ANNUAL PROGRESS REPORT - FY 1975

Army Medical Research Institute of Infectious Diseases
Frederick, Maryland

1 July 1975

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FREDERICK, MARYLAND 21701

ANNUAL PROGRESS REPORT

FISCAL YEAR 1975

RCS-MEDDH-288(R1)

Project 3A762760A834

1 July 1975

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Biological warfare	Bacterial diseases	Pathology
Vulnerability	Rickettsial diseases	Defense
Therapy	Host	Metabolism
Prophylaxis	Parasite	Virus diseases
Identification	Biochemistry	

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

A report of progress on the research program of the U. S. Army Medical Research Institute of Infectious Diseases on Medical Defense Against Biological Agents (U) for Fiscal Year 1975 is presented.

PRICES SUBJECT TO CHANGE

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

FOREWORD

This FY 1975 Annual Progress Report is a general review of research activities of the U. S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland, conducted under Project 3A762760A834, Medical Defense Against Biological Agents (U) and a small effort under the In-House Laboratory Independent Research Program (ILIR).

3A762760A834 01 - Pathogenesis of Infection of Military Importance.
3A762760A834 02 - Prevention and Treatment of Biological Agent Casualties.
3A762760A834 03 - Laboratory Identification of Biological Agents.

Ten contracts were in effect with educational institutions or industrial firms. Reports are available through Defense Documentation Center.

Tasks of the basic project are subdivided into work units, each identified by task number plus a 3-digit suffix. Subdivisions are identified in accordance with the following scheme:

General	001-099
Bacterial diseases	100-299
Rickettsial diseases	300-399
Viral diseases	400-699
Mycotic diseases	700-799
Intoxications	800-899
Contracts	900-999

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.

1 July 1975

AUTHOR INDEX

GLOSSARY

AkP	alkaline phosphatase
BHF	Bolivian hemorrhagic fever
CEC	chick embryo cell (culture)
CPE	cytopathic effect
DEC	duck embryo cell (culture
EEE	Eastern equine encephalitis (virus)
HA	hemagglutinins, hemagglutination
HI	hemagglutination inhibition
ID	intradermal(ly)
ID_{50}	median infectious dose(s)
IM	intramuscular(ly)
IP	intraperitoneal(ly)
IV	intravenous(ly)
JE	Japanese encephalitis
LD_{50}	median lethal dose(s)
LDH	lactic dehydrogenase
LEM	leukocytic endogenous mediator(s)
MMD	mass median diameter
mRNA	messenger RNA
NDV	Newcastle disease virus
PFU	plaque forming unit(s)
Pi	inorganic phosphorus
PMN	polymorphonuclear leukocytes
PR_{50} or PR_{80}	50% or 80% plaque reduction
PRNT	plaque reduction neutralization test
RBC	red blood cells
RES	reticuloendothelial system
RMSF	Rocky Mountain spotted fever
rRNA	ribosomal RNA
SC	subcutaneous(ly)
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B

SEC	staphylococcal enterotoxin C
SGOT	serum glutamic oxaloacetic transaminase
SGPT	serum glutamic pyruvic transaminase
SLE	St. Louis encephalitis (virus)
VEE	Venezuelan equine encephalomyelitis (virus)
VSV	vesicular stomatitis virus
WBC	white blood cells
WEE	Western equine encephalitis (virus)

ANNUAL PROGRESS REPORT - FY 1975

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PROJECT NO. 3A762760A834: MEDICAL DEFENSE AGAINST BIOLOGICAL AGENTS (U)

* Completed.

* Completed.
** Terminated.

* Completed.

VIRAL DISEASES

APPENDICES

** Terminated.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6412	75 07 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
74 07 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	62760A	3A762760A834	01	003
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Tissue enzyme changes in infectious disease of military medical importance

12. SCIEN. AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 10	75 07	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 75	2.0	240.7
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Physical Sciences Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Zenser, T. V.

TELEPHONE: 301 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Petrella, V. J.

NAME: George, D. T. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Cyclic AMP, (U) Adenylate cyclase, (U) Thymocyte, (U) Cholera toxin, (U) Adenosine, (U) Military medicine

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 serial changes in tissue enzyme systems during the course of experimental infections. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Studies of the adenyl cyclase system are conducted to determine what effect infections have on this endocrine-metabolic parameter.

25 (U) 74 07 - 75 06 - Experiments were conducted which demonstrated that cholera toxin (CT)-stimulation of thymocyte adenylate cyclase activity occurs gradually following the initial lag phase. Not only was CT-stimulation of adenylate cyclase kineticaily different from NaF-stimulation but addition of NaF to CT-stimulated cyclase was inhibitory.

Mechanisms involved in mediation of lymphocytic immunoresponsiveness were also examined. The immunosuppressive action of adenosine was correlated with its ability to increase thymocyte cyclic AMP. These increases in cyclic AMP were specific for adenosine and time- and concentration-dependent. Experiments have been designed to further elucidate the mechanisms of immunoresponsiveness and CT.

Publications: J. Immunol. 113:151-160, 1974.
Infect. Immunity 10:503-509, 1974.
Amer. J. Physiol. 227:1299-1305, 1974.
In Methods in Enzymology, Vol. 38, p. 192-195, 1974.
In Advances in Cyclic Nucleotide Research, In press, 1975.

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 65 AND 1498-1, 1 MAR 69 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 003: Tissue Enzyme Changes in Infectious Disease of Military Medical Importance

Background:

I: The action of Vibrio cholerae in the small intestine is the result of an enterotoxin, cholera toxin (CT), elaborated by this organism.[1] The massive fluid and electrolyte losses which occur in clinical cholera infection are the result of CT-stimulation of intestinal adenylate cyclase. In addition to its effect on intestinal cells, CT has also been shown to increase adenylate cyclase activity in a number of tissues including thymocytes. Although certain physical characteristics of the enterotoxin such as its structure and binding to membranes have been well defined, the mechanism by which CT, once bound to receptor, stimulates adenylate cyclase has not been established. By contrast, kinetic analysis has distinguished hormonal from NaF stimulation of adenylate cyclase. In this regard, kinetic properties of mouse thymocyte adenylate cyclase under control and CT-stimulated conditions with or without NaF have been investigated, in the hope of elucidating the molecular mechanism through which CT acts.

II: Recent reports of deficient adenosine deaminase activity in patients with severe combined immunodeficiency disease[2] or acute lymphocytic leukemia[3] have led to speculation as to the role of adenosine in modulating the immunological activities of lymphocytes. Since one suggested function of the immune system is the destruction of neoplastic cells,[4] a similar immune deficiency may be a causative mechanism in both diseases. In this regard, a variety of immunosuppressive activities have also been ascribed to adenosine 3',5'-monophosphate (cyclic AMP) and agents which raise intracellular cyclic AMP levels.[5] Adenosine, AMP, and ATP were also shown to suppress both basal and mitogen-induced [^{3}H]thymidine incorporation in human peripheral lymphocytes and mouse thymocytes. The actions of ATP and AMP were attributed to adenosine since these nucleotides do not enter the cell. However, the mode of adenosine action in these systems was not known. Since adenosine has been shown to increase cyclic AMP in a variety of tissues, the present study was conducted to determine whether, and by what mechanism, exogenous adenosine could increase thymocyte cyclic AMP content.

Progress:

I: Kinetic properties of mouse thymocyte adenylate cyclase were investigated. This enzyme was stimulated with cholera toxin (CT) after preincubation with intact cells or with NaF by addition to the in vitro enzyme assay system. While NaF-stimulation was immediate, CT-stimulation proceeded gradually after an initial lag period. Maximally activated CT-stimulated plasma membranes (CT/SPM) were significantly inhibited 30% by addition of 10 mM NaF. NaF inhibition of CT/SPM was dependent on the length of preincubation of CT with intact cells; was observed at various concentrations of Mg^{2+} and ATP, and appeared to be immediate. NaF, when added to control membranes (CM), caused a significant decrease in K_m for ATP, but not when added to CT/SPM. The K_m values for CM and CT/SPM ± NaF were similar. In the presence of Mn^{2+}, the NaF inhibition of CT/SPM was lost. Furthermore, in the presence of 5 mM Mg^{2+} as little as 1.25 mM Mn^{2+} reversed NaF inhibition of CT/SPM. Mg^{2+} and Mn^{2+} requirements for CM + NaF were different from the other conditions. In addition, combination of 5 mM Mg^{2+} with various concentrations of Mn^{2+} resulted in dramatically increased activity in CM + NaF while CM and CT/SPM + NaF activities increased to a lesser degree. F^-, not Na^+, was shown to be not only responsible for NaF activation of CM but also NaF inhibition on CT/SPM activity. These data tend to indicate that the activity expressed by CT/SPM + NaF in the presence of 5 mM Mg^{2+} is that of CT, not that of NaF, and that the inhibition of NaF on CT/SPM is noncompetitive in nature.

II: The effect of adenosine on the mouse thymocyte adenylate cyclase-adenosine 3',5'-monophosphate (cyclic AMP) system was examined. Adenosine, like prostaglandin (PG) F_1, can cause ≥ 5-fold increases in thymocyte cyclic AMP content in the presence but not in the absence of certain cyclic phosphodiesterase inhibitors. Two nonmethylxanthine inhibitors potentiated the PGE_1 and adenosine responses, while methylxanthines selectively inhibited the adenosine response. Adenosine increased cyclic AMP content significantly within 1 min and was maximal by 10 - 20 min with approximately 2 and 10 μM adenosine being minimal and half-maximal effective doses, repectively. Combinations of PGE_1, isoproterenol, and adenosine appeared to be additive not synergistic. Of the adenosine analogues tested only 2-chloro- and 2-fluoro-adenosine significantly increased cyclic AMP. Thymocytes prelabeled with [^{14}C]adenine exhibited dramatic increases in cyclic [^{14}C]AMP 10 min after addition of adenosine or PGE_1 which corresponded to simultaneously determined increases in total cyclic AMP. Using [^{14}C]adenosine, the total cyclic AMP increase due to adenosine was only 16%. Adenosine was also shown to elicit a 40% increase in particulate thymocyte adenylate cyclase activity. Therefore, the increased content of cyclic AMP seen in mouse thymocytes after incubation with adenosine was due primarily to stimulation of adenylate cyclase and only partially to conversion of adenosine to cyclic AMP. The increased cellular content of cyclic AMP may be, in part, responsible for various immunosuppressive effects of adenosine.

Presentation:

Zenser, T. V., F. R. DeRubertis, D. T. George, and E. J. Rayfield. Infection-induced hyperglucagonemia and altered hepatic response to glucagon in the rat. Presented, 2nd Internat. Conference on Cyclic AMP, Vancouver, Canada, July 1974, (Advances in Cyclic Nucleotide Research 5: In Press, 1975.

Publications:

1. DeRubertis, F. R., T. V. Zenser, W. H. Adler, and T. Hudson. 1974. Role of cyclic adenosine 3',5'-monophosphate in lymphocyte mitogenesis. J. Immunol. 113:151-161.

2. Zenser, T. V., and J. F. Metzger. 1974. Comparison of the action of Escherichia coli enterotoxin on the thymocyte adenylate cyclase-cyclic adenosine monophosphate system to that of cholera toxin and prostaglandin E_1. Infect. Immunity 10:503-509.

3. Zenser, T. V., F. R. DeRubertis, D. T. George, and E. J. Rayfield. 1974. Infection-induced hyperglucagonemia and altered hepatic response to glucagon in the rat. Am. J. Physiol. 227:1299-1305.

4. White, A. A., and T. V. Zenser. 1974. Preparation and characterization of guanylate cyclase from bovine lung. p. 192-195. In Methods in Enzymology, Vol. XXXVIII (J. G. Hardman and B. W. O'Malley, ed.), Academic Press, New York.

5. Zenser, T. V. 1975. Effect of exogenous adenosine on mouse thymocyte cyclic AMP content. Clin. Res. 23:300A.

6. Petrella, V. J., and T. V. Zenser. 1975. Properties of the cholera toxin-stimulated adenylate cyclase from mouse thymocytes. Clin. Res. 23:310A.

LITERATURE CITED

1. Kimberg, D. V., M. Field, J. Johnson, A. Henderson, and E. Gershon. 1971. Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. J. Clin. Invest. 50:1218-1230.

2. Giblett, E. R., J. E. Anderson, F. Cohen, B. Pollara, and H. J. Meuwissen. 1972. Adenosine-deaminase deficiency in two patients with severely impaired cellular immunity. Lancet 2:1067-1069.

3. Zimmer, J., A. S. Khalifa, and J. J. Lightbody. 1975. Decreased lymphocyte adenosine deaminase activity in acute lymphocyte leukemic children and their parents. Cancer Res. 35:68-70.

4. Burnet, F. M. 1971. Immunological surveillance in neoplasia. Transplant Rev. 7:3-25.

5. Bourne, H. R., L. M. Lichtenstein, K. L. Melmon, C. S. Henney, Y. Weinstein, and G. M. Shearer. 1974. Modulation of inflammation and immunity by cyclic AMP. Science 184:19-28.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6413	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. SSIFN INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 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	62760A	3A762760A834	01	005
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Investigation of spontaneous diseases in laboratory animals

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002[illegible]00 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 08	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 75	1.0	47.5
FISCAL YEAR CURRENT 76	1.0	70.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Pathology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Stookey, J. L.

TELEPHONE: 301 663-7211

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: McLeod, C. G.

NAME: Hickman, R. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Laboratory animals; (U) Pathology; (U) Neoplasia; (U) Infectious diseases; (U) Parasites; (U) Military medicine

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

23 (U) Study spontaneous diseases occurring in the laboratory species utilized. This is an essential element in a comprehensive program for defense against BW agents.

24 (U) Investigate outbreaks of disease occurring in laboratory animals of the Institute.

25 (U) 74 07 - 75 06 - During the past year 3,126 laboratory animal necropsies were performed to ascertain the level of disease in the Institute's experimental laboratory animals. These necropsies were performed as result of individual research experiments and in support of the quality control program for procured laboratory animals. Several of the instances of spontaneous disease in laboratory animals were selected for further research and study, the results of which have been or will be published. These include individual cases of neoplasia and cerebral tuberculosis in rhesus monkeys, Tyzzer's disease and Encephalitizoon cuniculi in guinea pigs and Pneumocystis carinii pneumonitis in owl monkeys.

Publications: Vet. Pathol. 12: In press.
J. Wildlife Dis. 11:221-223, 1975.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 005: Investigation of Spontaneous Diseases in Laboratory Animals

Background:

Normal colony laboratory animals are seldom if ever completely free of disease. The vast majority of laboratory animals utilized by investigators in the Institute are procured from a variety of outside sources. It is imperative that a representative number of these animals be examined by veterinary pathologists in order to ascertain the quality of both the animals and their prior handling and environment. A variety of disease conditions can occur in laboratory animals, including infectious processes, parasitic infestations, nutritional imbalance and neoplasia. Some are unique to specific species while others may affect a variety of animals, including man. Some diseases are present in these animals at the time they are procured and others may be manifested after their introduction into the Institute. In the former case it is important to identify diseased animals so that the contractor and the investigator can be informed. In the latter case, it is imperative that these disease conditions be detected and either eradicated or if this is not practical, brought to the attention of the investigators. An investigator is entitled to the best animals available but he should also be aware of the endemic disease processes inherent in the species of laboratory animals he selects for his experiment in order that he will not confuse them with induced experimental lesions.

Progress:

This year a total of 3,126 laboratory animal necropsies were performed. These included a variety of animal species examined for numerous research projects from each research division in the Institute. The quality control program, to evaluate the health and ascertain the incidence of disease in all laboratory animals procured for use by investigators, has been very useful and rewarding. This continuing surveillance of newly purchased animals has pinpointed several conditions that may have compromised future research and has materially improved the quality of research laboratory animals. In addition to the more common and routine disease entities encountered in the daily pathology workload, several unusual and occasionally unique disease processes were observed; these are summarized individually.

An outbreak of tuberculosis occurred in a small group of colony rhesus monkeys, which in itself is not unusual, however, the index case was most unusual in that tuberculosis was not suspected until necropsy and then it was not the primary differential diagnosis. This monkey had been negative for all tuberculin tests since arrival and it was only because of severe CNS signs, which included repeated convulsions and a progressive posterior paresis, that the animal was euthanized. At necropsy the cerebrum contained numerous focal cavitating lesions. Upon histologic examination of tissues, acid-fast bacilli were demonstrated in brain, lung and lymph node granulomas. Tuberculosis involving the CNS of any animal is, to our knowledge, unreported. Subsequent tuberculin testing of all monkeys in contact with this index case turned up 4 monkey reactors, 2 of which contained lesions and acid-fast organisms compatible with a diagnosis of tuberculosis. Further tuberculin testing of the quarantined exposed monkeys failed to reveal any additional reactors.

A number of unexpected deaths occurred in both the exposed and control groups of mice being utilized in a research study. At necropsy many of these mice contained significant intestinal and hepatic lesions suggestive of Tyzzer's disease. Histologic examination of affected tissues confirmed this diagnosis, since numerous organisms compatible with Bacillus piliformis were demonstrated. An investigation was conducted to ascertain the source of the infection but results were inconclusive. Small, isolated additional outbreaks of Tyzzer's disease have sporadically been observed during the year. The most unique of these was its occurrence in a single guinea pig with chronic diarrhea. Necropsy and histopathology revealed severe colitis, although hepatic lesions were absent. Although Tyzzer's disease occurs commonly in mice, rats and rabbits it has not been reported in guinea pigs.

Another interesting and rare disease entity was observed in a group of owl monkeys (Aotus trivirgatus). The protozoan organism Pneumocystis carinii was demonstrated in pneumonic lesions in the lungs of 4 of these monkeys. Because of the uniqueness of this diagnosis it was confirmed by electron microscopic examination of affected lung.

Encephalitizoon cuniculi is a rather common protozoan parasite of the brain and kidney of rabbits and occasionally rats and mice. This parasite, with typical associated encephalitis, was observed in the central nervous system of a guinea pig utilized in a research study. The occurrence of this parasite in the guinea pig is unique in our experience.

The previously reported embryonal rhabdomyosarcoma from the hand of a rhesus monkey recurred and was surgically removed for the second time by amputation of the limb at the elbow. Biopsy and histological examination of the enlarged axillary lymph node failed to reveal any metastatic neoplastic cells. Tissue specimens of the recurrent neoplasm were fixed for both light

and electron microscopic examination. Examination of all tissue specimens has lead to a diagnosis of embryonal leiomyosarcoma. The monkey is alive and healthy at this time.

Other interesting and unusual disease entities encountered over the past year included dwarfism in a rhesus monkey and edema disease in a miniature swine. A male lamb, born twin with an apparently normal female, was found to be afflicted with the spinal malformation spina bifida. Radiographs and necropsy confirmed this diagnosis and in addition revealed cerebellar hypoplasia and congenital renal anomalies.

Several papers describing previously reported conditions and investigations are in press but have not been published.

Presentations:

1. Stookey, J. L. Iatrogenic lesions in laboratory animals. Presented at course, "Pathology of Laboratory Animals," Armed Forces Institute of Pathology, Washington, D. C., 16-20 Sep 1974.

2. Moe, J. B. Respiratory diseases of rats and mice. Presented at course, "Pathology of Laboratory Animals," Armed Forces Institute of Pathology, Washington, D. C., 16-20 Sep 1974.

3. Stookey, J. L. Besnoitia bennetti in two Mexican burros. Presented at the American College of Veterinary Pathologists meeting in Chicago, Ill., 4-6 Dec 1974.

4. Moe, J. B. Cerebral tuberculosis in a rhesus monkey. Presented at the American College of Veterinary Pathologists meeting in Chicago, Ill., 4-6 Dec 1974.

Publications:

1. Long, G. G., J. L. Stookey, T. G. Terrell, and G. D. Whitney. 1975. Fibrous osteodystrophy in an opossum (Didelphis marsupialis). J. Wildlife Dis. 11:221-223.

2. Moe, J. B., J. D. White, W. P. Czajkowski, and J. L. Stookey. 1974. Myxosarcoma in a young rhesus monkey. Vet. Pathol. 12: In Press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0E6410	75 07 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
74 07 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	62760A	3A762760A834	01	007
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Effect of infectious diseases on the coagulation mechanism

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 08	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 75	2.0	154.0
FISCAL YEAR CURRENT 76	1.5	312.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Pathology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Mosher, D. F.
TELEPHONE: 301 663-7351
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Wing, D. A.
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Infectious diseases; (U) Blood coagulation; (U) Monkeys; (U) Military medicine; (U) Rocky Mountain spotted fever

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

23 (U) Investigate the coagulation mechanism in infectious diseases. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Perform basic coagulation tests on samples from a variety of experimental infectious disease models. Correlate results with other indices of infection. Develop and test hypotheses concerning pathogenesis and biologic significance of observed changes. Evaluate effect of therapy.

25 (U) 74 07 - 75 06 - Modest to severe disseminated intravascular coagulation was found in rhesus monkeys infected with Rickettsia rickettsii, Salmonella typhi and Streptococcus pneumoniae. Similar hematologic changes occurred in guinea pigs infected with R. rickettsii and seemed to be a direct consequence of vasculitis; heparin prevented thrombosis and lysis of fibrin but not the severity of vasculitis or depression of pletelet counts. Studies of cold-insoluble globulin, a plasma protein of unknown function, suggest a possible role for this protein in the interactions occurring between fibrin and several cell types.

Publications: Lab. Invest. 32:452-453, 1975.
Fed. Proc. 34:498, 1975 (abstr.)
J. Biol. Chem. 250, In press, 1975.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 007: Effect of Infectious Diseases on Coagulation Mechanism

Background:

The coagulation of blood is a complex process involving activation of at least 9 proteolytic enzymes, the participation of 3 protein co-factors, multiple feedback pathways, control by 4 or 5 distinct protease inhibitors, and interactions of soluble factors with a variety of cell types, including platelets, polymorphonuclear leukocytes, lymphocytes, macrophages, erythrocytes, endothelial cells, and fibroblasts. The process results in the formation of a hemostatic plug composed of platelets and fibrin. In addition to effecting hemostasis, the plug serves as a substratum for the subsequent proliferation and differentiation of blood vessels and fibroblasts. Activation of coagulation mechanisms seems to be an integral part of the inflammatory response to infectious agents, and fibrin is commonly found around foci of infection. Activation may become widespread in severe infection, resulting in disseminated intravascular coagulation.

Progress:

I. Animal Studies

Rocky Mountain spotted fever. Coagulation studies were carried out on samples obtained serially from 27 rhesus monkeys infected with RMSF and 4 controls. Infected monkeys all had increased fibrinogen levels and circulating fibrin-split products at some point during their illness. Declining fibrinogen concentrations, thrombocytopenia, and lengthened partial thromboplastin times were found in some severely ill monkeys. Correlations could be made between the severity of clotting factor changes and death, but not between the severity of such changes and histopathological lesions or duration of illness.

