stature of females infected early in life may lead to problems in calving. We have observed the death of one of our self-cured experimental animals due to the inability to complete parturition because of her small pelvic diameter.

Animals undergoing infection and Berenil treatment also showed resistance upon syringe or tsetse challenge with the same strain. Most of these animals self-cured the first or second challenge infections. Premunity did not play a role

in this protection since the animals had been given curative therapy to terminate the primary infection. The resistance appeared to be associated with the duration of infection, the time elapsing between treatment and challenge and the number of infections the animal has been subjected to. The short period that effective levels of Berenil persist in the blood of the bovine precludes any complicating drug effect in these studies. Trials in our laboratory showed that Berenil (7 mg/kilo) had an effect on infectivity for 12 days and on the prepatent period for up to 18 days but not at 25 days or longer after injection (Unpublished data). This is in agreement with previously published work (Cunningham et al. 1964).

While we have shown that a substantial immunity can be induced experimentally by infection and cure, the reasons are not well understood why it has not been more apparent in nature. Since most of the failures to produce an immunity in animals in the field by this method have been in areas of high tsetse challenge, we believe that the interval between treatment and reinfection is important. It is known that the lymphoid system in T. congolense infected bovines undergoes atrophy and depletion of lymphocytes (Kaliner, 1974). Morrison and Murray (1979) have shown a marked depletion of immunoglobulin containing cells in the spleens of T. congolense infected mice and these findings are consistent with the reports of deficient immunologic responses to a variety of antigens in T. congolense infected hosts (Manisfield and Wallace (1974), Holmes et al. (1974). It has been suggested that the response to the trypanosome by the infected host may also be defective and could account in part for the parasites survival (Murray and Urquhart, 1977). Little is known about the repopulation and recovery of the lymphoid system of the infected bovine after treatment, but reinfection soon after therapy may find the animal in a poor condition to respond immunologically. In our experiments, animals were given relatively long periods to recover after treatment and under these circumstances were demonstrably resistant to challenge for relatively long periods.

It also appears that the antigenic composition of populations of T. congolense in nature is complex and the number of different strains or serodemes being transmitted in given areas at different times may play an important role in the development of immunity (Dar, et al. 1973; Wilson, et al. 1973). We detected no cross strain immunity either against blood or tsetse fly induced infections with a strain of T. congolense from a different area. Under these circumstances any cross species immunity would be extremely unlikely and the presence of different species and strains trypanosomes in the same host would probably complicate the acquisition of immunity.

The consistent induction of immunity to both blood and tsetse fly challenge over a relatively long period lends support for the postulate that alternate genes are responsible for the process of antigenic variation. Our studies indicate that there probably is a limitation imposed by the parasite genome on the occurrance of different antigenic types. This would not be consistant with a process depending on the selection of mutants (Seed, 1974). Grey (1975) showed that a relatively predictable series of predominant antigenic types of T. brucei appeared early in the course of infection in different hosts. He also described a reversion to a basic strain antigen which took place upon cyclical or syringe passaged transmission. It appeared, however, that some tsetse flies transmitted trypanosome with a mixture of both basic strain and variant antigen types. Other investigators have also provided evidence that metacyclic trypanosome populations are antigenically heterogenous (Leray, et al. 1978).

Our experiments tend to support these findings since our experimental animals which were immunized by either infection and self cure or infection and chemotherapeutic treatment showed a marked resistance to tsetse fly challenge with the same strain of trypanosome. The relatively brief periods of parasitemia which occurred in some immunized animals possibly were due to the partial waning of immunity against particular predominant variant antigens or because tsetse flies transmitted populations of parasites, a portion which possessed a variant antigen not previously experienced by some of the immunized animals. Eventhough infections were established in some immunized animals they were controlled and eradicated rapidly indicating that whatever the extent of metacyclic heterogeneity, their progeny for the most part, were antigenically similar to those of previous blood induced infections.

The phenomena of self cure in younger animals and in previously infected adults is interesting and poses some important questions. The progressively decreasing level of parasitemia associated with increasing periods of apatency suggests that either the host response is increasingly efficient or the parasites capability to produce different antigenic variants eventually becomes exhausted. Whether or not similar cross reacting groups which have been identified in surface antigens from different variants (Barbet and McGuire, 1978) play a role in this increasingly enhanced ability to control the level and frequency of parasitemia in both self cured and previously infected animals is yet to be determined. In our experience, however, it would be unlikely that this immunity would extend to trypanosomes another strain.

TABLE 1

THE EFFECT OF AGE ON TRYPANOSOMA CONGOLENSE INFECTIONS IN CATTLE

Age (Years)	Number of Animals	Median Survival* Time (Weeks-Days)	Range (Weeks-Days)	No. Self Cures (%)
0.3-1	11	> 78-0	6-8 to > 78-0	10 (91)
1-2	11	24-4	5-5 to 30-6	2 (11)
2-3	11	11-5	5-5 to 78-0	0 (0)
3-4	5	6-3	6-1 to 13-6	0 (0)
4-5	2	6-8	4-2 to 9-0	0 (0)
5-6	2	8-1	8-0 to 8-3	C (0)

* Based on time to treatment or day of death.

TABLE 2

RESULTS OF PRIMARY CHALLENGE OF PREVIOUSLY INFECTED, SELF-CURED ANIMALS

Initial Infection						Interval[3] (Wks-Days)	Primary Challenge ($1x10^4$/500Lbs.)		
An. No.	Age (Yrs)	Sex[1]	Dose per 500Lbs.	P.P.[2] (Days)	Last Patent Parasitemia (Wks-Days)		Age (Yrs)	P.P.[4] (Days)	Result[5]
1	0.5	M	$2.8x10^3$	8	54-4	25-0	2.0	N.P.	N.D.I.
2	1.3	MC	$1.0x10^4$	5	61-1	31-6	3.0	N.P.	N.D.I.
3	0.3	F	$1.0x10^4$	5	56-2	36-5	2.0	N.P.	N.D.I.
4	0.5	M	$2.9x10^3$	6	30-5	48-5	2.0	N.P.	N.D.I.
5	1.4	MC	$1.0x10^4$	5	38-2	54-5	3.1	N.P.	N.D.I.
Average of 3 control animals for primary challenge							3.1	4.7	T(9-3)

1. F - Female; MC - Male Castrated; M - Male. 2. P.P. - Prepatent Period.
3. Time between last patent parasitemia and challenge
4. P.P. - Prepatent Period; N.P. - Not Patent.
5. N.D.I. - No Detectable Infection; T - Treated (time since challenge).

TABLE 3

RESULTS OF PRIMARY CHALLENGE OF PREVIOUSLY INFECTED AND TREATED CATTLE

Initial Infection						Interval[3] (Wks-Days)	Primary Challenge ($1x10^4$/500Lbs.)		
An. No.	Age (Yrs)	Sex[1]	Dose per 500Lbs.	P.P. (Days)	Time to Treatment (Wks-Days)		Age (Yrs)	P.P. (Days)	Result[4] (Wks-Days)
6	1.0	F	$6.8x10^6$	3	7-0	28-6	1.7	14	S.C.(17-0)
7	4.4	F	$1.0x10^4$	6	9-0	30-0	5.2	10	S.C.(11-5)
8	2.7	F	$1.0x10^4$	5	11-5	42-2	3.8	18	S.C.(15-5)
9	2.6	F	$1.0x10^4$	5	6-6	47-1	3.7	13	T.(36-6)
10	1.9	F	$1.3x10^5$	5	28-0	71-4	3.9	14	S.C. (4-4)
11	1.6	MC	$8.4x10^3$	6	5-5	86-0	3.4	8	T.(21-3)
12	1.9	MC	$1.0x10^4$	6	5-5	86-0	3.7	6	T.(11-5)
13	2.3	MC	$1.9x10^4$	5	11-0	122-5	4.9	6	T.(27-0)
14	3.4	F	$1.3x10^4$	5	5-1	128-4	6.0	6	S.C.(29-2)
Average of 8 control animals for primary challenge							4.1	5.5	T. (9-4)

1. F - Female; MC - Male Castrated.
2. Prepatent Period.
3. Time between treatment and challenge.
4. S.C. - Self Cure (Time of last patent parasitemia after challenge).
   T - Treated (Time since challenge).

TABLE 4

RESULTS OF THE SECOND CHALLENGE OF BOVINES REQUIRING TREATMENT OR SELF-CURED AFTER PRIMARY CHALLENGE

Group	Animal (No.)	Interval[1] (Months)	Prepatent[2] Period (Days)	Result[3] (Weeks-Days)
Self cure	3	5-30	N.P.	N.D.I.
Treated	3	6-10	N.P.	N.D.I.
Control (Average)	6	-	5.5	T (8.0)

1. From last patent parasitemia or treatment.

2. N.P. - Note Patent.

3. N.D.I. - No Detectable Infection; T. - Treatment (time after challenge).

TABLE 5

RESULTS OF TSETSE FLY CHALLENGE OF ANIMALS IMMUNIZED AGAINST BLOOD FORMS OF TRYPANOSOMA CONGOLENSE

Group	Age (Range) Years	# Patent/ # Challenged	Prepatent Period (Range)	Days of[2] Patent Infection (Range)	Lowest[3] PCV(%) (Range)	Result[4]		
						NDI	SC	T
Immune	4.6(1.9-8.0)	7/12	20.1(6-56)	6.4(0-32)	29.1(25-34)	5	7	0
Partially[1] Immune	5.4(4.2-6.3)	3/3	15.3(13-19)	79.7(61-100)	20.3(18.5-22.5)	0	2	1
Controls	3.5(1.2-7.4)	9/9	10.8(10-13)	96.6(83-100)	17.3(15.5-19.5)	0	1	8

1. Animals having a single infection and treatment

2. First 100 days after day 10.

3. Packed cell volume.

4. NDI - No Detectable Infection
   SC - Self Cure
   T - Required Treatment.

TABLE 6

HISTORY OF ANIMAL 151

Procedure	Date	Infection Source[1]	Method[2]	Dose/ 500Lbs.	Prepatent[3] Period (Days) 151 (control)	Result[4] (Days)	Lowest[5] PCV (%)
Primary Infection	14-9-70	B	S	$1.0x10^5$	5 (5)	T.(196)	11.0
1st Challenge	11-8-72	M	S	$1.0x10^4$	14 (5)	S.C.(32)	31.5
2nd Challenge	5-3-75	M	S	$1.0x10^4$	N.P.(5)	N.D.I.	31.0
3rd Challenge	10-7-75	B (Relapse)	S	$1.0x10^4$	N.P.(5)	N.D.I.	31.0
4th Challenge	17-3-76	M	S	$1.0x10^4$	N.P.(5)	N.D.I.	30.0
5th Challenge	15-12-76	M	S	$1.0x10^4$	N.P.(5)	N.D.I.	29.0
6th Challenge	26-9-77	B	T	?	19 (11)	S.C.(21)	33.0

[1]B - Bovine; M - Mouse. [2]S - Syringe; T - Tsetse fly. [3]N.P. - Not Patent.

[4]T - Treated (Days since challenge); S.C. - Self Cure (last patent parasitemia).

[5]P.C.V. - Packed Cell Volume.

TABLE 7

RESULTS OF BLOOD AND TSETSE FLY INDUCED CHALLENGE OF ANIMALS IMMUNE TO THE TRANS-MARA I STRAIN OF TRYPANOSOMA CONGOLENSE WITH A HETEROLOGOUS STRAIN (YOANI-I)

Group	Type Challenge	No. Patent/ No. Challenged	Prepatent Period (Range)	Result[1]		
				NDI	SC	T
Immune	Blood	3/3	7.3(6-9)	0	0	0
Control	Blood	3/3	7.0(6-8)	0	0	3
Immune	Tsetse Fly	3/3	13.6(12-15)	0	0	3
Control	Tsetse Fly	3/3	13.6(13-14)	0	0	3

[1]NDI - No Detectable Infection; S.C. - Self Cure;
T - Required treatment.

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LEGENDS FOR FIGURES

FIGURE 1: The effect of numbers of Trypanosoma congolense on the prepatent period in bovines.

FIGURE 2: Clinical parameters of young animals who survived Trypanosoma congolense infections. Data points where ±SD overlap have not been plotted.

FIGURE 3: An example of the stunting effect of Trypanosoma congolense infection on young animals can be seen in the animal in the foreground. The control is in the background. At the time of infection, 13 months previously, both animals were of similar age, weight and stature.

FIGURE 4: Clinical parameters of adult animals which wither required treatment to survive or died from Trypanosoma congolense infection. Data points where ±SD overlap have not been plotted.

FIGURE 5: Parasitemias and packed cell volumes of an animal treated 82 days after primary Trypanosoma congolense infection and challenge 296 days later.

FIGURE 6: Parasitemias and packed cell volumes of an immune and control animal challenged with 1×10^7 Trypanosoma congolense from a relapse parasitemia obtained from a chronically infected bovine.

FIGURE 7: Patterns of parasitemia in 5 immunized bovines after tsetse fly challenge with Trypanosoma congolense.

FIGURE 8: Average parasitemia levels of immunized and control bovines after tsetse fly challenge with Trypanosoma congolense.

FIGURE 9: Average packed cell volumes of immunized and control bovines after tsetse fly challenge with Trypanosoma congolense.

FIGURE 10: Average thrombocyte levels of immunized and control bovines after tsetse fly challenge with Trypanosoma congolense.

FIGURE 11: Average leucocyte levels of immunized and control bovines after tsetse fly challenge with Trypanosoma congolense.

Fig. 1

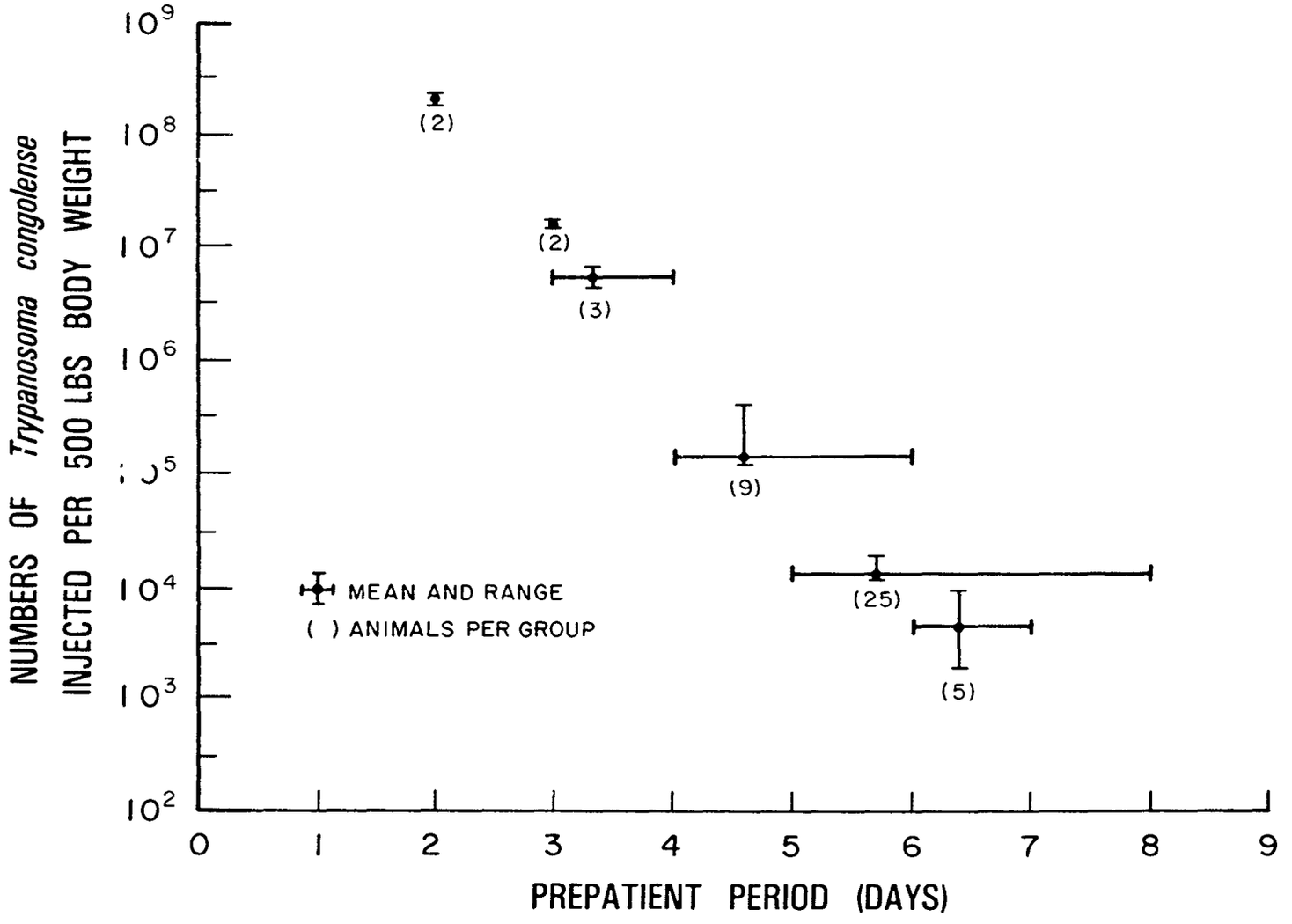

NUMBERS OF *Trypanosoma congolense* INJECTED PER 500 LBS BODY WEIGHT
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
(2)
(2)
(3)
(9)
(25)
(5)
MEAN AND RANGE
( ) ANIMALS PER GROUP
0 1 2 3 4 5 6 7 8 9
PREPATIENT PERIOD (DAYS)

Fig. 2

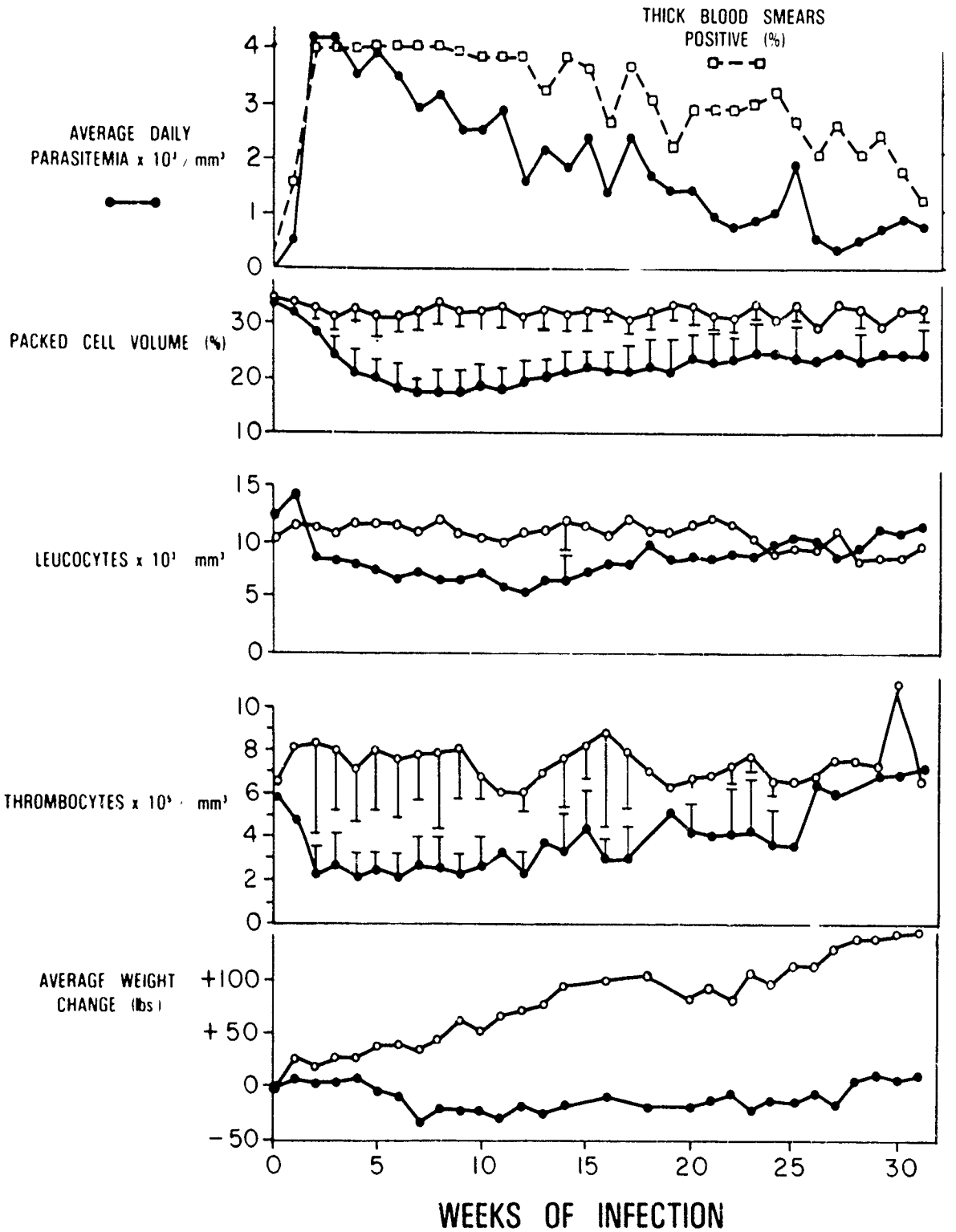
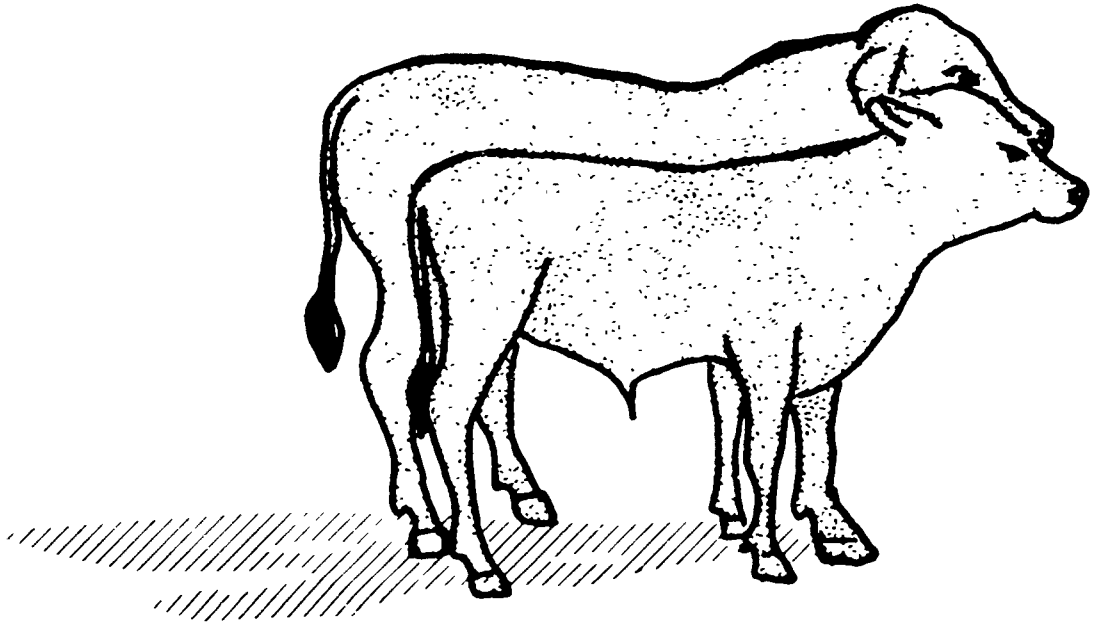

THICK BLOOD SMEARS POSITIVE (%)
AVERAGE DAILY PARASITEMIA x 10³ / mm³
PACKED CELL VOLUME (%)
LEUCOCYTES x 10³ mm³
THROMBOCYTES x 10³ / mm³
AVERAGE WEIGHT CHANGE (lbs)
+100
+ 50
0
- 50
0
5
10
15
20
25
30
WEEKS OF INFECTION

Fig. 3



605

Fig. 4

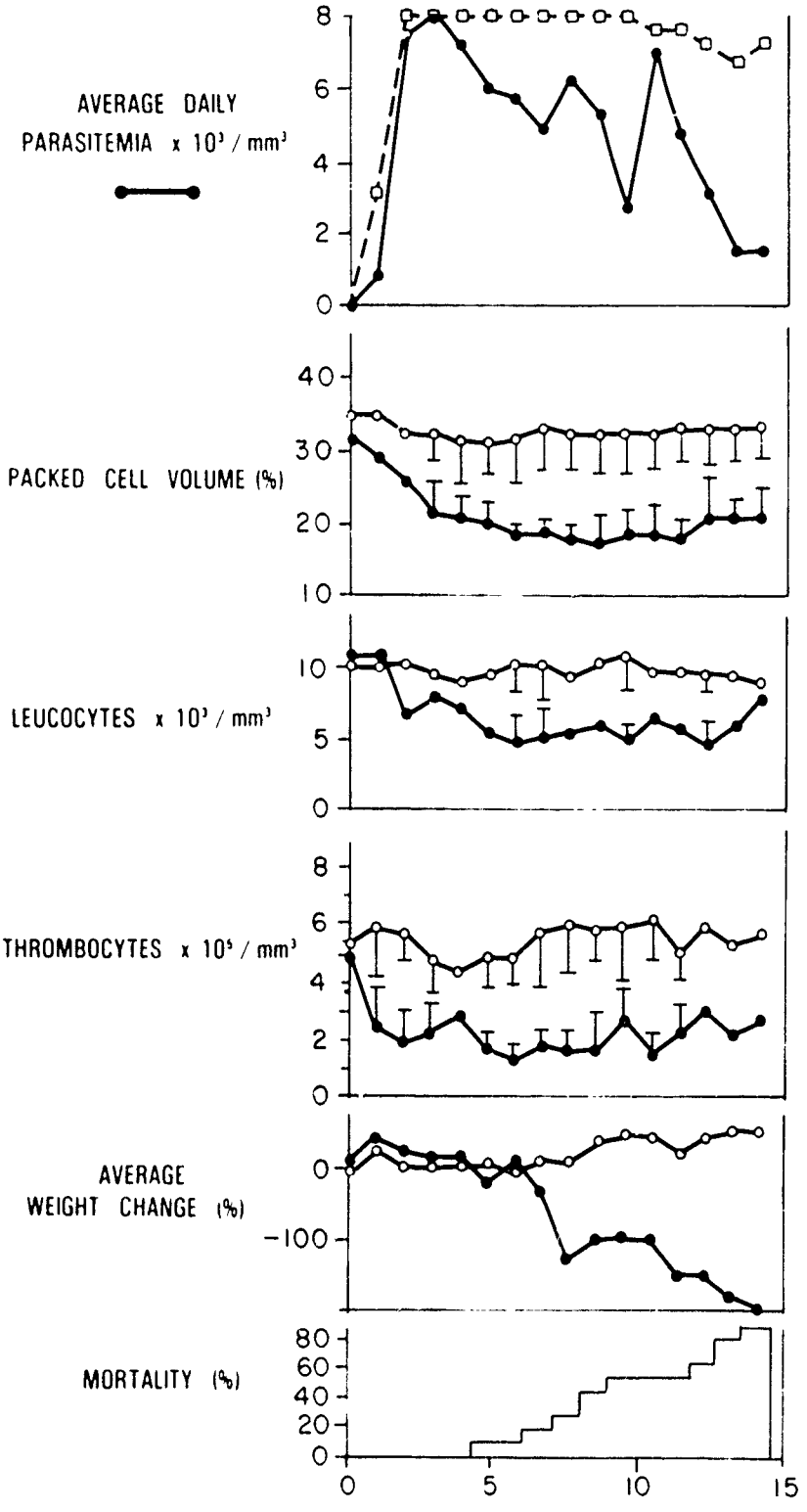

AVERAGE DAILY
PARASITEMIA x 10³ / mm³
8
6
4
2
0
PACKED CELL VOLUME (%)
40
30
20
10
LEUCOCYTES x 10³ / mm³
10
5
0
THROMBOCYTES x 10⁵ / mm³
8
6
4
2
0
AVERAGE
WEIGHT CHANGE (%)
0
−100
MORTALITY (%)
80
60
40
20
0
0
5
10
15
WEEKS OF INFECTION

Fig. 5

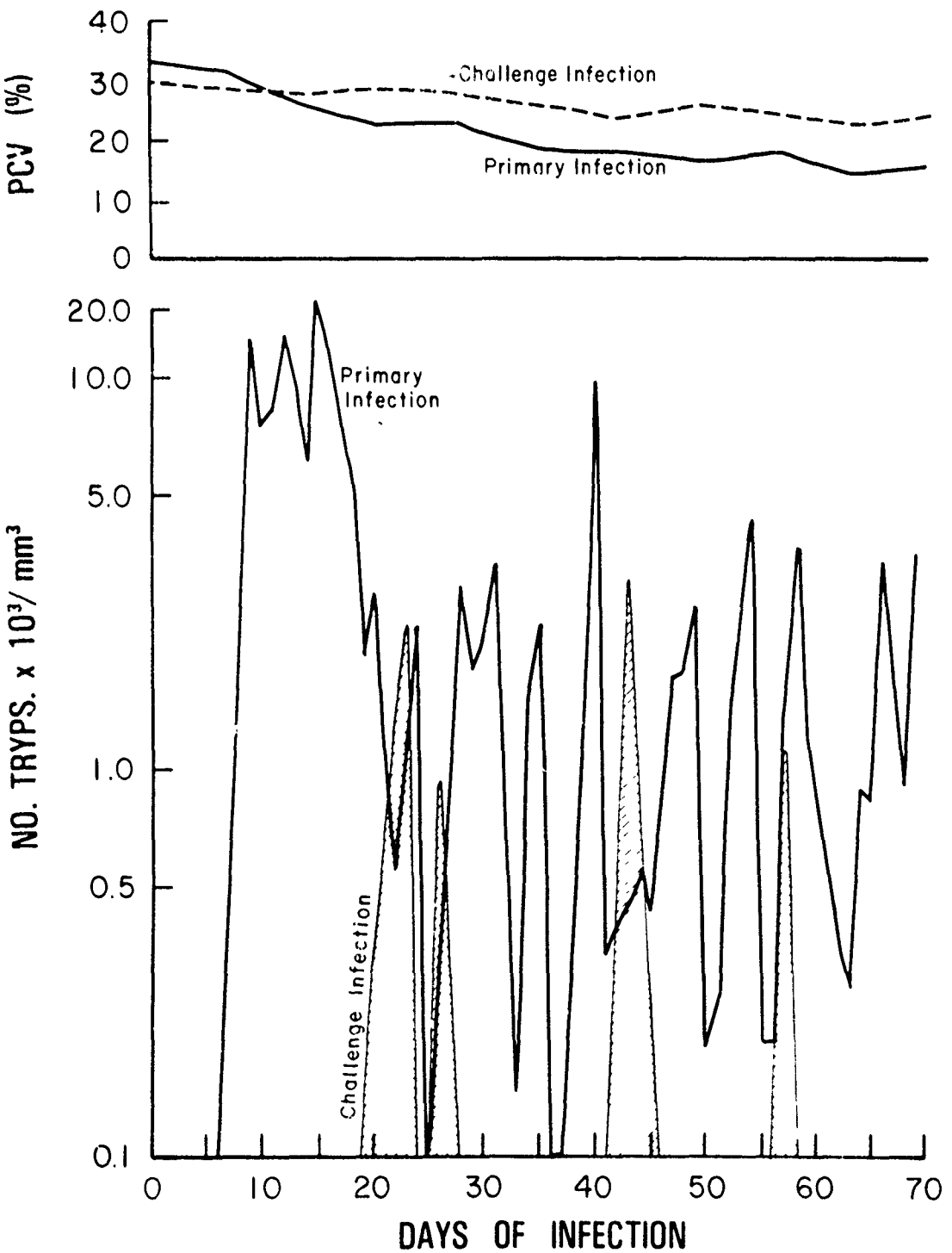

PCV (%)
40
30
20
10
0
Challenge Infection
Primary Infection
NO. TRYPS. x 10³/ mm³
20.0
10.0
5.0
1.0
0.5
0.1
Primary Infection
Challenge Infection
0
10
20
30
40
50
60
70
DAYS OF INFECTION

Fig.6

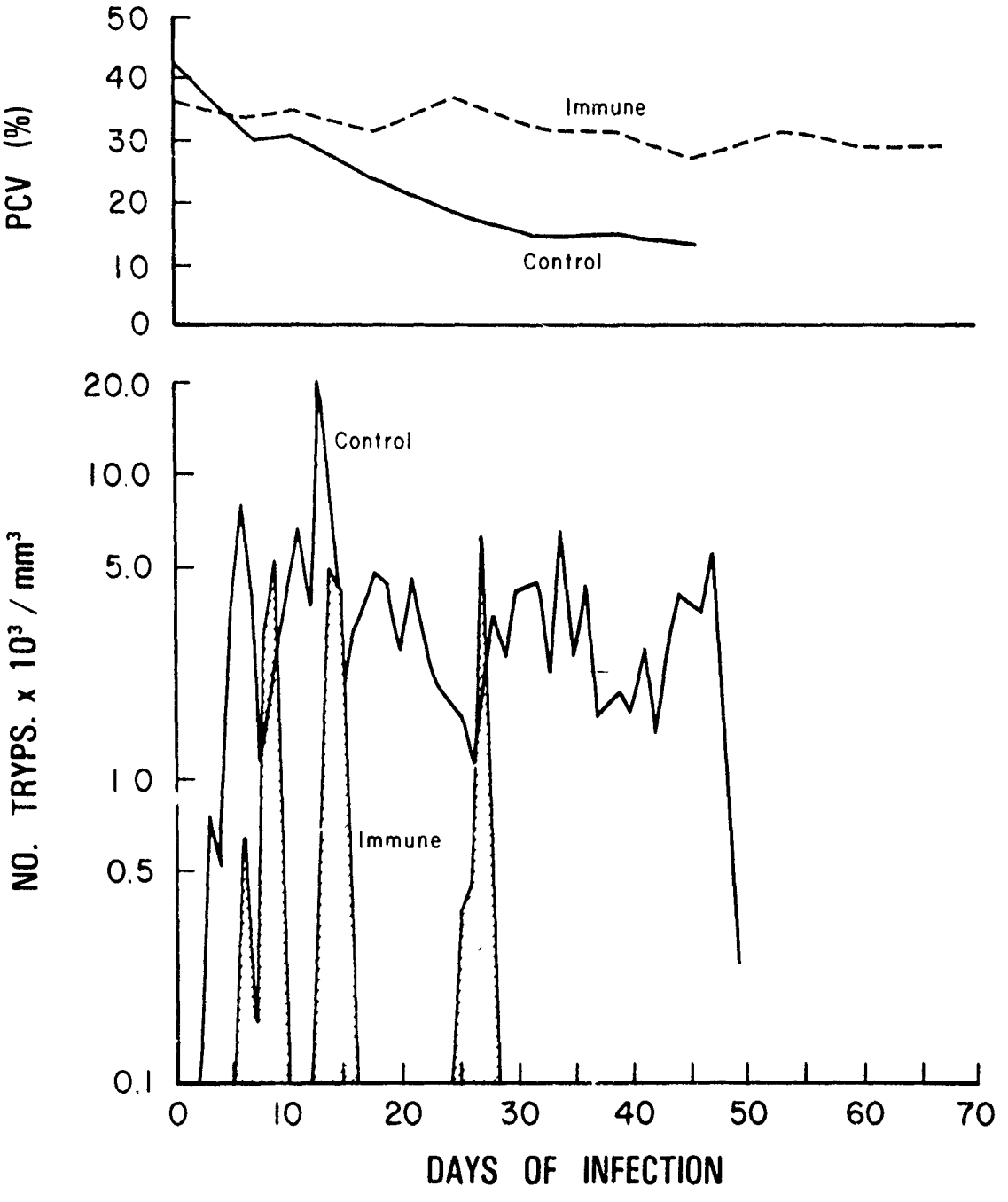
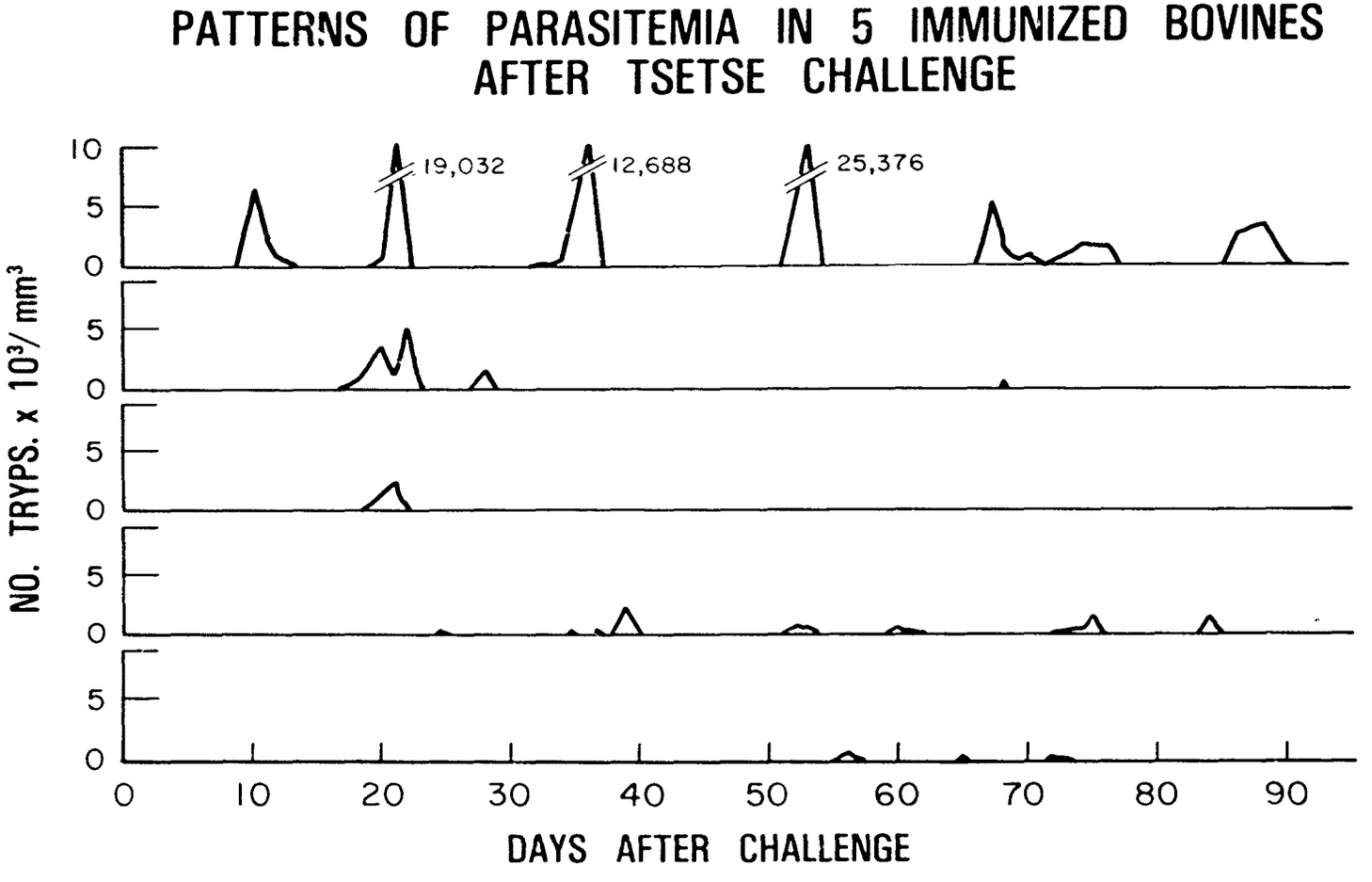

PCV (%)
50
40
30
20
10
0
Immune
Control
NO. TRYPS. x 10³ / mm³
20.0
10.0
5.0
1.0
0.5
0.1
Control
Immune
0
10
20
30
40
50
60
70
DAYS OF INFECTION

Fig. 7


PATTERNS OF PARASITEMIA IN 5 IMMUNIZED BOVINES
AFTER TSETSE CHALLENGE
19,032
12,688
25,376
10
5
0
NO. TRYPS. x 10³/mm³
0
10
20
30
40
50
60
70
80
90
DAYS AFTER CHALLENGE

609

Fig. 8

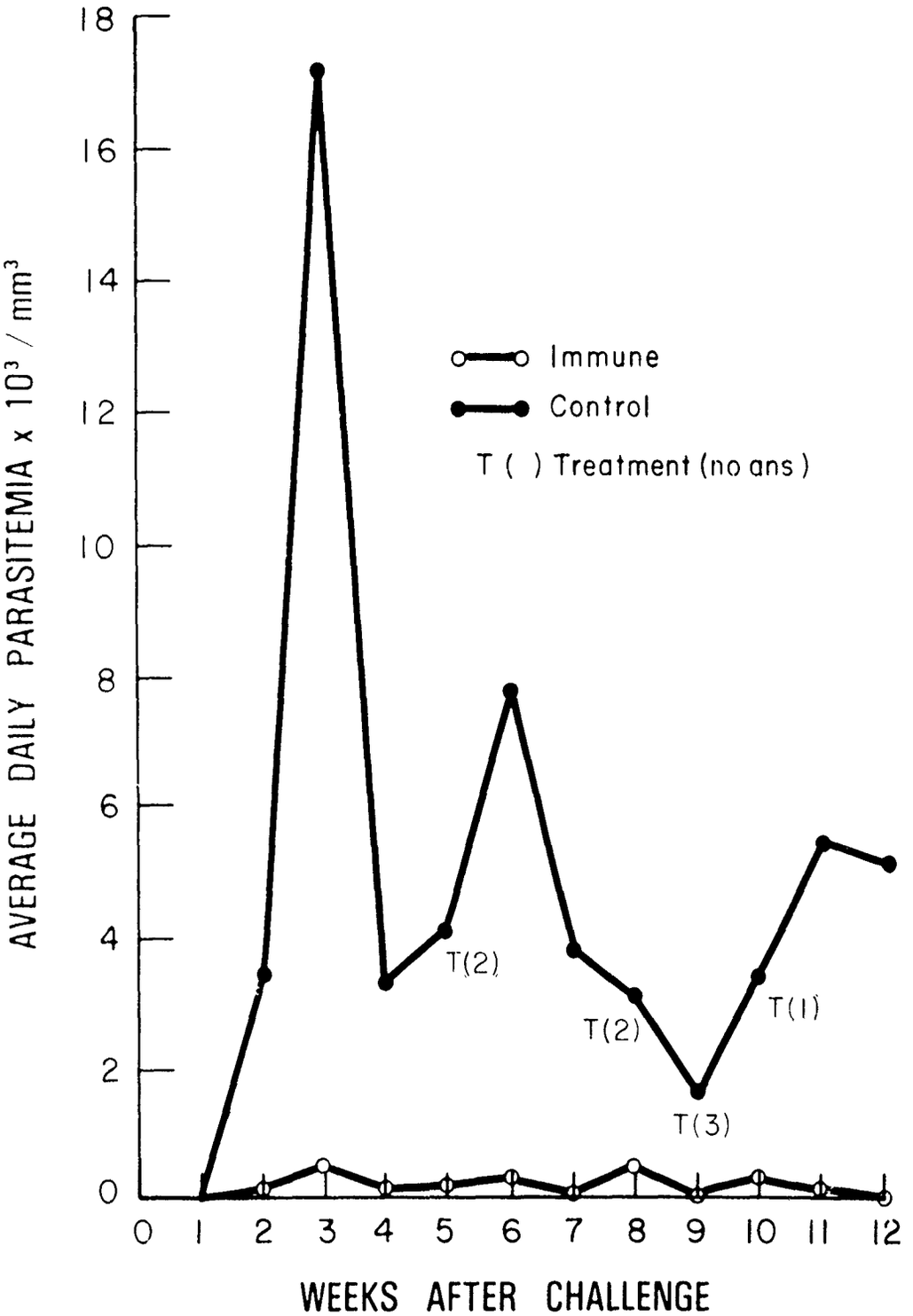

AVERAGE DAILY PARASITEMIA x 10^3 / mm^3
18
16
14
12
10
8
6
4
2
0
Immune
Control
T ( ) Treatment (no ans)
T(2)
T(2)
T(3)
T(1)
0 1 2 3 4 5 6 7 8 9 10 11 12
WEEKS AFTER CHALLENGE

Fig. 9

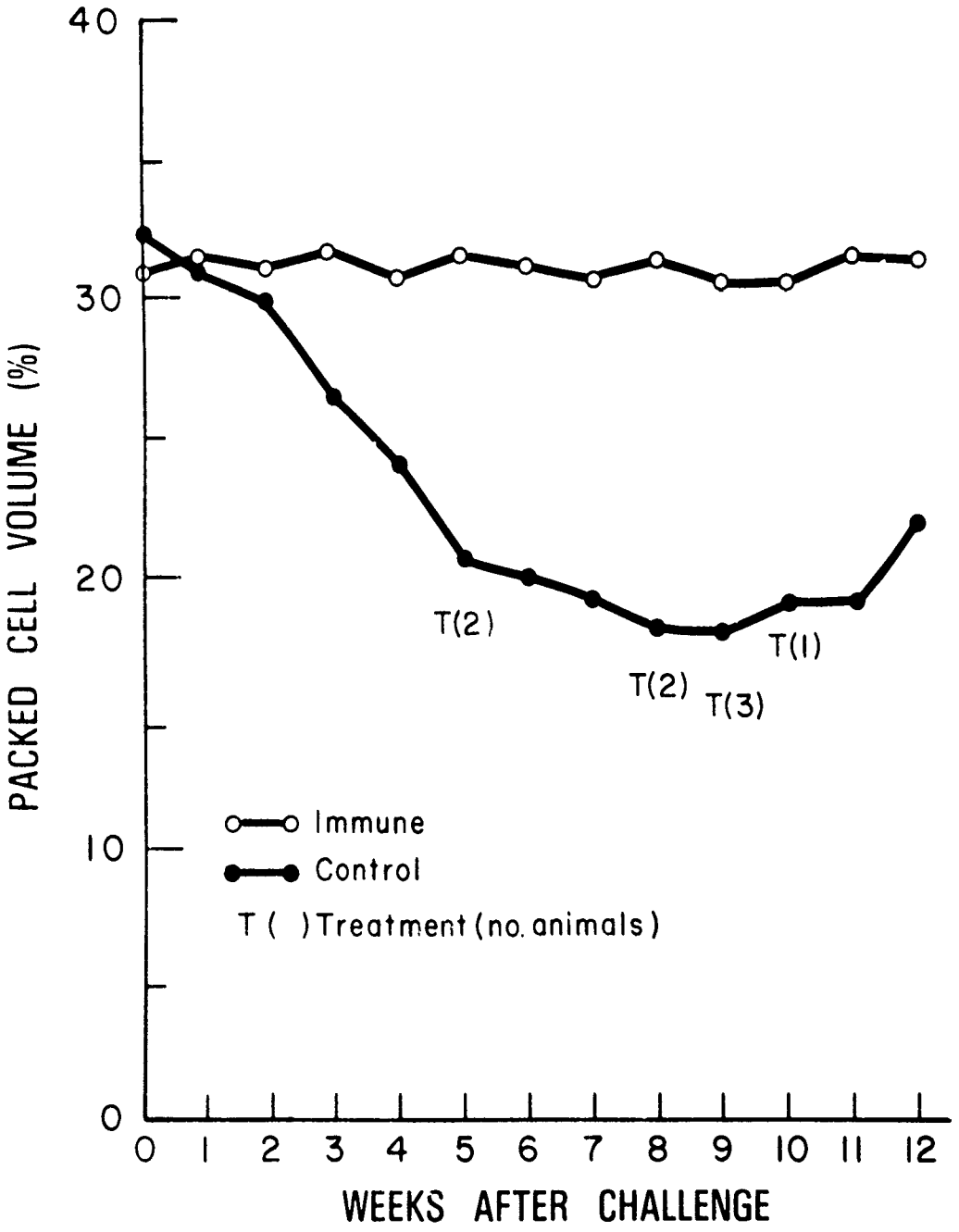

40
30
20
10
0
PACKED CELL VOLUME (%)
0 1 2 3 4 5 6 7 8 9 10 11 12
WEEKS AFTER CHALLENGE
T(2)
T(2)
T(3)
T(1)
Immune
Control
T ( ) Treatment (no. animals)

Fig. 10

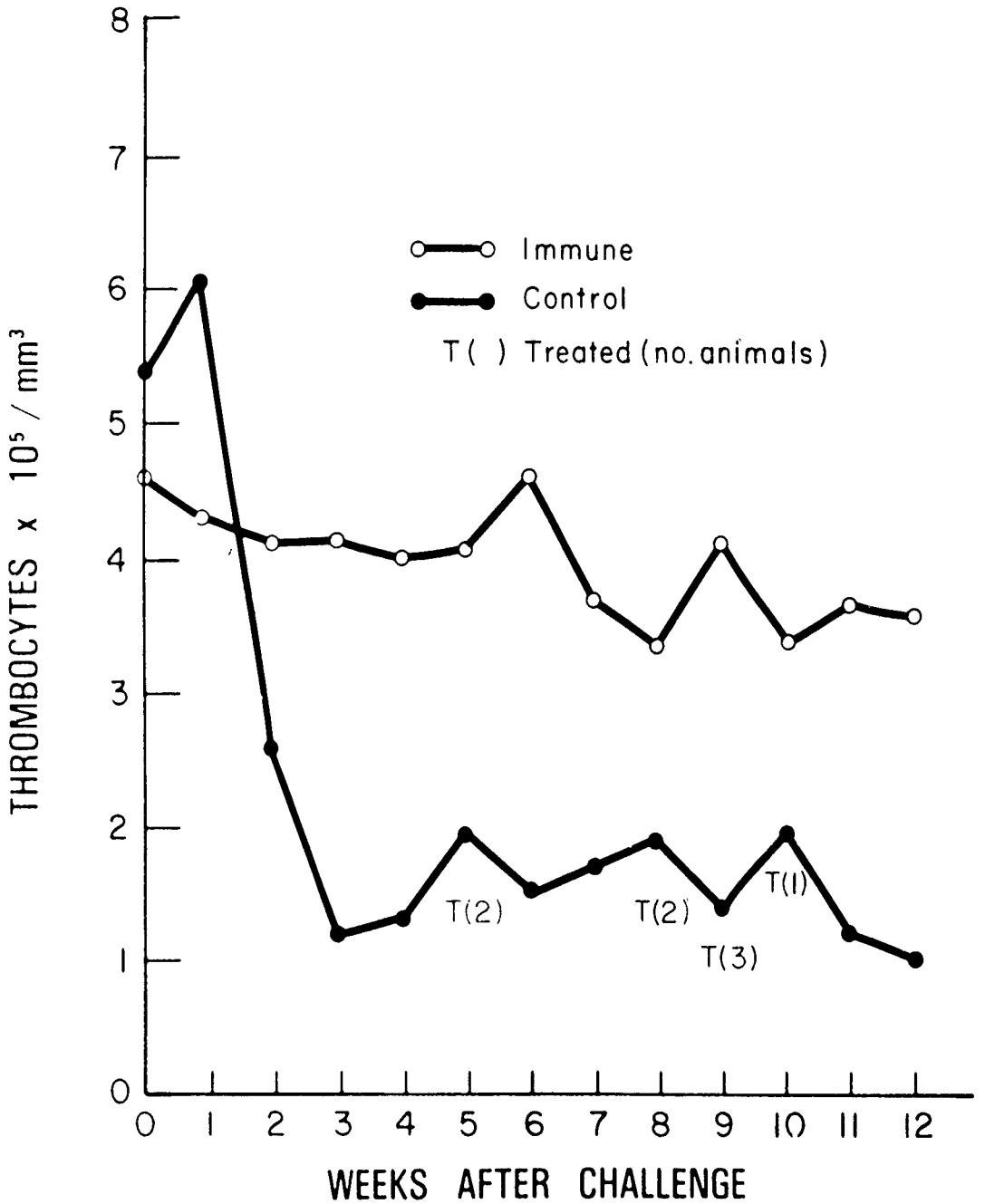
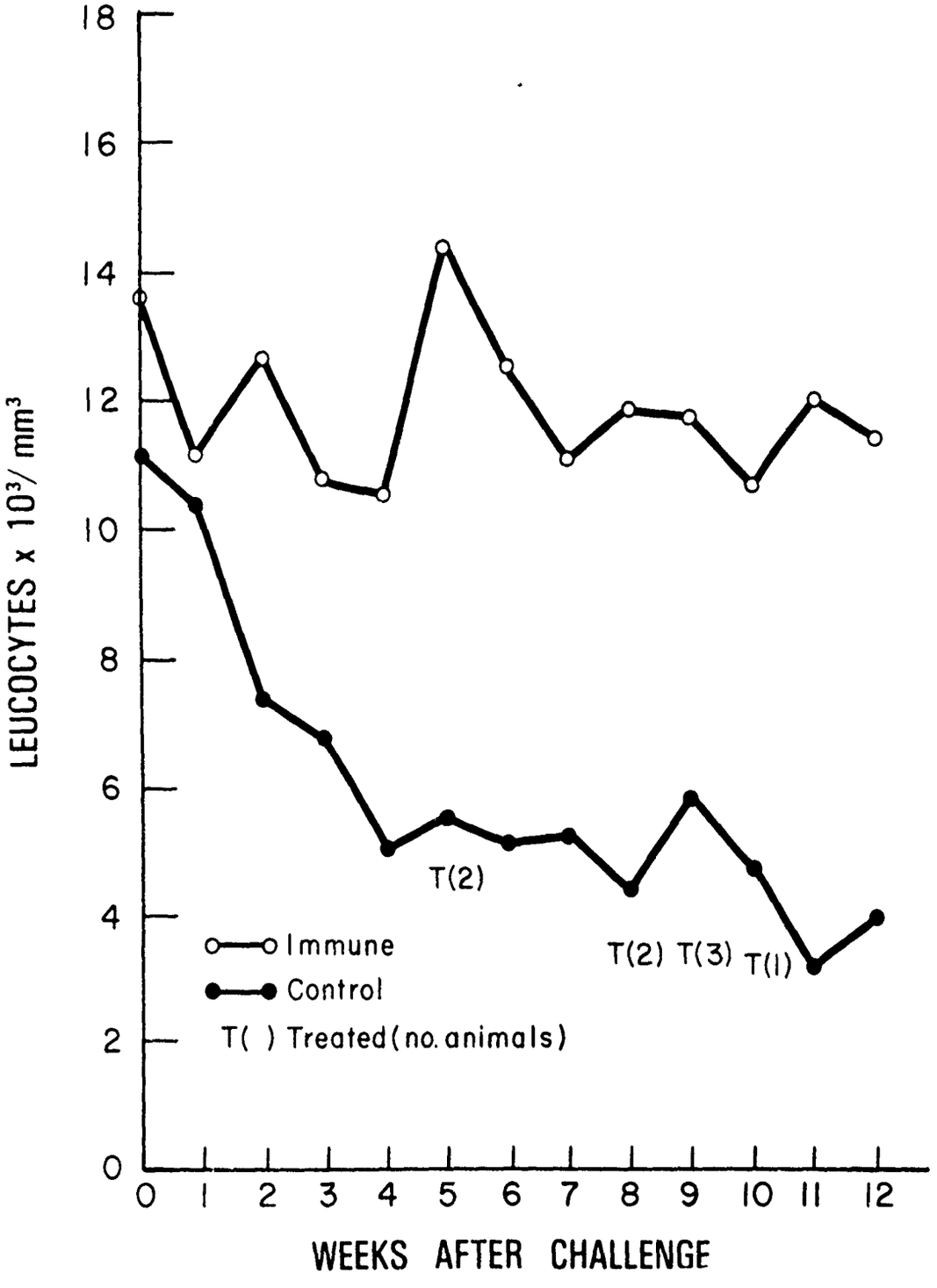

THROMBOCYTES x 10^5 / mm^3
8
7
6
5
4
3
2
1
0
Immune
Control
T( ) Treated (no. animals)
T(2)
T(2)
T(3)
T(1)
0 1 2 3 4 5 6 7 8 9 10 11 12
WEEKS AFTER CHALLENGE

Fig. 11


LEUCOCYTES x 10³/ mm³
18
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11 12
WEEKS AFTER CHALLENGE
Immune
Control
T( ) Treated (no. animals)
T(2)
T(2) T(3) T(1)

PART II: The Culture of Leishmania donovani in Schneider's Insect Medium: Its Value in the Diagnosis and Management of Patients with Visceral Leishmaniasis

INTRODUCTION

Diagnosis of visceral leishmaniasis has usually involved demonstration of the parasite in stained smears of various tissues or body fluids (Manson-Bahr, 1972). In most instances, the material is obtained by bone marrow or splenic aspirates. Alternatively, serologic techniques such as indirect hemagglutination, indirect fluorescent antibody, complement fixation and micro-Elisa are considered to be adequate (Kagan and Norman, 1976; Hommel, et al., 1978) for routine diagnosis but are rarely employed.

The first report of the culture of Leishmania donovani was made in 1904 using citrated blood (Rogers, 1904). In 1908, Nicolle reported a simplification of the Novy and MacNeal medium (Nicolle, 1908a; Nicolle, 1908b). This simplification became known as NNN and with minor variation has been the mainstay of leishmanial culture in clinical practice for the past 70 years. NNN consists of distilled water and blood agar but without peptones and beef. Cultures may become positive by the third day on NNN but often take as long as 3 to 4 weeks. The long delay in awaiting results for both diagnosis and assessment of cure has limited the value of this culture medium for the clinician. Various hemoprotozoa culture methods employing a variety of different media have been introduced during the past few years (Dwyer, 1972; Cross and Manning, 1973; Mansour et al., 1973; Miller, et al., 1974; O'Daly, 1975, Steiger and Steiger, 1976; Hendricks, et al., 1978) but for practical reasons or because of their cost, they have not yet gained widespread acceptance. The use of Schneider's insect culture medium, however, has been shown to be simple to use and valuable in the diagnosis of cutaneous leishmaniasis (Hendricks and Wright, 1979).

The incidence of visceral leishmaniasis appears to be on the increase in Kenya and transmission is occurring in areas remote from sophisticated medical support. Accordingly, it seemed worthwhile to explore the use of Schneider's medium in the diagnosis and management of visceral leishmaniasis. This study was itself part of a broader study of the clinical aspects of leishmaniasis including diagnosis and evaluation of standard antimony therapy (Kager et al., In Preparation; Rees, et al., In Preparation).

PATIENTS, MATERIALS AND METHODS

Patients

All patients seen by us over a period of 6 months at the Kenyatta National Hospital having visceral leishmaniasis were included in this study.

Splenic Aspirates

Splenic aspirates were performed using a standard technique (Kager, et al., In Preparation). An aspirate was performed prior to beginning treatment and at weekly intervals during the course of therapy. A small quantity of aspirate material, often amounting to little more than minute drops, was expressed into the culture medium from the aspirating syringe and needle. Careful aseptic technique was used to keep the aspirate and medium free from contamination. A further amount of splenic aspirate material was then expressed onto a glass microscope slide. A thick smear was made and stained with Field's stain and subsequently examined by light microscopy.

Culture Procedures

Commercially prepared Schneider's insect cell culture medium was used in all experiments (Schneider, 1974). Immediately prior to use the medium was modified by the addition of 30% (v/v) heat inactivated (56°C for 30 min) fetal bovine serum (FBS) and 100 IU penicillin and 100 ug streptomycin (final concentration/ml). Parasites were cultured in 16 x 10 mm plastic tissue culture tubes containing 3.0 ml of freshly prepared medium or vaccine vials containing freeze-dried Schneider's medium and FBS were reconstituted with 3 ml distilled water and used for diagnosis as described above.

The inoculated cultures were incubated at 26°C and examined daily for the presence of promastigotes by ordinary light microscopy. Promastigotes correspond morphologically to the sandfly stages of the leishmania life cycle and are extra-cellular, flagellated, aerobic and grow optimally at temperatures below 30°C. The culture tubes are ideally observed with an inverted phase contrast microscope but ordinary light microscope with a 10x objective is adequate.

The cultures were examined for 28 days before being considered negative.

RESULTS

Among 13 patients examined prior to treatment, smears of splenic aspirate material and parallel cultures of the same material were all positive. During the course of treatment, differences were noted between the two methods (Table 8). These techniques were compared 68 times during therapy, and in eight instances no parasites were detected by smear although the cultures were positive. A positive smear with a negative culture occurred only once.

The time required to detect parasites by culture of splenic aspirate material is shown in Table 9. Parasites were found within 72 hours in pretreatment cultures. When cultures were taken during the course of therapy, most cultures were positive on day 3 or 4 with none becoming positive later than day 5.

DISCUSSION

For many years most clinical laboratories have used blood based diphasic media such as NNN for the *in vitro* cultivation of leishmania. From the clinician's viewpoint, these media suffer from the disadvantage of often requiring at least two weeks before leishmania can be detected in the culture. In contrast, cultures rapidly become positive in Schneider's insect culture medium. Though the blood based media have been widely used to cultivate promastigotes for biochemical and immunologic studies, these media have serious deficiencies which limit their laboratory application as well. These include cultivation of parasites with red blood cell derived antigens, inconsistent growth of different species and strains of leishmania, and often 'ow yields of organisms.

Schneider's medium with 30% FBS has demonstrated its ability to support a wide array of leishmania species as well as geographic strains (Hendricks *et al.*, 1978). Recently this insect medium plus 30% (v/v) FBS has been compared to NNN in terms of its sensitivity as a culture medium. The increased sensitivity is presumably due to its ability to support *in vitro* transformation and multiplication of culture technique was used to diagnose and evaluate patients with cutaneous leishmaniasis and the procedure was shown to be more effective than either NNN or histopathologic examination (Hendricks and Wright, 1979). Similarly, this method is more sensitive than morphologic examination in patients with visceral leishmaniasis, as evidenced in this study by detection of parasites by culture in patients late in the course of therapy when the stained smears were negative.

We did not compare the two culture techniques in our studies because the standard diagnostic procedure utilized in Kenya is the examination of stained smears and not the use of NNN. Furthermore, it would have been difficult to aliquot the splenic aspirate material to properly and simultaneously evaluate the three techniques.

We believe that this culture technique offers an advantage over stained smears even in confirming a presumptive diagnosis primarily because of the ease of seeing large numbers of motile promastigotes in contrast to what can be a difficult and time consuming search for amastigotes in stained splenic aspirate smears. The greatest value of the culture lies in its use later in the course of therapy when it is quite sensitive in detecting small numbers of parasites and thus provides an objective criterion on which to judge the effectiveness of therapy.

TABLE 8

COMPARISON OF FIELD'S STAINED SMEARS OF SPLENIC ASPIRATE MATERIAL WITH PARALLEL CULTURE OF THE SAME MATERIAL

		Smear	
		Positive	Negative
Culture	Positive	60	8
	Negative	1	0

TABLE 9

TIME REQUIRED TO DETERMINE PRESENCE OR ABSENCE OF PARASITES BY CULTURE OF SPLENIC ASPIRATE MATERIAL

Cultures Done		Day Cultures Positive					
		1	2	3	4	5	6
Pre-RX	13	1	11	1	0	0	0
RX	68	3	37	15	11	2	0

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PART III: Trypanosoma congolense: Factors Affecting Infection and Transmission in Laboratory-Reared Glossina morsitans

INTRODUCTION

Factors known to influence tsetse fly infection rates include the species of fly and trypanosome, fly age at exposure to trypanosomes, temperature and the type of host animal. Buxton (1955) and Jordan (1976) suggested that there also may be certain genetic lines of tsetse flies within a species that become infected more readily than others. For a cyclically infected fly to transmit trypanosomes, sufficient quantities of infective metacyclic forms must be passed to a susceptible host during fly feeding. This report describes studies of some factors influencing both cyclic infection of Glossina morsitans Westw. with Trypanosoma congolense and subsequent transmission from fly to vertebrate host. Specific objectives were to determine (1) if the number of exposures to an infected host influences T. congolense infection in the fly; (2) if the sex of the individual infected flies during feedings to repletion on susceptible rats. Incident to these studies, a selective fly rearing program was initiated in an attempt to establish a genetic line more susceptible to cyclic infection with T. congolense.

MATERIALS AND METHODS

Fly Colony

The Glossina morsitans colony was established from puparia supplied by Dr. A.M. Jordan, Tsetse Researdh Laboratory, University of Bristol, Bristol, England. The Walter Reed colony became self-sustaining during FY 79, and shipments from Bristol were discontinued. Standard rearing procedures were used except that flies were not sexed, and males and females were held in the same cages for the entire adult life. Adults were kept in incubators at 26±1°C and 60±10% RH. Each Geigy-type cage of 20 flies was placed on a live bovine host for 15 minutes per day, 6 days per week.

Trypanosomes

The fly colony was infected with the Trans Mara I strain of Trypanosoma congolense. This strain was isolated from an infected cow in the Trans Mara area near the Kenya-Tanzania border in 1966 (Wellde, et al. 1974). The strain has never been cloned, and the infection has been maintained in an intensive fly-bovine - fly cycle since 1979. Randomly selected cages of 20 flies, 25-45 days old, were dissected periodically to determine infection rates in the colony.

Infection Experiments

Five groups of approximately 40 newly emerged (teneral) flies each were fed on a T. congolense-infected calf. On each subsequent day, one group was transferred to a negative animal while the remaining groups were refed on the infected animal. This was continued until day 6 when all cages were fed on a negative animal. Thus, group I fed on an infected animal once, group II fed twice on an infected animal, group III - 3 times, group IV - 4 times and group V - 5 times. Wet mounts prepared from blood of infected and negative animal were checked daily before flies were fed. Parasitemia of the infected animal was estimated daily by counting the numbers of trypanosomes per 100 leucocytes on thick blood films and relating these numbers to the total leucocyte counts per mm^3.

Fly Transmission to Rats

Three cages of 20 G. morsitans each were fed from emergence (24 hours old) until day 17 on a T. congolense infected calf. The cages were then fed on a non-infected bovine for 7 days to insure that subsequent transmission by individual flies was the result of a cyclic infection with T. congolense. Each fly was transferred to an individual plastic tube with a cork stopper at one end and screen mesh at the other. The flies were then fed singly on white rats to determine which flies could transmit a detectable infection to the rats. Infected flies that initially transmitted to rats were fed on a fresh rat each day for 10 consecutive feedings or as many as possible before the flied died. Wet blood smears from the host rats were examined daily from day 10 after fly feeding. Rats without parasitemia were challenged at day 30 with a consecutive feeding, the infected flies remaining alive were pooled for mating, and the progeny were maintained as a separate group from the main colony.

RESULTS

Fly Colony

No discernable differences were observed between flies reared from Bristol puparia and those from the Walter Reed colony. The colony produced an average of 3,616 flies per month (range: 3,014 to 4,426) during FY79. Pupal weight for randomly samples one day old pupae was 27.9 mg. (N = 2,830). Adult flies successfully emerged from 2,546 (90%) of the pupae. Flies invariably fed on the host animal at each feeding interval for the first week post emergence, but in 54 sequential host exposures of 10 cages of flies the overall feeding rate was 90.2%. Pregnant females just prior to larvi-

position often failed to feed during the 15-minute exposure to a host. We occasionally observed flies which had fed to repletion on the host, but appeared unable to digest the blood meal. Such flies ceased to feed during subsequent exposures on a host and died 2-3 days later. A few flies were observed to engorge to such an extent that they died within minutes after feeding.

T. congolense Infection

The dissection results for randomly selected cages of flies, 25-45 days old, is shown in Table 10. Male flies acquired cyclic infections more frequently than female flies. Midgut infections occurred more frequently than proboscis infections, and we did not find any proboscis infection without a midgut infection. Numbers of trypanosomes in the proboscis varied from 50 to 1,000.

Fly Infection Experiments

Feedings on a T. congolense infected host beyond the initial teneral feeding produced a greater number of cyclic infection in G. morsitans (Table 11). The rate of increase in female flies (17.2% with one exposure vrs. 30.4% with 4 exposures) was more pronounced than that for males (26% with one exposure vrs. 31.6% with 4 exposures). Host parasitemia during the 5 exposures was calculated to be 2×10^6 organisms per cc of blood.

Fly Transmission to Rats

Of the 60 flies fed individually on rats, 12 (20%) transmitted a detectable infection. Dissections demonstrated that 2 of the 48 flies which failed to transmit had trypanosome infections in the proboscis and midgut. Table 12 shows the results of individual refeedings of the 12 infected flies on laboratory rats. Five of the 12 transmitted T. congolense at each feeding, while 2 flies which transmitted in the initial feeding failed to transmit in any subsequent feedings. The prepatent period after fly feeding varied from 10-27 days. Of the rats developing a parasitemia after fly feeding 71 of 75 (94.5%) died from the infection. Those rats not showing a parasitemia were challenged with T. congolense blood forms harvested from mice. None survived challenge.

DISCUSSION

Potts (1933) reported an 87% emergence rate for G. morsitans puparia deposited in the laboratory. Buxton and Lewis (1934) compared the weight of one day old puparia to emergence of adult Glossina tachinoides and found the highest emergence (84.2%) from the heaviest group of puparia. It appears from rearing results with our colony that intensive feedings on a live bovine host is an ideal system in terms of G. morsitans production. Pregnant female flies just prior to larviposition should be avoided for studies requiring feeding on a host animal. These flies probably account in part for the decline in the feeding rate (100 - 92%) observed in the 54 sequential exposure of flies to a host.

In both the dissections of randomly selected cages of flies and refeeding of flies on an infected host we found higher cyclic infection rates in males than in females. Studies by Burtt (1964) with G. morsitans and T. rhodesiense indicated that in males, cyclical infection was 2.5 times as frequent as in females. In the present study, the difference was much less pronounced but was consistent. We determined only the presence or absence of trypanosomes in the proboscis and midgut. Additional dissections are needed to compare numbers of trypanosome per infection in males with those of females. As expected, the overall colony infection rate (30.8%) was much higher than the 0.6 to 2.7% infection rates reported by Buxton (1955) for field collected G. morsitans.

It appeared that repeated feedings on an infected host after the teneral feeding increased fly infection rates. This is in contrast to the report by Clarke (1969) who concluded that G. morsitans could only be infected by T. congolense in initial feedings. While host parasitemia has not been directly related to fly infection, it seems likely that a threshold level of blood forms must be met for a cyclic infection to become established. The relatively high parasitemia of the host animal in our experiments and the fact that the Trans Mara strain has been maintained in an intense fly-bovine fly cycle may contribute to the increased infection rate in flies exposed to multiple feedings.

Marked differences were apparent in the frequency of transmission to rats by flies known to be infected with T. congolense. Fairbain and Burtt (1946) found that

infected G. morsitans transmitted T. rhodesiense to rats with 99.2% efficiency. Variation in host susceptibility probably could not account for the differences we observed in fly transmission of T. congolense since the non-transmitting flies engorged on 17 different rats, none of which became parasitemic (Table 3). Titration of Trans Mara strain metacyclics in rats and an estimate of the number of metacyclics transmitted by infected G. morsitans are needed. Still, it appears that individual flies differ in their ability to transmit the Trans Mara strain to rats.

An alternative possibility was suggested by Jenni and Coworkers (1979). They found that rat sera lyses immature T. brucei metacyclics. If this is true for T. congolense, consecutive feedings on rats may possibly reduce the number of metacyclics in the proboscis and ultimately affect the ability of the fly to transmit; however, 5 flies were able to transmit to rats at each of the 10 feedings.