A number of experiments were done studying RMSF in guinea pigs. Although it has been difficult to obtain samples suitable for coagulation studies, we have found depression of platelet counts on days 2-10 of illness and appearance of fibrin-split products on days 4-10. Partial thromboplastin times were prolonged on days 2-4. Consistent changes in fibrinogen concentration were not seen. These findings correlated well with the histopathological appearance of vasculitis (days 1 or 2) and thrombosis (days 4-6).

The disease, assessed both histopathologically and hematologically, was not demonstrably different in genetically C4-deficient or in cobra venom factor-treated guinea pigs, suggesting that complement activation does not play a major role in the pathogenesis of the vasculitis. Heparin treatment did not affect the severity of the vasculitis but did prevent thrombosis in diseased vessels and the appearance of fibrin split products.

Bacterial infections. Rhesus monkeys infected with Streptococcus pneumoniae (12 animals) and Salmonella typhimurium (6 animals) were examined 2 days after IV inoculation. Low grade disseminated intravascular coagulation, manifested by appearance of fibrin-split products, was noted in all infected animals, and significant thrombocytopenia was found in the S. typhimurium group. Owl monkeys moribund after S. pneumoniae inoculation had no fibrin-split products. Thus, the propensity to develop fibrin-split products may be host species-dependent.

Viral infections. Samples were obtained from rhesus monkeys infected with VEE. Fibrinogen concentrations and platelet counts during illness did not differ from baseline or control value. Fibrin-split products were not detected in serum from infected monkeys using a radial immunodiffusion assay; this determination must be repeated using the more sensitive electroimmuno-assay.

Conclusions and future plans. Evidence for modest to severe disseminated intravascular coagulation was found in rhesus monkeys infected with a gram-positive bacterial, a gram-negative bacterial, and a rickettsial agent but not with a viral agent. These findings cannot be generalized to all infections. For instance, Macaca mulatta infected with the virus of simian hemorrhagic fever develop severe disseminated intravascular coagulation.[1] However, our findings provide a data base to which other infectious diseases in M. mulatta can be compared and identify a model illness (S. typhimurium) to study the pathogenesis of changes in coagulation mechanisms. The work is being prepared for publication, and a new proposal will be written based on the work to date.

Studies of M. mulatta and guinea pigs infected with RMSF have been more definitive. The coagulation changes seem to be a direct consequence of the vasculitis. The vasculitis in turn seems to be a direct toxic effect of the rickettsiae, since it is seen very early in the disease and is not affected by decomplementation or heparinization. These findings are being prepared for publication.

II. Studies of fibrinogen and cold-insoluble globulin. Cold-insoluble globulin is a plasma protein (CIG) (normal concentration 280 $\pm$ 60 µg/ml in humans) with the electrophoretic mobility of a β globulin and a sedimentation coefficient of 12-14S. Studies of this protein were begun because it is often found in precipitates of so-called "cryofibrinogen," thought to be a marker for hypercoagulability. We have established tht CIG: (1) consists of 2 subunits (molecular weight 2.0×10^5) held together by disulfide bonds;

(2) can be distinguished from factor VIII by amino acid analysis and molecular weight, although the 2 proteins have many properties in common, including subunit size; (3) is one of only 4 plasma proteins containing glutaminyl residues susceptible to the action of blood coagulation factor XIII; and (4) is covalently crosslinked by factor XIII to the α chain of fibrin and thus can be incorporated into a fibrin clot.

In addition, we have made several other observations on this protein. Plasma levels of CIG drop 2-3 fold during experimental RMSF in rhesus monkeys. Levels did not seem to change in other infections; however, these samples need to be re-analyzed. Antibody prepared against CIG reacts with a protein on the surface of fibroblasts; labeling with amino acid precursors that has suggested this protein is the precursor of CIG.[2] The fibroblast precursor protein is also a substrate for factor XIII; this reaction may be important for the migration and proliferation of fibroblasts into areas of inflammation. CIG may be on the surface of platelets and macrophages, closely associated with microfilaments.

Conclusions and future plans. These studies, by characterizing the interaction of factor XIII and fibrin with cold-insoluble globulin and its cellular precursor, suggest a variety of approaches to study the interaction of cells with fibrin after the initial burst of fibrin deposition.

Presentation:

Mosher, D. F. Labeling of cold-insoluble globulin and a fibroblast protein by fibrin-stabilizing factor. Presented, Annual Meeting FASEB, Atlantic City, N. J. 14-18 Apr 1975 (Fed. Proc. 34:498, 1975).

Publications:

1. Moe, J. B., D. F. Mosher, and R. H. Kenyon. 1975. Hematologic, pathologic, and vascular permeability changes during experimental Rocky Mountain spotted fever in guinea pigs. Lab. Invest. 32:452-453 (abstr.).

2. Mosher, D. F. 1975. Crosslinking of cold-insoluble globulin by fibrin-stabilizing factor. J. Biol. Chem. 250: in press.

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1. Giddens, Jr., W. E., D. Jessup, H. E. Branson, L. A. Mayer, D. R. Benjamin, and W. T. London. 1975. The pathogenesis of simian hemorrhagic fever: hematologic and histopathologic studies. Lab. Invest. 32:424 (abstr.).

2. Ruoslahti, E., and A. Vaheri. 1974. Novel human serum protein from fibroblast plasma membrane. Nature 248:789-791.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OA6414	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ENTR'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 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	62760A	3A762760A834	01	009
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code): (U) Host amino acid, protein and RNA metabolism during infectious disease of military medical importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

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: 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 75	2.0	179.5
FISCAL YEAR CURRENT 76	2.0	265.6

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Physical Sciences Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Wannemacher, Jr., R. W.
TELEPHONE: 301 663-7181
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Thompson, W. L.
NAME: Beisel, W. R. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Amino acids; (U) Hyperphenylalaninemia; (U) Phenylalanine load; (U) Catabolism; (U) Skeletal muscle; (U) RNA synthesis; (U) Protein synthesis; (U) Volunteers

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

23 (U) Study changes in amino acids of blood and tissues in infectious disease or conditions induced by other variables. Relate these changes with RNA and protein metabolism. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Free amino acid concentrations are determined by ion-exchange chromatography in plasma and tissue of experimental subjects infected with bacterial or viral organisms. Radioactive nonmetabolizable and metabolizable amino acid tracers are utilized to study amino acid flux. Labeled precursors of RNA and protein metabolism are used to study effects of infection on RNA and protein synthesis.

25 (U) 74 07 - 75 06 - The oxidation of phenylalanine is similar in both infected and control rats. Thus, the infection-related elevation in serum phenylalanine appears to be the result of an influx of this amino acid into the extracellular pool from breakdown of protein in skeletal muscle at a rate that is faster than this amino acid can be utilized by liver for increased synthesis of plasma proteins. Leukocytic endogenous mediator appears to influence hepatic protein synthesis of acute-phase globulin at both the transcriptional and translational sites, with the former requiring the permissive effect of the glucocorticoids.

Publications: In Proc. 9th Internat. Congress Nutrition, Vol. 2, p. 199-204, 1975.
In Total Parenteral Nutrition, p. 85-153, 1975.
Am. J. Clin. Nutr. 28:110-118, 1975.
Endocrinology 96:651-661, 1975.
Infect. Immunity 10:60-65, 1974; 11:873-875, 1975.
Fed. Proc. 34:620, 900, 1975 (abstr.).

*Available to contractors unless otherwise agreed.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORM 1498A 1 NOV 65
1 MAR 68 AND 1 MAR 69 FOR ARMY USE ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 009: Host Amino Acid, Protein and RNA Metabolism During Infectious Disease of Military Medical Importance

Background:

I. The infectious process is characterized by a flux of amino acids from tissues such as skeletal muscle to other body tissues such as liver where they are utilized for protein synthesis and gluconeogenesis.[1] This utilization of amino acids from skeletal muscle for many host defense mechanisms results in an increased loss of body nitrogen, which is associated with the catabolic aspect of infectious diseases.[2] The marked flux of amino acids results in a depression in most of the plasma free amino acids.[1,3] Paradoxically, in most of the experimental and naturally occurring infections that have been studied, plasma phenylalanine and the phenylalanine:tyrosine (Phe:Tyr) ratio are significantly increased.[3] Because of these differences studies were initiated to determine the effects of an infectious process on the oxidation and utilization of individual amino acids. In addition, experiments have been carried forth to clarify the mechanism by which infectious disease can lead to an accelerated rate of breakdown and loss of skeletal muscle.

II. A protein which is released from stimulated peritoneal leukocytes leukocytic endogenous mediator (LEM) has been shown to have marked stimulatory effects on the flux of amino acids, Zn and Fe into liver and on the synthesis of a number of acute-phase plasma globulins.[4] A similar heat-labile mediator has been observed in the serum of patients with various infectious diseases.[5] Because of these observations, studies have continued to elucidate further the regulatory role that LEM plays on hepatic amino acid, RNA, and protein metabolism.

Progress:

I. Several mechanisms can be suggested for the infection-related elevation in serum phenylalanine (Phe) concentrations. These include: inhibition of Phe hydroxylase; decreased utilization of Phe for protein synthesis; an increased rate of kidney reabsorption of Phe; and an increased rate of tissue breakdown. In order to study some of these possibilities, a Streptococcus pneumoniae infection was utilized in rats. For this model, the rats were injected SC with 10^5 organisms, following which bacteremia and fever developed in 16 hr, and death in 65 - 72 hr. Control rats received a similar injection of heat-killed organisms. Two hours before the rats were killed they received a pulse-dose of ^{3}H-Phe. All rats were killed at 24 hr and the serum was taken for determination of Phe concentrations and ^{3}H incorporation into serum proteins.

In addition, liver was analyzed for Phe hydroxylase. Serum Phe was significantly increased at 24 hr. Hepatic Phe hydroxylase was not altered by this infection. In addition, the incorporation of labeled Phe into serum proteins was significantly increased in infected rats. It has recently been reported in rats infected with the vaccine strain of Francisella tularensis, that serum Phe and the Phe:Tyr ratio were significantly increased, while hepatic Phe hydroxylase activity was only slightly decreased when compared to paired controls.[6] From these data it can be concluded that the infection-related elevation in serum Phe was not due to an alteration in hepatic Phe hydroxylase or an impaired utilization of Phe as a precursor of protein synthesis.

Since the conversion of Phe to Tyr requires, in addition to the enzyme Phe hydroxylase, a co-factor which is an intermediate electron donor, maintained in reduced form by an NADH-dependent enzyme, further studies on the oxidation and utilization of Phe were carried out to determine whether the infectious process has any physiological or stimulatory effects on the metabolism of this aromatic amino acid. At 16 hr after a SC injection of either live or heat-killed S. pneumoniae, rats were force-fed a load that contained 75 mg of Phe and 1 μCi of ^{14}C-Phe/dg body wt. The rats were immediately placed in a closed-circuit metabolic cage which allowed for complete collection of expired CO_2, urine and feces for the next 8 hr. The rate of oxidation of ^{14}C-Phe to CO_2 was similar in both infected and control rats. When compared on an hourly basis, the maximum rate of oxidation was observed 2 hr after force-feeding Phe. A comparison of the distribution of radioactivity 8 hr after an oral dose of ^{14}C-Phe indicated that in both the infected and control rats, 60-65% of the radioactivity was in the expired CO_2, 4%, in the urine, and 2%, in the feces. By difference this left 30-35% present in the body of the rat. Similar distributions of radioactivity have also been observed in rats infected with the live vaccine strain of F. tularensis. These data confirm the conclusion that the infection-related increases in serum Phe were not the result of decreased ability to oxidize Phe or an increased rate of kidney reabsorption of this amino acid.

The rate of Phe disappearance was studied in rats force-fed a Phe load; sequential samples of serum were analayzed for free Phe and Tyr. The disappearance of the former from the serum was similar in rats infected with S. pneumoniae and heat-killed controls. The half-disappearance rate of 30-32 min for Phe was in good agreement with that reported by other investigators. In contrast, serum Tyr decreased at a faster rate in the infected rat than their pair-fed controls. This difference may be related in part to the increase in tyrosine transaminase observed in liver of infected rats.

To determine whether the oral load or route of administration could influence the results, the control rats were studied after an oral or IP pulse-dose of 0.36 μg of ^{14}C-Phe. Only 10 - 13% of the label appeared in expired

CO_2 at the end of 8 hr in orally dosed rats, but again no difference was observed in the infected and control rats. Similarly an IP pulse-dose of phenylalanine resulted in only 5 - 7% of the label in the expired CO_2 at the end of 8 hr; again no difference was observed between infected and control rats.

At 8 hr after an oral dose only a small percentage of total Phe radioactivity in the body was found in the protein-free filtrates of liver, serum or muscle. While the total amount of radioactivity in the body was similar, the distribution in various tissues was significantly different in the infected as compared to the control rats. The radioactivity in the protein-free filtrates of the liver and skeletal muscle was significantly decreased while that in the serum was markedly elevated in the infected rats when compared to the controls. In both infected and control rats the liver proteins contained approximately 11% of the total body radioactivity. In the controls approximately 22% of the radioactivity was associated with serum proteins, while in the infected rats this was significantly increased to approximately 33%. In contrast, skeletal muscle in control rats represented approximately 17% of total body radioactivity which was significantly decreased in the infected rat to approximately 13%.

In the normal rat, the concentration of Phe in the extracellular pool is the result of an influx from dietary sources and the catabolism of body proteins and an efflux by losses in the urine and feces, and by utilization in various tissues of the body. Current evidence indicates that the majority of the amino acid utilized for protein synthesis probably comes from the extracellular pool. Thus, since nonhepatic tissues do not have the capacity to metabolize Phe, most Phe which is released as a result in metabolism of proteins in extrahepatic tissues will enter the extracellular pool for reutilization. In the liver, however, Phe can enter 3 pathways: (1) synthesis of plasma proteins; (2) synthesis of intracellular hepatic proteins; and (3) conversion to Tyr and subsequently to CO_2. The mechanisms which regulate the distribution of Phe within these 3 pathways of liver have not been elucidated. In the infected host, dietary Phe supply is usually decreased because of anorexia. Despite this decrease, the extracellular pool of phenylalanine is significantly elevated. No difference is observed in the rate of loss of phenylalanine from this pool in the urine or feces. The rate of utilization of this amino acid for protein synthesis in skeletal muscle is markedly decreased in the infected animal. In contrast, the catabolism of skeletal muscle is increased which results in an efflux of Phe from skeletal muscle to the extracellular pool. Amino acids are taken up by the liver at an elevated rate and enter into the pathway for an increased rate of synthesis of plasma proteins, especially the acute-phase proteins. Utilization of Phe by the liver for total hepatic protein synthesis or oxidation is not markedly influenced by the infectious process. Thus, an hypothesis for elevated serum Phe concentration in the infected animals can be offered. The increases result because of an accelerated influx of this amino acid into extracellular pool secondary to an increased breakdown of protein in skeletal muscle; the influx rate is faster than the rate of Phe utilization by liver, even in the presence of an accelerated synthesis of plasma proteins.

II. Previous data from infections in rats have led to the conclusion that the flux of amino acids to liver is associated with an increased synthesis of the acute-phase plasma globulins such as seromucoid, α_2-macrofetoprotein, haptoglobin, ceruloplasmin, and fibrinogen.[4] By use of an automated system for the detection of a specific protein of plasma by immunoprecipitin nephelometry, it is possible to determine 10 specific proteins (albumin, α_1 acid glycoprotein, (orosomucoid), α_1 antitrypsin, haptoglobin, α_2 macroglobulin, transferrin, C_3 complement, IgG, IgM, and IgA) in the plasma of man. In the collaborative study with Drs. DuPont and Hornick (the University of Maryland Contract No. DA 49-193-MD-2867) sequential plasma samples were analyzed for these 10 specific proteins in volunteers exposed to typhoid fever. In those volunteers who became ill, plasma α_1 acid glycoprotein, α_1 antitrypsin, haptoglobin and C_3 were significantly increased during illness and remained elevated for more than a week after admission to the hospital ward. In contrast, albumin and transferrin were significantly decreased. The plasma IgM concentrations were slightly increased by 14 days and were significantly different from control values by day 20. Little change was noted in IgG and only slight, not significant, increases were observed in IgA. In 10 exposed volunteers who did not become ill with typhoid fever no significant changes were observed in any of these specific proteins. The plasma of those volunteers who became ill contained a LEM-like mediator which was capable of stimulating the movement of amino acids into liver and depression of serum Zn when injected into recipient rats. Since LEM obtained from phagocytizing peritoneal leukocytes has been shown to stimulate an increased synthesis of the acute-phase globulins in rats, it may be concluded that a similar factor or hormone released from the peripheral cells of volunteers who were ill with typhoid fever may have been responsible for stimulating the increase in the 4 specific proteins.

To determine whether these changes in acute-phase globulins would be of diagnostic value for infectious disease, a collaborative study was carried forth with Dr. Klainer (University of West Virginia, Contract No. DADA 17-72-C-2151) in which serum samples from patients with known infections were sent to USAMRIID for analyses. In 94% of the samples α_1 acid glycoprotein was increased above maximum values found in normal controls. In addition, in 89 and 80% respectively, of the samples, α_1 antitrypsin and haptoglobin were increased above control values, while C_3 complement was increased in the serum from 63%. These preliminary results suggest that increases in these acute-phase globulins are indicative of an infectious process. Since other illnesses which involve tissue damage such as injury or myocardial infarction have been reported to also increase these plasma acute-phase globulins, samples from patients with these illnesses are currently being analyzed to see if the pattern of serum protein changes can be delineated from that observed during infection.

Previously, it has been reported that a single injection of LEM results in a marked increase in the synthesis of hepatic RNA.[3] By use of oligotide (dT) columns, it is possible to separate ribosomal RNA from messenger RNA,

which contains larger terminal sequences of poly A. By this technique, it can be demonstrated that crude LEM induces a marked increase in the synthesis of ribosomal RNA and a smaller but significant increase, by paired t test, in messenger RNA from both the free and bound ribosomal fractions of liver. Currently, techniques are being developed to separate messenger RNA into different weight classes by centrifugation upon linear sucrose gradients. By this technique, it is hoped that we can determine whether LEM stimulates a different profile of incorporation of orotic acid into messenger RNA. Eventually messenger RNAs purified by this technique will be added to an in vitro mRNA-dependent protein synthesizing system to determine whether certain proteins such as α_2 macrofetoprotein are synthesized in increased amounts in LEM-treated rats (in conjunction with Work Unit 834 01 020).

Adrenalectomy or hypophysectomy prevents the LEM-stimulated increase in the synthesis of hepatic RNA or serum α_2 macrofetoprotein.[3] However, when hypophysectomized or adrenalectomized rats were maintained on physiological doses of hydrocortisone (150-250 µg/day), an injection of crude LEM resulted in a significant increase in the synthesis of hepatic RNA and elevations in the concentrations of the serum α_2 macrofetoprotein. While these concentrations of hydrocortisone did not result in detectable concentrations of corticoids in serum, it did appear to meet the requirements necessary for transcription of new mRNA by liver. These data are an excellent biochemical illustration of a so-called "permissive" action of glucocorticoid hormones and demonstrate the essentiality of this hormone for the transcription of functional mRNA in liver tissue.

Recently the studies have been expanded to investigate the effects of adrenalectomy and hypophysectomy on the ability of LEM to stimulate the synthesis of serum haptoglobin. In contrast to the observations on serum α_2 macrofetoprotein, LEM did induce an increase in the serum concentration of haptoglobin in both experimental groups of rats. These observations suggest that LEM is capable of stimulating haptoglobin synthesis in the absence of the adrenal or pituitary glands.

Since the glucocorticoid hormones are necessary for LEM's stimulation of hepatic RNA and serum α_2 macrofetoprotein synthesis, the possibility still exists that LEM does not act directly on liver to produce some of these biochemical changes. To test this possibility the effects of LEM are being studied in a liver perfusion system. Livers from normal rats are perfused with a medium that contains oxygenated sheep red cells, glucose, amino acids in the concentration found in rat serum, glucagon, insulin, growth hormone, hydrocortisone, and either an equivalent amount of crude LEM or physiological saline. In addition, glucose, amino acids, all the hormones, and either LEM or saline are infused into the perfusate. The liver is allowed to equilibrate with this perfusion medium for 1 hr after which 1 µCi of ^{14}C-orotic acid is added to the perfusate. A sample of liver is then removed at either 1, 2, or 3 hr. Under these conditions the ^{14}C from orotic acid is rapidly incorporated into hepatic RNA including the nuclear, free and bound ribosomal and soluble fractions. Maximal labeling of the ribosomal RNA fractions is observed in 2-3 hr.

When crude LEM was added in the presence of hydrocortisone the radioactivity associated with the total and the bound ribosomal RNA fractions was increased at 2 and 3 hr above that found in the control rats. In the bound ribosomal fraction this increase was significantly different from that observed in the controls. When hydrocortisone was removed from the perfusate, no difference from controls was observed in the presence of LEM. These data suggest that LEM in the presence of glucocorticoids was able to act directly on the liver by stimulating an increased rate of incorporation of orotic acid into RNA associated with the bound ribosomal fraction.

As reported previously, crude LEM stimulates an increased uptake of amino acids by liver.[3,4] In addition, it also stimulates an increased synthesis of RNA especially that associated with the bound ribosomal fraction. A part of this increase in RNA synthesis requires the presence of the glucocorticoids. This increase in bound ribosomal RNA is associated with elevated production of acute-phase plasma proteins. The data indicate that the increased synthesis of some of the acute-phase proteins may not require stimulated RNA production or the presence of the glucocorticoids. Thus, it appears that LEM can influence protein synthesis at both the transcriptional and translational sites. The mechanisms by which LEM regulates plasma acute-phase protein synthesis is currently under investigation.

Presentations:

1. Wannemacher, Jr., R. W., R. E. Dinterman and M. C. Powanda. Phenylalanine metabolism in the infected rat. Presented, Annual Meeting FASEB, Atlantic City, N. J. 13-18 April 1975. (Fed. Proc. 34:900, 1975).

2. Thompson, W. L., F. B. Abeles, F. A. Beall, R. E. Dinterman, and R. W. Wannemacher, Jr. LEM-induced synthesis of hepatic RNA and acute-phase serum globulins. Presented, Annual Meeting FASEB, Atlantic City, N. J. 13-18 April 1975. (Fed. Proc. 34:620, 1975).

Publications:

1. Wannemacher, Jr., R. W. 1974. Amino acid losses and plasma changes during infection, p. 1-35. In Proc. Collaborative Study of Children Treated for Phenylketonuria, Snowmass-at-Aspen, Colorado.

2. Wannemacher, Jr., R. W., M. C. Powanda, and R. E. Dinterman. 1974. Amino acid flux and protein synthesis after exposure of rats to either Diplococcus pneumoniae or Salmonella typhimurium. Infect. Immunity 10:60-65.

3. Wannemacher, Jr., R. W. and M. C. Powanda. 1975. In vivo effects of inhibitors of protein synthesis on infection-related changes in serum amino acids, p. 199-204. In Proc. 9th Intern. Cong. Nutrition, Vol. 2, (A. Chavez, H. Bourges, S. Basta, ed.), S. Karger, Basel.

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5. Wannemacher, Jr., R. W., R. E. Dinterman, R. S. Pekarek, P. J. Bartelloni, and W. R. Beisel. 1975. Urinary amino acid excretion during experimentally induced sandfly fever in man. Am. J. Clin. Nutr. 28:110-118.

6. Wannemacher, Jr., R. W., R. S. Pekarek, W. L. Thompson, R. T. Curnow, F. A. Beall, T. V. Zenser, F. R. DeRubertis, and W. R. Beisel. 1975. A protein from polymorphonuclear leukocytes (LEM) which affects the rate of hepatic amino acid transport and synthesis of acute-phase globulins. Endocrinology 96:651-661.

7. Wannemacher, Jr., R. W., R. S. Pekarek, A. S. Klainer, P. J. Bartelloni, H. L. DuPont, R. B. Hornick, and W. R. Beisel. 1975. Detection of leukocytic endogenous mediator-like mediator of serum amino acid and zinc depression during various infectious illnesses. Infect. Immunity 11:873-875.

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1. Wannemacher, Jr., R. W., M. C. Powanda, and R. E. Dinterman. 1974. Amino acid flux and protein synthesis after exposure of rats to either Diplococcus pneumoniae or Salmonella typhimurium. Infect. Immunity 10:60-65.

2. Beisel, W. R., W. D. Sawyer, E. D. Ryll, and D. Crozier. 1967. Metabolic effects of intracellular infections in man. Ann. Intern. Med. 67:744-779.

3. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974, p. 17-24, Fort Detrick, MD.

4. Pekarek, R., R. Wannemacher, M. Powanda, F. Abeles, D. Mosher, R. Dinterman, and W. Beisel. 1974. Further evidence that leukocytic endogenous mediator (LEM) is not endotoxin. Life Sci. 14:1765-1776.

5. Wannemacher, Jr., R. W., H. L. DuPont, R. S. Pekarek, M. C. Powanda, A. Schwartz, R. B. Hornick, and W. R. Beisel. 1972. An endogenous mediator of depression of amino acids and trace metals in serum during typhoid fever. J. Infec. Dis. 126:77-86.

6. Powanda, M. C., R. E. Dinterman, R. W. Wannemacher, Jr., and G. D. Herbrandson. 1974. Distribution and metabolism of phenylalanine and tyrosine during tularemia in the rat. Biochem. J. 144:173-176.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6415	75 07 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
74 07 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	62760A	3A762760A834	01	010
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Procede with Security Classification Code)

(U) Effect of ionizing radiation on immunity and pathophysiology of infectious diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 014100 Radiobiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 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)
PRECEDING FISCAL YEAR 75	1.0	202.2
CURRENT FISCAL YEAR 76	1.0	128.7

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Animal Assessment Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Hilmas, D. E.

TELEPHONE: 301 663-7244

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Radiation; (U) Mice; (U) Infectious diseases; (U) Vaccine; (U) Military medicine; (U) Encephalomyelitis, equine (VEE)

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

23 (U) Investigate interrelationships between acute or chronic irradiation and disease processes. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Acute or protracted total-body irradiation is delivered to selected animal species before, simultaneously with, or after infection. Clinical and immune responses are observed and measured serially.

25 (U) 74 07 - 75 06 - Vascular clearance studies in rhesus monkeys have shown that initial clearance of TC-83 strain of VEE is not affected significantly by RES blockade, and 400 R total body irradiation of monkeys either 1 or 4 days before TC-83 infection also had no effect, either to enhance or delay clearance of virus. Killed VEE and killed VEE complexed with specific IgG are cleared similarly. Both appear to be cleared to some extent by the RES, since blockade delays their clearance. Therefore, prolonged viremia observed in irradiated monkeys given live, attenuated VEE virus is not associated with depressed initial clearance of virus in these animals.

A method has been developed to study cell-mediated immune response in monkeys using a dinitrochlorobenzene skin test with biopsy and histologic evaluation. The radioprotectant drug WR2721 had no effect on TC-83-immunzed mice. When WR2721 was given in combination with total-body irradiation, mice survived a normally 100% lethal exposure and exhibited severe immune suppression. Mice immunized with TC-83 and then irradiated chronically, 3 R/hr, 22 hr/day, 1320 R total, show much higher humoral antibody responses than similar vaccine controls not irradiated.

Publications: Infect. Immunity 11:481-487, 1975.
IRCS Med. Sci.:Immunol. Allergy 3:234, 1975, IRCS Med. Sci.:
Microbiol. 3:234, 1975; Infect. and Immunity 12, In press.

*Available to contractors upon ord

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 3A672760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 010: Effect of Ionizing Radiation on Immunity and Pathophysiology of Infectious Diseases

Background:

After various immunosuppressive measures, both potentiation[1] and non-potentiation[2] of experimentally induced virus infections have been described, but these phenomena have received little attention in irradiated monkeys. Results of acute (400 R) total-body radiation exposure studies in rhesus monkeys prior to vaccination with the VEE virus vaccine strain, TC-83, have shown that viremia was delayed and prolonged in this irradiated-vaccinated, nonhuman primate species.[3] Viremia titers tended to be higher and peaked later in irradiated-vaccinated animals. The appearance of humoral antibody in irradiated-vaccinated animals was delayed and prolonged. The delay in antibody appearance was consistent with prolonged viremia.[3]

Immune suppression associated with acute, total-body irradiation is well documented, particularly in small, nonprimate species, with various nonreplicating antigens.[4] However, little or no attention has been directed to studies of immune response, i.e., suppression or enhancement to any antigen given before, during or after chronic, long-term, low-dose, total-body irradiation exposure.

Progress:

Since the importance of the RES in initial clearance of VEE and in prolongation of viremia in irradiated rhesus monkeys was unknown, the following clearance study was undertaken: 12 monkeys of either sex, 3-4 kg weight, and negative for VEE serum-neutralizing antibody, were allocated at random into study groups as shown in Table I.

TABLE I. RATE CONSTANTS FOR TC-83 (k_v) THROUGH 10 MIN OF CLEARANCE IN MONKEYS

TREATMENT GROUP	k_v (10 MIN) Mean ± SE
TC-83	0.314 ± 0.011
RES block + TC-83	0.325 ± 0.006
400 R + TC-83	0.273 ± 0.069
400 R + RES block + TC-83	0.303 ± 0.010

After implanting intracardiac catheters into the left ventricle and right femoral vein, each monkey was placed in a restraint chair. One ml (10^9 PFU) of TC-83 virus was injected into the left ventricle. Venous blood was collected at intervals from 1-120 min after injection. Serum was assayed for virus on Vero cells. Irradiated monkeys were exposed to 400 R (30 R/min) of sublethal, total-body x-radiation ($LD_{50/30}$ = 550 R) from a 1-MeV x-ray generating unit 4 days prior to injection of TC-83. Our initial attempts to block the RES of 3 monkeys, using a suspension of thorium dioxide in dextrin, were not successful. This suspension, given by either the IV or IP route at concentrations 50% or less than has been used successfully in rodents, proved to be lethal or not effective. RES blockade prior to injection of TC-83 was finally effected by an IP injection of thorium dioxide (25% suspension in 10% dextrin, 2 ml/kg), given 18 hr prior to the 1st of 2 equal doses of colloidal C, containing 113 mg carbon/ml. Treatments were given IV, 2.5 ml/kg, 3 hr apart. TC-83 virus was given to RES-blockaded monkeys 45 min following the injection of the 2nd dose of carbon. Circulating C was detected in the blood throughout the experimental period. Clearance rates for carbon or virus were calculated from $k = 0.693/t_{\frac{1}{2}}$. The rates of carbon clearance (k_C, mean ± SE) following the 1st and 2nd doses of carbon were 0.035 ± 0.008 and 0.022 ± 0.005, respectively ($p < 0.10$). The rate constants for TC-83 (k_V) through 10 min of clearance, shown in Table I, were similar for all groups.

Next it was of interest to determine if the live TC-83 vaccine, formalin-killed VEE (KVEE) or KVEE complexed with specific IgG at equivalence produced the same or different clearance kinetics in "unblocked" and RES-"blocked" monkeys. To accomplish this, it was necessary to use a radioactive tag, since the KVEE could not be assayed in cell culture. An ^{125}I exogenous tag on the VEE virus was used because of previous success in other VEE studies. Twenty-two rhesus monkeys of either sex, 3-4 kg weight, and negative for VEE serum neutralizing antibody were allocated at random into treatment groups as shown in Table II. In each case, "blocked" animals were subjected to RES blockade as described above. Live or killed virus, or killed virus complexed with antibody, was given in a 1-ml bolus containing approximately either 10^9 PFU of virus and/or 10^8 cpm.

Graphic examination of the clearance data revealed that TC-83 virus and tagged virus followed an exponential clearance pattern with at least 2 different clearance rate kinetics, relatively fast clearance through the 1st 10 min and much slower clearance thereafter. For this reason, k-values were calculated for initial clearance through 10 min.

The 1st study showed that clearance of TC-83 virus in monkeys was rapid; by 10 min, > 95% of the injected was cleared from the circulation. Initial clearance of virus was similar in all 4 groups (Table I). RES impairment due to thorium dioxide and colloidal C treatments failed to affect clearance, suggesting that the RES is not important in initial clearance of TC-83 virus. In the 1st 10 min, virus particles may be cleared by other mechanisms, such as uptake by, or adherence to, a variety of cells which they contact. Sublethal, total-body x-irradiation 4 days before TC-83 infection had no effect on the initial clearance of virus, indicating that the prolonged viremia found in irradiated monkeys was not due to depressed clearance of virus. Further, RES

blockade in irradiated monkeys did not change the clearance kinetics of TC-83.

TABLE II. CLEARANCE RATES OF VEE IN MONKEYS ASSAYED BY CPM

TREATMENT	NO. ANIMALS	k-VALUE (10 MIN) Mean ± SE
TC-83	2	0.077 ± 0.008
XRT[1] + TC-83	2	0.096 ± 0.010
RES block + TC-83	2	0.092 ± 0.014
XRT + RES block + TC-83	2	0.088 ± 0.007
KVEE	4	0.128 ± 0.024
RES block + KVEE	2	0.079 ± 0.003
KVEE complexed with IgC	4	0.118 ± 0.030
RES block + KVEE complexed with IgG	4	0.082 ± 0.005

[1] XRT = x-radiation-treated.

Clearance kinetics by ^{125}I showed that approximately 45% of the injected, radiolabeled TC-83, KVEE, and killed, complexed TC-83 remained in the circulation by 10 min. Values of k in the 2nd study, Table II, indicate that clearance rates as measured by cpm were 3-4 times slower, on the average, than clearance of TC-83 as measured by PFU (Table I). Our only explanation for this discrepancy is that the exogenous label may be coming off the virus particles. However, both methods showed that initial TC-83 clearance was not affected significantly by RES blockade.

In one series of clearance experiments in the 2nd study, we attempted to measure virus clearance both by PFU and cpm from aliquoted blood samples from the same animal. It turned out that ^{125}I-tagged, live TC-83 did not exhibit any cytopathogenic effects in Vero cells, hence PFU could not be measured from these animals. Apparently the radioactive tag either killed or affected the TC-83 virus in some way so as to inhibit *in vitro* cell infection and plaque formation.

Another aspect of these studies was the determination of clearance patterns for KVEE and KVEE complexed with specific IgG. Although the numbers of animals are too few to draw definitive conclusions, certain important trends and tentative conclusions can be made. First, KVEE and KVEE complexed with specific IgG as measured by cpm are cleared similarly in "unblocked" normal monkeys during the 1st 10 min (Table II). Both KVEE and KVEE complex are

cleared slightly faster than TC-83. Blocking the RES resulted in slower clearance for both KVEE and KVEE complexed with specific IgG ($p < 0.20$). When k-values are combined for the "unblocked" KVEE- and KVEE complex-treated monkeys (total of 8) and compared to the k-values for RES-"blocked" KVEE- and KVEE complex-treated monkeys (total of 6), the differences in mean k-values are significant ($p < 0.05$). Whereas the RES does not appear to be involved significantly in clearance of TC-83, it may be important in clearance of KVEE and complexed KVEE.

In order to study the effects of irradiation on delayed hypersensitivity and infection, a method for quantitative evaluation of delayed hypersensitivity using 2-4-dinitrochlorobenzene (DNCB) was adapted for use in monkeys. When a sensitizing dose of DNCB (2,000-4,000 μg) was applied to the skin surface, little or no inflammatory response was obtained. When animals were challenged with DNCB in graded doses of 100-2,000 μg, minimal to very minimal reactions (erythema and induration) were seen. All challenge-dose sites were biopsied at 72 hr and submitted to Pathology Division for microscopic diagnosis. An additional site, on which croton oil had been applied 72 hr before, was also biopsied. The degree of histologic lymphoreticular infiltration for each challenge dose was graded as: none, 0; very minimal, +1; minimal, +2; moderate, +3; or severe, +4. For each of the 5 monkeys in the study, 100 μg of DNCB produced a mean reaction of +1; 250 μg, +2.4; 500 μg, +2.8; 1,000 μg, +3.0; and 2,000 μg, +3.4. Croton oil produced no response to a very mild reaction with a mean score for all 5 monkeys of +0.4. The rhesus monkey does not produce a good, consistent, spontaneous flare to DNCB which can be detected clinically with any degree of reliability; however, through the use of biopsies, the equivocality of the clinical reaction can be overcome and quantitated.

Preliminary studies in 2 monkeys designed to examine changes in a subpopulation of T-cells, namely, the active rosette-forming cells,[5] as a measure of immunocompetence, using DNCB contact sensitization, have shown remarkable correlation between cell-mediated immune response (delayed cutaneous hypersensitivity) and increased percentage of active rosette-forming cells.

In other immunosuppression studies, we have attempted to enhance the susceptibility of mice to Tacaribe virus using total-body irradiation and Cytoxan Cytoxan, 200 mg/kg, is 100% lethal to CD-1 mice when injected by the IP route, but at 25 mg/kg, 94% of the mice survived, the same percent survival observed in mice exposed to 500 R of total-body irradiation. The combination of Cytoxan at 50 mg/kg + 500 R of total-body radiation exposure resulted in only 17% survival. These findings indicate that Cytoxan, in combination with irradiation, must be given in very low doses to achieve significant immunosuppression without a large number of deaths; hence, it was concluded that the combination of Cytoxan and irradiation offered no advantage over total-body irradiation alone in immune suppression studies.

Preliminary studies in collaboration with Virology Division have been completed on 8 monkeys (2 irradiated, 400 R; 2 vaccinated with TC-83; 2 irradiated and vaccinated with TC-83; and 2 normal controls) to examine total T-lymphocytes, active T-cells, and response to mitogenic stimulation with

pokeweed, phytohemagglutinin, and concanavalin-A. In general, total and active T-cells were depressed significantly within 2 days in the irradiated and irradiated-vaccinated monkeys. Total and active T-cells were decreased in monkeys receiving TC-83 only when compared with the untreated controls. The transformation studies with various mitogens showed great variability among control values; irradiation had a marked depressive effect, and VEE showed no significant differences from control values.

Several preliminary experiments have been completed with WR 2721, S-2-amino propyl (amino ethyl) phosphoric acid, a radioprotective drug, to determine if the immune process can be altered significantly when higher radiation doses are given in conjunction with this compound. In CD-1 male mice, the acute radiation (1-MeV x-rays) $LD_{50/30}$ by probit analysis was 590 R (560 rads). When WR 2721, 0.3 mg/gm of body weight, was given 15 min before irradiation, the $LD_{50/30}$ was 1,140 R (1,080 rads), a dose-reduction factor of 2.11. When WR 2721 was given to CD-1 mice immediately or up to 4 days before vaccination with TC-83, there was no difference between the protection, i.e., resistance to virulent virus challenge, obtained when compared to TC-83 alone. When TC-83 was given before WR 2721, protection afforded these mice was also the same as that afforded mice given TC-83 alone. WR 2721 was also given 15 min before exposure of CD-1 male mice to 600 R of total-body x radiation, followed by vaccination with TC-83 in 1, 4 or 7 days. In contrast to mice given 600 R followed by TC-33, where protection was delayed a week or more, 42% were protected 3 days postvaccination (PV) in the WR 2721 + R-7 (irradiated 7 days before vaccination) day group, 25% at 3 days PV in the WR 2721 + R-4 (irradiated 4 days before vaccination) day group, and 8% at 3 days PV in the WR 2721 + R-1 (irradiated 1 day before vaccination) day group. This indicated that WR 2721 protected some target cells (lymphocytes?) from the indirect effects of radiation injury. Further, a sufficient number of these cells were protected so that the immune response to TC-83 was not greatly suppressed. An alternative hypothesis is that production of interferon may be stimulated by WR 2721 or, more likely, cells producing interferon are protected from indirect radiation injury, leading to this early resistance to virulent virus challenge.

Several long-term studies have been completed in which the responses of chronically irradiated mice to TC-83 were studied. When the index of response has been lethality, we found that a much higher radiation dose must be given to mice to achieve a degree of immune suppression similar to that found after acute, total-body, radiation exposure.[1] This was expected, but we did not anticipate that the magnitude of the chronic radiation exposure dose would be so great. That is, 600 R acute total-body radiation exposure of CD-1 male mice is approximately equal to 2700 R given chronically at the rate of 3 R/hr, 22 hr/day. Prolonged TC-83 viremia and increased serum virus levels reported after acute radiation exposure were also observed in the chronically irradiated model. Chronic irradiation treatment of TC-83-vaccinated mice resulted in enhanced humoral antibody responses (Table III). This enhancement occurred soon after vaccination because titers were essentially maximal by 7 days PV. Continued irradiation neither increased nor decreased the antibody responses. This finding is currently being studied in chronically irradiated swine.

TABLE III. MOUSE SERUM-NEUTRALIZING TITERS IN IRRADIATED-VACCINATED MICE

GROUP	RECIPROCAL TITER BY DAYS POSTVACCINATION					
	7	14	21	35	49	63
A (Irradiated for 20 days after TC-83, 3 R/hr, 22 hr/day. Total = 1,320 R.)	12,500	12,500	12,500	12,500	12,500	2,500
	12,500	12,500	12,500	12,500	12,500	12,500
	>12,500	>12,500	12,500	2,500	>12,500	>12,500
	2,500	12,500	12,500	>12,500	>12,500	2,500
	<20	12,500	12,500	>12,500		
	12,500	12,500	12,500	2,500		
B (Vaccine controls)	<20	100	100	500	500	500
	100	100	500	500	500	500
	100	500	500	500	500	500
	500	500	500	500	500	500

Publications:

1. Spertzel, R. O., D. E. Hilmas, J. R. Brown, and D. W. Mason. 1975. Response of irradiated mice to live-virus (TC-83) immunization. Infect. Immunity 11:481-487.

2. Hilmas, D. E., W. E. Houston, R. T. Faulkner, J. R. Brown, C. L. Crabbs, and R. O. Spertzel. 1975. Vascular clearance of live, attenuated VEE virus, TC-83. IRCS Med. Sci. :Immunol. Allergy 3:234, IRCS Med. Sci.: Microbiol. 3:234.

3. Hilmas, D. E., and R. O. Spertzel. 1975. Response of sublethally irradiated monkeys to a replicating viral antigen. Infect. Immunity 12: In press.

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3. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974. pp. 25-31. Fort Detrick, Md.

4. Spertzel, R. O., D. E. Hilmas, J. R. Brown, and D. W. Mason. 1975. Response of irradiated mice to live-virus (TC-83) immunization. Infect. Immunity 11:481-487.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6416	75 07 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
74 07 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	62760A	3A762760A834	01	011
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code): (U) Rapid electron microscopic assay for virus particles of diseases of military medical importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 02	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 75	1.0	33.2
	CURRENT 76	1.0	61.0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Physical Sciences Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Buzzell, A.
TELEPHONE: 301 663-7181
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Virus; (U) Electron microscopy; (U) Negative staining; (U) Military medicine

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 rapid electron microscopic assay for virus particles. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) The method is being adapted for use with Millipore filters with a view to increasing the sensitivity of the assay.

25 (U) 74 07 - 75 06 - Work is continuing on developing a rapid electron microscopic assay procedure of sufficient sensitivity for diagnostic purposes. In the procedure, modified from one described by Sharp in 1949 and still in use, virus particles are concentrated by ultracentrifugation onto a block of agar. To transfer virus from the agar to an electron microscope grid for counting, a new method has been devised which is simpler, more reliable, and requires less virus. Moreover, sensitivity of the assay can probably now be increased, using Millipores in place of agar in the centrifugation step, since most of the problems encountered in transferring virus from Millipores have been resolved. To facilitate viral identification, negative staining can be incorporated in the transfer step, using Millipores as well as agar, with damage minimal even for virus particles usually disrupted by negative staining. Using stains of different charge, further study of the strucutural alterations produced by anions in the bacteriophage test virus, described previously, have revealed new features of the structure not recognized before. It thus seems increasingly likely that virus particles might eventually be identified on the basis of structure alone, since structural alterations are reported to occur for many viruses.

Publication: Biophys. J. 15:201a, 1975 (abstr.).

*Available to contractors upon originator's approval.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 011: Rapid Electron Microscopic Assay for Virus Particles of Diseases of Military Medical Importance

Background:

In 1949, Sharp[1] showed that virus particles could be concentrated and partially purified by sedimenting them onto a block of agar in the ultracentrifuge, allowing particle counts to be made at concentrations of 10^6/ml. In Sharp's procedure, particles are retrieved for viewing in the electron microscope by the "pseudo-replica" method of forming a collodion film on the agar, a procedure which is tedious and not wholly reliable. Furthermore, negative staining has to be done as a subsequent step,[2] by procedures which can further disrupt the particles, or mask them, the stain generally being distributed unevenly.[3]

An easier and more reliable procedure, described in previous reports has been devised for retrieving particles from agar, which incorporates negative staining in the transfer step, minimizing damage to the virus and allowing smaller samples to be used. With the new procedure, particles can probably be transferred from Millipore filters, a potential advantage in the centrifugation step. Far greater concentrations would be possible if the particles could be funneled down through a small orifice onto a Millipore. Since the receptor would have to maintain a seal at the orifice, ordinary agar gels cannot be used, since gels sediment during ultracentrifugation.