It also seems possible that the observed differences in vector capacity are to some extent genetically influenced. Selection of progeny from infected flies for comparison with the main colony is still in progress. Selection for increased or decreased vecgor capacity has been successful in other anthropods, most notably mosquitoes. Continued interbreeding of infected flies is planned in an attempt to establish a genetic line of flies with increased infection and transmission rates. High colony infection rates are obviously desirable for future immunization attempts with metacyclics and for fly challenges of "immunized" or drug treated hosts.

TABLE 10

TRYPANOSOMA CONGOLENSE (TRANS MARA I) INFECTION IN RANDOMLY SELECTED LABORATORY REARED GLOSSINA MORSITANS WESTW.*

	No. dissected	No. Positive	
		Proboscis	Midgut
Females	118	32 (27.1%)	36 (30.5%)
Males	116	40 (34.5%)	46 (39.6%)
Total	234	72 (30.8%)	82 (35.0%)

* Entire cages of 25-45 day old flies were sampled. The teneral feeding and 11 or more additional consecutive feedings were on a T. congolense infected host.

TABLE 11

CYCLIC TRYPANOSOMA CONGOLENSE INFECTION IN GROUPS OF GLOSSINA MORSITANS WESTW. EXPOSED TO VARYING NUMBERS OF FEEDINGS ON AN INFECTED BOVINE*

No. Times Exposed	Proboscis Infection: No. Positive/No. Examined		
	Female	Male	Total
1	5/29 (17.2%)	4/15 (26.7%)	9/44 (20.5%)
2	5/22 (22.7%)	7/17 (41.2%)	12/39 (30.8%)
3	4/20 (20.0%)	5/17 (29.4%)	9/37 (24.3%)
4	7/23 (30.4%)	6/19 (31.6%)	13/42 (30.9%)
5	5/18 (27.8%)	5/17 (29.4%)	10/35 (28.6%)

* Flies were fed on a non-infected host for a minimum of 18 consecutive feedings before dissection.

TABLE 12

CONSECUTIVE FEEDINGS OF INDIVIDUAL TRYPANOSOMA CONGOLENSE INFECTED* GLOSSINA MORSITANS WESTW. ON LABORATORY RATS

Fly No.	No. rats parasitemic/ No. exposed	Avg. days to Parasitemia	Range in days
1	4/10 (40%)	20.3	16-23
2	1/10 (10%)	20.0	-
3	10/10 (100%)	18.0	14-22
4	0/7**(0%)	-	-
5	10/10 (100%)	15.2	10-18
6	9/10 (90%)	19.0	16-21
7	10/10 (100%)	17.8	15-21
8	0/10 (0%)	-	-
9	9/10 (90%)	20.3	17-24
10	10/10 (100%)	19.3	18-23
11	2/10 (20%)	21.5	21-22
12	10/10 (100%)	22.6	20-27
Totals	75/117 (64.1%)	19.4	10-27

* All flies had transmitted prior detectable infections to individual rats.

** Fly died after 7 feedings.

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PART IV: *Trypanosoma congolense*: Thrombocyte Survival in Infected Steers

INTRODUCTION

Thrombocytopenia is a characteristic of many host animals undergoing natural or experimental trypanosome infections. Previous studies have shown that cattle infected with *Trypanosoma congolense* develop a pronounced thrombocytopenia (Maxie & Lossos, 1976), (Wellde, Kovatch, Chumo and Wykoff, 1978), (Forsberg, Valli, Gentry and Donsworth, 1979). Thrombocytopenia was most severe during periods of high parasitemia and curative therapy of acutely infected animals induced a rapid thrombocytosis. In chronically infected animals which underwent intermittent parasitemia, there was an inverse relationship between levels of trypanosomes and thrombocytes. During periods of remission in parasitemia thrombocytes usually were found at normal or elevated levels. These findings indicated that an increased production and destruction of thrombocytes occurred in infected animals (Wellde *et al.* 1978). In an effort to clarify the etiology of the thrombocytopenia we determined the apparent survival times of ^{51}Cr labelled thrombocytes in normal steers and in steers acutely infected with *Trypanosoma congolense*.

MATERIALS AND METHODS

Animals

Ten steers of a predominant Charolais breed, ranging in age from 14 to 16 months and weighing between 170 to 200 Kg were used in these studies. The care and pre-experimental treatment of our animals has been previously described (Preston, Wellde and Kovatch, 1979). Infected and control animals were transferred to metabolism cages before transfusion with radioisotopically labelled platelets.

Parasites

The Trans Mara I strain of *Trypanosoma congolense*, the origin and maintenance of which is described elsewhere (Wellde, et al. 1974) was used to infect the experimental animals. Trypanosomes were collected from infected mice, counted in a hemocytometer and diluted with phosphate buffered saline (pH 7.8) containing 5% glucose and 10% fetal calf serum. Cattle were infected I.V. with 1×10^4 *T. congolense* per 240 Kg.

Preparation of labelled thrombocytes

Twelve hundred ml. of blood was collected from healthy non-infected donor animals into a flask containing 200 ml of acid citrate dextrose solution (Aster and Jandl, 1964). The blood was transferred to 400 ml centrifuge bottles and centrifuged at 400 g for 15 minutes. The supernatant plasma (thrombocyte rich) was then placed in 50 ml tubes and centrifuged for 5 minutes at 500 g to further remove contaminating erythrocytes and leucocytes. The resulting supernatant was centrifuged for 15 minutes at 1300 g, the supernatant decanted and the thrombocytes resuspended in normal plasma. The thrombocyte suspension was incubated with 1mCi ^{51}Cr as sodium chromate (high specific activity) for 15 min. with occasional mixing. The suspension was then centrifuged at 1300 g and the supernatant removed. The labelled platelets were then washed twice with 50 ml of normal plasma before resuspension in 30 ml of plasma containing 60 mg of ascorbic acid prior to injection. All procedures were carried out at room temperature.

Injection and sampling

Known numbers of ^{51}Cr-labelled thrombocytes ($2.1\text{-}3.6 \times 10^{11}$) in suspension were injected into experimental and control steers by means of a jugular catheter. Blood samples (10 ml) were collected into EDTA from the contralateral vein 30 minutes after injection and daily for 7 days. Aliquots of blood (5 ml) were diluted in 10 ml 0.01NaOh for scintillation counting.

Transfusions of labelled thrombocytes from a donor to another animal are referred to as heterologous transfusions. Thrombocytes obtained from a donor, and after labelling, returned intravenously to the same donor are described as autologous transfusions.

Thrombocyte counts were done using the method described by Brecker and Cronkite (1950).

Calculations and Expression of Results

The radioactivity of each blood sample was expressed as a percentage of the value at 30 minutes after injection. The apparent thrombocyte half-life ($T_{\frac{1}{2}}$) was obtained by regression analysis of the disappearance curve over a 7-day period.

RESULTS

Thrombocyte levels

Prepatent periods of infected animals ranged from 5 to 6 days and thrombocyte levels in these animals began to decline shortly before trypanosomes were detected in the blood. From a level of 6 x $10^5/mm^3$ on the third day after infection the average thrombocyte levels in infected animals progressively decreased to 1 x $10^5/mm^3$ on the eleventh day post infection. Thrombocyte levels in infected animals usually remained between 1 x 10^5 and 3 x 10^5 during the remainder of the experiment, while thrombocyte levels in control animals did not decrease over pre-infection values (Fig 12).

Thrombocyte survival

In the first experiment thrombocytes were obtained from a normal healthy donor, labelled with ^{51}Cr, and separated into three aliquots. One aliquot of labelled thrombocytes was returned to the donor animal while the other two aliquots were injected into a normal control and an infected animal respectively. The results of this experiment (Fig.13) indicated that transfusions of labelled thrombocytes into autologous and heterologous normal animals resulted in similar thrombocyte half lives while the thrombocyte half life in the infected animal was substantially reduced. In subsequent experiments thrombocytes were harvested from normal animals, labelled and transfused into heterologous infected and control animals. The apparent half life of labelled thrombocytes in 5 infected animals ranged from 0.8 to 2.1 days. Survival times of thrombocytes in 5 non-infected animals, however, ranged from 2.9 to 4.5 days (Table 13). Both infected and control animals tolerated the thrombocyte transfusions without untoward reactions.

DISCUSSIONS

We have shown that there is a marked reduction in the apparent survival time of thrombocytes in bovines infected with Trypanosoma congolense. The apparent half-life of normal bovine platelets labelled with ^{51}Cr-chromate was 1.3±0.5 (2SE) days in infected animals compared to 3.7±0.5 days in controls. This supports our previous report that the thrombocytopenia observed in bovines infected with Trypanosoma congolense appeared to result from increased

production and destruction rather than from a suppression or inhibition of production (Wellde, et al. 1978). These results are similar to those of Robbins-Browne, Schneider and Metz (1975) who described a shortened apparent survival time of thrombocytes in humans infected with Trypanosoma rhodesiense. Other authors, however, have characterized the thrombocytopenia in Trypanosoma congolense infected calves as being due to an ineffective thrombopoiesis since platelet life span, as measured by ^{35}S-mentionine incorporation was normal, in spite of an increased megakaryocytic mass (Forsberg, Valli, Gentry and Donworth, 1979).

Thrombocyte transfusions in our experiments were done without regard to antigens present on the thrombocytes. Heterologous thrombocyte transfusions in normal animals resulted in similar survival times to that of the autologous transfusion. However, the autologous transfusion produced the longest apparent survival time (4.5 days). Whether or not the somewhat shortened survival times resulting from heterologous transfusions were the result of incompatability of antigens on the thrombocytes is not known. In man, A and B blood group antigens are present on thrombocytes and a shortened thrombocyte survival was noted when A thrombocytes were transfused into O recipients (Aster, 1965). Some clinicians, however, do not consider ABO incompatability to be a major drawback in the treatment of thrombocytopenic patients (Brecker and Aster, 1972). Both thrombocyte specific antigens and HL-A antigens on thrombocytes can induce antibody formation in the recipient and sensitization may follow repeated transfusions resulting in greatly shortened thrombocyte survival times (Wintrobe, 1975). Since our experimental animals received only a single transfusion and were studied for a relatively short period, it seems unlikely that these antigens played an important role in the thrombocyte survival time.

The mechanism of thrombocyte destruction associated with thrombocytopenia in Trypanosoma congolense infected cattle remains to be defined. We have shown that there is a mild coagulopathy associated with experimental infections in bovines infected with the Trans Mara I strain of Trypanosoma congolense (Wellde, et al. 1978) which may have been initiated by thromboplastic substance which were generated by the persistent destruction of thrombocytes. It did not appear, however, that the coagulation process initiated the thrombocytopenia.

Rat thrombocytes aggregated when trypanosomes or supernatants from lysed Trypanosoma rhodesiense were added to thrombocyte suspensions (Davis, Robbins, Weller and Braude, 1974). A heat labile factor presumably of parasite origin apparently facilitated aggregation, sequestration and destruction of thrombocytes in their experimental rats.

Thrombocyte pooling in the spleen has been identified as an important factor in the thrombocytopenia of human trypanosomiasis (Robbins-Browne, Schneider and Metz, 1975). Thrombocytopenia, however, is prominent in spectomized T. congolense infected calves (M.S. Bhogal and B.T. Wellde, Unpublished data). However, this data did not indicate whether thrombocyte pooling occurred elesewhere in the reticuloendothelial system.

Labelled erythrocytes also have a decreased apparent survival time in calves infected with T. congolense (Preston, Wellde and Kovatch, 1979) and the purpose of destruction may be similar to that of thrombocytes. Further efforts in our laboratory to elucidate the effects of trypanosomes and their products on erythrocyte and thrombocyte viability are in progress.

TABLE 13

APPARENT THROMBOCYTE SURVIVAL TIMES OF STEERS INFECTED WITH *TRYPANOSOMA CONGOLENSE* AND NON-INFECTED CONTROLS

Group	Animal No.	Prepatent Period (Days)	Cr^{51} Day of Thrombocyte Transfusion[a]	Recipients Thrombocyte level at Transfusion ($x10^3$)	Apparent Thrombocyte Survival ($T_{\frac{1}{2}}$)
Infected	1	5	10	100	0.8
	2	5	14	148	1.6
	3	6	15	200	1.2
	4	5	21	182	0.9
	5	6	25	348	2.1
	Mean $\pm$2SE	5.4	-	195.6 ± 83.5	1.3 ± 0.5
Control	6	-	-	1042	2.9
	7	-	-	1154	3.7
	8	-	-	1304	4.1
	9	-	-	580	4.5[b]
	10	-	-	910	3.5
	Mean $\pm$2SE	-	-	998.0 ± 245.9	3.7 ± 0.5

[a] Days post infection.

[b] Autologous transfusion.

LEGENDS FOR FIGURES

FIGURE 12 Thrombocyte level in five Charolais steers infected with Trypanosoma congolense and five non-infected control steers (±2SE).

FIGURE 13 Apparent thrombocyte survival times ($^{51}CrT_{\frac{1}{2}}$) of labelled thrombocytes in three steers:

(a) an autologous transfusion in a normal steer (0)

(b) a heterologous transfusion in a normal steer (0)

(c) a heterologous transfusion in a Trypanosoma congolense infected steer ( ).

Fig. 12

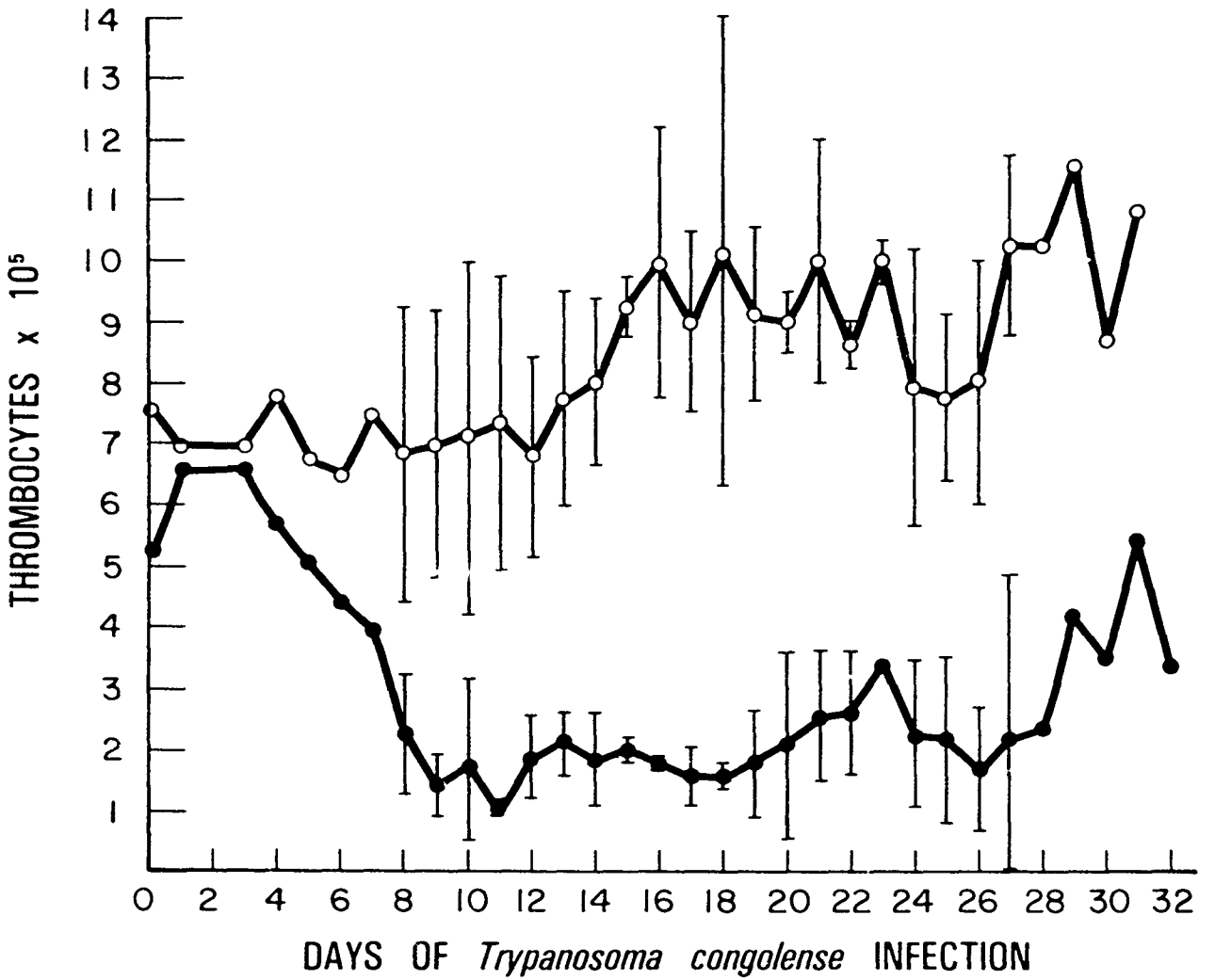
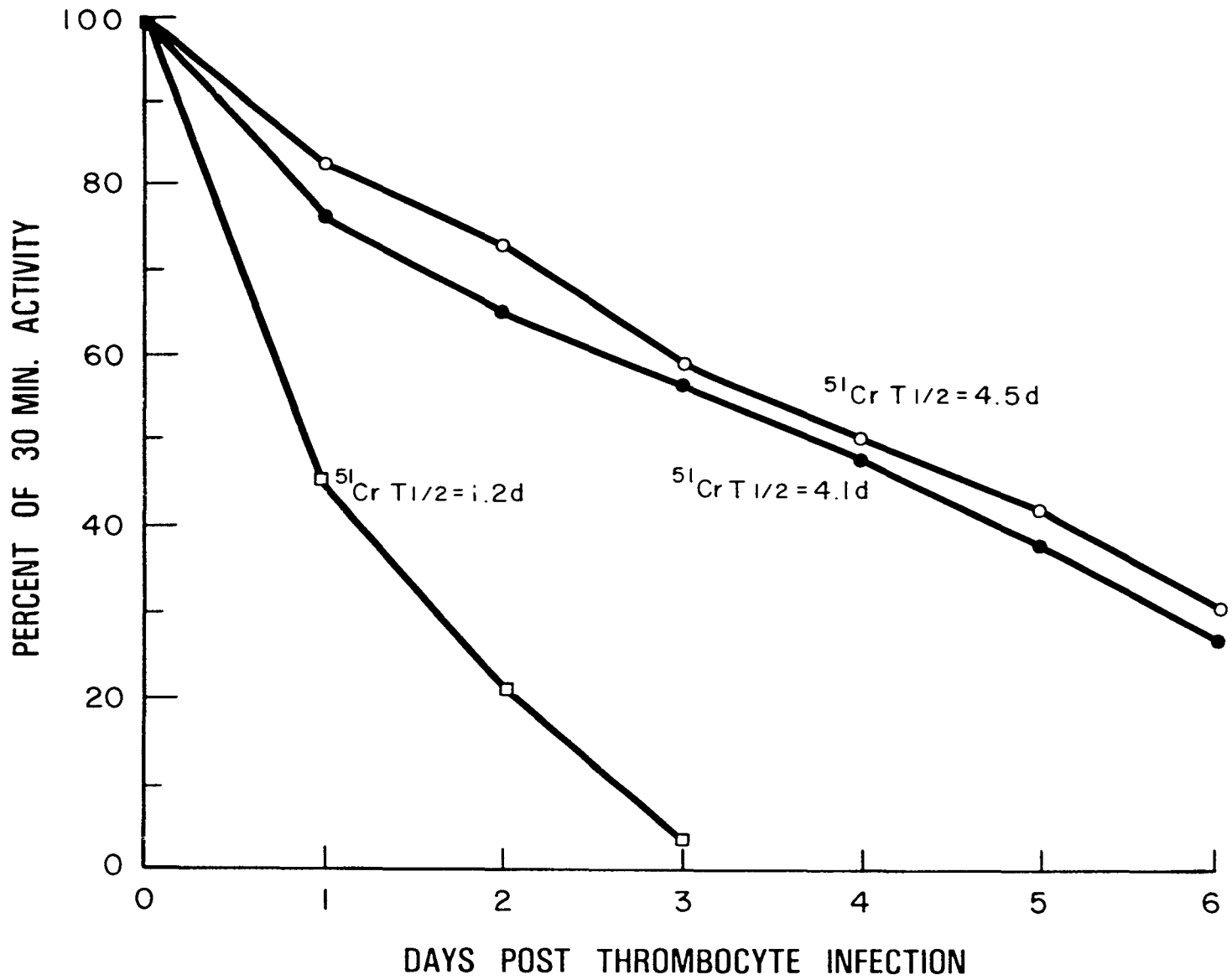

THROMBOCYTES x 10^5
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
DAYS OF *Trypanosoma congolense* INFECTION

Fig. 13


100
80
60
40
20
0
PERCENT OF 30 MIN. ACTIVITY
0
1
2
3
4
5
6
DAYS POST THROMBOCYTE INFECTION
51Cr T1/2 = 4.5d
51Cr T1/2 = 4.1d
51Cr T1/2 = 1.2d

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OA 6436	79 10 01	DD DRAF(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	139
b. CONTRIBUTING				
c. [redacted]	Cards 114F			

11. TITLE (Precede with Security Classification Code)

(U) Microbial Genetics and Taxonomy

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
63 08	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER:

c. TYPE d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	4	359
FISCAL YEAR CURRENT 80	4	377

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NAME: DA

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Vaccine; (U) Enteric Bacteria; (U) Antigens; (U) Virulence; (U) Salmonella; (U) Plasmids

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number preceded text of each with Security Classification Code.)

23. (U) Definition in genetic and molecular terms of the properties of gene transfer antigenicity, and virulence of pathogenic enteric bacteria which because of their disease producing capabilities, are of importance to military medicine concerned with the prevention and treatment of such infections in Army personnel. We anticipate that it will be possible to genetically modify enteric bacteria to any desired antigenic structure and pathogenicity to serve as vaccine strains or as tools to study the infectious process.

24. (U) Use of genetic recombination between strains of enteric bacteria. Where possible, the genetic results are extended to include study of the informational macromolecules involved.

25. (U) 78 10 - 79 09 Two components of the Citrobacter freundii Vi antigen were distinguished by genetic and serological procedures and their genetic determinants, as well as the gene controlling reversible form transition of the antigen, were mapped at the viaB locus. Citrate utilization in an atypical Escherichia coli strain isolated from a clinical specimen was found to be determined by plasmid-borne genes. An Escherichia coli strain containing eight different sized plasmid species was characterized to serve as a size reference source in agarose gel electrophoretic analysis. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 139 Microbial Genetics and Taxonomy

Investigators:
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Description:

1. Two components of the Citrobacter freundii Vi antigen were distinguished by genetic and serological procedures, and their genetic determinants, as well as the gene controlling reversible form transition of the antigen, were mapped at the viaB locus.

2. Citrate utilization in an atypical Escherichia coli strain isolated from a clinical specimen was found to be determined by plasmid-borne genes.

3. An Escherichia coli strain containing eight different sized plasmid species was characterized to serve as a size reference source in agarose gel electrophoretic analysis.

Progress:

1. Genetic investigation of reversible expression of the Vi antigen in Citrobacter freundii.

a. Expression of the Vi antigen by certain strains of the genus Citrobacter differs strikingly from the expression of this antigen by bacteria of the genus Salmonella. In Salmonella, Vi antigen expression by strains of S. typhi, S. paratyphi C, and S. dublin is a relatively stable characteristic, and, in routine culturing of these organisms, strains that express the Vi antigen are not observed to give rise to variants that do not express it. Likewise, when Vi-negative mutants of these strains are obtained, they are not readily revertible to the Vi-positive state. In marked contrast, streaking Vi expressing colonies (referred to as V forms) of Citrobacter freundii, or C. intermedius on solid culture media generates colonies which express the antigen as well as colonies which do not express it. These Vi non-expressing colonies, called W forms, which are visually as well as serologically distinguishable from the V forms, may constitute as much as 50% of the progeny of a single V form. Similarly, restreaking of a W form colony also gives rise to a mixture of V and W forms, with Vi antigen expression reappearing in 10 to 30% of the progeny colonies. The rapid, reversible transition between expression

and nonexpression of the Vi antigen in Citrobacter strongly suggests the operation of some mechanism for regulation of gene expression. In Salmonella, we have shown previously (Annual Report, WRAIR, 1977) that genes at two widely separated chromosomal loci, viaA and viaB, are involved in the expression of the Vi antigen. These genes occupy identical chromosomal locations in S. typhi and S. paratyphi C, and we expected that the Citrobacter genes determining Vi antigen expression would be similarly located. The present investigation was undertaken to determine whether genes at either the viaA or viaB loci are involved in the reversible expression of the Vi antigen of C. freundii.

b. C. freundii donor strains WR7005 and WR7006 were constructed from C. freundii WR7004 by introduction of the E. coli K-12 sex factor, F. Strains of S. typhi, unable to express the Vi antigen because of genetic defects in either viaA or viaB genes were employed as recipients. The C. freundii donor WR7005 was used to transfer its melibiose utilization (mel^+) genes, to which the $viaB^+$ genes are linked, to S. typhi WR4226 (genotype mel, viaB); this donor, however, was not able to transfer its histidine synthesis determinants (his^+), to which the $viaA^+$ gene is linked. Transfer of his^+ and $viaA^+$ to S. typhi WR4205 (genotype his, viaA) was accomplished by the C. freundii donor WR7006, which is unable to transfer its mel^+ determinants. In his^+ selected hybrids of S. typhi that also inherited the C. freundii $viaA^+$ gene, the continuous, nonreversible expression of the Vi antigen normally seen in Vi expressing S. typhi strains was restored. Thus, the viaA locus of C. freundii did not appear to be involved in the reversible expression of the antigen.

c. Twelve of 200 mel^+ selected hybrids of S. typhi WR4226 were found to have received the C. freundii $viaB^+$ determinants, as indicated by their agglutination in antiserum prepared against C. freundii (which is Vi specific for S. typhi because no other surface antigens are shared by these organisms); each of these twelve hybrids retained its native somatic and flagellar antigens and no unselected characters other than Vi antigen expression were detected in them. One of these Vi expressing hybrids, designated 5-12, was seen to alternate, when streaked on solid culture media, between two morphologically distinguishable colonial forms. The first form, called Type I closely resembled the V form colonies of the C. freundii parent. The second form, called Type II, more closely resembled the W form colonies of the S. typhi parent, but was, nevertheless, morphologically distinguishable from them. The Type II form colonies of hybrid 5-12 were identical in appearance to the only colonial form produced by the other eleven Vi expressing hybrids. Restreaking of either a single Type I form colony or a single Type II form colony of hybrid 5-12 gave rise to a mixture of both colonial forms. Subsequently, four of the eleven hybrids that initially produced only the Type II colonies were seen to give rise to

Type I colonies. Upon restreaking, these Type I colonies gave rise to both Type I and Type II colonies, each of which, on subsequent replatings, now displayed the same reversible form transition seen with hybrid 5-12. Quite fortuitously, it was found that Type II colonies, although agglutinated by antiserum against C. freundii, were not agglutinated by antiserum prepared against a Vi expressing strain of C. intermedius, whereas Type I colonies were agglutinated by the antiserum against C. intermedius as well as by antiserum against C. freundii. No W form colonies, which would not have been agglutinated by either of these antisera, were observed among these hybrids.

d. From the agglutination of Type II S. typhi hybrid colonies in antiserum against C. freundii but not in antiserum against C. intermedius, it appeared that these hybrids expressed a component of the C. freundii Vi antigen that is not present in the Vi antigen of C. intermedius. This proposition was confirmed by our finding that absorption of C. freundii antiserum with three times the number of C intermedius cells required to remove all agglutinins against C. intermedius removed none of its agglutinating titer against the Type II hybrid colonies. This absorbed antiserum was likewise unchanged in its ability to agglutinate Type I hybrid colonies, implying that they too express, in addition to a C. freundii Vi component that is expressed also by C. intermedius, a C. freundii Vi component that is not expressed by C. intermedius. We found also that, using one of the S. typhi hybrids that produced only Type II colonies (and showed no agglutination in antiserum against C. intermedius), we were able to absorb from C. freundii antiserum all agglutinins against C. intermedius. It appears, therefore, that this hybrid, although showing no evidence of Type I colony production, and not producing a sufficient amount of the Vi component that is common to C. intermedius to be agglutinated in C. intermedius antiserum, has, nevertheless, at least some capability for synthesis of this component. Thus, in some divergence from the V to W transition that occurs in the C. freundii parent, the reversible expression seen in these S. typhi hybrids appears to involve only one of two serologically identifiable components of the C. freundii Vi antigen, namely, the one that is common to the C. intermedius antigen. In type I colonies, this component is synthesized in sufficient quantity to permit its detection by agglutination in C. intermedius antiserum; in Type II colonies its synthesis is reduced, and agglutination in C. intermedius antiserum is not observed.

e. Because of the infrequency with which S. typhi hybrids inheriting the viaB genes from the C. freundii WR7005 donor were recovered, we were not able to get a clear picture of the linkage

relationships of the genes determining synthesis of the Vi antigen components and the gene or genetic element responsible for reversible expression of the component common to C. intermedius. In order to improve this situation, we converted the S. typhi hybrid 5-12 to an Hfr strain, and used it to transfer its C. freundii mel$^+$ and viaB$^+$ genes to Vi-negative (viaB) S. typhi WR4227. Unselected marker inheritance was greatly increased in this cross, with 74 of 103 mel$^+$ selected hybrids exhibiting Type II colonies that agglutinated in C. freundii antiserum, but not C. intermedius antiserum. Initially, only 21 of these 74 Type II hybrids also produced Type I colonies that agglutinated in both C. freundii and C. intermedius antisera, but, over a period of several months, we were able to obtain Type I colonies from all but eight of them. These eight, however, were found to be capable of absorbing C. freundii antiserum of all its agglutinins against C. intermedius, thus indicating their inheritance of the genes determining synthesis of the C. freundii Vi component common to C. intermedius. All hybrids exhibiting Type I colonies also exhibited the reversible form expression described earlier for hybrid 5-12.

f. As the genes determining synthesis of both Vi antigen components were inseparable by recombination in these experiments and were inherited together at the viaB locus, so too, we believe, was the gene or genetic element controlling the reversible Type I expression. Although the appearance of reversible Type I colonies was not observed in some Type II hybrids, we think that the presence in them of the genes determining synthesis of the C. intermedius-common Vi antigen component argues in favor of the presence also of the controlling gene. As the function of such a gene, or regulatory element, would be to prevent expression of the genes determining synthesis, it would be expected that hybrids in which this element became separated, by recombination, from the viaB genes would be nonreversible Type I forms, continuously synthesizing both antigen components. No hybrids of that type, however, were observed. We propose, therefore, that in those Type II hybrids in which Type I colony forms were not observed, the regulatory gene or genetic element is present but, in some manner, altered in function so that it continuously prevents expression of the genes determining synthesis of the C. intermedius-common Vi antigen component.

2. Molecular, genetic, and biochemical characterization of plasmid-mediated, atypical utilization of citrate by Escherichia coli.

a. Certain biochemical traits of bacteria are employed as key diagnostic aids in the clinical microbiology laboratory. Over the past 15 years, a variety of diagnostically important functions, such as hydrogen sulfide or urease production, as well as the fermentation of lactose or sucrose, have been demonstrated by genetic and molecular

means to be plasmid-mediated. Though Escherichia coli are typically citrate-nonutilizing, Washington and Timm, in 1976 (2), reported the isolation of several E. coli strains with the ability to utilize citrate. Subsequently, we decided to examine the nature of citrate-utilizing ability in one such atypical E. coli strain, V414, obtained from the Center for Disease Control, Atlanta, Ga. This strain exhibited typical E. coli reactions in diagnostic biochemical tests, with the exception of its ability to utilize citrate. In addition, it was found to be resistant to both tetracycline and chloramphenicol.

b. During routine subculturing of strain V414, citrate-nonutilizing derivatives appeared frequently among its progeny, suggesting that a plasmid might be involved in its ability to utilize citrate. In mating experiments with E. coli recipient strain 2340, strain V414 was found to transfer the citrate-utilization character at a frequency of 10^{-6} to 10^{-7} per donor cell. All citrate-utilizing transconjugants also acquired resistance to tetracycline and chloramphenicol. Subsequently, the citrate-utilization character was transferred from strain 2340 to other E. coli strains at about the same frequency, and those strains likewise acquired also the resistances to chloramphenicol and tetracycline. As expected, when transfer of tetracycline resistance was the selected trait, chloramphenicol resistance and citrate-utilization were also coinherited.

c. Strain V414 and several citrate-utilizing transconjungants from the mating with E. coli 2340 were examined for the presence of plasmid DNA by centrifugation of cell lysates in cesium chloride-ethidium bromide gradients. Covalently-closed circular plasmid DNA bands were never observed in DNA preparations made from the plasmid-free recipient strain, 2340, but were detected in the lysates of V414 and of the transconjugants. The plasmid DNA preparations obtained from V414 and its transconjugants were examined on agarose gels, and each was found to contain a single plasmid band that appeared identical in all preparations and was approximately 125 Mdal in size. More accurate sizing was obtained by contour length measurements in the electron microscope and showed the plasmid in strain V414 and in each of the transconjugants to have a molecular weight of 130 Mdal.

d. Normally E. coli does not utilize citrate, apparently, because citrate can not be transported into the cell. Metabolic studies were performed to see if radiolabled citrate could be incorporated into these citrate-utilizing E. coli cells, perhaps via a plasmid-mediated permease. Cells pregrown in a casamino acids-basal salts medium with or without citrate were washed and adjusted to a set cell density in fresh basal salts medium containing ^{14}C-1-5 labelled citrate. Samples were taken periodically for 120 minutes and examined for ^{14}C-label incorporation with TCA precipitable cellular material, radiolabled

carbon dioxide respiration, and also total protein to monitor cell growth. With strain V414, as well as a citrate-utilizing 2340 transconjugant, very little of the ^{14}C-label was incorporated into TCA-precipitable material over this time span. However, the majority of the utilized radiolabelled citrate carbon could be accounted for as expired carbon dioxide. In addition, the cells pregrown in citrate showed a 3-fold increase in the initial rate of ^{14}C-labelled carbon dioxide respired over that seen with cells pregrown in the absence of citrate. As expected, the plasmid-free E. coli 2340 strain neither incorporated label nor respired labelled carbon dioxide under these conditions. These results indicate that citrate is being metabolized extracellularly or, perhaps, at the cell surface, and that decarboxylation of citrate occurs prior to or during uptake. Although preliminary, these data are consistent with the system proposed by Sachan and Stern (1) for the uptake of citrate by Enterobacter aerogenes and Salmonella typhimurium. In that system, a membrane-bound oxaloacetate decarboxylase acts additionally as a transport protein for citrate.

3. Characterization of a multiple plasmid-containing Escherichia coli strain to serve as a source of size reference plasmid molecules.

a. The utility of agarose gel electrophoresis in the study of bacterial plasmids has been well established during the past few years. Plasmid size may be estimated conveniently by this method using either purified plasmid DNA or plasmid-containing cell lysates that have been cleared of most chromosomal DNA by high speed centrifugation. Such size estimations are made possible by the inclusion of plasmid molecules of known molecular size in the same electrophoretic run. It has been shown that there is a linear relationship between the logarithm of the migration of covalently closed circular DNA molecules and the logarithm of the plasmid molecular size. Availability of circular reference molecules of known size is usually limited by the necessity of utilizing several bacterial strains, each containing a single molecular species, in order to obtain a full complement of molecular sizes. We have analyzed a strain of Escherichia coli, designated V517 in our laboratory, that contains multiple plasmid species of 8 distinct sizes. We describe here the characterization of these plasmids and suggest that this strain will be a useful single source of plasmid reference molecules.

b. Plasmid DNA was routinely isolated from 500 to 100 ml of mid-logarithmic cells (grown in Antibiotic Medium No. 3, Difco Labs, Detroit, MI) by dye-buoyant desity centrifugatin of cleared cell lysates according to the method of Clewell and Helinski. Optimal cell lysis of V517 was obtained by allowing the lysozyme-spheroplasts to

remain in the presence of Brij-58 at 4°C for several hours. Such Brij-58 treated suspensions were placed in a 60°C water bath for 3 minutes, and then centrifuged to remove chromosomal DNA. Standard reference DNA for gel analysis was as follows: R1drd19, 62 X 10^6 daltons (62 Mdal); ' 5K, 26 Mdal; pSC101, 6.02 Mdal; and pMB8, 1.8 Mdal. pSC185 (mole .ar size 9.2 Mdal) was used as an internal contour length reference in electron microscopic analysis. Migration distances of the DNA components in agarose slab gel electrophoresis experiments were measured directly from photographs of the gels. The distance from the bottom of the well to the leading edge of the band was always measured. Standard curves and size estimates of the plasmids of V517 were generated by linear regression analysis. CsCl-ethidium bromide analysis of crude cell lysates, preparation of DNA samples for electron microscopy and measurements of open circular molecules photographed in the electron microscope were performed by standard procedures.

c. Agarose gel analysis of cleared concentrated cell lysates of strain V517 revealed multiple plasmids. Covalently closed circular plasmid DNA, purified by dye-buoyant density centrifugation, was analyzed by both agarose gel electrophoresis and electron microscopy. These analyses revealed the presence of eight different size classes of circular DNA in V517. The size determinations of the eight plasmids are presented in Table 1 and relatively good agreement is seen for values obtained by the two methods. Standard deviation for the size values obtained from gel determinations ranged from 4 to 11% while the range was from 2 to 5.8% for the values obtained by contour length measurements. We feel that subtle variations in band curvature and band size account for the significantly higher standard deviation of gel measurements relative to the values obtained by electron microscopy. We thus accept values obtained by the latter method as standards. Plasmid isolation and migration properties (from several independent plasmid preparations) have been found to be highly reproducible.

d. At this time, we are not able to ascribe phenotypic functions of any of the plasmids of E. coli V517. Nevertheless, we present this strain as a convenient single source of a range of covalently closed circular plasmid molecules useful as references in agarose gel analysis. The molecular sizes of these plasmids span the range of most naturally-occurring plasmids isolated from a number of species including the staphylococci and streptococci and, further, encompass the size range of most plasmids constructed by recombinant DNA methods.

TABLE 1. SIZE ESTIMATES OF PLASMIDS IN E. coli V517

PLASMID DESIGNATION	SIZE (Mdal)±STANDARD DEVIATION		
	AGAROSE GEL ANALYSIS [a]	ELECTRON MICROSCOPY [b]	
		n[c]	size
pVA517A	32.04±3.60	5	35.84±1.0
pVA517B	5.19±0.27	18	4 82±0.11
pVA517C	3.48±0.20	54	3.67±0.08
pVA517D	3.03±0.18	29	3.39±0.09
pVA517E	2.24±0.09	19	2.63±0.07
pVA517F	1.69±0.10	34	2.03±0.06
pVA517G	1.51±0.09	76	1.79±0.07
pVA517H	1.24±0.09	61	1.36±0.08

a. Based on the results of 4 independently performed analyses using R1drd19, PSC101 and pMB6 as CCC reference markers.

b. Size estimates made from open circular contour lengths using pSC185 as an internal reference.

c. n=number of molecules measured.

Summary:

1. Citrobacter freundii donor strains exhibiting reversible Vi antigen expression were employed to transfer their viaA$^+$ and viaB$^+$ genes to Vi non-expressing Salmonella typhi recipients with genetic defects at either of these two loci. S. typhi hybrids inheriting the C. freundii viaA$^+$ gene were restored to the continuous, non-reversible expression of the Vi antigen normally seen in S. typhi. Inheritance of the C. freundii viaB$^+$ genes, however, produced hybrids that were seen to alternate between two morphologically distinguishable colonial forms, designated Type I and Type II. Type I colonies were agglutinated by antiserum prepared against either C. freundii or against Vi expressing C. intermedius, whereas Type II colonies were agglutinated by antiserum against C. freundii, but not by antiserum against C. intermedius. Antiserum absorption studies revealed that Type I hybrid colonies synthesize at least two serologically distinguishable components of the C. freundii Vi antigen, one which is also expressed by C. intermedius, and one which is not expressed by C. intermedius. Transition from the Type I form colony to the Type II form colony was found to involve a reduction in the synthesis of that Vi antigen component which is expressed also by C. intermedius, while synthesis of the other component remained unaffected. It was concluded from the results of genetic experiments that the genes determining synthesis of both antigen components reside at the viaB locus, and that the gene or genetic element controlling reversible form expression also is situated at or very near this locus.

2. Citrate utilization was examined in an atypical Escherichia coli strain, V414, isolated from a clinical specimen. Plasmid-mediated citrate assimilation was suspected because citrate-non-utilizing derivatives of this citrate utilizing (Cit$^+$) strain appeared frequently among its progeny during subculturing. The Cit$^+$ character of strain V414 was found to be transmissible, by conjugation, to an E. coli K-12 recipient, which, in turn, transferred it to other E. coli strains. Purified plasmid DNA from strain V414 and from Cit$^+$ transconjugants was examined by agarose gel electrophoresis and electron microscopy. A single 130 megadalton conjugative plasmid was found to encode the ability to assimilate citrate. Although E. coli cells are normally unable to transport exogenous citrate, they can catabolize it intracellularly. Metabolic studies of strain V414 indicated that intact citrate may not be incorporated directly into the cells, but may be partially metabolized at the cell surface before uptake and assimilation.

3. Escherichia coli strain V517 was shown to contain eight distinct plasmid species. Seven of these plasmids range in size from 1.36 to 4.82 megadaltons, whereas the remaining plasmid is 35.8 megadaltons. This strain can be employed as a convenient single source of covalently closed circular DNA molecules of different sizes for use as references in agarose gel electrophoretic analysis.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 139 Microbial Genetics and Taxonomy

Literature Cited:

References:

1. Sachan, D.S., and Stern, J.R. Studies of citrate transport in Aerobacter aerogenes: binding of citrate by a membrane bound oxaloacetate decarboxylase. Biochem. & Biophys. Res. Commun. 45: 402-408. 1971

2. Washington, J.A., and Timm, J.A. Unclassified citrate-positive member of the family Enterobacteriaceae resembling Escherichia coli. J. Clin. Microbiol. 4: 165-167. 1976.

Publications:

1. Diena, B.B., Lior, H., Ryan, A., Krol, P., Johnson, E.M., and Baron, L.S. Mouse protective capabilities of Escherichia coli hybrids expressing Salmonella typhi antigens. Infect. Immunity 24: 90-93. 1979.

2. Friedman, D.I., Baumann, M., Baron, L.S., Carter, C., Flamm, E., and Ziegler, S. NUS: E. coli mutants that fail to support the action of the N antitermination function of λ. Abst. 23rd Ann. Bacteriophage Meeting, Cold Spring Harbor. p.42. 1979.

3. Kopecko, D.J., Baron, L.S., and McCowen, S.M. Molecular genetic and biochemical characterization of plasmid-mediated atypical utilization of citrate by Escherichia coli. Abst. Ann. Meeting, A.S.M., p.131. 1979.

4. Kopecko, D.J., Holcombe, J., and Formal, S.B. Molecular characterization of plasmids from virulent and spontaneously occurring avirulent colonial variants of Shigella flexneri. Infect. Immunity 24: 580-582. 1979.

5. Kopecko, D.J., Johnson, E.M., and Baron, L.S. Genetic and molecular aspects of bacterial heredity. p.157-211. In B.A. Freeman, Burrows Textbook of Microbiology, 21st ed., W.B. Saunders, Co., Philadelphia. 1979.

6. Kopecko, D.J., Vickroy, J., Johnson, E.M., Wohlhieter, J.A., Casey, T., and Baron, L.S. Molecular analysis of plasmids responsible for lactose catabolism in Salmonellae. Plasmid 2: 298. 1979.

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6. Kopecko, D.J., Vickroy, J., Johnson, E.M., Wohlhieter, J.A., Casey, T., and Baron, L.S. Molecular analysis of plasmids responsible for lactose catabolism in Salmonellae. Plasmid 2: 298. 1979.

7. Macrina, F.L., Kopecko, D.J., Jones, K.R., Ayers, D.J., and McCowen, S.M. Multiple plasmid-containing Escherichia coli: a simple method for preparing plasmid roference molecules. Va.J. Sci. 29: 52. 1979.

8. Snellings, N.J., Johnson, E.M., and Baron, L.S. Genetic investigation of Vi antigen variation in Citrobacter ballerup. Abst. Ann. Meeting, A.S.M., p.48. 1979.

9. Yamamoto, N., Numa, S., Gemski, P., Wohlhieter, J.A., and Baron, L.S. Isolation of hybrid phages between coliphage ϕ80 and Salmonella phage P22. Abst. Ann. Meeting, A.S.M., p.24. 1979.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
				DA OA 6450	79 10 01	DD DR&E(AR)636

3 DATE PREV SUMRY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 REGRADING	8A DISP'N INSTR'N	8b SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10 NO /CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	140
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11 TITLE (Precede with Security Classification Code)
(U) Military Hematology

12 SCIENTIFIC AND TECHNOLOGICAL AREAS
008800 Life Support 02600 Biology 003500 Clinical Medicine

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMANCE METHOD
58 05	CONT	DA	C. In-House

17 CONTRACT/GRANT
a. DATES/EFFECTIVE: NA EXPIRATION
b. NUMBER
c. TYPE d. AMOUNT:
e. KIND OF AWARD f. CUM. AMT.

18 RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	10	417
FISCAL YEAR CURRENT 80	10	542

19 RESPONSIBLE DOD ORGANIZATION
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RESPONSIBLE INDIVIDUAL
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Michael J. Haut, LTC, MC
TELEPHONE: (202) 576-3358
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: DA
NAME:

21 GENERAL USE
Foreign intelligence not considered

22 KEYWORDS (Precede EACH with Security Classification Code)
(U) Coagulation; (U) Malaria; (U) Blood; (U) Anemia

23 TECHNICAL OBJECTIVE. 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23.(U) To define the hematologic pathophysiology of infectious diseases of military importance, and of trauma, burns and shock, and to identify modalities to restore hemostasis.

24.(U) Procedures include biochemical, immunologic, and cell culture methods; in vitro cell-free and membrane-dependent systems; large and small laboratory animal models; and studies of human subjects.

25.(U) Studies of protein and platelet aspects of coagulation, demonstrated that heavy exercise produces activation of clotting via both the protein cascade and platelets, and a balanced activation of clot lysis. An original method for gentle tritiation of proteins, activation of complement C3 to C3b was found to involve exposure of a free thiol group. This was involved in the firm covalent binding of C3b to target cells, probably via a thiolester. An N-formyl peptide activator of white cell chemotaxis was shown to activate multiple functions of the alveolar macrophage. Differences between nucleotide metabolism of malaria and red cells were discovered and delinated to identify points of specific susceptibility of malaria to chemotherapy. Evidence was found suggesting that a much higher adenosine deaminase activity in P.falciparum may be a susceptible step in parasite metabolism. Malaria parasites were shown to lack signifi- capacity to synthesize coenzyme B_6. Investigations are in progress to determine whether the polymorphism for low red cell pyrdoxal kinase activity will limit P. falciparum growth in vitro and whether this organism will be sensitive to B_6- antagonists. Pyridoxal and pyridoxal-P were shown to enter red cells, react covalently with hemoglobin, increase or decrease oxygen affinity of hemoglobin, and inhibit sickling. These vitamins are promising agents for treatment of sickle cell trait, sickle cell anemia, and for the modification of oxygen transport in red cells. For Technical Report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78-30 Sep 79.

*Available to contractors upon originator's approval

PROJECT 3M161102BS01 **BASIC RESEARCH ON MILITARY INJURY AND DISEASES**

WORK UNIT 140 - Military Hematology

The following studies are under investigation:

1. The Reaction of Pyridoxal and Pyridoxal Phosphate with Valine and Lysine
2. Modification of Intracellular Hemoglobin by Vitamin B_6
3. Inhibition of Erythrocyte Sickling by Pyridoxal Phosphate
4. Familial Polycythemia due to Hemoglobin Walter Reed
5. The Optimum Hemoglobin Level in two Contrasting Types of Polycythemia
6. Rapid Method for Fluorometric Measurement of Human Erythrocyte Pyridoxal Kinase (PLK)
7. Absence of Pyridoxal Kinase (PLK) Activity in Plasmodia
8. Vitamin B_6 Metabolism in the Hematopoietic System of Patients Receiving Isoniazid
9. Vitamin B_6 Metabolism in the Hematopoietic System of Patients with Sideroblastic Anemia
10. Medical Treatment of Amenorrhea-Galactorrhea Syndrome with Vitamin B_6 (Pyridoxine)
11. An Animal Model for Quantitative Study of Reversible Subacute Lead Encephalopathy
12. Production of Sideroblastic Anemia in Animals by Selective Deprivation of Vitamin B_6
13. Abnormalities of Lipid Metabolism in the Nervous System and Blood Cells of Vitamin B_{12}-Deficient Rhesus Monkeys

14. A Study of Delta Aminolevulinic Acid Synthetase and Ferrochelatase Activities in Anemia

15. A Study of Trace Metal Screening Procedures for Lead, Platinum, and other trace metals Using Protein Perturbants

16. The Carbohydrate Dependence of Platelet Surface Interactions in Hypercoagulable States

17. The Special Roles of the Coagulation Laboratory

18. The Effect of RBC Biochemical Genetic Polymorphisms on in vitro Malaria Cultures: The Role of Vitamin B_6

19. Biochemistry of the Host-Parasite Relationship in Malaria: Study of Purine and Pyrimidine Metabolism Utilizing in vivo and in vitro Models

A. TITLE: "The Reaction of Pyridoxal and Pyridoxal Phosphate With Valine and Lysine"

B. INVESTIGATORS:

Principal: LTC John A. Kark, M.D., MC

C. OBJECTIVES: To determine conditions affecting the Schiff base formation between the B_6 aldehydes and these amino acids, the acceptor amines on hemoglobin. These reactions serve as a model for the more complex reaction between these compounds and hemoglobin.

D. MATERIALS AND METHODS: Pyridoxal and valine were mixed in different buffers, at varied pH and temperature. The reaction was followed by scanning in a double beam, corrected spectrum spectrophotometer. The effect of sodium borohydride on the Schiff base (reduction to an aldimine) was also examined.

E. RESULTS: Buffers acting as reaction inhibitors or catablysts were discovered. Specific spectral shifts identified the free pyridoxal, the Schiff base and the reduced aldimine.

F. RELEVANCE TO THE ARMY: These observations provide a theoretical background for the therapeutic modification of hemoglobin.

A. TITLE: "Modification of Intracellular Hemoglobin by Vitamin B_6"

B. INVESTIGATORS:

Principal: LTC John A. Kark, M.D., MC

Associate: Cecil U. Hicks, Medical Technologist

CPT Rudolphi Bongiovanni, MSC
Clinical Investigation Service, WRAMC

C. OBJECTIVES: To determine practical conditions for the modification of hemoglobin within intact red cells, for the purposes of 1) improving stored red cells for transufsion in shock and for exchange transfusion, and 2) treatment of sickle cell anemia and prophylaxis for sickle cell trait.

D. MATERIALS AND METHODS: (See WRAMC Clinical Investigation Protocols #9019 and 9020, approved 16 October 1979, Office of the Surgeon General).
Synopsis: whole blood or suspended red cells were incubated under varied conditions with pyridoxal or pyridoxal phosphate. Extent of uptake was measured fluorometrically, formation of adducts were identified by IEF and measured by HPLC. The effect on red cell oxygen dissociation curves was examined with the Aminco Hem-O-Scan.

E. RESULTS: Extensive binding to hemoglobin was achieved (70 to 95%) under conditions reasonable for parenteral administration in vivo for pyridoxal or for extracorporeal treatment of blood for pyridoxal and pyridoxal phosphate.

Publication:

Kark, J.A., Hannah, J.S., Kale, M.P., Hicks, C.U., and Bongiovanni, R. Modification of intracellular hemoglobin by Vitamin B_6. Blood, November, 1979. (abstract).

F. RELEVANCE TO THE ARMY: These vitamins may provide a means for restoring normal oxygen affinity to stored red cells in situations where in vivo metabolic restoration in 4 to 8 hours is too slow, e.g., in treatment of hemorrhagic shock, in treatment of severe anemia (such as bone marrow radiation injury), and exchange transfusion. Modification of hemoglobin oxygen affinity also might improve performance of soldiers rapidly adapting to high altitude.

A. TITLE: "Inhibition of Erythrocyte Sickling by Pyridoxal Phosphate"

B. INVESTIGATORS:

Principal: LTC John A. Kark, M.D., MC

Associate: Cecil U. Hicks, Medical Technologist
Lawrence Lessin, M.D., Professor of Medicine
George Washington University School of Medicine,
Washington, DC

C. OBJECTIVES: To determine the efficacy of pyridoxal phosphate as an inhibitor of red cell sickling with different mechanisms of action from pyridoxal.

D. MATERIALS AND METHODS: (See protocol #9019, WRAMC, Clinical Investigation)

E. RESULTS: Preliminary tests suggested some effect but were unclear due to 1) excessive incubation time, resulting in loss of full sickling change with deoxygenation and, 2) blood samples inadvertently taken from patients with rare variant sickle cell disorders - later identified in the IEF examination of hemoglobins performed with each clinical study.

F. RELEVANCE TO THE ARMY: Development of a non-toxic antisickling agent for prophylactic use in troops with sickle cell trait. These studies are unique in that they deal with natural intermediates of cellular metabolism, which could possibly be non-toxic antisickling agents.

A. TITLE: "Familial Polycythemia Due to Hemoglobin Walter Reed"

B. INVESTIGATORS:

Principal: MAJ William Butler, M.D., MC
LTC John A. Kark, M.D., MC
Robert M. Winslow, M.D.
Chief, Hemoglobin Section,
CDC, Atlanta, GA

C. OBJECTIVES: To define the properties and describe the pattern of inheritance of a new very high affinity hemoglobin variant. To improve the management of health problems for the propositus and his relatives.

D. MATERIALS AND METHODS: Fresh blood samples from a soldier with polythythemia and from six siblings, his mother, and a nephew and niece were examined by iso-electric focussing, by stability tests, by use of the Hem-O-Scan, by red cell antigen testing, and by conventional hematologic laboratory procedures. Sequencing, to identify the exact changes in beta-chain amino acid sequence, is being performed by Schmidt, et al. at the CDC.

E. RESULTS: Five people had a bi-phasic left shifted oxygen dissociation curve with a P50 of 11 (the lowest abnormal P50 reported is 10). Twenty-four percent of the hemoglobin was identified as abnormal by HPLC. The abnormal hemoglobin has been purified and separated from Hb A.

F. RELEVANCE TO THE ARMY: Identification and description of this mutant hemoglobin will assist in studies of the effect of altered P50, polycythemia, and high altitude exposure on exercise tolerance. We are assisting this soldier and his family to understand and adjust to the minor medical problems created by this genetic variant.

A. TITLE: "The Optimum Hemoglobin Level in Two Contrasting Types of Polycythemia"

B. INVESTIGATORS"

Principal: Robert M. Winslow, M.D.
Chief, Hemoglobin Section
CDC, Atlanta, GA

MAJ William Butler, M.D., MC

LTC John A. Kark, M.D., MC

Harvey Klein, M.D.
Clinical Center Blood Bank
National Institutes of Health
Bethesda, MD

C. OBJECTIVES: To determine the difference between submaximal work performance at elevated hemoglobin level versus normal hemoglobin level in a man with a high affinity hemoglobin and a man with familial elevation of erythropoietin. These studies will examine the relative importance of increased viscosity versus oxygen-carrying capacity in submaximal work performance.

D. MATERIALS AND METHODS: Two closely matched patients with very different extremely rare causes of secondary polycythemia were recently identified at Walter Reed. They afford a unique opportunity to compare the importance of decreased efficiency of cardiac pumping, due to increased blood volume and hematocrit, versus the importance of oxygen-carrying capacity, which is reduced for the man with a high affinity hemoglobin. The facilities of the NIH are being used to carry out exercise testing before and after bleeding to a normal hemoglobin level under an NIH clinical protocol.

Reference:

Miller, D.M., Winslow, R.M., Klein, H., Wilson, K.C., Brown, F.L., and Statham, N.J. Exercise in the Evaluation of Therapy in Sickle Cell Anemia. Submitted to the NEJM.

E. RESULTS: The first patient, with high-affinity hemoglobin, did not benefit from phlebotomy. This documentation of the physiologic effect of phlebotomy in this disease is probably the first such report. The Second patient is being studied, and results are not complete.

F. RELEVANCE TO THE ARMY: These studies will assist in understanding of the management and pathophysiology of polycythemia, a universal response to high altitude. These studies may have important implications for management of troops at high altitude.

TITLE: "A Rapid Method for Fluorometric Measurement of Human Erythrocyte Pyridoxal Kinase (PLK)"

B. INVESTIGATORS:

Principal: LTC John A. Kark, M.D., MC
LTC Michael J. Haut, M.D., MC

C. OBJECTIVES: To develop and test a rapid, reproducible assay for erythrocyte synthesis of coenzyme B_6.

D. MATERIALS AND METHODS: (See manuscript)

E. RESULTS: (See manuscript). The objections raised when this paper was submitted in 1975 are now answered by further experimentation. The methods, results, introduction, and discussion sections are finished. The references and typing remain to be completed.

F. RELEVANCE TO THE ARMY: This technique has provided methods for investigation of an hypothesis relating the African low PLK variant to protection against malaria.

A. TITLE: "Absence of Pyridoxal Kinase (PLK) Activity in Plasmodia"

B. INVESTIGATORS:

Principal: LTC John A. Kark, M.D., MC

Samuel K. Martin, M.D.
Laboratory of Parasitic Diseases
National Insitutes of Health
Bethesda, MD

Louis H. Miller, M.D.
Chief, Malaria Section
Laboratory of Parasitic Diseases
National Institutes of Health
Bethesda, MD

C. OBJECTIVES: To determine whether certain species of malaria, including P. falciparum, are capable of synthesis of coenzyme B_6, or might depend on host red cells for this activity.

D. MATERIALS AND METHODS: (See publication of same title, Blood, December 1978, abstract).
Synopsis: plasmodia were raised in animals, and the animals bled. Plasmodia were taken from red cells after saponin lysis, and the PLK of the red cell fraction compared with the plasmodia fraction.

E. RESULTS: Several experiments demonstrated no PLK activity for plasmodia. Controls included ability to measure plasmodial PK, good recovery of pyridoxal and PLP from plasmodia fractions, and failure of plasmodia fractions to inhibit red cell PLK.

F. RELEVANCE TO THE ARMY: This is an important step in establishing whether plasmodia depend on host red cells for PLK activity: a possible point of metabolic attack for therapy, since mature cells do not require PLK for survival.

A. TITLE: "Vitamin B_6 Metabolism in the Hematopoietic System of Patients Receiving Isoniazid"

B. INVESTIGATORS: LTC John A. Kark, M.D., MC

Peter G. Tarrassoff, M.D.
Department of Medicine
Walter Reed Army Medical Center

C. OBJECTIVES: To determine the mechanism of dyserythropoiesis in patients taking isoniazid. To develop a means for monitoring isoniazid therapy. To understand the role of Vitamin B_6 in the production of hypoplastic anemias.

D. MATERIALS AND METHODS: (See Clinical Investigation Protocol #9010) Plans are being made to purify erythrocyte PLK for more precise studies of enzyme inhibition.

E. RESULTS: No new results were obtained this FY, but experiments were planned.

F. RELEVANCE TO THE ARMY: To improve the use of INH as a treatment for tuberculosis. To gain further knowledge of nutritional and metabolic hypoplastic anemias.

A. TITLE: "Vitamin B_6 Metabolism in the Hematopoietic System of Patients With Sideroblastic Anemia"

B. INVESTIGATORS:

Principal: LTC John A. Kark, M.D., MC

LTC Michael J. Haut, M.D., MC

C. OBJECTIVES: To compare the response of steps in vitamin B_6 metabolism to B_6 therapy and the hematologic response in refractory sideroblastic anemias.

D. MATERIALS AND METHODS: (See Clinical Investigation Protocol #9010).

E. RESULTS: A manuscript is in preparation summarizing results presented at meetings, e.g., Kark, et al. The Dissociation of Biochemical and Hematologic Responses to Pyridoxine in Patients with Sideroblastic Anemia. Blood 48:966, abstract, 1976.

F. RELEVANCE TO THE ARMY: An understanding of the mechanism of "primary" hypoplastic anemia may provide important insight into the mechanism of radiation hypoplastic anemia.

A. TITLE: "Medical Treatment of Amenorrhea-Galactorrhea Syndrome with Vitamin B_6 (Pyridoxine)"

B. INVESTIGATORS:

Principal: MAJ Gerald Kidd, M.D., MC
MAJ Robert A. Vigersky, M.D., MC
Endocrinology Service
Walter Reed Army Medical Center

LTC John A. Kark, M.D., MC
LTC Michael J. Haut, M.D., MC

C. OBJECTIVES: To determine whether this syndrome is due to a pituitary tumor and to compare the response of idiopathic versus adenoma-related disease, when high oral doses of pyridoxine are given for three months.

D. MATERIALS AND METHODS: (See WRAMC Clinical Investigation Proposal #1362, 1977).

E. RESULTS: Four patients entered the study. Measurements of prolactin, GH, and other hormones suggested that two patients had small pituitary adenomas. The other two patients had a complete cessation of lactation and return of menses with the first month of pyridoxine treatment. All four showed an identical major increase in plasma PLP, red cell PLP, and red cell pyridoxal kinase activity while taking pyridoxine.

Unfortunately, the referring group of doctors from OB-GYN lost interest, and Dr. Kidd and Dr. Haut have left WRAMC. This promising study is temporarily inactive.

F. RELEVANCE TO THE ARMY: Pyridoxine could be useful in the management of this syndrome in young women. Pyridoxine is extremely safe at this dose level, having no known side effects.

A. TITLE: "An Animal Model for Quantitative Study of Reversible Subacute Lead Encephalopathy"

B. INVESTIGATORS:

Principal: Daryl Hawkins, Ph.D.
Department of Neuropyschiatry
Walter Reed Army Institute of Research

LTC John A. Kark, M.D., MC

C. OBJECTIVES: To develop a reproducible animal model of lead encephalopathy, with a time course of about one month, reversible, and a means for measurement of the severity of the neurologic defect.

D. MATERIALS AND METHODS: Drinker strain rats were used to provide mother nursing rats who would accept high lead levels in the water. Audiogenic seizure sensitive rats were used to provide newborn rats capable of seizure response in a reproducible fashion to a measured audiogenic stimulus.

(See WRAIR animal investigation protocol #M-19-77)

E. RESULTS: A reproducible, measured threshold for audiogenic seizures was obtained for newborn rats. Administration of lead via a Drinker nursing mother resulted in a reproducible decrease in threshold, which lasted for 3 to 5 weeks after cessation of lead poisoning.

F. RELEVANCE TO THE ARMY: This animal model provides an excellent means for examing the production of neurotoxicity by a number of agents. It could be used to define neurotoxicity of metals, environmental pollutants and chemical agents. It could be used to test modes of therapy of lead poisoning and other agents causing encephalopathy.

A. TITLE: "Production of Sideroblastic Anemia in Animals by Selective Deprivation of Vitamin B_6"

B. INVESTIGATORS:

Principal: LTC John A. Kark, M.D., MC
Mr. Harold Williams, Chemist
LTC Michael J. Haut, M.D., MC

C. OBJECTIVES: To define the changes in electron microscopic morphology, stem cell activity, and vitamin B_6 levels in animals deprived of vitamin B_6.

D. MATERIALS AND METHODS: (See WRAIR animal protocol: Sideroblastic Anemia I and II.)

E. RESULTS: (See WRAMC animal investigation protocol: Sideroblastic Anemia I and II. A manuscript is in preparation).
Synopsis: For the first time, iron-laden mitochondria were observed in erythroblasts from B_6 deficient anemic animals. Defects in BFU-e and CFU-e were characterized. Although a major loss of ALA-synthetase activity occurred in marrow cells, ferrochetalase remained intact. Rhesus monkeys failed to develop atherosclerosis, but did develop severe, sometimes fatal, neutropenia.

F. RELEVANCE TO THE ARMY: These studies may shed some light on the pathogenesis of bone marrow failure, including the aplastic anemia which results from radiation injury.

A. TITLE: "Abnormalities of Lipid Metabolism in the Nervous System and Blood Cells of Vitamin B_{12}-Deficient Rhesus Monkeys"

B. INVESTIGATORS:

Principal: Judy S. Hannah, M.S.
Graduate Student, Department of Nutritional Biochemistry
University of Maryland
College Park, MD

LTC John A. Kark, M.D., MC

Mary Catherine McKenna
Post Doctoral Fellow, Nutritional Biochemistry
National Institutes of Health
Bethesda, MD

Associate: Maurice Victor, M.D.
Professor of Neurology
Case Western Reserve University School of Medicine
Cleveland, OH

John W. Harris, M.D.
Professor of Medicine
CWRUSM at Cleveland Metropolitan General Hospital
Cleveland, OH

C. OBJECTIVES: To define the abnormalities of lipid metabolism which result in demyelinating disease, as expressed in vitamin B_{12} deficiency disease.

D. MATERIALS AND METHODS: Discussed in full in NIH Grant "Vitamin B_{12} Deficiency in Rhesus Monkeys".
Synopsis: Samples from control and deficient monkey nervous system, distributed between affected and unaffected regions of the spinal cord and brain have been collected and frozen during the period, 1973-1976. Tissues are analyzed by Folch-Lees extraction, liquid column chromatography, thin layer chromatography, derivatization with BF_3 and GLC.

E. RESULTS: Normal spinal cord and brain lipids have been characterized in detail. Normal red cell and white cell lipids have also been examined incompletely.

F. RELEVANCE TO THE ARMY: Important basic research which is expected to contribute to fundamental understanding of the biochemistry and function of the spinal cord. (This work was discussed in full, and approved in a letter from COL Buescher when I arrived at WRAIR in 1973). Eighty percent of working time is carried out by the visiting scientist, Judy S. Hannah.

A. TITLE: "A Study of Delta Aminolevulinic Acid Synthetase and Ferrochelatase Activities in Anemia."

B: INVESTIGATORS: Harold L. Williams
Deadre J. Johnson
John Kark
Jeffrey Berenberg

C: OBJECTIVE: To evaluate more comprehensively the blood forming role of delta aminolevulinic acid synthetase and ferrochelatase in the normal and anemic states and to accurately define the levels of these enzymes.

D: MATERIALS AND METHODS: Bone marrow will be collected from normal and controls and patients in preservative-free heparin and processed in a manner adapted after Bottomley (1) and analyzed for ALAS after a modified method of Ebert (2) and ferrochelatase measured by the technique of William, et al. (3)

E: RESULTS: Preliminary work in this laboratory in measuring ferrochelatase activity in bone marrow of human patients indicate a level of enzyme activity at least one order of magnitude higher than that reported by Bottomley. We have reason to question the validity of the absolute values of this enzyme's activity reported by previous investigators due to their analyzing for this enzyme under conditions which were not optimal.

F: MILITARY RELEVANCE: The understanding and proper treatment of disease processes provides a soldier who should be in better physical and mental health and more able to effectively carry out his/her military mission.

A. TITLE: "A Study of Trace Metal Screening Procedures for Lead, Platinum, and other trace metals using Protein Perturbants."

B. INVESTIGATORS: Harold L. Williams
Deadre J. Johnson

C. OBJECTIVE: To provide a means for analyzing for Platinum, Lead, and other trace metals by spectrophotometric means, or other colorimetric procedures to provide a less expensive analysis for lead poisoning and for Platinum when used in cancer research.

D. MATERIALS AND METHODS: In vitro testing of blood samples spiked with grated amounts of the metals.

Williams, Harold L., et al., Simultaneous spectrophotometry of Fe^{+2} and Cu^{+2} in serum denatured with guanidine hydrochloride. Clin. Chem. 23(2):237, 1977.

Johnson, Deadre J., et al., Improved colorimetric determination of serum zinc. Clin. Chem. 23(7):1321, 1977.

Golla, Edward D. and Gilbert H. Ayres, Spectrophotometric determination of platinum with o-phenylenediamine. Talanta, 20:199, 1973.

Khan, Amanullah, editor, The Third International Symposium on Platinum Coordination Complexes in Cancer Chemoterapy, Part I. Journal of Clinical Hematology and Oncology. Wadley Institutes of Molecular Medicine, Dallas, Tx., 1977.

E. RESULTS: Pending

F. RELEVANCE TO ARMY: A better understanding of metal mediated physiological responses.

A. TITLE: "The Carbohydrate Dependence of Platelet Surface Interactions in Hypercoagulable States."

B. INVESTIGATORS:

Principal: MAJ Salvatore Scialla, M.D., MC

Associate: LTC Michael J. Haut, M.D., MC
MAJ Grant Taylor, M.D., MC
LTC Jeffrey Berenberg, M.D., MC

C. OBJECTIVES: To examine platelets and plasma from selected individuals with hypercoagulable states to determine if altered carbohydrate and carbohydrate synthesizing capacity is present.

D. MATERIALS AND METHODS:

1. Platelets and plasma are separated from blood drawn from controls and cancer patients.

2. Coagulation profiles are performed on the plasma sample which includes a detailed analysis of Factor VIII complex.

3. Detailed analysis of the Factor VIII complex includes a separation procedure by chromatography using agarose. Sialic Acid content of Factor VIII is determined by the Warren Method.

4. Platelet surface sialyltransferase is determined by a C^{14} Sialic Acid incorporation assay with platelets as the enzyme source.

5. Platelet surface sialic acid is determined by incubation with neuriminidase and subsequently the Sialic Acid assay by Warren.

6. The above studies are correlated with platelet aggregation photometric method.

E. RESULTS: The mechanism of Sialyltransferase Activity (STA) and platelet function is being clarified. An inhibitor of the platelet release reaction (Prostaglandin E_1) does not inhibit collagen stimulated STA. Ristoaten does inhibit surface platelet STA. The work this year is to clarify the interaction of Factor VIII, STA, and Sialic Acid.

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Kessel, D. and Allen, J. Elevated Plasma Sialotransferase in the Cancer Patient. Cancer Research 35:670-672 (1975).

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Legaz, M.E., Schmer, G., Counts, R.B. and Davie, E.W. The Isolation and Characterization of Human Factor VIII (anti-hemophilia factor). The J. of Biolo. Chem. 248:3946-3955 (1973).

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F. RELEVANCE TO THE ARMY: The biochemistry of platelet membranes is very important in understanding the hemostatic potential of the body during periods of injury. Platelet transfusion and preservation are also dependent on platelet membrane biochemical interactions.

COAGULATION LABORATORY

The Coagulation Laboratory serves a dual purpose. It performs a clinical function for WRAMC, and it serves as the major coagulation research laboratory for the Department of Defense.

1. Clinical Role: We have the opportunity to study bleeding and clotting problems in active duty personnel. One patient was found to have a Factor XII deficiency that was unique in having antigenic content. We have collaborated with Dr. Hidehiko Saito of the University Hospitals of Cleveland to present and publish this variant of Hagman Trait.

   a. Presentation to American Society of Hematology, December 4, 1978.

      CRM^t Variants of Hagman Trait. H. Saito, J.G. Scott, H.Z. Movat and S.J. Scialla.

   b. Abstract submitted to 1979 ASH Isolation and Properties of Non-Functional Hagman Factor (HF, Factor XII) from a CRM^t Hagman Trait Plasma H. Saito and S.J. Scialla.

   c. Published Article

      CRM^t Variants of Hagman Trait. J. Lab. Clin. Med. 94:256 (1979).

2. Research Role:

   a. We have studied the role of coagulation factors, platelet function, and platelet membrane activity in various states of enhanced clotting (esp. patients with cancer as a model of hypercoagulability).

      1) Publication

         Scialla, S.J., Speckart, S., Haut, M., and Kimball, D.B. Alterations on Platelet Surface Sialyltransferase Activity and Platelet Aggregation on a Group of Cancer Patients with a High Incidence of Thrombosis. Cancer Research 39:2031 (1979).

      2) Abstract for Presentation to American Federation of Clinical Research - Eastern Regional Section - 19 October 1979. Boston. Stimulated Sialyltransferase Activity, Rebose Reaction, An Aggregation in Washed Platelets Using Prostaglandin E_1. Scialla, S.J. and Berenberg, J.L.

b. We have collaborated with Earl Ferguson, Department of Biochemistry, Uniformed Services, University of Health Sciences for the Study of Exercise on Coagulation and Platelet Function.

1) Presentation at 3rd National Conference on Thrombosis and Hemostasis - November 1978. Reduction of Platelet ADP Content by Exercise. E.W. Ferguson and C.F. Barr. Abstract 536.

2) Fibrinogonolysis and Fibrinolysis with Strenuous Exercise. E.W. Ferguson, C.F. Barr, and L.L. Bernier. J. of Applied Physiology (in press).

A. TITLE: "The Effect of RBC Biochemical Genetic Polymorphisms on *In Vitro* Malaria Cultures: The Role of Vitamin B_6."

B. INVESTIGATORS: LTC June M. Whaun
CPT Horace K. Webster
LTC Michael J. Haut
PFC Teresa L. Harter
Mr. Vincent Okoye
LTC John Kark
MAJ David Haynes

C. OBJECTIVES: To determine the role of Vitamin B_6 in the nutrition of the parasite and the effect of Vitamin B deprivation (nutrient deprivation or addition of Vitamin B_6 analogues) on *in vitro* p. falciparum growth.

Malaria is an important world-wide parasitic disease. Its incidence has increased recently because of failures in vector control as well as the emergence of chloroquin-resistant strains in South America, S.E. Asia, and now, Africa.

Vitamin B_6 is necessary for transamination, decarboxylation and racemization reactions, and as the malaria parasite lacks the ability to make purines and pyrimidines *de novo*, it would be expected to be essential for parasite growth. Pyridoxine must be converted to its physiologically active form pyridoxal phosphate by pyridoxine Kinase (Pnk). Because blacks have decreased levels of red cell pyridoxine kinase (Chern and Beutler, 1975), some investigators (Martin et al, 1978) have examined the association of low Pnk as a protective mechanism against malaria.

To test the hypothesis that red cell polymorphisms are protective against malaria, blood from donors with low Pnk will be used in an *in vitro* p. falciparum culture to see if they support malarial growth as well as blood from donors with normal Pnk. To examine the mechanism of Vitamin B_6 deprivation on malarial growth and red cell metabolism, nucleotide profiles and Vitamin B_6 metabolites will be obtained on components of this culture system. Vitamin B_6 deprivation in culture will be obtained through either nutrient deprivation or the addition of Vitamin B_6 analogues.

D. STUDY PLAN: METHODS

1. *In vitro* p. falciparum cultures with FCR_3 (Rockefeller drug-sensitive strain) after the method of Haynes et al (1976) and Trager and Jensen (1976).

2. Ancillary techniques of ^3H - hypoxanthine uptake and quantitation of parasites.

3. Pyridoxal phosphate assay after Chabner and Livingston (1970) as modified by Lumeng and Li (1974).

4. Pyridoxine kinase assay after Chern and Beutler (1975) and Karawya and Fonda (1978).

5. Separation and quantitation of Vitamin B_6 compounds by high voltage electrophoresis (McCoy, Colombini and Strynadka, 1979).

6. Culture arrangements:

   Parasite: P. falciparum drug sensitive cf resistant strain
   Host: Normal Rbc cf Pnk low Rbc
   Media: Medias Vitamin B_6 sufficient cf deficient media (Vitamin B_6 analogues will be used if media cannot be rendered free of Vitamin B_6)

7. Synchronous cultures (Lambros and Vanderberg, 1979

E. RESULTS

1. We have successfully maintained this FCR_3 strain in continuous culture in both Vitamin B_6 sufficient and Vitamin B_6 'deficient' media for 7 months and 6 months respectively. No significant growth inhibition has been noted in the Vitamin B_6 'deficient' medium.

   Subsequently, we found RPMI 1640 medium to contain riboflavin, a necessary cofactor for pyridoxal phosphate oxidase (which converts pyridoxine to pyridoxal phosphate), and tryptophan. These 2 components will be omitted in future media.

2. We have added Vitamin B_6 analogues to our cultures. Preliminary results show 50% inhibition of growth of parasites at 1mM and 5mM with pyridoxal and pyridoxal phosphate derivatives analogues respectively.

   The effects of these analogues on purine and pyrimidine metabolism, particularly an apparent accentuation of ^3H - hypoxanthine uptakes are still under study.

   The assay for pyridoxine kinase is being set up and screening for low Pnk subjects for in vitro cultures will proceed.

Future Direction of this Study:

This study of Vitamin B_6 on host and parasite growth and nutrition is only getting underway. We would like to examine the relationship between Vitamin B_6 and phospholipid metabolism in addition to our studies on nucleotide metabolism in both host and parasite.

Particularly relevant in malaria research is the need to know more about drug resistant P. falciparum strains. These must have different nucleotide and phospholipid metabolisms.

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F. RELEVANCE TO THE ARMY:

Malaria research is essential for any military activity in tropical areas where malaria is endemic, particularly in areas where drug-resistant strains are present. Increased knowledge of the biochemistry of malarial growth and manipulation of the parasite environment by natural polymorphisms, host immunity or by drug interaction will afford reasonable means to control malaria.

Vitamin B_6 is involved in many aspects of intermediary metabolism, including amino acid and protein metabolism, carbohydrate and fat metabolism. Such a key coenzyme should have a role in host and parasite nutrition.

G. PREPARATIVE WORK

Prior to acquisition of our lab, we spent several months learning lipid techniques in Dr. C. Alving's Lab of Membrane Biochemistry. These methods included thin layer chromatography, phospholipid analyses, TLC quantitation by scanning, liposome preparation. This and other lipid techniques will be used in our future in vitro culture work.

A. TITLE: "Biochemistry of the Host-Parasite Relationship in Malaria: Study of Purine and Pyrimidine Metabolism Utilizing *in vivo* and *in vitro* Models."

B. PRINCIPAL INVESTIGATOR: CPT Kyle Webster, Ph.D.

CO-INVESTIGATORS: LTC Michael J. Haut, M.D.
LTC June M. Whaun, M.D.
Mr. Sylvester Slater, DAC

C. OBJECTIVES: To determine whether (a) the *in vivo* level of purine and pyrimidine nucleotides, nucleosides and nucleobases, or (b) the level of activity of enzymes involved in the synthesis of nucleotides from precursors via salvage and interconversion pathways change in erythrocytes or plasma of rhesus monkeys parasitized with *P. knowlesi* during the course of a synchronous infection. To determine whether such changes are related to the *Plasmodia* parasites intraerythrocytic (IE) growth and differentiation during schizogony.

To determine whether *in vivo* perturbations of parasitized erythrocyte nucleotide metabolism has implications for impaired host defense to infection - specifically immune response (lymphocyte activation) and hematopoiesis.

To determine whether selected anti-malarial agents affect the *in vivo* metabolism of purine and pyrimidine compounds at the level of nucleotide synthesis in infected monkey erythrocytes, and to determine what part this might play in relation to parasite viability and progression through the IE growth cycle.

To determine whether *Plasmodium knowlesi* can be grown synchronously *in vitro* continuous erythrocyte culture thereby allowing systematic study of nucleotide metaolism and potential delineation of alternate metabolic pathways unique to the parasite.

D. MATERIALS AND METHODS: Measurement of levels of purine and pyrimidine metabolites was by high performance liquid chromatography (HPLC). Both Anion-exchange and reverse - phase gradient chromatography were used.

Measurement of the activity of purine and pyrimidine pathway enzymes was done in blood hemolysates using methods described by Henderson (1972).

In vivo studies utilized a chaired monkey model based on the design of Mason (1958).

In vitro continuous erythrocyte culture of malaria parasites was based on the method developed by Trager and Jensen (1976).

E. RESULTS: Changes in the in vivo levels of purine and pyrimidine nucleotides have been described for P. knowlesi infected rhesus monkeys (Webster, 1978 and Webster et al. 1979).

Methods have been refined and developed for HPLC analysis using anion - exchange and reverse phase approaches. Work is currently underway directed at a single-column technology for separation of nucleotides, nucleosides and nucleobases employing a ternary gradient system and stop - scan spectroscopic analysis.

A pilot study employing two rhesus monkeys infected with P. knowlesi provided evidence for changes in the level of plasma nucleosides and nucleobases and for elevation of purine salvage enzymes in infected erythrocytes.

In vitro culture capability has been established by LTC Whaun.

(It is important to note that the principal investigator's military duties required his absence from WRAIR for 8 of the past 12 months).

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Webster, H.K., Haut, M.J., Martin, L.K. and Hildebrandt, P.K. Nucleotide Profiles During in vivo Eucaryote Differentiation: Synchronous Malaria Infection in Rhesus Monkeys. (submitted to Nature).

F. RELEVANCE TO THE ARMY: Malaria is a major disease threat to combat and support personnel deployed in endemic regions of the world. The emergence of resistant strains of Plasmodia dictates the need for improved means of chomoprophylaxis and chomotherapy. Knowledge of the biochemistry of the host-parasite relationship in malaria infection has direct relevances to design of anti-malarial agents and rational treatment of infection based on understanding of the biochemical basis of pathophysiological consequences.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A 6464	79-10-01	DD DR&T(AR)636

3 DATE PREV SUM'RY	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 REGRADING	8a DISP'N INSTR'N	8b SPECIFIC DATA-CONTRACTOR ACCESS	9 LEVEL OF SUM
78-10-01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A WORK UNIT

10 NO /CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	141
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11 TITLE (Precede with Security Classification Code)
(U) Pathogenesisof Renal Disease of Military Importance

12 SCIENTIFIC AND TECHNOLOGICAL AREAS
012900 Physiology 003500 Clinical Medicine 016200 Stress Physiology

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMANCE METHOD
54 09	CONT	DA	C. In-house

17 CONTRACT/GRANT
a. DATES/EFFECTIVE: NA — EXPIRATION:
b. NUMBER:
c. TYPE: — d. AMOUNT:
e. KIND OF AWARD: — f. CUM. AMT.

18 RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	9	638
FISCAL YEAR CURRENT 80	9	509

19. RESPONSIBLE DOD ORGANIZATION
NAME: Walter Reed Army Institute of Research
ADDRESS: Washington, D.C. 20012

RESPONSIBLE INDIVIDUAL
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21 GENERAL USE
Foreign Intelligence not considered

20 PERFORMING ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER
ASSOCIATE INVESTIGATORS
NAME: DUARTE, LTC, MC, C.
NAME: WEISMANN, MAJ, W.
NAME: JOHNSON, LTC, J.P.

22 KEYWORDS (Precede EACH with Security Classification Code)
(U) Renal Failure; (U) Renal Hemodynamics; (U) Heat Stress; (U) Shock; (U) Fluid and Solute Homeostasis; (U) Dialysis; (U) Kidney Function

23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To investigate mechanisms for maintaining fluid, electrolyte and hemodyhamic homeostasis in response to disease, injury and environmental stresses of military significance, such as acute renal failure, shock, infectious disease, heat stress, and gastrointestinal disorders, in order to provide rational basis for prevention and treatment.

24. (U) Clearance methods, dialysis, isotope dilutions, experimental models, in vivo micropuncture, in vitro renal microperfusion, membrane transport, tissue culture, radioimmunoassay, light and electron microscopy, and chromatography.

25. (U) 7810-7909 the pathogenesis of acute renal failure was further investigated in a new model of exertional rehabdomyolysis. The evidence and severity of renal failure were directly correlated with the preexisting fluid intake and volume status of the animals. Production and release of renin, a mediator of tubuloglomerular feedback and purported modulator of acute renal failure, was found to be exquisitely sensitive to hemorrhage. Volume expansion on the other hand decreased renin release rapidly but did not acutely affect renin production suggesting that chronic volume expansion may be necessary to suppress renin production and possibly to prevent acute renal failure. Regulation of water balance requires a finite sensitivity of the kidney to vasopressin, the antidiuretic hormone. Vasopressin effectiveness in the anuran urinary bladder was demonstrated to be dependent upon the redox state of the serosal membrane at the level of the hormone-receptor-adenyl cyclase complex. Alterations in redox state might facilitate or inhibit the vasopressin response and serve as a mechanism for modulating vasopressin responsiveness. A previously unidentified calcium dependent protein-kinase regulatory protein was also isolated from rat kidney and may serve to modulate the cyclic adenosine monophosphate system at a more distal step. Anuran epithelial cells have been successfully grown in tissue culture and their responsiveness to hormonal modulators and diptheria and cholera toxin have been characterized.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 141 Pathogensis of Renal Disease of Military Importance

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Description

Studies are directed at investigations of mechanisms for maintenance of body fluid, electrolyte and hemodynamic homeostasis or their correction in response to disease, injury and environmental stress of military significance such as acute or chronic renal failure, shock, heat stress, physical stress, infectious diseases, drug toxicity and gastrointestinal disorders. The role of adaptive homeostatic mechanisms, including renal and systemic mechansims, whereby body fluid and solute balance are maintained in the face of stress, has been emphasized in order to provide a rational basis for the development of improved methods for prevention and treatment of altered fluid, electrolyte, and hemodynamic states, and acute and chronic renal failure induced by these alterations.

Progress

1. Acute Renal Failure

a. Although the clinical settings predisposing to the development of acute renal failure have been well defined the mechanisms responsible for the induction and maintenance of acute renal failure remain subject to controversy (1,2). In addition where pathogenetic mechanisms have been defined in the experimental animal their relevance to the clinical setting has frequently been questioned because of the dissimilarity of the events leading to the renal injury. The development of acute renal failure in the patient usually follows some overwhelming stress such as hemorrhagic or septic shock, rhabdomyolysis, extensive surgical procedures with attendant anesthetic administration, the administration of nephrotoxic antibiotics and drugs, or ingestion of nephrotoxins etc. Except for the latter situations the experimental models have differed significantly from the clinical setting. Despite this divergence of precipitating factors a number of observations in the experimental model have been made which reveal a certain degree of consistency in mechanisms responsible for the renal injury. Most experimental models reveal increases in renal vascular resistance with decreased renal blood flow early in the course of renal failure, although these changes do not appear necessary for maintenance of the injury once it has occurred.

Similarly alterations of tubular transport characteristics with increased fractional sodium excretion and loss of renal concentrating ability precede the development of histologic alterations. Tubular obstruction from swollen cells and cellular debris is also observed in some models. Later, with alterations in cell membrane characteristics and with cellular death and loss of cellular continuity, there is increased permeability of the tubular wall so that back diffusion of glomerular filtrate may occur with resultant further retention of nitrogenous wastes. Except where injury is excessive and irreparable, healing begins in three to five days with return of renal function to normal (provided death has not occurred in the interim from the ravages of uremia or other complications of the inciting event).

Additional humoral factors have been invoked to account for the decreased renal blood flow and GFR seen early in acute renal failure. Local release of renin at the juxtaglomerular level as a result of increased sodium delivery to the macula densa has been postulated to result in the observed vasoconstriction. (3) Although this may play a role in certain circumstances recent evidence has dissociated the renin lowering effect of various protective manipulations from the protection afforded. (2) Other humoral or renal factors may however still be involved.

In order to attempt to bridge the gap between the divergence of experimental models and the clinical induction of acute renal failure we have attempted to develope several new models of acute renal failure, in addition to completing studies in progress with nephrotoxic models. These have included a) the evaluation of plasmocid, (an 8-aminoguinolone with known muscle toxicity) in the induction of rhabdomyolysis and renal failure; b) the evaluation of endotoxin and hemorrhagic hypotension, separately and together; and c) the evaluation of aminoglycoside nephrotoxicity with chronic gentamicin administration. Results of these studies will be discussed below but only the latter two models have been successful thus far. Both of these however have significant relevance to the clinical setting of acute renal failure.

a. Rhabdomyolysis.

Attempts were made to establish a rhabdomyolysis induced model of acute renal failure. In these studies rats were potassium depleted by a potassium deficient diet for three weeks and then exercised in rotating drums or by swimming in a tank. Potassium deficient rats did not exercise sufficiently to produce rhabdomyolysis. Therefore an attempt was made to superimpose the effects of a muscle toxin on the exercising potassium-deficient model. In these studies following completion of exercise, plasmocid, an 8-aminoguinolone compound was administered. While muscle necrosis of extensive degree was noted in the heart, diaphragm and non-injected skeletal muscle, renal functional impairment was not noted. Attempts at development of this model have been postponed.

b. Hemorrhage and Endotoxin.

Because of the frequent occurence of renal failure following both hemorrhagic hypotension and endotoxemia a study was begun to evaluate their combined effects on the induction of acute renal failure. In these studies rats were maintained on a regular diet up until the time of study with the exception that water intake was restricted for 24 hours prior to study. Rats were then subjected either to hemorrhage (15% of estimated blood volume), administration of E. coli. endotoxin i.v. at LD_{50} doses or a combination of the two. The mortality rate was /5% in those receiving endotoxin, 80% in those that were hemorrhaged and 35% in those which received both experimental procedures. In those animals surviving 24 hours there was no change in urine volume, sodium excretion or osmolality and no change in plasma creatinine in those animals subjected to hemorrhage or endotoxin. In animals subjected to both hemorrhagic hypotension and endotoxin administration urine volume increased and urinary osmolality and sodium excretion decreased; all unrelated to fluid intake. These alterations persisted for 7 days and then returned to normal. Plasma creatinine concentration doubled by 24 hours post hemorrhage and endotoxin administration and was declining by day 3 and had returned to normal by day 7. No histologic study has been performed.

These studies strongly suggest that the combination of endotoxin and hemorrhagis hypotension result in a reversible model of renal failure similar to that which occurs in the clinical setting. Future studies will attempt to define the model more precisely and to characterize those factors which potentiate or protect against development of nephrotoxicity.

2. Hormonal mediators of renal function

a. Renin-Angiotensin System- Previous studies have documented that maneuvers which suppress renin release, such as chronic loading with either sodium or potassium chloride, are capable of preventing the nephrotoxicity associated with heavy metal administration. (4) Increased renin release results in increased Angiotensin II production with consequent vasoconstriction. Local intrarenal formation of Angiotensin II may also mediate glomerular afferent and efferent arteriolar constriction and modulate both renal blood flow and glomerular filtration rate. Knowledge of the factors involved in the modulation of both plasma and renal renin content are therefore important to our understanding of glomerular filtration and acute renal failure. In previous studies we demonstrated that chronic sodium chloride loading suppresses both plasma and juxtaglomerular apparatus (JGA) renin levels and that acute saline loading resulted in a decrease in only plasma renin. (5)

Additional studies were performed in the last year to further evaluate the control of renin production and release. In these studies the effect of acute hemorrhage on renin secretory rate and JGA renin activity was evaluated. Dogs were subjected to controlled hemorrhage of 15 ml/kg body weight and the renin secretory rate and JGA renin activity measured over a 100 minute period. In control animals after 30 minutes renin secretory rate (RSR) was 385 ± 48 angiotensin I units/min;100 min RSR was 354 AIU/min. Corresponding JGA renin activity was 11.5 ± 1.9 and 9.7 ± 1.4 AIU, respectively. Hemorrhage resulted in significant decreases in mean arterial pressure and renal blood flow. Renin secretory rate increased from 379 ± 54 to 2214 AIU and JGA renin was 31.5 ± 5.6 AIU. Hemorrhage produced significant increases in both RSR and JGA renin showing that hemorrhage can acutely raise renin production and that renin secretory rate may be directly mediated by changes in JGA Renin content.

3. Regulation of Sodium and Water Homeostasis

a. Effect of Pentothal Anesthesia on renal excretion of sodium and water.

General anesthesia in both animals and man regularly results in an anti-diuretic response which has been postulated to be secondary to catecholamine induced release of ADH. Recent studies, however, reveal that neither ADH nor catecholamines are released by anesthesia alone and are compatible with early studies which reported a depression of sympathetic activity after induction of anesthesia. (6) Other observations have revealed that less anesthetic agent is required for induction of anesthesia when circulating catecholamine levels are depressed (7).

Recent studies from this laboratory (8) revealed, contrary to the popular belief, that administration of thipental (Pentothal) produced a consistant and significant diuresis and natriuresis. These studies were performed in well trained animals as judged by their calm acceptance of the laboratory and by their persistent bradycardial and sinus arrythmias during the experimental procedure. The latter suggesting that sympathetic activity was depressed.

Studies are currently in progress to define the mechanism of the natriuresis and diuresis in response to thiopental anesthesia. These studies have confirmed our earlier findings and have expanded them as described below.

Only 15 mg/kg of thiopental was required to induce anesthesia in the well trained dog. This is roughly 50% of the usual dose cited in the literature and is consistent with the observations noted above that anesthetic requirements are less when circulating catecholamines are low.

Induction of anesthesia produced no change in glomerular filtration rate of renal blood flow but increased sodium excretion from 127 μ Eq/min

to 297 μ Eq/min (p= < .02) and urine flow from 0.56 to 1.59 ml/min (p= <.001). This natriuresis and diuresis was not altered by prostaglandin synthetase inhibition but was abolished by prior alpha adrenergic blockade with phenoxybenzamine (1 mg/kg). Plasma renin activity was not elevated either before or after anesthesia (0.35 ng AI/ml/hr) these observations suggest that alpha adrenergic receptors may be involved in the diuretic response to thiopental. Circulating levels of epinephrine and nor-epinephrine are currently being obtained before and after anesthesia to evaluate this possibility. Alternatively, the inhibition of the thiopental mediated diuresis by alpha adrenergic blockade may be mediated by local renal sympathetic responses. A comparison of the response with the denervated kidney is planned. Comparative studies with sodium pentobarbital anesthesia (30 mg/kg)did not result in a diuresis or natriuresis suggesting that the response is agent specific.

c. Aminoglycoside induced acute renal failure.

Gentamicin and other aminoglycosides produce renal failure with a frequency of 6-22% in patients to whom they are administered and account for up to 25% of cases of acute renal failure in severely ill patients. We have recently initiated studies in the dog and rat to evaluate, in depth, the mechanism of this nephrotoxicity. Only preliminary results in the rat are available but they indicate a virtual 100% incidence of non-oliguric acute renal failure with chronic gentamicin administration.

b. Cellular Action of Vasopressin

Vasopressin, the mammalian antidiuretic hormone, is intimately involved in the regulation of body water homeostasis. The weight of evidence to date suggests that the primary effect of vasopressin to increase the permeability of the distal nephron to water is mediated by cyclic adenosine monophosphate (cyclic-AMP) acting as a second messenger. The proposed mechanism of action of vasopressin involves attachment of the hormone to its receptor site on the basolateral cell surface with subsequent activation of membrane-bound adenylate cyclase which in turn catalyzes the conversion of adenosine triphosphate (ATP) to 3'-5' adenosine monophosphate (cyclic-AMP). Cyclic AMP subsequently stimulates the activation of a cytosolic protein kinase which in turn causes the phosphorylation of mucosal membrane proteins and intracellular microtubules, release of membrane bound calcium, and increase in permeability of the mucosal membrane to water, sodium and polar non-electrolytes (9).

We have previously demonstrated that the nephrotoxic heavy metal, mecuric chloride, reversibly inhibits the action of vasopressin in the urinary bladder of the toad, Bufo marinus, and prevents the increase in water permeability and sodium transport (10). Similar observations have been recently noted with gentamicin, another nephrotoxin (11). We have proposed that this inhibitory effect would readily explain the rapid

decrease in renal concentrating ability seen prior to evidence of histologic abnormalities in experimental, nephrotoxic acute renal failure. As heavy metals such as mercuric chloride have high affinity for sulfhydryl groups and as sulfhydryl groups have been associated with the vasopressin receptor site (12) it is likely that at least this aspect of mercuric chloride nephrotoxicity is related to its sulfhydryl binding ability.

Also, it has previously been demonstrated, that dithiothreitol, a sulfhydryl stabilizing agent and heavy-metal chelator, can dramatically reduce the incidence and severity of both mercuric chloride and uranyl nitrate nephrotoxicity (13). Dithiothreitol was also previously shown to reverse the inhibitory effects of these agents on membrane sodium transport in the turtle urinary bladder (14) and on vasopressin induced osmotic water flow in the urinary bladder of the toad.

To further investigate the mechanism of action of the reducing agent, dithiothreitol (DTT), on renal membrane function we studied its effects on basal and vasopressin-stimulated osmotic water flow and sodium transport in the urinary bladder of the toad. Serosal DTT had no effect on basal osmotic water flow but produced dose dependant inhibition of the response to vasopressin (10 mU/ml) which was complete at 2×10^{-3} M. This inhibition was partially reversible (34 %) by washing the membranes in DTT-free Ringers or by addition of supra maximal (300 mU/ml) doses of vasopressin (75%). DTT also inhibited the osmotic water response to theophyllin (10 mM) but not to exogenous cyclic AMP (10 mM) suggesting that its site of action was proximal to the generation of cyclic-AMP.

In addition DTT (2.0 mM) caused transitory stimulation followed by depression of sodium transport (as measured by the short circuit technique) and inhibited the short-circuit response to vasopressin, but not to cyclic-AMP, confirming the results of the water flux studies.

These studies with DTT suggested that vasopressin responsiveness might be regulated by the relative redox state of the urinary membrane either by influencing binding of the hormone to its receptor or the translation of the activation signal from receptor to adenylate cyclase. To investigate this possibility we evaluated the ability of several oxidizing agents (dehydroascorbic acid and potassium oxalate) to reverse the inhibitory effects of DTT. Dehydroascorbic acid was investigated more thoroughly. First it was determined that dehydroascorbic acid, at higher concentrations than DTT (20 mM) could reversibly inhibit the hydroosmotic water response to vasopressin and to theophyllin but not to exogenous cyclic AMP, suggesting a site of action near to the sight of action of DTT. These concentrations also produced significant inhibition of sodium transport. Secondly, it was demonstrated that smaller concentrations of dehydroascorbic acid and potassium oxylate (1.0-3.0 mM), which in themselves were non-inhibitory, could reverse the inhibitory effects of DTT on both vasopressin stimulated osmotic water

flow and sodium transport, as well as theophyllin stimulated osmotic water flow. These observations lend strong support to the contention that alterations of redox potential at some site in the membrane receptor-cyclase complex is crucial for stimulation of adenylate cyclase by vasopressin. They further suggest that vasopressin responsiveness in vivo might be modulated by administration of oxidizing and reducing agents.

Further studies are currently in progress to define the mechanism of action of reducing and oxidizing agents on membrane adenylate cyclase. Preliminary results in cultured urinary epithelial cells suggest that these agents alter vasopressin-stimulated but not basal levels of adenylate cyclase which would indicate an effect prior to the enzyme itself, either at the membrane receptor or on a protein regulator of adenylate cyclase.

As glutathione, an agent with similar reducing properties, is produced in similar concentrations in the kidney and as the ascorbic-dehydroascorbic acid system is also present, these or similar compounds might play a comparable role in vivo.

c. Effect of glucocorticoids on renal water handling.

A number of observations have documented impaired water excretion in adrenal insufficiency (15). Although this defect may be partially corrected by mineralocorticoids, an effect thought to be secondary to maintenance of extra cellular fluid volume, considerable evidence suggests that gluco-corticoids are necessary for maximum urinary dilution and normal excretion of free-water load.

To investigate the mechanism of impaired water excretion glucocorticoid deficiency we studied the renal handling of water in eight dogs which had undergone surgical adrenalectomy 4 weeks prior to study. The animals were maintained on a high sodium intake (120 meq/d) in addition to their regular diet and received desoxycorticosterone (DOCA) 0.5 mg per day throughout. In addition the animals received replacement doses of hydrocortisone until 48 hours prior to study. Each animal was studied in the glococorticoid in sufficient and replete states and the studies were separated by at least 2 weeks. (Details of the studies are currently being prepared for publication).

These studies revealed a decreased ability of glcocorticoid insufficient dogs to excrete a water load (5.6 ±0.7 ml/min) when compared to the glucocorticoid replete state (10.6 ±0.7 ml/min) and to maximally dilute the urine (71 ±13 mOsm/l vs 48 ±6 mOsm/l, respectively). These changes in free water clearance were not accompanied by any significant change in either mean arterial blood pressure, renal blood flow, or glomerular filtration rate, suggesting that they were not due to hemodynamic alterations. This was further documented by the finding of supressed plasma renin activity in all animals suggesting that intra-

vascular volume was well maintained.

The possible mechanisms for decreased free-water clearance in glucocorticoid insufficiency included persistence of ADH secretion despite serum hypotonicity and a direct tubular effect of glucocorticoids. Plasma vasopressin levels were obtained in the first six studies and were all suppressed below 0.4 μIU/ml, excluding a persistent ADH effect. To evaluate the potential role of prostaglandins in mediating a tubular effect of glucocorticoids, the animals were also studied after prostaglandin inhibition with meclofenamate in both the glucocorticoid deficient and glucocorticoid replete state. Prostaglandin inhibition had no effect on free water handling in the glucocorticoid replete state but significantly increased free-water clearance and decreased maximum urinary osmolality in glucocorticoid insufficient dogs.

These studies document a glucocorticoid effect on renal water handling which appears to be at the tubular level and which appears to be mediated by prostaglandins.

d. Role of Calcium as an Intracellular Modulator of Hormone Function.

The importance of calcium ion as a mediator of intracellular events is now recognized as a universal phenomenon (16). While a great deal is known regarding the requirements for intracellular calcium (Ca^{++}) in stimulus-excitation and secretion coupling in muscle, exocrine and nervous tissue little is known about the mechanisms which control homeostasis of Ca^{++} (17).

Recent studies using Ca^{++} uptake blockers in acute renal failure models have shown dramatic amelioration of the usual observed renal insufficiency. Whether these effects are due to changes in smooth muscle tone, renin release, prostaglandin production or glomerular permeability are unknown. The current series of studies are designed to look at the mechansims by which Ca^{++} may be regulated in renal cortical (non vascular) tissue with particular reference to known renal hormones (PTH, ADH, PGE_2 renin) which may be active in acute renal failure.

Previous work has demonstrated that cytoplasmic Ca^{++} as maintained at 10^{-6}-10^{-7} M presumably by the active Ca^{++} uptake mechanisms in mitochondria and smooth endoplasmic reticulum. (18) When the Ca^{++} is increased several fold with the ionophore A23187 or cholinergic agents there are profound effects on sodium transport, cAMP, cGMP and ADH sensitivity. (19) (20)

The presence of a Ca^{++} activated ATP-ase in renal cortical microsomes and their ability to accumulate Ca^{++} suggests a possible role for these structures in the control of cytoplasmic Ca^{++} analogous to the sarcoplasmic reticulum of muscle.

The following experiments explore the role of cAMP dependent protein kinase (PK), the known intracellular mediator of cAMP in renal tissue, on the release of Ca^{++} from rat renal cortical microsomes.

(1) Purified cAMP protein kinase has been prepared from cortical homogenates of renal tissue after the removal of subcellular particles and debris by differential ultra-centrifugation. The ammonium sulfate precipitation of the crude kinase preparation has been further purified using DEAE sephadex exchange chromotography which results in a 5-6 fold enhancement of activity. The preparation has been followed with polyacrylamide gel gradient electrophoresis. Currently the kinase preparation contains 8 protein bands. The major band of MW 80-100,000 appears to represent the kinase activity consisting of regulatory and catalytic sub-units. Two distinct protein bands in the 30-40,000 NW range are enhanced with cAMP suggesting these are the dissociated regulatory and catalytic subunits. A cGMP dependent protein kinase has also been identified and appears to be present in quantities equal to the cAMP kinase. The calculated K-max. for cGMP of .51 p moles/m gm protein compares with a K-max of .48 p moles/m gm protein for cAMP. The cGMP kinase elutes with the cAMP kinase fractions on DEAE sephadex. Studies performed by Dr. Chester Amedia in this laboratory utilizing a 5'AMP-sephadex affinity resin and a pH gradient elution have resolved the cAMP and cGMP activity into two distinct peaks. This suggests distinct kinase entities rather than the same kinase which responds to both cAMP and cGMP.

(2) Microsomes essentially free of contaminating plasma membranes and mitochondria were prepared by differential ultracentrifugation. The microsomes were rapidly phosphorylated in the presence of the partially purified rat renal cAMP PK (900 μ gm/ml) reaching a maximum after 5 minutes of 0.5 p moles /mg protein. The phosphorlation was stimulated approximately 4 fold in the presence of .5 μM cAMP. cAMP concentrations above 1.5 μM were inhibitory. Varying (Ca^{++}) from 0 to 100 μM had no effect on the rate or amount of phosphoryation. Microsomes were then incubated in Ringers containing 2.5 mMATP and 100 μ M(Ca^{++}) labeled with ^{45}Ca. The uptake of ^{45}C was followed until a steady state was obtained after approximately 45 minutes. These ^{45}Ca loaded microsomes were then suspended in ^{45}Ca-free Ringers with and without the PK and EGTA 1.0 mM. The release of ^{45}Ca was determined by the amount of ^{45}Ca remaining in aliquots of microsomes washed with 1 mM EGTA on Millipore filters and expressed as a percent control at time 0. Control microsomes lost 14, 22, 37, and 45% of initial Ca^{++} after 1, 3, 5, 10 minutes. PK treatment of the microsomes in the presence of cAMP markedly enhanced the release of bound ^{45}Ca so that 28, 65, 72, and 89% of initial Ca was lost in the same time course. These differences were statistically different after 1 min ($p < .05$) and were most dramatic over the first 5 minutes of exposure to the PK. The action of PK was unaffected by the presence of EGTA 1 mM or EGTA buffered (Ca^{++}) up to 100 μM. Likewise the effect of PK was dependent on cAMP at a concentration of 5 μM. cAMP concentrations above 25 μ M diminished the effect of PK on ^{45}Ca release. Micro-

somes suspended in Ringers containing Aequorin, a Ca^{++} sensitive ('μ Molar Ca^{++}) luminescent protein, were observed in a photomultiplier apparatus after the addition of PK ± cAMP. The presence of PK and cAMP induced a rapid release of nascent Ca^{++} similar to the ^{45}Ca studies confirming an effect on total non radioactive Ca^{++} release.

(3) Assuming a relationship between the Ca^{++} stimulated ATP-ase of renal microsomes and Ca^{++} uptake (on the basis of an absolute ATP requirement for Ca^{++} uptake) the following experiments were performed to determine if phosphorylation of microsomal membranes affected the activity of this enyzme. The rationale being that if this enyzme were inhibited uptake of Ca^{++} would cease and Ca^{++} would leak out of the microsomes along a concentration gradient.

The ATP-ase was measured in the absence of Na^{+} and K^{+} and in the presence of ouabain 10^{-3}M and NaAzide 10^{-4}M to inhibit any contaminating Na^{+}-K^{+} ATPase or mitochondial ATPases that might be present. Baseline Ca^{++} stimulated ATPase was completely inhibited in the presence of cAMP and PK. PK alone inhibited from 30-50% of control activity while cAMP alone had no effect on baseline activity. Current studies into the kinetics of this inhibition are being performed to further define the nature of this interaction.

The present studies demonstrate that renal cortical microsomes can be phosphorylated by a cAMP dependent PK and that this phosphorylation appears to enhance the release of $^{45}Ca^{++}$ from sites insensitive to EGTA. These data suggest that the activation of PK by cAMP may increase cytoplasmic (Ca^{++}) by releasing stored (Ca^{++}) from smooth endoplasmic reticulum and thereby modulating various enzymatic and transport processes. The data further suggests that the enhanced Ca^{++} release is a result of inhibition of the Ca^{++} - ATPase.

Further studies are in progress designed to explore the reversibility of this process, the effects of released Ca^{++} on prostaglandin synthesis and the role of the cGMP stimulated kinase.

4. Renal effects of Potassium Depletion

a. Effect of potassium depletion on magnesium balance.

Disorders of potassium balance have profound effects on body homeostasis and renal function. Potassium depletion has been noted to result in nephrogenic diabetes insipidus, cardiac arrythmias, rhabdomyolysis with acute renal failure. Potassium depletion in basic trainees has been incriminated in the etiology of exertional rhabdomyolysis and renal failure. To evaluate the systemic effects of potassium depletion on body mineral homeostasis we studied the effects of potassium depletion with and without desoxycorticosterone acetate (DOCA) administration on magnesium balance and plasma magnesium. Rats were fed either a control diet, a potassium deficient diet or a potassium deficient

supplemented with DOCA, 0.5 mg/100 g BW BID x 8-12 d. Plasma potassium decreased from 3.9 ± 0.09 meq/L in controls to 2.2 ± 0.8 in the DOCA rats and 2.2 ± 0.1 meq/L in the low K rats. Fractional excretion of potassium decreased from 24 ± 3% in the controls to 3 ± 0.6% in the low potassium rats and remained inappropriately high (28 ± 3%) in the DOCA rats indicating persistent potassium wasting. Administration of DOCA to potassium depleted rats further reduced plasma potassium to 1.4 meq/L but did not increase fractional potassium excretion further.

Accompanying these changes in potassium homeostasis plasma Mg^{++} increased significantly in low potassium rats but not in DOCA rats (Control= 1.6 ± 0.06 meq/L; DOCA = 1.7 ± 0.1 meq/L; low potassium = 2.2 ±0.1 meq/L). DOCA administered to potassium depleted rats resulted in a partial reversal of hypermagnesemia to 1.9 ± 0.1 meq/L. This hypermagnesemia reduction of plasma magnesium seen in potassium depleted rats given DOCA was accompanied by significant reductions in hematocrit and increases in body weight and muscle water content suggesting that hemodilution may have contributed to the decrease in magnesium levels.

These observations, as well as parallel studies in progress with other major cations, suggest that potassium depletion may significantly alter the metabolism of other cations important in body homeoastasis. Further studies are needed to determine if potassium depletion per se or the accompanying disturbances in cationic metabolism are responsible for the various systemic abnormalities associated with potassium depletion.

5. Evaluation of the Culture of Urinary Epithelial Cell as a model of Tubular Function.

In order to support and extend observations which have been made with whole animals and intact organ systems this laboratory has developed a cell culture system derived from urinary epithelia which allows more detailed analysis of physiologic and pathophysiologic mechanisms at a cellular level. Efforts during the last year have been threefold. (1) To characterize the model system with respect to it's basic transport physiology. (2) To study the effect of a variety of hormones and toxins on cells in culture and (3) to develop new continuous cell lines with different properties for study.

a. Transport Physiology

Two continuous cell lines derived from toad urinary bladder epithelial cells have been studied in detail. This tissue has the same developmental orgin and functional characteristics as the mammalian collecting duct. Both cell lines form epithelia in culture characterized by high electrical resistance. Typical orientation and polarization of the cells has been documented morphologically by electron

microscopic analysis and physiologically by the observation that amiloride inhibits transport only when added to mucosal side and that oubain inhibits transport only when added to serosal aspect of cells in culture. The cells can be grown on semipermeable supports and studied by standard electrophysiologic techniques in modified Ussing chambers. In both cell lines a short circuit current of 2-20 $\mu AMP/cm^2$ develops depending on experimental conditions, and bidirectional isotopic sodium fluxes have established that this short circuit current is the equivalent of net Na^+ transport. Basal urea permeability in both cell lines is 10^{-6} cm/sec and basal water flow is less than 1 $\mu L/hr/cm^2$.

b. Effect of hormones and toxins on cells in culture

Toad urinary bladder has been used extensively to study the effects of hormones and transport inhibitors as a model of the distal portion of the mammalian kidney. We have been able to demonstrate that these cells grown in continuous culture retain many characterisitcs found useful in modulating mammalian kidney responses and develop interesting new properties as well. Toad urinary bladder epithelial cells in culture do not maintain their response to vasopressin, the mammalian antidiuretic hormone. Studies with ^3H-lysine vasopressin demonstrate that this is due to loss of specific receptors. Efforts are now underway (vide infra) to develop cell lines which retain this characteristic in culture. Cyclic AMP induces sharp increases in urea permeability and net sodium transport but has no effect on net water flow. This striking separation of transport properties is associated with the inability to demonstrate cyclic AMP stimulated granule aggregation in freeze fracture etchings of the mucosal membranes of the cells. Furthermore, cyclic AMP induced increments in sodium transport are insensitive to Amiloride, in contrast to aldosterone induced increments in sodium transport which are sensitive to amiloride. This suggests that these agents may stimulate sodium transport through two fundamentally different sites. Aldosterone stimulates sodium transport which is amiloride sensitive and the time course, dose response curve and steroid analog affinity have been characterized in both cell lines.

Several other agents affect Na^+ transport in these cells and their varying sensitivity to amiloride suggests an interesting hypothesis. Cholera toxin and PGE_1 which stimulate cyclic AMP production cause amiloride insensitive transport. MK-196 a steroid analogue and diptheria toxin, which do not stimulate cyclic AMP, cause amiloride sensitive increments in sodium transport. Further toxins which affect transport will be studied in collaboration with Dr. J. Sadoff of the Dept. of Infectious Diseases.

c. Development of new cell lines

Attempts to develop cell lines with mutant transport responses are underway. Cell populations are enriched by separating cell types before culture with isopycnic centrifugation on continuous and discontinuous gradients. One cell line which appears to retain vasopressin responsiveness has already been developed. New conditions for primary culture are being investigated including the growth of cells on semipermeable translucent supports which will enable cells to be bathed by media on both sides.

Several primary cultures of turtle urinary bladder epithelial cells -used as models of acidification by distal mammalian nephrons - have demonstrated promising growth but as yet have not developed into continuous well characterized cell lines.

The above effects have clearly demonstrated that epithelial cells in culture may prove a useful and easily manipulated model for the effects of hormones and toxins on urinary epithelial.

The culmination of these studies should allow for isolation and characterization of the function and toxic susceptibility of the several renal epithelial cell types, and should allow for assessment of factors required for optimum regeneration following cellular injury.

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Work Unit 141 Pathogensis of Renal Disease of Military Importance

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB 7532	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	142
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Pathophysiology of Systemic Responses to Shock and Trauma

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

012600 Stress Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE — EXPIRATION

b. NUMBER

c. TYPE — d. AMOUNT

e. KIND OF AWARD — f. CUM AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	2	113
FISCAL YEAR CURRENT 80	2	150

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Graeber, MAJ, Geoffrey

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) .45 caliber pistol; (U) Model development; (U) Surgical management; (U) Penetrating renal trauma

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) The most common war-related injuries of the urinary tract are pentrating missile wounds of the kidney. More than half of these renal injuries required nephrectomy. In view of the high risk of acute renal failure following major abdominal trauma, it is imperative in precisely these cases than renal tissue be maximally conserved. Determination of optimal surgical management requires an animal model with a contralateral nephrectomy. A randomized block design was employed to determine the best surgical management of battle casualties.

24 (U) Since an adequate model to deliver a reproducible injury with significant kinetic energy had not been described, we modified an existing captive bolt weapon. This Supercash Mark 2 is lightweight (1.3 kg), easily disassembled for sterilization, and powered by a 2 grain .22 caliber charge. The .45 caliber captive bolt projectile is propelled at a velocity of 170 fps and retracts by its buffers after traversing 7.5 cm. The ballistics of this injury compares favorably with current law enforcement sidearms. Three canine treatment groups are being evaluated: standard in situ repair, ex vivo repair and autotransplantation and a control group.

25 (U) 78 10 - 79 09 At this writing, the results are preliminary (21 animals), but striking. Though no gross differences in morbidity or renal function is apparent, the mortality rates merit mention. Despite a very significant lower pole renal injury, and no attempt to provide hemostasis, all animals in the control group have thus far survived (7/7). The only treatment these animals received was local hypothermia during randomization (<10 min) and closure of the perirenal fascia. The mortality rate from standard therapy was 14% (1/7) and in sharp contrast to the extracorporeal surgery - transplant group which exceeds 85% (6/7). For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78 - 30 Sept 79.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 142 Pathophysiology of Systemic Responses to Shock and Trauma

Investigator
Principal: LTC William D. Belville, MC
Co: MAJ Geoffrey M. Graeber, MC

I. Model Development for Penetrating Renal Injury

A. Background and Statement of the Problem. The most common war-related injuries of the urinary tract are penetrating missle wounds of the kidney. Salvatierra and associates,[1] in a review of 252 urological injuries, noted that thirty-one percent involved the kidney. More than half of these renal injuries required nephrectomy. In most cases, nephrectomy was necessary to control renal hemorrhage associated with extensive parenchymal damage. Nephrectomy is also frequently required when penetrating injuries to the kidney are associated with trauma to the gastrointestinal tract. In view of the high risk of acute renal failure following major abdominal trauma, it is imperative in precisely these cases that renal tissue be maximally conserved.[2] Despite this need for renal parenchymal conservation, the use of extracorporeal surgery and autotransplantation after renal trauma have been reported infrequently. Experimental work in this area has been limited,[3] as measurement of function in the repaired kidney is difficult in the presence of a normal contralateral kidney because of renal counter-balance.[4] This phenomenon is seen frequently in the clinical setting and has been well shown experimentally to suppress the reparative process.[5] Determination of optimal surgical management, therefore, requires an animal model with a contralateral nephrectomy. No prior report has described an adequate model which considers the ballistics of a penetrating injury.

Since an adequate model to deliver a reproducible injury with significant kinetic energy had not been described, our initial approach was to design a weapon powered by carbon dioxide (CO_2). Research in this area, however, led to a general agreement that the velocity of such a system would be inadequate and complete reproducibility of these injuries would be questionable. It was decided that a powder charge was required to fulfill both of these needs. However, most weapons utilizing such a system employ a projectile which would be hazardous to personnel and would disallow adequate sterilization. It then became apparent that a captive bolt system was required. Such a system was available from Koch Supplies Inc., Kansas City, Missouri as the "Supercash Mark 2." This captive bolt stunner was developed for the humane stunning of large animals during meat processing.

B. Experimental Approach. This Supercash Mark 2 is lightweight (1.3 kg), easily disassembled for sterilization, and powered by a 2 grain .22 caliber charge. The .45 caliber captive bolt projectile is propelled at a velocity of 170 fps and retracts by its buffers after traversing 7.5 cm. The relatively large mass of the projectile allows 211 Joules of energey to be transmitted to the kidney and renal vessels. This compares favorably to current law enforcement sidearms (.38 Smith and Wesson 176 Joules at 50 yards). A stainless steel device was designed that bolted to the operating table and allowed fixation of both the kidney and weapon in a reproducible fashion.

C. Results and Discussion. Fifteen (L) canine kidneys were evaluated in this device and the injury was 100% reproducible. The weapon system's positive safety and silencing mechanism made it's use in the operating theater comfortable. The consistency of the injuries allows a meaningful comparison of various treatment modalities.

D. Conclusion and Recommendations. With minor modification, an existing readily available captive bolt projectile weapon has allowed development of a safe producible model for studying penetrating tissue injury in a sterile environment. The ballistics of the injury is analogous to that created by a projectile fired from many law enforcement sidearms. The system can be modified to duplicate most any low velocity penetrating injury. By changing the leading surface of the projectile the application of this producible system to blunt trauma seems tenable. The system is being considered for patent and an abstract has been submitted for presentation to the Annual Meeting of the Association of Academic Surgery (November 1979).

II. Management of Penetrating Renal Trauma

A. Background and Statement of the Problem. Application of the aforementioned model lends itself to the study of penetrating renal injury. In a sterile environment, the feasibility of extracorporeal renal surgery with autotransplantation could be studied. Extracorporeal renal surgery with hypothermia facilitates meticulous vascular and parenchymal repair. The procedure provides a totally bloodless field, increases the allowable ischemia time, allows easy application of the operating microscope, and permits accurate angiographic studies prior to replantation.[6] In renovascular injury, particularly renal artery branch lesions, extracorporeal repair with autotransplantation may be the only method for kidney salvage.[7] In addition, autotransplantation may protect the kidney from contamination from the missile tract or chemical injury associated with adjacent gastrointestinal trauma. Lastly, this method can save the otherwise lost kidneys secondary to subtotal ureteral compromise.[8]

B. Experimental Approach. Seven to ten days following a contralateral right nephrectomy and after baseline renal function had been measured, the canines were ready for the experimental surgery. Each dog was anesthetized with sodium thiopental, intubated and maintained with halothane. Through a transperitoneal approach the (L) kidney was completely mobilized, placed without tension in the captive bolt device, and the injury inflicted. A randomized block design subsequently placed each animal into one of three treatment categories. One third were managed conventionally by in situ repair, one third by ex vivo repair and autotransplantation and no treatment (control) in one third. Surgical technique was standardized and identical supportive post operative therapy employed.

C. Results and Discussion. At this writing, the results are preliminary (21 animals), but striking. Though no gross differences in morbidity or renal function is apparent, the mortality rates merit mention. Despite a very significant lower pole renal injury, and no attempt to provide hemostasis, all animals in the control group have thus far survived with the mortality rate being 0/7. The only "treatment" these animals received was local hypothermia during randomization (<10 min) and closure of the perirenal fascia. It is known that this facial envelope in the human can provide a significant tamponade effect, however, the diminutive and incomplete development of this structure in the canine was not expected to play such a significant role. The mortality rate from standard therapy is 14% (1/7) and in sharp contrast to the extracorporeal surgery - transplant group which exceeds 85% (6/7).

D. Conclusions and Recommendations. It appears currently that there is little place for renal autotransplant in the management of such experimental renal trauma. The tremendous homeostatic-reparative capability of the canine in this setting lends experimental evidence for the conservation approach in penetrating renal injury. The current trend in the management of blunt renal trauma is non-operative, and this impressive and unexpected preliminary experimental evidence is being prepared for publication.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 142 Pathophysiology of Systemic Responses to Shock and Trauma

Literature Cited.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1 AGENCY ACCESSION* DA 0A 6467	2 DATE OF SUMMARY* 79 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3 DATE PREV SUM'RY 78 10 01	4 KIND OF SUMMARY D. Change	5 SUMMARY SCTY* U	6 WORK SECURITY* U	7 RE-GRADING* NA	8a DISP'N INSTR'N NL	8b SPECIFIC DATA-CONTRACTOR ACCESS ☒ YES ☐ NO	9 LEVEL OF SUM A. WORK UNIT

10 NO./CODES*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	143
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)*

(U) Gastrointestinal Responses to Shock and Trauma

12 SCIENTIFIC AND TECHNOLOGICAL AREAS*

008800 Life Support 016200 Stress Physiology

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY			16 PERFORMANCE METHOD
63 09	CONT	DA			C. In-House

17 CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION

b. NUMBER*

c. TYPE d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18 RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 79	2	137
	CURRENT 80	2	150

19 RESPONSIBLE DOD ORGANIZATION

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NAME: Graeber, MAJ, Geoffrey

21 GENERAL USE

Foreign intelligence not considered

22 KEYWORDS (Precede EACH with Security Classification Code) (U) Gastric mucosal barrier; (U) Abdominal visceral Blood Flow; (U) Septic shock; (U) Portacaval shunt; (U) Stress ulcer; (U) Shock

23 TECHNICAL OBJECTIVE,* 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) The technical objective was to study surgical gastrointestinal problems associated with combat casualties. Specific objectives were to study the pathophysiology of stress ulcers, to study the ability of Dacron patches to close bowel wounds, to identify enzyme markers of abdominal visceral ischemia, to develop a model of septic shock in monkeys; and to study the effect of portacaval shunts on splanchnic blood flow.

24 (U) The methods used include canine Heidenhain pouch studies of the gastric mucosa; a rabbit small bowel model for Dacron patches; dog, rabbit, and human serum LDH and CPK isoenzyme studies for visceral ischemia; and a septic gall bladder monkey model for septic shock; radioactively tagged microspheres were used to study splanchnic blood flow.

25 (U) 78 10 - 79 09 Dacron patch grafting of the rabbit ileum proved to be a satisfactory way to close large defects of the bowel without stenosis in 50 rabbits studied to date. This method worked consistently. It has not been used previously to our knowledge. The electrophysiologic, light, and electron microscopic results show that the bowel mucosa which grows over the Dacron patch has characteristics of normal mucosal Studies of histamine Type I and II blockers, as well as of 16,16 dimethyl prostaglandin E2 failed to document a cytoprotective effect beyond the well known ability of these agents to diminish gastric acid secretion. A model for canine visceral release of LDH and CPK enzymes by ischemia was established. The similarity of the human and canine distribution of these enzymes in the bowel was proven. Initial efforts to establish a septic shock model in monkeys gave very inconsistent results. In our protacaval shunt studies we documented various compensatory changes in blood flow following shunting. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sept 79.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 143 Gastrointestinal Responses to Shock & Trauma

Investigator
Principal: MAJ John W. Harmon, MC
Co: MAJ Geoffrey M. Graeber, MC
MAJ Clayton H. Shatney, MC

I. Pathophysiology of Gastric Stress Ulcers

A. Background and Statement of the Problem. Bile, with ischemia and acid, have been implicated in the etiology of gastric stress ulcers. Gastric stress ulcer bleeding is a major cause of morbidity and mortality among combat casualties. Our laboratory has studied the effects of various bile acids on the gastric mucosa of dogs to more precisely understand their injurious effects. A paper from our laboratory entitled "Gastric Stress Ulceration: Current Concepts of Pathophysiology and Therapy" which appeared in Military Medicine, 144:291-296, 1979, provides extensive background on this subject.

B. Experimental Approach. Dogs were prepared with chronic Heidenhain pouches with 2 cannulas each for through and through perfusion. Acid loss was measured with a pH stat and transmucosal electrical potential difference was measured with a voltmeter, KCL agar bridges, and Calomel electrodes.

Reduction in potential difference and increased permeability to hydrogen ion were the indices of injury measured. During the time period of this report two studies were brought to completion utilizing this model and a third project studying the effects of antrectomy on the dogs release of a new candidate hormone, pancreatic polypeptide was also completed.

C. Results and Discussion. 1) Investigation of cytoprotective effect of 16,16 dimethyl prostaglandin E_2: To better understand the cytoprotective properties of PGE2 we studied the effects of this agent on ion fluxes before, during, and after, exposure to taurodeoxycholic acid (TDC). Heidenhain pouches in 5 awake dogs were studied. Net flux of hydrogen ion (JH+) was measured with a pH stat. Transmucosal electrical potential difference (PD) was measured with a voltmeter. Net fluxes of volume (H_2O) were determined by direct measurement. Net flux of Na was determined by flame photometer and calculation. PGE2 reduced histamine stimulated acid secretion 56% at 0.6 µg/kg-hr and 99% at 2.4 µg/kg-hr. The effects of saline, PGE2 0.6 µg/kg-hr, and PGE2 2.4 µg/kg-hr on bile (5mM TDC) induced gastric injury at pH 2 was assessed. Exposure of the gastric mucosa to TDC without PGE2 infusion resulted in luminal disappearance

of hydrogen ion, reduction in PD, and entry into the lumen of Na and H_2O. During 2 hours of recovery without bile these parameters returned to normal. Infusion of PGE2 had no consistent effect on JH+. PD was diminished in all periods of study by PGE2 averaging 88% of control with 0.6 µg/kg-hr and 73% of control with 2.4 µg/kg-hr. With PGE2 Na flux continued into the lumen in the second hour of recovery while it had returned to normal in the control animals. Similarly movement of H_2O into the lumen continued in the PGE2 experiments while H_2O absorption returned to exceed control levels in the control dogs. In summary, these findings demonstrate that PGE2 did not significantly prevent bile acid induced hydrogen ion back diffusion. PGE2 reduced PD and caused secretion of Na and H_2O. These changes could be related to its cytoprotective effect.

2) Investigation of the Effect of Blockade of Types I and II Histamine Receptors on Bile Salt Injury of the Stomach: We tested the hypothesis that histamine mediates the changes in cation permeability and potential difference (PD in mV) characteristic of gastric mucosal barrier disruption. Canine Heidenhain pouches were exposed to 100 mM HCl + 20 mM Na taurocholate (TC) both with and without combined histamine H_1 and H_2 receptor blockade with diphenhydramine 2 mg/kg and metiamide 10 µmoles/kg-hr. Histamine blockade failed to attenuate the decrease in PD and increase in net H^+ flux (ΔH^+ in mmole/30 min) due to bile salt:

Group	Hour	H^+	PD
Control	1	-1.13±.12	-13.4±3.2
H_1 + H_2 block		-1.14±0.24	-19.6±4.8
Control	2	-0.68±0.11	-17.6±1.5
H_1 + H_2 block		-0.65±0.09	-19.2±3.5
Control	3	-0.44±0.05	-15.5±1.7
H_1 + H_2 block		-0.48±0.07	-16.8±3.5

Histamine receptor blockade also failed to attenuate the changes in net ion fluxes of Na^+, K^+, Cl^-, and volume. When luminal pH was kept at 2.0 with pH-stat and TC was varied from 0 to 20 mM, combined histamine blockade also failed to attenuate the changes due to bile salt including mucosal H^+ permeability. Failure to block the changes characteristic of gastric mucosal barrier disruption with the use of histamine H_1 and H_2 receptor blockade is a strong argument against histamine being the mediator of these changes.

3) Investigation of the Effect of Antrectomy on the Release of Pancreatic Polypeptide: The PP response to a standard meal was measured in 5 dogs immediately before and then 1-2 months (early) and 4-6 months (late) after antrectomy without vagotomy. Three

other dogs had the same studies performed before and after a sham operation in which the dogs were anesthetized, a laparotomy performed, the bowel handled but no resection performed. The results given as mean ± SE are summarized in the table. The mean basal serum PP concentration and mean postprandial response (pM) are given for each study.

	ANTRECTOMY (n = 5)	
	BASAL	POSTPRANDIAL
Pre-op	36.7±2.6	89.8±18.6
Early Post-op	32.1±4.0	43.8±10.2*
Late Post-op	17.6±1.4*	26.9±1.7*

*$p<0.01$ significantly different from pre-op

	SHAM-OP (n = 3)	
	BASAL	POSTPRANDIAL
Pre-op	35.5±1.7	83.8±16.7
Early Post-op	41.9±7.1	86.7±17.9
Late Post-op	33.6±8.1	61.2±18.3

Antrectomy significantly reduced the PP response to food in both the early and late postoperative periods, but significantly reduced basal serum PP concentration only in the late postoperative study. Sham-operation did not significantly affect basal or postprandial PP concentrations. We have previously shown that physiologic doses of gastrin do not release PP suggesting that antrectomy impairs the PP response to food by a mechanism other than through diminished gastrin release. Other explanations include: 1) removal of an antral hormone other than gastrin, 2) interruption of the vagal innervation of the pancreas. In summary, these studies show that antrectomy, without a truncal vagotomy, inhibits the PP response to food.

II. Pathophysiology of Reflux Esophagitis

A. Background and Statement of the Problem. The human esophagus may be exposed to acid and/or alkaline gastric juice during periods of gastroesophageal reflux. The acid pH of refluxed gastric juice results from gastric secretion of HCl, while the alkaline pH persumably results from the pyloric reflux of alkaline duodenal contents into the stomach. This exposure of the esophageal mucosa to gastric contents is thought to result in esophagitis. One

argument in support of this contention is the observation that reducing gastroesophageal reflux, by surgically creating a competent valve at the gastroesophageal junction, leads to disappearance of esophagitis. This incriminates the refluxed gastric contents as the cause of the esophagitis. Experimentally, it has been shown that esophagitis can be produced in dog, monkey, and rabbit esophagus by exposing the esophageal mucosa to various components of gastric juice. It has also been shown in man, dog, and rabbit that increased hydrogen permeability of the esophageal mucosa is seen in the early phases of esophageal injury.

B. Experimental Approach. In the present experiments we measured hydrogen ion loss from the rabbit esophagus with a pH stat autoburette system. We used increased hydrogen permeability as an index of esophageal mucosal injury. The rabbit esophagus normally neither absorbs nor secretes acid. When the mucosa is injured a flux of hydrogen ion down the concentration gradient from the lumen to the blood is seen, with disappearance of hydrogen ion from the esophageal lumen. We performed most of our experiments at pH 2. At pH 2 minimal loss of hydrogen ion from the lumen normally occurs for up to four hours of observation.

C. Results and Discussion. 1) The Effect of HCL and Bile Acids on the Hydrogen Ion Permeability of the Rabbit Esophagus: A 6 cm length of esophagus was perfused with isotonic acid test solutions (ATS). In 28 experiments net acid flux (NAF) of hydrogen ion leaving the esophageal lumen was less than 10 μEq/10 min at pH's 7, 5 and 2. At pH 1 NAF equalled 70±11 μEq/10 min. In these experiments, a threshold for the back diffusion of hydrogen ion existed between pH 1 and 2. Furthermore, at pH 2 back diffusion of hydrogen ion remained consistently low over one hour in 6 animals with NAF equal to 1±1 μEq in the first 30 minutes and 2±1 μEq/10 min in the second 30 minutes. At pH 1 however, in 5 animals, NAF increased from 35±14 μEq/10 min in the first 30 minutes to 105±15 μEq/10 min in the second 30 minutes. These data suggest that exposure to pH 1 HCL caused progressive increases in the hydrogen ion permeability of the esophageal mucosa while exposure to pH 2 HCL did not. The effect of ATS containing 5 mM taurodeoxycholic acid at pH 2 was evaluated for period of exposure from 1 to 60 minutes in 5 animals. NAF increased with the time (t-min) of exposure such that $NAF = 1.25t + 10.8$ ($r = .93$, $p<.05$). The effect of varying bile acid concentration [B] from 1 to 5 mM was tested for 60 minutes of exposure at pH 2. $NAF = 12.7\ [B]-0.8$ ($r = .96$, $p<.05$). These experiments show that the hydrogen ion permeability of the rabbit esophagus can be increased by exposure to HCL at pH 1 or to bile at pH 2. The increases in NAF caused by bile acid correlated directly and significantly with the time of exposure to bile and with the concentration of the bile.

2) The Effect of 16,16 Dimethyl PGE2 on Bile Acid Induced Injury of the Rabbit Esophagus: Exposure of the rabbit esophagus to bile acid results in increased rates of hydrogen ion disappearance from the esophageal lumen. We investigated the possibility that PGE2 could increase the resistance of the esophageal mucosal barrier to hydrogen ion back diffusion. The PGE2 we used reduced histamine stimulated gastric acid secretion by 99% at 2.4 µg/kg-hr intravenously in our laboratory. Fifteen rabbits were anesthetized with Innovar and tested in vivo with the esophagus cannulated proximally and distally. A 6 cm length of esophagus could thereby be studied. Acid test solutions (ATS) containing HCL at pH 2 and made isotonic with NaCl were perfused through the esophagus. Net acid flux (NAF) was measured with an in-line pH stat autoburette. The amount of acid added to keep pH constant equalled the amount of acid lost. The system was calibrated daily and its coefficient of variation titrating 48 µEq of NaOH at pH 2 was ± 2 µEq. The experimental format consisted of 4 experimental hours as follows: 1) ATS without bile, 2) ATS with 5 mM tauro-deoxycholic acid, 3) ATS without bile, 4) ATS without bile. In this way bile injury could be observed, followed by perfusion without bile when recovery could occur. Three series of experiments, each with 5 rabbits, were performed: 1) perfusion of the esophagus with ATS without bile to determine the effect of ATS itself, 2) perfusion of the esophagus with bile, with saline intravenous infusion, 3) perfusion of the esophagus with bile, with PGE2 µg/kg-hr intravenous infusion. The table below shows our experimental results. NAF is in µEq/10 min ± SEM.

	ATS	ATS	ATS	ATS
No bile injury	2±1	4±2	8±3	9±3
	ATS	ATS + BILE	ATS	ATS
Saline control	5±2	55±11	57±8	31±5
PGE2 2.4 µg/kg-hr	2±1	45±3	39±1	44±8

We conclude from these experiments that PGE2 does not significantly reduce the ability of bile to increase the hydrogen ion permeability of the rabbit esophagus.

III. The Use of Prosthetic Material to Repair Small Bowel Defects

A. Background and Statement of the Problem. Injury to the intestine occurs frequently in combat injury. Massive loss of small intestine is a debilitating problem. We have been investigating strategies for expanding the absorptive area of the small bowel. In the present series of experiments we studied the ability of the small bowel mucosa to regenerate over a patch of woven Dacron prosthetic material. Further we studied the electrophysiological

properties of the neogut which grew over the Dacron patch.

B. Experimental Approach. New Zealand white rabbits weighing 4 to 7 pounds were prepared surgically. The anti-mesenteric surface of the distal ileum was opened for a length of 7 cm. A patch of woven Dacron vascular prosthetic material 7 cm x 1.5 cm was then sewn in place in the defect with running 4-0 Tevdek suture. As the diameter of the rabbit ileum is about 1.5 cm this represented a 1/3 enlargement of the bowel circumference. Rabbits returned to regular diet after 5 days of fasting. The following observations were made: 1) Mortality, 2) gross examination of the patched ileum at 3 weeks (1 rabbit), 2 months (1 rabbit), or 3 months (5 rabbits), 3) light microscopic evaluation of the neogut mucosa with hemotoxylin and eosin staining in 3 rabbits, 4) evaluation of the electrophysiologic properties of the neogut which grew over the Dacron patches in comparison to normal ileum. Two to three pieces of the neogut from each of 4 rabbits were studied. Mucosa in Ussing chambers separates two small reservoirs containing test solutions and agar bridges. Using electrodes and a voltmeter the potential difference and short circuit current were measured and the conductance was calculated.

C. Results and Discussion. Mortality: Seven of eight rabbits with patch grafts survived and the one death was not related to the patch graft. Attempts to use longer and wider grafts resulted in death from leakage in 3 rabbits.

Gross examination: At 3 weeks the serosal surface of the graft was covered with newly grown bowel. The Dacron patch was entirely exposed on the mucosal surface. At 2 months the Dacron graft was attached at one end but hanging free in the bowel lumen at the other end. A full thickness of bowel wall had grown behind the Dacron, without incorporating the Dacron. At 3 months, 1 rabbit still had the Dacron patch partially attached, but 4 others had extruded the patch leaving the bulbous enlargement in the bowel with no Dacron present.

Light microscopy: The neogut had mucosal, muscular, and serosal layers in normal relationship. Compared with adjacent bowel, the villi were shorter, but the cellular composition of the mucosa was normal. Otherwise, the neogut was similar to adjacent bowel.

Electrophysiology: The results below demonstrate that the neogut mucosa's physical properties were very similar to normal mucosa because the conductances (G) were virtually identical. Active transport mechanisms in the neogut produced lumen negative trans-mucosal electrical potential difference (PD) values of smaller magnitude than in normal gut. This with the short circuit current

(ISC) results suggest that active transport mechanisms in the neogut were similar but less active than in normal gut.

	Isc (μEq/hr-cm^2)	PD (millivolts)	G (milli-mho/cm^2)
Control	2.2±.5	2.6±.5	22±1
Neogut	1.4±.5	1.5±.5	21±4

Isc = short circuit current, PD = transmucosal electrical potential difference, G = conductance

Discussion: These experiments demonstrate that the rabbit ileum has the capacity to grow over a Dacron patch graft. The neogut that results, judged by microscopic and electrophysiologic evaluation, appears to be functional.

IV. Comparison of Total Hepatic Arterial Blood Flow Using Gamma-Labelled Microspheres Following Porta-Systemic Shunting

A. Background and Statement of the Problem. Portacaval shunts have been extensively utilized in the treatment of bleeding esophageal varices secondary to hepatic cirrhosis. This procedure also has a potential role in the treatment of intestinal schistosomoiasis and in the reconstruction of the traumatized portal system.[1] Although portacaval shunts are effective in controlling variceal hemorrhage, hepatic failure following shunting can occur acutely or chronically and appears in a significant percentage of shunted patients.[2] Hepatic decompensation has been attributed to the loss of portal inflow to the liver by the shunt. Because of this possibility the interposition (H-graft) mesocaval shunt was developed, and its proponents claimed that it was hemodynamically superior to the standard portacaval shunts.[3]

This laboratory previously studied the portal hemodynamics of three porta-systemic shunts using gamma-labelled microspheres to evaluate portal blood flow.[4] A comparison was made between the hepatopetal portal blood flow following end-to-side and side-to-side portacaval shunts and mesocaval interposition shunts. The microsphere technique showed that about 60% of the portal flow to the liver was preserved by the mesocaval shunt and virtually none by the side-to-side portacaval shunt. The results indicated therefore, that the side-to-side porta-caval shunt was identical to the end-to-side shunt with regards to portal hemodynamics. Since the incidence of hepatic failure has been reported to be less after side-to-side portacaval shunting, changes in portal blood flow could not solely be responsible for hepatic decompensation after portasystemic shunts.

Another possible contributing factor in post-shunt hepatic failure was the volume of arterial blood flowing to the liver. It

has been postulated that with the loss of portal inflow there is a compensatory increase in hepatic arterial blood flow in order to provide sufficient oxygen to the liver for its metabolic needs. However, some studies have shown increased arterial flow, while others have demonstrated a reduction in both hepatic arterial blood flow and oxygen consumption following portacaval shunting.[7,8] We initially attempted to resolve the question of arterial blood flow to the liver after porta-systemic shunts with the use of gamma-labelled microspheres, which allowed us to determine the total input to the liver from all arterial sources.

Our studies showed that total hepatic arterial blood flow (HABF) significantly increased immediately after mesocaval or side-to-side portacaval shunting, but within 3 weeks hepatic arterial blood flow returned to preshunt levels. The increase in HABF was independent of changes in cardiac output or circulating blood volume, as gauged by pulmonary artery pressure, and was apparently a transient compensatory response to the reduction in hepatic portal flow. In contrast to side-to-side portacaval shunting, which permanently reduced total hepatic blood flow (THBF), THBF was preserved by the mesocaval H-graft procedure.

These investigations answered our initial questions, but they also left some issues unanswered. Specifically, we wondered about the response of the hepatic arterial and total hepatic blood flows after the distal splenorenal (Warren) shunt, which is now very popular.[9] We also wondered if the reduction in hepatic perfusion following side-to-side or end-to-side portacaval shunting could be alleviated by the use of a graft from the aorta to the portal vein above the shunt. Accordingly, our most recent efforts have been in these directions.

B. Experimental Approach. Mongrel dogs anesthetized with Halothane were randomly assigned to several groups: 1) sham operated, 2) Warren shunt, 3) side-to-side portacaval shunt, 4) side-to-side shunt with arterialization of the portal vein 5) end-to-side portacaval shunt and 6) end-to-side with arterialization.

Pulmonary artery pressure, cardiac output, hepatic arterial blood flow and visceral blood flow were determined prior to 1 hour and 3 weeks after shunting, when the animals were sacrificed. Cardiac output was measured by thermodilution and organ blood flow with 50±3 μ radioactive microsphere (^{51}Cr, ^{85}Sr, ^{141}Ce) injected into the left ventricle.