Progress:

Continued work on the problem of adapting the transfer procedure for use with Millipore filters has revealed 2 sources for the erratic results with negative staining. As described last year, a high charge on negative stained anions appears to impede diffusion through millipores, since results were generally better for silicotungstate than for phosphotungstate, partial hydrolysis giving the latter a charge of -7. It was later discovered, by further search of the literature and consultation with a specialist (Dr. Michael Pope, Georgetown University), that silicotungstate can be hydrolyzed too. Even brief exposure to high pH causes the charge to increase from -4 to -8; low pH is also deleterious. Since solutions of silicotungstate were made by titrating the acid with a base, more rapid titration should eliminate this source of trouble.

A second source of erratic results proved to be air bubbles in the filter. With bubbles present the stain solution cannot flow down under gravity to wash the virus free, nor can salts diffuse into the filter from the virus solution. It is all too easy to introduce bubbles after the filters are wet, for drying is rapid. But if reliable means can be found for keeping fluid levels constant, indications are that virus transfer from Millipores will be quantitative and negative staining good, possibly even with stains of higher charge. A centrifuge cell has therefore been designed for funneling virus down onto a Millipore, similar to one described previously but modified for use at higher speed, in a 50-L swinging bucket rotor.

Pending solution of the problems with Millipores, work was continued with agarose gels to see if gels of higher concentration could be used, as a possible substitute for them in the centrifugation step. The viscosity of agarose at higher concentrations made it necessary to plate solutions between microscope slides, separated by spacers. With this procedure, based on that of Beale and Mason,[4] a 10% gel can be made as thin as a Millipore, strong yet flexible enough for the peeling process used in virus transfer. At this concentration gels should retain even the smallest virus particles.[5] But difficulties with virus transfer, only recently resolved, have prevented further testing, or trials in the ultracentrifuge. Hydrolysis of the agarose proved to be the problem, high concentration magnifying the ill effects, with more sites provided for adsorbing virus and more fragments freed to impair the negative staining.

Hydrolysis can make agarose harder to dissolve initially. Therefore, when overheating at the vessel walls was avoided, which was relatively more important with the volume small, agarose could be dissolved near 80 C, eliminating the stickiness characteristic of hydrolysis. The temperature of plating also affects behavior of the gels. At > 60 C strands can evidently become oriented with hydrophobic sides outward, making gels impermeable even for tiny drops of virus solution. Gels plated near 60 C are hydrophilic, however, even large drops penetrating readily. Thus, stain can now be added after plating, making it easier to use a variety of stains, some of which interfere with gel formation.

With stains of different charge, the structural alterations induced by anions in a bacterial virus could be manipulated further to reveal features of the structure not previously recognized. Manipulations of this kind may well prove useful in virus identification, as suggested previously, since structural alterations are reported to occur for a variety of viruses.

Presentation:

Buzzell, A. Electron microscopic assay for virus particles for early diagnosis. Presented, Annual Meeting, Biophysical Society, Philadelphia, PA. 18-21 Feb 1975. (Biophys. J. 15:201a, 1975).

Publications:

None

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1. Sharp, D. G. 1949. Enumeration of virus particles by electron micrography. Proc. Soc. Exp. Biol. Med. 70:54-59.

2. Smith, K. O., and J. L. Melnick. 1962. A method for staining virus particles and identifying their nucleic acid type in the electron microscope. Virology 17:480-490.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6418	75 07 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
74 07 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	62760A	3A762760A834	01	013
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Host lipids in infectious and toxic illnesses for defense against BW

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 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 75	1.0	165.5
FISCAL YEAR CURRENT 76	0.3	36.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Physical Sciences Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Kaufmann, R. L.

TELEPHONE: 301 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede each with Security Classification Code)

(U) Lipids; (U) Cholesterol; (U) Triglycerides; (U) Postheparin lipolytic activity; (U) Carbohydrate-lipid interrelations; (U) Bacterial illness; (U) Endotoxin

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

23 (U) Study changes in lipid dynamics during infectious illness. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Measure serum triglyceride and cholesterol levels during infectious illness and determine the activity of lipoprotein lipase.

25 (U) 74 07 - 75 06 - Lipid disposal mechanisms have been found to be significantly inhibited 48 hr after inoculation with Salmonella typhimurium. Intolerance to exogenous lipid loading orally or intravenously has been demonstrated during S. typhimurium sepsis along with decreased lipoprotein lipase activity at 1, 2 and 6 min postheparin. Increased plasma triglycerides along with impairment of lipid disposal were also observed after treatment with S. typhimurium endotoxin.

Livers of rats infected with S. typhimurium demonstrate increased free fatty acids and triglycerides 48 hr after inoculation. This phenomenon has not been demonstrated using endotoxin.

These studies support the concept that lipid metabolism is significantly altered during infection by affecting lipid disposal as well as synthetic mechanisms.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 013: Host Lipids in Infectious and Toxic Illnesses for Defense Against BW

Background:

The role of impaired lipid disposal mechanisms has been evaluated in male rhesus monkeys in order to determine mechanisms producing elevated triglyceride concentrations observed during bacterial sepsis.[1] Impaired clearing of IV lipid has been demonstrated during Salmonella typhimurium, but not Streptococcus pneumoniae, sepsis. Endotoxin has also been shown to increase triglyceride concentrations.[2] Impaired lipid disposal has also been demonstrated after endotoxin by decreased postheparin lipolytic activity and impaired clearing of IV administered lipid. However, endotoxin added to heparin activated plasma in vitro does not reduce the assayable activity of lipoprotein lipase. This evidence supports the concept that endotoxin plays a significant role in the hypertriglyceridemia observed during gram negative sepsis.

Lipid biochemical studies in rat liver have been undertaken to determine the sequential changes in various lipid constituents during the course of experimental gram negative and gram positive sepsis.

Progress:

Significant evidence has accumulated to demonstrate that lipid disposal mechanisms are significantly impaired particularly during gram negative sepsis produced by S. typhimurium in the rhesus monkey 48 hr after inoculation. At this time monkeys are intolerant to exogenous lipid given by nasogastric tube, 4 gm/kg, or IV, 0.5 gm/kg, over 30 min showing higher triglyceride concentrations after lipid loading than noninfected controls. Likewise, S. typhimurium-infected monkeys demonstrated significantly decreased lipoprotein lipase activity at 1, 2, and 6 min after IV heparin. In addition, endotoxin (7 mg/kg) has been shown to increase free fatty acid and triglyceride concentrations and lead to decreased clearing of IV lipid with significantly reduced lipolytic activities at 1, 2, 6 and 10 min postheparin in rhesus monkeys.

Hepatic lipids were analyzed 24 and 48 hr after IP injection of S. typhimurium or S. pneumoniae in rats. Triglycerides, free fatty acids and esterified cholesterol were higher in S. typhimurium-infected rats at 48 hr, while these changes were not observed in S. pneumoniae-infected rats. However, 3 and

6 hr after IP injection of 2 mg endotoxin significant increments in serum or liver free fatty acids or triglycerides were not observed.

Publications:

None.

LITERATURE CITED

1. Fiser, R. H., J. C. Denniston, and W. R. Beisel. 1972. Infection with Diplococcus pneumoniae and Salmonella typhimurium in monkeys: changes in plasma lipids and lipoproteins. J. Infect. Dis. 125:54-60.

2. Fiser, R. H., J. C. Denniston, and W. R. Beisel. 1972. Endotoxemia in the rhesus monkey: alterations in host lipid and carbohydrate metabolism. Pediatr. Res. 8:13-17.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6420	75 07 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
74 07 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	62760A	3A762760A834	01	015
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code): (U) Cardiovascular and cardiac electrophysiological effects of infections of military significance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 012900 Physiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 03	75 08	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 75	1.0	118.3
FISCAL YEAR CURRENT 76	1.0	72.7

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Animal Assessment Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Pettit, G. W.
TELEPHONE: 301 663-2238
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Hilmas, D. E.
NAME: Liu, C. T. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Myocardium; (U) Physiology; (U) Infectious diseases; (U) Military medicine; (U) Myocardial contractility; (U) Electrophysiology

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

23 (U) Determine to what extent various infectious diseases cause changes in myocardial function. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Using conventional methods of quantitating myocardial contractility, determine baseline values and then measure changes induced by infections induced in rhesus monkeys. In addition, other cardiovascular parameters will be measured.

25 (U) 74 07 - 75 06 - Others detected myocardial depressant factors (MDF) in plasma from animals in shock; we are attempting to detect MDF in plasma from infected animals. We have developed an in vitro technique utilizing an isolated rabbit papillary muscle for detection of MDF. In vivo myocardial contractility in rhesus monkeys is periodically measured throughout the course of infection, and plasma, obtained at times when contractility is depressed, is applied to the in vitro assay system. Conclusive data have not been obtained in the first 2 experiments; refinements in methods should improve results.

In other work we are determining if SEA and SEB have direct actions on myocardium. In vivo electrocardiographic studies in rabbits revealed no changes other than tachycardia.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 015: Cardiovascular and cardiac electrophysiological effects of infections of military significance

Background:

Boucher et al.[1] indicated that myocardial depression occurred 12-36 hr after IV infection of monkeys with about 10^8 Streptococcus pneumoniae organisms. A dialyzable myocardial depressant factor (MDF) is released during shock and has been isolated and studied extensively.[2] Very recently, a non-dialyzable depressant factor, passively transferrable lethal factor (PTLF), has been detected.[3] Since Boucher et al. found a short period of depressed myocardial contractility coincident with tachycardia in infected monkeys, we reasoned that MDF may be released and might account for some of the myocardial depression found during infection.

Also, data recently gathered by Dr. C. T. Liu (Animal Assessment Division) indicate that there may be myocardial depression during shock induced by IV staphylococcal enterotoxin B (SEB); circulating MDF might be responsible for part of this depression. Since myocardial depression has been found during SEB shock and during sepsis with S. pneumoniae, the present investigators feel that myocardial depression due to circulating factors (e.g., MDF and PTLF) may be important in the pathogenesis of infections. Since large amounts of MDF have been shown to be formed by damaged pancreas,[2] investigation of other infections, particularly infections which attach the pancreas (e.g., VEE) will also be warranted.

Progress:

Surgical procedure has been modified for implantation of intraventricular micromanometers in monkeys. In this procedure, the transducer is implanted initially without externalization of the connecting cable, and the monkey is permitted to recover in his cage. The cable is finally externalized when the monkey is chaired 2-3 wk after surgery.

Further refinements were made in a technique utilizing an isolated rabbit papillary muscle for bioassay of cardiodepressive substances in plasma. Experiments were performed in which myocardial contractility was measured at intervals in a chaired monkey during various pharmacological interventions known to alter contractility. At the same intervals, plasma samples were taken from the monkey and bioassayed on isolated rabbit papillary muscle. It was encouraging to find that when monkeys had received small dosages of isoproterenol (0.02 mg/kg) their plasma had an appreciable positive inotropic action on the isolated rabbit papillary muscle. Contractile force increased 33% even when no effect on contractility was observed in the chaired monkey;

this indicated that our bioassay is very sensitive to circulating inotropic agents. Unfortunately, we found that arrhythmias developed when normal or drug-containing monkey plasma was applied to the isolated papillary muscle.

Two monkeys instrumented for measurement of left ventricular pressure were chaired, catheterized, and infected IV with about 2 x 10^8 organisms of S. pneumoniae Type I, A5. Heart rate, blood pH, IM temperature, mean arterial blood pressure, left ventricular pressure, and maximum velocity of contractile element shortening (V_{max}) were periodically monitored when serial plasma samples were withdrawn from the monkeys during the course of the infection. Unfortunately, in the 2 monkeys studied we were unable to reproduce the striking decrease in V_{max} coincident with tachycardia observed by Boucher et al.[1] In 1 monkey, heart rate increased 18% and contractility increased 17.5% above preinfection levels. In the other monkey, heart rate increased 31% and contractility increased 33% above preinfection levels. Furthermore, none of the plasma samples drawn exhibited any myocardial depressant activity. In future experiments we hope to make our results more definitive by infecting monkeys with other S. pneumoniae Type I strains and by analyzing raw plasma, ultrafiltrates of plasma and nonfiltrable fractions for myocardial depressant activity. Also, since Boucher et al.[1] found myocardial depression to peak around 16 hr after challenge with bacteria, we are presently collecting 1 sample for assay of MDF approximately 16 hr after S. pneumoniae infection, at a time when we verify that myocardial contractility is depressed. Since only 2 plasma samples are taken (control and at 16 hr postinfection), we can take larger samples (about 20 ml) which will be sufficient for liquid chromatographic analysis (by Dr. T. Yamada, Bacteriology Division). This allows more precise characterization of low-molecular weight polypeptides which have been shown to be myocardial depressant substances.[4]

We are conducting studies in order to investigate the possibility that the rabbit produces high levels of cardiodepressive substances during hemorrhagic shock; to date, dogs and cats are the only animal models for MDF studies. Using a hemorrhagic shock model,[4] we found that rabbit plasma exerts an increased myocardial depressant activity during shock. In results from 1 experiment we found 17% reduction in contractile force of an isolated rabbit papillary muscle when preshock plasma ultrafiltrate was applied, and 50% reduction when shock ultrafiltrate was applied. These initial results are consistent with those obtained in cats by Lefer and Martin.[4] The ultrafiltrate has been processed further, using liquid chromatographic techniques developed by them.[4] Seven milliliters of ultrafiltrate from 20 ml of plasma was applied to a Biogel P-2 column, and optical densities of eluted fractions were read at 230 mμ. Using ultrafiltrate of normal rabbit plasma, we found the same general pattern of optical density vs. eluted volume as found by Lefer and Martin;[4] we found peaks in optical density at roughly the same elution volumes. Also, the pattern of occurrence of MDF activity in the various eluted fractions was similar to their patterns; most myocardial depressant activity resided in a peak which occurred at an elution volume of about 125 ml, designated peak "D."[4]

Experiments designed to assess the direct action of SEB on myocardium are presently in progress. Initial experiments consisted of periodic monitoring

of 6-lead (frontal plane) electrocardiograms (EKG) after administration to rabbits of large doses of SEB (2 mg/kg, IV, n = 4) and SEA (n = 2). The only demonstrable EKG change after the enterotoxins was tachycardia; heart rate increased an average of 51%. Vector axes, magnitudes, and intervals were unchanged. These findings indicate that the toxins have no effect on the conduction system of the heart. Experiments on isolated papillary muscles, patterned after the work of Kutner and Cohen,[5] will allow us to expand these studies by assessing direct cardiac actions of SEB on myocardium in vitro.

Publications:

None.

LITERATURE CITED

1. Boucher, J. H., D. E. Hilmas, C. T. Liu, W. P. Czajkowski, and R. O. Spertzel. 1974. Myocardial depression during Diplococcus pneumoniae infection in monkeys. Proc. Soc. Exp. Biol. Med. 145:112-116.

2. Lefer, A. M., and J. A. Spath, Jr. 1974. Pancreatic hypoperfusion and the production of a myocardial depressant factor in hemorrhagic shock. Ann. Surg. 179:868-876.

3. Nagler, A. L., and S. M. Levenson. 1974. The nature of toxic material in the blood of rats subjected to irreversible hemorrhagic shock. Circ. Shock 1:251-264.

4. Lefer, A. M., and J. Martin. 1970. Relationship of plasma peptides to the myocardial depressant factor in hemorrhagic shock in cats. Circ. Res. 26:59-69.

5. Kutner, F. R., and J. Cohen. 1966. Effect of endotoxin on isolated cat papillary muscle. J. Surg. Res. 6:83-86.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6424	75 07 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
74 07 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	62760A	3A762760A834	01	016
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Studies of sequential respiratory infections in a model host

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 005900 Environmental biology

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: 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 75	0.5	101.0
	CURRENT 76	0.5	117.8

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Aerobiology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Berendt, R. F.

TELEPHONE: 301 663-2439

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Influenza; (U) Pneumonia; (U) Airborne infection; (U) Synergism; (U) Interference

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

23 (U) Assess suitability of the rhesus monkey for sequentially acquired respiratory infections and as a model system for studies of particle-size effects, pathogenesis, respiratory immunization, etc. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) After adaptation of an influenza virus and Streptococcus pneumoniae to monkeys, infect these animals with the virus followed by the bacterium. Determine the effects by usual parameters.

25 (U) 74 07 - 75 06 - Squirrel monkeys were given 8.2 logs median egg infectious doses of influenza virus by intratracheal instillation followed 102 hr later by 770 Streptococcus pneumoniae organisms. Three of 4 monkeys died within 40 hr, the fourth was very ill. Characteristic clinical findings following the bacterial challenge were paroxysmal cough, increased respiratory rate, anorexia, bacteremia, prostration and death. Influenzal-control monkeys had increased respiratory rates, coughing and coryza. Bacterial-control monkeys were asymptomatic. Pathologic changes noted in sequentially-infected monkeys were severe exudative pneumonia; in viral-control monkeys, tracheobronchitis and bronchopneumonia; and pneumococcal-controls, minimal histopathologic changes.

Publication: Infect. Immunity 10:369-374, 1974.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 016: Studies of Sequential Respiratory Infections in a Model Host

Background:

In the last annual report[1] we discussed the persistence of type 1 Streptococcus pneumoniae in rhesus monkeys following influenza virus infection. Since none of the monkeys developed clinical signs of illness, the model was not considered suitable. Two lines of research seemed to be the most promising: (1) screening of other monkey species for susceptibility to the influenza virus and pneumococcal strains already employed and (2) investigate clinical response to recent isolates of influenza virus. This report presents data obtained principally from study of other monkey species plus some work done with recent viral isolates.

Progress:

Screening of type A influenza virus, Aichi 2/68 (H3N2), and of type 1 S. pneumoniae (ATCC 6301) was carried out by intratracheal inoculation of selected monkey species. The rhesus (Macaca mulatta) and cynomolgus (Macaca fasicularis) monkeys were refractory to both organisms; the capuchin monkey (Cebus capucinus) reacted clinically to both organisms, but was found at necropsy to be heavily infected with lung parasites and therefore was unsuitable for detailed pathogenesis and therapy studies. Owl monkeys (Aotus trivergatus) had very mild clinical reactions to influenza virus; however, they were very susceptible to pneumococcal challenge even at low doses and died of overwhelming bacteremia. The squirrel monkey (Saimiri sciureus) was found to be susceptible to challenge with both organisms and was selected for further study.

Initial experiments were directed towards determining dose-response characteristics and an evaluation of selected clinical parameters following challenge to the 2 microorganisms administered separately. Results of virus-challenge experiments are given in Table I and pneumococcal challenge in Table II. The data showed that both organisms produced readily detectable clinical signs after challenge. Influenza virus administration was followed by fever, coryza, rapid respiration and sneezing or coughing (Table I). Pneumococcal challenge was lethal in 6-7 days at doses of $\geq 10^5$ cells. Monkeys given 770 cells were markedly ill in 3-4 days (Table II), but did not die.

Sequential infection (Seq) experiments were performed by inoculating 4

TABLE I. CLINICAL SIGNS IN SQUIRREL MONKEYS FOLLOWING INTRATRACHEAL CHALLENGE WITH TYPE A INFLUENZA VIRUS

DOSE (EID_{50})	SIGN	NO. POS./2	MEAN DAY OF ONSET	MEAN DURATION (days)
$10^{8.2}$	Fever[a] (1)	2	1.0	1.0
	(2)	2	6.0	4.0
	Coryza	2	3.5	6.5
	Rapid respiration	2	3.5	2.8
	Dyspnea	2	3.5	4.8
	Sneezing and/or coughing	2	3.5	6.0
$10^{6.2}$	Fever	2	1.0	3.0
	Coryza	2	2.0	6.5
	Rapid respiration	2	4.0	2.0
	Dyspnea	2	4.0	3.0
	Sneezing and/or coughing	0	NA	NA
$10^{4.2}$	Fever	2	1.0	4.0
	Coryza	2	5.5	4.5
	Rapid respiration	2	7.0	2.0
	Dyspnea	2	8.0	3.0
	Sneezing and/or coughing	2	8.0	3.0

a. Fever is defined as $\geq$1.5 F above baseline. There were 2 distinct episodes of fever in high-dose monkeys, only one in the 2 lower dose groups.

TABLE II. RESPONSE OF SQUIRREL MONKEYS TO INTRATRACHEAL CHALLENGE WITH SELECTED DOSES OF TYPE 1 STREPTOCOCCUS PNEUMONIAE

NO. CELLS ADMINISTERED	SIGN	NO. POS./2	MEAN DAY OF ONSET	DURATION (days)
$6x10^8$	Fever[a]	2	1.0	5
	Bacteremia	2	1.0	6
	Lethargy	2	1.0	6
	Anorexia	2	4.5	1.5
	Coughing	2	3.0	3
	Dyspnea	2	3.0	3
	Death	2	-	(6,6)[b]
10^5	Fever	2	2.0	1.0
	Bacteremia	2	2.0	6
	Lethargy	2	4.5	3
	Anorexia	2	4.0	3
	Coughing	2	5.0	1.5
	Dyspnea	2	4.0	2
	Death	2	-	(6,7)
770	Fever	2	2.0	1.0
	Bacteremia	2	1.5	5
	Lethargy	2	3.0	5
	Anorexia	2	4.0	3
	Coughing	2	6.5	7.5
	Dyspnea	2	4.0	3.5
	Death	0	-	-

a. >1.5 F above baseline.
b. Day of death of each monkey.

monkeys with $10^{8.2}$ EID_{50} of virus followed 102 hr later by 770 pneumococci. Four viral (VC) and 4 bacterial control (BC) monkeys were also inoculated; 4 monkeys were inoculated with broth as sham-controls at the appropriate times and showed no clinical response. Results are given in Table III. One influenza-virus control monkey died on day 3. Necropsy indicated that influenza may have been complicated by spontaneous bacterial infection. Three of the sequentially infected monkeys died about 40 hr after bacterial challenge. The 4th monkey was very ill, and along with 2 each of the control animals, was killed and necropsied. Viral control monkeys had a tracheobronchitis and bronchopneumonia, whereas the sequentially infected monkey had severe exudative pneumonia. Pneumococcal controls, however, showed no marked pathological response. In another experiment 2 monkeys were given 770 cells, followed until they were clinically ill, and killed and necropsied. The major post mortem observation was massive bacteremia rather than pneumonia; in fact,

TABLE III. RESPONSE OF SQUIRREL MONKEYS TO SEQUENCE OF INFLUENZA FOLLOWED BY *STREPTOCOCCUS PNEUMONIAE*[a]

PARAMETER	% OF MONKEYS RESPONDING BY DAY AFTER INITIAL EXPOSURE							MONKEY GROUP
	0	1	2	3	4	5	6	
Bacteremia	0	0	0	0	0	0	0	VC
	0	0	0	0	0	100	100	BC
	0	0	0	0	0	100	100	Seq
Anorexia	0	0	0	25	0	0	0	VC
	0	0	0	0	0	0	0	BC
	0	0	0	25	25	75	100	Seq
Cough	0	0	0	25	67	67	67	VC
	0	0	0	0	0	0	25	BC
	0	0	0	50	75	100	100	Seq
Lethargy	0	0	25	50	100	100	33	VC
	0	0	0	0	0	0	0	BC
	0	0	0	25	75	100	100	Seq
Cumulative % mortality	0	0	0	25	25	25	25	VC
	0	0	0	0	0	0	0	BC
	0	0	0	0	0	0	75	Seq

a. *S. pneumoniae* inoculated afternoon of day 4.

there was little if any histological changes noted in the lungs. Thus, initial viral infection apparently not only enhanced response to subsequent pneumococcal challenge, but also enabled the bacteria to multiply in the lung.