C. Results and Discussion. Data from the Warren Shunt and the Sham groups are tabulated below:

HEPATIC FLOW DYNAMICS IN CANINE PORTASYSTEMIC SHUNTS

SHAM

	Baseline	1 Hour	3 Weeks
Hepatic Arterial Blood Flow (ml/100 gms/min)	19.3±4.4	33.0±14.1	22.9±10.0
Cardiac Output (l/min)	5.41±1.10	4.56±0.75	5.12±1.14
Hepatoportal Blood Flow (ml/100 gms/min)	83.3±12.6	85.1±18.9	80.6±15.0
Total Hepatic Blood Flow (ml/100 gms/min)	102.6±12.0	118.1±25.8	103.5±14.0

WARREN SHUNT

	Baseline	1 Hour	3 Weeks
Hepatic Arterial Blood Flow (ml/100 gms/min)	15.2±3.8	16.7±6.9	9.9±3.4
Cardiac Output (l/min)	4.48±0.40	2.91±0.16*	4.82±0.26
Hepatoportal Blood Flow (ml/100 gms/min)	86.8±10.9	41.2±10.8*	58.4±11.5
Total Hepatic Blood Flow (ml/100 gms/min)	102.0±9.9	57.9±15.7*	68.2±10.0*

*Differs from baseline at $p<.05$

Unlike our previous shunted dogs, the cardiac output decreased significantly one hour after the completion of the distal splenorenal shunts. Again in contrast to the previous shunt studies, neither the absolute hepatic arterial blood flow nor the percent cardiac output going to the liver via the hepatic artery increased signifi-

cantly one hour after the completion of the Warren shunt. By the 3-week assay point, hepatic arterial blood flow was less than the baseline value. Furthermore, total hepatic blood flow was significantly reduced and remained so after distal splenorenal shunting. Thus, the hepatic arterial response to distal splenorenal shunting differs from that seen after the other types of portasystemic shunts. We wondered if the reason for this difference was the extensive dissection in the lesser omental area associated with the traditional Warren shunt. We therefore repeated this group of dogs and minimized any dissection in the area of the hepatic artery and porta hepatitis. The flow results were identical to our first group. Thus, the difference in the hepatic arterial response after distal splenorenal shunting, versus that seen following other types of porta-systemic shunts, is apparently not due to surgical trauma in the porta hepatitis.

The arterialization studies have just been completed, and the data has not been collated and statistically analyzed at this time. However, we can say that the addition of an arterial graft to either end-to-side or side-to side portacaval shunts does preserve total hepatic blood flow. The arterialization does not, however, prevent either the persistent increase in blood ammonia concentration or the progressive weight loss seen after porta-systemic shunting without arterialization. Whether or not arterialization has a positive effect on the incidence of hepatic encephalopathy could not be determined in our studies.

D. Conclusion and Recommendations. Following either side-to-side portacaval or H-graft mesocaval shunting there is a transient, significant increase in hepatic arterial blood flow and a long-term increase in blood flow to the small intestine. After Warren distal splenorenal shunting these changes do not occur. The addition of portal vein arterialization to either end-to-side or side-to-side portacaval shunting preserves total hepatic blood flow, but does not prevent the appearance of elevated blood ammonia levels or the progressive weight loss seen after shunting without arterialization. Further studies are indicated on the long-term hepatic chemical and histological effects of portacaval shunting in association with portal vein arterialization.

V. Changes in the Integrity and Function of Primate Gastric Mucosa During Gram-Negative Septic Shock: Development of a Model System

A. Background and Statement of the Problem. Gram-negative septic shock remains a serious threat in our hospitals, despite a large body of knowledge gained through clinical and experimental investigations. The incidence of gram-negative-rod bacteremia has steadily increased during the past two decades through the combination of a number of host, agent and transmission factors.[10,11] Renal

failure, respiratory insufficiency, heart failure, and gastrointestinal bleeding are frequent complications of septic shock. Approximately 30% of these patients experience at least one episode of upper gastrointestinal bleeding, which is due to hemorrhagic (stress) gastritis in the vast majority of cases.[12]

Septic shock and its complications have been extensively investigated during the past 20 years. Endotoxin shock has been the primary method of studying the pathophysiology and treatment of septic shock. However, due to numerous pathophysiologic differences between human septic shock and experimental endotoxin shock, the latter has frequently been criticized as being an inadequate reproduction of the clinical situation.[13,14]

The need for an experimental preparation of septic shock which closely approximates the clinical setting prompted a series of investigations in our laboratory. Using a new canine model of septic shock based on the creation of an ischemic, infected (E. coli) gallbladder,[15] we attempted to develop a satisfactory preparation in Rhesus monkeys. In addition, we attempted to evaluate the effect of this form of septic shock on gastric mucosal integrity. Initial investigations indicated that a dosage of E. coli containing 10^8 and 10^6 organisms per kg in the monkey led to death in 24 hours. These investigations demonstrated the feasibility of this technique for the production of septic shock in the Rhesus monkey. However, a dosage of 10^4 E. coli per kg appeared to be ideal for this species, since it is perferable that the animals remain septic for approximately 2-4 days before succumbing. Accordingly, 10^4 E. coli/kg was the dose employed for our subsequent studies.

B. Experimental Approach. Rhesus monkeys weighing 4-8 kg were tranqualized (2 mg/kg Sernylan IM) and brought to the operating room. Prior to anesthesia gastric potential difference (P.D.), an indication of mucosal integrity, was measured endoscopically using transendoscopic electrodes. The monkeys were then anesthetized with Halothane. Arterial and venous cannulas were introduced, as well as Swan-Ganz catheters. After baseline hemodynamic and hematologic studies, the abdomen was opened and the cystic artery and duct ligated. A suspension of E. coli (Type ATCC 25922) containing 10^4 organisms per kg was injected into the gallbladder. The abdomen was then closed, and the animal returned to the cage. Thereafter gastric, hemodynamic and hematologic studies were repeated daily until the monkey succumbed from septic shock.

C. Results and Discussion. Five monkeys were studied using the above technique. In contrast to the canine model, all monkeys exhibited a low-flow (hypodynamic) form of shock throughout the entire course of sepsis. Monkeys survived 1 1/2 to 3 days prior to death, but they never developed the hyperdynamic circulatory status seen in patients with early septic shock. Thus, although the animals

developed bacteremia and shock the model was unacceptable to us. In addition, the transendoscopic P.D. measurements were not helpful, since there was considerable variability in the readings in each monkey and between monkeys.

D. Conclusion and Recommendations. The use of an ischemic, infected (E. coli) gallbaldder preparation in Rhesus monkeys is a good model of low-flow septic shock. Overall, it is one of the best such models that has yet been devised. However, because of a high-flow phase of shock does not occur, this preparation does not simulate human septic shock. Furthermore, the technique of measuring gastric mucosal potential difference using a transendoscopic electrode does not generate reproducible, reliable data. Since the ischemic, infected gallbladder model does not produce satisfactory results in the primate, a study of the effects of cecal ligation is worthy of pursuit in this species.

VI. Defining a Serum Marker of Severe Ischemic Injury to the Gut (such as that seen with hemorrhagic shock). Determining an Appropriate Model

A. Background and Statement of the Problem. At the current time there are no laboratory aids in determining the presence of severe ischemic gut injury. The enzyme systems of creatine phosphokinase (CPK) and lactic dehydrogenase (LDH) are potential markers of such injury because they are easy to measure and they have particular isoenzymes which are present in the bowel in large quantities. The first step was to determine a suitable laboratory animal for study. The canine emerged as the animal of choice.

B. Experimental Approach. Fresh postmortem specimens from both canine and human subjects were obtained from random autopsy material. One gram samples of large and small bowel were taken from each of the major segments of each organ. These samples were homogenized in a blender in 10 cc of Ringer's lactate, centrifuged, and the supernatant was sent for analysis. The isoenzymes were determined by agarose gel electrophoresis. Total enzyme activity was determined by spectrophotometric analysis.

C. Results and Discussion. 1) The LDH system. LDH appears to be present in equal amounts in all levels of the small and large bowel. LDH_2, LDH_3, and LDH_4 are the predominant isoenzymes present. Only small amounts of LDH, and LDH_5 are present. The canine and the human have comparable levels of the total enzyme and of the respective isoenzymes in all segments of bowel studied. 2) CPK system. Once again the canine and the human were found to be completely analogous. Equal levels of total enzyme were found in all levels. All three isoenzymes were found in equal concentrations in both the small and large bowel.

	Small bowel supernatant	Large bowel supernatant
CPK (IU)	9813±3575	10311±2449
CPK MM (%)	33±2	30±3
CP MB (%)	36±2	37±3
CPK BB (%)	34±2	33±2

D. Conclusion and Recommendations. The canine is an appropriate animal model for the study of these isoenzymes as markers of ischemic gut injury such as that seen in hemorrhagic shock.

VII. Defining a Serum Marker of Severe Ischemic Injury to the Gut: Acute Mesenteric Artery Occlusion

A. Background and Statement of the Problem. Observations by ourselves and others[15] have shown that intestinal necrosis may be associated with the appearance of the CPK BB isoenzyme in the serum. The presence or changes in LDH have not been studied.

B. Experimental Approach. Using a randomized block design, canine subjects were assigned to one of three groups: laparotomy as control, laparotomy with peritonitis, and laparotomy with superior mesenteric ligation. Serum were drawn for 30 hours after each procedure and analyzed with spectrophotometry for totals and agarose gel electrophoresis for isoenzymes.

C. Results and Discussion. 1) LDH system. Total LDH levels remain relatively consistent at preoperative levels. The isoenzymes show a change in pattern in that LDH_3 and LDH_4 become more prominent in mesenteric necrosis. 2) CPK system. Total CPK becomes elevated for the first 12 hours and starts to fall towards preoperative levels at 24 hours. In the dogs suffering an infarction the CPK total rises again between 24 and 30 hours. The isoenzymes of CPK associated with bowel (MB and BB) appear in the serum with increasing peaks between 24 and 30 hours.

D. Conclusion and Recommendations. The CPK and LDH isoenzymes systems are appropriate markers for severe ischemic injury to the gut. Changes in these systems can be monitored in the peripheral serum. How hemorrhagic shock alters the levels of these enzymes will be determined in the near future.

VIII. Studies of CPK and LDH After Surgery

A. Background and Statement of the Problem. In order that the limits of reliability of CPK and LDH as markers of gut necrosis can be determined, their levels in injury must be determined. Surgical patients undergoing routine and emergency procedures have been used as adequate subjects for the evaluation of these serum enzymes. Patients have been used under the close supervision of the Human Use Committee at Walter Reed Army Medical Center.

B. Experimental Approach. After obtaining written consent from each patient, initial serum samples are obtained from the patients before surgery. Subsequent serum samples are obtained on a daily basis until the enzyme and the isoenzymes return to normal levels.

C. Results and Discussion. 1) Patients undergoing abdominal procedures. In none of the patients did either the CPK or LDH system change in a manner analagous to mesenteric or myocardial necrosis.

	Norm	MI	Hernia	Laps	Mus Spl
CPK (IU)	10-130	478±53	84±23	533±324	270±73
MB band	absent	present	absent	absent	absent
LDH (IU)	50-110	227±134	95±33	138±44	86±18
LDH_1 LDH_2	absent	present	absent	absent	absent

2) Thoracic patients. The changes noted in these patients sera showed that the total and isoenzyme patterns were those of injury to skeletal muscle alone. The presence of CPK-MB or BB bands in their sera were never seen. In a similar fashion LDH_2 remained the most dominant isoenzyme in their sera.

C. Conclusion and Recommendations. Surgery in itself does not cause changes in the CPK and LDH systems analagous to the changes seen in myocardial or mesenteric necrosis. Those specific changes remain valid for diagnosing the presence of either myocardial or mesenteric infarction.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA 6527	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISS'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	144
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Control Mechanisms of Regional Circulation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical Medicine 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY		16. PERFORMANCE METHOD
65 07	CONT	DA		C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION

b. NUMBER

c. TYPE: d. AMOUNT:

e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	1	133
	CURRENT 80	1	150

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Cutaneous blood flow; (U) Microspheres; (U) Pig; (U) Skin grafts

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) To define the changes which occur in cutaneous blood flow when various surgical techniques are used to prepare skin grafts. This approach could help allow surgeons to cover major war wounds more effectively.

24 (U) Blood flow to cutaneous skin grafts was measured in porcine skin using 15 micron radioactively tagged microspheres. Fifty and 15 micron sphere flow was simultaneously measured to identify shunting.

25 (U) 78 10 - 79 09 Blood flow in the delayed skin flaps increased with increasing delay intervals, reaching maximal levels after one week. This increase was paralleled by an increase in surviving flap length. A marked difference in 50 and 15 μ flow was identified in a pilot study with 15 μ flow being about 50% of 50 μ flow. This suggests a 50% arteriovenous shunt. Blood passing through arteriovenous shunts would presumably not be oxygenating the tissue in the flap. This initial finding needs to be confirmed and studied because of its potential clinical importance. For technical report see Walter Reed Army Institute of Research Annual Progress Report 1 Oct 78 - 30 Sept 79.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 144 Control Mechanisms of Regional Circulation

Investigator
Principal: MAJ Alexander M. Guba, Jr., MC

I. Regional Hemodynamics in Delayed Skin Flaps

A. Background and Statement of the Problem. The physiologic alterations occurring with a surgical delay in skin flaps have not been quantitated. Using a pig model, flaps were designed and blood flow to the skin flaps was determined.

B. Experimental Approach. Temperature, blood gases, cardiac output were measured. Blood flow was then measured using 15 micron radioactively tagged microspheres at three times; prior to any surgical manipulation, after increasing periods of delay (2 days to 14 days), and 24 hours after final raising of the skin flaps.

C. Results and Discussion. Blood flow in the delayed skin flaps increased with increasing delay intervals, reaching maximal levels after one week, $p \leq 0.05$. This increase paralleled an increase in surviving flap length.

These studies quantitate two important physiologic aspects of the delay phenomenon: an increase in nutrient capillary flow during the delay period and subsequent decrease in capillary flow while enhanced tissue survival persists.

II. Shunting in Delayed Skin Flaps

A. Background and Statement of the Problem. An hypothesis has been made that surgical delay of a skin flap causes a decrease in arteriovenous shunting thereby increasing nutrient blood flow and ultimate tissue survival.

B. Experimental Approach. Four skin flaps were delayed in each of 10 pigs. After 2 days delay, 50 and 15 micron radioactively tagged microspheres were administered. After 7 days of delay, additional 50 and 15 microspheres were given. Fifty, 15 and 50-15 blood flows were determined.

C. Results and Discussion. Total blood flow, nutrient blood flow and calculated arteriovenous shunting increased in delayed skin flaps during the interval from 2 to 7 days after surgical delay.

Changes in arteriovenous shunting cannot explain increased time survival. Increases in total flow and nutrient flow appear to be more important in this regard.

III. Vascular Access: Additional Therapeutic Utilization

A. Background and Statement of the Problem. Vascular access procedures have been performed to allow renal dialysis in renal failure patients. Such procedures can also be useful in patients requiring multiple transfusions, chemotherapy, and treatment of diabetic ketoacidosis.

B. Experimental Approach. Records were reviewed in the Vascular Clinic in WRAMC of patients who had undergone vascular access procedures for non-dialysis purposes.

C. Results and Discussion. Thirteen patients underwent these procedures and successfully obtained medical therapy via these access procedures.

This study emphasizes the utility of vascular access in many medical diseases. In addition, the variety of surgical technical options is presented.

IV. Coverage of Soft Tissue Defects

A. Background and Statement of the Problem. Civilian and military casualties are a major concern of reconstructive surgeons. Large defects of skin and subcutaneous tissues require optimal therapy in order to return the individual to a functioning status.

B. Experimental Approach. Methods available to treat large, soft tissue defects were reviewed.

C. Results and Discussion. Skin grafts, dressings and skin flaps can be utilized for coverage purposes.

This study emphasizes the critical factors involved in the selection of the optimal treatment modality for a particular injury.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 144 Control Mechanisms of Regional Circulation

Literature Cited.

Publications:

1. Guba, A.M., Collins, G.J., Kozloff, L., McDonald, P.J., Rich, N.M.: Vascular access: Additional therapeutic utilization. Amer J Surg In press.

2. Guba, A.M., Hoopes, J.E.: Coverage of soft tissue defects. Military Medicine 144(10), 655-658, 1979

3. Guba, A.M., Callahan, J.: Delayed axial pattern skin flaps. Plast Reconstr Surg 64(3), 373-376.

4. Guba, A.M.: Changes in cutaneous arteriovenous shunting with surgical manipulation. Submitted for presentation.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1 AGENCY ACCESSION	2 DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6458	79 10 01	DD DR&E (AR)636

3 DATE PREV SUM'RY	4 KIND OF SUMMARY	5 SUMMARY SECTY	6 WORK SECURITY	7 REGRADING	8a DISTR INSTR'N	8b SPECIFIC DATA CONTRACTOR ACCESS	9 LEVEL OF SUM
78 10 01	Termination	U	U	NA	NL	[X] YES [ ] NO	A WORK UNIT

10 NO / CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a PRIMARY	61102A	3M161102BS01	00	145
b CONTRIBUTING				
c CONTRIBUTING	Cards 114F			

11 TITLE (Include with Security Classification Code): (U) The Effects of Specific Environmental Pollutants on the Biosynthetic Functions of Mammalian Cells in vitro; A search for Structure/Activity Relationships

12 SCIENTIFIC AND TECHNOLOGICAL AREAS: 002600 Biology 005900 Environmental Biology

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMANCE METHOD
77 08	79 10	DA	C. In-house

17 CONTRACT/GRANT
a DATES/EFFECTIVE — EXPIRATION
b NUMBER
c TYPE — d AMOUNT
e KIND OF AWARD — f. CUM. AMT.

18 RESOURCES ESTIMATE	a PROFESSIONAL MAN YRS	b FUNDS (In Thousands)
FISCAL YEAR PRECEDING 79	1.0	80
FISCAL YEAR CURRENT 80	0	0

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NAME: Altstatt, COL, L.B. DA
NAME:

21 GENERAL USE
Foreign intelligence not considered

22 KEYWORDS (Precede EACH with Security Classification Code)
(U) Environmental Toxicology; (U) In Vitro Toxicology, (U) Munitions Pollution

23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To develop in vitro systems for toxicology screening of polluting products of the manufacture of munitions, to determine the mechanism of action of the toxic effect, and to find structure-activity relationships with predictive value. By executive order, the Army has been charged with complying with EPA regulations and policies, EPA requires toxicologic studies of manufacturing wastes to establish standards.

24. (U) Replicate in vitro hemoglobin synthesis including heme and globin synthesis, erythroblast formation and maturation. Compounds of interest (initially benzene, nitrobenzene and nitrotoluenes) to the Army will be introduced in vitro, and mode of action determined.

25. (U) 77 10-79 09. In vitro systems for examination of heme synthesis (rat liver homogenates), globin synthesis (rabbit reticulocyte lysate), and erythroid cell proliferation (plasma clot culture of dog bone marrow) were established and characterized. Special efforts were made to standardize each of the systems as much as possible to insure comparability of results obtained at different times. Several vehicles for delivery of the benzene and toluene derivatives were compared. Comparative toxicity data were obtained for a limited number of compounds, at concentrations ranging from 0.000001 to 001, using all three test systems. The compounds examined in detail include: benzene, o-, m-, and p-dinitrobenzenes and 2,3; 2,4; 2,5; 2,6; 3,4; and 3,5 dinitrotoluenes. For Technical Report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sep 79.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 68 AND 1498 1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

PROJECT 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 145 The Effects of Specific Environmental Pollutants on the Biosynthetic Function of Mammalian Cells In Vitro
A search for Structure Activity Relationships.

Investigators

Principal: LTC Michael J. Haut, MC

Associate: COL Leslie B. Altstatt, MC; Mr. Harold L. Williams GS-13; Ms. Deadre Johnson GS-11; Kenneth Goldstein, MD (Associate Professor of Medicine, George Washington University School of Medicine); Peter Parsons, Ph.D. (Associate Professor of Biochemistry, Mount Holyoke College, Massachusetts).

Description

The basic goal of this research project is to determine whether relative in vitro toxicities of a group of compounds arising from breakdown of a particular pollutant can be predicted by (a) examining the relative in vitro toxicities of a small number of such derivative compounds with different structural characteristics, and (b) using structure-activity relationships to predict which others will be most toxic in the in vitro system. The specific test systems we are using are heme synthesis by rat liver homogenates and rabbit reticulocyte lysates, globin synthesis by a cell-free rabbit reticulocyte lysate system, and erythroid cell proliferation in a plasma clot culture system.

The second year's work concentrated on two areas: (a) optimizing and validating, in as thorough a manner as possible, the methodology established during the first year; and (b) obtaining reproducible toxicity data for benzene, the nitrobenzenes, the nitrotoluenes, dinitrotoluenes, trinitrotoluenes, and amino-dinitrotoluenes, using each of the three test systems. Progress in these two major efforts is discussed below.

Progress

A. Heme synthesis

Heme synthesis studies during the past year have focused on (a) optimizing and standardizing our array procedures for ALA synthetases and ferrochelatases, with special attention paid to examination of the ferrochelatase reaction, and (b) re-examining, in a more rigorous manner, the effect of the dinitrobenzenes, dinitrotoluenes, trinitrotoluenes, and amino-dinitrotoluenes on the activity of these two enzymes.

1) Optimization and standardization of our arrays for ALA synthetases and ferrochelatase

The quantity of heme formed was linearly related to time elapsed during the course of the reaction; this relationship was maintained up to 1.5 h (Figure 4).

We evaluated the effect of excess heme on the conversion of porphyrin to heme. Table 2 shows the effect of additional heme on ferrochelatase activity. At very low concentrations of added heme, there was little effect on the enzyme's activity. As the concentration of added heme approached within 0.5% of heme synthesized, enzyme activity diminished by about 30%. This suggests that heme, in addition to functioning as a negative feedback inhibitor to the production of delta-aminolevulinate, also functions as a product inhibitor to the additional production of heme.

We preferred a radiochemical procedure over colorimetry or spectrofluorometry because of its greater sensitivity, especially when labeled materials of high specific activity were used. The incorporation of iron into mesoporphyrin was linearly related to amount of iron to 500 nmol.

2) Effect of the dinitrobenzenes, dinitrololuenes, trinitrotoluenes, and amino-dinitrotoluenes on the activity of ALA synthetase and ferrochelatase

Tables 3 and 4 show dose response curves for the effect of these hydrocarbons on ALA synthetase activity and heme synthetase activity, respectively. These data are depicted in graphic form in Figures 5-7.

B. Globin synthesis

Work in this area during the first part of the past year focused on optimizing the reticulocyte lysate cell-free protein synthesis system. During the latter part of the year, the optimized system was used to examine the effect of the various isomers of DNT and TNT.

1) Optimization and standardization of the reticulocyte lysate cell-free protein synthesis system

Optimum concentrations of the assay components for the reticulocyte lysate cell-free protein synthesis system were determined to be the following:

lysate = 40 µl/100 µl array
creatine phosphate = 0.011M
KCl = 0.11M
$Mg\ Cl_2$ = 0.53mM
unlabeled amino acids = 0.11mM each
creatine kinase = 0.04 mg/ml
^{3}H-leucine = 0.044mM (5Ci/m mole)

Figures 8 through 12 show the studies done to arrive at these optimum concentrations.

The majority of our efforts on enzyme optimization and standardization during the past year were directed toward the ferrochelatase (heme synthetase) reaction. Our efforts were concentrated on this enzyme because the results of our arrays for ALA synthesis agrees closely with those of Ebert et al (Biochem Biophys Acta 208:236-250, 1970), whereas the results of our ferrochelatase arrays were consistently higher than those obtained by other investigators such as Bottomley (Blood 31:314, 1968) or Borkowsky et al (J Clin Invest 56:1139, 1975).

The method we use for measuring ferrochelatase activity is a radiochemical one in which the in vitro incorporation of ^{59}Fe into mesoporphyrin-IX to form mesoheme is determined. Optimization of the reaction involved strict maintenance of anaerobic, reduced, iron-free conditions.

Figure 1 shows the relationship between heme formation and iron content when the concentration of porphyrin is held constant. The optimum amount of iron for this assay with a 50 g/L rat-liver homogenate was about 500 nmol. When this amount of iron was greatly exceeded--e.g., 750 nmol--substrate inhibition occurred. To optimize porphyrin concentration, we used mesoporphyrin-IX in methanolic hydrolysates up to 2600 µmol/L. Figure 2 shows the relationship between mesoporphyrin concentration and its conversion to mesoheme at constant iron. The calculated K_m values for iron and mesoporphyrin were 5.48×10^{-7} mol/L, respectively.

The possible inhibitory effect of methanol on ferrochelatase activity was evaluated. In addition to the 0.5 mL of methanol in the solubilized porphyrin solution, we added more methanol to the test system and found no effect on the reaction if the total volume of methanol in the system did not exceed 0.75 mL. Amounts over 0.75 mL drastically inhibited enzyme activity (as shown in Figure 3).

The relationship between protein concentration and the quantity of heme formed is essentially linear up to 15.4 mg of protein in the reaction mixture.

Within-day and day-to-day measurements were performed on a homogenate of five pooled rat livers. Table 1 shows the results of eight replicate analyses performed on this specimen on different days and indicates that there were no significant differences in the within-day and day-to-day variation ($p<0.05$).

We continued this aspect of the study for over a month. Later studies on unselected samples indicated that the specimens were stable for more than six months if kept at -70°C or lower temperatures.

The range of values for rat-liver ferrochelatase activity was considerable, from 10 to 40 µmol of heme produced in 45 min per gram of protein. Typical mean values for normal rat-liver ferrochelatase activity were 19-21 µmol of heme formed in 45 min per gram of protein.

Figure 8 demonstrates the smallest amount of lysate which results in an essentially linear response with respect to time and at the same time results in levels of ^{3}H-leucine incorporation that are well above zero time or background radioactivity.

Figure 9 demonstrates that 0.011M creatine phosphate is the highest concentration which results in a linear response with respect to time.

Figure 10 demonstrates that:

a) 0.08 mg/ml creatine kinase may result in slightly better incorporation than 0.04 mg/ml.

b) ATP cannot replace creatine phosphate as an energy source.

c) the addition of ATP to the system which already contains creatine phosphate results in marked inhibition of the system. The amount of ATP present shows a positive correlation to the extent of inhibition. Thus, we have kept the creatine phosphokinase-creatine phosphate ATP regenerating system in preference to ATP.

We thought perhaps the higher levels of ATP in Figure 3 were chelating the Mg which may be needed in a free form. Thus, we titrated for Mg Cl_2 using either creatine phosphate or ATP. Figure 11 shows increasing Mg Cl_2 concentration does improve incorporation with ATP as energy source at least up to a 1:1 ratio of Mg Cl_2:ATP. Above this is inhibitory. Increasing the Mg Cl_2 concentration is inhibitory when creatine phosphate is the energy source, and 0.53 mM Mg Cl_2, 0.01M creatine phosphate gave the best results.

Figure 12 reveals the optimum KCl level in the system. The optimum was found to be 0.11M. Also an increased Mg Cl_2 concentration seems to inhibit somewhat.

2) Effect of various DNT and TNT isomers on cell-free protein synthesis in the reticulocyte lysate system

Studies on the effect of various DNT and TNT isomers on globin biosynthesis consisted of two efforts. In the earlier part of the year, the effects of these toxic hydrocarbons on globin synthesis was examined using the cell-free protein synthesis system utilized by Dr. Fournier during the first year of our project. This effort was directed at completing a comprehensive examination of all the DNT and TNT isomers. During the latter half of this year, work was focused on repeating our previous studies of the toxicity of these hydrocarbons on globin synthesis, using the optimized, standardized reticulocyte lysate cell-free protein synthesis system developed this year.

Figures 13-19 show the effect of a number of specific hydrocarbons on globin synthesis, using the system utilized last year. The compounds studied included the dinitrotoluenes, trinitrotoluenes, aminodinitrotoluenes, and the chlorotoluenes.

Figure 20 graphs the inhibition of globin synthesis (expressed as percent control) for the isomers of DNT and TNT.

Figures 21-30 show the time course of inhibition of globin biosynthesis by each of the DNT and TNT isomers.

C. Erythroid colony formation

The CFU-E culture system established and standardized the previous year was refined, and the optimized system was restandardized. In brief, the method is as follows. Nucleated cells are isolated from bone marrow aspirated from healthy dogs. Nucleated cells are isolated from the aspirated marrow by centrifugation in Ficoll-hypaque, placed in a defined culture medium, and incubated in microtiter wells for 48 hours at 39°C in a humidified incubator with 4% carbon dioxide level. Following incubation, the plasma clots are placed on glass slides and stained. Erythroid colonies are then counted using the microscope; these colonies are defined as groups of eight or more hemoglobin staining cells.

1) Effect of graded doses of various DNT and TNT isomers on erythroid colony formation

The major part of this past year's effort has been on utilizing this refined, standardized assay system to examine the effect of various doses of TNT and DNT derivatives on erythroid colony formation in an organized manner. The data obtained in their toxicologic studies are shown in Figures 31-46. Because of the inescapable variability between cultures done on different days, the data are shown in different ways. For each compound studied more than once, a comparative graph of colonies formed vs concentration of toxic agent is plotted, in which the number of colonies formed in the presence of a given concentration of inhibitor is expressed as per cent of control value obtained with no inhibitor. This is followed by plots of raw data for each individual study.

By the end of last year, only 2,6-DNT and 3,4-DNT had been studied using this system, and these had been studied only once in a duplicate (3,4-DNT) or triplicate (2,6-DNT) cultures. During the past year, the effect of the dinitrotoluenes (2,3-DNT; 2,4-DNT; 2,5-DNT; 2,6-DNT; 3,4-DNT; 3,5-DNT) and the trinitrotoluenes (2,4,6-TNT; 2,3,4-TNT) on erythroid colony formation have been examined.

Figure 31A shows composite results for two runs in which colony formation was determined as a function of 2,3-DNT concentration. Although results of the two runs are not completedly superimposable, they are similar; and both show complete inhibition

at 10^{-4} M 2,3-DNT. Figures 31B and 31C show data for the individual runs.

Figure 32A shows results of a single study (done with three replicate cultures) examining the effect of 2,4-DNT on erythroid colony formation. Inhibition by this compound is relatively small, reaching only 20% inhibition at 10^{-4} M.

Figure 33A shows results of two runs examining the effect of 2,5-DNT. Inhibition of colony formation was significant only at 10^{-4} M. Figures 33B and 33C show data for the individual runs.

Figure 34A shows results of three runs examining the effect of 2,6-DNT. Results of studies using this compound were not as consistent as with the other compounds. However, significant inhibition was noted in all three runs at 10^{-5} M and 10^{-4} M concentration of 2,6-DNT. Figures 34B, 34C, and 34D show data for the individual runs.

Figure 35A shows results of three runs examining the effect of 3,4-DNT on erythroid colony formation. Reproducibility here was excellent. A profound decrease in number of colonies is seen as the concentration of this toxic agent increases from 10^{-6} M to 10^{-4} M. Figures 35B, 35C, and 35D show data for the individual runs.

Figure 36A shows results of two runs examining the effect of 3,5-DNT on erythroid colony formation. Both runs show minimal inhibition of colony formation, even at 10^{-4} M concentration of the toxic agent. Figures 6B and 6C show data for the individual runs.

Figure 37A shows results of two runs examining the effect of 2,4,6-DNT on colony formation. Results of the two runs are similar, both essentially showing no inhibition at concentration of toxin less than 10^{-4} M. Figures 37B and 37C show data for the individual runs.

Figure 38A shows results of two runs examining the effect of 2,3,4-TNT on colony formation. Results of the two runs are similar, both showing complete inhibition at 10^{-5} M and 10^{-6} M. Figures 38B and 38C show data for the individual runs.

2) Effect of specific small molecules in modulating the effects of toxin hydrocarbons on erythroid colony formation

A number of small molecules have been shown to be involved in control of heme synthesis, globin synthesis (directly or through their action on the heme controlled repressor), or erythroid colony formation. To determine whether these compounds could alter the effect of the toxic hydrocarbons under study, we examined the effect of cyclic AMP, pyridoxal HCl, and ALA on colony formation, both alone and in the presence of benzene, a known inhibitor of colony formation for which we have extensive data.

Figure 39A shows the results of two runs examining the effect of cAMP concentration on erythroid colony number; Figures 39B and 39C show data for the individual runs. Similarly, Figures 40A and 41A show results of two runs each, in which the effect of pyridoxal HCl and ALA on erythroid colony number are examined; Figures 10b, 10c, 11b, and 11c show data for the respective individual runs.

Figure 42A shows the results of three runs examining the effect of benzene on erythroid colony formation; Figures 42B, 42C, 42D show data for the individual runs. Figure 43 shows that incubation of cells for 10 minutes before washing twice and inoculating the cultures gives eventually as much inhibition as preincubating cells for 30 minutes.

Figures 44 and 45 show preliminary experiments in which the effect of cAMP and pyridoxal HCl on inhibition of erythroid colony formation by benzene were examined.

Figure 46 shows the averaged results for six runs (performed with triplicate cultures) in which the effects of cAMP, pyridoxal HCl, and ALA on inhibition of colony formation were examined. Raw data for each of these runs is presented in Table 5.

Tables 1,2

Table 1. Within-Day and Day-to-Day Study of Ferrochelatase Activity

	Activity, μmol heme formed/g protein			
	Day 0	Day 1	Day 29	Day 33
	12.1	13.4	12.4	12.4
	11.4	14.9	10.8	12.9
	10.8	12.5	10.6	13.3
	10.9	13.8	10.9	12.7
	10.9	13.5	11.3	11.6
	11.4	15.0	10.0	14.2
	11.4	13.8	11.6	12.7
	10.5	14.0	10.3	12.6
Mean	11.2	13.9	11.0	12.8
SD	0.51	0.81	0.77	0.74
SEM	0.18	0.29	0.27	0.26

Table 2. Effect of Added Heme on Ferrochelatase Activity

Added heme, nmol	Heme formed, μmol/g of protein in 45 min
0	40.1
4	39.7
8	38.4
20	33.4
40	27.4
80	36.9
160	31.1
200	28.5

TABLE 3

CONCENTRATION EFFECTS OF AROMATICS ON DELTA-ALA SYNTHETASE ACTIVITY

Concentration with respect to aromatic	Percentage of Control		
	I	II	III
o-Dinitrobenzene			
10^{-3} M	43	49	55
10^{-4} M	84	84	78
10^{-5} M	104	101	102
10^{-6} M	105	101	104
m-Dinitrobenzene			
10^{-3} M	83	83	82
10^{-4} M	94	99	103
10^{-5} M	118	113	108
10^{-6} M	100	98	95
p-Dinitrobenzene			
10^{-3} M	41	48	42
10^{-4} M	50	54	52
10^{-5} M	81	78	92
10^{-6} M	93	88	95
2,3-DNT			
10^{-3} M	55	60	51
10^{-4} M	81	96	83
10^{-5} M	97	112	101
10^{-6} M	104	114	108
2,4-DNT			
10^{-3} M	92	94	92
10^{-4} M	100	97	92
10^{-5} M	102	103	101
10^{-6} M	99	98	104

TABLE 3 (CON'T)

Concentration with respect to aromatic	Percentage of Control		
	I	II	III
2,5-DNT			
10^{-3} M	43	39	43
10^{-4} M	91	80	80
10^{-5} M	89	98	107
10^{-6} M	90	109	112
2,6-DNT			
10^{-3} M	77	75	91
10^{-4} M	87	92	102
10^{-5} M	100	102	103
10^{-6} M	97	110	101
3,4-DNT			
10^{-3} M	56	53	69
10^{-4} M	86	84	77
10^{-5} M	97	110	88
10^{-6} M	99	105	85
3,5-DNT			
10^{-3} M	91	84	98
10^{-4} M	93	93	92
10^{-5} M	96	99	94
10^{-6} M	97	99	95
2,3,4-TNT			
10^{-3} M	26	16	29
10^{-4} M	69	106	75
10^{-5} M	101	110	100
10^{-6} M	99	118	91

TABLE 3 (CON'T)

Concentration with respect to aromatic	Percentage of Control		
	I	II	III
2,4,6-TNT			
10^{-3} M	86	89	86
10^{-4} M	93	98	87
10^{-5} M	103	111	93
10^{-6} M	105	98	96
4-Amino-2,6-DNT			
10^{-3} M	82	74	87
10^{-4} M	87	116	83
10^{-5} M	93	106	93
10^{-6} M	89	94	103
4-Amino-3,5-DNT			
10^{-3} M	89	80	87
10^{-4} M	94	86	91
10^{-5} M	107	98	105
10^{-6} M	107	85	107

TABLE 4

CONCENTRATION EFFECTS OF AROMATICS ON FERROCHELATASE ACTIVITY

Concentration with respect to aromatic	Percentage of Control		
	I	II	III
o-DNB			
10^{-3} M	143	148	164
10^{-4} M	148	125	140
10^{-5} M	146	96	123
10^{-6} M	105	89	123
m-DNB			
10^{-3} M	149	164	139
10^{-4} M	118	117	114
10^{-5} M	119	116	94
10^{-6} M	104	105	96
p-DNB			
10^{-3} M	152	183	127
10^{-4} M	180	143	147
10^{-5} M	127	128	131
10^{-6} M	112	114	109
2,3-DNT			
10^{-3} M	130	216	228
10^{-4} M	103	145	175
10^{-5} M	85	124	129
10^{-6} M	99	128	158
2,4-DNT			
10^{-3} M	179	201	212
10^{-4} M	80	138	189
10^{-5} M	92	122	202
10^{-6} M	96	116	182
2,5-DNT			
10^{-3} M	139	190	132
10^{-4} M	102	180	131
10^{-5} M	82	135	113
10^{-6} M	90	118	103

TABLE 4 (CON'T)

Concentration with respect to aromatic	Percentage of Control		
	I	II	III
2,6-DNT			
10^{-3} M	117	194	135
10^{-4} M	96	138	113
10^{-5} M	89	116	101
10^{-6} M	84	104	94
3,4-DNT			
10^{-3} M	131	229	137
10^{-4} M	119	155	133
10^{-5} M	87	117	93
10^{-6} M	94	116	94
3,5-DNT			
10^{-3} M	166	205	138
10^{-4} M	130	164	124
10^{-5} M	110	116	108
10^{-6} M	107	115	100
2,3,4-TNT			
10^{-3} M	104	126	144
10^{-4} M	119	156	116
10^{-5} M	96	122	79
10^{-6} M	100	113	85
2,4,6-TNT			
10^{-3} M	150	144	179
10^{-4} M	136	215	143
10^{-5} M	117	140	107
10^{-6} M	119	120	100
4-Am.-2,6-DNT			
10^{-3} M	137	115	102
4-Am.-3,5-DNT			
10^{-3} M	152	132	140

TABLE 5

THE EFFECTS OF BENZENE ERYTHROID COLONY GROWTH WITH/WITHOUT PYRIDOXAL HCl, cAMP and ALA

	Experiment 1	Experiment 2	Experiment 3
Control (nothing added)	.434 ± 44*	478 ± 72	472 ± 28
Benzene (10^{-4} M)	323 ± 26	372 ± 65	261 ± 45
Pyridoxal HCl (10^{-6} M)	405 ± 16	497 ± 25	492 ± 26
cAMP (10^{-7} M)	447 ± 41	533 ± 49	509 ± 28
ALA (10^{-5} M)	464 ± 39	118 ± 53	442 ± 46
Benzene (10^{-4} M + pyridoxal 10^{-6} M)	331 ± 60	357 ± 11	521 ± 36
Benzene (10^{-4} M + cAMP 10^{-7} M)	217 ± 11	339 ± 39	699 ± 56
Benzene (10^{-4} M + ALA 10^{-5} M)	322 ± 38	308 ± 75	512 ± 47

*Erythroid colony numbers ± SEM

NOTE: Cells were incubated with 10^{-4} M benzene before adding ALA or cAMP or pyridoxal

TABLE 5 (cont'd)

	Experiment 4	Experiment 5	Experiment 6
Colony (nothing added)	430 ± 30	411 ± 21	193 ± 23
Benzene (10^{-4} M)	284 ± 30	254 ± 21	121 ± 9
Pyridoxal HCl (10^{-6} M)	507 ± 22	412 ± 23	200 ± 27
cAMP (10^{-7} M)	504 ± 36	423 ± 34	203 ± 13
ALA (10^{-5} M)	455 ± 17	438 ± 39	143 ± 19
Benzene (10^{-4} M + pyridoxal 10^{-6} M)	513 ± 18	342 ± 35	133 ± 16
Benzene (10^{-4} M + cAMP 10^{-7} M)	495 ± 36	428 ± 56	177 ± 9
Benzene (10^{-4} M + ALA 10^{-5} M)	460 ± 34	464 ± 89	154 ± 26

Figures 1-4

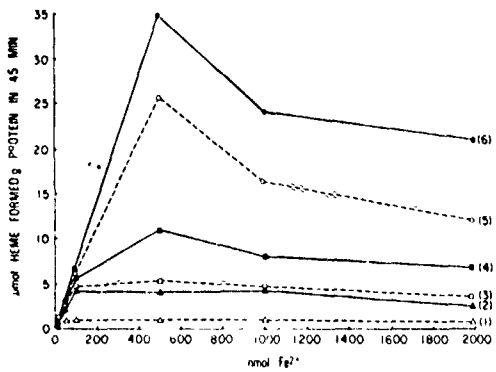


Fig 1 Iron incorporation into mesoporphyrin-IX in presence of various amounts of porphyrin

Curves 1-6 represent 20, 100, 200, 500, 1000, and 1500 nmol of mesoporphyrin-IX, respectively, used in the assay

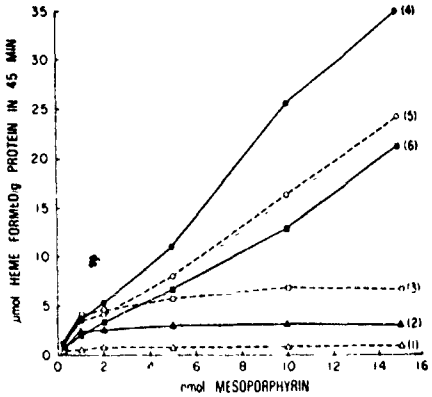


Fig. 2. Effect of different concentrations of iron on the ability of ferrochelatase to convert mesoporphyrin-IX into mesoheme

Curves 1-6 represent 10, 50, 100, 500, 1000, and 2000 nmol of iron, respectively, used in the assay

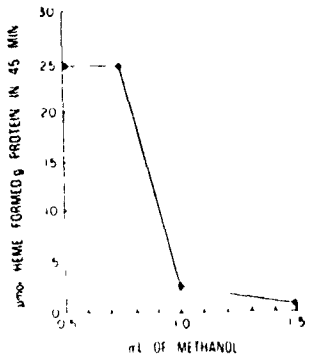


Fig 3 Effect of methanol on ferrochelatase activity

The assay was performed with use of 500 nmol of iron and 1000 nmol of porphyrin

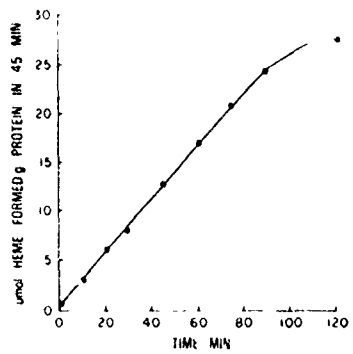
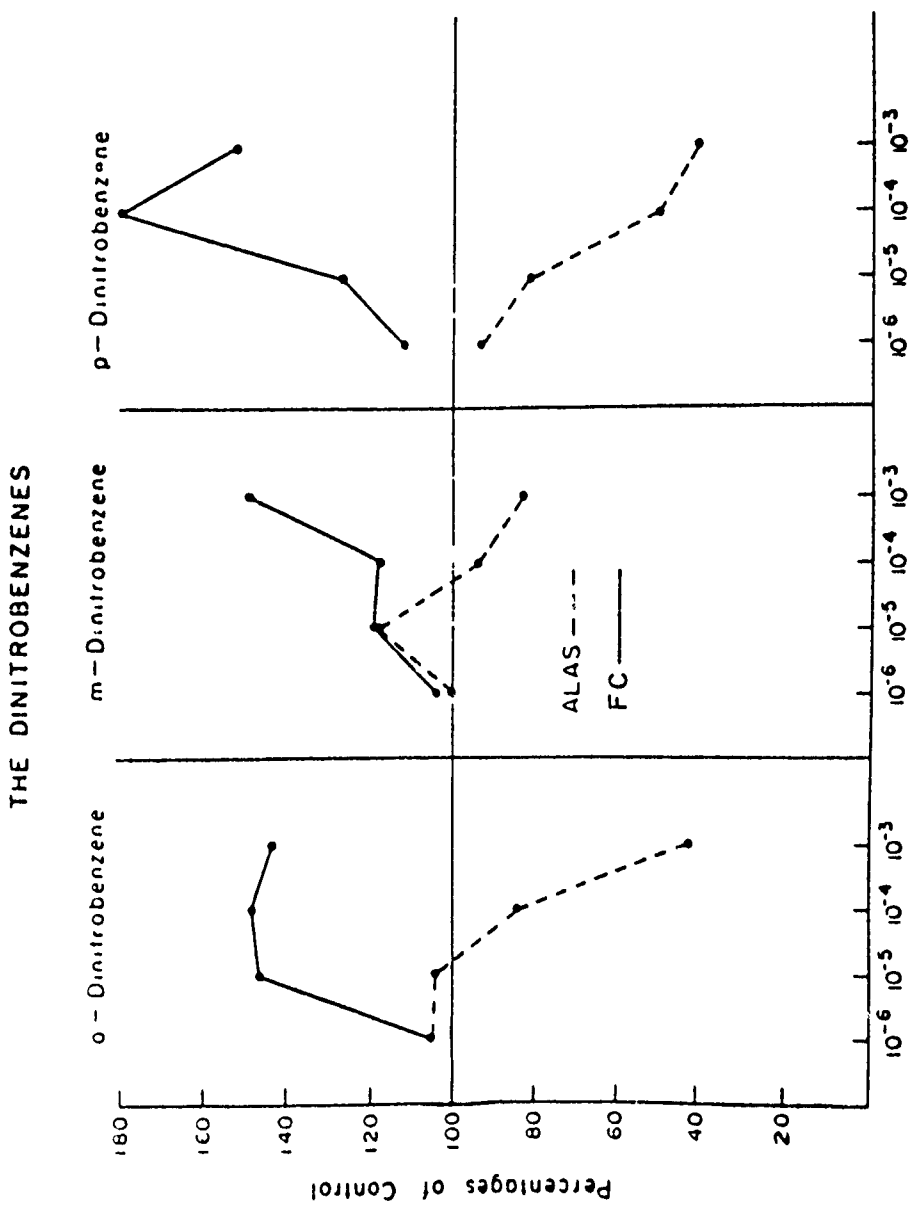


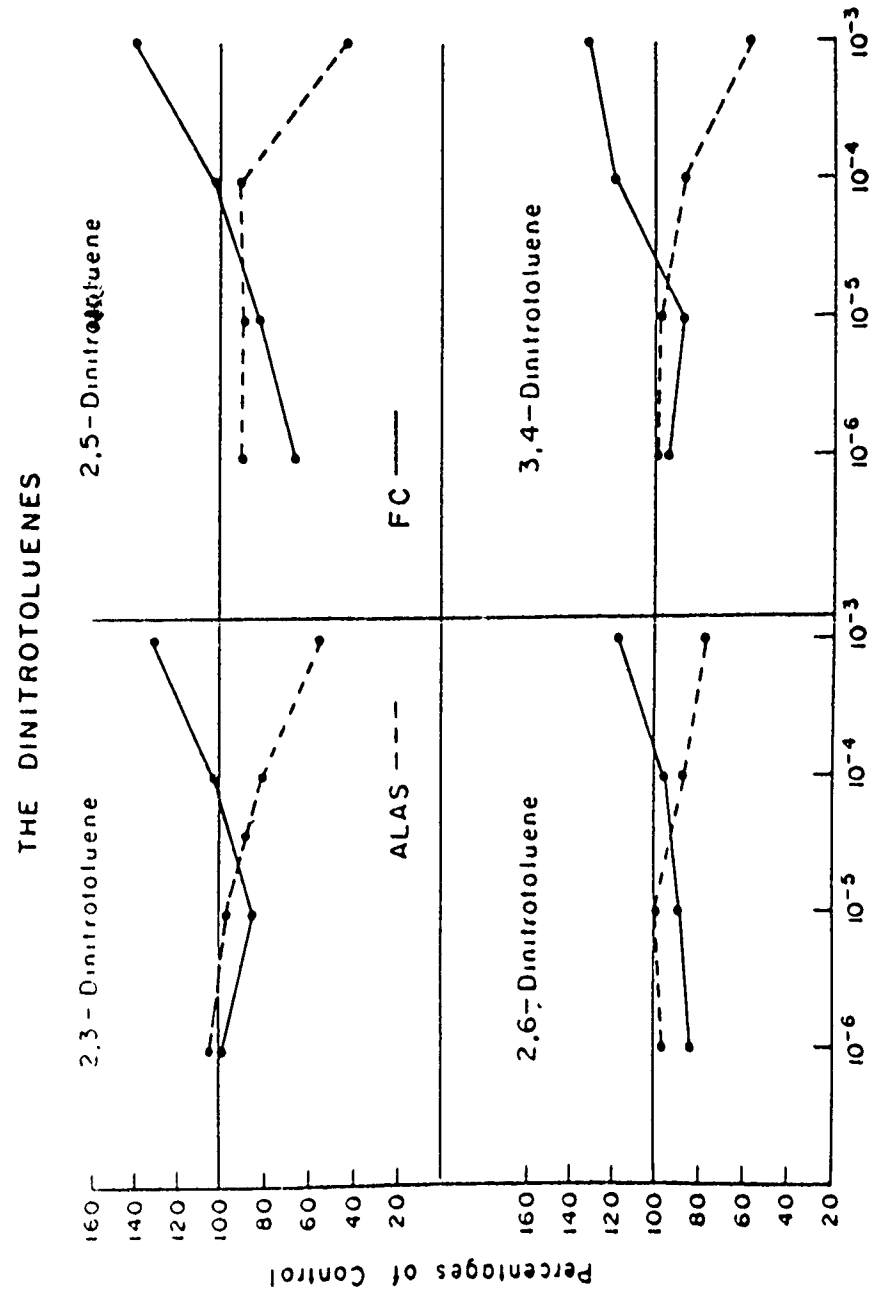
Fig 4 Relation between ferrochelatase activity and time

FIGURE 5



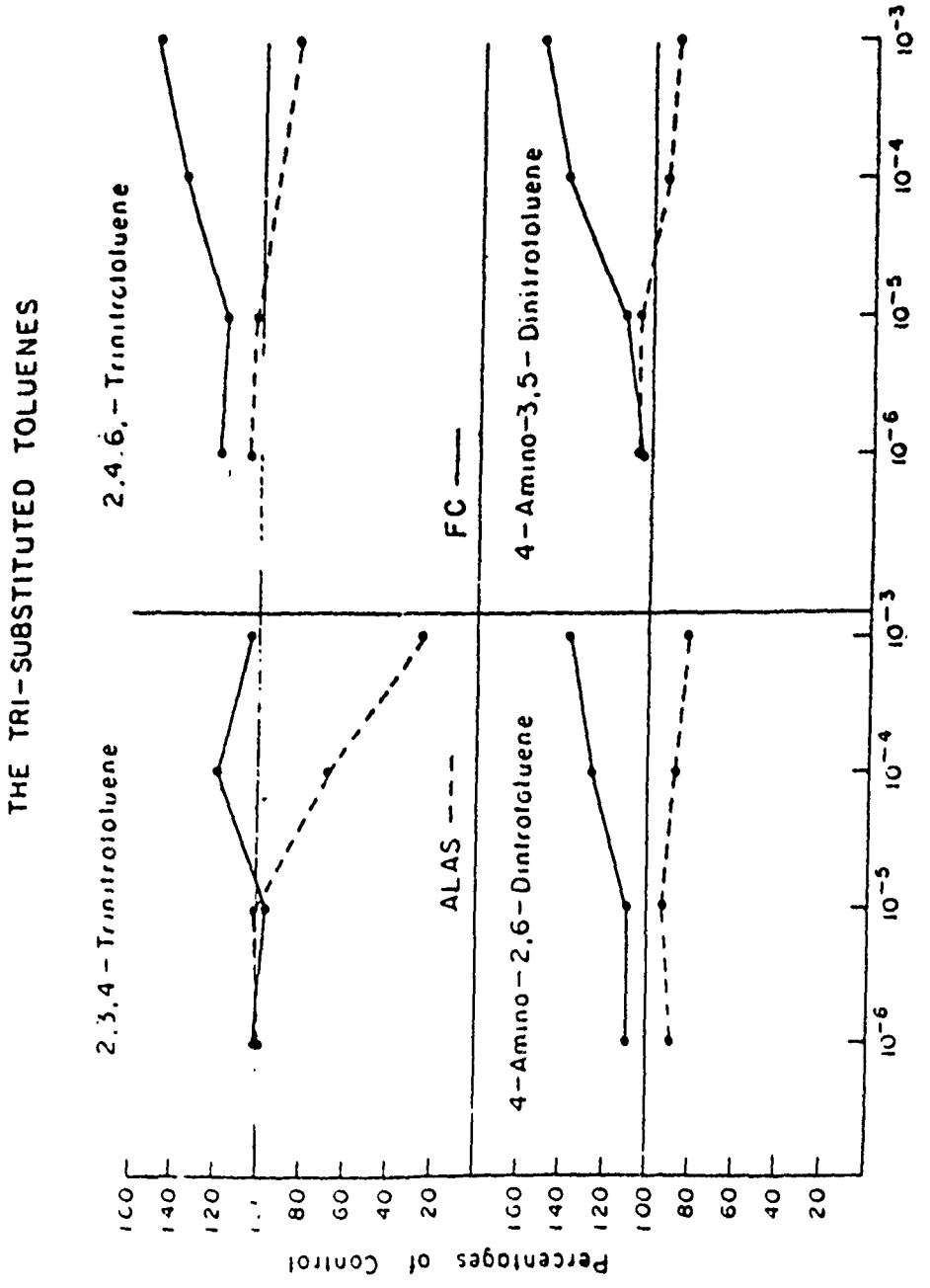
The effect of the dinitrobenzenes on ALAS and FC activities. Note the inverse relationship at higher concentrations resulting in a "V" shaped curve.

FIGURE 6



A comparison of the effect of the dinitrotoluenes on ALAS and FC activities. Note the coincidence of activity in the 10^{-5} to 10^{-4} mol/l concentration range of the aromatic resulting in an "X" shaped curve.

FIGURE 7



A comparison of the effect of tri-substituted toluenes on the enzyme systems studied producing a "Y" shaped curve.

FIGURE 8

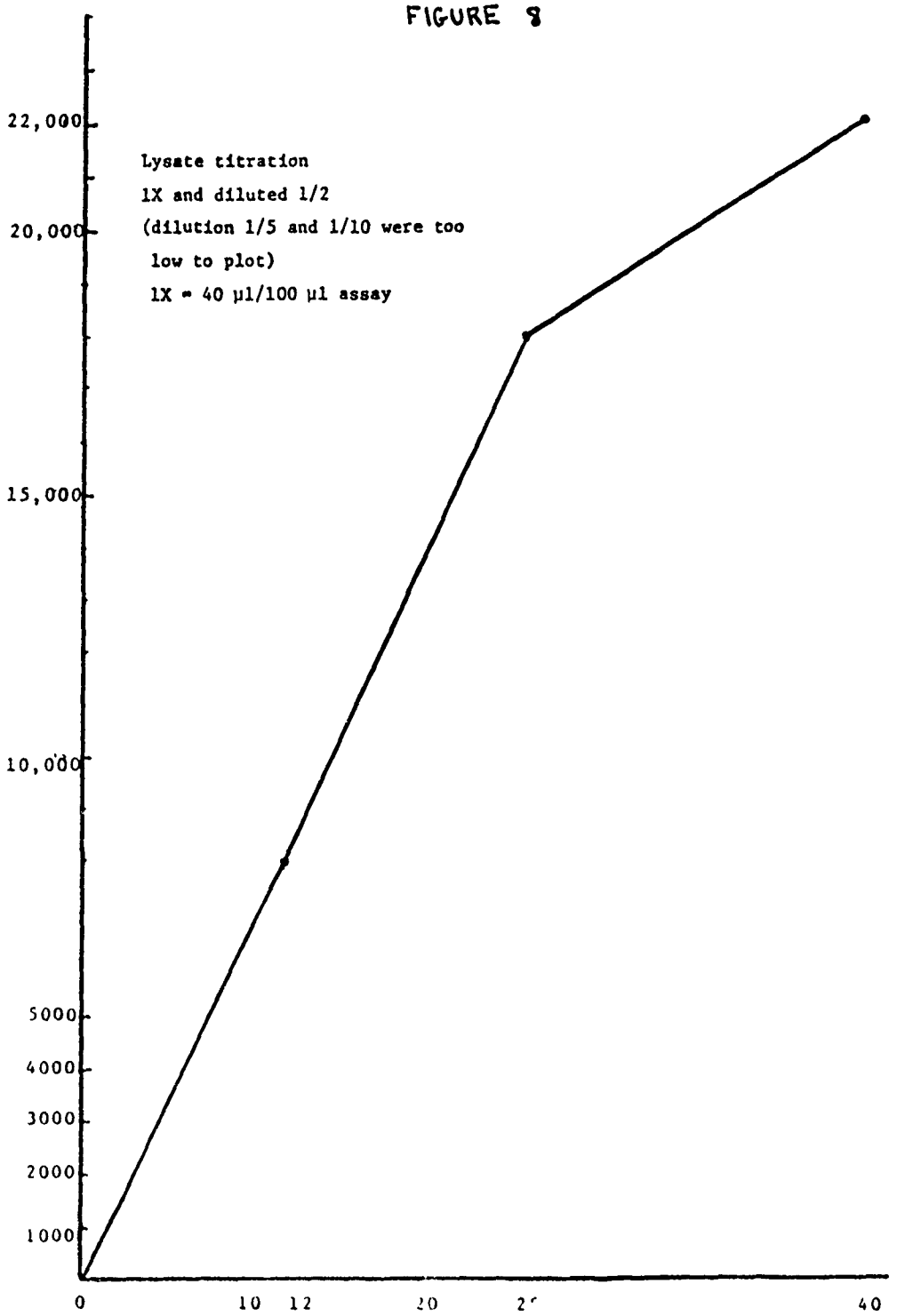

22,000
20,000
15,000
10,000
5000
4000
3000
2000
1000
Lysate titration
1X and diluted 1/2
(dilution 1/5 and 1/10 were too
low to plot)
1X = 40 µl/100 µl assay
0
10
12
20
40

FIGURE 9

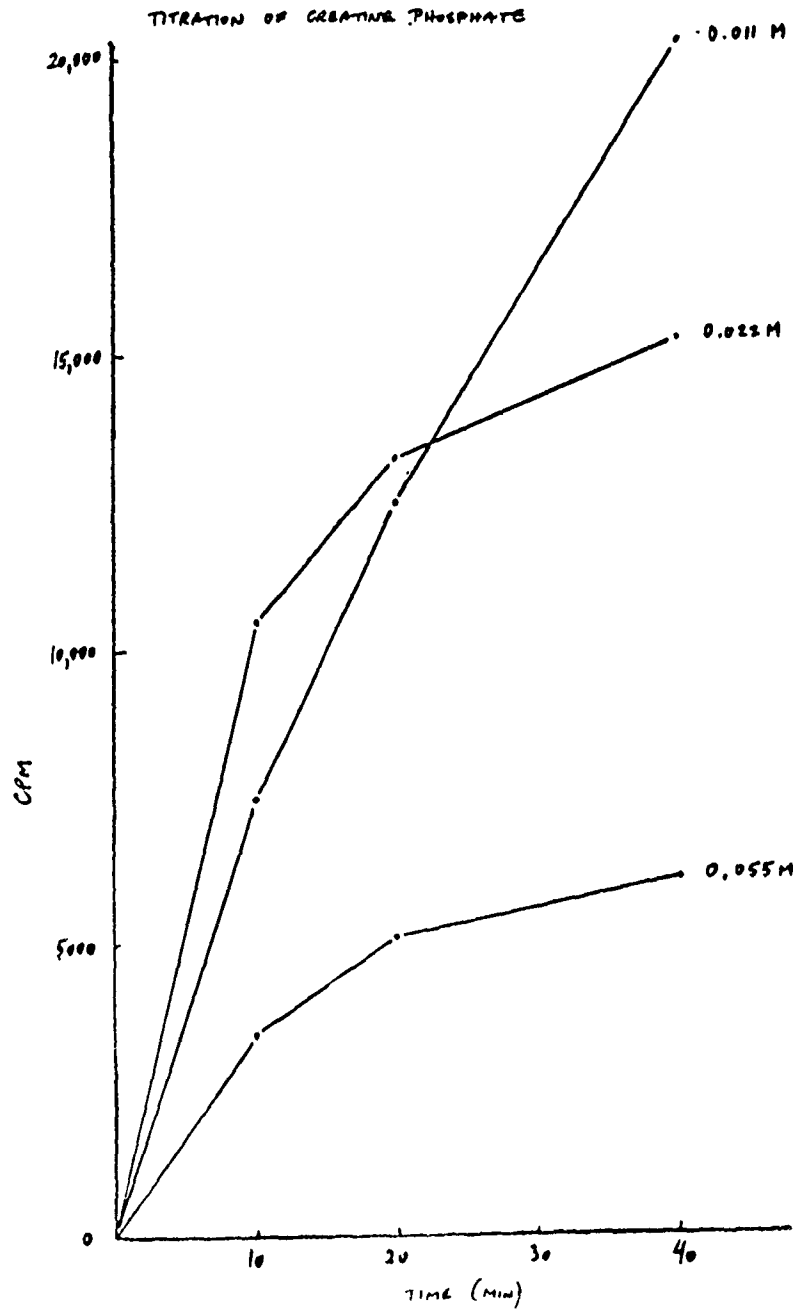

TITRATION OF CREATINE PHOSPHATE
20,000
15,000
10,000
5000
0
CPM
0.011 M
0.022 M
0.055 M
10
20
30
40
TIME (MIN)

FIGURE 10

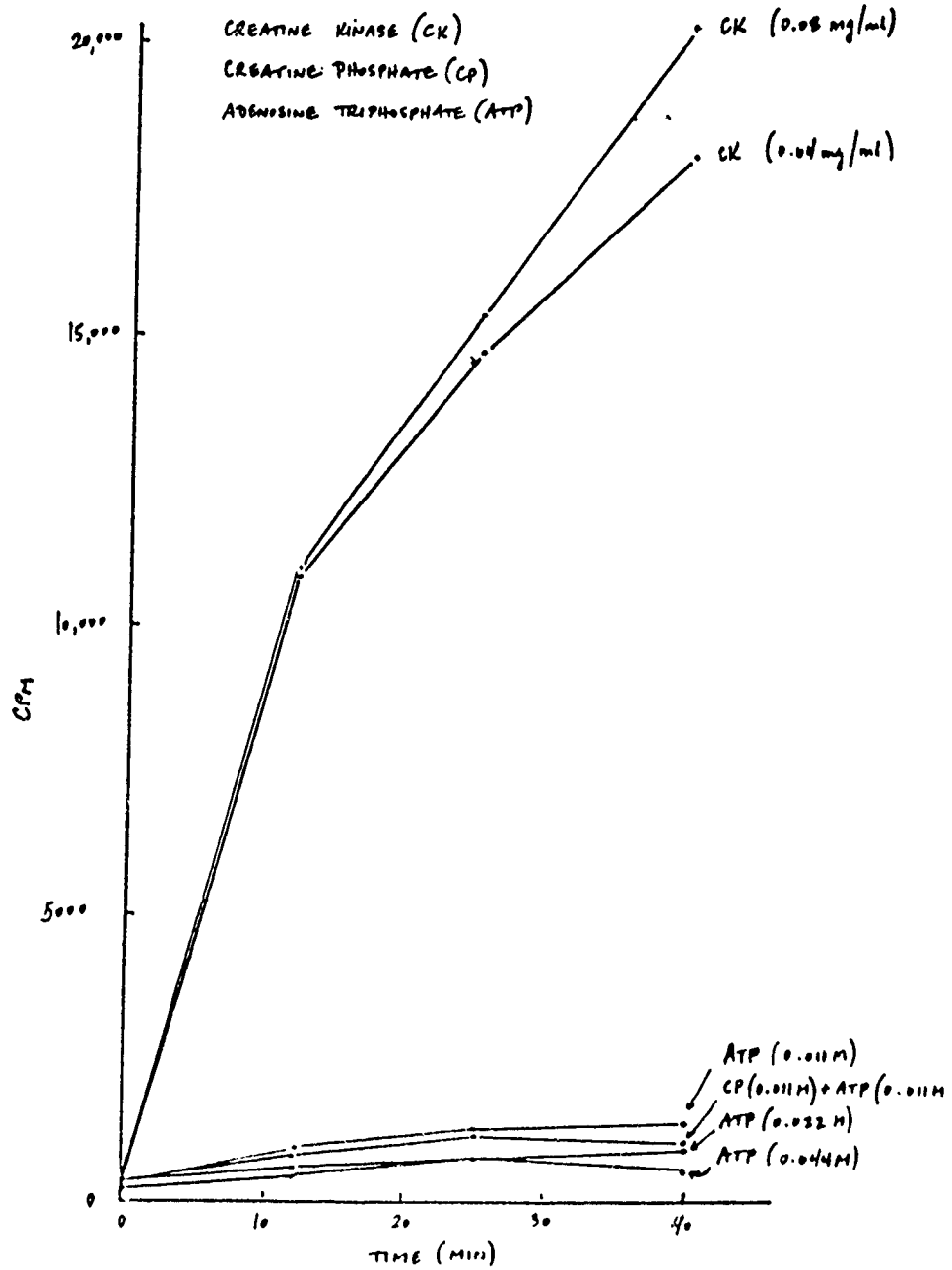

CREATINE KINASE (CK)
CREATINE PHOSPHATE (CP)
ADENOSINE TRIPHOSPHATE (ATP)
CK (0.08 mg/ml)
CK (0.04 mg/ml)
ATP (0.011 M)
CP (0.011 M) + ATP (0.011 M
ATP (0.022 M)
ATP (0.044 M)
CPM
20,000
15,000
10,000
5000
0
0
10
20
30
40
TIME (MIN)

FIGURE 11

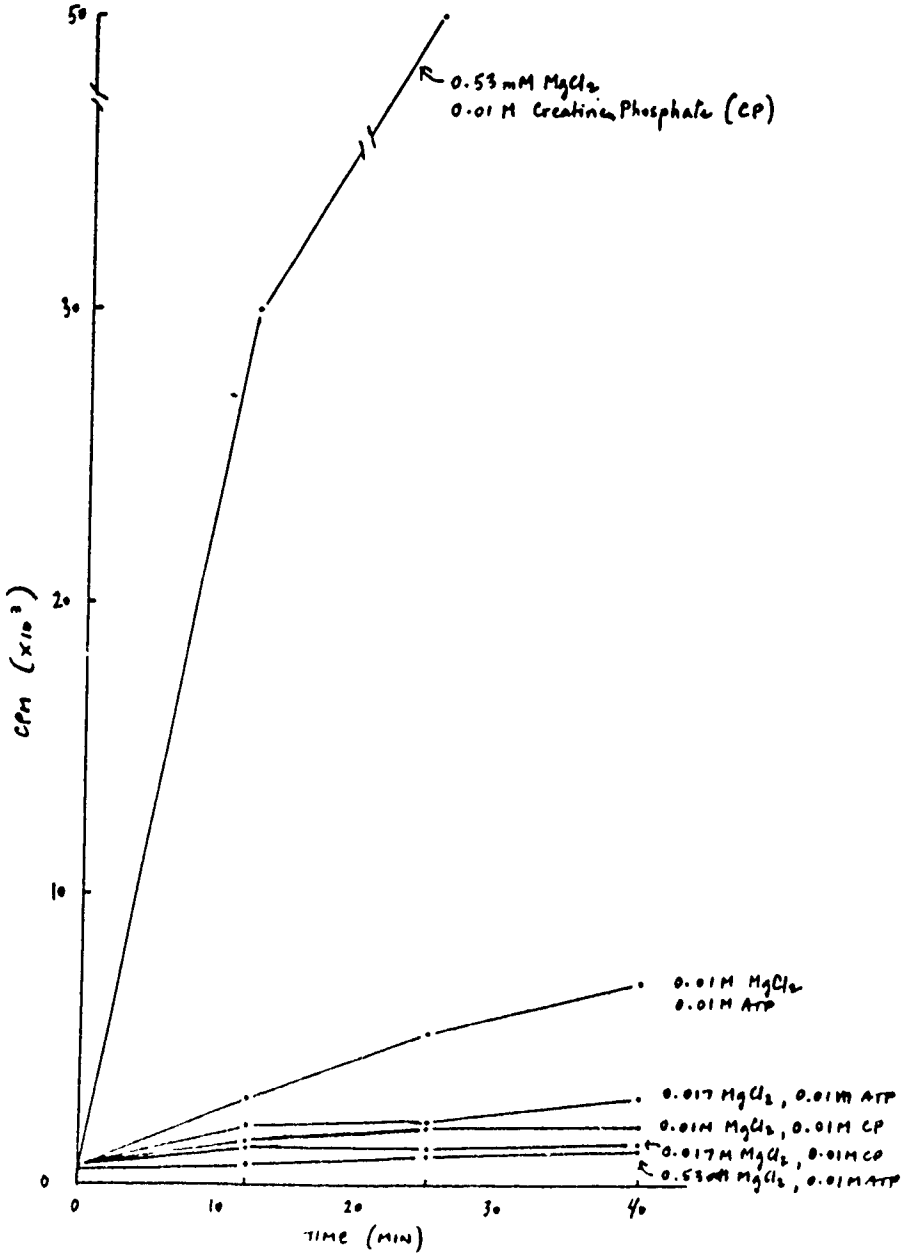

50
30
20
10
0
CPM (x10³)
0.53 mM MgCl₂
0.01 M Creatine Phosphate (CP)
0.01M MgCl₂
0.01M ATP
0.017 MgCl₂, 0.01M ATP
0.01M MgCl₂, 0.01M CP
0.017M MgCl₂, 0.01M CP
0.53mM MgCl₂, 0.01M ATP
0
10
20
30
40
TIME (MIN)