In an effort to simulate more closely naturally occurring infection, monkeys have been exposed to aerosols of the Aichi strain of influenza virus. The calculated inhaled dose was 10^5 EID_{50}. A clinical response characterized by anorexia, increased respiration and coughing was seen which was identical to that seen after intratracheal challenge at comparable doses.

A strain of influenza virus more recently isolated than the Aichi has been tested by intratracheal inoculation of 2 monkeys. This particular strain of influenza virus is employed for human challenge by Murphy and co-workers.[2] The monkeys responded with essentially the same signs of disease that were seen with the Aichi strain, but the clinical illness was more marked at comparable doses. Aerosol evaluation of this strain (Warren, H3N2) is in progress.

Future plans include completion of aerosol testing with the Warren strain and attempts to establish a sequence of influenza virus and Staphylococcus aureus. Although tentative plans also include extensive use of the influenza aspect of the model, future research will be carried out under another work unit, and this work unit should be completed by the end of FY 1975.

Presentations:

1. Berendt, R. F., and J. S. Walker. The squirrel monkey as a primate model for clinical influenza. Presented, Workshop on Antivirals in Influenza. National Institutes of Health, Bethesda, Md. 12-13 Dec 1974.

2. Berendt, R. F., and G. G. Long. A squirrel monkey model for sequential respiratory infection. Presented, Annual Joint Meeting of the Maryland-Washington, D.C. Branches of the American Society for Microbiology. Fort Detrick, Frederick, Md. 19 Apr 1975.

Publications:

Berendt, R. F., W. E. McDonough, and J. S. Walker. 1974. Persistence of Diplococcus pneumoniae after influenza virus infection in Macaca mulatta. Infect. Immunity 10:369-374.

LITERATURE CITED

1. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974, pp. 47-56. Fort Detrick, Md.

2. Murphy, B. R., E. G. Chalhub, S. R. Nusinoff, and R. M. Chanock. 1972. Temperature-sensitive mutants of influenza virus. II. Attenuation of ts recombinants for man. J. Infect. Dis. 126:170-178.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6422	75 07 01	DD-DR&E(AR)636

3. RATE 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
74 07 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	62760A	3A762760A834	01	020
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e)	(f)		

11. TITLE (Precede with Security Classification Code)

(U) Acute phase protein dynamics in the rat during infection

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

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: 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 75	1.0	119.0
	CURRENT 76	1.0	119.0

19. RESPONSIBLE DOD ORGANIZATION

NAME:* USA Medical Research Institute of Infectious Diseases

ADDRESS:* Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME:* Physical Sciences Division USAMRIID

ADDRESS:* Fort Detrick, MD 21701

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

NAME:* Abeles, F. B.

TELEPHONE: 301 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Rats; (U) Protein; (U) Infectious diseases; (U) Inflammation; (U) Military medicine

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

23 (U) Investigate changes in acute-phase proteins during infection. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Infect normal and surgically altered rats with a variety of organisms and measure acute-phase proteins and their kinetics.

25 (U) 74 07 - 75 06 - Progress has been primarily in the direction of furthering our understanding of the physiological activity of leukocytic endogenous mediator (LEM) as well as mechanisms concerned with its synthesis. The pancreas is an additional target organ for LEM with the result that an increase in serum glucagon and insulin follows treatment of rats with LEM. Whether or not other physiological functions of the pancreas, such as digestive enzymes, can be regulated by LEM remains to be seen. It is suggested that other potential target sites for LEM can be studied using unpurified LEM. The other significant fact emerging is the observation that Venezuelan equine encephalomyelitis turned off LEM synthesis while bacterial infections did not; the latter are known to stimulate PMN and cause a secretion of LEM. What this means in terms of the etiology of viral infections compared to bacterial infections is unknown although an interesting distinction in terms of host response.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 020: Acute Phase Protein Dynamics in the Rat During Infection

Background:

The objective of the work reported here is to understand the regulation and function of serum proteins secreted by the liver during infection and inflammation. The diseased state is characterized in part by changes in the concentration of certain serum proteins. These changes can be represented by increased levels of fibrinogen. C- reactive protein, α_1 acid glycoprotein, α_1-antitrypsin, α_2-acute-phase globulin (rat), α_1-acute-phase globulin (rat), ceruloplasmin, haptoglobin, and seromucoid, and decreased albumin.[1] The purpose of this study is to obtain information on the metabolism and function of these types of serum proteins in disease. Specifically most of the work has focused on the changes and physiology of α_2-macrofetoglobulin (α_2-MFG). In the rat this acute-phase reactant increases $\geq$ 100-fold from levels which are barely detectable by radial immunoassay techniques. The work during the period covered by this report has dealt with the regulation of acute-phase reactants by bacterial and viral infections, the role of leukocyte endogenous mediator (LEM) as a regulatory intermediate mechanism in this process and finally the attempt to develop a system of translating liver mRNA which codes for α_2-MFG.

Progress:

Polymorphonuclear leukocytes (PMN) when stimulated by phagocytic activity release endogenous mediators which alter amino acid, Zn and Fe distributions, increase acute-phase protein synthesis, blood PMN counts, and fever. We found that LEM also increased serum glucagon and insulin levels and simultaneously decreased serum glucose and free fatty acids in fasted rats. The changes in these serum components were first observable 1 hr after injection and reached a maximum at approximately 5 hr. Heating the leukocyte extract to 56C for 30 min did not dissipate its activity, although 100C for 30 min did. The factor was stable to dialysis and remained inside the dialysis tubing which indicated it had a molecular weight of $\geq$ 10,000 or greater (in collaboration with Work Units 834 01 401 and 834 01 801).

The role of LEM on other aspects of rat physiology were also studied. We found that LEM caused increased rates of incorporation of ^{14}C-orotic acid into hepatic RNA and elevations in serum concentrations of acute-phase globulins such as α_2-MFG and haptoglobin. Adrenalectomy and hypophysectomy prevented the ability of LEM to induce α_2-MFG or stimulate RNA synthesis. However, the ability to regulate haptoglobin synthesis was retained in rats lacking these endocrine glands. Hydrocortisone (500 µg) injected daily into adrenalectomized rats acted as a replacement for the adreal glands since LEM regained the ability to increase α_2-MFG and stimulate RNA synthesis. (Collaborative work with Work Unit 834 01 009).

Changes in the levels of α_2-MFG were used as an indication of the degree of illness in rats treated with a respiratory pathogen Klebsiella pneumoniae. Intranasal inoculation of 10^7 K. pneumoniae in rats engenders moderate to severe pneumonia and splenitis by 24 hr, interstitial pneumonia at 72 hr, and an overall mortality of 20-30% within 2 wk. Infected rats were killed at various times up to 2 wk. Mean spleen weight and white cell count increased up to 6 days. Preliminary analysis of the data indicates that increases with acute-phase reactants and decreases in serum albumin and Zn are due to biological sepsis. Serum α_2-MFG increased during the final stages of the disease and were diagnostic of rats with high bacterial counts and marked severity of illness. These studies were done with Work Units 834 01 016, 834 01 401; 834 01 803 and 834 02 107.

As discussed above, α_2-MFG is found in the serum of normal neonatal or adult rats with bacterial infections, artifically induced inflammation (turpentine abscess), or following inoculation with LEM. Unlike bacterial infections a VEE infection was found to cause only a small increase in the levels of this acute-phase reactant. In addition, VEE-infected rats also failed to synthesize α_2-MFG when injected with turpentine but responded normally when treated with LEM. Since the action of turpentine on the synthesis of acute-phase reactants is thought to be mediated by the activation of PMN, the observation that VEE infection blocked the rise of α_2-MFG normally seen after an injection of turpentine suggested that one effect of VEE infection was a disruption of normal PMN physiology.

On the assumption that the release of LEM into serum might be inhibited during VEE infection, the effect of LEM as replacement therapy following lethal VEE virus infection was tested. Rats injected SC with LEM 24 hr after VEE virus infection had enhanced survival rates as compared to controls given normal saline solution, heat-inactivated LFM, or LEM 24 hr preinfection (Work Unit 834 03 405).

The synthesis of α_2-MFG can be studied by following the translation of α_2-MFG in a cell-free protein synthesizing system. This part of the work has not been productive. It has been possible to develop a cell-free system from rabbit reticulocytes which is mRNA-dependent in terms of the translation of poly(u) into polyphenylalanine. However, attempts to extract mRNA from rat liver capable of activating this system has not been successful. The purpose of this study is to prove that infection, via LEM, and the permissive regulation of the adrenal-pituitary axis, controls acute-phase reactant synthesis of which α_2-MFG is one only one, by increasing mRNA synthesis. An effort to resolve the problem will involve the development of less degradative mRNA extraction procedures as well as the use of other cell-free protein synthesizing systems,[2] e.g., Ascites-Krebs II preparation (Collaboration with Work Unit No. 834 01 009).

Recently Riley and Rafter[3] described the activation of ATPase by LEM. Because of this we have initiated similar studies to evaluate the possibility that this system could be used as an in vitro assay for LEM. I found that crude LEM enhanced ATPase activity. However, heating LEM to 90C for 30 min failed to destroy the ATPase activating capacity of LEM in 3 of 4 experiments even though the typical biological properties of LEM were lost. The original report noted that heating LEM caused a 50% loss in activity. The cause for the observed variability and differences observed between the original report and work done here is unknown.

Presentations:

1. Abeles, F. B. Ethylene: plant hormone and urban air pollutant. Presented, Annual Meeting, American Chemical Society, Atlantic City, N. J. 24 July 1974.

2. Abeles, F. B. Chemistry of ethylene and its fate in soils. Presented, American Society of Agronomy, Chicago, Ill., 11 November 1974.

Publications:

None.

LITERATURE CITED

1. Koj, A. 1974. Acute-phase reactants. Their synthesis, turnover and biological significance. p. 73-132. In Structure and Function of Plasma Proteins (A. C. Allison, ed.) Plenum Press, New York.

2. Moldave, K., and L. Grossman (ed.) 1974. Section V. Messenger RNA and protein synthesizing systems. In Methods in Enzymology, Vol 30F, p. 605-761.

3. Riley, R. S., and G. W. Rafter. 1975. Activation of rabbit brain microsomal ($Na^+ + K^+$)-dependent ATPase by a leukocytic product. Biochim. Biophys. Acta 381:120-127.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6412	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. SUM'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 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	62760A	3A762760A834	01	022
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Effect of infectious disease on gluconeogenesis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

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: 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 75	1.0	157.4
FISCAL YEAR CURRENT 76	1.0	177.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Physical Sciences Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Neufeld, H. A.

TELEPHONE: 301 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Gluconeogenesis; (U) Nicotinamide adenine dinucleotides; (U) Lactate; (U) Pyruvate; (U) Acetoacetic acid; (U) Beta-hydroxybutyric acid; (U) Military medicine

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

23 (U) Study the effect of infection on the process of gluconeogenesis. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Microanalytical methods for the study of metabolites involved with computations used to estimate the direction of the gluconeogenic process.

25 (U) 74 07 - 75 06 - Using the concentration of lactate and pyruvate the ratio of NAD to NADH in the cytoplasm has been studied; the same ratio in mitochrondria has been determined by the precise determinations of acetoacetate and beta-hydroxybutyric acid. In only pneumococcal infection did there appear to be any alteration of this cytoplasmic ratio. Pneumococci caused a lowering of the ratio, indicating that gluconeogenesis, in its cytoplasmic compartment, was not inhibited. All infections, but not endotoxin, caused an inhibition of the marked rise in the mitochondrial concentrations of acetoacetate and beta-hydroxybutyrate which is found in fasting monkeys. This alteration resulted in an increase in the NAD:NADH ratio in the mitochondria suggesting a deficiency of NADH. These metabolic alterations can lead to at least two speculative proposals for explaining the phenomenon: (1) the lack of NADH in the mitochondria might explain the inhibition of gluconeogenesis observed by others and (2) the failure of acetoacetate and beta-hydroxybutyrate to increase in fasted infected animals might be due to an impairment in the sick animal of its ability to utilize fatty acids properly.

Publication: Experientia 31:391-392, 1975

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 022: Effect of infectious disease on gluconeogenesis

Background:

In unpublished results Curnow, from this laboratory, found that livers from rats infected with Streptococcus pneumoniae or Salmonella typhimurium demonstrated a marked derangement in gluconeogenesis when precursors, such as pyruvate or alanine, were used in liver perfusion experiments. It was theorized by him that this impairment of gluconeogenesis during infection was the result of a deficiency of hepatic reducing equivalents. It has been the purpose of this study to attempt to explain this apparent impairment of gluconeogenesis during infection by means of biochemical analysis of intracellular metabolites.

Progress:

Since the gluconeogenic process is governed to a large extent by the ratio of NAD^+:NADH in both the cytoplasmic and mitochondrial cell compartments, assays were set up for the determinations of lactate, pyruvate, acetoacetate, and β-hydroxybutyric acid. Since the stability of the system requires that the liver be isolated in $\leq$ 10 sec by means of quick freezing in liquid nitrogen, it is not possible to make direct determinations of NAD^+ or NADH in either the cytoplasm or mitochondria. By the judicious choice of enzyme systems, it is possible to measure this ratio in the cytoplasmic and mitochondrial compartments. A reduction in the magnitude of the ratio would signify an abundance of NADH or reducing equivalents and an increase in the ratio would indicate a lack or a reduction of reducing equivalents. The use of the lactic dehydrogenase system permits determination of the cytoplasmic NAD^+:NADH ratio and the use of the β-hydroxybutyric dehydrogenase system permits the determination of the ratio in mitochondria. It is important to note that the cell does not permit the passage of NAD^+ or NADH from cytoplasm to mitochondria or the reverse.

The first step in this investigation was to adapt assays obtained from the literature so that they could be reliably and conveniently handled in our laboratory. Assays for lactate, pyruvate, total NAD^+, total NADH, acetoacetate, and β-hydroxybutyrate were adapted and revised where needed.

The general procedure for studying the response of these parameters to stress was the following: (1) fasted, fed, or infected rats were killed by cervical dislocation and as much of the liver as possible was excised and quickly placed between aluminum blocks which had been previously cooled in liquid nitrogen. The frozen tissue were stored at -70C until required for assay.

The effect of the following infections have been studied: S. pneumoniae, S. typhimurium and Francisella tularensis as well as intoxication with endotoxin. The organisms were selected so that the effects of a gram positive infection (S. typhimurium) could be compared with infection by a gram negative organism containing endotoxin (S. typhimurium) and a gram negative organism which contains no endotoxin (F. tularensis).

Table I shows the effect of the various treatments on the cytoplasmic NAD^+:NADH ratios. It seems that only in the case of S. pneumoniae can any effect be noted. The lowering of the ratio points to an increase in cytoplasmic NADH, a fact which suggests sufficient reducing equivalents to maintain the process of gluconeogenesis.

TABLE I. EFFECT OF INFECTION ON THE CYTOPLASMIC NAD^+:NADH RATIO OF RATS

GROUP[a] (n)	HOUR RATS KILLED	RATIO NAD^+:NADH ± SE[b]
Controls		
Fed (37)	48	335 ± 33
48-hr fast (15)	48	255 ± 16
S. pneumoniae		
Control (12)	48	190 ± 35
Live (14)	48	107 ± 14
F. tularensis, LVS		
Control (12)	24	305 ± 79
Live (12)	24	341 ± 61
S. typhimurium		
Control (8)	48	420 ± 80
Live (14)	48	339 ± 60
Endotoxin		
Saline (4)	24	398 ± 82
Toxin, 5 mg (3)	24	226 ± 69

a. Bacterial controls are heat-killed.

b. No significant difference at $P < 0.001$.

Of greater interest is the observation on the effect of infection on the concentration of acetoacetate and β-hydroxybutyrate in the mitochondria (Table II).

TABLE II. EFFECT OF INFECTION ON THE CONCENTRATION OF ACETOACETATE AND β-HYDROXYBUTYRATE AND ON THE MITOCHONDRIAL NAD^+:NADH OF RATS

GROUP[a]	mg/gm LIVER (WET WT.) ± SE (n)		RATIO ± SE
	Acetoacetate	β-hydroxybutyrate	NAD^+:NADH
Controls			
Fed	83 ± 8 (37)	188 ± 21* (39)	3.62 ± 0.40
24-hr fast	134 ± 10* (10)	739 ± 36* (10)	3.06 ± 0.53
48-hr fast	196 ± 12* (15)	1,170 ± 80* (15)	3.60 ± 0.36
S. pneumoniae			
Control-24	210 ± 20* (11)	810 ± 136* (11)	8.58 ± 2.45
Live-24	137 ± 17* (20)	385 ± 38* (21)	9.60 ± 2.47
Control-48	261 ± 26* (12)	1,067 ± 81* (12)	5.15 ± 0.55*
Live-48	131 ± 22* (14)	338 ± 55* (20)	11.01 ± 1.81*
F. tularensis			
Control-24	204 ± 32* (12)	896 ± 72* (12)	6.98 ± 0.07*
Live-24	155 ± 17* (12)	309 ± 58* (13)	13.17 ± 1.90*
S. typhimurium			
Control-24	233 ± 17 (7)	457 ± 113 (7)	14.03 ± 3.57
Live-24	136 ± 24 (11)	357 ± 75 (11)	11.20 ± 1.52
Control-48	266 ± 8* (8)	999 ± 47* (8)	5.37 ± 0.28*
Live-48	129 ± 14* (14)	312 ± 54* (14)	8.53 ± 1.05*
Endotoxin, 5 mg			
Saline-6	182 ± 7 (10)	309 ± 45 (10)	15.24 ± 2.75
Toxin-6	190 ± 5 (13)	387 ± 52 (13)	12.66 ± 1.81
Saline-12	281 ± 37 (4)	582 ± 68 (4)	10.51 ± 2.37
Toxin-12	235 ± 13 (4)	423 ± 88 (4)	12.52 ± 1.68
Saline-24	236 ± 24 (4)	956 ± 97 (5)	4.65 ± 0.67
Toxin-24	201 ± 15 (3)	662 ± 34 (3)	6.19 ± 0.50

* $P < 0.001$ compared to own control.

a. Bacterial controls are heat-killed. Rats killed at 6, 12, 24 or 48 hr.

In the normal rat subjected to the stress of fasting both the liver and the blood show large increases in the concentrations of acetoacetate and β-hydroxybutyric acid. These increases are due to the fact that fasting in normal rats causes lipid mobilization and a high influx of fatty acids to the liver for oxidation. A by product of this is the accumulation of acetoacetate and β-hydroxybutyric acid which spills out into plasma for use by peripheral tissues.

The significant data contained in Table II points to the fact that in every instance livers from infected rats fail to show the normal fast-induced increase in acetoacetate and β-hydroxybutyric acid. Each of the infections studied, S. pneumoniae, S. typhimurium, which contains endotoxin, and LVS strain of F. tularensis which lacks endotoxin demonstrated a similar response. Of equal interest is the fact that livers from rats subjected to endotoxemia tended to show a normal response with respect to concentrations of aceto-acetate and β-hydroxybutyric acid.

Another significant observation is the fact that in livers from infected rats the NAD^+:NADH ratio in the mitochondria showed marked increases pointing to a deficiency in mitochondrial NADH.

The implication of the observations concerning the failure of aceto-acetate and β-hydroxybutyrate to be produced in the liver of fasting, infected rats is that there is some impairment in fatty acid metabolism which is directly attributable to the presence of an infectious organism. The data so far obtained have not permitted any valid speculations as to metabolic mechanisms.

Concurrently with the work reported done on the effect of infection on whole rats, similar studies are in progress on perfused livers. The study of the perfused liver from the view point of this research plan is important because of Dr. Curnow's observations concerning the possible impairment of gluconeogenesis. In a limited number of experiments involving liver from rats infected with S. pneumoniae and perfused with pyruvate the following has been observed: the level of acetoacetate and β-hydroxybutyrate are depressed as compared to livers from rats injected with heat-treated organisms. In addition the mitochondrial NAD^+:NADH ratio is increased suggesting a deficiency of reduced NADH in the mitochondria. These observations tend to confirm the effects noted in livers from whole rats.

Publications:

Neufeld, H. A., R. D. Towner, and J. Pace. 1975. A rapid method for determining ATP by the firefly luciferin-luciferase system. Experientia 31:391-392.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6411	75 07 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
74 07 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	62760A	3A762760A834	01	109
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Respiratory disease mechanisms and pathogenesis in airborne infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 02	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

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING 75	1.0	95.7
CURRENT 76	1.0	173.8

19a. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

19b. PERFORMING ORGANIZATION

NAME: Aerobiology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Larson, E. W.

TELEPHONE: 301 663-2439

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Dominik, J. W.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Bacterial disease; (U) Viral disease; (U) Aerosols; (U) Respiratory physiology; (U) Influenza virus

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

23 (U) Examine respiratory disease mechanisms, including penetration, retention, clearance, and replication, of airborne organisms in a suitable host system. This is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Challenge experimental animal hosts with infectious microorganisms by 3 primary methods. Assess effects postexposure by quantitation from segments of the respiratory tract and peripheral organs.

25 (U) 74 07 - 75 06 - Two preparations of D/Sendai/52 virus were compared on the basis of their ability to replicate in eggs, virion concentration and morphology, lethal dose response characteristics in mice, and the virus population dynamics in mouse respiratory tissues. While the unadapted virus replicated to high concentrations in embryonated eggs as measured by both electron microscopy and egg infectivity, it lacked morphological integrity and was not lethal for mice challenged by the respiratory route. The adapted virus did not replicate as well in embryonated eggs; however, the virus possessed morphological integrity and produced lethal infections in mice. Virus populations were measured in respiratory tissues following small particle aerosol challenge with both unadapted and adapted virus, both with and without antiviral therapy.

Publication: Appl. Microbiol. 28:929-934, 1974.
In: Developments in Industrial Microbiology, Vol. 15, p. 33-37, 1974.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infections of Military Importance

Work Unit No. 834 01 109: Respiratory Disease Mechanisms and Pathogenesis in Airborne Infections

Background:

The continuing objective of the research under this work unit is to investigate the pathogenesis of respiratory infections including the penetration and deposition properties of airborne microorganisms in the respiratory tract of experimental animals and the characterization of microbial population dynamics in respiratory tissues during infection. The working hypothesis is that the mathematical characteristics of the microbial populations might explain some of the fundamental relationships between the host and infecting microorganisms.

Previous reports presented the results of technology development studies[1,2] and definitive investigations of influenza virus dynamics in the respiratory tissues of experimentally infected mice.[3] In this report period, parainfluenza type 1, Sendai, virus in the mouse was developed as a second model of respiratory viral infection. Efforts were directed to the characterization of the virus, enhancement of mouse virulence, and initial studies on Sendai virus population dynamics in mouse respiratory tissues.

Progress:

Two primary virus suspensions were developed from a Sendai virus seed, strain D/Sendai/52, acquired from the American Type Culture Collection (ATCC). The first preparation, referred to as unadapted virus, was prepared as an allantoic fluid harvest of embryonated eggs inoculated with the original seed. The second suspension, referred to as mouse-adapted virus, was also prepared as allantoic fluid harvest, but the eggs were inoculated with infected mouse lung homogenates after 10 passages.

Screening studies among Swiss outbred and various strains of inbred mice indicated that the AKR inbred mouse gave uniform responses when challenged with these Sendai virus suspensions by the respiratory route. Table I contrasts the unadapted and mouse-adapted virus preparations on the basis of virus concentrations as measured by EID_{50}, and AKR mouse respiratory dose responses when challenged by IN instillation and both small- (SPA) and large- (LPA) particle aerosols.

It can be seen that the mouse-adapted virus is markedly less infectious for embryonated eggs than the unadapted virus. The latter virus was not lethal

TABLE I. CHARACTERISTICS OF UNADAPTED AND MOUSE-ADAPTED D/SENDAI/52 VIRUS

CHARACTERISTIC	LOG_{10}	
	Unadapted	Mouse-adapted
Titer (EID_{50}/ml)	8.7	5.0
Mouse Resp. LD_{50} (EID_{50})		
IN	>5.0	0.26
SPA	>5.0	0.46
LPA	>2.4	>0.38

for AKR mice at the highest aerosol doses achievable or at $10^{5.0}$ EID_{50} by IN instillation. The mouse-adapted virus, however, was uniformly lethal for AKR mice at low doses when the virus was deposited mainly in the lungs either by IN or SPA challenge. Lethalities were not produced with LPA, upper respiratory tract challenges.

Electron microscope studies were performed by Virology Division (CPT Gangemi) to compare the virus suspensions on the basis of virus particle concentrations and morphologies. A virion density of approximately 10^{10}/ml in the unadapted virus preparation was 10-fold the virion concentration in the preparation of adapted virus. The contrast of these measurements with the egg infectivity titrations suggested that 10^4 virus particles represented 1 EID_{50} with mouse-adapted virus while $10^{1.3}$ virus particles represented 1 EID_{50} for the unadapted virus. The 2 preparations had differing virus particle morphologies. Numerous thread-like structures were seen to radiate from the virus particles of unadapted virus; these structures were not present in the mouse-adapted material. Based on the observations of others,[4] the thread-like structures are considered indicative of nucleocapsid leakage from the virus particles, suggestive of a lack of envelope integrity. The possible importance of these observations to the pathogenesis of Sendai virus in the mouse model is not presently known.

Studies were performed to investigate Sendai virus population dynamics in the respiratory tissues of AKR mice as a function of time after respiratory challenge. In 3 studies mice were challenged with uniformly lethal doses of mouse-adapted virus as SPA to deposit the virus primarily in the lung. In 2 of these studies, separate groups of the infected mice were placed on continuous aerosol therapy with ribavirin, an antiviral compound, starting 24 hr after the virus challenge and continuing through 72 hr. In another experiment, AKR mice were exposed to a high dose ($10^{5.4}$ EID_{50}) of unadapted virus, also as SPA. In each experiment, groups of 3 mice from each treatment group were withdrawn periodically from their holding cages and necropsied for assays of the virus concentrations in the lungs, trachea, and nasopharynx of each mouse.

Table II presents the mean virus concentrations in the lungs of mice as a function of time after challenge.

TABLE II. EID_{50} OF SENDAI VIRUS IN WHOLE LUNGS OF MICE FOLLOWING SPA CHALLENGE

HR POST-CHALLENGE	LOG_{10} EID_{50}		
	Unadapted Virus	Adapted Virus	
		No Therapy	Ribavirin Therapy
0	2.06	≤0.98	≤0.98
6	0.86	≤0.98	≤0.98
24	4.60	1.97	1.86
48	5.00	2.45	2.17
72	5.79	4.15	2.64
96	5.39	5.76	4.71
144	4.94	5.66	4.78
192	2.92	5.29	3.83
240	1.36	a	≤0.98
Pooled SE	0.19	0.25	0.33

a. No surviving mice to assay.

With unadapted virus, the mouse lungs contained measurable quantities of virus immediately after exposure. Exponential virus replication commenced by 6 hr and high concentrations were observed by 24 hr. Peak virus levels equal to those seen with lethal mouse-adapted virus were reached by 72 hr. Virus disappearance commenced by 96 hr and virus levels in the lungs returned to near baseline by 240 hr. With the mouse-adapted challenge, lung virus concentrations were below detectable levels both immediately after challenge and at 6 hr. The 24-hr samples contained the first measurable virus. Without therapy, virus concentrations in the lungs increased exponentially through 96 hr and remained level or decreased slightly through 144 and 192 hr. In the absence of therapy, mice failed to survive beyond 192 hr.

Lung virus populations developed later and exponencial growth was slower in mice treated with ribavirin when compared to untreated mice. Peak concentrations in treated mice were reached at approximately the same time but were a log less than in untreated mice.

Lung virus concentrations alone did not account for the severity of the Sendai infections. Despite relatively high lung virus titers, the unadapted virus did not produce extensive lung consolidation. Minimal consolidation

amounting to approximately 25% of the total lungs was seen in the mice necropsied at 192 and 240 hr after challenge. With adapted virus in untreated mice, however, nearly total lung consolidation was seen by as early as 144 hr. In ribavirin-treated mice, no consolidation was observed through 144 hr, but lungs showed approximately 75% consolidation at both 192 and 240 hr.

Tables III and IV present the mean virus titers measured in the trachea and nasopharyngeal tissues. The patterns of virus recoveries in these tissues were similar to those observed in the lungs. With adapted virus, however, in both untreated and ribavirin treated mice, the peak virus titers were lower than in the lungs. In contrast, peak virus titers in these tissues with unadapted virus were markedly higher than with adapted virus and equal to those seen in the lungs.

TABLE III. EID_{50} OF SENDAI VIRUS IN TRACHEA OF MICE FOLLOWING SPA CHALLENGE

HR POST-CHALLENGE	LOG_{10} EID_{50}		
	Unadapted Virus	Adapted Virus	
		No Therapy	Ribavirin Therapy
0	1.64	$\leq$0.98	$\leq$0.98
6	$\leq$0.80	$\leq$0.98	$\leq$0.98
24	3.89	1.27	1.25
48	4.96	2.30	1.49
72	5.47	2.98	1.81
96	5.37	2.40	3.11
144	4.41	3.38	2.71
192	3.02	2.30	2.13
240	1.19	a	$\leq$0.98
Pooled SE	0.38	0.27	0.28

a. No surviving mice to assay.

TABLE IV. EID_{50} OF SENDAI VIRUS IN THE NASOPHARYNX OF MICE FOLLOWING SPA CHALLENGE

HR POST-CHALLENGE	LOG_{10} EID_{50}		
	Unadapted Virus	Adapted Virus	
		No Therapy	Ribavirin Therapy
0	2.32	≤0.98	≤0.98
6	1.36	≤0.98	≤0.98
24	4.66	1.05	1.03
48	4.68	1.77	1.48
72	6.02	2.95	1.69
96	6.08	4.26	3.32
144	5.74	3.45	3.40
192	5.72	3.10	2.41
240	1.83	a	≤0.98
Pooled SE	0.39	0.24	0.35

a. No surviving mice to assay.

Mice which were programmed to study survival and time-to-death properties in these experiments yielded the results which are summarized in Table V.

TABLE V. MOUSE SURVIVAL AND TIME-TO-DEATH PROPERTIES FOLLOWING SPA CHALLENGE WITH SENDAI VIRUS

CHALLENGE TREATMENT	NO. SURVIVING/TOTAL (%)	MTD[a] days
Unadapted virus and untreated	13/13 (100)	-
Adapted virus and untreated	0/88 (0)	7.9
Adapted virus and treated[b]	33/63 (52)	9.6

a. Mean time-to-death.
b. Continuous aerosol therapy with ribavirin from 24 through 72 hr after virus challenge.

In contrast to untreated groups, 52% of mice challenged with adapted virus and treated with ribavirin survived and the mean time-to-death among those that died was 9.6 days. Hence, treatment with ribavirin not only dramatically improved survival, but death was delayed among those that died.

These data provide a base of information on which to initiate mathematical studies in developing expressions to identify and delineate host and virus relationships and mechanisms, and have been submitted to Computer Science Office for modeling studies. Of particular interest will be any differences between the models required to describe the virus population dynamics of unadapted as opposed to adapted virus. Experimentation in the immediate future will be focused on the sequential histopathological changes that occur in mouse lungs following infection with both unadapted and adapted virus. In comparison studies, attempts will be made to quantitate sequentially intracellular virus replication by immunofluorescence assays of thin sections of infected lungs. As additional experimental variables, it may be worthwhile to further examine the nasopharynx as the site of initial virus deposition as well as attempt to further alter the infections both prophylactically, such as by immunization, and therapeutically, and by extending ribavirin treatment beyond 72 hr.

Presentations:

1. Larson, E. W., J. W. Dominik, and A. H. Rowberg. Virus population dynamics and their mathematical form in influenza infections of mice following respiratory exposure. Presented, 14th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., 11-13 Sep 74.

2. Larson, E. W. Experimental aerobiology and the epidemiological evidence for airborne transmission of respiratory infections. Guest lecture presentation, Maryland Branch, American Society for Microbiology, Towson, Md., 30 Oct 74.

Publications:

1. Young, H. W., E. W. Larson, and J. W. Dominik. 1974. Modified spinning top homogeneous spray apparatus for use in experimental respiratory disease studies. Appl. Microbiol. 28:929-934.

LITERATURE CITED

1. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1973. Annual Progress Report, FY 1973, pp. 97-101. Fort Detrick, Md.

2. Young, H. W., E. W. Larson, and J. W. Dominik. 1974. Modified spinning top homogeneous spray apparatus for use in experimental respiratory disease studies. Appl. Microbiol. 28:929-934.

3. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974, pp. 93-102. Fort Detrick, Md.

4. Kingsbury, D. W., A. Portner, and R. W. Darlington. 1970. Properties of incomplete Sendai virions and subgenomic viral RNAs. Virology 42:857-871.

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
74 07 15	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
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19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Animal Assessment Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Liu, C.T.
TELEPHONE: 301 662-2769
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

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22. KEYWORDS (Precede each with Security Classification Code)
(U) Renal function; (U) Staphylococcal enterotoxin B (SEB); (U) Body fluids; (U) Hepatic responses; (U) Cardiovascular responses; (U) Respiratory response

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

23 (U) Study renal function and fluid compartment dynamics in rhesus monkeys. This is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Initially, examine SEB distribution and clearance, expanding on previous studies at this Institute.

25 (U) 74 07 - 75 06 - Blood volume and total body water decreased 5 hr post-SEB (1 mg/kg). When SEB was given IV at 1.0 or 0.05 mg/kg, the following responses were observed within 5-6 hr: (1) glomerular filtration rate, effective renal plasma flow, total renal blood flow, osmolal clearance, tubular concentration of solute-free water, and tubular maximum secretion of para-aminohippurate decreased. Renal resistance, arteriorenal venous oxygen difference and extraction ratio in the kidney increased; (2) despite unchanged blood pressure, cardiac output, stroke volume, and cardiac work decreased. Tachycardia and vasoconstriction were present; (3) tidal volume and respiratory rate increased slightly without change in functional residual capacity and oxygen consumption; and (4) hepatic secretory function was impaired, as evidenced by a decreased disappearance rate of injected cardiogreen. Oral SEB (1 mg/kg) failed to show any cardiorenal changes in the presence of gut toxicity. Dibenzyline, isoproterenol and plasma albumin prolonged the survival time in SEB shock.

Publication: Fed. Proc. 34:225, 1975 (abstr.).

*Available to contractors upon originator's approval.

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 110: Mechanisms of Renal Responses during Toxemias and Infectious Diseases

Background:

It has been well documented that IV injection of as little as 25 μg/kg of SEB produces shock and death in monkeys. When 1 mg/kg SEB is administered orally, only vomition and occasional diarrhea occur. Rapoport et al.[1] observed that 80-90% of IV injected SEB disappeared rapidly from the circulation within 30 min. By using the radioiodinated SEB, Morris et al.[2] were able to demonstrate the percent distribution of the injected SEB in various organs in the monkey. Renal cortex and liver were found to be the major sites for SEB accumulation (30-45%). The heart contained only 0.5% of the injected SEB. It was unknown whether there was any correlation between the amount of SEB accumulation in each organ and its toxicity.

The clinical signs were described as decreases in aortic flow, mean stroke volume, central venous pressure, mean blood pressure and F cell ratio. No significant changes were observed in the heart rate and total peripheral resistance.[3,4] Elsberry, et al.[3] and Beisel[5] proposed a theory that peripheral pooling of blood in the capillary bed is the main reason for producing SEB shock in monkeys.

The purpose of this investigation was to study early renal, cardiovascular, respiratory, and hepatic responses to IV or oral administrations of SEB for a period of 5-6 hr. Changes in body fluid compartments following IV SEB were also determined. Based upon the evidence of physiological changes post-SEB, a hypothesis for the development of SEB shock was proposed. Further, pharmacological approaches for the treatment of SEB-induced shock were initiated.

Progress:

Immediately following the IV injection of SEB (1 mg/kg), there were no significant changes in all body fluid compartments except for a decrease in total body water. Four monkeys receiving IV SEB at the same dose demonstrated 5 hr post-SEB marked decreases in plasma volume, blood volume, true circulatory blood volume, and total body water. The intracellular water remained constant, and only a trend toward decreases in RBC volume, extracellular water, interstitial water, and F cell ratio was found in the SEB monkeys (Table I).

TABLE I. EFFECT OF IV SEB (1 mg/kg) ON BODY FLUID COMPARTMENTS IN RHESUS MONKEYS (MEAN ± SE)

PARAMETER (cc/kg)	CONTROL (n = 17)	SEB	
		Immediate (n = 5)	5 hr (n = 4)
Plasma volume	49.2 ± 1.5	51.7 ± 3.9	40.5 ± 1.1*
Blood volume	60.7 ± 1.8	62.6 ± 3.7	50.8 ± 5.0*
RBC volume	24.2 ± 1.2	23.2 ± 0.97	21.3 ± 2.3
True circulatory blood volume	74.1 ± 2.3	74.8 ± 4.3	60.8 ± 2.7*
Circulatory hematocrit (%)	31.9 ± 0.87	31.2 ± 1.7	34.2 ± 2.0
Hematocrit x 0.96 (%)	30.1 ± 1.4	30.5 ± 2.6	34.6 ± 0.88
F cell ratio	0.91 ± 0.01	0.89 ± 0.02	0.86 ± 0.05
Total body water	653 ± 10.7	600 ± 9.6*	507 ± 29.1*
Extracellular water	266 ± 11.0	289 ± 6.7	218 ± 36.1
Intracellular water	331 ± 25.1	314 ± 3.2	338 ± 9.2
Interstitial water	218 ± 10.6	237 ± 3.6	176 ± 36.4

*$P < 0.05$ compared to control.

No changes in renal functions were observed after oral SEB at a high dose of 1 mg/kg (Table II). The results indicate that little or no SEB could be transported into the general circulation from the gastrointestinal tract. It is highly possible that SEB molecules could get into the portal circulation, but the absorbed enterotoxins may be removed or metabolized by the liver.

Although only the high dose of SEB (1 mg/kg) produced hypotension (90 mm Hg) and the low dose of SEB (0.05 mg/kg) maintained a relatively constant mean blood pressure (BP) (115 mm Hg) within 5 hr post-SEB, both doses depressed cardiac output and various renal functions (Table II). Glomerular filtration rate (GFR), effective renal plasma flow (ERPF), total renal blood flow (TRBF), osmolal clearance (C_{osm}), tubular concentration of solute-free water (T^CH_2O), urine flow, and tubular maximum secretion of para-aminohippurate (TmPAH) were decreased. However, renal resistance and plasma osmolality (P_{osm}) were increased. Extraction ratio of PAH (E_{PAH}), filtration fraction (FF), tubular maximum reabsorption of glucose (TmG), arteriorenal venous (A-RV) differences of plasma glucose, protein and osmolality across the kidney, as well as % renal reabsorption of Na^+, Cl^- and HCO_3^- did not show any

TABLE 11. EFFECTS OF SEB ON RENAL FUNCTIONS IN RHESUS MONKEYS

PARAMETER	SEB GROUP	(n)	DOSE OF SEB (mg/kg)	CONTROL	MEAN + SE BY HOURS POST-SEB 3	4	5
GFR cc/min/kg	Control	(39)	0	3.2 ± 0.1	3.2 ± 0.1	3.4 ± 0.2	3.2 ± 0.1
	Oral	(13)	1.0	2.8 ± 0.3	3.3 ± 0.1*	3.5 ± 0.3*	3.6 ± 0.4*
	IV	(22)	1.0	3.3 ± 0.2	2.2 ± 0.2*	2.2 ± 0.1*	2.2 ± 0.2*
	IV	(26)	0.05	3.4 ± 0.2	2.6 ± 0.2	2.4 ± 0.1	2.3 ± 0.2
ERPF cc/min/kg	Control	(8)	0	29 ± 2	28 ± 2*	27 ± 2*	25 ± 4*
	IV	(9)	1.0	31 ± 2	19 ± 1*	18 ± 1*	17 ± 1*
	IV	(8)	0.05	29 ± 3	18 ± 3	17 ± 2	17 ± 3
TRBF cc/min/kg	Control	(8)	0	50 ± 3	41 ± 2*	40 ± 3	39 ± 6
	IV	(9)	1.0	50 ± 4	31 ± 2*	30 ± 1*	28 ± 2*
	IV	(8)	0.05	50 ± 7	28 ± 4	26 ± 3	27 ± 4
TmG mg/min/kg	Control	(18)	0	5.5 ± 0.6	7.2 ± 0.7	7.0 ± 0.7	6.9 ± 0.8
	Oral	(5)	1.0	6.0 ± 0.5	6.6 ± 2.4	9.5 ± 1.6	6.0 ± 0.9
	IV	(7)	1.0	7.4 ± 1.6	6.5 ± 0.8	5.4 ± 1.6	9.1 ± 0.9
	IV	(8)	0.05	5.7 ± 0.7	6.7 ± 0.8	6.2 ± 1.0	8.9 ± 1.2
TmPAH mg/min/kg	Control	(12)	0	4.4 ± 0.2	4.3 ± 0.2	4.3 ± 0.2	4.3 ± 0.2
	Oral	(6)	1.0	4.8 ± 0.5	4.4 ± 0.7*	4.7 ± 0.4	4.9 ± 0.6*
	IV	(7)	1.0	3.9 ± 0.1	2.9 ± 0.4*	3.2 ± 0.2	3.1 ± 0.3*
	IV	(8)	0.05	4.2 ± 0.3	3.9 ± 0.2	3.6 ± 0.2	3.4 ± 0.2
C_{osm} cc/min/kg	Control	(39)	0	0.37 ± 0.01	0.37 ± 0.01	0.37 ± 0.01	0.37 ± 0.02
	Oral	(12)	1.0	0.38 ± 0.02	0.38 ± 0.02	0.39 ± 0.02*	0.39 ± 0.02*
	IV	(23)	1.0	0.38 ± 0.01	0.29 ± 0.02*	0.31 ± 0.01*	0.31 ± 0.02*
	IV	(26)	0.05	0.37 ± 0.20	0.31 ± 0.02	0.28 ± 0.02	0.30 ± 0.02
$T^{C}H_2O$ cc/min/kg	Control	(39)	0	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.15 ± 0.01
	Oral	(12)	1.0	0.15 ± 0.01	0.16 ± 0.01*	0.16 ± 0.01*	0.15 ± 0.02*
	IV	(22)	1.0	0.14 ± 0.01	0.07 ± 0.01*	0.08 ± 0.01*	0.08 ± 0.01*
	IV	(26)	0.005	0.13 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01

Mean BP pressure mm Hg	Control	(38)	0	121 ± 3	116 ± 2	116 ± 3	116 ± 3
	Oral	(11)	1.0	119 ± 4	117 ± 5	116 ± 6	118 ± 5
	IV	(22)	1.0	118 ± 3	99 ± 4*	97 ± 4*	94 ± 4*
	IV	(14)	0.05	131 ± 2	125 ± 2	120 ± 5	116 ± 3*
Urine flow cc/min	Control	(39)	0	0.89 ± 0.05	0.87 ± 0.05	0.90 ± 0.05	0.86 ± 0.05
	Oral	(13)	1.0	0.85 ± 0.05	0.81 ± 0.06*	0.82 ± 0.05	0.89 ± 0.05
	IV	(23)	1.0	0.97 ± 0.07	0.82 ± 0.06*	0.89 ± 0.07*	0.88 ± 0.07*
	IV	(26)	0.05	0.91 ± 0.06	0.79 ± 0.04*	0.75 ± 0.04*	0.71 ± 0.04*
P_{osm} mosm	Control	(39)	0	310 ± 2	311 ± 2	311 ± 3	312 ± 3
	Oral	(12)	1.0	312 ± 2	312 ± 3*	314 ± 3*	315 ± 4*
	IV	(23)	1.0	312 ± 2	320 ± 3*	326 ± 5*	328 ± 6*
	IV	(26)	0.05	310 ± 2	317 ± 3*	319 ± 3*	320 ± 3*
U_{osm} mosm	Control	(39)	0	504 ± 13	500 ± 12	497 ± 13	512 ± 13
	Oral	(12)	1.0	524 ± 25	540 ± 14*	540 ± 12	514 ± 24
	IV	(23)	1.0	499 ± 20	436 ± 11*	451 ± 13	458 ± 14
	IV	(26)	0.05	486 ± 16	464 ± 11	481 ± 10	483 ± 9
Renal resistance dyne-sec/ cm^5 (x 10^5)	Control	(6)	0	0.55 ± 0.03	0.64 ± 0.03	0.65 ± 0.04	0.66 ± 0.06
	IV	(7)	1.0	0.57 ± 0.03	0.68 ± 0.11	0.78 ± 0.04	0.80 ± 0.03
	IV	(8)	0.05	0.54 ± 0.07	0.73 ± 0.13	0.78 ± 0.14	0.81 ± 0.14
Cardiac output[a] L/min/kg	Control	(10)	0	0.28 ± 0.01			0.27 ± 0.02*
	IV	(15)	1.0	0.28 ± 0.02			0.20 ± 0.02*
	IV	(11)	0.05	0.22 ± 0.02			0.17 ± 0.01
Renal fractions %	Control	(3)	0	17 ± 1			15 ± 2
	IV	(8)	1.0	15 ± 2			10 ± 1*
	IV	(7)	0.05	23 ± 9			14 ± 1
E_{PAH}	Control	(7)	0	93 ± 1	94 ± 1	95 ± 1	94 ± 1
	IV	(9)	1.0	94 ± 1	89 ± 3	84 ± 4	86 ± 5
	IV	(8)	0.05	89 ± 4	88 ± 4	90 ± 2	90 ± 3

* $P < 0.05$.

a. Cardiac output was only measured prior to and 5 hr after SEB administration.

significant changes.