FIGURE 12

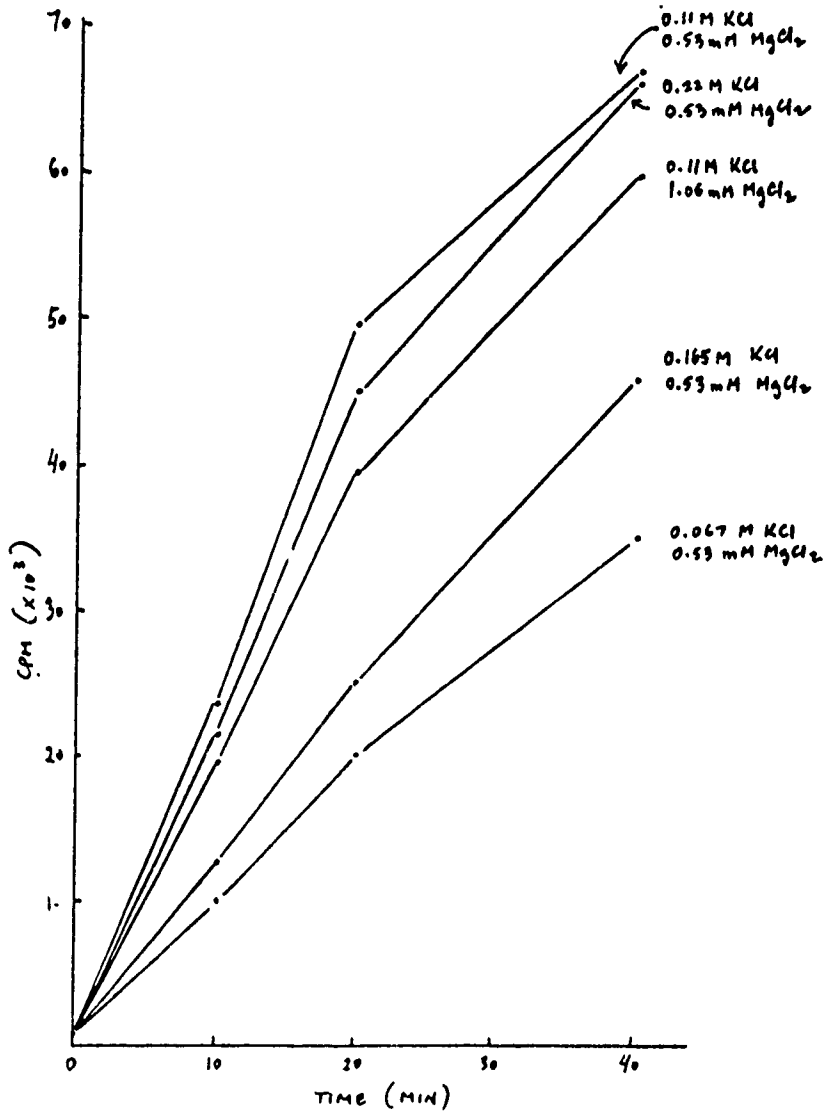

0.11 M KCl
0.53 mM MgCl2
0.22 M KCl
0.53 mM MgCl2
0.11 M KCl
1.06 mM MgCl2
0.165 M KCl
0.53 mM MgCl2
0.067 M KCl
0.53 mM MgCl2
CPM (x10³)
TIME (MIN)
0
10
20
30
40
50
60
70
1
2
3
4

FIGURE 13

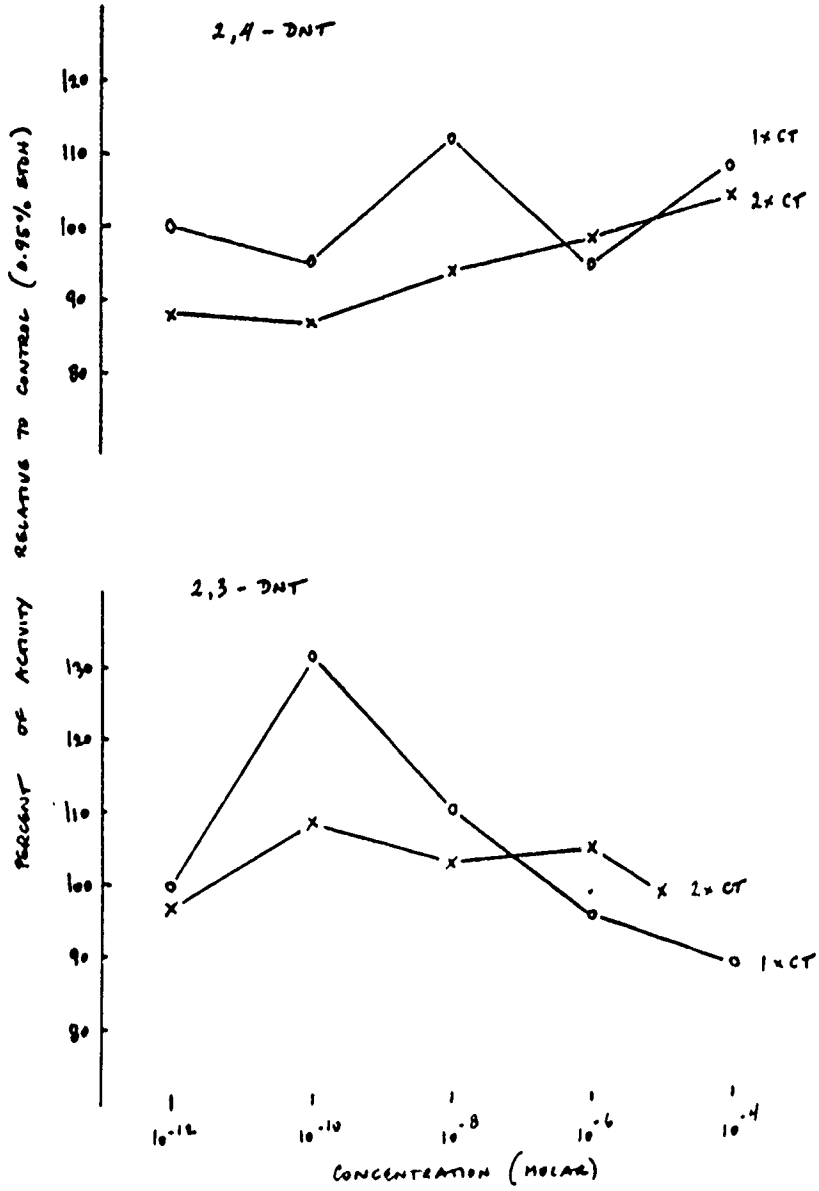

2,4 - DNT
2,3 - DNT
PERCENT OF ACTIVITY RELATIVE TO CONTROL (0.95% EtOH)
120
110
100
90
80
130
1× CT
2× CT
10^{-12}
10^{-10}
10^{-8}
10^{-6}
10^{-4}
CONCENTRATION (MOLAR)

FIGURE 14

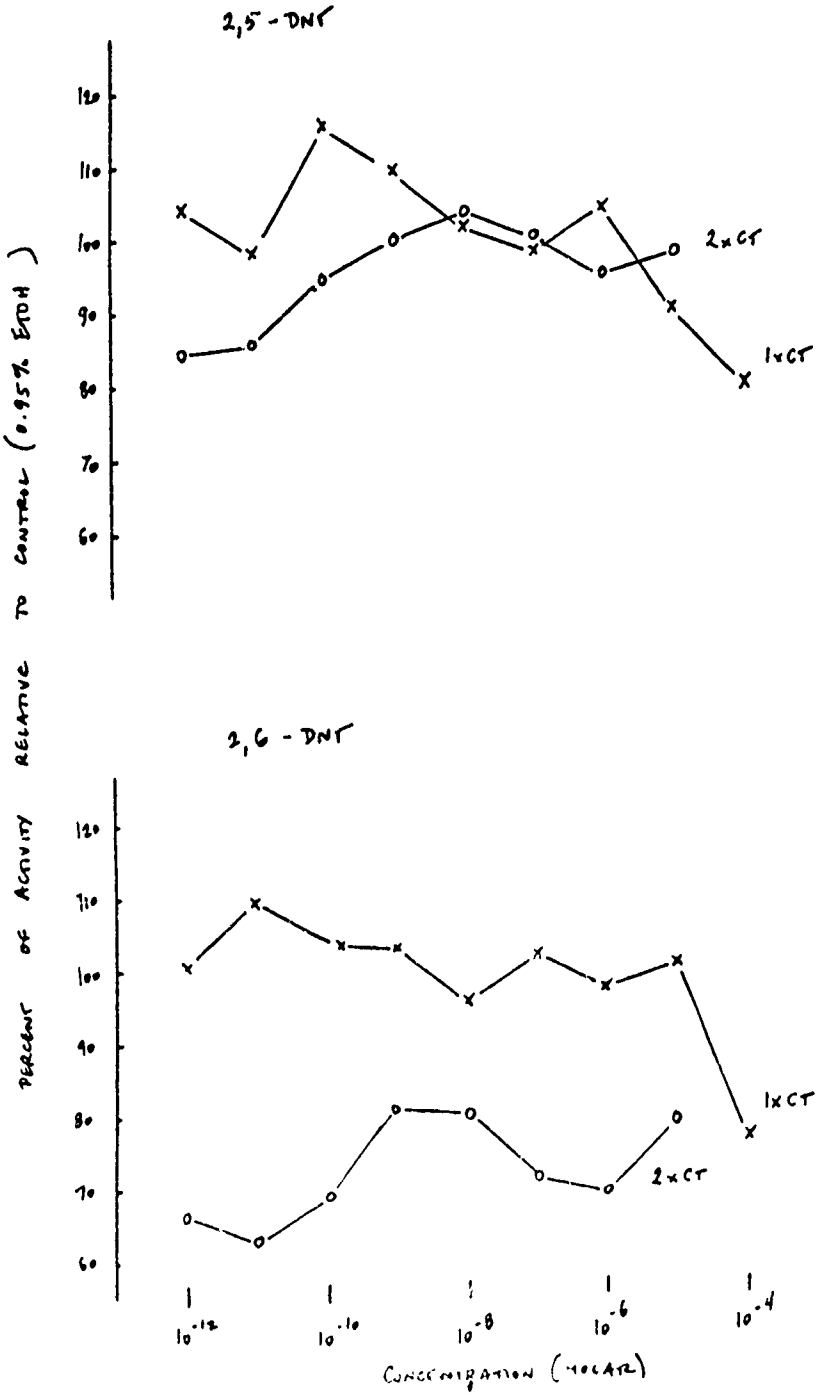

2,5 - DNT
2,6 - DNT
PERCENT OF ACTIVITY RELATIVE TO CONTROL (0.95% ETOH)
120
110
100
90
80
70
60
2xCT
1xCT
1xCT
2xCT
10^{-12}
10^{-10}
10^{-8}
10^{-6}
10^{-4}
CONCENTRATION (MOLAR)

FIGURE 15

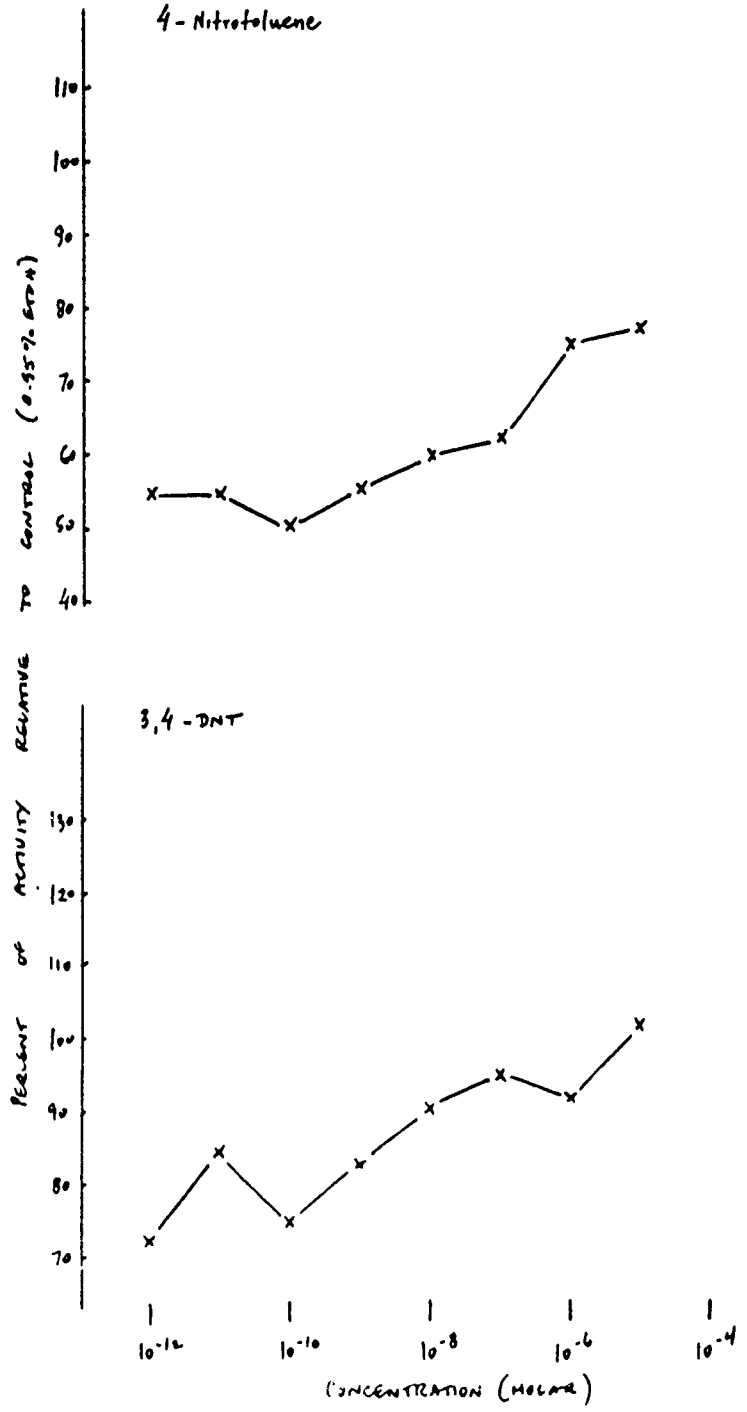

4-Nitrotoluene
110
100
90
80
70
60
50
40
PERCENT OF ACTIVITY RELATIVE TO CONTROL (0.95% EtOH)
3,4-DNT
130
120
110
100
90
80
70
10^{-12}
10^{-10}
10^{-8}
10^{-6}
10^{-4}
CONCENTRATION (MOLAR)

FIGURE 16

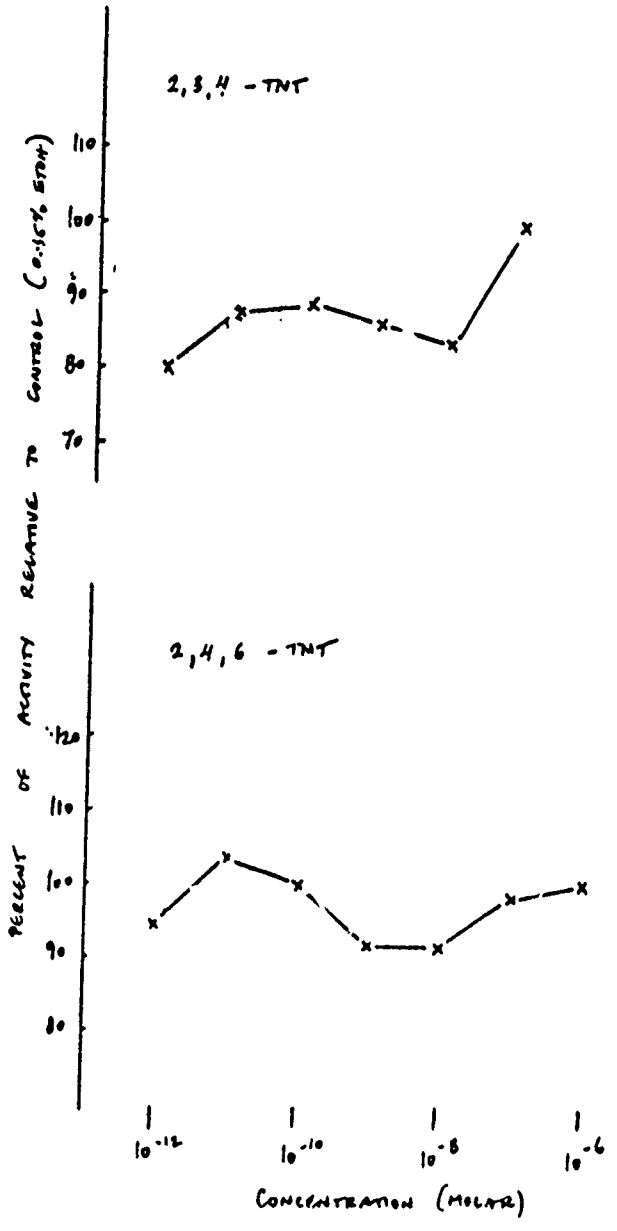
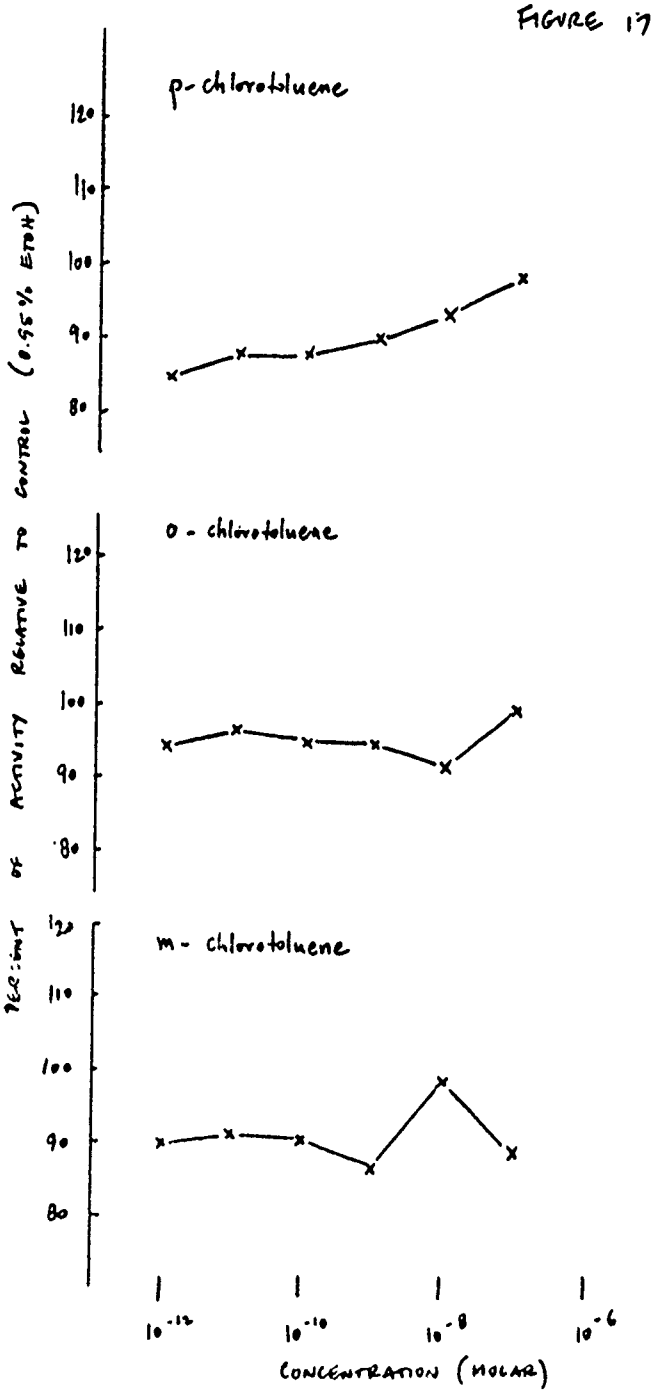

2,3,4 - TNT
2,4,6 - TNT
PERCENT OF ACTIVITY RELATIVE TO CONTROL (0.15% EtOH)
110
100
90
80
70
120
110
100
90
80
10^{-12}
10^{-10}
10^{-8}
10^{-6}
CONCENTRATION (MOLAR)

FIGURE 17


p-chlorotoluene
o-chlorotoluene
m-chlorotoluene
PERCENT OF ACTIVITY RELATIVE TO CONTROL (0.95% ETOH)
120
110
100
90
80
10^{-12}
10^{-10}
10^{-8}
10^{-6}
CONCENTRATION (MOLAR)

FIGURES 18 & 19

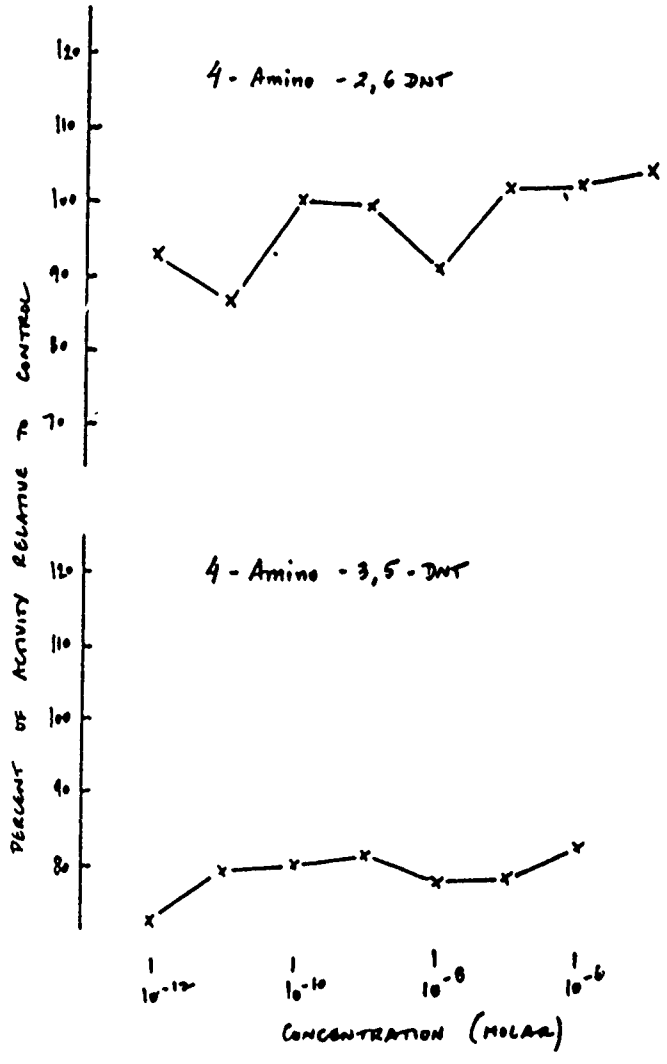


FIGURE 20

PERCENT OF CONTROL AFTER 40 MINUTE INCUBATION

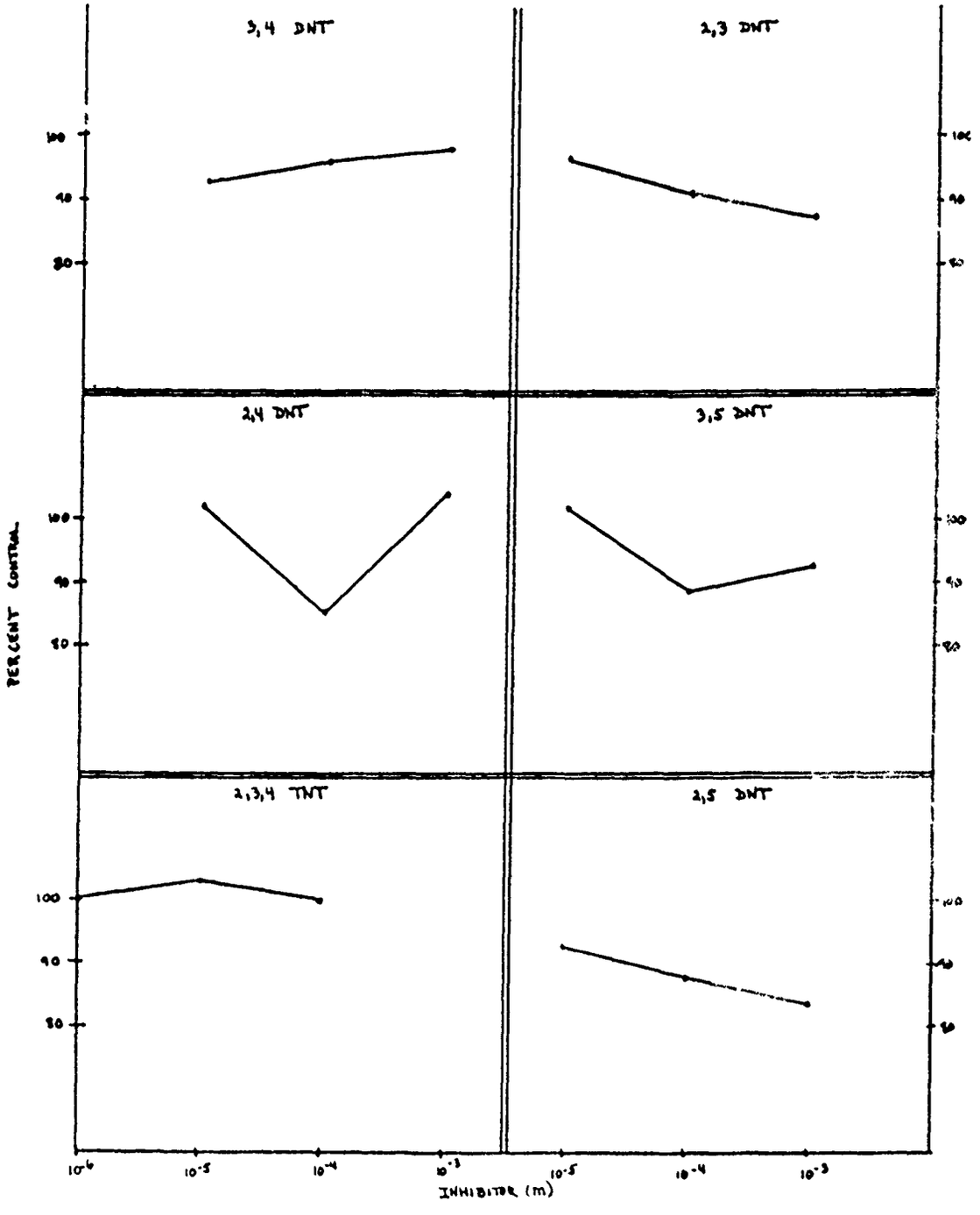


FIGURE 21

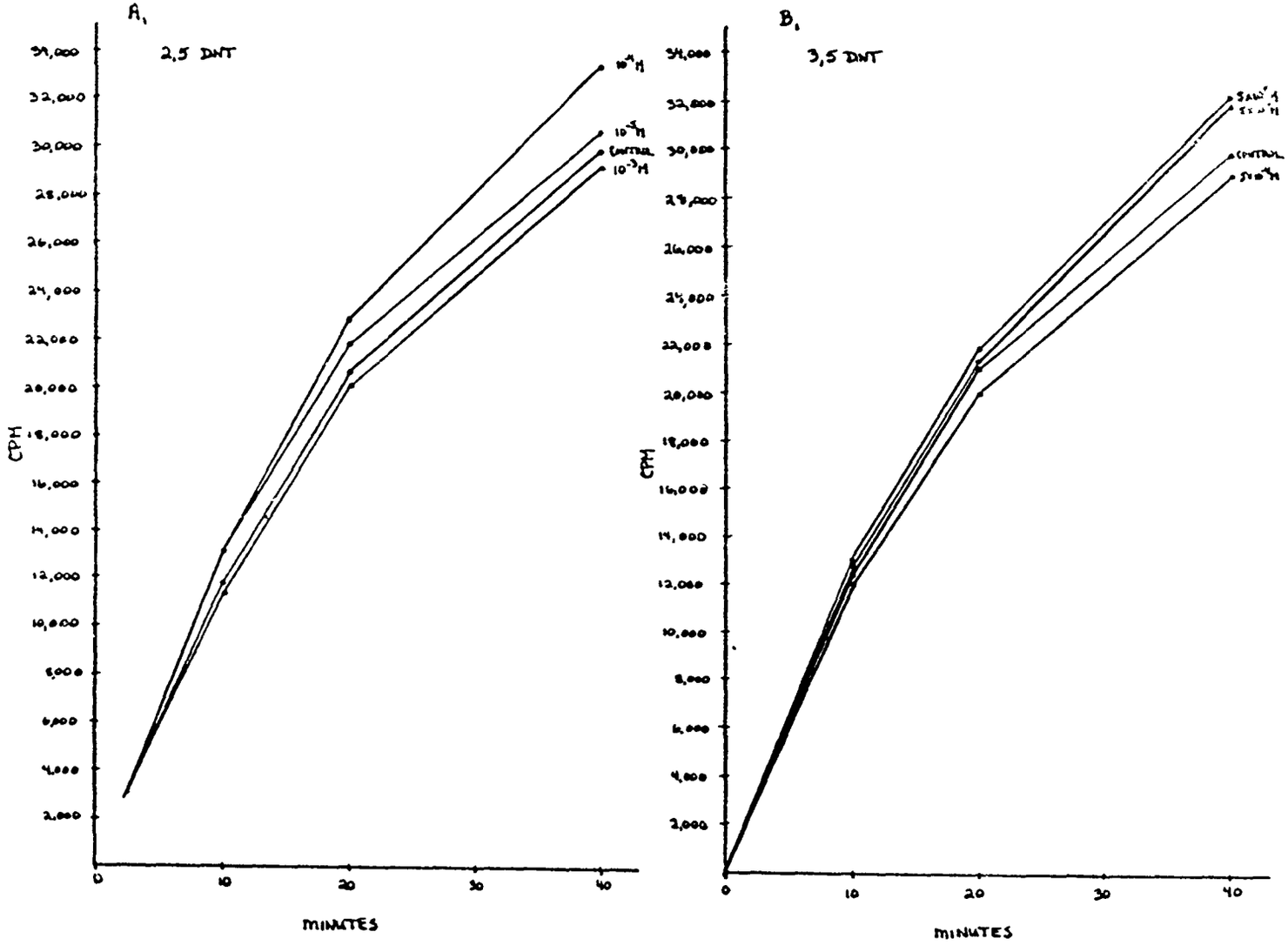


FIGURE 22

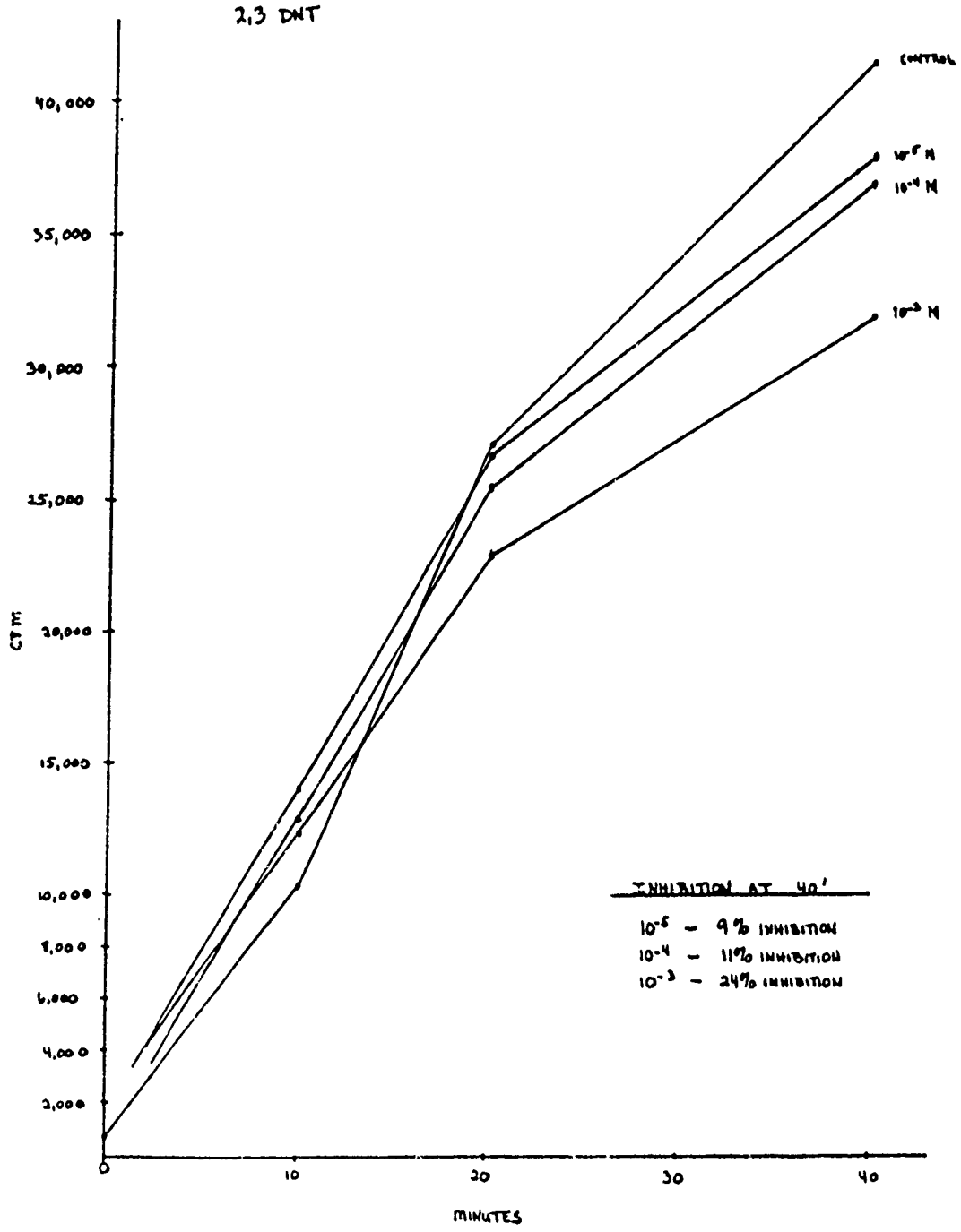

2,3 DNT
CONTROL
10⁻⁵ M
10⁻⁴ M
10⁻³ M
40,000
35,000
30,000
25,000
20,000
15,000
10,000
8,000
6,000
4,000
2,000
CPM
0
10
20
30
40
MINUTES
INHIBITION AT 40'
10⁻⁵ – 9% INHIBITION
10⁻⁴ – 11% INHIBITION
10⁻³ – 24% INHIBITION

FIGURE 23

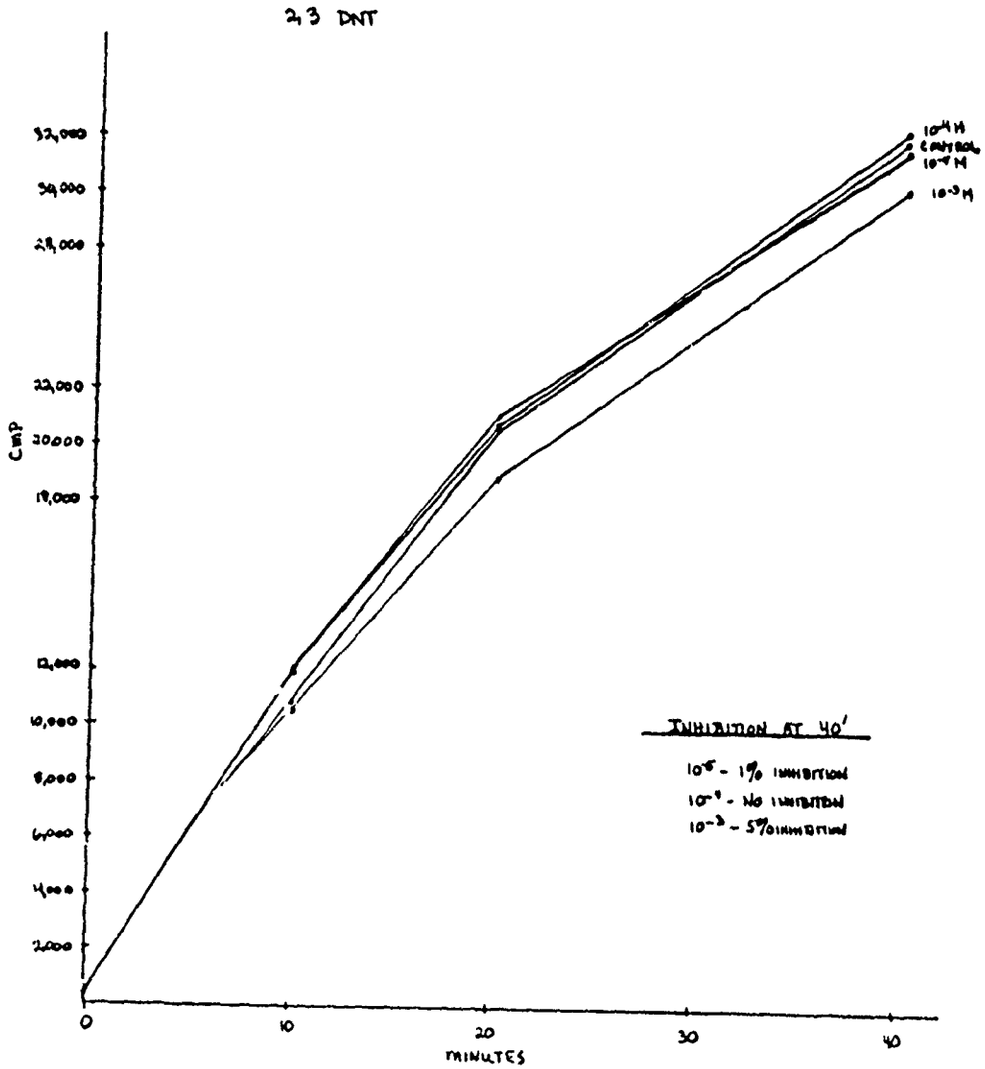

2,3 DNT
32,000
30,000
28,000
22,000
20,000
18,000
12,000
10,000
8,000
6,000
4,000
2,000
CmP
0
10
20
30
40
MINUTES
10⁻⁴ M
CONTROL
10⁻⁶ M
10⁻³ M
INHIBITION AT 40'
10⁻⁶ - 1% INHIBITION
10⁻⁴ - NO INHIBITION
10⁻³ - 5% INHIBITION

FIGURE 24

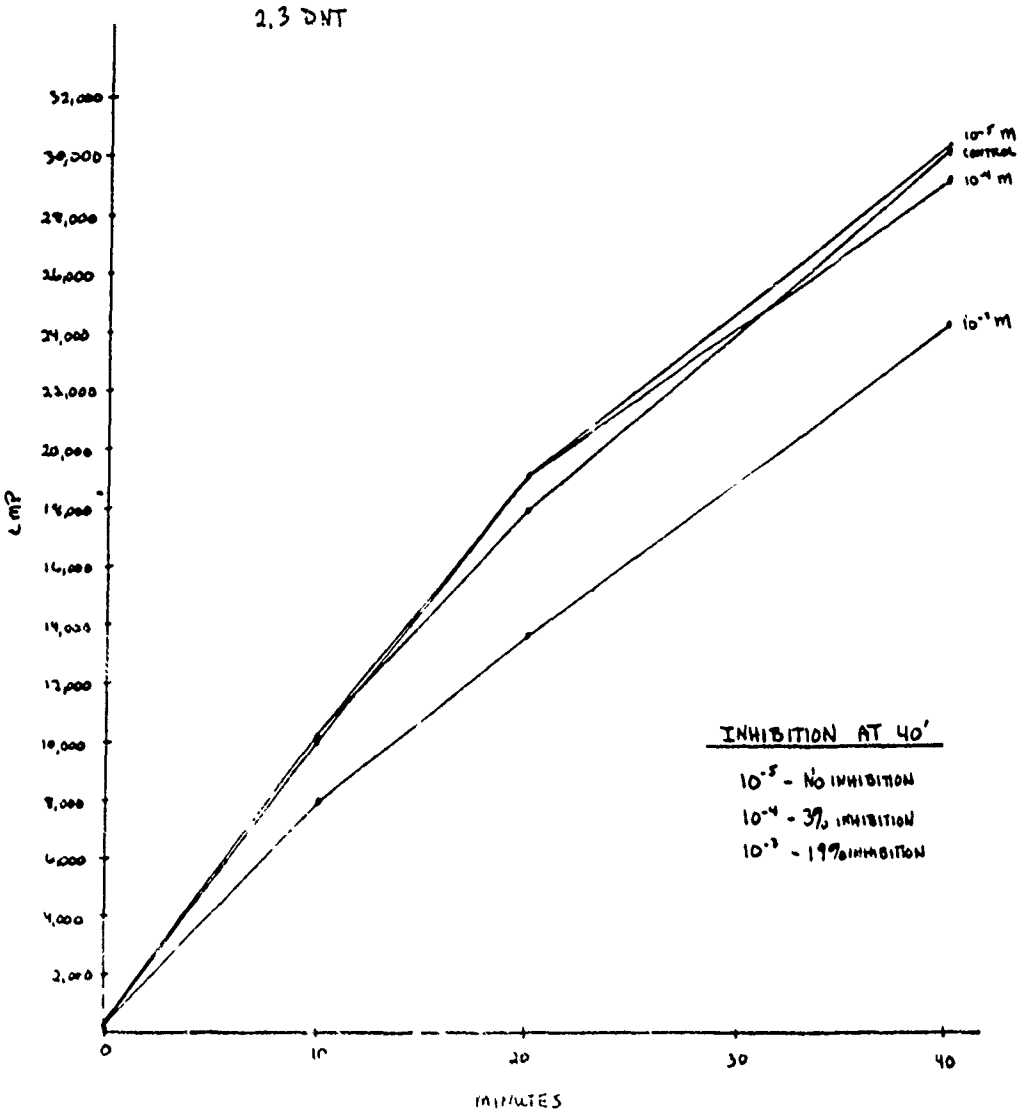

2,3 DNT
32,000
30,000
28,000
26,000
24,000
22,000
20,000
18,000
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
CMP
0
10
20
30
40
MINUTES
10^{-5} M
CONTROL
10^{-4} M
10^{-3} M
INHIBITION AT 40'
10^{-5} - NO INHIBITION
10^{-4} - 3% INHIBITION
10^{-3} - 19% INHIBITION

FIGURE 25

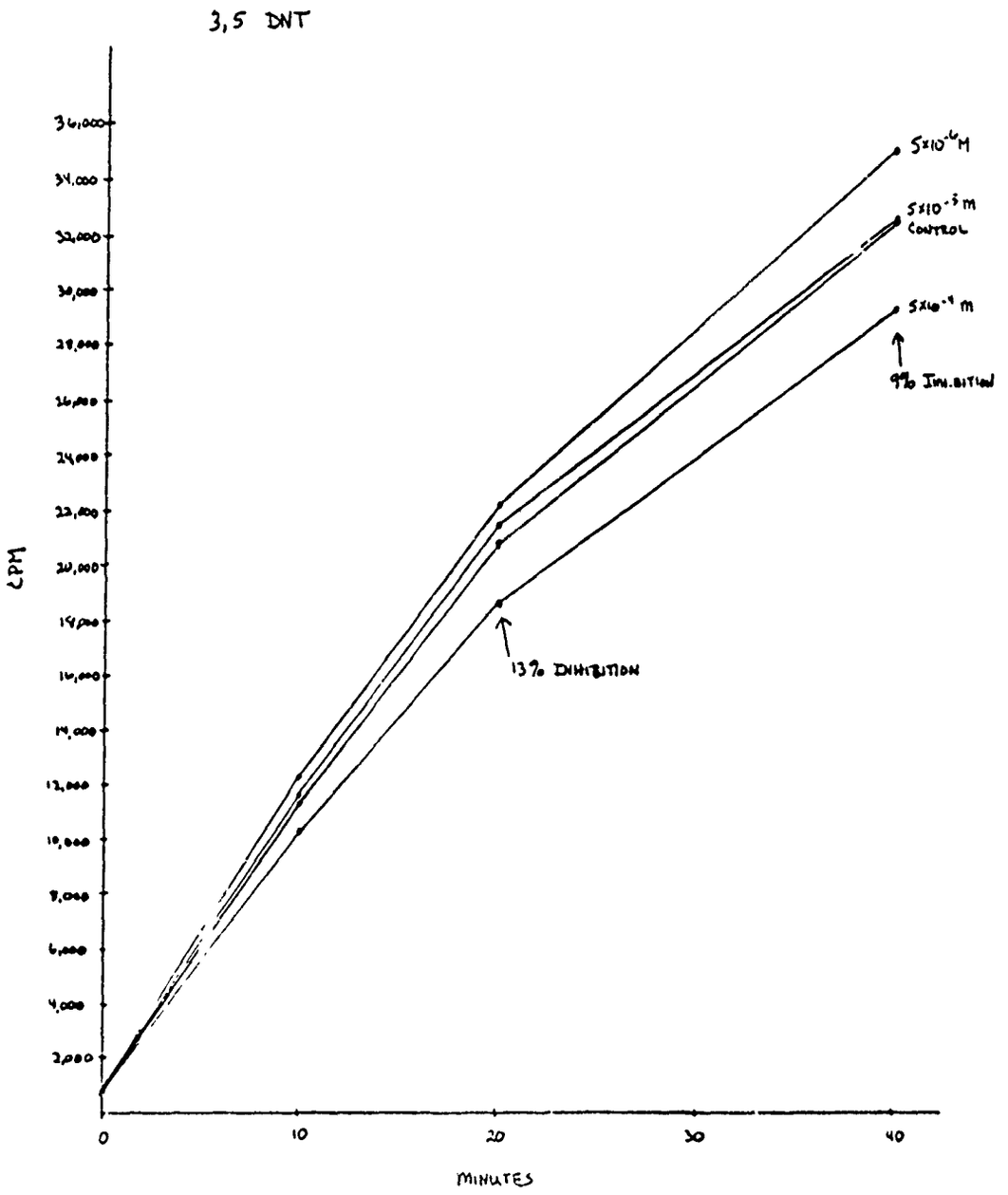

3,5 DNT
CPM
36,000
34,000
32,000
30,000
28,000
26,000
24,000
22,000
20,000
18,000
16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
0
10
20
30
40
MINUTES
5×10^{-6}M
5×10^{-5}M
CONTROL
5×10^{-4}M
9% INHIBITION
13% INHIBITION

FIGURE 26

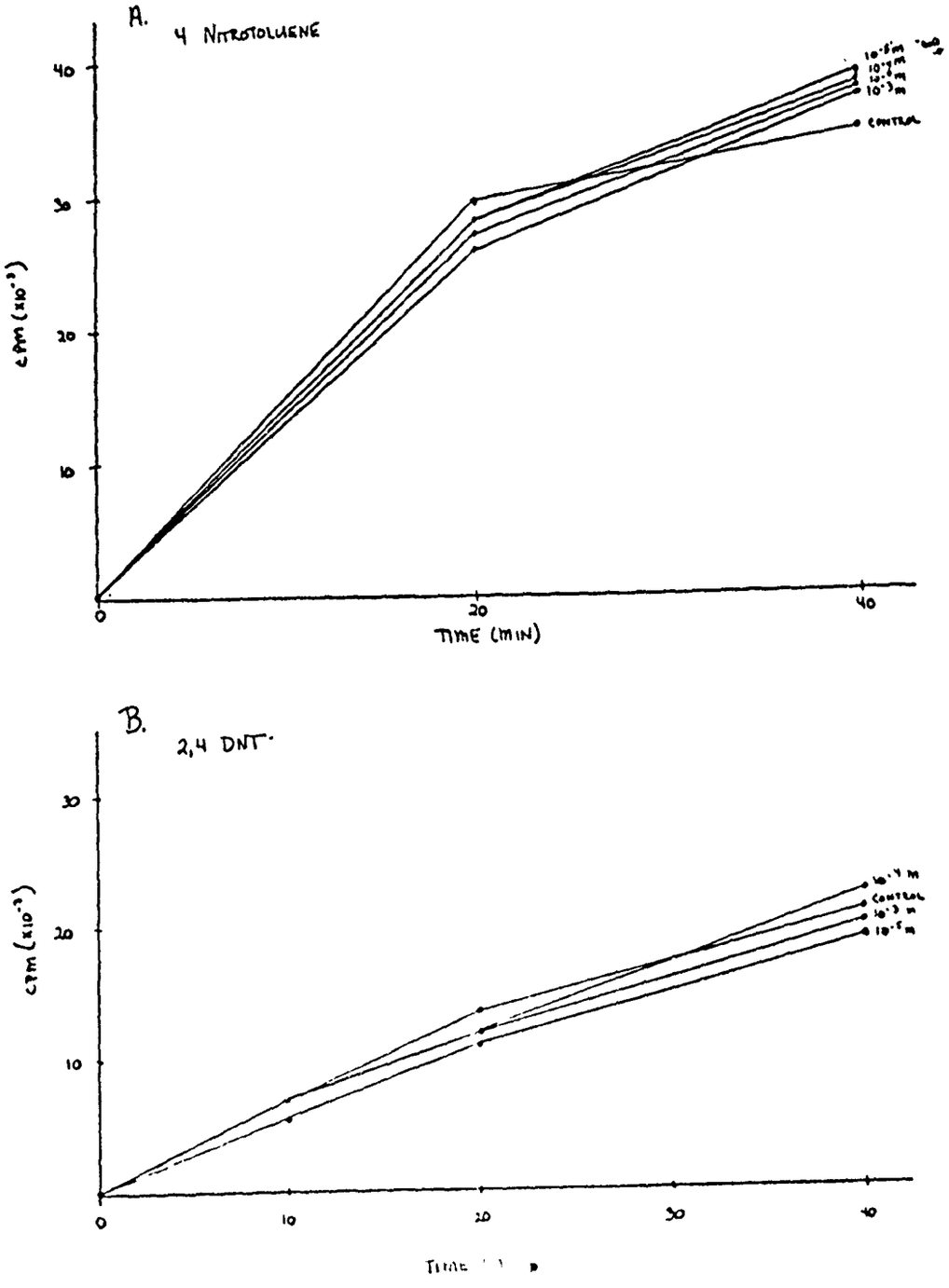

A.
4 NITROTOLUENE
40
30
20
10
CPM (x10⁻³)
0
20
40
TIME (MIN)
CONTROL
B.
2,4 DNT
30
20
10
CPM (x10⁻³)
0
10
20
30
40
CONTROL

FIGURE 27

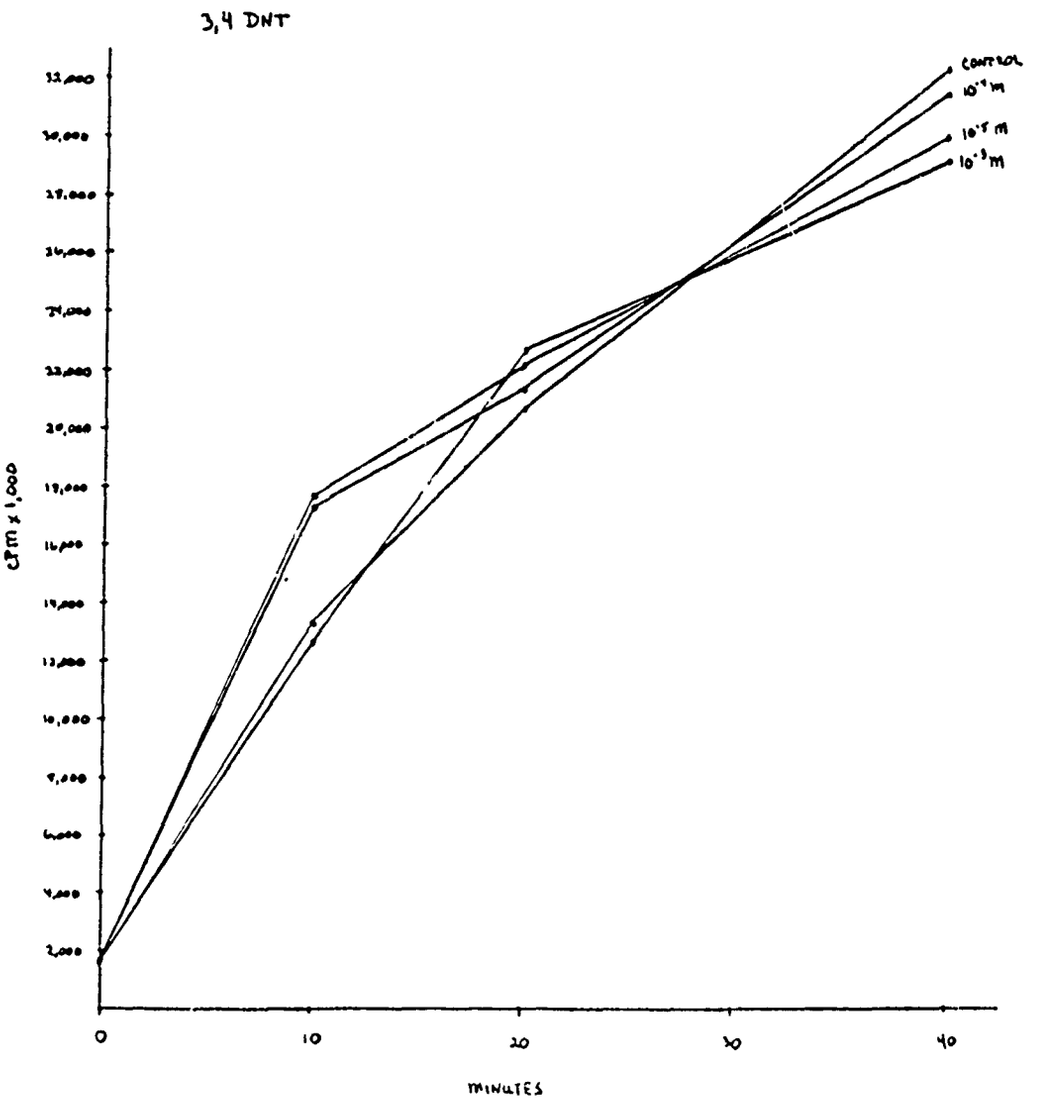


FIGURE 28

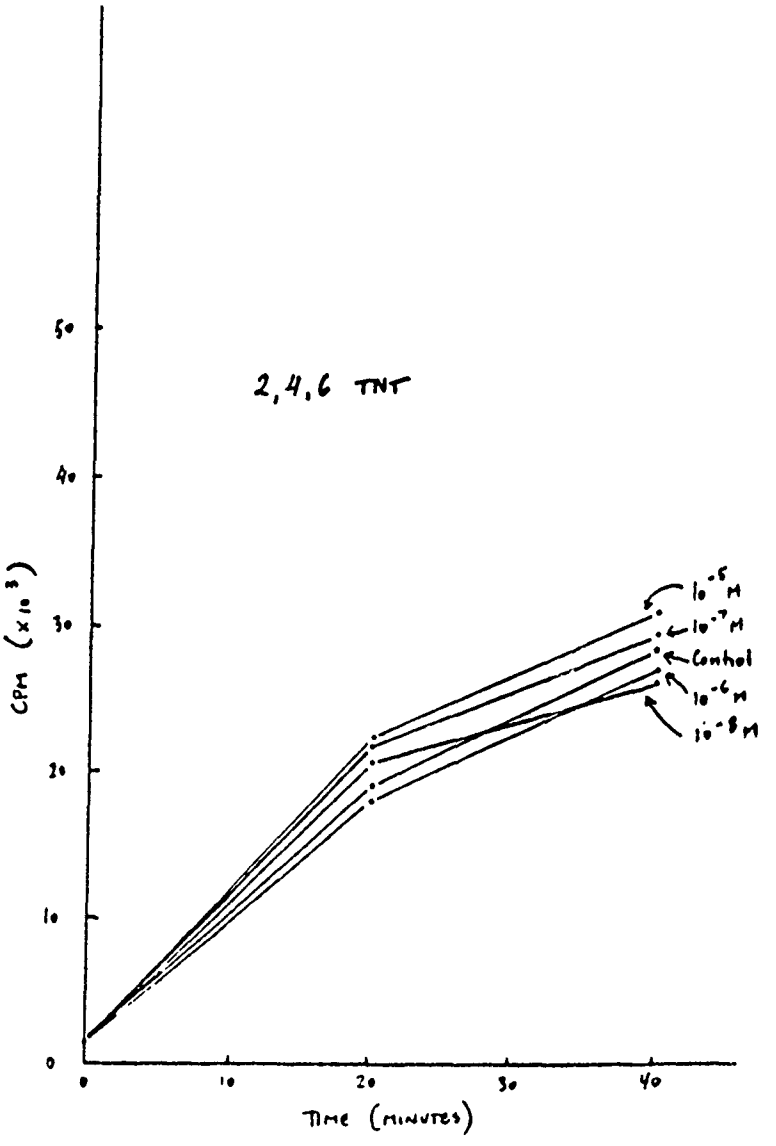

2,4,6 TNT
CPM (x10³)
50
40
30
20
10
0
0
10
20
30
40
TIME (MINUTES)
10⁻⁵M
10⁻⁷M
Control
10⁻⁶M
10⁻⁸M

FIGURE 29

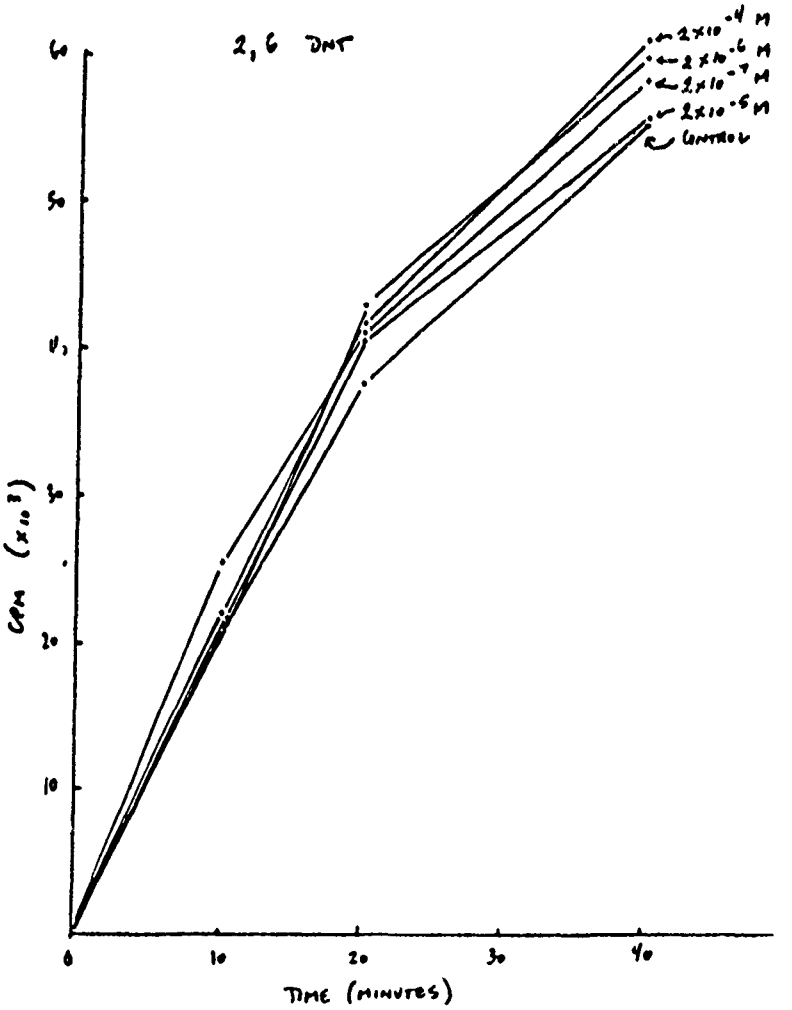

2,6 DNT
2x10-4 M
2x10-6 M
2x10-7 M
2x10-8 M
CONTROL
CPM (x10²)
60
50
40
30
20
10
0
10
20
30
40
TIME (MINUTES)

FIGURE 30.

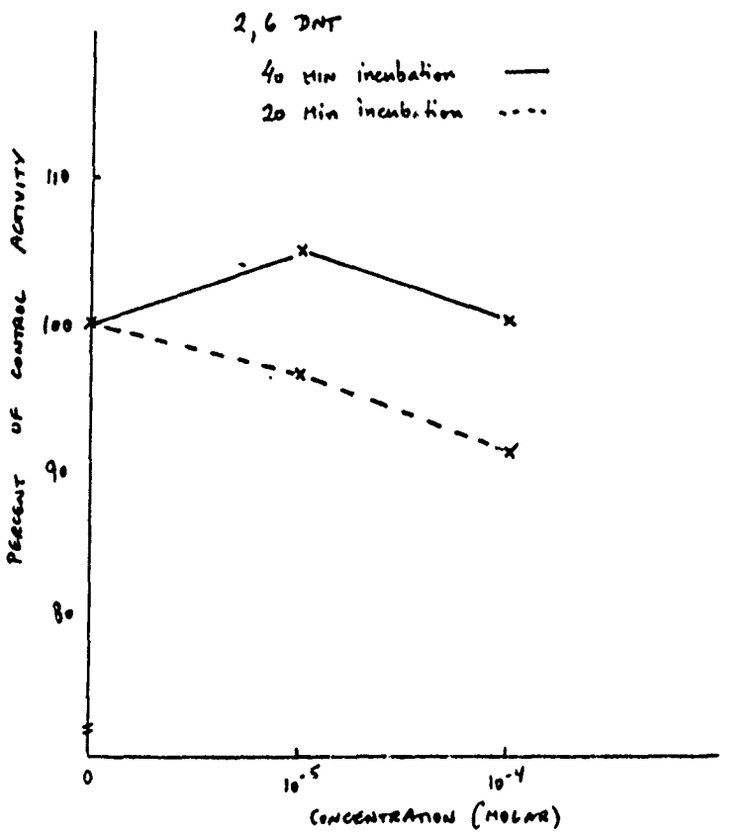
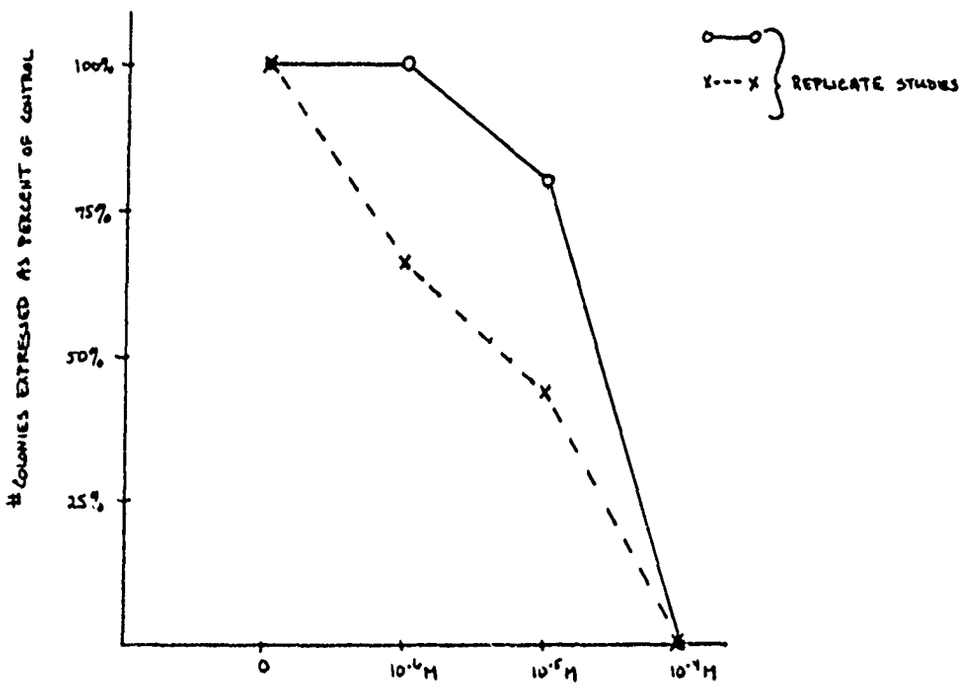

2, 6 DNT
40 MIN incubation
20 MIN incubation
PERCENT OF CONTROL ACTIVITY
110
100
90
80
0
10^{-5}
10^{-4}
CONCENTRATION (MOLAR)

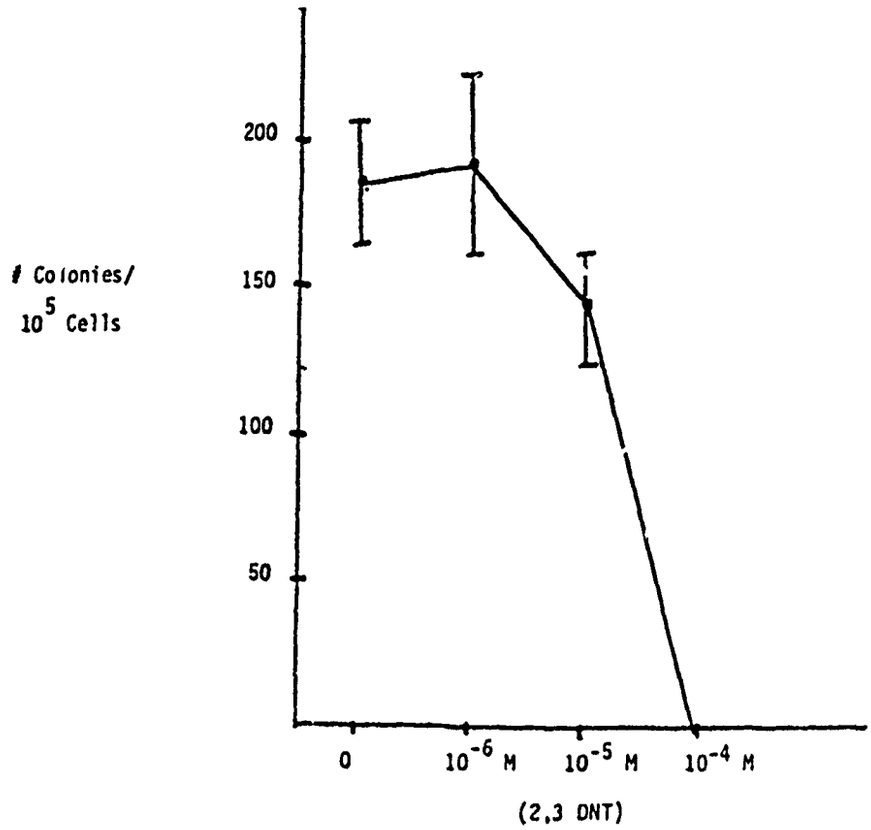
FIGURE 31-A



2, 3 - DNT

FIGURE 31B

Effect of Concentration of 2,3 DNT
on Erythroid Colony Number

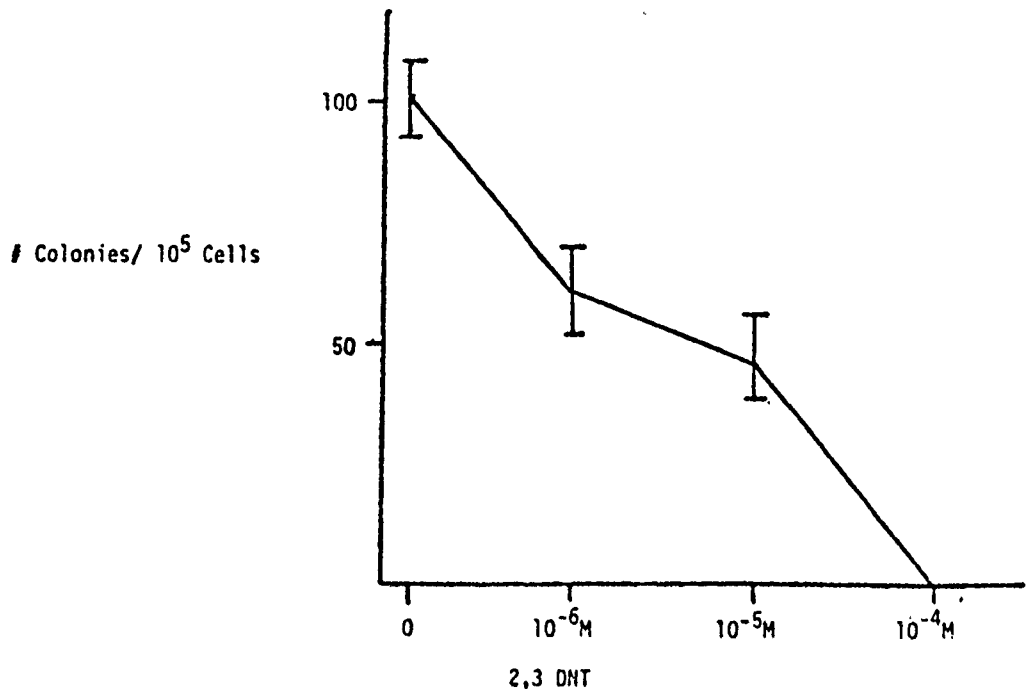


final (2,3 DNT)	# Colonies/10^5 Cells	
0	187 (± 20)	
10^{-6} M	194 (± 30)	
10^{-5} M	147 (± 18)	21% inhib
10^{-4} M	0	100% inhib

[a] Value = mean (± S.E.M.) of three replicate cultures.