Because renal GFR, hemodynamics, tubular secretory functions and capacities in urine concentration were all depressed in rhesus monkeys receiving the low dose of IV SEB (0.05 mg/kg), it is clear that the SEB-induced renal impairment can be produced independently and not necessarily as a secondary response to circulatory collapse.

Renal metabolism was studied in a separate group of 8 monkeys injected with 0.05 mg/kg of SEB for a period of 5 hr. Increases in A-RV O_2 difference and the O_2 extraction ratio in the kidney were observed. Renal O_2 consumption and respiratory quotient (RQ) were not significantly altered. There was only a transient increase in renal total CO_2 output 3 hr post-SEB.

Certain biochemical changes in the kidney (cortex, outer medulla and inner medulla) were analyzed 7 hr following IV SEB (1 mg/kg). The following were found: (1) Na^+ concentrations were doubled with little or no change of K^+ contents; (2) total lipids increased in the cortex and outer medulla, but decreased in the inner medulla; and (3) water and solute contents remained constant.

These observations may be interpreted as follows: (1) the universal phenomenon of an increased concentration gradient of Na^+ from cortex to inner medulla was also found in the kidney of rhesus monkeys; (2) the accumulation of Na^+ in the renal tissue post-SEB suggests that the integrity of renal tubular cells was modified; (3) the mechanism of a decrease in urine-concentrating power following IV SEB is not due to a disturbance of Na^+ concentration gradient. The decrease in antidiuretic hormone (ADH) release from the hypothalamus and neurohypophysis might be one of the possible mechanisms for the renal defects in urine concentration post-SEB; and (4) the changes in total lipid contents of the kidney after SEB may reflect changes of renin and prostaglandins (lipid) activities. Based on this indirect evidence, renin activity may be increased and prostaglandins content may be decreased.

When experiments were carried out in rhesus monkeys without appreciable blood loss or surgical trauma, the mean BP and transit time (femoral vein to femoral artery) did not show any significant changes within 6 hr post-SEB (Table III). However, tachycardia, vasoconstriction and cardiac depression (including decreases in cardiac output, stroke volume, cardiac work, and mean cardiac power) were observed in a few hours following IV SEB (1 mg/kg). Oral SEB (1 mg/kg) did not show any changes on the measured cardiovascular parameter.

Impairment of hepatic function was observed in monkeys receiving oral or IV SEB (1 mg/kg) as shown by a prolonged half-life of cardiogreen in the plasma, and slower elimination of the injected dye by the liver. However, the degree of hepatic depression was less severe after oral SEB as compared to IV injection at the same dose level. These results from oral studies may be interpreted that SEB molecules are absorbed through the intestinal mucosa and reach the liver via the portal vein. Although SEB exerts some influence directly on the liver to impair its function, the absorbed SEB may be destroyed

TABLE III. EFFECTS OF IV (1 mg/kg) ON HEMODYNAMICS

PARAMETER	GROUP	MEAN + SE BY HOURS POST-SEB				
		CONTROL	3	4	5	6
Mean BP, mm Hg	Control[a]	132 ± 4	131 ± 6	132 ± 7	125 ± 7	127 ± 9
	SEB[b]	135 ± 10	131 ± 4	126 ± 3	132 ± 4	125 ± 4
Cardiac output cc/min/kg	Control	268 ± 31	229 ± 28	245 ± 26*	257 ± 24	276 ± 33*
	SEB	267 ± 18	209 ± 18	185 ± 12*	165 ± 8*	166 ± 10*
Heart rate beat/min	Control	178 ± 7	171 ± 6	179 ± 5	181 ± 6*	195 ± 5*
	SEB	176 ± 8	241 ± 8*	222 ± 14*	228 ± 7	226 ± 3
Stroke volume cc/beat/kg	Control	1.5 ± 0.1	1.3 ± 0.1*	1.4 ± 0.2*	1.5 ± 0.2*	1.5 ± 0.1*
	SEB	1.5 ± 0.1	0.8 ± .04*	0.9 ± 0.1*	0.8 ± 0.1	0.7 ± .04*
Cardiac work J/beat	Control	0.10 ± 0.01	0.09 ± 0.01*	0.09 ± 0.01*	0.09 ± 0.01*	0.09 ± 0.01*
	SEB	0.11 ± 0.01	0.06 ± 0.004*	0.06 ± 0.01*	0.05 ± 0.01*	0.05 ± 0.01*
Mean cardiac power J/sec	Control	0.29 ± 0.03	0.25 ± 0.03	0.27 ± 0.03	0.27 ± 0.03*	0.30 ± 0.03
	SEB	0.34 ± 0.04	0.24 ± 0.02	0.21 ± 0.02	0.20 ± 0.02*	0.19 ± 0.02*
Total peripheral resistance dyne-sec/cm^5 (x 10^2)	Control	113 ± 15	130 ± 18	122 ± 19	104 ± 13*	103 ± 19*
	SEB	102 ± 13	129 ± 12	146 ± 15	154 ± 14*	151 ± 11*
Central blood volume, cc/kg	Control	41.6 ± 4.4	35.6 ± 2.9	41.2 ± 4.8*	37.3 ± 2.8*	38.0 ± 4.7*
	SEB	39.3 ± 2.8	29.5 ± 1.2	25.3 ± 1.3*	24.5 ± 2.5*	27.2 ± 2.8*
Transit time (sec)	Control	5.3 ± .26	6.0 ± .59	5.5 ± .15	5.4 ± .30	5.2 ± .23
	SEB	5.3 ± .46	5.1 ± .52	5.1 ± .36	4.9 ± .39	5.8 ± .46

* Statistically significant ($P < 0.05$).

a. N = 5.

b. N = 6.

or removed by the liver. Consequently, little or no SEB could enter the circulation from the liver. Further, the failure of oral SEB to alter cardiovascular and renal functions supports the hypothesis that the liver inactivates SEB.

Both the total hepatic and portal blood flow were increased after oral administration of SEB (1 mg/kg). This finding suggests that the rate of cardiogreen elimination following oral SEB is not due to a reduced hepatic blood flow, rather that certain hepatic cellular functions are impaired.

Within 6 hr following IV SEB injection (1 mg/kg) in monkeys anesthetized with ketamine and nembutal, there were no changes in O_2 consumption and functional residual capacity (FRC) except that the respiratory rate, tidal volume and minute volume were slightly increased.

The changes in blood pH and gases were measured in conscious monkeys during renal studies for a period of 5 hr post-SEB. Slight acidosis with decreases in P_{CO_2} and HCO_3^- were observed. However, the significant change in total CO_2 (T_{CO_2}) could not be demonstrated. Blood P_{O_2} was maintained relatively constant.

In studies of treatment of SEB shock, experimental evidence has indicated that the main causes of death following IV SEB might be due to vasoconstriction, decreases in cardiac output and regional blood flow, as well as losses of plasma protein and plasma volume from the circulation. In order to reverse these clinical conditions, the combinations of dibenzyline (α-adrenergic blocker), isoproterenol (β-adrenergic stimulator), human plasma albumin (plasma expander), glucose, and NaCl were used for the treatment of SEB-induced shock. The survival time of rhesus monkeys receiving IV SEB (1 mg/kg) was significantly increased from 8-15 hr, to a mean value of 44.6 hr. Although diarrhea persisted in some monkeys, vomition was completely abolished in all monkeys throughout the entire experimental period following medication (Table IV).

Despite the fact that no monkey has recovered permanently, the results do suggest that monkeys die from severe vasoconstriction associated with diminished cardiac functions. Adjusting the challenge doses of IV SEB and drugs, or starting treatment at earlier time intervals post-SEB may test the validity of the proposed hypothesis concerning death mechanisms from SEB toxicity.

TABLE IV. TREATMENT OF SEB SHOCK (1 mg/kg)

MONKEY NO.	DRUG[a]	DOSE	TREATMENT POST-SEB (hr)	SURVIVAL TIME (hr)	POSTMEDICATION Vomition	POSTMEDICATION Diarrhea
X-836	Dibenzyline Isuprel	1.2 mg/kg 0.05 μg/kg/min	- 1.5 +24.0	55.3	No	Yes
X-670	Dibenzyline Isuprel	1.0 mg/kg 0.25 μg/kg/min	+ 1.0	31.3	No	No
X-898	Dibenzyline Isuprel Albumin	0.8 mg/kg 0.125 μg/kg/min 6.0 mg/min	+ 8.0	41.0	No (vomited 2 hr post-SEB)	Yes
X-884	Dibenzyline Isuprel Albumin	0.5 mg/kg 0.125 μg/kg/min 8.3 mg/min	+ 1.0	51.0	No	Yes

[a]Drugs were mixed in 2.5% glucose + 0.45% NaCl and infused at 0.5 cc/min except Monkey X-884 (0.2 cc/min). Dibenzyline was infused for a 1-hr period as a single dose. Isuprel was infused with dibenzyline and then alone continuously after 1 hr postmedication.

Presentations:

1. Beisel, W. R., and C. T. Liu. Renal depression induced by intravenous staphylococcal enterotoxin B in conscious rhesus monkeys. Presented, Annual Meeting, FASEB, Atlantic City, N. J., 13-18 April 1975. (Fed. Proc. 34:364, 1975).

2. Liu, C. T., and R. T. Faulkner. Cardiohepatic responses to staphylococcal enterotoxin B in conscious rhesus monkeys. Presented, Annual Meeting, FASEB, Atlantic City, N. J., 13-18 April 1975. (Fed. Proc. 34:225, 1975).

LITERATURE CITED

1. Rapoport, M. I., L. F. Hodoval, E. W. Grogan, V. McGann, and W. R. Beisel. 1966. The influence of specific antibody on the disappearance of staphylococcal enterotoxin B from blood. J. Clin. Invest. 45:1365-1372.

2. Morris, E. L., L. F. Hodoval, and W. R. Beisel. 1967. The unusual role of the kidney during intoxication of monkeys by intravenous staphylococcal enterotoxin B. J. Infect. Dis. 117:273-284.

3. Elsberry, D. D., D. A. Rhoda, and W. R. Beisel. 1969. Hemodynamics of staphylococcal B enterotoxemia and other types of shock in monkeys. J. Appl. Physiol. 27:164-169.

4. Rhoda, D. A., D. D. Elsberry, and W. R. Beisel. 1970. Fluid compartment alterations in the monkey with staphylococcic B enterotoxemia. Am. J. Vet. Res. 31:507-514.

5. Beisel, W. R. 1972. Pathophysiology of staphylococcal enterotoxin, type B, (SEB) toxemia after intravenous administration to monkeys. Toxicon 10:433-440.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6423	75 07 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
74 07 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	62760A	3A762760A834	01	111
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Lysosomal activation in polymorphonuclear neutrophils

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 012300 Biochemistry

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: 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 75	1.0	105.6
FISCAL YEAR CURRENT 76	1.0	86.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Rausch, P. G.

TELEPHONE: 301 663-7341

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Dangerfield, H. G.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Specific granules; (U) Salmonella typhimurium; (U) Alkaline phosphatase; (U) Myeloperoxidase; (U) Leukocytes; (U) Lysosomes; (U) Military medicine

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

23 (U) Define the response of lysosomal enzymes in circulating polymorphonuclear leukocytes (PMN) during infection and/or immunization. This work is an essential element in a comprehensive program for defense against BW agents.

24 (U) Prepare specific antisera in goats against alkaline phosphatase (AkP) and myeloperoxidase (MPO). Use them as reagents for evaluation of changes in rabbits after challenge with bacteria.

25 (U) 74 07 - 75 06 - An electroimmune assay for rabbit PMN AkP was developed using goat antisera, but despite enhancement by autoradiography, intracellular levels of AkP were below the sensitivity of the assay. Further analysis of the isolated enzyme by immunodiffusion indicated that a nonenzymatic cellular and one serum contaminant were still present. Attempts are now underway to remove them.

A phylogenetic comparison of PMN levels of beta-glucuronidase, MPO, lysozyme (LZM) and AkP has been completed. Avian PMN are deficient in MPO; monkey, cow, goat, sheep, cat and hamster PMN lack LZM; PMN from rhesus monkeys, cats, chickens and geese are deficient in AkP.

In an attempt to develop a noninfectious model for AkP induction in the rhesus monkey, progesterone (known to induce rabbit PMN AkP) was given in varying dose levels. Serum AkP was increased 2-fold, but no effects were observed on AkP or other granule marker enzymes in the PMN. These data suggest that activation of AkP in the 2 species is controlled by different mechanisms.

Publications: Fed. Proc. 34:861, 1975 (abstr.).
Infect. Immunity, in press, 1975.
Blood, in press, 1975.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infections of Military Importance

Work Unit No. 834 01 111: Lysosomal Activation in Polymorphonuclear Neutrophils

Background:

Following ingestion of microorganisms, rapid degranulation of PMN occurs as cytoplasmic granules fuse with phagocytic vacuoles and hydrolases and bactericidal proteins are discharged. Many studies evaluating bactericidal and digestive mechanisms of PMN have therefore concentrated on the biochemistry and morphology of these granules. The heterophilic nature of PMN granules has long been suspected; however, only recently have techniques been developed to permit their separation and analysis. Rabbit neutrophils have been studied most extensively; nonetheless, the results are applicable to most other species. These data indicate that there are at least 2 and possibly 3 types of granules. The larger, azurophilic (primary) granules appear electron dense and are formed during the promyelocyte stage of cellular maturation. They contain myeloperoxidase (MPO), lysozyme (LZM), anti-bacterial proteins and acid hydrolases which classify them as true lysosomes. The smaller, less dense specific (secondary) granules appear during the later myelocyte stage of cellular maturation and contain LZM, alkaline phosphatase (AkP), lactoferrin (LF) and in some animals, collagenase.[1]

While the biochemical content of PMN granules in other species is generally similar to that of rabbit, a number of notable exceptions have been reported. For example, in human granulocytes, AkP is localized to a 3rd cytoplasmic particle.[2] Chicken leukocytes have been reported to lack MPO, while those of cattle are deficient in LZM. The histochemical absence of AkP has also been reported in leukocytes of cats, dogs, mice and rhesus monkeys.[3]

Since PMN are frequently the first cells involved in the host response to infection, and the cellular complement of granular enzymes is undoubtedly important in this response, it is necessary to elucidate the mechanisms responsible for enzyme induction during infection and to understand the functional significance of individual enzymes as they contribute to the bactericidal capacity of the cell.

Progress:

In order to clarify enzymatic responses of PMN to infection, the relationship between total intracellular content, hydrolytic activity, and histochemical localization of selected marked enzymes had to be determined. AkP was initially selected as the marker for specific granules. Attempts were made to isolate this enzyme in sufficient purity for development of a quantitative immunoassay. Rabbits were selected as the experimental model.

Rabbit PMN AkP was isolated by a modification of Fishman's technique as reported last year; this material was used to immunize rabbits and a goat. Antibody response was quantitated by microhemagglutination using tanned sheep RBC; titers in the goat reached 1:8192 on day 42. Rabbits produced only low titers of antibody.

Analysis of Ouchterlony immunodiffusion plates indicated that the antibody was specific for rabbit AkP because no cross-reactions with monkey, human, goat, sheep, horse, burro, rat or hamster enzyme could be detected.

An electroimmunoassay with the goat antiserum was developed according to the methods of Laurell.[3] While the technique was effective in quantitating standards of purified antigen, its sensitivity was not sufficient to detect the low levels of enzyme present in unstimulated PMN. Attempts to increase sensitivity with radioautography were similarly unsuccessful.

The potential increase in sensitivity afforded by radioimmunoassay would be advantageous; however, this technique requires monospecific antiserum, and therefore further studies of the isolated AkP and antiserum were initiated.

Analysis of the goat antiserum by Ouchterlony immunodiffusion demonstrated 3 to 4 precipitin lines against the enzyme preparation and cellular homogenates. Absorption of the antiserum with normal rabbit serum and rabbit PMN homogenates indicated that 2 minor bands represented cellular protein, a 3rd band was probably albumin; the major precipitin band contained all of the AkP activity.

Consequently, attempts were made to remove contaminants from the enzyme so that monospecific antisera could be produced. Cellular components were eliminated with DEAE cellulose ion exchange chromatography. Ion exchange in pH gradients and affinity chromatography are being explored to effect removal of albumin. Dialysis of active fractions obtained from the DEAE columns against distilled water occasionally resulted in the formation of fine needle-like crystals. While this material appears to be pure AkP, relative insolubility in standard buffers may limit its use.

Investigations of the biochemical and physical properties of the original enzyme isolated were also undertaken. Chromatography on Bio-Gel A, 1.5 M, gave an estimated molecular weight of 190,000 daltons. The enzyme was inactivated at 56 C and inhibited by numerous heavy metals and amino acids (Table I). These results are in agreement with data previously published for AkP from other sources.

TABLE I. INHIBITION OF RABBIT PMN AkP.

INHIBITOR	MOLARITY ($\times 10^{-4}$)	% INHIBITION AT 30 MIN
$ZnCl_2$	1	95
$CuSO_4$	5	31
$FeCl_2$	5	92
Ha Azide	5	4
NaF	5	8
EDTA	5	100
NH_4Cl	5	0
NaK-tartrate	5	5
L-Glutamine	3	15
L-Cystine	3	98
DL-Histidine	3	0
DL-Arginine	3	18
Glycine	10	0
Tryptophan	3	3

That the enzymes in PMN granules contribute to the overall bactericidal capacity of the cell has long been assumed, but only in the case of MPO and LZM have specific functions been identified. The functional importance of other enzymes has been difficult to determine because of their multiplicity and the inability to study them individually.

Deficiencies of one or more enzymes in the PMN of man have been reported periodically, and investigations of such patients afford one approach to functional analysis. However, the occurrence of such deficiencies are infrequent and the variety of studies which can be performed are limited. Laboratory animals in which enzyme deficiencies naturally occur, offer another opportunity to evaluate enzymatic function. Unfortunately, identification of suitable models for investigation has been difficult since few interspecies comparisons have been published. Accordingly, the phylogenetic comparisons reported previously have been expanded to include additional species and enzymes.

Neutrophils were harvested from anticoagulated blood by Isopaque-Ficol centrifugation and diluted to 5×10^6 PMN/ml. Sonicates of cell preparations were analyzed for β-Glucuronidase (β-G), MPO, LZM and AkP. Marked interspecies variations were noted (Table II).

TABLE II. ACTIVITY OF GRANULAR ENZYMES IN PMN FROM VARIOUS ANIMAL SPECIES

	SPECIES	NO. TESTED	UNITS/5 x 10^6 PMN/MIN ± SE			
			β-G (μg)	MPO (ΔOD)	LZM (μg)	AkP (nmoles)
Primates	Human	7	232 ± 73	109 ± 44	86 ± 11	3 ± 1
	Monkey--Rhesus	7	90 ± 40	38 ± 7	<1	<1
	Cynomolgus	5	9 ± 2	68 ± 5	<1	30 ± 8
	Squirrel	3[a]	12 ± 4	127 ± 9	<1	23 ± 1
Ungulata	Cow	4	12 ± 7	27 ± 2	<1	10 ± 2
	Goat	9	17 ± 4	6 ± 1	<1	7 ± 3
	Sheep	9	33 ± 11	19 ± 3	<1	8 ± 1
	Horse	3	31 ± 9	39 ± 10	16 ± 1	124 ± 20
	Burro	11	50 ± 11	63 ± 9	8 ± 1	141 ± 53
Carnivora	Cat	3	89 ± 21	9 ± 1	<1	<1
	Dog	6	18 ± 6	95 ± 26	9 ± 2	2 ± 1
Lagomorpha	Rabbit	5	22 ± 6	13 ± 6	15 ± 3	26 ± 12
Rodentia	Rat	10[a]	154 ± 19	46 ± 4	10 ± 2	25 ± 9
	Guinea pig	6	62 ± 7	11 ± 3	15 ± 3	124 ± 24
	Mouse--AKR	2[a]	21	18	10	<1
	C57	2[a]	29	20	5	<1
	Hamster	3[a]	20 ± 2	16 ± 3	<1	72 ± 6
Aves	Chicken	3	34 ± 15	0	84 ± 14	<1
	Goose	4	28 ± 1	0	24 ± 4	<1

a. Number of pools tested.

β-G was present in PMN of all species tested. Chicken and goose PMN were found to lack MPO. PMN of all monkeys, cattle, goats, sheep, cats and hamsters were deficient in LZM. AkP was undetectable in rhesus monkeys, cats, 2 strains of inbred mice, chickens and geese. Cats and rhesus monkeys were deficient in both LZM and AkP.

Total AkP and MPO histochemical scores, determined according to the method of Kaplow,[4] correlated closely with enzyme activity data for all species examined. AkP activity was restricted to neutrophils except for burros where it was also seen in eosinophils. MPO was visualized in neutrophils, eosinophils, and to a lesser extent, in monocytes except for chickens and geese, where it occurred only in eosinophils.

These enzyme variations can be explained by either true interspecies differences in enzyme content or by biochemical differences between enzymes which require methodological differences in assay conditions. To test these possibilities, metal requirements and pH optima were determined for maximal expression of AkP activity from all sources. Metal requirements were determined by the inclusion of 0.01 and 0.1 mM Mg^{2+}, Zn^{2+}, or Fe^{2+} in the incubation medium. Percentage change was calculated from the activity determined without metal addition. The deficiency in AkP observed for the rhesus monkey, cat, mouse, chicken and goose, was not artifactual since the enzyme was undetectable regardless of pH or metal changes. Data for all other species are summarized in Table III. Results indicate that while rabbit and sheep require Mg^{2+}, other species are maximally activated by Zn^{2+}. Because of limited sample volumes, similar studies were not performed for MPO, β-G or LZM, but presumably the same conditions apply.