FIGURE 31C

Effect of 2,3 DNT Concentration
on Erythroid Colony Formation



2,3 DNT	# Colonies/ 10^5 Cells
----	102 (± 6)
10^{-6}M	67 (± 10) 34% inhibition
10^{-5}M	45 (± 8) 56% inhibition
10^{-4}M	0 (± 0) 100% inhibition

FIGURE 32A

Effect of 2,4 DNT on Erythroid
Colony Number

final (2,4 DNT)	# Colonies/10^5 Cells	
-	320 (± 34)[a]	
10^{-6} M	284 (± 39)	11% inhib
10^{-5} M	271 (± 32)	15% inhib
10^{-4} M	255 (± 16)	20% inhib

[a] Value = mean (± S.E.M.) of three replicate cultures

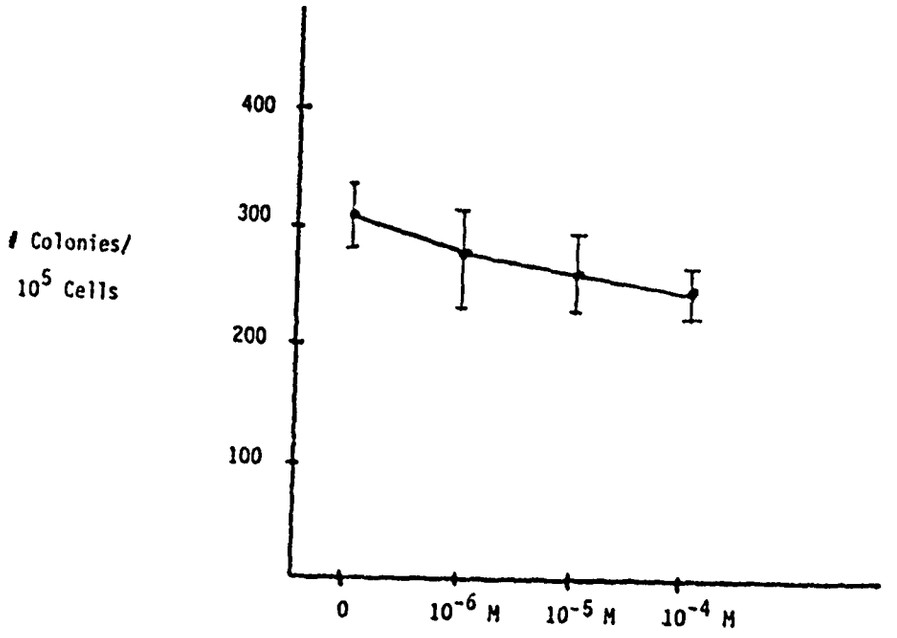
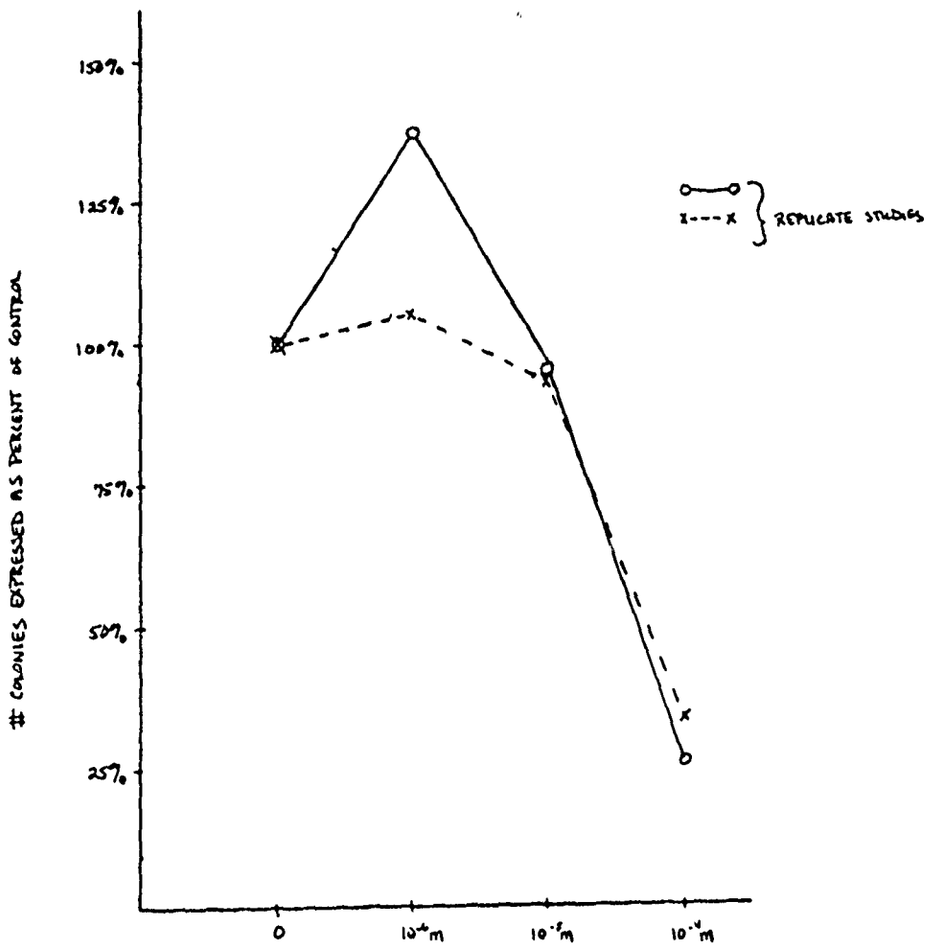


FIGURE 33-A



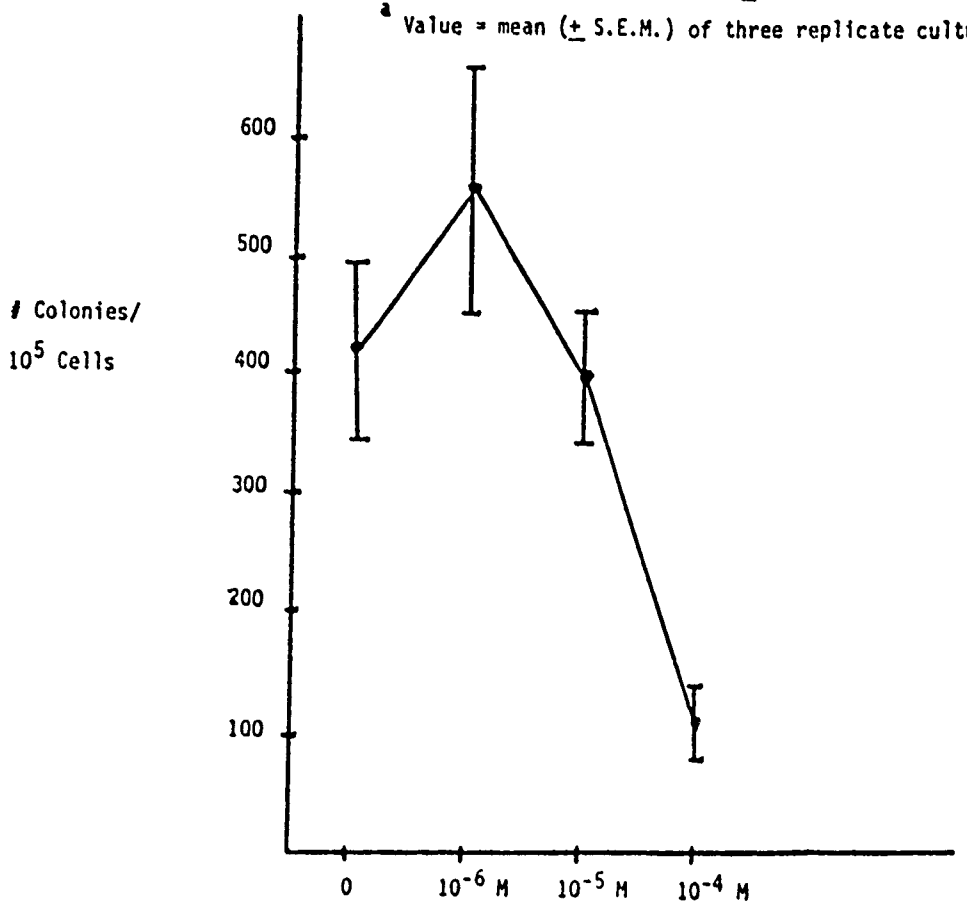
2,5 - DNT

FIGURE 33B

Effect of Concentration of 2,5 DNT
on Erythroid Colony Number

(2,5 DNT)	# Colonies/10^5 Cells
-	422 (± 74)[a]
10^{-6} M	563 (± 118)
10^{-5} M	401 (± 54)
10^{-4} M	108 (± 30) 74% inhib

[a] Value = mean (± S.E.M.) of three replicate cultures

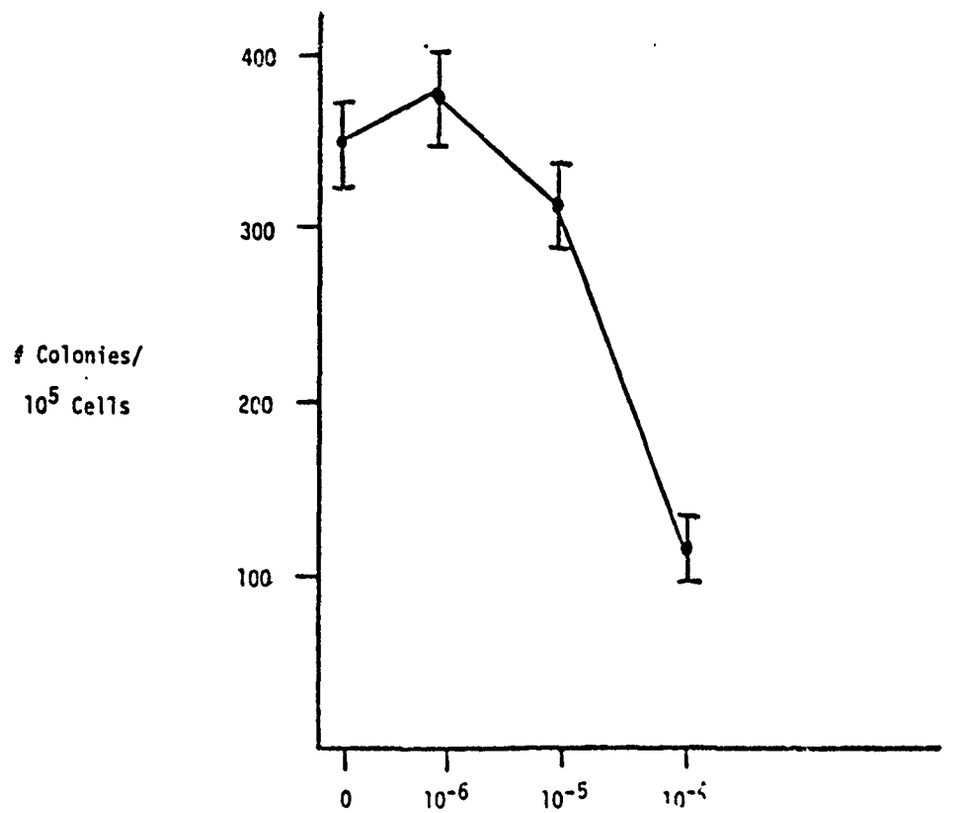


Effect of 2,5 DNT

on

Erythroid Colony Number

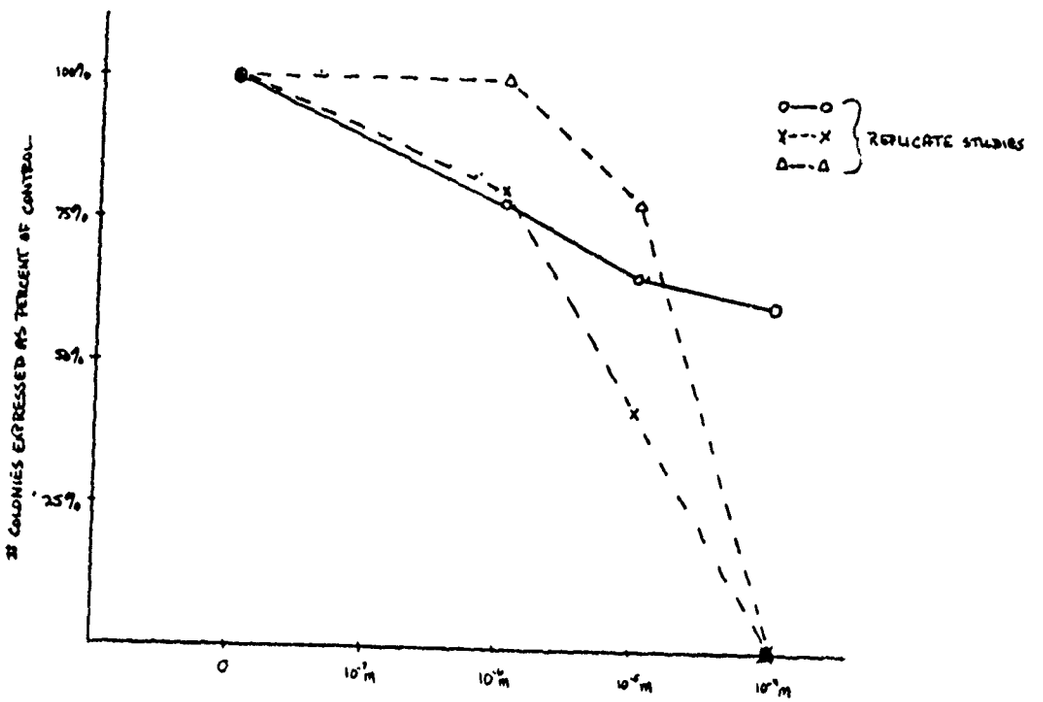
FIGURE 33C



Final (2.5 DNT)	# Colonies/10^5 Cells
-	348 (± 64)[a]
10^{-6}	369 (± 82)
10^{-5}	324 (± 47)
10^{-4}	119 (± 21)

[a]Value = mean (± S.E.M.) of three replicate cultures

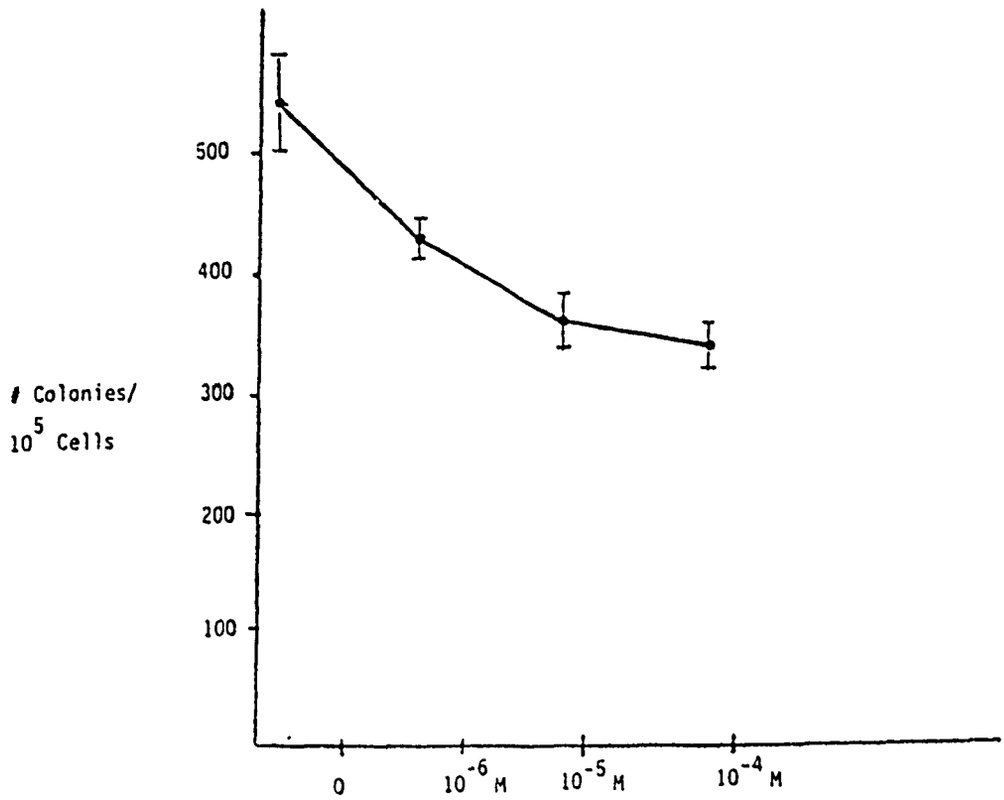
FIGURE 34-A



2,6 - DNT

FIGURE 34B

Effect of Concentration of 2,6 DNT on Colony Number



(2,6 DNT)	No. of Colonies/10^5 cells (initial inoculum)	
0	550 (± 42)[a]	
10^{-6} M	433 (± 13)	21% inhibition
10^{-5} M	365 (± 15)	34% inhibition
10^{-4} M	339 (± 19)	38% inhibition

[a] Value = mean (± S.E.M.) of 3 replicate cultures

FIGURE 34C

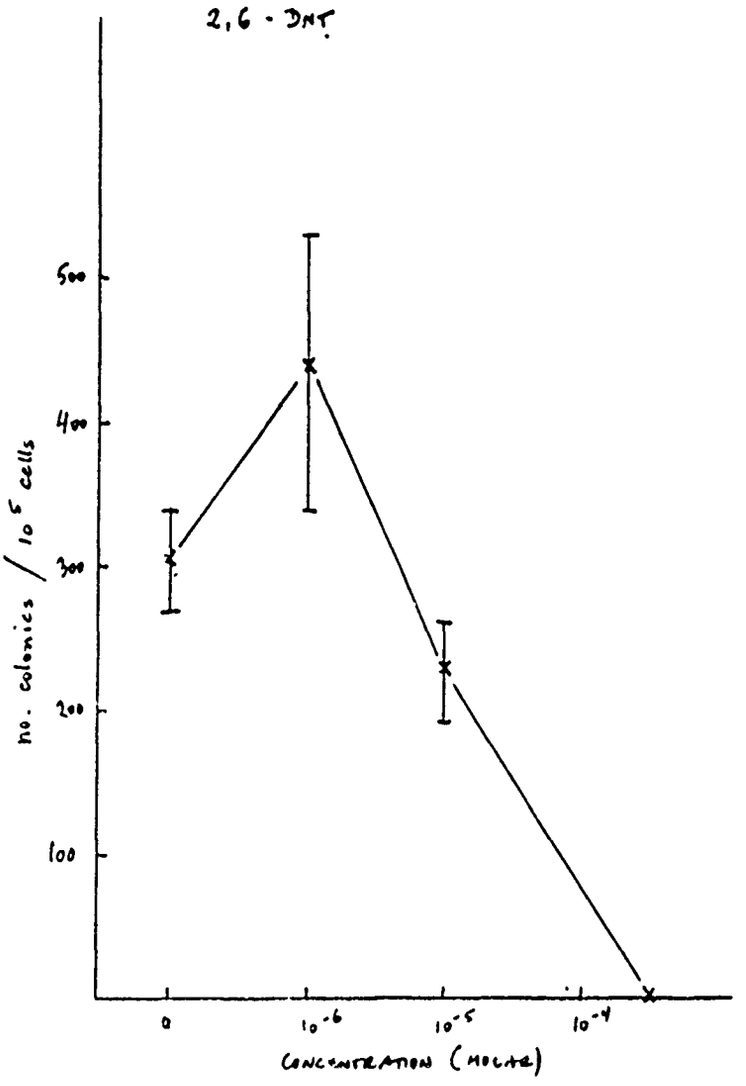

2,6 - DNT
500
400
300
200
100
no. colonies / 10^5 cells
0
10^{-6}
10^{-5}
10^{-4}
CONCENTRATION (MOLAR)

FIGURE 34D

2,6 - TAT

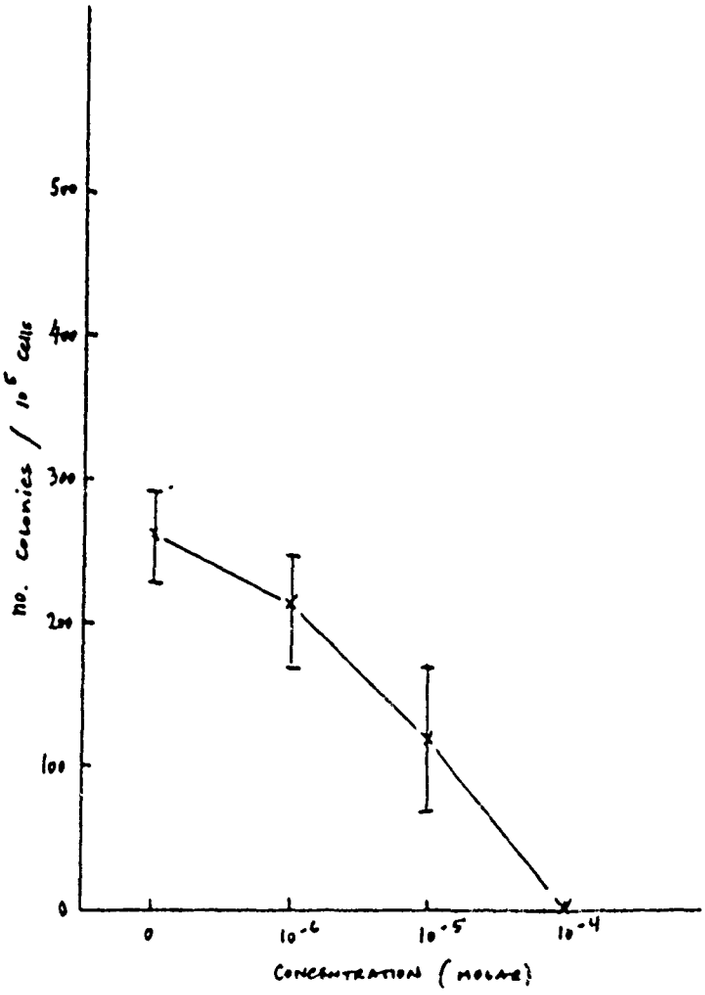
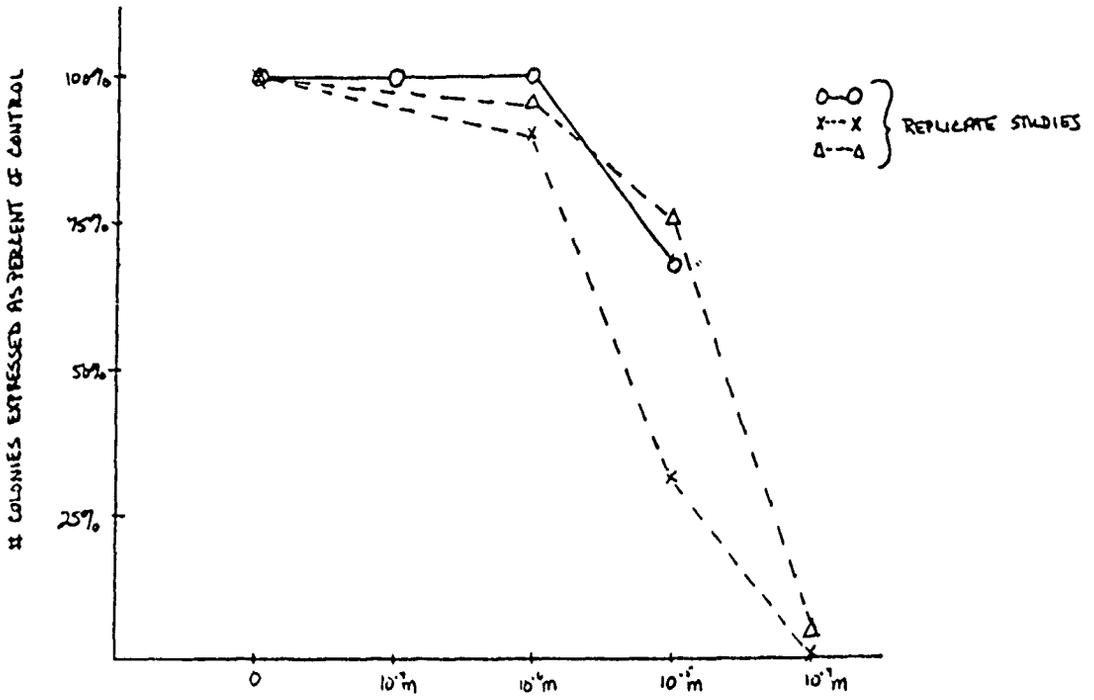


FIGURE 35-A



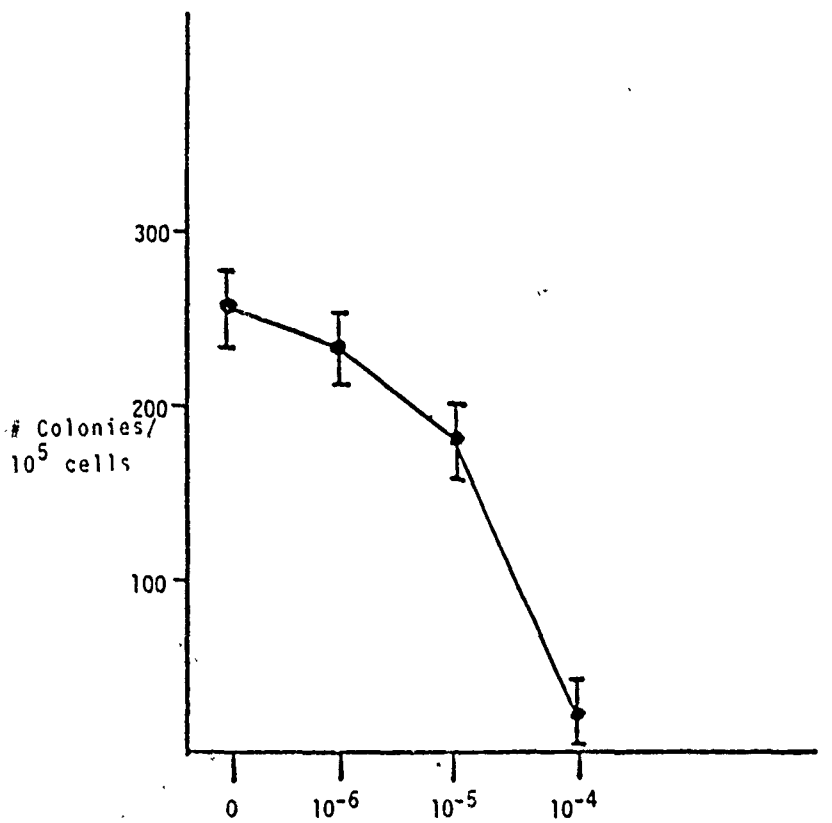
3,4 - DNT

Effect of 3,4 DNT

on

Erythroid Colony Number

FIGURE 35B

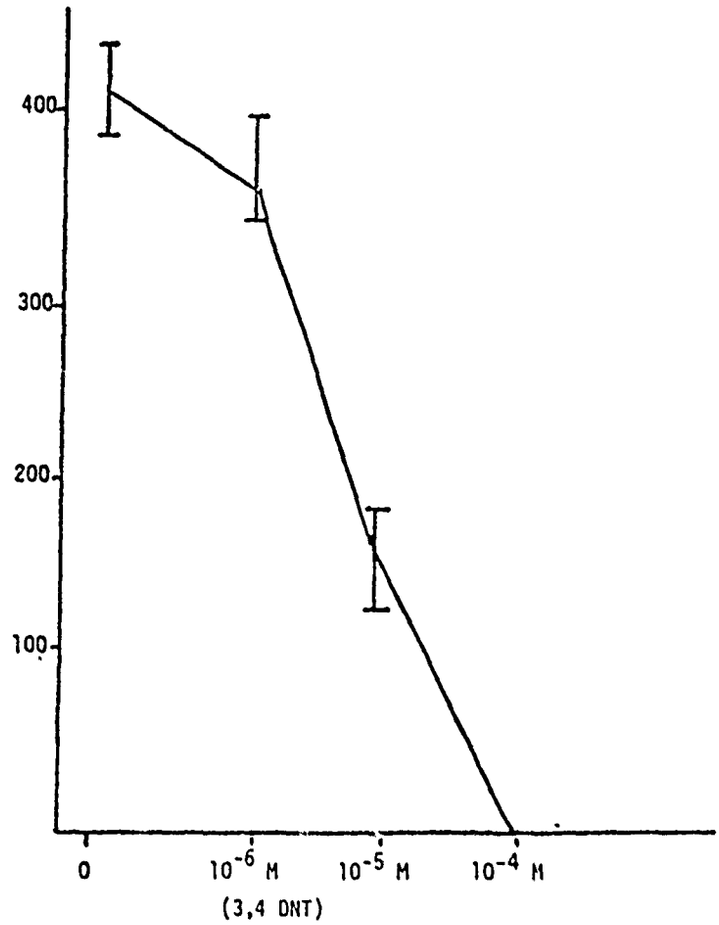


Final (3,4 DNT)	# Colonies/10^5 Cells
-	256 (± 48)[a]
10^{-6}	243 (± 51)
10^{-5}	195 (± 36)
10^{-4}	14 (± 8)

[a] Value = mean (± S.E.M.) of three replicate cultures

FIGURE 35C

Effect of the Concentration of 3,4 DNT on the Growth of Erythropoietic Colonies

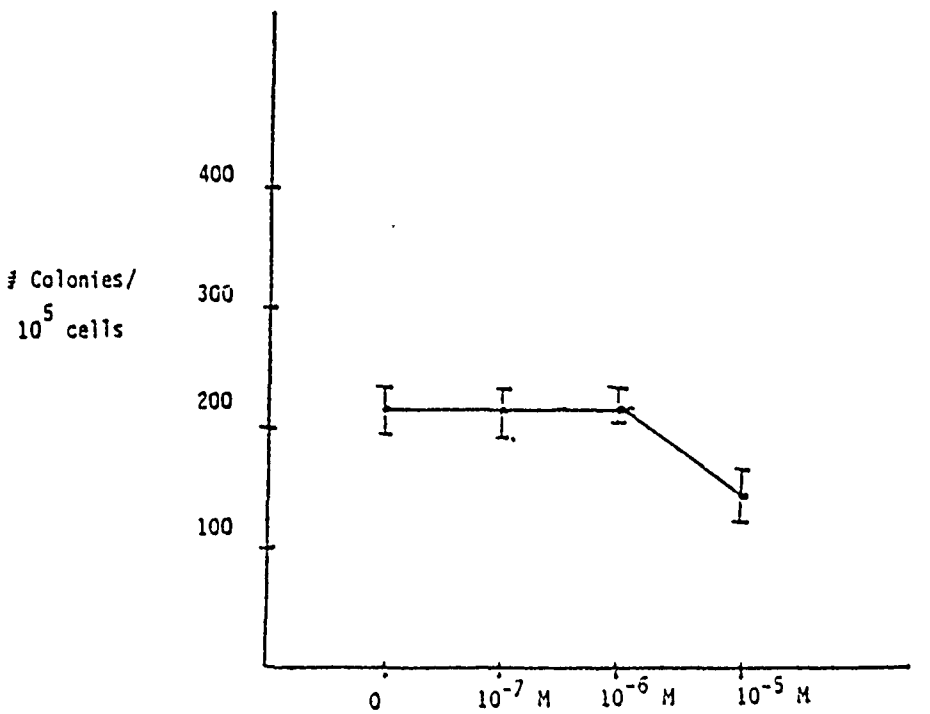


(3,4 DNT)	# Colonies/ 10^5 Cells	
----	418 (± 29)	
10^{-6} M	378 (± 25)	10% inhib
10^{-5} M	152 (± 25)	64% inhib
10^{-4} M	0 (± 0)	100% inhib

FIGURE 35D

Effect of Concentration of 3,4 DNT

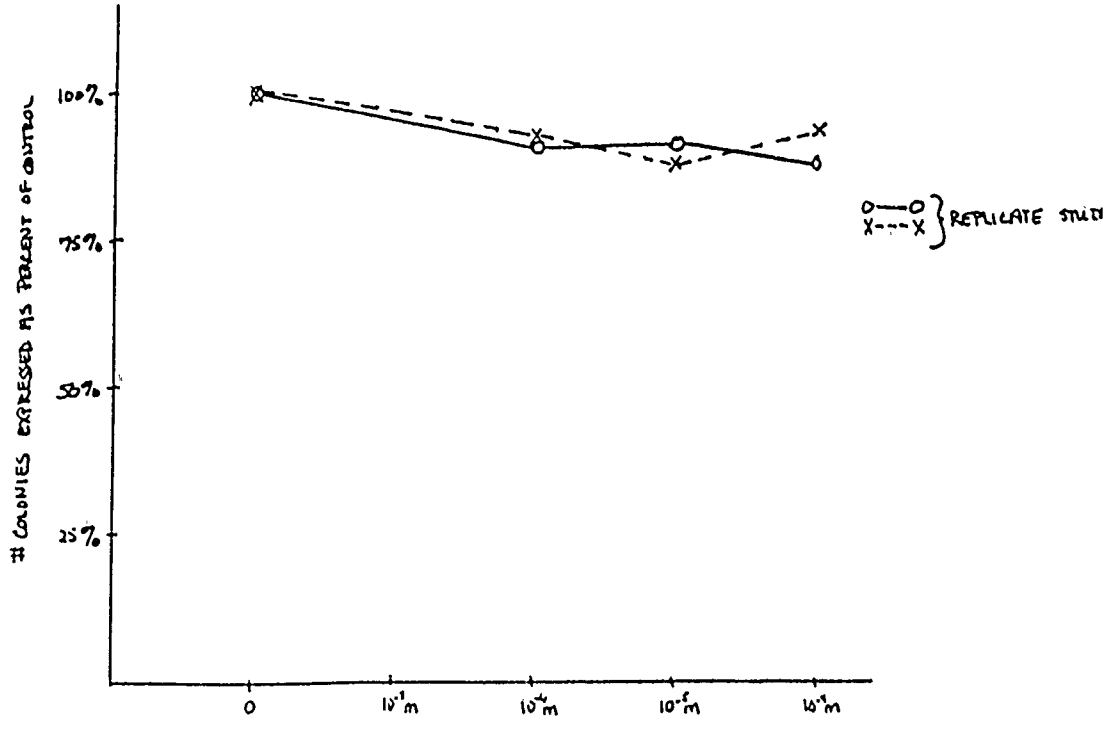
on Colony Number



final (3,4 DNT)	#/10^5	
-	217 (± 19)[a]	
10^{-7} M	215 (± 19)	
10^{-6} M	217 (± 9)	
10^{-5} M	145 (± 23)	33% inhibition

[a] Value = mean (± S.E.M.) of three replicate cultures

FIGURE 36-A



3,5 - DNT

FIGURE 36B

4 Amino 3,5 DNT

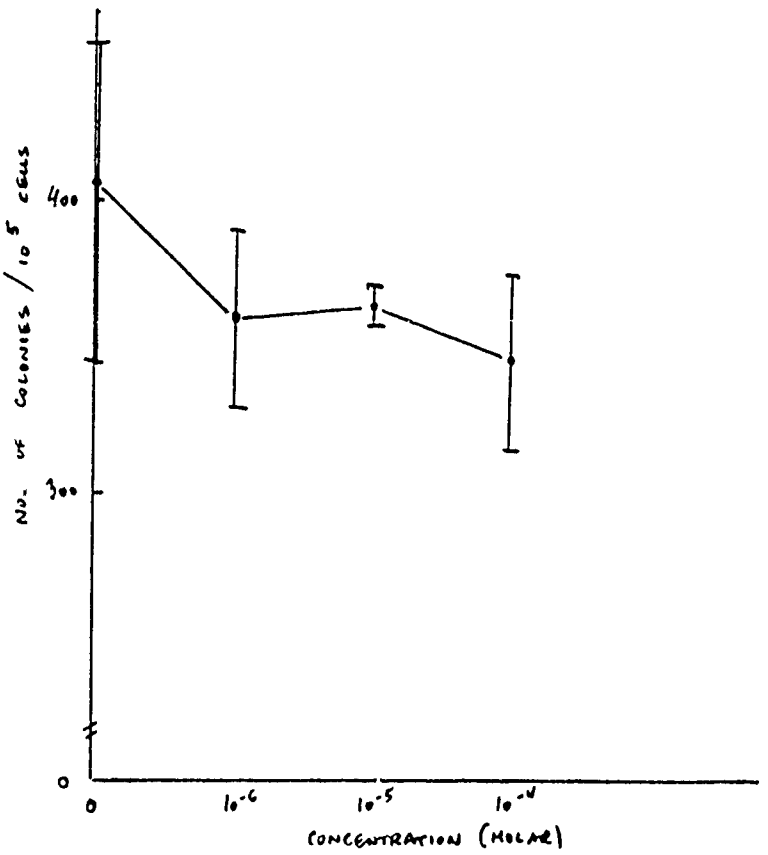
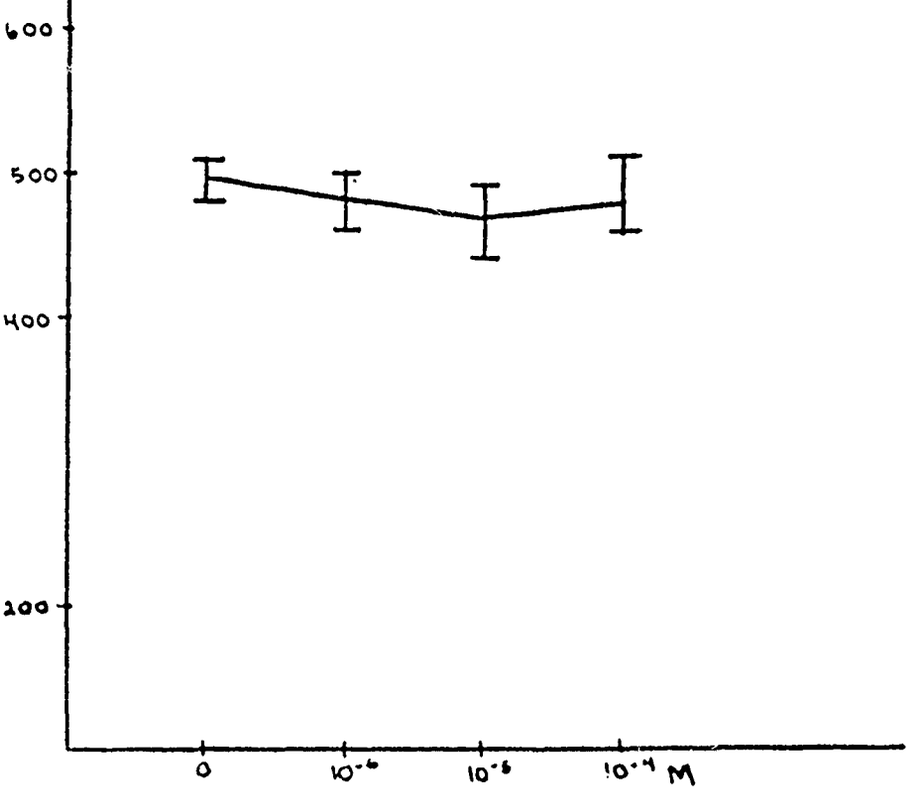


FIGURE 36C

Final Conc. 4 Amino 3,5-DNT		No. Colonies/10^5
—		500 ≠ 6.9
10^{-4} M	7% inhibition	466 ≠ 21.8
10^{-5} M	10% inhibition	451 ≠ 25.5
10^{-6} M	5.4% inhibition	473 ≠ 18.5



CONCENTRATION 4 AMINO
3,5-DNT (Molar)

FIGURE 37A
2,4,6-TNT

Final Conc. 2,4,6 TNT	No./10^5	
control	268 ± 10.9	
10^{-6} M	296 ± 23.5	10.4% inhibition
10^{-5} M	30.7 ± 32.1	12.9% inhibition
10^{-4} M	33.5 ± .4	7.5% inhibition

Value = Mean (± SEM) of 3 replicate cultures

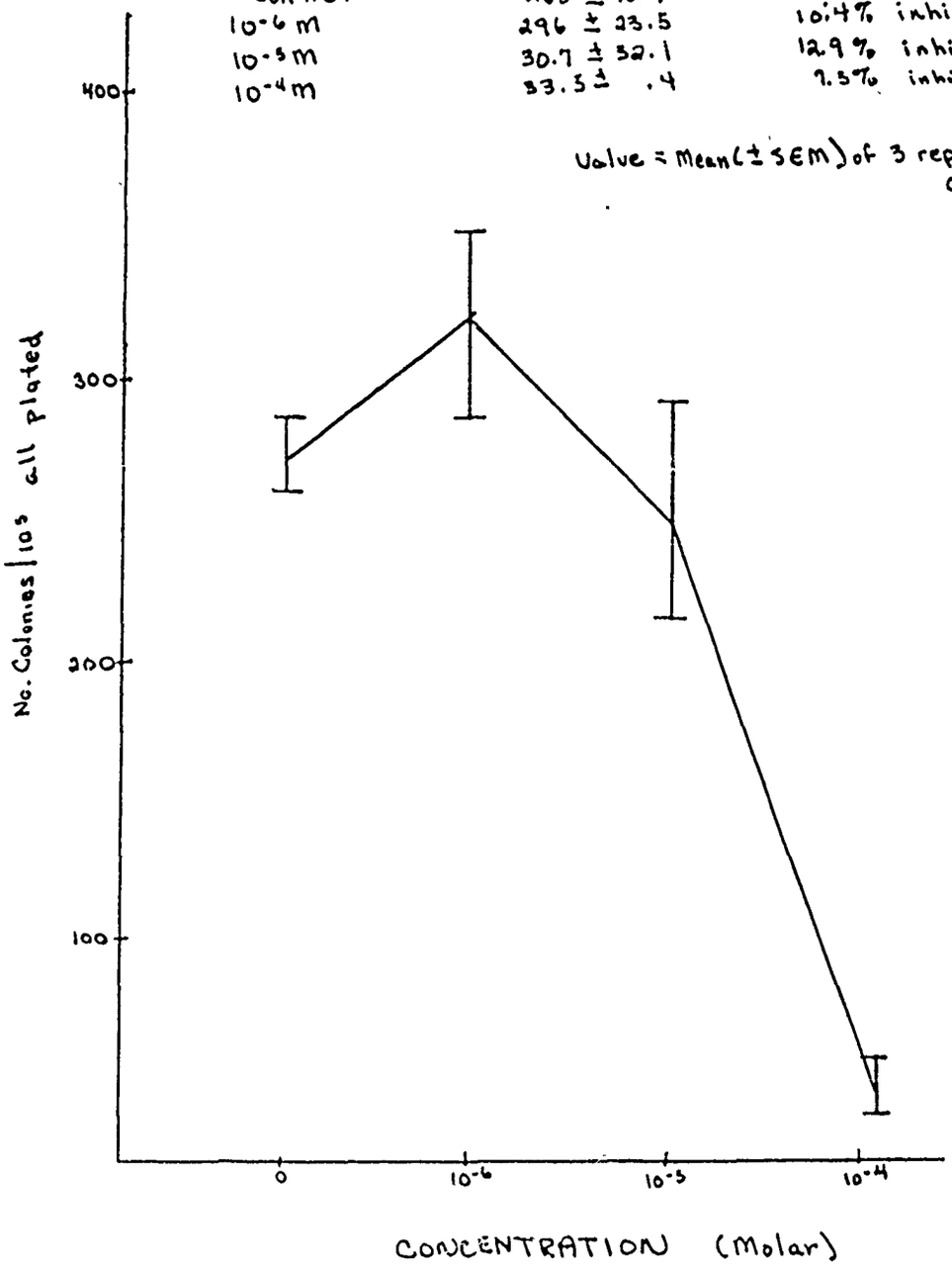


FIGURE 37B 2,4,6 TNT

Final Conc. 2,4,6 TNT	No. Colonies/10^5 cells Plated	
—	239.8 ± 51.4	
10^{-5} M	305.3 ± 19.3	39.8% stimulation
10^{-4} M	257.1 ± 18.9	7.2% stimulation
	31.8 ± 8.3	86.7% inhibition

Value = Mean (± SEM) of 3 replicate cultures

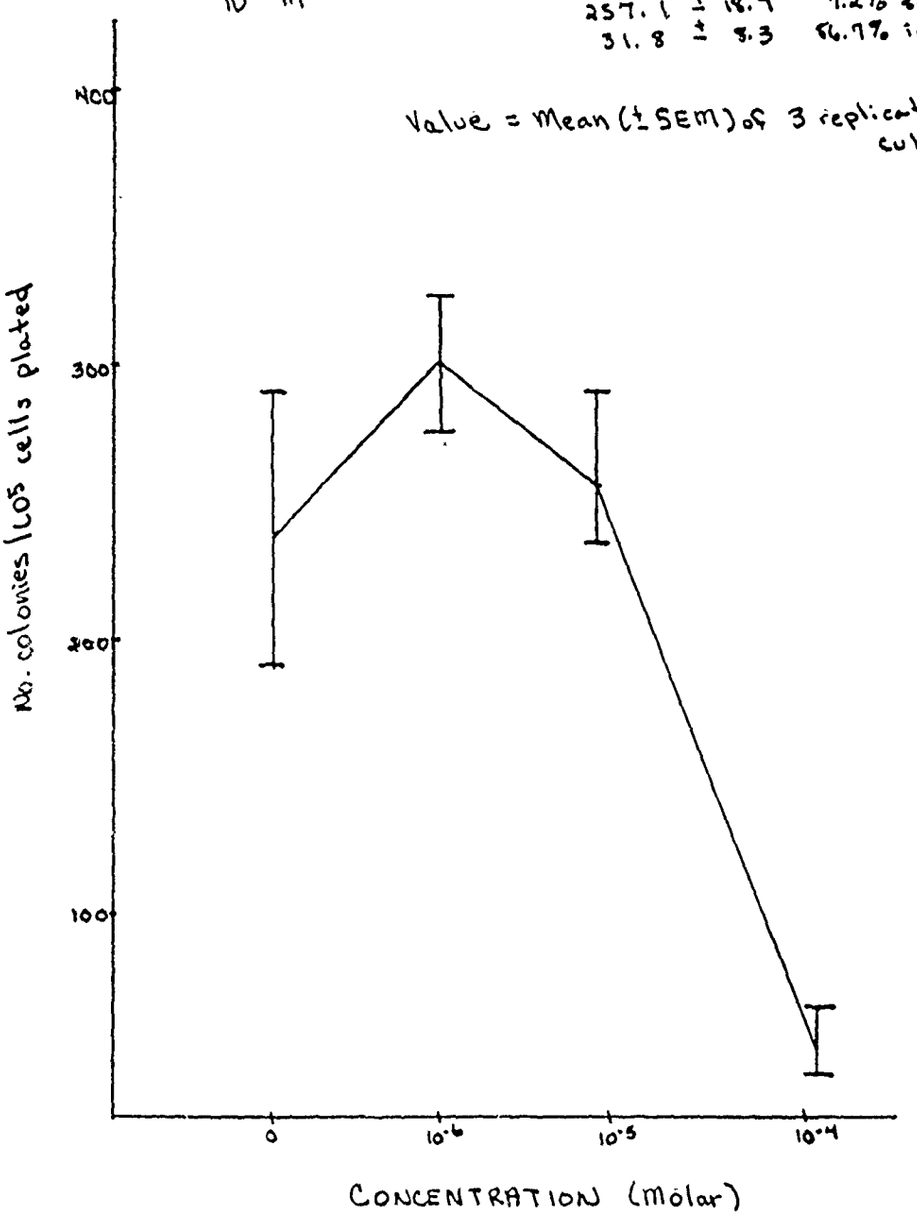


FIGURE 38A
2,3,4 TNT

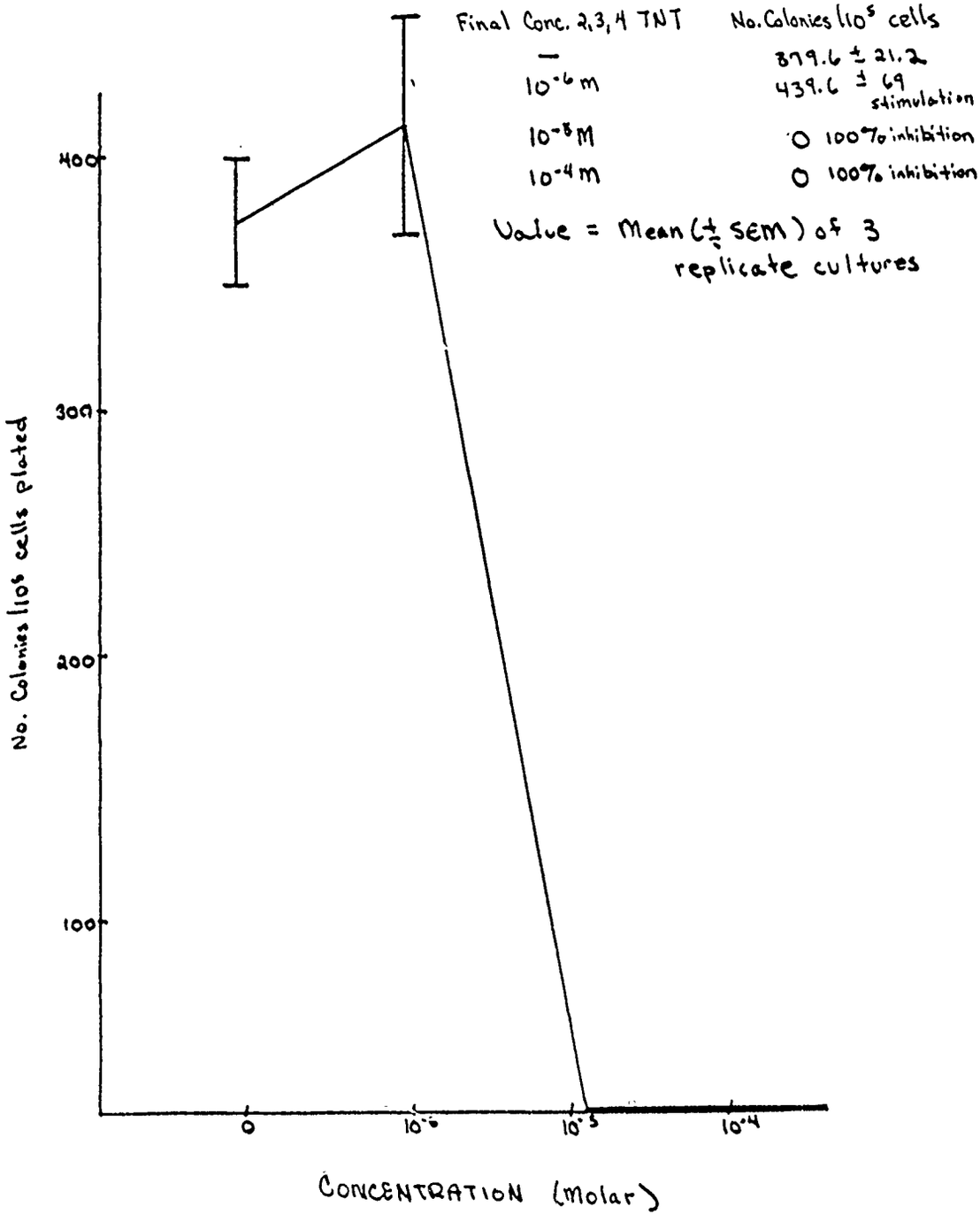


Figure 38B.
2,3,4 TNT

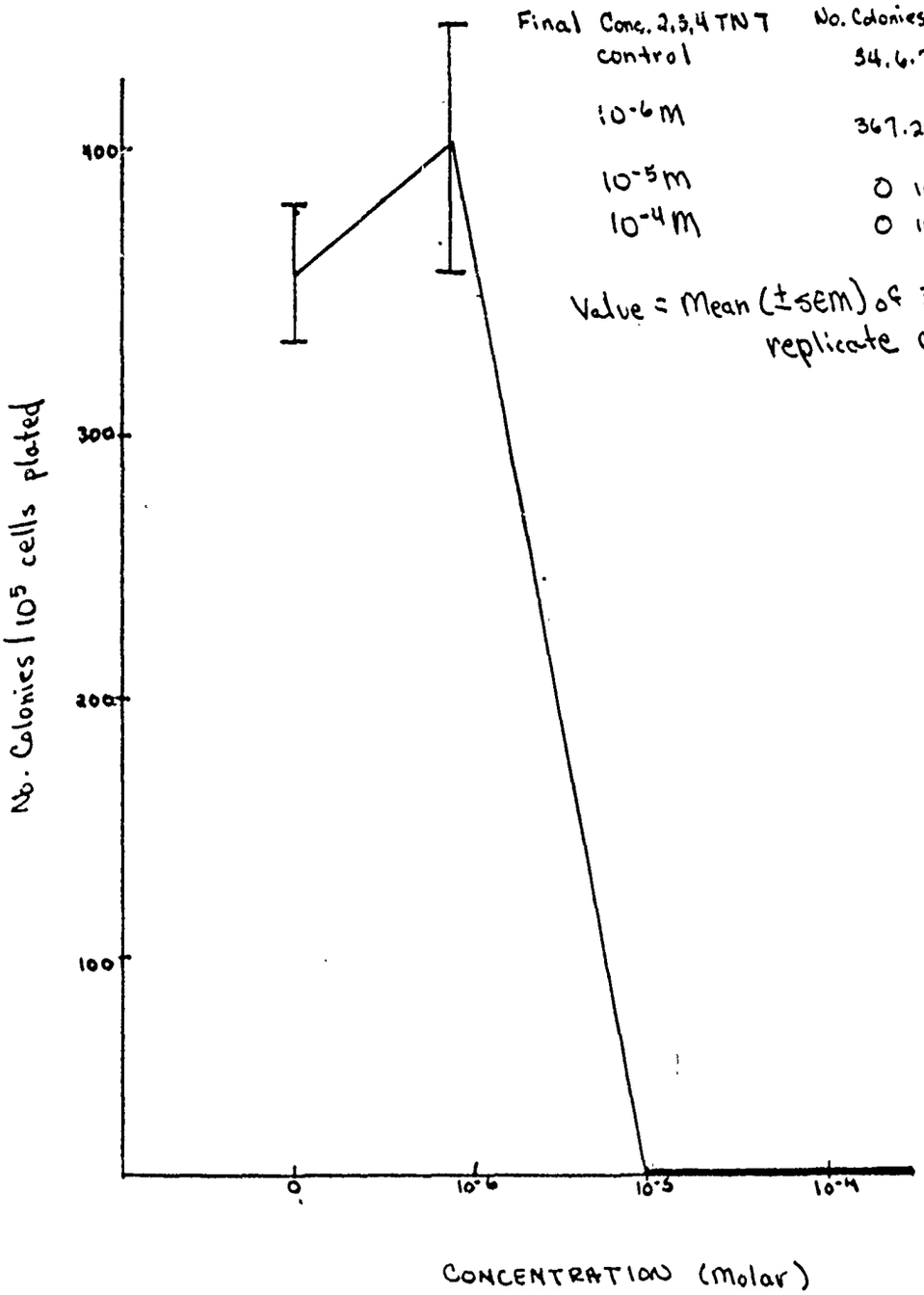


FIGURE 39 A

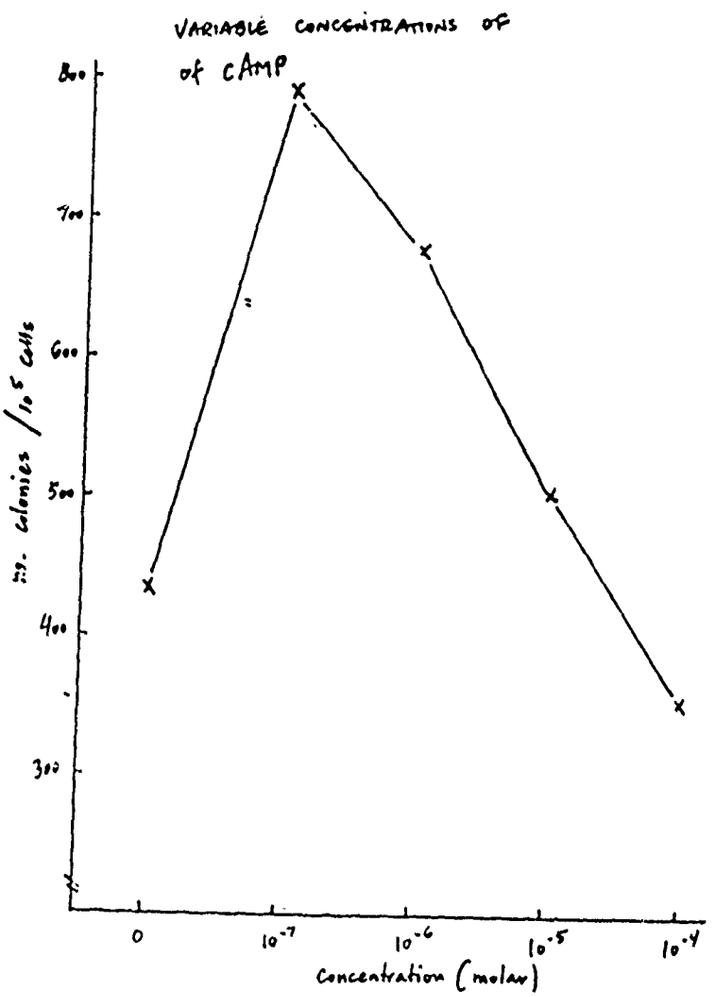
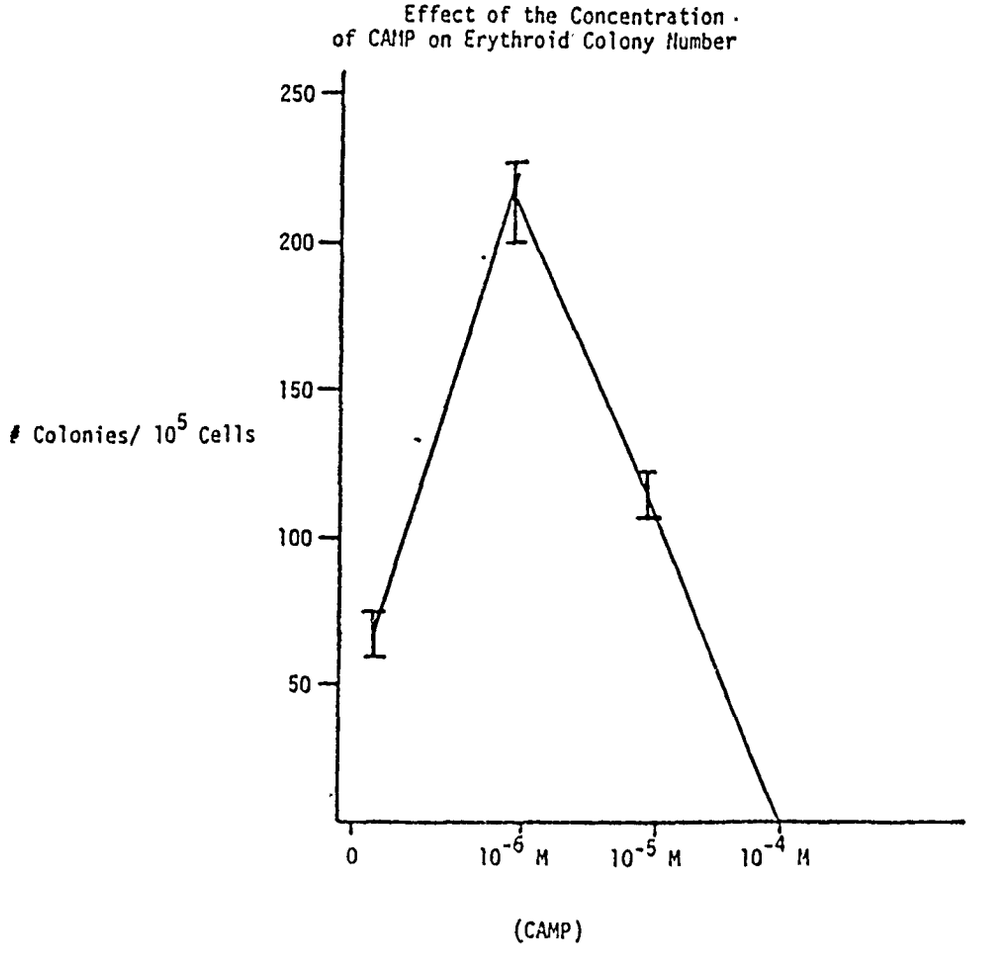

VARIABLE CONCENTRATIONS OF
of cAMP
800
700
600
500
400
300
No. colonies /10^5 cells
0
10^-7
10^-6
10^-5
10^-4
Concentration (molar)

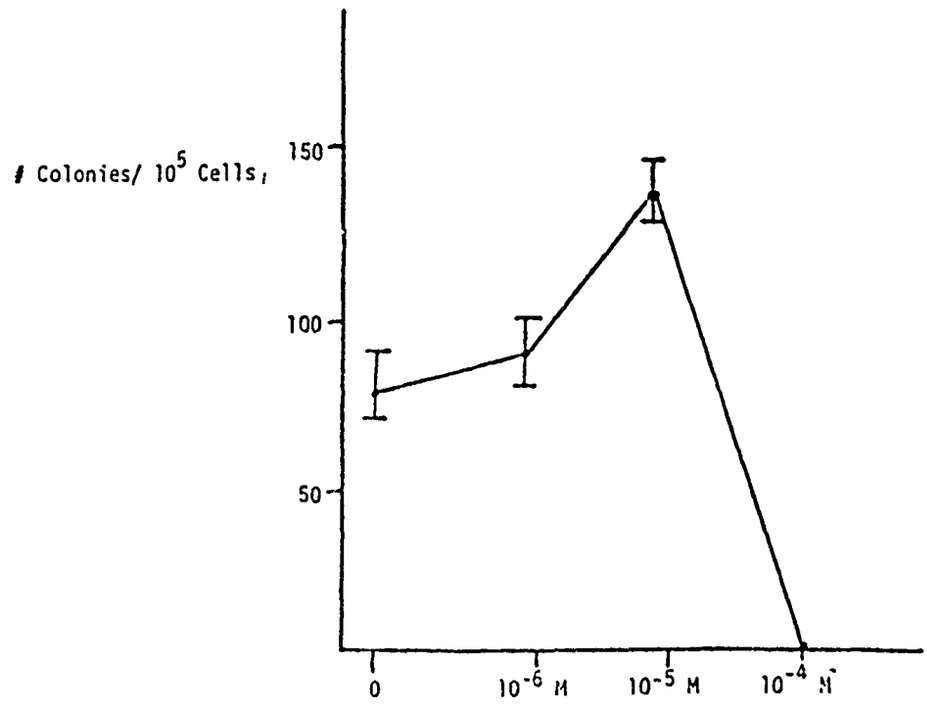
FIGURE 39B



(dibutyryl) (CAMP)	# Colonies/ 10^5 Cells
----	65 (± 6)
10^{-6} M	213 (± 13) 228% stim
10^{-5} M	115 (± 5) 77% stim
10^{-4} M	0 100% inhib

FIGURE 40A

Effect of Conc. of Pyridoxal HCL
on Colony Number



(Pyridoxal HCL)	# Colonies/ 10^5 Cells	
----	81 (± 9)	
10^{-6} M	91 (± 11)	12% stim
10^{-5} M	134 (± 8)	65% stim
10^{-4} M	0	100% inhib

FIGURE 4o B

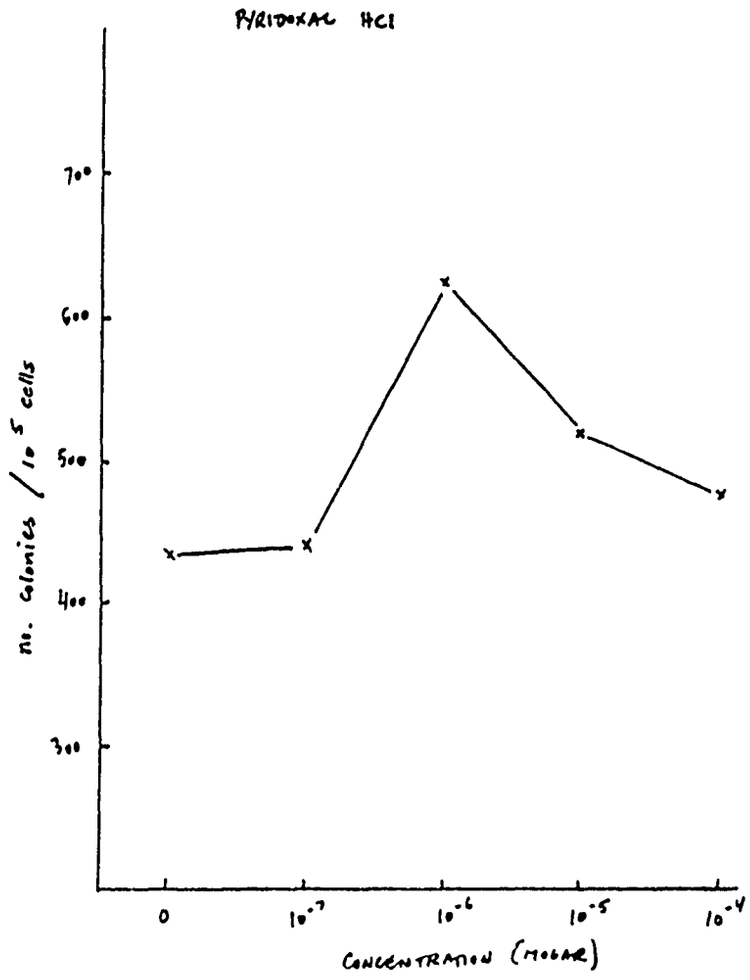

PYRIDOXAL HCl
700
600
500
400
300
no. colonies / 10^5 cells
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
CONCENTRATION (MOLAR)

FIGURE 41A

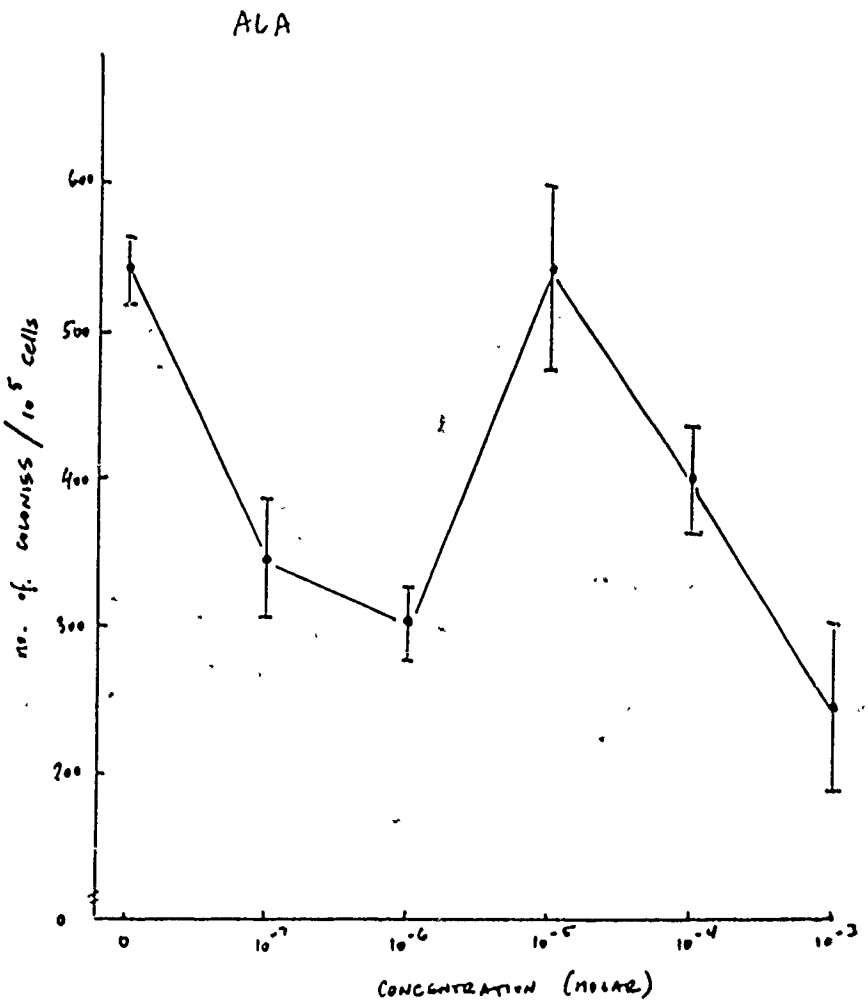

ALA
600
500
400
300
200
0
no. of colonies / 10^5 cells
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
CONCENTRATION (MOLAR)

FIGURE 41B

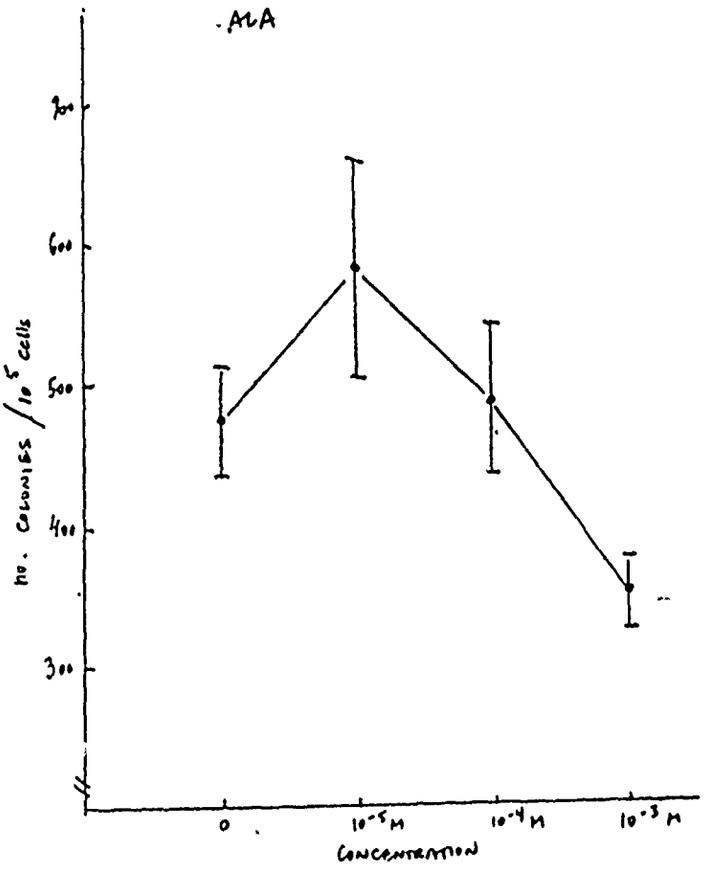

ALA
700
600
500
400
300
no. colonies / 10^5 cells
0
10^{-5} M
10^{-4} M
10^{-3} M
CONCENTRATION

FIGURE 42A

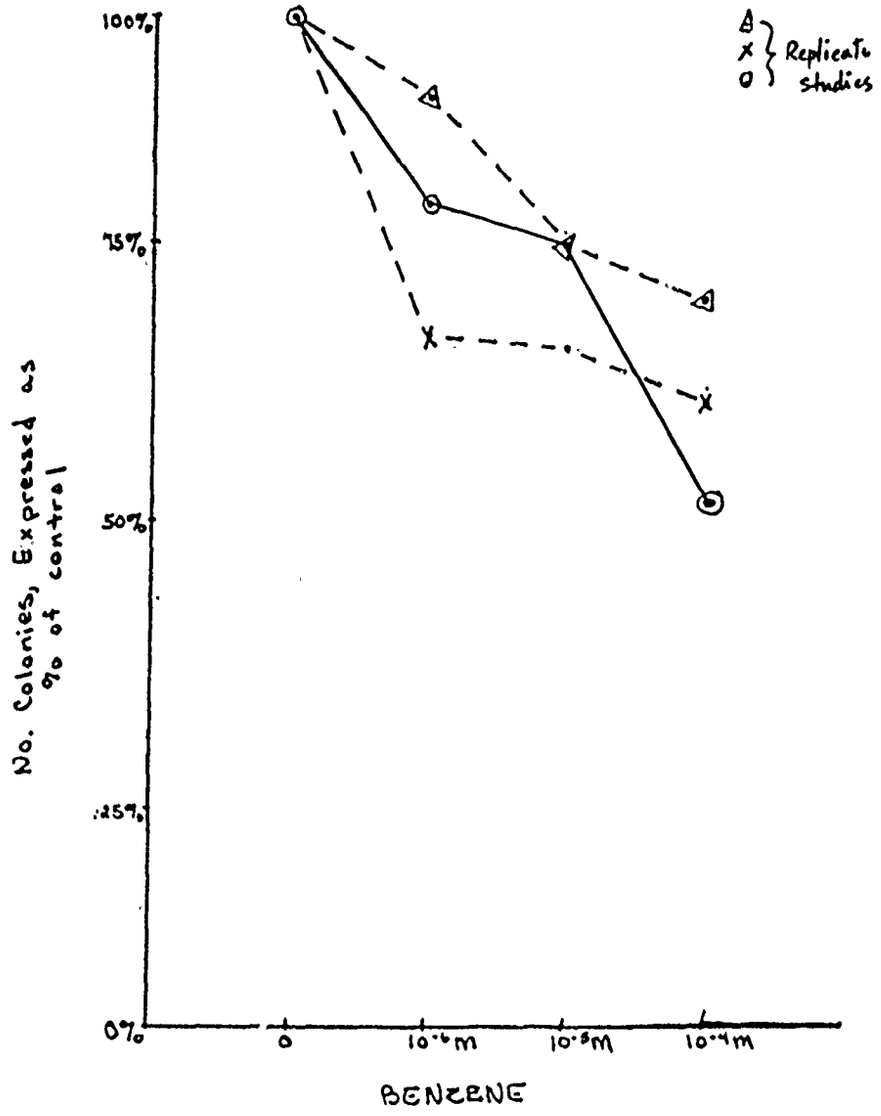
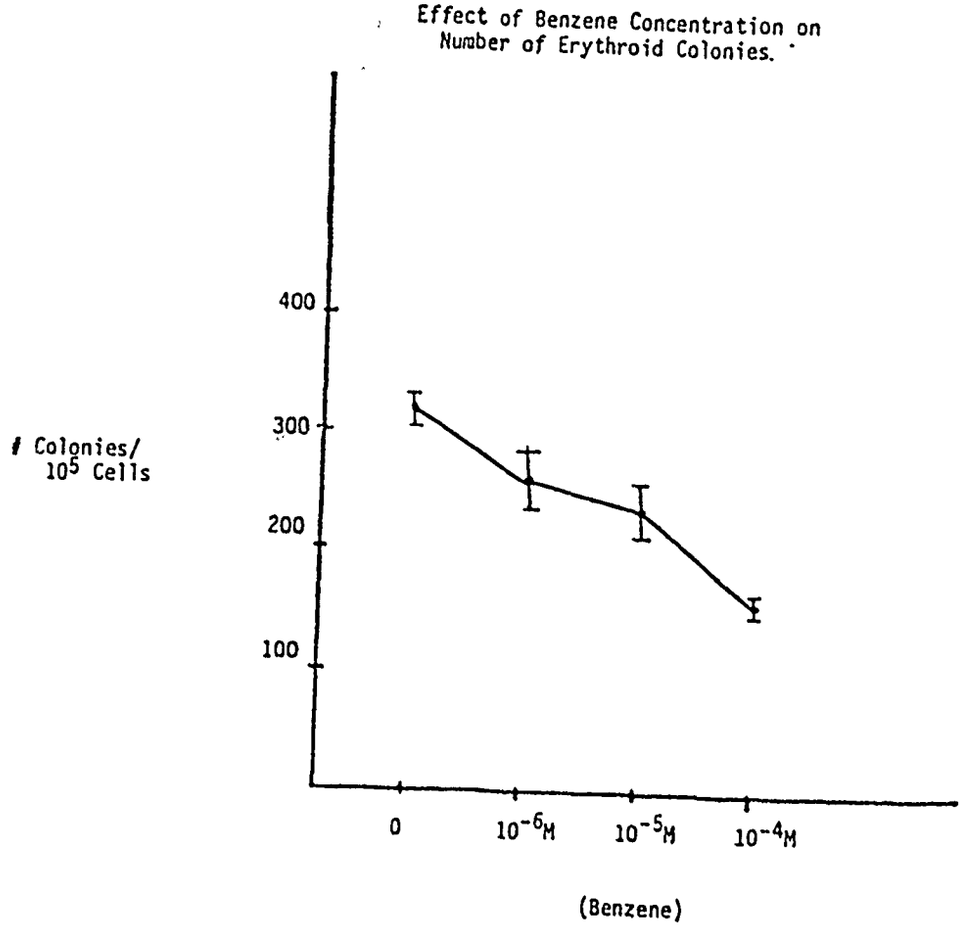

100%
75%
50%
25%
0%
No. Colonies, Expressed as % of control
0
10⁻⁶M
10⁻⁵M
10⁻⁴M
BENZENE
Replicate studies

FIGURE 42B

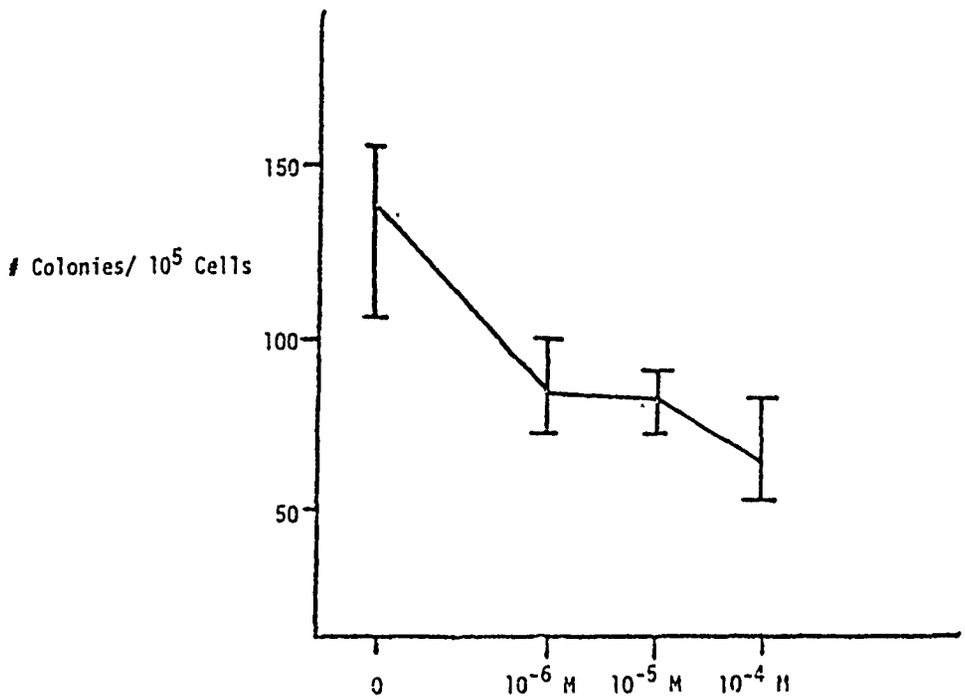


(Benzene)	# Colonies/10^5 Cells	
-	322 (± 10)[a]	
10^{-6}M	263 (± 19)	18% inhib
10^{-5}M	240 (± 15)	25% inhib
10^{-4}M	165 (± 5)	49% inhib

[a] Values = mean (± S.E.M.) of three replicate cultures

FIGURE 42C

Effect of Benzene Concentration
on Erythroid Colony Number



(Benzene)	# Colonies/ 10^5 Cells
----	130 (± 23)
10^{-6} M	90 (± 10) 31% inhib
10^{-5} M	89 (± 8) 32% inhib
10^{-4} M	75 (± 10) 42% inhib

FIGURE 42 D

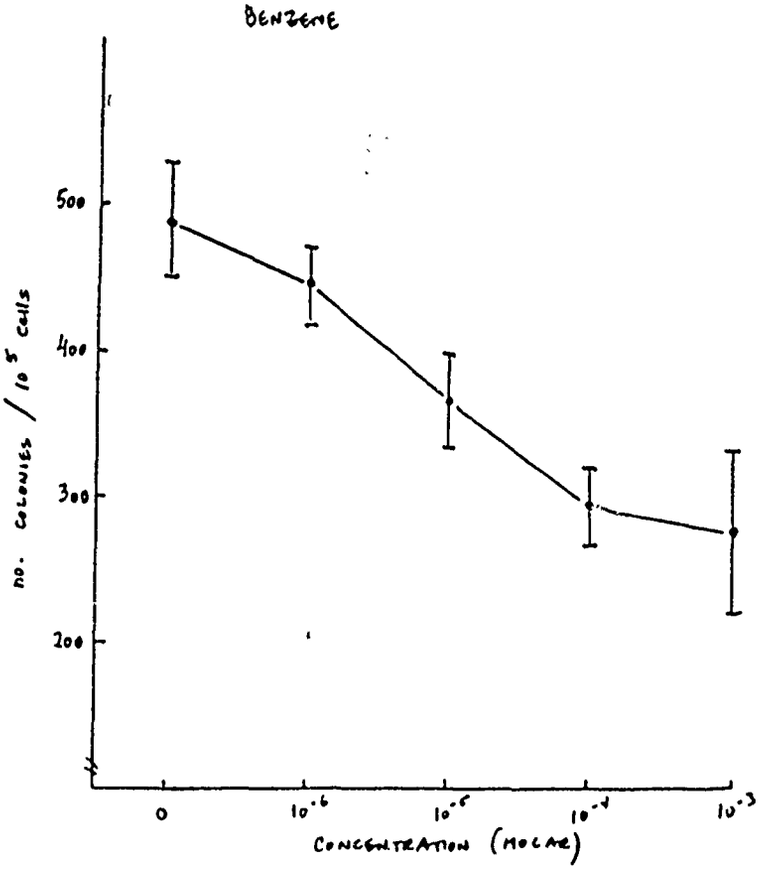

BENZENE
500
400
300
200
NO. COLONIES / 10^5 CELLS
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
CONCENTRATION (MOLAR)

FIGURE 43

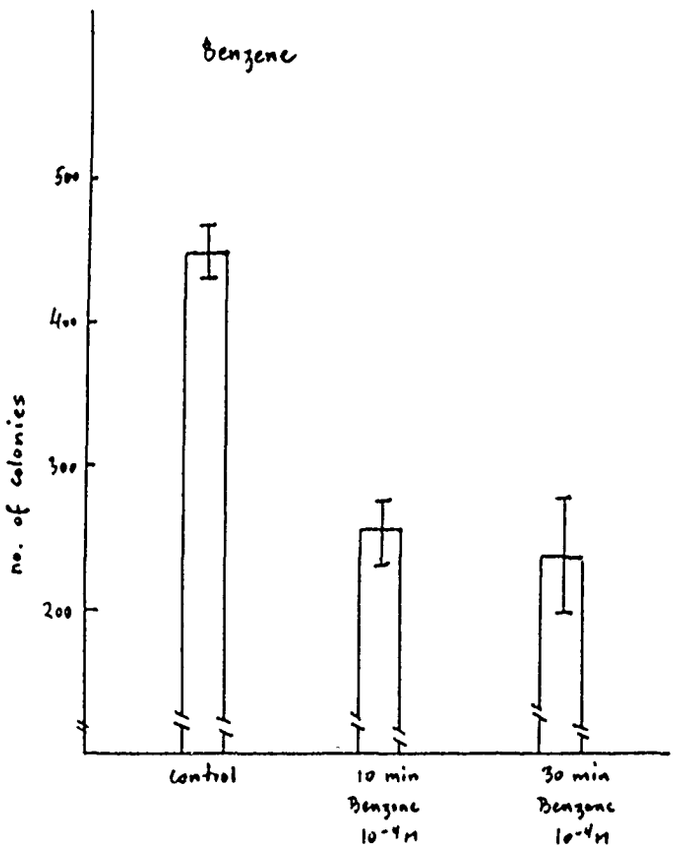
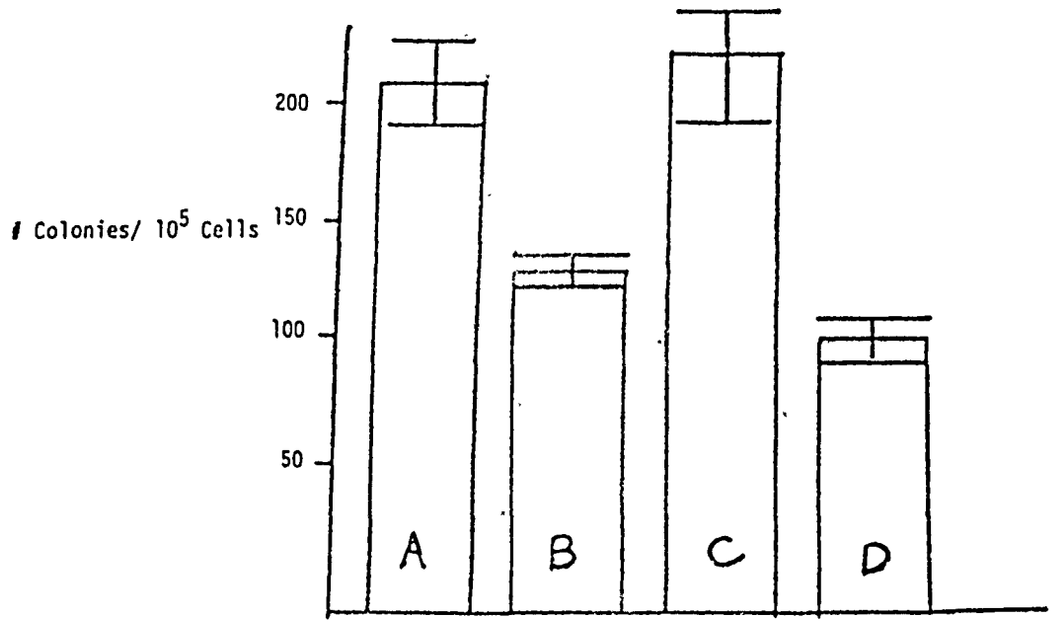

Benzene
500
400
300
200
no. of colonies
control
10 min
Benzene
10⁻⁴M
30 min
Benzene
10⁻⁴M

FIGURE 44

Effect of the Addition of CAMP on
Inhibition of Erythroid Colony Formation
by Benzene



A - Control - 212 (± 22)[a]

B - Control + Benzene 10^{-4}M - 124 (± 5)
41% inhibition of A

C - dibutryl CAMP 10^{-6}M - 224 (26)

D - dibutryl CAMP 10^{-6}M + Benzene 10^{-4}M
97 (± 8) 57% inhibition of C

a Value = mean (± SEM) for triplicate cultures

FIGURE 45

EFFECT OF PYRIDOXAL HCl ON
INHIBITION OF ERYTHROID COLONY GROWTH

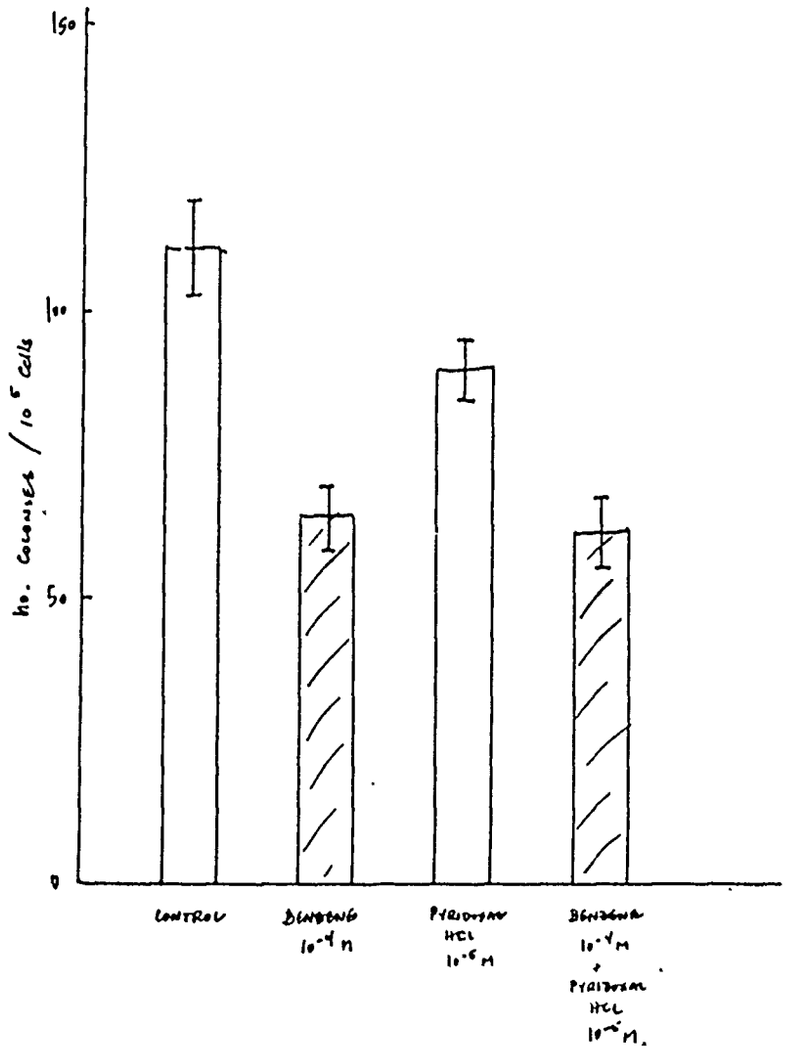
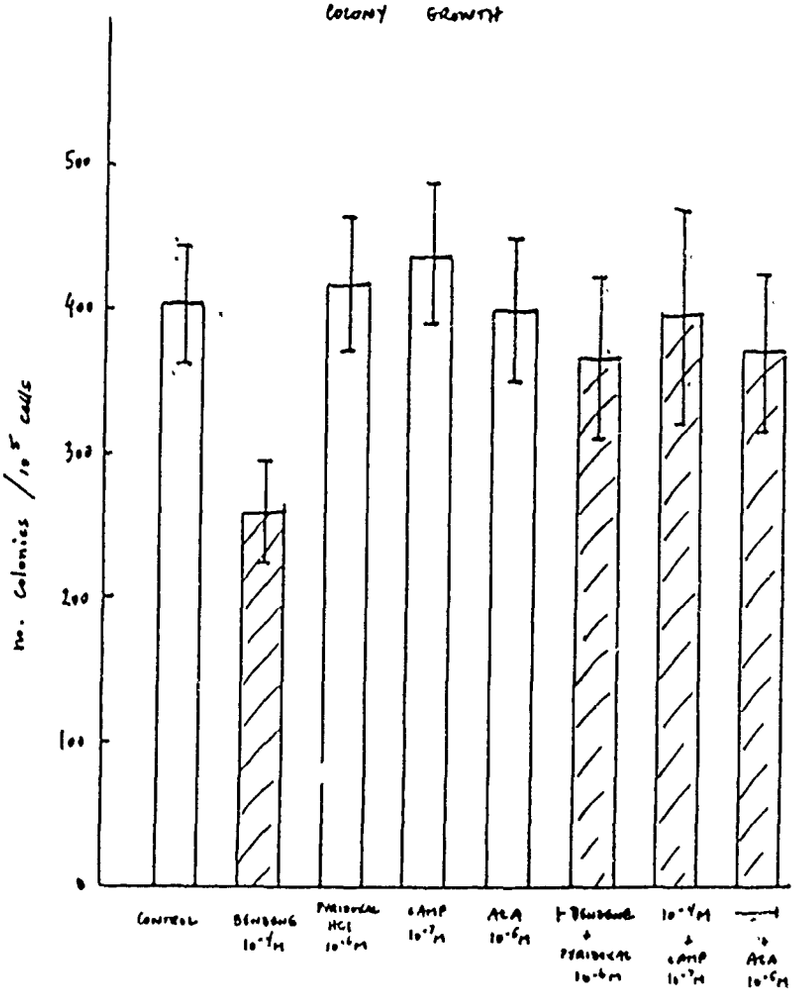


FIGURE 46

EFFECTS OF BENZENE ON ERYTHROID COLONY GROWTH



RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6473	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY S_TY	6. WORK SECURITY	7. REGRADING	8A. DISS'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161602BS01	00	146
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Military Stress: Non-Invasive Monitoring of Health and Performance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

01620 Stress Physiology 013400 Psychology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 10	CONT	DA	C. In-House

17. CONTRACT/GRANT: NA

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR — PRECEDING 79	4.0	231
FISCAL YEAR — CURRENT 80	4.0	221

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Redmond, LTC D.P.

NAME: Genser, LTC S.G.

21. GENERAL USE

Foreign intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Electrophysiology; (U) Psychophysiology; (U) Psychophysics; (U) Stress; (U) Performance; (U) Human Volunteer

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Objective is the development of non-invasive human psychophysiological monitoring technology in support of field studies of stress in military environments.

24. (U) Approach is to exploit advances in signal acquisition and processing technologies to enlarge the scope of psychophysiological measurements than can be made under field conditions. Techniques are validated in the laboratory prior to deployment in controlled field trials.

25. (U) This Work Unit provides the technology base for Work Unit 048, Military Stress: Circadian Ultradian Factors (Accession Number DA OC 6457). Development and field deployment of a self-contained Rest/Activity monitory device, the Mark I Actigraph, was completed. Delivery of a second-generation, or Mark II, prototype occurred in Sep 79, and represents technological improvements based on experience and study of the first device. Field deployable device read-out systems were engineered and constructed. Development of systems for cardiovascular monitoring, core temperature recording, and cognitive performance assessment continue. For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 OCT 78-30 SEP 79.

*Available to contractors upon originator's approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 146 Military Stress: Non-Invasive Monitoring of Health and Performance

Investigators.

Principal: Frederick W. Hegge, Ph.D.

Associate: LTC Daniel P. Redmond, MC; LTC Sander G. Genser, MC; David R. Thorne, Ph.D.; Michael P. Toman, Ph.D.; Alison L. Lee; Stanley Hall; Jacob Karen; Helen Sing, M.S.; John Jackson; Joseph Fritz; SSG Kathryn Elhardt; SSG Norman Cook; SP5 Patricia Muldrow; SP5 Arthur Ferreira.

Description

The soldier is subject to numerous influences which taken together may impose a critically important margin of performance decrement and probability of casualty. Central concerns are sleep deprivation and disruption associated with deployment, and physical and mental stresses in sustained combat, under conditions of spatial and temporal dislocation, under life threat, and with the contingency of decisive consequences of behavior. This work unit provides the supportive technology base for Work Unit 048, Military Stress: Circadian and Ultradian Factors (Accession Number DA OC 6457), designed to address both in the field and in the laboratory these central problems of modern combat stress. The goal of work herein is to exploit, refine, and apply rapidly improving techniques of physiologic data acquisition and performance assessment. The research strategy utilizes laboratory studies for technical improvements and design of data processing and analytic approaches, coupled with field studies for validation and for the development of applications minimizing the intrusion of the research, or the researcher, into realistic operations.

Progress

1. Rest/Activity Monitoring: The Actigraph

Background. Electrographic (EEG) determination of sleep/wake cycles in naturalistic settings presents formidable difficulties in terms of quality, and cost of data acquisition, reduction and analysis. The use of wrist-mounted motion detection for such purposes is finding increased application in the investigation of psychomotor pathology and sleep disturbance. During the last year, this laboratory has routinely fielded such devices for studies involving transmeridial temporal adjustments, circadian cycles in normal and diseased humans, shift work schedules, and sleep disruption. Improving on the constraints inherent in the

initial devices constructed and deployed, a second generation of instruments reached final development stage.

Mark I Actigraph. In 1978, 50 Mark I units were constructed following plans for a device developed at the National Institutes of Health, modified for extended battery life and rugged field use. As built, the devices are capable of storing accumulated activity counts in 15 minute epochs for a total of 64 hours. In 1979, most applications were for pilot purposes, to develop and to validate the feasibility of the devices, as well as to obtain relevant data within the experimental settings. The listing of applications below is a lineal history of improvement in reliability and data quality, until, after over 12,000 hours of continuous human rest/activity monitoring, the device is a tool of proven utility. Applications included:

a. Reforger '79 - Several devices were applied to subjects flying from Ft. Riley, Kansas to West Germany. Device failure, data retrieval problems, freezing temperatures and rugged use combined to narrowly limit the number of subjects from whom meaningful baseline and transition data were acquired. However, numerous remediable problems were identified during this valuable field test.

b. Firefighter's Study - After initial testing of maintenance and repair procedures designed to assure reliability, the Actigraph was used to log, continuously, the rest/activity patterns of 13 Montgomery Fire Department Radio Dispatchers for the 28 eight days of their rotating shift schedule. Furthermore, 6-10 days of activity were monitored in 15 firefighters and emergency personnel in the field. The adjustment of sleep patterns to shiftwork in the former, and late night sleep disruptions of the latter were clearly documented. Analysis of these data is in an early phase, but the Actigraph data in raw form have proven compatible with extant methods of circadian and ultradian rhythm analysis.

c. Chicago - May 79 - In collaboration with investigators from Loyola University, and the Universities of Arkansas and Minnesota, twelve subjects were closely monitored for changes in multiple variables over a single 24-hour cycle. The Actigraph recorded the expected circadian rhythm as well as disruptions induced by experimental

design. Of singular interest in this study is the fact that seven subjects were simultaneously monitored by another, similar commercially available device, hence offering a unique opportunity for cross-validations. Reporting the comparative results awaits a final exchange of raw data between investigators.

d. Renal Dialysis/Transplant Patients - In support of a collaboration between the Department of Neurosciences and the Nephrology Service, WRAMC, this Department provided Actigraphic monitoring for a study of the interaction of therapy with sleep and psychomotor behavior patterns in renal patients.

Limitations. The expanse of recent experience with the Mark I Actigraph has served to identify a number of electromechanical problems with the present model, even during the collection of valuable data. Most of these were anticipated and incorporated in the recently acquired Mark II Actigraph. Most serious of these is the limited dynamic range of the device across several states of normal activity, ranging from sleep (too insensitive) to hyperactivity (over-sensitive and subject to saturation). The problem of measurements during sleep has been let by USAMRDC contract to a sleep physiology laboratory. For analysis of ultradian rhythms, or high frequency transitions in active state, the deficiencies at both extremes cause distortions in results of normal methods of time series analysis. Another problem is the calibration or uniformity of sensitivity across devices. Rest/activity data consist of essentially arbitrary, relative units, so far without demonstrable relationship to standard physical units of work, energy, or force. Comparison of data across subjects (in terms of amplitude but not frequency) is difficult, and hampered by the lack of uniformity experienced so far. The Mark II Actigraph provides, at least, uniformity in components and construction, and is mechanically simpler. Improved cross-calibration technique will be one of the first goals in deployment of the new device. Finally, the definition of device characteristics in environmental extremes, especially the cold, awaits further study.

The Mark II Actigraph. The Department completed and extended an interagency agreement with Harry Diamond Laboratories to construct 50 second-generation actigraphs using hybrid circuit technology. This second version offers improved temporal resolution (10 minute vs. 15 minute sample epochs), wider dynamic range (12 bit vs. 8 bit word length, for a maximum count of 3072 vs.

256) and a longer recording period (one week vs. 64 hours). The new units are physically smaller and lighter than the original, can be activated in the field by the wearer, and can be worn in the bath, shower or rain.

The first prototype unit has been received and initial testing is underway, to include circuit level debugging, software debugging, temperature cycling, immersion, pressurization and depressurization, power drain, battery life, and field performance. The latter will require trial-and-error studies to determine the transducer/amplifier settings that make the most use of the increased sensitivity and range.

In addition to supplying 50 complete units Harry Diamond Labs is to supply photo masks, art work and information so that additional actigraphs can be constructed by outside manufacturers. In anticipation of this, and with knowledge of semiconductor supply lags, memory chips for a second set of actigraphs have been ordered.

Field Deployable Actigraph Readout Systems. An important element of field utility of the Actigraph device, with its limited span of free-running data collection, is the ability to "turn-around" the devices - that is, to read and retain the data stored, then to reset and redeploy each unit in a matter of minutes. This serves to obviate in a most practical way the problem of comparability across devices - since the same device may be worn by a given subject for the full length of battery life. Each turn-around, furthermore, provides an immediate qualitative check of the function of the Actigraph. This year, two such field systems were engineered and constructed by the Department, each serving separate needs.

a. The Datalogger. A commercially acquired portable digital tape machine was modified to record an image of Actigraph memory contents for later processing. It was further modified to record on cassette tape, in twelve separate data groups, absolute counts or elapsed times (to 0.1 second resolution) within presetable epochs of 1-30 minutes. Finally, it was built to accept and record data from certain digital-display sphygmomanometers. This logging device was designed specifically for use in the Montgomery County Emergency Operations Center, where it still resides monitoring quantity of emergency radio/telephone traffic per five-minute epoch. Taped data are read off line at the laboratory.

b. Suitcase-Enclosed Readout/Hardcopy System - A microprocessor controlled device extracts Actigraph data,

records it on cassette tape and provides a written listing and a graphic display of time-series data. The unit is constructed within a rugged aluminum suitcase, is fully portable, and has the versatility of accepting, with appropriate programming, any digital memory based set of data.

2. Canadian Core Temperature Telemetry System

Field and laboratory studies of activities in the long term depend upon measures of continuous physiologic processes. In the relevant literature, the commonest of these is core temperature as recorded by rectal probe connected to either a belt-worn recorder or an umbilical cable. The disadvantages of long term use of rectal probes are obvious. The Canadian Defense and Civil Institute of Environmental Medicine has developed an inexpensive, capsule-sized, and disposable temperature transmitter, which can be swallowed and used for telemetered data (resolution less than 0.1 degree Celsius) for the duration of gut transit time. Prototype devices are in hand, and adaptation of extant receiving and recording equipment is underway. Negotiations concerning technical interactions and supply of devices are proceeding.

3. Assessment of Cognitive Performance

The impact of deployment and battlefield stress factors on higher order and complex cognitive performances are of central importance. In the Jet Lag II field research, as well as in laboratory studies now under protocol review, several tests of cognitive performance, memory, subjective fatigue and affect have been assembled, and to varying degree applied. Several of these tests have been transferred to software by Department programmers, allowing automated application, data acquisition and display graphics. Important collaborative interactions, to ensure standardization of performance measures and inter-laboratory validation, have been established. The continuing process of assembly, refinement and automation, and the rendering of such tests fully field portable is leading to the construction of a Performance Assessment Battery of high sensitivity and applicability to such problems as the temporal structure of performance, the impact of various stressors, such as sleep deprivation or pharmacologic agents, on performance, and the evaluation of interventive measures.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6744	79 10 01	DD-DRAF(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	147
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Protocol with Security Classification Code)

(U) Immunological and Biochemical Aspects of Membranes

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry, 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 10	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:
b. NUMBER:
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	2	90
FISCAL YEAR CURRENT 80	2	119

19. RESPONSIBLE DOD ORGANIZATION

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20. PERFORMING ORGANIZATION

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Washington, D.C. 20012

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TELEPHONE: (202) 576-3248
SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME: Owens, Roberta L., Ph.D, DAC
NAME:

21. GENERAL USE

Foreign Intelligence not considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Parasites: (U) Bacteria; (U) Toxins; (U) Antibodies; (U) Antigens; (U) Drug Carriers

23. TECHNICAL OBJECTIVE. 24. APPROACH. 25. PROGRESS (Furnish individual paragraphs identified by number, precede text with Security Classification Code.)

23. (U) This work unit has five major objectives. First, preparation of synthetic lipid membranes (liposomes) containing entrapped drugs for treatment of leishmaniasis. Second, preparation of "empty" liposomes (containing only saline) that have incorporated glycolipids for the treatment of exoerythrocytic forms of malaria. Third, preparation of liposomes that might serve as combined adjuvants and antigens for protection against infectious diseases. Fourth, identification and characterization of receptors for toxins. Fifth, study of membrane-associated immunological mechanisms and complement activation.

24. (U) The approach will involve preparation of liposomes from purified lipid mixtures. Substances, such as drugs or proteins, will be trapped in the aqueous spaces separating the lipid membranes, or in the membranes themselves. Analysis of the effectiveness of the liposome-encapsulated materials will be performed by appropriate standard means, such as the cure rate of leishmania-infected, or malaria-infected, animals; or production of specific anti-microbe antibodies; or specific interactions of toxins, or antibodies, with receptors or antigens; etc.

25. (U) We have demonstrated that liposomes containing certain glycolipid, but in the absence of additional drugs, cause a radical cure of exoerythrocytic forms of malaria in mice. The therapeutic index of this treatment for malaria is greater than a million. The mechanism of the effect has been partially revealed, by the nature of the experiments, as an interference, perhaps by competition, with the passage of the parasite through the hepatocyte. We have shown that a toxin (tetanolysin) acts by specifically binding the mechanism by which an acute phase reactant (C-reactive protein) interacts with phosphatidylcholine as a receptor and activates complement. We have described activation of the alternative pathway of human complement by protein-free lipid membranes.

For technical report see WRAIR Annual Progress Report, 1 Oct 78 - 30 Sep 79.

(Available to contractors upon originator's approval)

DD FORM 1 MAR 66 1498 PREVIOUS EDITIONS OF THIS FORM AND 1498-1, 1 MAR 66 (FOR ARMY U

814 ETE. DD FORMS 1498A, 1 NOV 65 OLETE.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 147 Immunological and Biochemical Aspects of Membranes

Investigators:
Principal: Bhupendra P. Doctor, Ph.D
Assistant: Carl Alving, M.D., LTC, MC;
Roberta Richards (Owens), Ph.D, Glenn Swartz;
SSG Piyush Gandhi; PFC Elizabeth Graves;
PFC Lucious White

1. Sporozoite-Induced Malaria: Therapeutic Effects of Glycolipids in Liposomes.

Liposomes containing neutral glycolipids with a terminal glucose or galactose, when injected intravenously, prevented the appearance of erythrocytic forms of malaria (Plasmodium berghei) in mice previously injected with sporozoites. Inhibitory glycolipids included glucosyl, or lactosyl ceramide. Inhibition was not observed with liposomes containing ceramide, phosphocholine ceramide, sulfogalactosyl ceramide (sulfatide), or ganglioside G_{M1}. Liposomes containing glycolipids did not inhibit infection transmitted by injecting blood containing erythrocytic stages of malaria. These results may have therapeutic implications in the treatment of malaria. Analysis of the mechanism of interference with the life cycle of malaria by liposomal glycolipids may yield information about the interactions of parasites with cellular membranes.

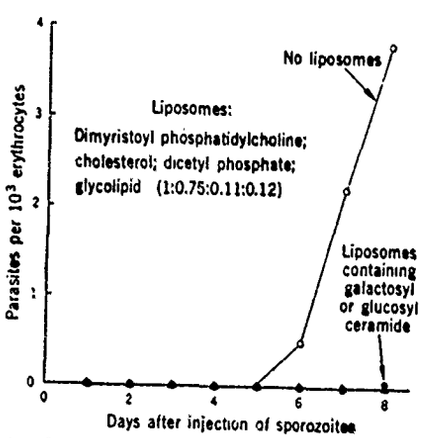

Fig 1 Inhibition of erythrocytic parasites after injection of liposomes containing glycolipids. The animals were injected intravenously with liposomes 1 day after sporozoite injection. Each point represents the mean of 10 or 11 animals.

Table 1

Influence of saccharide moieties on inhibitory effects of ceramide lipids in liposomes. The liposomes consisted of dimyristoyl phosphatidylcholine, cholesterol, and dicetyl phosphate, plus the indicated lipid. Controls received only saline.

Ceramide lipid	Number of animals*		Patency (%)	Average prepatent period (days)†
	With patent infections	Injected		
Control(no liposomes)	49	62	79	5.1 ± 1
Glucosyl ceramide	2	28	7.4	6.0
Galactosyl ceramide	6	40	15	7.3 ± 1.6
Lactosyl ceramide	1	7	14	6.0
Ceramide	6	7	86	6.3 ± 0.8
Phosphocholine ceramide (sphingomyelin)	5	7	71	6.2 ± 0.4
Sulfogalactosyl ceramide (sulfatide)	5	7	71	5.8 ± 0.8
Ganglioside G_{m1}	5	7	71	6.4 ± 0.9

*Animals were examined for potency daily during 3 to 8 days after injection of sporozoites. Potency was defined as at least 1 parasite per 10^5 erythrocytes. †Values given as mean ± standard deviation.

2. Cholesterol-Dependent Tetanolysin Damage To Liposomes.

Tetanolysin caused membrane damage, resulting in release of trapped glucose from liposomes containing cholesterol. Maximum glucose release occurred from liposomes that contained 50 mol% cholesterol. At higher or lower levels of cholesterol, glucose release was reduced and glucose release did not occur at all below 40 mol% cholesterol. The apparent activity of tetanolysin was not influenced by temperature (24°C compared to 32°C) or by liposomal phospholipid fatty acyl chain length. We conclude that tetanolysin caused cholesterol-dependent lysin mediated damage to liposomes, possibly by means of a pore consisting of a complex of toxin and cholesterol.

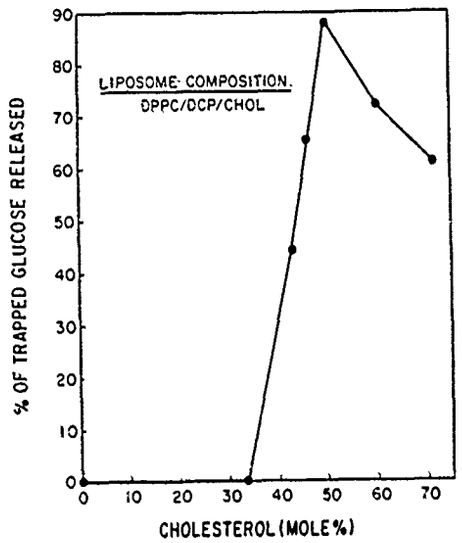

Fig. 2 Effect of cholesterol concentration on tetanolysin damage to liposomes. The liposomes (5 μl) having the indicated lipid composition were incubated at 24° C with 6000 hemolytic units of tetanolysin in a total volume of 1.0 ml. DPPC, dipalmitoyl phosphatidylcholine; DCP, dicetyl phosphate, CHOL, cholesterol.

3. Choleragen (Cholera Toxin): A Bacterial Lectin.

Choleragen (cholera toxin) agglutinated erythrocytes and liposomes containing the toxin receptor, galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl)-galactosyl-glucosylceramide (ganglioside G_{M1}). Cells that had been exposed to G_{M1} were agglutinated, but agglutination was not observed when cells had been exposed to other gangliosides (GM_2, GM_3, GDla, GD_{1b}). Choleragen-dependent agglutination of liposomes was slightly less specific, because liposomes containing either GM1 or GD_{1b}, but either G_{M1} or GD_{1b}, but neither G_{M2}, GD_{1a} nor G_{M3} were agglutinated. The oligosaccharide isolated from GM_{M1} inhibited both the agglutination of cells and liposomes containing G_{M1} and the binding of choleragen to liposomes containing G_{M1}.

Galactose and sialic acid were less effective inhibitors of liposomal agglutination and did not inhibit cellular agglutination or binding of cholerágen to liposomes. Liposomal agglutination was dependent on choleragen concentration and occurred with the B but not the A protomer of choleragen. These results suggest that choleragen, through its binding to the oligosaccharide portion of a glycolipod, exhibits lectin-like activity, which results in agglutination of liposomes and erythrocytes.

4. Liposomes As Model Membranes For Ligand-Receptor Interactions: Studies with Choleragen And Glycolipids†

Abstract: Binding of (^{125}I) choleragen to liposomes containing G_{M1} (galactosyl-N-acetylgalactosaminyl-(N-acetylneuraminyl) galactosylglucosylceramide) had the characteristics previously described for the binding of the toxin to cells and membranes Binding was rapid, not readily reversible, and saturable. Half-saturation occurred at 10^{-10} M choleragen, and similar concentrations of unlabeled toxin blocked the binding of (^{125}I) choleragen to the liposomes. Binding was highly specific for liposomes containing G_{M1}; only small amounts of toxin bound to liposomes containing the homologous glycolipids G_{D1b}, G_{A1}, and G_{M2}. Choleragen effectively protected the G_{M1} of liposomes from external labeling by sequential treatment with galactose oxidase and NaB^3H_4; incorporation of ^3H into the galactose of G_{M1} was reduced by 9%. Liposomal G_{D1b}, GA1, and G_{M2} were protected to a lesser extent. Binding of choleragen also reduced the labeling of the sialic acid residue of liposomal G_{M1} by $NaIO_4$ and NaB^3H_4. These results are similar to those reported for G_{M1} in intact cells. Thus, the interaction of choleragen with G_{M1} incorporated into lipid model membranes mimicked the characteristics and specificity noted with biological membranes. Liposomes appear to be useful as model membranes to explore the interaction of ligands with glycolipids incorporated into the liposomal membranes.

5. Production Of Antibodies Against Phosphocholine, Phosphatidylcholine, Spingomyelin, And Lipid A By Injection of Liposomes Containing Lipid A.

Liposomes were investigated as a combined carrier and adjuvant to promote the immunogenicity of lipid A derived from endotoxin. Lipid Acontaining liposomes, or lipid A alone, were injected once into rabbits either subcutaneously or i.v. Antiserum activity was measured by complement-dependent damage to appropriate indicator liposomes containing trapped glucose. Lipid A in liposomes produced a greater immune response against lipid A than that obtained by injecting identical amounts of lipid A alone, and the response to the liposomelipid A combination was equivalent to that obtained by a frequently used alternative

method, in which the antigen consisted of acid treated bacterial cells coated with lipid A.

Antiserum, or purified IgG antibodies, from rabbits immunized with liposomes containing lipid A also reacted with liposomes lacking lipid A. The antibodies were inhibited by phosphocholine, and the activity was adsorbed by phosphatidylcholine, or ceramide phosphochonine (spingomyelin). The anti-phosphocholine antibodies did not cross-react with lipid A, but their binding to liposomes was inhibited by the presence of anti-lipid A antibodies on the surface of the liposomes. An "anti-liposome" response was not observed when either lipid A alone, acid-treated bacterial cells coated with lipid A, or liposomes lacking lipid A were injected.

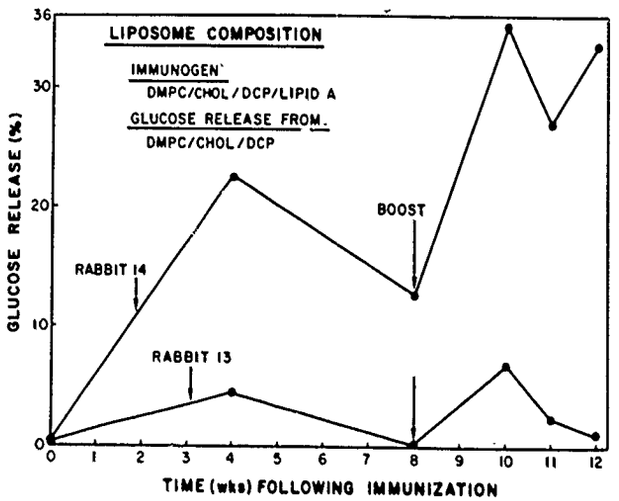

Fig 3. Complement-mediated immune damage against liposomes lacking lipid A after immunization with liposomes containing lipid A. The liposomes used for glucose release contained 20 μg of lipid A per μmole of DMPC.

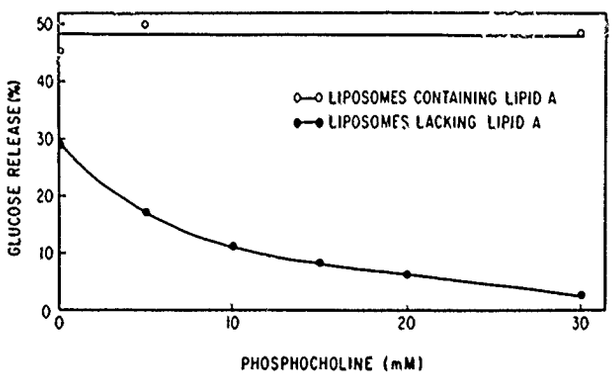

Fig 4. Inhibition by phosphocholine. Glucose release from liposomes lacking lipid A or containing lipid A (20 μg per μmole DMPC) was measured in the presence of 50 μl of antiserum from rabbit 14 4 weeks after immunization Phosphocholine was added to the assay cuvette before the addition of C.

We concluded that one injection of liposomes containing lipid A produces an immune response against lipid A that is equivalent to that produced by other methods of immunization. Injection of liposomes containing lipid A also produces an immune response against phosphocholine, phosphatidylcholine, and sphingomyelin.

6. Activation Of Human Complement By Liposomes: A Model For Membrane Activation Of The Alternative Pathway.

Liposomal model membranes were found to activate the alternative pathway of human complement. Activation was measured by C3 conversion and component consumption in serum that has been incubated with liposomes. C3 conversion did not require C1 or C2 of the classical pathway, since it was observed in serum from a C1r-deficient patient, serum from a C2-deficient patient, and normal serum in buffer containing EGTA and $MgCl_2$. The incubation of liposomes with C2-deficient serum resulted in consumption of components C3 through C9 with no consumption of C1 or C4 in a profile typical of alternative pathway activation. The reaction was further shown to require alternative pathway factor D, and to be independent of antibody.

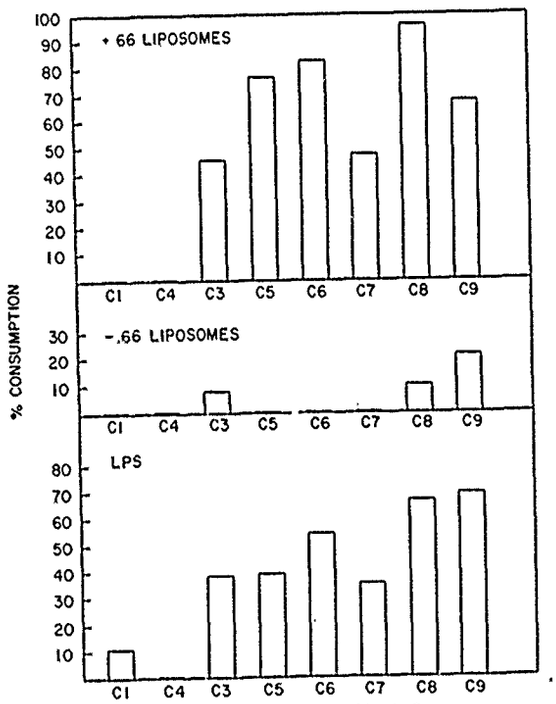

Fig 5. Consumption profile for individual C components after incubation of C2-deficient serum with 600 μg LPS, or with 20 μl of liposomes containing $C(14:0)_2PC$/CHOL/DCP (−0.66 liposomes) or SA (+0.66 liposomes)/GC in molar ratios of 2/1.5/0.66/0.37.

Activation of the alternative pathway was dependent on the membrane composition of the liposomes. A positive charge was required for liposomes to produce C3 conversion. Liposomal cholesterol concentration and phospholipid fatty acyl chain length and unsaturation all influence activation, suggesting the importance of membrane fluidity. Positively charged liposomes containing dimyristoyl phosphatidylcholine and cholesterol required the presence of certain glycolipids for C3 conversion. The activation of the alternative complement pathway by liposomes of defined membrane composition may provide a suitable model for the study of alternative pathway activation by cellular membranes.

7. Interactions of C-Reactive Protein And Complement With Liposomes.

Influence of Membrane Composition

We found previously that interaction of C-reactive protein (CRP) with liposomal model membranes resulted in complement(C)-dependent membrane damage. In the present study, we investigated the influence of membrane composition on the interactions of CRP and C with liposomes. Adsorption experiments showed that binding of CRP was greatest to strongly positive liposomes. A lesser, but still substantial, extent of CRP binding also was observed with negative liposomes, but negligible amounts of CRP bound to neutral or weakly positive liposomes. CRP-mediated consumption of hemolytic C, and C-dependent glucose release from liposomes both were strongly influenced by liposomal charge, positive being superior to negative. Glucose release and, to a lesser extent, consumption of hemolytic C were inversely related to phospholipid fatty acyl chain length. Phospholipid fatty acyl unsaturation and liposomal cholesterol concentration both had strong influences on C consumption and glucose release. The data suggest that CRP-mediated C consumption and membrane damage require an optimum membrane fluidity.

Complement damage in the presence of CRP was enhanced by certain sphingolipids and also by digalactosyl diglyceride, but not by sphingomyelin. Our results thus demonstrate that CRP-mediated C consumption and C-dependent membrane damage both are influenced by the liposomal membrane composition.

8. Immune Reactivities Of Antibodies Against Glycolipids.

Natural Antibodies

Naturally-occurring antibodies against simple glycolipids have appeared in scattered reports in the literature. About 75% of all normal humans have complement-activating anti-Forssman activity,

and a monoclonal Waldenstrom macroglobulin IgM antibody (McG) having specificity for Forssman glycolipid was derived from the plasma of a patient. Some normal, or abnormal human sera have anti-digalactoslyl diglyceride antibodies. Rrecently we reported the occurrence of "natural" antibodies, apparently autoantibodies, with specificity against di- and trihexosyl ceramide haptens (CDH and CTH), in normal rabbit sera. We also found natural anti-ganglioside G_{M1} antibodies in normal human, guinea pig, and rabbit sera.

The major purpose of the present study was to describe, and to quantify, the widespread occurrence of natural complement-fixing autoantibodies against numerous simple glycolipids. We show that every individual rabbit and human serum tested had complement fixing autoantibodies against glycolipids that are widely distributed in circulating blood cells and other tissues.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 147 Immunological and Biochemical Aspects of Membrane

Publications

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA OC 6472	2. DATE OF SUMMARY* 79 10 01	REPORT CONTROL SYMBOL DD-DRAE(AR)636
3. DATE PREV SUM'RY 78 10 01	4. KIND OF SUMMARY D Change	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8a. DISP'N INSTR'N NL	8b. SPECIFIC DATA CONTRACTOR ACCESS ☒ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES.*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	148
b. CONTRIBUTING	62773A	3E162773A818	00	041
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)*

(U) Medical Effects of Blast Overpressure: Applied Studies

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

017100 Weapons Effects 013300 Protective Equipment 00200 Acoustics

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 03	Cont	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: NA EXPIRATION:

b. NUMBER*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	7	0
FISCAL YEAR CURRENT 80	7	600

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21. GENERAL USE

Foreign Intelligence Not Considered

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ASSOCIATE INVESTIGATORS

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NAME: JAEGER, James J., CPT, MSC DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Impulse noise; (U) Blast Overpressure; (U) Human Volunteer; (U) Temporary Threshold Shift; (U) Bioacoustics

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To define the physiological effects upon the human of blast overpressure generated by firing Army weapons systems in terms of: (a) the physical characteristics of the pressure wave responsible for injury; (b) the interaction between the wave and susceptible organs; (c) the threshold for injury of the various organ systems; and (d) potential means of prophylaxis and treatment of blast overpressure injury.

24. (U) Procedures include the field measurement, recording, analysis, and interpretation of the time-history and frequency content of the blast wave generated by firing extended range weapons systems. Attempts are currently underway to predict the pressure field around the cannon by computer simulation.

25. (U) 7810 - 79 09. Extensive field measurements have been made of the M198/M203 and M109/M203. Peak overpressures and B durations of the M198/M203 were applied to the criteria set forth in MIL-STD 1474A (MI) and safety recommendations were offered to maintain crew exposures within acceptable limits during the M198 FDTE. Similar evaluation of the M109/M203 revealed bizarre waveforms with extremely long B durations in the all hatch closed configuration. These waveforms appear capable of more biological damage than the "classical" waveform encountered in the free field situation, and the recommendation was made that the M109/M203 not be fired in this configuration. Analysis of TECOM recorded M110 data allowed firing recommendations to be made for this system. A blast tube simulated M198/M203 blast wave (Lovelace Foundation) has been further refined and is undergoing final analysis.

For technical report see Walter Reed Army Institute of Research Annual Progress Report, 1 Oct 78 - 30 Sept 79.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 148 Medical Effects of Blast Overpressure: Applied Studies

Investigators
Principal: MAJ Gary E. Sander, MC
Associates: CPT James Jaeger, MSC,
Dr. Marvin Stein, Ph.D.
CPT Jeffrey Hess, VC,
Dr. James H. Patterson, Ph.D.

Description

Studies are directed toward definition of the physiological effects upon the human of blast overpressure generated by firing extended range Army weapon systems. This in turn requires investigation of (A) the physical characteristics of the pressure wave responsible for injury; (B) the interaction between the blast wave and susceptible organs; (C) the threshold for injury of the various organ systems; and (D) potential means of prophylaxis and treatment of blast overpressure injury. These are long range goals and will require extensive work.

Short terms goals are directed toward meeting the needs of the weapons community in attempting to insure crew safety while operating strategically necessary extended range weapons. These goals have been met by recording and analyzing overpressure data in crew areas of the M110, M198, and M109 while firing top zone charges, carefully analyzing the data records, applying the finalized data to MIL-STD 1474 (1), and recommending firing limitations such as to maintain operator exposure within the limits set forth in the MIL-STD. The participation of the Sensory Physiology section of the USAARL and a civilian contractor, Jaycor (C 3362), have been instrumental in accomplishing this task.

In an effort to develop an experimental procedure to achieve controlled blast overpressure exposures to evaluate nonauditory injury in animals and auditory risk in human volunteers, the blast tube facility at the Lovelace Inhalation Toxicology Research Institute in Albuquerque, New Mexico is being utilized to simulate the M198/M203 pressure patterns. The accuracy of this simulation is currently under evaluation. Because

a very preliminary study exposing sheep to simulated blast waves suggested the possibility of non-auditory (lung) injury in several of these animals (2), a field study exposing sheep to the actual M198/M203 has been approved and is scheduled for November 1979.

One means of reducing hearing loss is by identification of individuals unusually susceptible to impulse noised induced TTS prior to exposure such that they can be occupationally screened from an impulse noise environment. Dr. John Erdreich of the University of Oklahoma Health Sciences Center is exploring possible means of accomplishing this (C-8074).

Progress

Susceptibility to Hearing Loss. Dr. John Erdreich (4) is currently conducting the study "Factors Associated with Noise Induced Temporary Threshold Shifts." The initial goal of this investigation was to examine the relation between the non-linear amplitude response of the ear and susceptibility of an individual to TTS. Early in the study several individuals exhibited relatively high TTS to fatiguing stimuli of 90-95 dB SPL; the only discernible factor uniting these individuals was a history of chronic smoking. Further specific study of smoking suggests that smokers generally exhibit higher TTS in response to a fatiguing tone than do non-smokers. Early data for 1 KHz and 2 KHz fatiguing support the relation between amplitude non-linearity and TTS susceptibility.

M198 Overpressure Measurements: Overpressure data and field mapping is now available from recordings made at Yuma in April 1977 (5) and Aberdeen in December 1978 (6,7). These data confirm that the pressures in certain operator positions exceeds the Z-line of MIL-STD 1474, and define the pressure pattern around the M198 (Figure 1). Based on the preliminary results of the Yuma data, safety recommendations were made for firing the M198/M203 in training situations (8). These recommendations, including use of a 25 ft. lanyard, were followed during the M198 FDTE conducted at Ft. Bragg between September 1978 and February 1979. An article summarizing the risks of auditory and non-auditory injury resulting from blast overpressures, with emphasis on the M198/M203, was prepared for the artillery community (9).

M110: PM 110E2 requested consultation from the Blast Overpresure Program on safety recommendations for firing this weapon

system. Because of prior time and personnel commitments, BOP was unable to directly participate in the overpressure measurements during the test conducted at Yuma Proving Ground in November 1978, but agreed to evaluate the TECOM test plan, instrumentation, and methodology, and to base safety recommendations on the TECOM data. After evaluation of the test data, all indications were that the test was properly conducted and the data points were accurate and consistent within the limits of the current state-of-the-art. These pressure and B duration measurements were then interpreted according to the guidelines of MIL-STD 1474, and the requested safety recommendations provided (10). These guidelines permit firing of all charges by operators in standard crew positions wearing appropriate hearing protection, but do limit the total number of charges fired.

M109: In an attempt to extend the range of the M109A2/A3 SPH, the Artillery School at Ft. Sill wishes to fire the M203 from the M109. The ARRADCOM Safety Office has requested BOP recommendations on M109/M203 firings, based on data collected by USAARL and Jaycor at APG in December 1978. Prior TECOM data had suggested that the overpressures exceeded the Z-line both inside the turret and in crew positions behind the gun. The December test was conducted with a number of different hatch and door configurations, including one with all hatches and doors closed ("buttoned up" configuration) in an attempt to determine if such manipulations might reduce pressures to levels below the Z-line. It was also designed to provide sufficient field mapping to determine how far behind the weapon the Z-line actually extended. Preliminary Jaycor and USAARL data are now available, and based on this information recommendations have been forwarded to ARRADCOM suggesting that a 50 ft. lanyard be used during all M109/M203 firing (11). The Z-line is exceeded within the turret in all configurations in which any door or hatch is open, and up to distances 50 ft. to the rear of the M109 (Figure 2). The pressure waves generated within the turret in the "buttoned up" configuration (Figure 3) are complex, with multiple high pressure reflected waves and total B durations in excess of 300 msec. Clemedson (12) has reported that such complex pressure waves may produce lung injury in rabbits at one-fifth the peak overpressure required to produce similar lung injury in the open field "classical-type" situation. Based upon such considerations BOP has recommended that crewman not be exposed inside the turret until more information is available on the injury potential of such complex waveforms.

Blast Tube/M198 Correlations: BOP is participating in a contract between AFRRI and the Lovelace Inhalation and Toxicology Re-

search Institute in order to make use of the blast tube facility located at Lovelace. The goal is to adequately simulate with the blast tube the pressure pattern in crew positions of the M198. Availability of a simulated waveform in an experimental environment is necessary to generate animal exposure data to assess the risks of non-auditory injury from such waves and to ultimately extend such studies to hearing protection validation in humans if safety from non-auditory injury can be adequately demonstrated. Lovelace scientists have produced a waveform (which appears similar to the M198/M203 wave at 180 dB and 185 dB in time-history and frequency content) by detonating double primer cord charges within the six foot diameter section of the tube. This wave could be reproduced at 20 sec intervals for 25 shots. However, when unexpected lung lesions were detected in sheep exposed at 185 dB (2). it became necessary to re-evaluate the closeness of fit of the actual and simulated waveforms. Jaycor was contracted to assist USAARL in evaluating this issue and to further explore calibration and reproducibility difficulties experienced by Lovelace. Jaycor has reported (6) that pressure gauge calibration issues will require further resolution since USAARL calibration techniques lead to peak pressures 10% below Lovelace computations. Furthermore, cross correlations between entire waveforms may not be an accurate method of comparison, suggesting that further correlation analysis should focus on very specific characteristics relating to the waveform, such as the initial 20 msec segment, the rise time, or the total peak pressures. By correlating the initial several msec rather than the entire time history, correlation coefficients drop from near unity to 0.5 to 0.6. However, even here it is difficult to know the significance of a correlation coefficient since there is no precedent for this type of comparison in the scientific literature. Work is still ongoing in this area. The Lovelace scientists now feel that they have produced a more stable, reproducible waveform by utilizing a single primer cord charge and a reflecting plate, and by increasing the interval between shots to 60 sec. Analysis of this revised pressure wave is currently in progress at Jaycor and USAARL.

Animal Exposure Study. Apparent lung injuries were observed in sheep exposed to 185 dB blast tube simulated waves. But because of several still unanswered questions about the experimental design, there is still considerable question as to significance of these lesions (2). In an attempt to directly assess the possibility of injury to animals exposed to the M198/M203, an experimental protocol "Pilot Study for Evaluation of Risk of Lung Injury to Sheep Exposed to M198/M203" has been approved by the WRAIR LAIRB (01979) and will be executed

in late November 1979. Sheep will be exposed to multiple rounds of M203 in high pressure positions in which injury is expected, in control positions in which no injury is expected, and in the highest pressure crew position. It is anticipated that this study will provide a good indication of the likelihood of injury in that operator position.

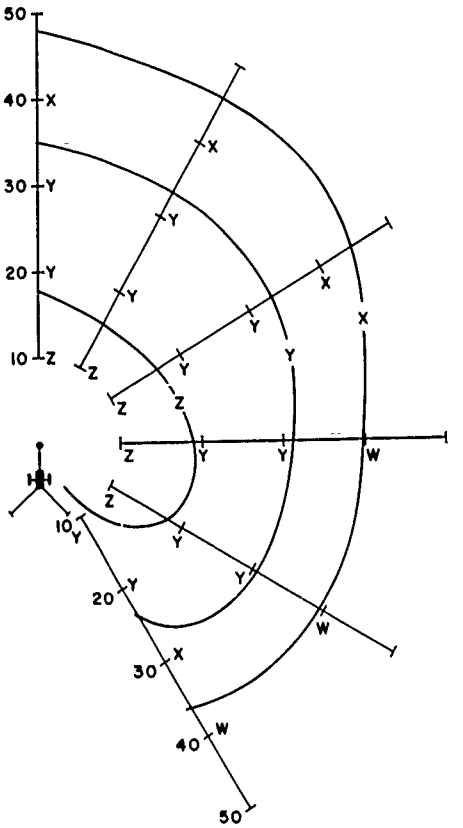

Figure 1. Overpressure field map around M198/M203. The X,Y, and Z-lines denote firing restrictions as defined in MIL-STD 1474A (MI).

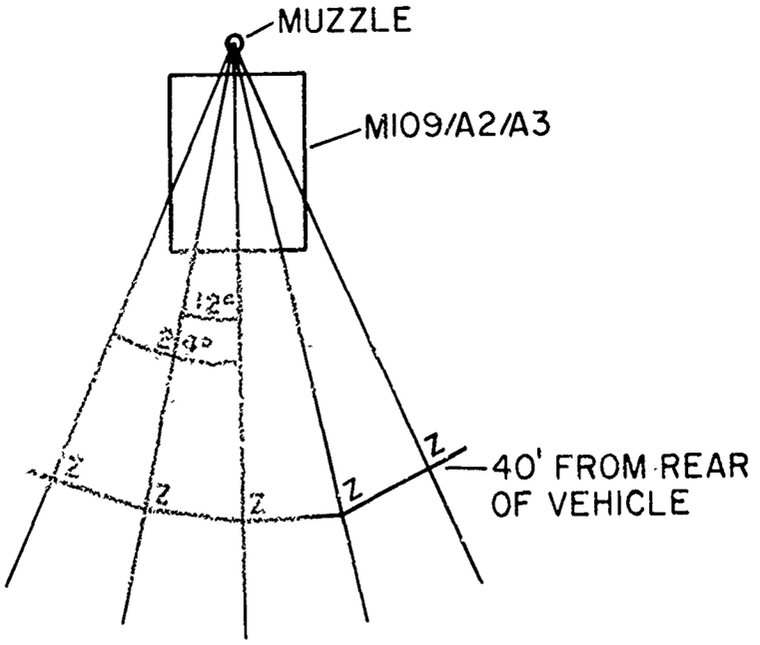

Figure 2. Overpressure field map for M109/M203, showing distance of Z-line from the weapon.

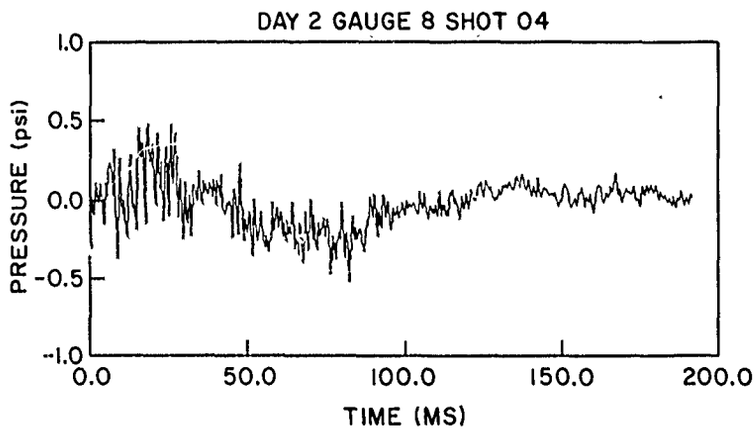

Figure 3. Time history for M109/M203 within the turret in the "buttoned up" configuration.

PROJECT 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 148 Medical Effects of Blast Overpressure: Applied Studies

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC 6435	79 10 01	DD-DRAP(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. RESEARCHING	7a. CHEM'N INSTR'N	7b. SPECIFIC DATA (CONTRACTOR ACCESS)	8. LEVEL OF SUM
78 10 01	D. Change	U	U	NA	NL	(X) YES ( ) NO	A. WORK UNIT

10. NO./CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS01	00	149
b. CONTRIBUTING	62770A	3M162770A803		083
c. [illegible]	CARDS 114F			

11. TITLE (Precede with Security Classification Code)

(U) Protective Immunity in Protozoan Diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

002600 Biology 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 07	CONT	DA	C. In-House

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: N/A. EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PREVIOUS 79	7.0	687
FISCAL YEAR CURRENT 80	7.0	672

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NAME:

21. GENERAL USE

Foreign Intelligence not Considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Antigens, (U) Protozoa, (U) Immunity, (U) Tropical Medicine, (U) Antibodies

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23 (U) The objective of the WU 149 is to elucidate the protective mechanisms involved in immunity to malaria and African sleeping sickness. Malaria is a disease which has repeatedly impeded military operations and African sleeping sickness has a high potential for doing so should there be troops in the endemic area.

24 (U) The approach used in these studies is to study in both animal models and through the use of in vitro techniques the response elicited by the immune system, to determine the roles of cellular and molecular mediators in these processes, and to design experimental immunogens which will provide the basis for future vaccine development programs.

25 (U) 78 10 - 79 09 Analysis of African trypanosomes derived from mice infected with the metacyclic forms of African trypanosomes reveals great heterogeneity of antigenic types. However, some immunity to challenge can be induced by immunization through short term, metacyclic initiated, drug terminated infections. Monoclonal antibodies have been prepared against common and variant specific trypanosome antigens. Different antigenic types of African trypanosomes have similar lectin reactivity. Complement mediated cytotoxicity against trypanosomes can be effected with both IgG1 and IgG2 as well as IgM murine antibodies. Antibody dependent killing of trypanosomes is also mediated by macrophages. Colloidal silica has proven to be an effective medium for density gradient fractionation of malaria parasitized erythrocytes. Adoptive transfer of immunity to sporozoite induced malaria with T lymphocytes is accompanied by antibody production in recipients. For technical report see Walter Reed Army Institute of Research Annual Progress Reports, 1 Oct 1978 - 30 Sep 1979.

*Available to contractors upon originator's approval

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 149 Protective Immunity in Protozoan Diseases

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I. Studies on the feasibility of polyvalent immunization with multiple variable antigen types (VATs) of Trypanosoma rhodesiense.

Objective: This study was designed to test the feasibility of using multiple VATs of T. rhodesiense simultaneously to elicit a protective immune response to challenge by individual or multiple VATs.

Description: The ability of African trypanosomes to undergo antigenic variation poses a major obstacle to production of an effective vaccine. One potential solution to this problem is the use of a polyvalent vaccine having specificity for several different antigenic types of trypanosome. Additional data are needed on the number and prevalence of different trypanosome VATs occurring in endemic areas before the feasibility of such an approach can be tested. However, the efficacy of immunization with a mixture of VATs to provide a protective immunity to challenge by the individual or multiple homologous VATs can be determined using available animal models. For this purpose, the previously described WRATat series of defined VATs of T. rhodesiense was used. Five of the 18 available WRATats were chosen for this study.

Progress: Male C57BL/6J mice were immunized with either one or two intraperitoneal injections of one or 5 different VATs. Each mouse received 0.5 ml of a suspension of 60 KR irradiated trypanosomes (from frozen stabilates) adjusted to contain 1x10 organisms of a single WRATat or 1x10 organisms of each of 5 WRATats (total of 5x10 trypanosomes). The mice were challenged, i.p., 21 days later with 2x10 infectious organisms of a single WRATat or a mixture of the 5 WRATats containing 2x10 organisms of each

(total of $1x10^3$ trypanosomes). Mice not developing a patent parasitemia or splenomegaly within 30 days were considered to have been immune to challenge.

The results of this work are shown in Table 1. Of the 5 groups of mice immunized with a single WRATat, only 3 showed complete protection to homologous challenge. The failure to completely protect animals in groups A1 and A2 had previously been observed and was attributed to the presence of low numbers (0.01-1 %) of minor VATs in the parasite preparations.

In general, the animals having received a single immunization containing 5 WRATats (groups B1 - B5) demonstrated less resistance to homologous challenge than the animals in the "A" group immunized with a single WRATat. The reasons for this observation are not yet clear.

Group B-6 having received both the 5 WRATat immunization and challenge showed only partial resistance. This is not surprising as one would not expect this group to show a higher degree of resistance to challenge than the least resistant group challenged with a single WRATat. However, group B-7 which received two immunizing doses 3 weeks apart, was completely resistant to challenge.

Discussion: The effectiveness of a single immunization with either a single, or several mixed antigenic types of T. rhodesiense appears to vary among the WRATats. This could be attributed to any of the following factors: 1. Differences in immunogenicity among the antigenic types used, 2. Some variation in the quality of the antigenic preparations used as immunogen, 3. Differences in the numbers of minor VATs present in the WRATat used. Previous data suggest that the last possibility is a major factor in causing this variation.

However, despite these problems, the resistance to a polyvalent challenge by group B-7 clearly demonstrates the feasibility of polyvalent immunization using 5 different VATS of T. rhodesiense. Therefore, although the data are admittedly preliminary, it does suggest that other studies should be pursued to determine which antigenic types of trypanosome would be encountered by the host during challenge by infected tsetse fly. These antigen types could then be used to prepare a polyvalent vaccine.

Table 1

Resistance of immunized mice to challenge.

Animal Group		WRATat[a] Immunization	Challenge	Number[b] of mice developing Parasitemia	Splenomegaly
A	1	2	2	2	2
	2	4	4	2	2
	3	6	6	0	0
	4	9	9	0	0
	5	12	12	0	0
B	1	2,4,6,9,12	2	0	0
	2	"	4	4	4
	3	"	6	0	0
	4	"	9	0	2
	5	"	12	0	1
	6	"	2,4,6,9,12	3	3
	7 (2 doses, 3wks apart)	"	2,4,6,9,12	0	0

a. Walter Reed Army Trypanozoon antigen type.

b. The values given represent the number of mice out of 5 developing parasitemia or splenomegaly within 30 days post challenge. In all cases, 5/5 control animals developed parasitemia and splenomegaly upon challenge.

II. Studies on models of Trypanosoma rhodesiense infection.

Objective: To determine the feasibility of using Cynomolgus monkeys in place of Rhesus monkeys as hosts for experimental infections of T. rhodesiense in primates. Also, to collect sera and parasites for use in the development of an enzyme-linked immunosorbent assay (ELISA) as ·y for human trypanosomiasis.

Description: Previous studies have demonstrated that experimental infections of Rhesus monkeys with T. rhodesiense result in a series of remissions and relapses with associated antibody formation similar to that seen in humans. Also, Rhesus immunoglobulins are reactive with anti-human immunoglobulin reagents, making the Rhesus monkey suitable as a source of antibody for use in the development of the ELISA for a human system.

Given the current shortage and uncertain future availability of Rhesus monkeys, the use of a different primate would be desirable. Therefore this study was designed to provide both useful reagents and a comparison of the course of infection in Rhesus and Cynomolgus monkeys.

Initially, one Rhesus and one Cynomolgus monkey was infected by intravenous inoculation of $1x10^4$ trypanosomes of a clone of tsetse fly passaged T. rhodesiense (LVH strain). At a later time, an additional Rhesus monkey was infected with a different fly passaged clone of T. rhodesiense. Parasitemias were monitored daily with stabilates being made during, and serum obtained after, each peak parasitemia. Also, thin blood smears were prepared at appropriate times for later use in immunofluorescence studies on the antigenic composition of the parasite populations present.

Progress: The course of parasitemic in the Rhesus (No. 29) and the Cynomolgus (No. 97) monkeys infected with WRATat 14 are shown in Figure 1. The Rhesus monkey experienced a peak of high parasitemia during the acute phase followed by generally very low, often subpatent parasitemia. In contrast, the Cynomolgus monkey showed no large initial peak of parasites but did develop a series of patent parasitemic peaks for at least 60 days. A second Rhesus monkey (No. 561) infected with WRATat 15 developed a course of parasitemia intermediate between the two previous monkeys as shown in Figure 2.

The collection of trypanosomes for stabilates, the preparation of blood smears for immunofluorescence assay and the

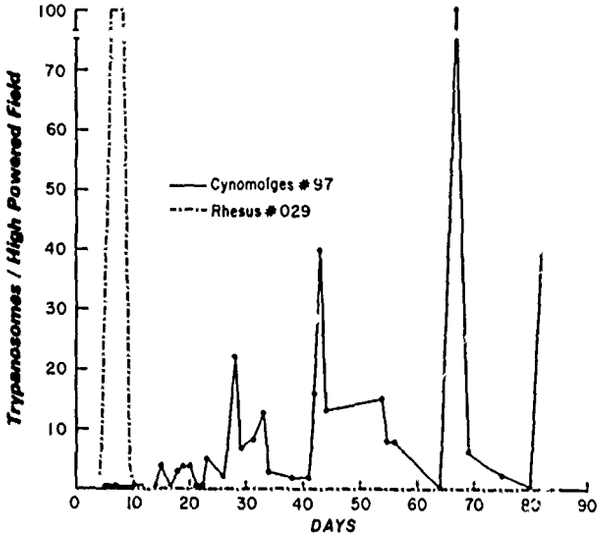

Figure 1. The course of parasitemia in monkeys infected with WRATat No. 14.

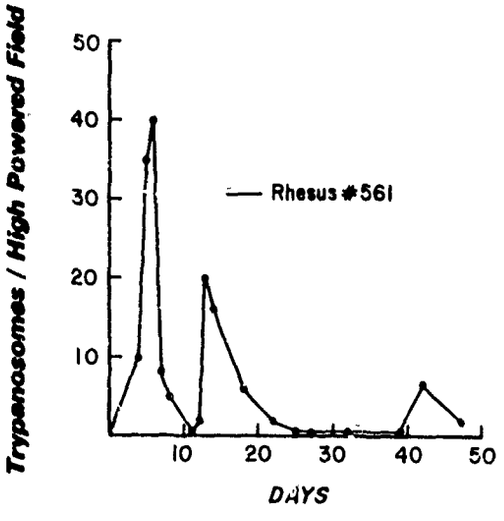

Figure 2. The course of parasitemia in monkeys infected with WRATat No. 15.

collection of serum following peaks of parasitemia were facilitated by the recrudescent nature of the infection in monkeys 97 and 561. After the initial peak of parasitemia, only serum and no parasites could be collected from monkey 29.

Discussion: The courses of infection observed in this study indicate that the Cynomolgus monkeys may be suitable hosts for future experimental primate infections with the LVH strain of T. rhodesiense. The Cynomolgus monkey used was susceptible to infection and developed a course of parasitemia which made the collection of the needed parasites and serum possible.

Analysis of the material collected from all three monkeys is currently underway to further determine the relative suitability of the monkeys for use as models of human trypanosomiasis. Also, sera and parasites obtained from this study are being used to set up and evaluate an ELISA assay intended for future use in screening humans for present or past exposure to T. rhodesiense.

III. Studies on the prevalence of human trypanosomiasis in the Lambwe Valley Area of Kenya.

Objective: To determine the feasibility of a large scale prospective epidemiological study of human trypanosomiasis in the Lambwe Valley of Kenya.

Description: For the past 9 years, WRAIR personnel stationed in Kenya have collected parasites and, when possible, serum from trypanosome infected humans presenting themselves at the Homa Bay Hospital in Kenya. This collection procedure has provided the parasites used for the development of the WRATat serodeme of T. rhodesiense (previously described) and also has provided the reagents which allowed a limited assessment of the trypanosome variable antigen types (VATs) prevalent in the Lambwe Valley. However, most of the work has dealt with material obtained from patients seeking treatment. No active epidemiological survey of the endemic area had been undertaken within the past 8 years and consequently no accurate estimate of the prevalence of the disease can be made. Therefore a small scale epidemiological survey of the Lambwe Valley was undertaken to determine the present state of human trypanosomiasis in this endemic area. Sera were obtained from approximately 600 residents of the Lambwe Valley for testing by available assays for reactivity with trypanosome antigens. Pending the completion of the development of an ELISA assay for the trypanosome system, an indirect immuno-

fluorescence assay (IFA), using trypanosomes of the WRATat serodeme as antigen, was used to screen the sera. Previous work with known positive cases of trypanosomiasis consistently showed the greatest reactivity in this assay system in conjunction with a fluorescence conjugated anti-human IgG reagent; therefore, this procedure was also used throughout this study. All sera were tested at a 1:5 dilution and the intensity of the fluorescence reactions observed was scored qualitatively as strong, intermediate, weak, equivocal or nonreactive.

Progress: The results of the IFA on 340 sera are shown in Table 2.

Discussion: A total of 42 sera were reactive in the IFA. Only 8 of these correlated with known positive cases. Also, an additional 16 known positive cases were not reactive in the assay.

At this time the reasons for the observed discrepancy are not clear. It does appear that the IFA, at least as it was applied here, may not be an appropriate assay for the detection of cases of human trypanosomiasis. The ELISA assay, with the use of the appropriate trypanosome antigens will hopefully be both more specific and more sensitive.

Additionally, as only two confirmed positive cases of previously undetected trypanosomiasis were found in the epidemiological survey, it does not appear that a large scale survey would be useful at this time. However, the continued collection of parasites and sera from cases presenting at the Homa Bay Hospital will be useful to facilitate monitoring of the prevalent trypanosome VATs in the endemic area. This VAT prevalence data will be useful if vaccine development appears feasible.

IV. Development of monoclonal antibodies specific for trypanosome antigens.

Objective: To develop a system for the production of trypanosome antigen specific monoclonal antibodies which will be used in the identification and purification of discrete trypanosome antigens.

Description: Investigations of antigenic variation have recently dealt with the antigenic composition of the blood stream form of the parasite both through immunofluorescence and biochemical analysis, but little has been done with the infective metacyclic form of the parasite from the tsetse fly vector. The limiting factor in work with metacyclic forms has been the low

Table 2
Reactivity of Lambwe Valley human sera in the IFA assay[a]

Source of Human Sera	Number Tested	Number of Sera Reactive				Number Non reactive
		Strong[b]	Intermed.	Weak	Doubtful	
All Persons in sample group	340	2	19	16	6	298
Persons in the sample group with a history of trypanosome infection	24	1	5	2	0	16

a. Indirect fluorescent antibody assay using a mixture of WRATat serodeme trypanosomes as antigen.

b. The intensity of the observed fluorescence was grades qualitatively as indicated.

numbers of organisms which can be obtained for analysis or for preparation of specific antisera. Also the specificity of some antisera to trypanosome antigens has been questioned in view of recent descriptions of common determinants in the variable antigens of different variable antigen types (VATs) from the same trypanosome serodeme.

Recently, techniques have become available to allow the preparation of monoclonal antibodies specific for a given antigen by formation of hybrids between mouse myeloma cells and antibody secreting spleen cells. These techniques provide the means with which to prepare antisera of discrete specificity for a trypanosome antigen. Also, these antisera can be obtained without the need for large number of parasites. This study deals with the application of these techniques to the identification of specific trypanosome antigens in both blood stream and metacyclic forms of T. rhodesiense.

Progress: BALB/c plasmacytoma cells (P3/X63-Ag8) were fused with spleen cells from either BALB/c or C57BL/6 mice which had previously been exposed to T. rhodesiense organisms. For the preparation of variable antigen specific monoclonal antibodies to blood stream forms, mice were infected with trypanosomes, drug cured with Berenil and subsequently boosted with purified variable antigen prior to the fusion of spleen and plasmacytoma cells. Alternatively, mice were infected, drug cured on day 6 and the spleens taken for fusion on day 8.

For the preparation of antibodies to metacyclic forms, spleen donor mice were repeatedly exposed to bites of infective tsetse flies followed each time by drug cure with Berenil. Trypanosome specific antibody producing hybrid cells were selected on the basis of the reactivity of culture supernatants in an indirect immunofluorescence assay using fixed, intact trypanosomes as antigen. The majority of the cell clones obtained were found to produce antibody to common trypanosome antigens as demonstrated by reactivity with the trypanosome flagellum, nucleus, kinetoplast and other internal cell antigens of trypanosomes of several different VATs. However, hybrid cells producing VAT specific antibody were also found as demonstrated by culture supernatant antibody reactive with trypanosomes of only a single VAT.

Discussion: The reagents and methodologies described have potential for defining the discrete antigenic determinants relevant to a protective immune response and also for the purification of specific antigens. The relationship between the hybri-

doma forms of T. rhodesiense is currently being investigated.

V. Extraction of variant specific surface coat antigens from Trypanosoma rhodesiense.

Objective: The objective of these studies was to compare methods for the extraction of surface coat antigen from African trypanosomes for use as experimental immunogens.

Description : A variety of techniques for the extraction of the surface antigens of trypanosomes have been used both in our laboratory and elsewhere. These include the homogenization of organisms by freezing and thawing followed by extrusion through a syringe needle, the extraction of intact organisms using hypertonic KCl and the recovery of antigen from the supernatant fluid after incubation in isotonic saline. We have compared these preparations with respect to their heterogeneity as determined by isoelectric focusing on polyacrylamide gel and by their reactivity with antiserum.

The Wellcome CT strain of T. rhodesiense was used. For the comparisons, cells were harvested from lethally irradiated mice infected 3 days previously and purified by DEAE chromatography. Aliquots were extracted by the 3 procedures.

An antiserum was obtained from a rabbit immunized with saline extract antigen. It was monospecific in the sense that it gave a single line by conventional immunoelectrophoresis with any of the antigen preparations. Multiple reacting components, however, were demonstrable by isoelectric focusing. This antiserum was used to prepare Mancini plates for a quantitative determination of the amount of reactive material in each extract. In addition it was used to locate active fractions after preparative isoelectric focusing. The antiserum was shown to be highly active in mouse neutralization tests and in vitro cytotoxic assays against the organisms. In preparative isoelectric focusing experiments, the position and intensity of precipitin bands was used to estimate the fractions which had peak activity.

Progress: Figures 3 and 4 show representative isoelectric focusing experiments using 2 of the extracts. The two optical density (OD) curves are similar in that the peak activity lies between pH 8 and pH 9 in each case. In addition, maximum precipitation in gel is observed with fractions near and slightly to the right of these OD peaks. Examination of these fractions by analytical isoelectric focusing (data not shown) has repeatedly demonstrated several components in this isoelectric point range.

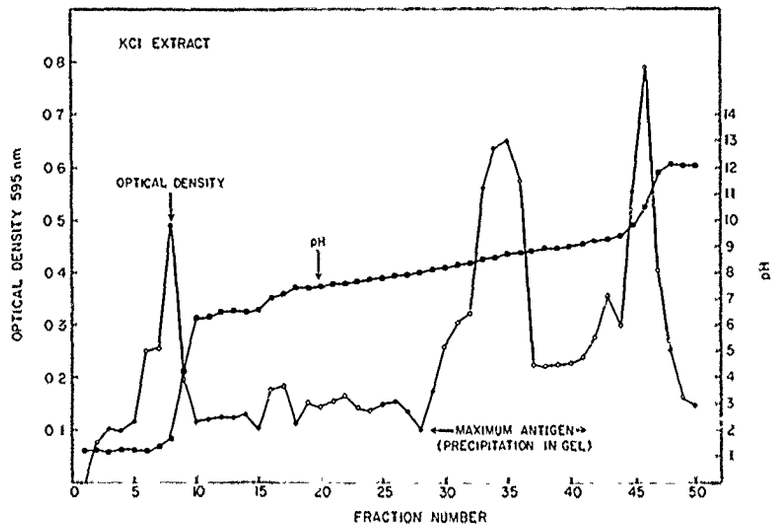

Figure 3. Preparative isoelectric focusing of T. rhodesiense soluble antigen extract.

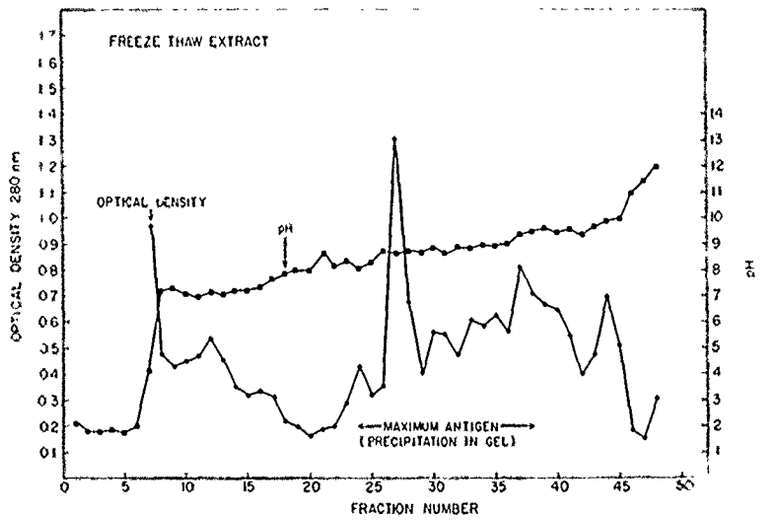

Figure 4. Preparative isoelectric focusing of T. rhodesiense soluble antigen extract.

Figure 5 illustrates two experiments in which the analytical isoelectric focusing pattern of all 3 extracts is shown. The actual measured pH's observed along the gel are indicated for each experiment. It can be seen that KCl and saline extracts exhibit two major bands at around an isoelectric point of 8, whereas the freeze-thaw extract contains multiple components in this range. However, in both experiments the two major KCl bands seem also to be present in the freeze-thaw but the two bands in the saline extract appear to have differing isoelectric points, one greater than either of the major KCl bands and one less than either of these.

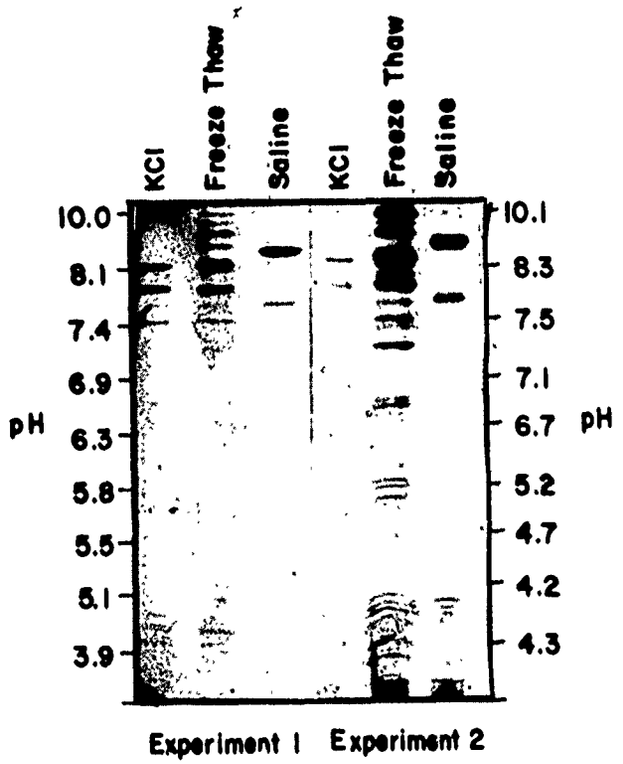

Figure 5. Comparison of various soluble antigen preparations of T. rhodesiense by isoelectric focusing in polyacrylamide gel.

Analysis of antigen concentrations by radial immunodiffusion of the three types of preparations are illustrated in Table 3. It can be seen that the total recovery of antigen is greater in the freeze-thaw preparation, whereas the specific activity in terms of units per milligrams of protein is higher for the KCl extract.

Table 3

Antigenic activity of extracts of Trypanosoma rhodesiense.

Extraction method	Units*/10^9 cells		Units/mg Protein	
	Expt 1	Expt 2	Expt 1	Expt 2
Freeze/thaw	853	215	1457	1205
KCL	751	168	2228	5485
Saline	542	103	1695	1375

*A unit of activity was determined by comparing the diameter of an unknown from radial immunodiffusion to a purified reference standard.

Discussion and Recommendations: These data indicate that reproducible differences in the constituency of T. rhodesiense occur as a function of the extraction procedure. For most purposes it would appear that the KCl extraction procedure is preferable to the preparation of homogenates by freeze-thawing in terms of the complexity of the mixture obtained by the latter procedure. Furthermore, the saline extracts appear to be qualitatively different from the other two preparations as judged from the isoelectric focusing patterns. It is tempting to speculate that KCl extracts contain the intact native molecule. Saline extracts may only contain that portion which protrudes above the membrane proper, and that this is somehow broken during the saline extraction procedure. These data will be useful for the further characterization of trypanosome antigens for a variety of experimental purposes.

VI. Immunoglobulin classes responsible for antibody and complement mediated cytotoxicity against Trypanosoma rhodesiense.

Objective: The objective of this project is to determine the immunoglobulin classes and subclasses of murine antibody which are responsible for the killing of T. rhodesiense in collaboration with the alternative pathway of the complement system.

Description: Previous studies in our laboratory have demonstrated that immune serum derived from animals immunized against gamma irradiated T. rhodesiense kills viable organisms in the presence of complement. This cytotoxic reaction can proceed through the alternative complement pathway as demonstrated through the use of C4 deficient serum as a source of complement and the failure of EGTA, a chelater of calcium, to abrogate the reaction. Alternative pathway activation is thus in this case associated with antigen-antibody complexes, and it is of interest to determine which immunoglobulin classes are involved.

The approach used to evaluate this question was to use affinity columns prepared with monospecific anti-murine immunoglobulin to deplete sera from immunized mice of one or more classes of immunoglobulin. In addition to control serum from nonimmunized animals, serum taken 3 days or 1, 2 or 4 weeks after a single immunizing dose of irradiated parasites were studied. Anti-IgM was prepared in rabbits through immunization with commercially obtained IgM followed by

absorption using an IgG-sepharose affinity column. The IgG in this instance was obtained from normal mouse serum by staphylococcal protein A sepharose affinity chromatography. Monospecific anti-IgG antiserum was obtained commercially from Litton Bionectics, Kensington, Maryland. Antiserum reactive with both IgG_{2a} and IgG_{2b}, but not IgG_1 was obtained by immunization of rabbits with commercially obtained IgG_{2a} myeloma protein followed by absorption with an affinity column prepared with IgG_1 myeloma protein. The IgG_1 protein was purified from ascitic fluid by binding the total IgG to staphylococcal protein A followed by stepwise elution with buffers of decreasing pH. Evaluation of monospecificity of antisera and of completeness of depletion of sera of specific immunoglobulin classes or subclasses was performed by Ouchterlony analysis.

Estimates of the activity of the sera were made using the cytotoxic assay previously described (1). Fresh frozen rat serum was used as a source of complement. In order to compare assays done on different days, a single standard hyperimmune rat antiserum directed against the parasite was titrated with each assay, the ED_{50} for all antisera calculated, and the potency of each experimental serum expressed as a ratio of that of the standard hyperimmune rat serum.

Progress: Table 4 summarizes the results to date. As can be seen, activity could not be detected in three days, whereas after one week maximal activity was observed. This persisted at lower levels during the 2nd and 4th weeks. Absorption with anti-IgM removed a substantial proportion of activity from all three of the sera. Additional experiments will be performed to determine whether or not activity persists in the IgM depleted one week serum sample. This was clearly the case at weeks 2 and 4. As can be seen, there was a further depletion of activity when the 2 and 4 weeks sera were absorbed with anti-IgG ; in fact, it was reduced to levels too low for easy detection in the case of the 4 week serum. The 2 week serum clearly had activity remaining even after all detectable IgM and IgG were removed. An absorption with anti-IgG removed the remaining activity from this serum.

It can be noted that activity in the "blank" control column treated sera declined as a function of the number of absorptions as well, although this effect was much less pronounced than that observed with the specifically absorbed sera. This is most likely due to dilution of the serum during the manipulation and in fact a rather marked decrease was observed in the optical density at 280 nm meters of the serum obtained after 2 weeks of immunization after 4 absorptions. If this decreased

Table 4

Relative potencies of immune sera cytotoxic to Trypanosoma rhodesience after immunoglobulin depletion.

Time after Immunization (weeks)	Number of Absorptions: 1		2		4	
	IgM Depleted	* Control	IgM IgG_1 Depleted	Control	IgM + IgG_1 + IgG_2 Depleted	Control
0	<0.2	<0.2	—**	—	—	—
1/2	<0.2	<0.2	—	—	—	—
1	<1	1.7	—	—	—	—
2	0.4	0.8	0.2	0.5	<0.04	>0.33
4	0.4	0.7	0.2	0.2	—	—

*"blank" column prepared with normal rabbit IgG
** assay not done.

absorbance is taken to be a function of dilution and the relative potency of this fourfold absorbed serum recalculated, it is even higher than that observed with the singly absorbed fraction of the same antiserum (relative potency greater than 1.7).

Discussion and Recommendations: These studies demonstrate cytotoxic activity in IgM, IgG_1 and IgG_2 fractions of antisera taken at various times after immunization. It is further suggested that the bulk of the activity is in the IgM fraction at one week but that higher proportions of the activity are due to IgG antibodies later, as might be expected. Additional studies are recommended to explore the extent to which IgG_1 as opposed to IgG_2 is active in complement dependent killing of trypanosomes through the alternative complement pathway. In addition, since phagocytosis and killing of trypanosomes by macrophages may be another important mechanism of killing of these organisms, the roles of IgM, IgG_1 and IgG_2 in this reaction should also be investigated.

VII. Strain-dependent specificity of mice to lethal effects of infection with Trypanosoma rhodesiense (EATRO 1886).

Objective: This investigation was designed to determine the relative susceptibilities of various highly inbred mouse strains to infection with the EATRO strain of 1886 T. rhodesiense.

Description: Many inbred strains of mice can be separated into animals resistant or susceptible to infection with certain parasites, including viruses or bacteria. Investigators have previously reported that trypanosome infection is not correlated with a specific H-2 complex, yet the evidence points to a genetic basis for the great variability of death seen among diverse mouse strains. With a better understanding of the genetics involved in trypanosome infections of mice, one can go on to investigate the various cellular and humoral factors involved in the disease. Male or female mice 4-10 weeks of age were either purchased from The Jackson Laboratory, Bar Harbor, ME or bred in-house. Mice were injected with either 10^3 or 10^5 trypanosomes and checked daily for deaths. As shown in the previous report, a determination of the more resistant strains and the dose-related nature of the parasite infection was established.

Progress: The completed data (Figures 6, 7) of the partially presented work of the last report demonstrated that C57BL/6J male mice were most resistant to death due to T. rhodesiense infections and C3H/HeJ, DBA/2J and BALB/cJ, were most susceptible when infected with 10^3 trypanosomes/mouse. Based on these results, highly inbred RI (recombinant inbred) lines

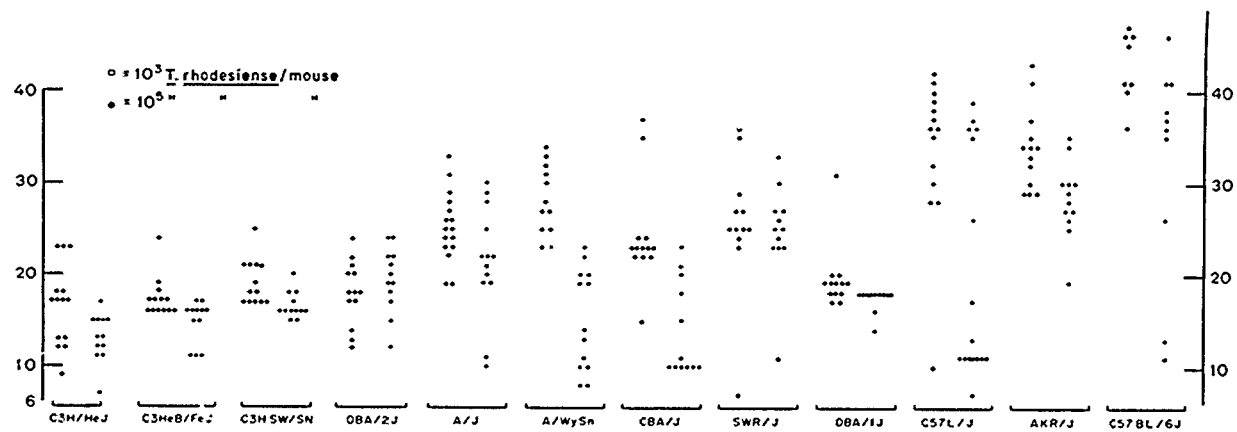

Figure 6. Strain-dependent specificity of varied mouse strains to injections of 10^3 and 10^5 T. rhodesiense.

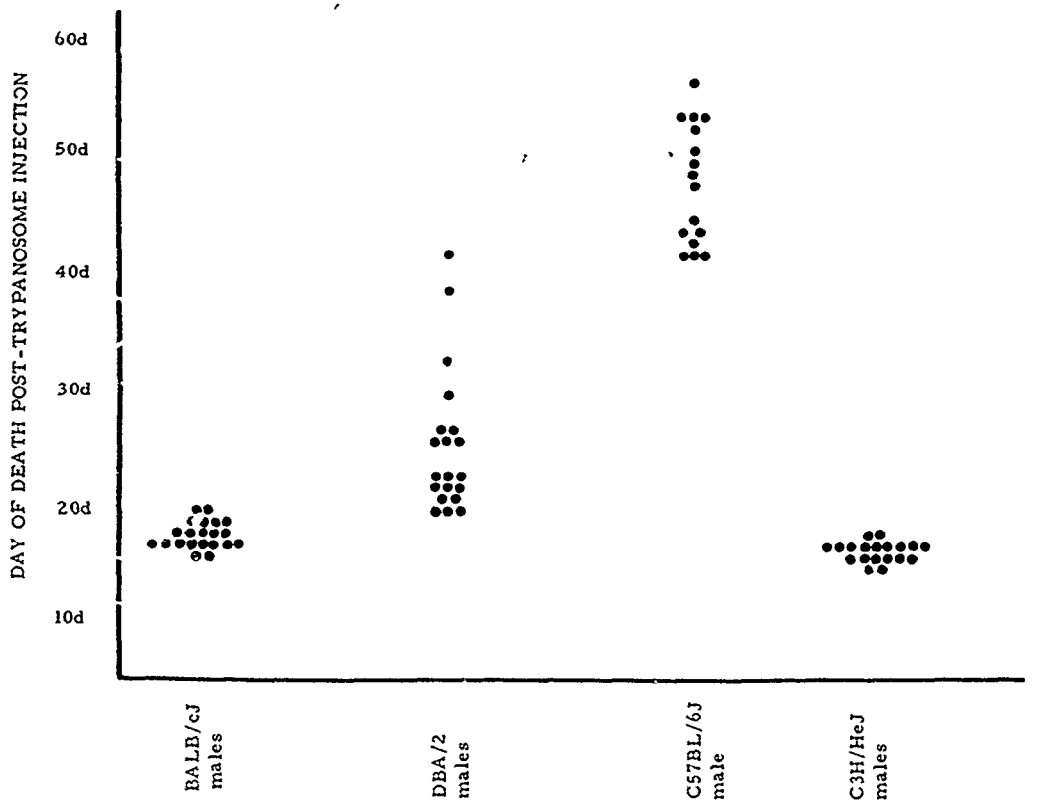

Figure 7. Parental mouse strains: susceptibility and relative resistance to injection with 10^3 T. rhodesiense.

derived from crosses of the susceptible BALB/cBy and the relatively resistant C57BL/6By were obtained. The results of infection of this CXB series (Figure 8) established for the first time the existence of mouse strains, CXBH and CXBJ, that are more resistant to infections with the EATRO 1886 type of T. rhodesiense than their progenitor strain C57BL/6By.

Discussion and Recommendations: It is clear from results obtained that there is strong genetic control over the responses of mice to infections with trypanosomes. Some of the highly resistant RI mice represent another sphere to aid in our understanding of resistance to this parasitic infection. The use of other RI strains which are derived from crosses of susceptible with resistant strains is recommended. BXD and BXH strains are derived from crosses of C57BL/6J with susceptible strains DBA/2J and C3H /HeJ, respectively. Data from infections of these mice, incorporated with currently generated data on deaths of F1 and backcross mice, should result in an understanding of the patterns of inheritance of relative susceptibilities and resistance among mice to T. rhodesiense infection. Alloantigens which characterize these various inbred strains may help us identify individual loci, and perhaps lead to a map describing the major loci which are the genetic determinants of resistance to infection of mice by T. rhodesiense.

VIII. Adherent murine peritoneal exudate cell mediated killing of trypanosomes in vitro in the presence of anti-trypanosomal serum.

Objective: This study was designed to delineate the factors necessary for the in vitro killing of T. rhodesiense (Wellcome strain) by mouse peritoneal macrophages.

Description: In the presence of serum from animals immunized against experimental African trypanosomiasis by injection of gamma irradiated organisms, normal resident peritoneal washout cells from mice will bind and phagocytize living trypanosomes in vitro. The present study examines the requirements of this system.

All studies were carried out on 72 hour adherent mouse peritoneal monolayers. Normal or immune sera and parasites were layered on these cell preparations.

Progress: It was determined that a trypanosome to macrophage ratio of 50:1 - 100:1 in the presence of 1/5-1/10 immune serum resulted in maximal attachment of trypanosomes to

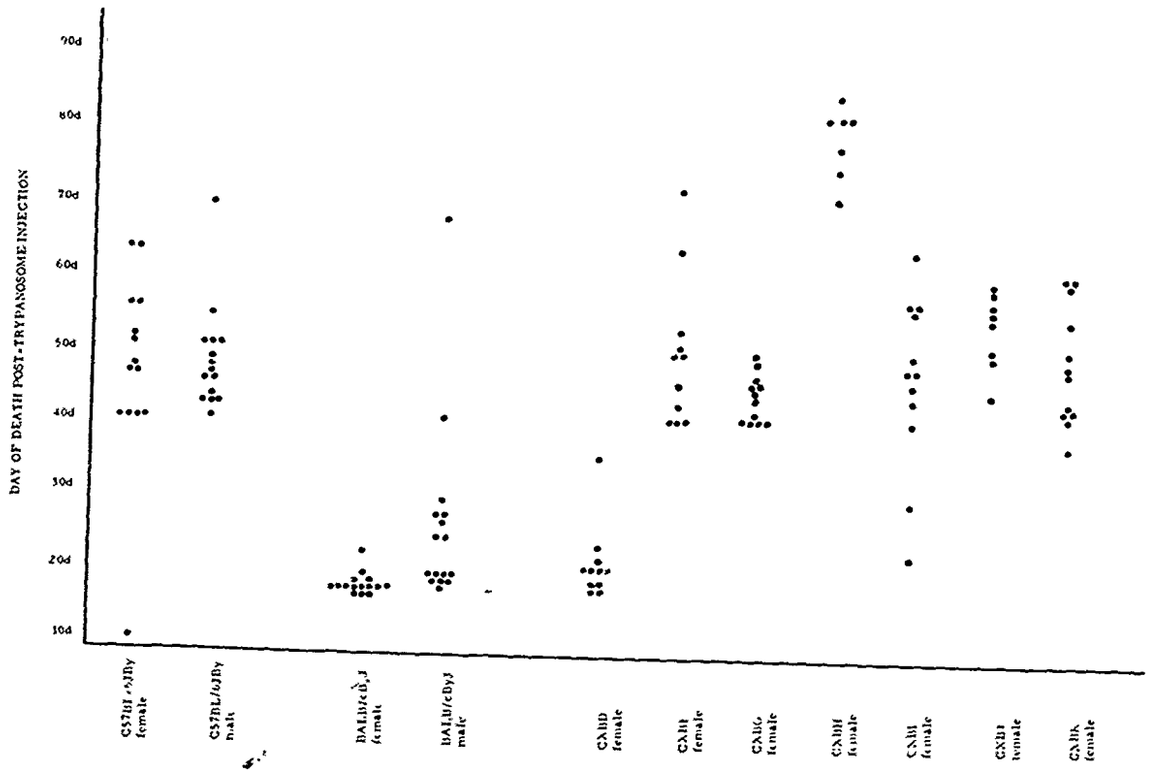

Figure 8. RI mouse strains: susceptibility and relative resistance to T. rhodesiense.

adherent cell during a 30 minute incubation of cultures in a 5% CO_2 humidified incubator. Fewer externally bound trypanosomes were seen at 120 minutes. No attachment of trypanosomes occurred in the presence of serum from noninfected (normal) mice. Trypanosomes in the presence of anti-trypanosomal serum bound equally well to normal wash-out cells as to cells from infected animals.

The data in Table 5 demonstrate that trypanosomes will not attach to cells treated with immune serum and extensively washed to remove excess antibody. This indicates that cytophilic antibody is not involved in the attachment of trypanosomes to adherent cells. However, the data does indicate that trypanosomes sensitized with antibody and then washed will readily adhere to cells. The presence of such an opsonic antibody can be consistently demonstrated.

Discussion and Recommendations: It is planned to continue the effort toward characterization of the phenomenon by which trypanosomes attach, in the presence of immune serum, to adherent cells. The immunoglobulin class specificity, as well as the immunoglobulin fragment required for this parasite-to-cell interaction, will be examined.

IX. Studies on the identity and location of lectin-binding saccharides of Trypanosoma rhodesiense antigenic variants.

Objective: To determine: (1) If several cloned population of T. rhodesiense of known, distinct variant antigen type (VAT) have lectin binding saccharides (sugar-binding protein) on their intact or enzyme-modified surface, and (2) If the presence or absence of the saccharides can be correlated with the VAT of each cloned population.

Description: Repeated antigenic variation by African trypanosomes allows the parasites to escape the host's response and poses a major obstacle to effective vaccine production. Trypanosome variant antigens are cell surface glycoproteins of marked amino acid heterogeneity (at least in their N-terminal regions). Carbohydrates of different antigens vary quantitatively and qualitatively but little is known of saccharide contribution to the antigenic characteristics of trypanosomes. Conceivably, location and type of carbohydrate groups could influence the antigenic and serological characteristics of variant antigens.

This study was designed to exploit the sugar-binding properties of a group of proteins termed lectins, to determine

Table 5

Percent trypanosome attachment to adherent murine peritoneal cells.

	Tr adhering/100 cells
Sensitized Cells* (cytophilic antibody)	0
Sensitized Trypanosomes** (opsonic antibody)	74±16
Control*** (simultaneous additions)	100

* 0.15 ml of immune serum at 1:5 dilution was added to a cell monolayer and incubated at 27 C for 45 min. The serum was removed by aspiration, and the cells washed three times with warm RPMI 1640 before addition of 0.3 ml of trypanosomes.

** 0.5 ml of trypanosomes and an equal volume of immune serum at 1:5 were incubated at 0 C for 45 minutes and washed before 0.3 ml of treated parasites were placed upon the monolayer.

*** 0.3 ml of RPMI 1640 was placed upon a monolayer at 27 C. After 45 min, the monolayer was washed and 0.15 ml trypanosomes and 0.15 ml of serum at 1:5 dilution was added.

if certain sugars are on the intact or enzyme modified cell surface of living and/or fixed trypanosome variants.

Twenty-two cloned antigenic variants of T. rhodesiense were prepared in pure suspension, free of host blood cells, then incubated in solutions of each of 5 different lectins. Lectin-induced cell agglutination patterns were examined after a 60 minute incubation period. Control tests were conducted to confirm the accuracy of the results. Since the cloned parasite stocks were of known antigenic types with known serological cross-reactivity patterns, the lectin-induced parasite agglutination results were studied to determine if lectin binding could be used to differentiate serologically distinct antigenic variants of T. rhodesiense.

Ultrastructural visualization of lectin binding sites was carried out to determine the location and spatial distribution of trypanosome saccharides.

As described in the 1978 Annual Report, trypanosome variant antigen isolation by Concanavalin A-lectin affinity chromatography, was one of the best methods for obtaining antigens for biochemical and immunological analysis. The results of the lectin agglutination tests described herein, identify other lectins which could be used to isolate trypanosome antigens.

Progress: Lectin agglutination tests were conducted with the lectins Concanavalin A (Con A, specific for α-D-mannose), Phytohemagglutinin P (Phyto P, specific for n-acetyl-D-galactosamine), Soybean agglutinin (SBA, specific for n-acetyl-D-galactosamine and D-galactose). Wheat germ agglutinin (WGA, specific for n-acetyl-D-glucosamine), and Fucose binding protein (FBP, specific for α-L-fucose). Trypanosomes were incubated with lectins (at 250, 125, 100, 50, 10, 0 μg/ml) in sealed, rotating microtiter plates at 26 C for one hour, with and without 50 mm of the appropriate competing sugar. Agglutinations , scored visually by inverted light microscopy, ranged from strong (3+), moderate (2+), weak (1+) to none (0). All tests were repeated twice with two replicates per test and all positive results were photographed with Tri X Pan 35 mm film. Control agglutination tests with appropriate red blood cell types indicated that all lectins were functional prior to their use with trypanosome stocks.

Results of the Con A and Phyto P induced agglutination of 22 stocks of living, intact T. rhodesiense variants are summarized in Tables 6 and 7, respectively. No spontaneous agglutination occurred in the absence of the lectins. The addition of

Table 6

Concanavalin A-induced agglutination patterns of *T. rhodesiense*

	Microgram lectin concentration						
Variant	250	125	100	50	10	5	0
LVH 18A	3+	3+	3+	2+	0	0	0*
1D 12A	3+	3+	3+	2+	0	0	0
2D 12A	3+	3+	2+	2+	1+	0	0
2D 12B	3+	3+	2+	1+	0	0	0
3D 13A	2+	2+	1+	1+	0	0	0
3D 13B	3+	3+	2+	1+	0	0	0
3D 13C	3+	3+	3+	2+	0	0	0
3D 13D	3+	3+	2+	1+	0	0	0
4D 13A	3+	3+	3+	2+	0	0	0
5D 13A	3+	3+	3+	2+	0	0	0
6D 13A	3+	3+	3+	3+	2+	1+	0
6D 13B	3+	3+	3+	2+	0	0	0
6D 13C	3+	3+	3+	2+	1+	0	0
6D 13D	3+	2+	2+	1+	0	0	0
7D 12A	3+	3+	3+	2+	1+	0	0
7D 12B	3+	3+	3+	2+	0	0	0
8D 31A	3+	2+	2+	2+	0	0	0
8D 31B	3+	2+	2+	2+	0	0	0
8D 31C	2+	2+	1+	1+	0	0	0
CMFMA	3+	3+	3+	2+	1+	0	0
CMFMB	2+	2+	2+	2+	1+	0	0
CMFMD	2+	2+	2+	2+	1+	0	0

* No agglutination was present when 5-mM ∝-D-mannose was added.

Table 7

Phytohemagglutinin P induced agglutination patterns of

T. rhodesiense antigenic variants.

	Microgram lectin concentration						
Variant	250	125	100	50	10	5	0
LVHI8A	1+	1+	1+	0	0	0	0*
ID12A	0	0	0	0	0	0	0
2D12A	2+	2+	1+	1+	0	0	0
2D12B	0	0	0	0	0	0	0
3D13A	0	0	0	0	0	0	0
3D13B	0	0	0	0	0	0	0
3D13C	0	0	0	0	0	0	0
3D13D	0	0	0	0	0	0	0
4D13A	0	0	0	0	0	0	0
5D13A	0	0	0	0	0	0	0
6D13A	0	0	0	0	0	0	0
6D13B	0	0	0	0	0	0	0
6D13C	0	0	0	0	0	0	0
6D13D	0	0	0	0	0	0	0
7D12A	3+	3+	3+	2+	0	0	0
7D12B	1+	1+	1+	0	0	0	0
8D31A	0	0	0	0	0	0	0
8D31B	0	0	0	0	0	0	0
8D31C	0	0	0	0	0	0	0
CMFMA	1	0	0	0	0	0	0
CMFMB	0	0	0	0	0	0	0
CMFMD	3	2	2	1	0	0	0

*No agglutination was present when 50 mM n-acetyl-D-galactosamine was added.

the appropriate competing saccharide reduced agglutination, thus indicating the specificity of the results. Both Con A (Table 6) and Phyto P (Table 7) agglutinated trypanosomes in a concentration-dependent manner. Con A agglutinated all 22 variants while Phyto P only agglutinated 6 variants. While there were variations in the agglutination patterns, no correlation between the serologic type of a variant and its lectin reactivity could be determined. None of the other lectins (WGA, SBA, FBP) agglutinated any of the 22 T. rhodesiense variants.

Discussion: Results summarized in Tables 6 and 7 indicate that all 22 variants of T. rhodesiense have D-mannose-like sugars on their intact cell surfaces and some of these variants also have surface saccharides resembling n-acetyl-D-galactosamine. Possibly these, and other sugars, are present within the surface coat material of T. rhodesiense. Work in progress with limited proteolytic enzyme digestion of the trypanosome surface, followed by lectin agglutination tests, will address this point. Additionally, ongoing work is being conducted to confirm that the lectins are binding to sugar residues. Several glycosidic enzymes are being used to cleave sugar groups from the parasite. After such treatment the trypanosome will be tested for lectin agglutination.

The ultrastructural localization of lectin binding sites of T. rhodesiense is also being investigated. Histochemical tests have been conducted with trypanosomes and epon blocks of material, ready for sectioning and subsequent electron microscopic analysis, have been prepared.

The results to date indicate that, in addition to Concanavalin A, Phytohemagglutinin P might be a suitable lectin for affinity chromatographic isolation of trypanosome antigens. While no direct correlation between variant serotype and lectin agglutination has yet been demonstrated, it may be possible to demonstrate this if limited proteolytic digestion of the surface of the variants should reveal additional saccharides which exhibit multiple lectin binding properties.

X. Mechanisms of immunity to Plasmodium berghei sporozoites.

Objective: Various investigators have demonstrated the possibility of immunizing rodents and primates, including man, with malaria sporozoites, the mosquito transmitted infective stage of this parasite. Malaria remains an infection of major importance in large areas of the world; and chemoprophylaxis has not proven entirely satisfactory due to, among other reasons, the develop-

ment of drug resistant parasite strains. During combat operations in malarious areas, especially where Plasmodium falciparum is the dominant species, malaria could generate a large number of casualties with a resultant decrease in combat effectiveness and an increased burden on medical treatment resources which would normally be used for combat-related casualties. Effective immunization against this parasite would be an undeniable advantage over present methods of prophylaxis and/or treatment. Since the mechanisms of immunity to malaria sporozoites are not understood, a precise identification of these mechanisms might lead to a safe, more effective immunization.

Description: Since the demonstration by Nussenzweig et al (2) that inoculation with X-irradiated P. berghei sporozoites protects mice against subsequent challenge, attempts have been made to elucidate the immune mechanisms involved. The demonstration of precipitating and neutralizing antibodies in the sera of sporozoite immunized rodents raised the possibility of humoral immunity being a primary protective mechanism. The demonstration that protective immunity in the early stages of response is largely independent of the presence of these humoral factors seemed to rule out this possibility in rodents. This issue is less clear in man, as precipitating antibodies accompany the development of protective immunity after exposure to irradiated sporozoites of P. vivax and P. falciparum.

Recent studies have concentrated on potential roles of cellular immunity to sporozoites. Two approaches have been used: (1) passive transfer of lymphocytes from immunized rodent hosts to sublethally irradiated recipients and challenge of the recipients and (2) examination of the effects of immunization of T cell or B cell deficient mice. Both approaches have led to the conclusion that T cells, but not B cells, are necessary for the development of anti-sporozoite immunity.

Earlier experiments in this laboratory failed to demonstrate a role for either direct T cell cytotoxicity or T cell derived lymphokine in anti-sporozoite immunity in the mouse P. berghei model. Since refinement of fluorescent antibody techniques have allowed for detection of antibody at an earlier period in the immune response, it was decided to exploit this technique to examine the response of irradiated recipients of sporozoite sensitized lymphocyte grafts to sporozoite challenge.

Sensitized lymphocytes were recovered from the spleens of female BALB/c mice which had received an initial intravenous immunization of gamma irradiated (15,000 rads) P. berghei

sporozoites followed by 3 weekly boosters of 1×10^4 irradiated sporozoites. The spleens were expressed through wire mesh into L-15 tissue culture medium with 10% fetal calf serum (FCS), washed three times and counted. T and B cells were separated on nylon wool columns after initially incubating the splenocyte suspension for 1 hour at 37 C in a plastic Petri dish to remove adherent cells. The number of living cells was determined by Trypan blue dye exclusion and the cell concentration adjusted to 5×10^6 cells per ml. Recipient animals were 8-10 week old female BALB/c mice which received 500 rads of gamma irradiation 6 hours prior to cell transfer. Each recipient was inoculated i.v. with 2.5×10^6 cells followed 24 hours later by a boost of 1×10^4 irradiated sporozoites. This protocol has been shown to be effective in completely protecting passive transfer recipients against subsequent sporozoite challenge. Recipient mice were serially bled from the ocular orbit using 50 µl microhematocrit tubes on days 3, 6, 10 and 14 after cell transfer. Sera were collected by centrifugation and stored at -70 C until tested. After the final bleeding, all mice were challenged with $1x 10^4$ sporozoites and monitored to determine if infection resulted.

The indirect fluorescent antibody test (IFA) was conducted according to standard procedures using frozen sporozoites for antigen and FITC conjugated rabbit anti-mouse IgG. Sera were initially screened at a dilution of 1:5. Sera which were reactive at this dilution were titrated in fourfold dilutions from 1:10 to 1:2560.

Progress: Table 8 lists the number of sera which were IFA positive on given days after cell transfer and the number of mice in each group which survived a sporozoite challenge as evidenced by failure to develop a patent infection. Denominator discrepancies are a result of insufficient serum or serum lost in centrifugation. All mice which developed a parasitemia did not survive. Sera from intact mice receiving a single direct immunization of 7.5×10^4 irradiated sporozoites (Group 1) were tested and included in the table for comparison with irradiated groups which received cell transfers. Fifty percent of the directly immunized group demonstrated detectable IFA titers by the third day after immunization, and 100% of the group was reactive by day 10. None of the cell recipient mice were IFA reactive on the third day after cell transfer, but a majority of mice which received immune cells were reactive on day 7. Some of the mice in groups that received normal, non-sensitized cells demonstrated IFA reactivity, but at very low titers (Groups 3 and 5). This antibody is probaby a response to the "boosting" inoculum and is independent of the

Table 8

Proportion of immunized and graft recipient mice IFA reactive and protected.

Group	Treatment	Proportion IFA Reactive on Day				Protected Against challenge
		3	7	10	14	
1	Primary*	4/8	6/7	9/9	9/9	9/9
2	Immune T&B cells** + 10^4 sporozoites	0/9	4/8	7/9	8/9	9/9
3	Normal T&B cells + 10^4 sporozoites	0/8	0/8	1/9	1/9	2/9
4	Immune T cells + 10^4 sporozoites	0/10	6/9	8/10	8/10	9/10
5	Normal T cells + 10^4 sporozoites	0/10	2/9	1/10	0/10	0/10
6	10^4 sporozoites	0/10	1/10	4/10	—	— ●

* Intact mice receiving a single inoculum of 7.5 x 10^4 irradiated sporozoites and no cells.
**All recipient mice received 500 rads whole body irradiated prior to cell transfer and a "boosting" dose of 10^4 irradiated sporozoites after cell transfer.
● all mice died prior to challenge

cells transferred, since the irradiated mice in Group 6 did not receive any type of cells but were capable of producing antibody at low titers in response to a "boosting" inoculum. All individuals within the latter group died prior to day 14, undoubtedly as a result of radiation effects upon the hematopoetic system. Mice receiving T cell enriched populations (< 10% surface IgG positive) were as capable of making antibody as mice receiving unfractionated splenocytes, (> 60% surface IgG positive), and there is little difference in mean antibody titers between these two groups (Table 9) on the days observed. In comparing groups of mice receiving normal cells with those receiving sensitized cells, it can be observed that the presence of detectable antibody can be correlated with protection, but it can not be deduced that this antibody is the mechanism of protection.

Discussion and Recommendations: Utilization of IFA techniques has resulted in considerable improvements in the ability to detect anti-sporozoite antibodies in experimental hosts. The detection of such antibodies at a time in the course of infection when protection against sporozoite challenge is operative reopens the question as to the possible role of humoral factors in anti-sporozoite immunity. Passive transfer studies and attempts at immunization of athymic mice have demonstrated the necessity of T cells for the development of anti-sporozoite immunity. In view of these data, it can be hypothesized that donor T cells are performing a "helper" function in the production of antibody in recipients of sensitized T cell grafts. Antibody forming cells could be derived from either or both of two sources, (1) contaminating B cells in the inoculum of donor T cells, or (2) antibody precursor cells in the irradiated recipient animal itself, since it has been shown that unsensitized lethally irradiated host mice may be a major source of B cell activity in the presence of various types of donor cells. Despite the fact that antibody has been shown to be sufficient to protect certain hosts against sporozoite challenge investigators have demonstrated that it is not necessary. One criticism of the latter study is that these investigators looked for sporozoite neutralizing antibody and circumsporozoite precipitating antibody and did not employ IFA techniques. It can only be concluded that the mechanisms of anti-sporozoite immunity in the intact host remain unknown. The present studies have shown, however, that passive transfer models are not totally reliable for ruling out a possible role for antibody in anti-sporozoite immunity.

Table 9

IFA titers of immunized and graft recipient mice.

Group	Treatment	GMRT* on Day			
		3	7	10	14
1	Primary	320	113	137	254
2	Immune T&B cells + 10^4 Sporozoites	—	17	49	113
3	Normal T&B cells + 10^4 Sporozoites	—	—	<10	<10
4	Immune T cells + 10^4 Sporozoites	—	50	95	159
5	Normal T cells + 10^4 Sporozoites	—	<10	<10	—
6	10^4 Sporozoites	—	<10	<10	—

*GMRT= Geometric mean reciprocal titer

Further studies of this problem should refine techniques of lymphocyte separation by employing anti-IgG and anti-thymocyte sera in addition to or instead of column separation. In addition, the role of T cells in the activation of phagocytic cells should be investigated since the reticuloendothelial system (RES) has shown to bc possibly involved in anti-sporozoite immunity. The value of pursuing studies of immunity to sporozoites in view of recent encouraging advances in the production of erythrocytic stages in vitro and the advent of increased availability of antigen lies in the difference in what Meuwissen et al (3) have called causal and suppressive prophylaxis. Since immunity to pathogenic erythrocytic stage is merely suppressive, immunity to the sporozoite (causal) stage is preferable.

XI. Purification of malaria parasitized erythrocytes on colloidal silica gradients.

Objective: The objective of this study was to design a rapid and effective method for the purification of the mature forms of malaria parasites from the blood of infected animals or from cultures. Such methods are of great importance as steps in the purification of antigens for experimental immunization.

Description: Although a number of methods have been described for the separation of malaria infected erythrocytes of different stages of parasite maturation, all available methods have disadvantages and further improvements in these technologies are required in order to satisfy the requirement for a method which can result in good yields of the desired forms with low levels of contamination by other forms or by extraneous material. Recent developments have exploited the availability of colloidal silica for cell separations. The material we have used (Ludox, E.I., DuPont, De Nemours and Co., Inc.) is heterodisperse with respect to sedimentation velocity, and therefore forms a series of layers of differing densities when subjected to an ultracentrifugal field. The material is toxic to some cells, but this toxicity can be prevented by the admixture of polyvinyl pyrrolidone (PVP) with the silica. We therefore usc mixtures of this type in all of the work performed.

Two sources of malaria parasitized cells were used: human erythrocytes infected with P. falciparum derived from cultures and murine erythrocytes infected with Plasmodium yoelii derived from infected animals. In the case of the murine blood, white cells were removed by passage of the blood over a cellulose column (50% microcrystalline cellulose fiber, and 50% alpha cellulose fiber, both products of Sigma Chemical Company). The P. falciparum cultures do not contain leuko-

cytes since the blood is stored for at least one week prior to use, resulting in death and autolysis of the large majority of these cells.

The density of the silica/PVP preparations was conveniently measured by refractive index, which was found to be a linear function of density.

Most experiments were performed by admixture of silica/PVP with infected erythrocytes followed by ultracentrifugation. In this way erythrocytes of differing densities separated simultaneously with the formation of the various layers of silica. Fractions were then collected and the density and the constituency with respect to stages of parasitized cells were determined. Parasites with three or more nuclei were considered to be schizonts for purposes of enumeration.

Progress: The results of a typical experiment are illustrated in Figures 9 through 11. All three figures show the density of the silica in the respective fractions. The density curves were found to be highly reproducible between experiments. Figure 9 also illustrates both the total percent parasitemia and the percentage of schizonts. It can be seen that the fraction nearest the meniscus contains a very high proportion of parasitized cells and that most of these were schizonts. Fractions obtained closer to the bottom of the the tube contain progressively more parasites and fewer schizonts. Figure 11 illustrates that the trophozoite concentration peaked near the middle of the tube. Small ring forms were preferentially found near the bottom of the tube as were uninfected erythrocytes. The starting material for these experiments ranged from 2 to 6% total parasitemia.

Entirely similar results were obtained for P. yoelii in murine blood.

The information obtained from these studies has allowed us to design a method in which cells are overlayered on a colloidal silica suspension of a single density which allows the collection of schizonts at the interface.

Discussion and Recommendations: This method has proved to be very useful in the purification of the mature forms of malaria parasites and is being used for a number of experimental purposes. Although less mature forms exhibit greater contamination with uninfected erythrocytes, the enhanced homogeneity with respect to parasite stage may be useful for many experimental purposes.

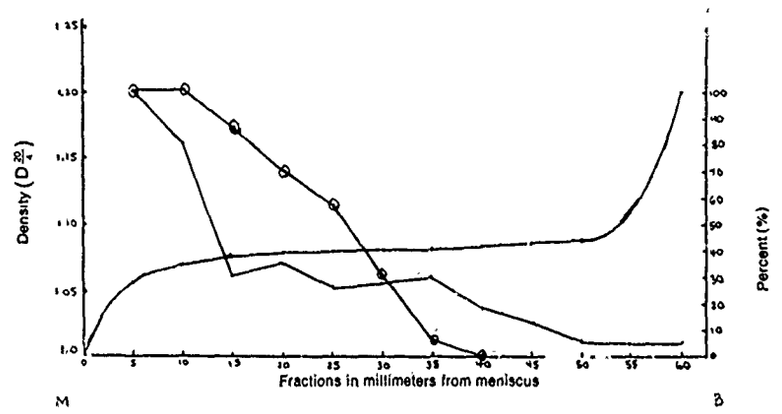

Figure 9. Parasitemia and schizont stage of cultured P. falciparum (Rockefeller strain) fractionated by continuous colloidal silica gradient.

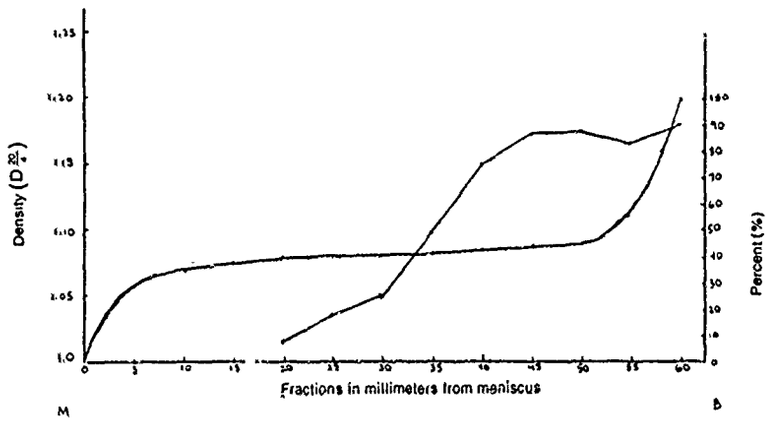

Figure 10. Ring forms of cultured P. falciparum (Rockefeller strain) fractionated on continuous colloidal silica gradient.

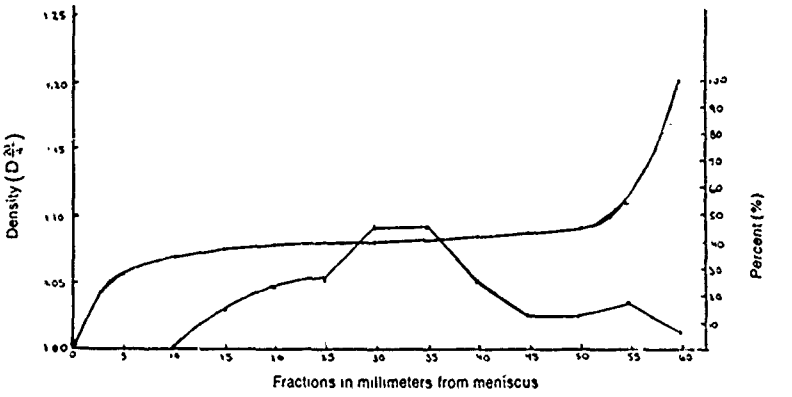

Figure 11. Trophozoite stage of culture P. falciparum (Rockefeller strain) fractionated on continuous colloidal silica gradient.

Like other methods for malaria parasite purification, the current technique has some disadvantages. In addition to its inability to provide immature forms with low contamination by uninfected erythrocytes, the possible disadvantages of the presence of the silica itself have not been fully evaluated. Additional methods for the purification of malaria parasites should therefore be explored.

XII. Mechanisms of resistance to Plasmodium falciparum in the chimpanzee.

Objectives: There are multiple objectives of this study. In general terms, the immune response of each animal during primary and secondary infection with malaria will be compared with its own status prior to infection and with other animals prior to their infection. Serum and cellular responses will be studied as detailed in the description section below.

Description: Only a relatively small degree of immunity to challenge is observed after a single episode of P. falciparum malaria in man (4). On the contrary, repeated attacks over a long period of time are usually observed before a clinically important degree of immunity can be appreciated (5). In contrast a single episode of malaria with the development of relatively high levels of parasitemia is sufficient for the acquisition of a very striking immunity in the owl monkey (Aotus trivirgatus) (6). Although there are many possible reasons

for this difference, it is striking to observe that owl monkeys develop a degree of immunity similar to that seen in humans when the primary parasitemia is limited in extent and in time. It can therefore be postulated that the difference in the typical human pattern versus the typical owl monkey pattern is due to a difference in the amount of the appropriate antigen(s) which is presented to the immune system. Chimpanzees represent another important species, susceptible to P. falciparum, in which the immune response can be characterized. Although some information is available on this subject, it is quite sketchy, and for the most part has been obtained from animals which were splenectomized to enhance the degree of parasitemia (7). Further information on cellular and humoral aspects of the immune response of this host species to P. falciparum should help to clarify the immune response to this parasite in general.

After a preliminary observation period each animal will be infected with 10^6 P. falciparum infected erythrocytes (Malaysian Camp strain) previously adapted to the chimpanzee. The animals will be infected sequentially in such a way as to be able to compare 5 pairs of animals undergoing primary and secondary infection. Bleedings will be performed at weekly intervals. Serum samples will be stored at -70 C.

A complete array of clinical pathological observations will be made on each animal on each bleeding occasion. Weekly review of the data will be made to monitor clinical status of the animals. Measurements will include serum antiparasitic effects in vitro, parameters of lymphocyte and macrophage functions, complement levels, clinical parasitologic measurements and other parameters as outlined below.

Detailed procedures:

1. Antibody and complement assays

    a. Study of serum for parasite growth inhibitory activity.
    b. Hemolytic complement assays.

2. Macrophage activation

    a. Phagocytosis of sheep erythrocytes.
    b. Lymphocyte activating factor (LAF) assays.

3. Studies on lymphocytes

    a. Sheep erythrocyte rosette formation.

b. Occurence of lymphotoxins.
c. Lectin and antigen induced blastogenesis.
d. Immunoglobulin levels (total and class specific) and polyclonal activation.
e. Suppressor (T) activity.

Progress: Data collection has not yet been initiated. Work up to the present has dealt primarily with accomplishing the extensive administrative and collaborative details required of a study of this sort. At present our colleagues at the National Institutes of Health and at Washington University, St. Louis are prepared to integrate their efforts with those of staff at the WRAIR. Preliminary baseline data now being developed with the first animal (in its normal state) is very similar to results obtained in normal humans. This first chimpanzee is scheduled for its primary infection shortly.

XIII. Isolation and evaluation of protective antigens of Plasmodium yoelii (lethal strain) in BALB/c mice.

Objectives: The primary project objective is to establish which stage(s) of the malaria blood forms is protective against infection. In addition we hope to develop general information on the minimum protective dose required for and the duration of this protection.

Description: Several approaches have been employed in the induction of protective immunity against rodent malaria. These approaches have included drug modification of active infection (8), in vitro attenuation of virulent strains of the parasite (9) and injection with extracts of whole infected blood (10, 11).

More recently, inbred mice have been immunized against lethal malaria by a single injection of irradiated infected blood (12). In combination with this approach, this department now has the expertise to study the immunogenic potential of discrete forms of the blood stages of the malaria parasite (13). It is anticipated that data from the present study will have considerable impact on work with human malaria. Specifically, findings from this study will influence the initial orientation of related investigations of falciparum malaria and will serve as the first step in the definitive isolation of protective malaria antigens. The latter is an essential step in the continuum toward the production of a malaria vaccine.

Methodology incorporated for infected whole blood challenge, routine passage and immunization is as described in reference 7 with minor modification. Briefly, BALB/c donor mice are infected with 2×10^7 infected erythrocytes. These mice are etherized 3-4 days later and are exsanguinated by retro-orbital bleeding. The blood is suspended in RPMI 1640 and standardized with a hemocytometer count of stained cells. Challenge is by infection of 1×10^5 infected cells. Optimal whole infected blood immunization is by the injection of 1×10^9 irradiated (2×10^4 rads) infected red cells. The standard interval between immunization and challenge is 1 week.

Discrete stages of the parasite are isolated as potential immunogens through colloidal silica gradients. Parasitized erythrocytes are mixed with a suspension of silica and polyvinyl-pyrrolidone in medium RPMI 1640 and centrifuged at 5.6 x 10 x g for 30 minutes. The selective distribution of the forms occurs by age such that the younger the parasite the more dense and thus more distal from the meniscus. Experimental mice will be immunized with 1×10^9 discrete irradiated parasitic forms. Challenge will be with 1×10^5 non-irradiated whole infected red cells.

Immunogenic effect is to be evaluated by comparing mean parasitemia of the immunized groups with those immunized with irradiated whole blood and normal controls. The cumulative mortality of these groups will also be compared.

Progress: This project was recently initiated. Initial problems involving undesirable survival of nonimmunized mice challenged with the parasite appear to have been overcome. Infections from frozen stabilates now result in uniform lethality among certain age groups of mice. Once the relationship, if any, between age and resistance is determined and the model (reference 7) reestablished, no problem is anticipated in accomplishing these steps.

XIV. Mechanisms of protective immunity to Plasmodium yoelii in mice.

Objective: These investigations were performed to study the conditions required for adoptive transfer of immune protection to P. yoelii in BALB/c mice in order to gain insight into immune regulatory mechanisms responsible for potentiation and/ or suppression of immune responses during malaria infection.

Description: BALB/c mice can be protected against the normally lethal malaria, P. yoelii, by vaccination with irradiation attentuated infected red blood cells. Immunization results in complete protection against a normally lethal challenge of the parasite as early as three days following vaccination (Table 10). However, mechanism(s) of immune protection are poorly understood. Using the inbred BALB/c mouse model the adoptive transfer of immune elements between animals can be accomplished to provide a better understanding of the immune response and its control. Although the adoptive transfer of lymphoid cells has been successful for many years, the transfer of immunity by these cells has not always been successful. Prior to beginning extensive studies using this model system, we investigated the conditions under which immunity to malaria can be transferred by lymphoid cells from an immune animal to a normal animal. Briefly, the technique involves the intravenous inoculation of a single cell suspension of spleen cells from a vaccine protected donor animal to a host animal prior to infection with the parasite.

Progress: Immune spleen cells from immunized mice, both unchallenged and challenged recovered, were used to test for for adoptive transfer of immunity. The results, presented in Table 11, showed no evidence of immune transfer with spleen cells. Since it has been shown in other experimental models of immune transfer that irradiation pretreatment of the recipient animal may be necessary in order to demonstrate immunity by transferred cells. We repeated the above experiments using mice, sublethally irradiated with 500 rads, as hosts. Irradiated mice showed a significantly different course of infection and survival pattern from their unirradiated counterparts (Table 12 compared to Table 11). Both groups of spleen cells proved to possess protective ability. However, spleen cells from challenged recovered donors produced the most dramatic effect. Parasitemias were low and rapidly cleared and all mice survived. Currently, titration of immune cells is being studied.

Discussion: The requirement for irradiation pretreatment of host animals for successful adoptive transfer of immunity is surprising. It is not required in all adoptive transfer models, including some malaria models. Those systems most thoroughly studied, the requirement for irradiation is directly related to its effect on the host's immune system. However, in malaria models, the issue is complicated by the dependency of

Table 10

Parasitemia and survival in BALB/c mice immunized with 10^8 irradiated P. yoelii infected erythrocytes.

Group	Time to challenge* (Days)	Parasitemia (Days after challenge) 4	6	8	10	20	30	30 day survival
1	1 hour	13±3	42±9	38±10	29±15	0	0	7/10
2	3	11±5	24±6	5±5	0	0	0	10/10
3	7	12±2	29±4	10±10	3±3	0	0	8/10
4	challenge control (no immunization)	15±5	61±10	+	-	-	-	0/10

*challenged with 10^5 viable P. yoelii infected erythrocytes.

+ all mice dead.

Table 11

Parasitemia and survival in BALB/C mice engrafted with spleen cells from various donors*

Group	SPC Donor	Parasitemia (Days after challenge) 4	6	8	10	30 day survival
1	Normal	16±10	62±10	+	_	0/10
2	Immune Unchallenged	11±7	58±6	+	_	0/10
3	Immune Challenged	15±6	60±5	+	_	0/10
4	Challenge control (no SPC)	18±5	65±7	+	_	0/10

*engrafted with 5 x 10^7 spleen cells 1 hour before challenge with 10^5 P. yoelii infected erythrocyte.

+ all mice dead.

Table 12

Parasitemia and survival in irradiated BALB/c mice engrafted with spleen cells from various donors*.

Group	SPC Donor	Parasitemia (Days after challenge) 4	6	8	10	20	30	30 days survival
1	Normal	12±5	60±5	+	-	-	-	0/10
2	Immune Unchallenged	4±2	14±6	12±4	22±10	5±15	0	8/10
3	Immune challenge	2±4	8±3	3±5	1±2	0	0	10/10
4	Challenge Control (no SPC)	15±5	65±8	+	-	-	-	0/10
5	Irradiation challenge control (no SPC)	3±1	12±5	28±6	62±10	+	-	

*engrafted 1 hour before challenge with 10^5 P. yoelii infected erythrocytes.

+ all mice dead.

the parasite on hematopoietic components other than immune cells, for its survival. Beneficial effects of irradiation could be related to its suppressive effects on red blood cell precursors, delaying or preventing parasite invasion and/or replication. Until this matter is resolved, it is difficult to speculate on the importance of our observations towards elucidating the immune responses in malaria. However, with the adoptive transfer model now available, this question as well as many others related to immune function and regulation can be studied more easily.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 149 Protective immunity in protozoan diseases

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Publications:

Wells, R.A., Pavanand, K., Zolyomi, S., Permapanich, B. and MacDermott, R.P., 1970. Loss of circulating T lymphocytes with normal levels of B and "null" lymphocytes in Thai adults with malaria. Clin. Exp. Immunol. 35:202-209.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

DA OC 6490 | 79 10 01 | DD PROGRAM/BASE

3 DATE PREV SUMM	4 KIND OF SUMMARY	5 SUMMARY SCTY	6 WORK SECURITY	7 RECRUITING	8 EXEMPLAR SYSTEM	9a SPECIFIC DATA CONTAINED THIS WORK UNIT	9b LEVEL OF SUM	A WORK UNIT
78 10 01	D, Change	U	U	NA	NA	Yes No		

10 MC CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62770A	3M161102BS01	00	150
b. CONTRIBUTING	62770A	3M162770A803	00	093
c. CONTRIBUTING	CARDS 114F			

11 TITLE (Precede with Security Classification Code)

(U) Research on Nerve Agent Antidotes

12 SCIENTIFIC AND TECHNOLOGICAL AREAS

002300 Biochemistry 012100 Organic Chemistry 012900 Physiology

13 START DATE	14 ESTIMATED COMPLETION DATE	15 FUNDING AGENCY	16 PERFORMANCE METHOD
78 10	CONT	DA	In-House

17 CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNTS:

e. KIND OF AWARD: f. CUM. AMT.

18 RESOURCES ESTIMATE	a PROFESSIONAL MAN YRS	b FUNDS (In Thousands)
PRECEDING FISCAL YEAR 79	1	75
CURRENT FISCAL YEAR 80	9	471

19 RESPONSIBLE DOD ORGANIZATION

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21 GENERAL USE

Foreign Intelligence not considered

22 KEYWORDS (Precede EACH with Security Classification Code)

(U) Biochemistry (U) Neurochemistry (U) Cell Biology (U) Nerve Agent Antidote

23 TECHNICAL OBJECTIVE, 24 APPROACH, 25 PROGRESS (Furnish individual paragraphs identified by number, precede text of each with Security Classification Code)

23. (U) The technical objectives of this work unit are (1) to investigate new approaches for the prophylaxis and chemotherapy of chemical agents, (2) to establish an in vitro method for the testing of potential nerve agent antidotes, (3) to expand the in vivo testing system to an in in vivo animal model, (4) to determine the physical properties, distribution, and binding of nerve agent antidotes, and (5) to devlop analytical methods for the analysis of these antidotes, their breakdown products and metabolites. This work unit supports the Army's program of medical defense for chemical agents.

24. (U) The objectives will be met by establishing cell culture and animal models, by the use of enzyme analysis and kinetics, binding studies, membrane permeability, and cellular metabolism. The stability of the nerve agent antidotes under simulated field and storage conditions will be determined. Analytical method using high performance liquid chromatography, gas chromatography, mass-spectometry and spectrophotometric will be developed as needed.

25. (U) Long term studies were initiated to determine the stability of Benactyzine under various conditions of temperature, pH. packaging, with or without plasticizer, and in combination with the other TAB components. The kinetics of degradation and the catalytic effects of other factors are being followed. Methods were developed for the analysis of Aprophen which used high performance liquid chromatography, gas chromatography and mass spectrometry. For technical report see WRAIR Annual Progress Report 1 Oct 78 to 30 Sept 79.

*Available to contractors upon government approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

Project 3M161102BS01 BASIC RESEARCH ON MILITARY INJURY AND DISEASE

Work Unit 150 Research on Nerve Agent Antidote

Investigators:
Principle: Bhupendra P. Doctor, Ph.D.
Associate: Nesbitt D. Brown, M.S.; Gray, R. Richard, M.S.; Leo Kazyak, B.S.; SFC Evelyn Moore; H. Kenneth Sleeman, Ph.D.

The objective of this work was to study the components of nerve agents antidotes with respect to stability under various conditions of storage, i.e, pH, temperature, and packaging, to isolate and identify degradation products, and to detemine the kinetics of breakdown. New analytical methods were developed as needed to conduct the project.

1. The stability of Benactyzine.HCl under various storage conditions.

2. The development of methodology for the determination of Aprophen.

1. The Stability of Benactyzine.HCl under various storage conditions.

Investigations were initiated to study and improve the stability of Benactyzine under simulated field and storage conditions. Solutions of Benactyzine.HCl were prepared for us and packaged in either sealed glass vials or styrettes. The glass vials contained Bactyzine.HCl plasticizer, and combined with the other components of TAB. The styrettes contained Benactyzine with pH adjusted to pH 2.7, no pH adjustment, with 40% propylene glycol added, and in combination with the other components of TAB. The vials and styrettes were apportioned into groups and stored at 5°, 25° and 54°C. The groups are analyzed biweekly for 6 months and the results tabulated and analyzed. The analytical methods used are high performance liquid chromatography and gas chromatography. The study will be completed in Feb. '80.

2. The development of methodology for the determination of Aprophen.

Two methods were developed for the analysis of Aprophen. One involved tne use of ion-pair reverse phase high performance liquid chromatography which can be used for Aprophen solutions or formulations without sample preparation. The other gas-liquid chromatography which can be used for Aprophen solutions, formulation and biological specimens. Aprophen is extracted quantatively from

solutions by chloroform at pH 6.0 to 7.0 and when added to blood can be recovered at 93.1 ± 6.4% efficiency. The high performance liquid chromatography method was used to show that Aprophen was unstable in alkaline solutions and the hydrolyzed product was 2,2' diphenylpropionic acid.

*U.S. GOVERNMENT PRINTING OFFICE : 1980 0-311-017/115