Ebadi and McCoy[5] have shown that progesterone produces markedly increased AkP activity in rabbit PMN. In an attempt to develop a noninfectious model for AkP induction in the rhesus monkey, a study of steroid effects on rhesus monkey PMN AkP was performed in cooperation with CPT Collins (Animal Resources Division). After the determination of baseline enzyme levels, 8 rhesus monkeys were treated according to the following protocol:

Group 1 (3 animals) progesterone 1 mg/kg IM for 3 days.

Group 2 (2 animals) progesterone 10 mg/kg IM for 3 days.

Group 3 (2 animals) normal saline, 1 ml IM for 3 days.

The animals were bled at 6, 12, 24, 48 and 72 hr following the 1st injection and serum and PMN levels of MPO, β-G, LZM and AkP determined. Results indicate that progesterone produced a 2-fold increase in serum AkP but had no effect on the PMN enzyme. Similarly, it did not affect the activity of PMN MPO, β-G or LZM. These data indicate that AkP induction in the rhesus monkey may be mediated by a mechanism different from that operative in rabbits.

TABLE III. INTERSPECIES COMPARISON OF pH OPTIMA AND HEAVY METAL EFFECTS ON PMN AkP AT OPTIMAL pH.

SPECIES	pH OPTIMUM	% CHANGE[a]			
		Mg^{2+}	Zn^{2+}	$Mg^{2+} + Zn^{2+}$	Fe^{2+}
Primates					
Human	9.8 - 10.0	+ 5	+ 4	+ 9	0
Cynomolgus monkey	-	0	+ 340	+ 270	- 20
Ungulata					
Cow	9.8 - 10.0	0	+ 200	+ 100	+ 30
Goat	-	- 50	+ 120	- 40	0
Sheep	9.0 - 9.2	+ 5	- 35	- 10	0
Horse	9.0 - 9.2	- 30	+ 260	+ 210	- 30
Burro	9.8 - 10.0	- 5	+ 160	+ 100	- 10
Lagomorpha					
Rabbit	9.8 - 10.0	+ 5	- 15	0	- 15
Rodentia					
Rat	9.8 - 10.0	+ 2	+ 15	+ 15	- 10
Guinea pig	9.8 - 10.0	+ 80	+ 680	+ 515	+ 65

a. $\frac{\text{Activity with 0.1 mM cation-activity without cation}}{\text{Activity without cation}} \times 100$

Presentations:

1. Rausch, P. G. Lysosomal enzymes in polymorphonuclear leukocytes. Presented, Professional Staff Conference, John Wesley County Hospital, University of Southern California, School of Medicine, Los Angeles, Calif. 20-26 Nov 1974.

2. Rausch, P. G. Granular enzymes of mature poly-leukocytes. Presented, Tufts University, School of Medicine, Hematology Seminar, Boston, Mass. 3 Feb 1975.

3. Rausch, P. G. Granular enzymes of mature polymorphonuclear leukocytes: a phylogenetic comparison. Presented, Annual FASEB Meeting, Atlantic City, N. J. 13-18 Apr 1975. (Fed. Proc. 34:861, 1975).

Publications:

1. Rausch, P. G., and T. G. Moore. Granular enzymes of polymorphonuclear neutrophils: a phylogenetic comparison. Blood. In Press.

2. Rausch, P. G., and P. G. Canonico. Characterization of monkey peripheral neutrophil granules during infection. Infect. Immunity.12: In press.

LITERATURE CITED

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2. Spitznagel, J. K., F. G. Dalldorf, M. S. Leffell, J. D. Folds, I. R. H. Welsh, M. H. Cooney, and L. E. Martin. 1974. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Lab. Invest. 30:774-785.

3. Laurell, C.-B. 1972. Electroimmunoassay. Scand. J. Clin. Lab. Invest. 29(Suppl 124):21-37.

4. Kaplow, L. S. 1968. Leukocyte alkaline phosphatase-cytochemistry: applications and methods. Ann. N. Y. Acad. Sci. 155:911-947.

5. Ebadi, M., and E. McCoy. 1966. Progesterone-mediated increases of leukocyte alkaline phosphatase in rabbits. Biochim. Biophys. Acta 130: 502-510.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6425	75 07 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
74 07 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	62760A	3A762760A834	01	300
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)
(U) Pathogenesis of the vascular lesions of Rocky Mountain spotted fever

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 07	75 07	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 75	1.0	47.5
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Pathology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Moe, J. B.
TELEPHONE: 301 663-7211
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Kenyon, R. H.
NAME: White, J. D. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Rocky Mountain spotted fever; (U) Pathogenesis; (U) Fluorescent antibody technique; (U) Electron microscopy; (U) Military medicine

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 pathogenesis of vascular lesions during experimental Rocky Mountain spotted fever (RMSF) in guinea pigs. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Develop techniques and conduct sequential studies to elucidate morphologic and functional changes in blood vessels of infected guinea pigs.

25 (U) 74 07 - 75 06 - Pathogenesis of vascular lesions of RMSF in guinea pigs was studied by light microscopy utilizing immunofluoresence, routine histology, electron microscopy, rickettsial plaquing, carbon particle permeability and blood coagulation techniques. Morphologic lesions of RMSF and increased vascular permeability were temporally correlated with presence of detectable levels of fibrin split products in blood, reduction of platelet numbers and rickettsiae in circulating blood. Blocking of the complement system or anticoagulant therapy with heparin failed to prevent lesions of RMSF. Vasculitis during the early stages of RMSF appeared to be a direct effect, which occurred in the absence of intact complement and coagulation systems.

Publications: Lab. Invest. 32:452-453, 1975 (abstr.).
Am. J. Vet. Res., In press, 1975.
Vet. Pathol., In press, 1975.

* 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.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 300: Pathogenesis of the Vascular Lesions of Rocky Mountain Spotted Fever

Background:

The morphologic lesions of RMSF in naturally-acquired and experimentally-induced infections are well documented.[1,2] Most gross and microscopic lesions of RMSF are attributed to endothelial swelling and proliferation, with resultant thrombosis and vascular occlusion.[3] Although rickettsiae have been demonstrated within vascular structures, their role in the genesis of vascular lesions remains unknown. Investigation of experimental RMSF in guinea pigs has suggested that vascular lesions of RMSF are immune-mediated, but the total pathogenesis has not been elucidated.[4] Current investigations were undertaken to correlate morphologic lesions, functional changes in vascular permeability, coagulation changes and alterations in inflammatory mediators using appropriate techniques.[5]

Progress:

Initial effort this year was directed toward completing the study of morphologic and vascular permeability changes, and correlating these with hematologic, coagulation and rickettsemia determinations. One hundred twenty-five male guinea pigs (average weight 300 gm) were inoculated IP with 1 ml 10% chicken egg yolk sac suspension containing approximately 10^7 Rickettsia rickettsii. The following studies were performed on days 1, 2, 3, 4, 5, 8, 10, and 12 postinoculation:

Morphologic pathology and vascular permeability. Four infected guinea pigs and 1 noninfected guinea pig were injected IV with 0.6 ml of colloidal carbon (Pelikan^R) and killed 1/2 hr later. Cremaster muscle, testicle and epididymis were collected and processed for flat mount and routine histologic examination. Results of these examinations are shown in Table I.

TABLE I. RMSF VASCULAR LESIONS IN GUINEA PIGS.[a/]

PARAMETER	NUMBER AFFECTED/TOTAL EXAMINED BY DAYS							
	1	2	3	4	5	8[c/]	10	12
Increased carbon permeability	4[b/]	4	4	4	4	3	4	4
Vasculitis	4[b/]	4	4	3	4	3	4	4
Lymphocytic perivascular infiltrate	0	1	2	3	4	3	4	4
Thrombosis	0	0	1	1	1	2	4	4
Hemorrhage	0	3	1	0	1	1	4	3
Total	4	4	4	4	4	3	4	4

a. Noninfected controls were not affected.

b. Generally minimal changes.

c. Complete tissues from only 3 guinea pigs available for study.

Although venules and capillaries were most severely affected in the early stages of infection, after day 5 all vessels were affected.

Hematologic studies. Blood was collected via cardiac puncture, examined to determine WBC count, differential count and platelet count. Results of those tests are shown in Table II.

TABLE II. HEMATOLOGY IN RMSF IN GUINEA PIGS.

PARAMETER	MEAN VALUE ± SD							
	1	2	3	4	5	8	10	12
WBC Count								
Infected	3548 ±844	3520 ±888	6125 ±1689	3438 ±1197	2658 ±887	6332 ±3715	10678 ±4111	9792 ±1764
Control (1 guinea pig/day)	16940	15180	5750	5000	3630	6490	12100	5750
	(All days = 8855 ± 5112)							
% PMN Infected	35 ±13	35 ±15	59 ±8	26 ±12	52 ±14	62 ±14	61 ±14	43 ±11
% Lymphocytes Infected	51 ±22	39 ±13	40 ±9	46 ±27	46 ±13	35 ±14	34 ±15	53 ±11
Platelets - $x10^3$								
Infected	486 ±57	345 ±108	393 ±237	299 ±94	246 ±76	192 ±94	322 ±82	551 ±85
Control (1 guinea pig/day)	1199	325	405	831	342	471	627	624
	(All days = 603 ± 295)							
Hematocrit %								
Infected	29.8 ±3.7	35.9 ±5.3	36.5 ±6.4	33.5 ±6.9	37.0 ±5.9	30.6 ±3.6	34.4 ±5.6	39.3 ±3.0
Control (1 guinea pig/day)	29.5	41.0	39.0	40.0	37.5	42.5	43.0	40.0
	(All days = 39.1 ± 4.3)							

Coagulation studies. To determine possible involvement of the clotting factors in pathogenesis of RMSF, prothrombin time (PT), activated partial thromboplastin time (PTT), fibrinogen quantitation and fibrin split products (FSP) tests were performed. Results are tabulated in Table III.

TABLE III. COAGULATION STUDIES IN RMSF IN GUINEA PIGS.

PARAMETER	MEAN VALUE ± SD BY DAYS							
	1	2	3	4	5	8	10	12
PT time (sec) Infected	34.2 ±4.7	35.6 ±6.9	38.5 ±3.1	32.8 ±4.4	35.2 ±4.4	32.3 ±7.3	35.0 ±5.0	35.7 ±4.0
Control (1 guinea pig/ day)	28.1	32.2	27.4 (All days = 29.3 ± 3.2)	30.4	23.7	29.9	46.9[a]	33.2
PTT (sec) Infected	39.0 ±4.2	35.6 ±6.9	58.1 ±8.6	55.0 ±11.8	48.9 ±8.0	48.8 ±7.4	47.7 ±9.9	34.5 ±5.3
Control (1 guinea pig/ day)	49.1	35.4	42.6 (All days = 40.3 ± 5.8)	38.5	31.2	42.3	68.1[a]	42.9
Fibrinogen µg/ml Infected	324 ±47	301 ±78	400 ±79	387 ±30	283 ±83	244 ±40	247 ±78	315 ±39
Control (1 guinea pig/ day)	400	291	192 (All days = 280 ± 84)	224	198	279	610[a]	379
FSP Infected No. with FSP / No. examined	1/6	3/5	2/5	4/6	4/6	5/6	5/5	5/5
FSP, µg/ml	40	33	76	61	30	62	53	58
Control No. affected (1 guinea pig/day)	0	0	0	0	0	0	1[a]	0

a. Pneumococcal infection, febrile for 2 days before bleeding, 32 µg/ml FSP. No values included in average calculations.

Rickettsemia study. Blood samples were collected and placed on tissue cultures to estimate the number of viable rickettsiae by PFU. Results of this study with fever data are listed in Table IV.

TABLE IV. RICKETTSEMIA AND FEVER IN RMSF IN GUINEA PIGS.

PARAMETER	VALUES BY DAYS								
	1	2	3	4	5	8	10	12	16
Rickettsemia									
Infected									
No. positive / No. examined	0/6	4/6	4/4	6/6	6/6	6/6	3/5	2/6	0/3
PFU/ml blood (mean of positives)	0	115	262	385	820	298	195	15	0
Control (1 guinea pig/day)	All negative								
Rectal Temperature °F									
Δ Infected - noninfected	-0.12	2.11	1.91	1.41	1.35	1.00	-0.43	-0.63	
P value (Student's T)	ns	0.001	0.001	0.01	0.001	0.05	ns	ns	

Mortality. Twenty infected guinea pigs were used as mortality controls and observed for 21 days. A total of 14 guinea pigs died of RMSF, with the first death being recorded on day 4; the remainder occurred at various times during the next 17 days.

From these studies it was concluded that significant hematologic and clotting changes occurred concurrently with functional and morphologic changes during experimental RMSF in the guinea pig.

The following studies were aimed at determining possible pathogenetic mechanisms which might mediate these changes.

Studies in C4-deficient and complement-depleted guinea pigs. Nine C4-deficient guinea pigs were inoculated IP with $\sim 10^7$ *R. rickettsii*. Lesions in these 9 guinea pigs were compared with tissues from 1 noninfected C4-deficient, 9 infected, complement-intact, and 1 noninfected, complement-intact guinea pigs. In summary, vasculitis, increased vascular permeability, hematologic and clotting changes were essentially similar in infected C4-deficient and complement-intact guinea pigs. Therefore, this suggested that the classical complement pathway was not involved in RMSF in guinea pigs.

To further investigate the possible role of complement, 11 guinea pigs were inoculated IP with R. rickettsii and treated with cobra venom factor (CVF). These guinea pigs had vascular lesions and coagulation changes similar to those seen in infected, saline-control guinea pigs.

Since RMSF lesions develop in the absence of a functional complement system, complement does not appear to be required in lesion development.

Therapy studies. One hundred forty guinea pigs were inoculated IP with 10^7 R. rickettsii and subjected to various therapeutic regimens on days 2-5 postinoculation.

Tetracycline HCl given IP reduced mortality to 10%, compared to 70% in untreated, infected guinea pigs, and reduced fever within 24 hr of initiation of treatment. The treated guinea pigs which died had typical vascular lesions of RMSF. Tetracycline therapy of RMSF in guinea pigs gives results very similar to those in humans, in whom mortality is reduced, but not prevented.

There was no reduction in fever or mortality from RMSF following therapy with chloramphenicol. However, this drug did not prevent Francisella tularensis infection following IP treatment of guinea pigs in a later study, suggesting poor absorption from the peritoneal cavity in this species.

Heparin therapy prevented formation of detectable FSP in blood and development of thromboses in tissues, but did not reduce mortality or prevent other vascular lesions from developing.

Dexamethasone, a synthetic glucocortcoid, was of no value in preventing fever, reducing mortality or preventing development of lesions.

Immunofluorescence and electron microscopic studies. Using indirect immunofluorescence microscopy techniques, rickettsiae were demonstrated. These were mainly in the exudate adherent to the cremaster muscle on days 1-3 and in deeper structures by days 7 and 8, when rickettsiae were abundant in the walls of some blood vessels.

Location of rickettsiae by electron microscopy has been more difficult. In only one guinea pig (day 5) have rickettsiae been definitely identified. The rickettsiae were in nuclei and cytoplasm of circulating monocytes in lumens of blood vessels, and in cytoplasm of an endothelial cell in a venule in the cremaster muscle.

Studies in rhesus monkeys. Necropsy results from 62 rhesus monkeys infected IV or IP with R. rickettsii were tabulated and organized into a manuscript for publication. Monkeys inoculated IV with large numbers of organisms ($> 10^4$) died on days 3-6 postinoculation, while those receiving small numbers of organisms ($< 10^3$) IV or large numbers IP died on day 7 or later. There was no significant difference in incidence of lesions between

the early- and late-death groups. Vasculitis and thrombosis occurred most frequently in the nares, pinna of the ear, perineal skin and testicle. Adrenal cortical necrosis was caused by capillary thrombosis. Rickettsiae, demonstrated by plaquing on tissue culture or immunofluorescence, were most numerous in the lung and spleen. Results are detailed more thoroughly in Tables V and VI.

TABLE V. TIME OF DEATH IN RHESUS MONKEYS INOCULATED IV OR IP WITH VARIOUS NUMBERS OF R. RICKETTSII.

DAY OF DEATH	ROUTE OF INOCULATION	LOG_{10} INOCULATED (Range)	NO. OF MONKEYS	GROUP
3	IV	6.0-7.5	11	
4	IV	6.0-7.5	17	
5	IV	4.0-7.5	12	Early death
6	IV	6.0-7.5	4	
	IP	7.5	1	
7	IV	1.5-2.0	4	
	IP	7.0	2	
8	IV	1.0-2.0	4	
	IP	7.0	2	
9	IV	2.0	1	Late death
	IP	8.0	1	
12	IP	7.5	1	
13	IV	7.5	1	
23	IV	1.0	1	

TABLE VI. INCIDENCE OF LESIONS OF EXPERIMENTAL RMSF IN RHESUS MONKEYS.

LESION	NO./TOTAL EXAMINED		TOTAL (%)
	Early Death (Day 3-6)	Late Death (Day 7-23)	
Vasculitis and thrombosis			
Nares	17/18	10/10	96
Larynx and trachea	22/43	9/17	52
Tongue	17/43*	13/17*	50
Testicle and epididymis	22/27	14/16	84
Skeletal muscle (psoas)	2/39[a]	4/15	11
Ear (pinna)	38/43	16/16	92
Skin (perineal and other)	37/44	15/16	87
Hepatic necrosis	12/45	7/17	31
Adrenitis	19/44	9/17	46
Adrenal cortical necrosis	6/44	3/17	15
Testicular and epididymal necrosis	4/27	5/16	21
Splenitis	10/43	1/16	19
Interstitial pneumonia	20/44	9/17	48
Total	226/504[a]	115/207	48

a. $P>0.05 <0.10$, χ^2 test.

* $P<0.025$, χ^2 test.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6423	75 07 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
75 03 24	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	62760A	3A762760A834	01	301
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Pathogenesis of Rocky Mountain spotted fever in the rhesus monkey

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 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)
FISCAL YEAR	PRECEDING 75	1.0	168.6
	CURRENT 76	1.0	119.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Rickettsiology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Sammons, L. S.
TELEPHONE: 301 663-7465
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Kenyon, R. H.
NAME: Pedersen, Jr., C. E. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pathogenesis; (U) Rocky Mountain spotted fever (RMSF); (U) Rickettsia; (U) Military medicine

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 pathogenesis of Rocky Mountain spotted fever in the rhesus monkey model. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Examine the pathophysiology and pathogenesis of RMSF in order to study prophylaxis and therapy.

25 (U) 74 07 - 75 06 - Serum constituents were measured in monkeys infected with RMSF. Glucose, potassium, calcium and SGPT were not altered during infection; total protein, SGOT, and LDH increased in all ill monkeys except those transiently ill; at the same time amylase and BUN decreased. In some monkeys showing signs of RMSF the sodium, chloride, phosphorus, and alkaline phosphatase decreased; total and direct bilirubin decreased.

Two vaccine studies were conducted. The first compared the 2 embryo cell-culture vaccines to the commercial yolk sac-grown vaccine. The cell-source vaccines afforded relatively better protection in monkeys than the commercial vaccine. Five different vaccination schedules were employed in monkeys; 2 inoculations 2 weeks apart appeared to be the best schedule.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 301: Pathogenesis of Rocky Mountain Spotted Fever in the Rhesus Monkey

Background:

RMSF is the most prevalent rickettsial disease in the U. S. Pathological lesions seen in RMSF in humans have been reported to be similar to those seen in the monkey infected with Rickettsia rickettsii.[1] Other investigators have reported that monkeys exposed to aerosolized R. rickettsii exhibit clinical signs which compare closely to those seen in man.[2] Because of the similarity of signs and lesions between man and monkey, and since eradication of rickettsiae in endemic areas is thought to be improbable, the monkey would serve as a useful model to study pathophysiology and vaccine protection.

Work at USAMRIID has defined the rhesus monkey infected with R. rickettsii in terms of clinical signs, hematologic data, coagulation and complement values.[3] Because of the generalized nature of the lesions of RMSF, pathophysiologic studies have been initiated to elucidate the disease process.

Progress:

Monkeys inoculated with varying concentrations of viable R. rickettsii by the IV and SC routes exhibited the same signs of illness. However, the SC route appeared to increase the length of illness and incidence of rash while it decreased mortality (Table I).

Monkeys showing signs of RMSF had an increase in rectal temperature which stayed elevated for the period of illness. During the febrile response, the white blood cell counts tended to increase. This rise was accounted for by the absolute increase in neutrophils. With onset of fever a decrease in lymphocytes was observed. Cell counts returned to normal in those monkeys which died, but were not significantly altered in ill or asymptomatic monkeys. Hematocrit values of ill monkeys appeared to decrease more than those showing no signs.

TABLE I. CLINICAL RESPONSE OF MONKEYS TO INOCULATION WITH YOLK-SAC-GROWN *R. RICKETTSII*

CHALLENGE			NO./TOTAL(%)			DAYS (RANGE)		
Route	PFU/ml	N	Ill	Dead	Rash	Incubation	Illness	TTD[a]
IV	10^3	7	7(100)	4(57)	3(43)	5.3 (4-7)	3.9 (2-7)	8.5 (8-10)
	10^2	7	7(100)	6(85)	4(57)	5.6 (4-7)	4.4 (3-8)	9.3 (8-10)
	10^1	8	6(75)	5(62)	3(38)	5.8 (5-8)	4.0 (2-5)	9.8 (9-13)
	10^0	6	1(16)	1(16)	0	9.0	3.0	12.0
	10^{-1}	3	0					
SC	4	4	3(75)	0	2(50)	8.0 (7-9)	5.3 (5-6)	-
	3	12	10(83)	8(66)	9(75)	4.9 (3-7)	4.7 (3-6)	8.8 (7-12)

a. Time to death.

Of those serum components measured, glucose, K, Ca and SGPT were not altered in ill monkeys. Those components which changed with onset of fever were BUN, SGOT, LDH, total protein, and amylase. Maximal deviations were noted by the 6th day of fever, and in Table II are presented as group means $\pm$ SE of the peak value. Alkaline phosphatase, Pi, Cl and Na levels decreased while total and direct bilirubin increased in infected monkeys on one or more days; their mean minimum values $\pm$ SE are shown. A manuscript is in preparation on the findings.

The monkey model was used to study the efficacy of 3 *R. rickettsii* vaccines, 2 of which were developed at USAMRIID. Monkeys (6 per group) were vaccinated SC with undiluted, 1:10, or 1:100 dilutions of each of the vaccines. One month later 9 groups of vaccinated, 4 nonimmune control and 5 naturally immune monkeys were challenged IV with 10^3 PFU/ml yolk sac-grown *R. rickettsii*. Response of

TABLE II. EFFECT ON SERUM COMPONENTS OF R. RICKETTSII INFECTION IN MONKEYS

COMPONENT	MEAN PREINFECTION VALUE (± SD)[a]	NO. ILL	NO. ILL SHOWING CHANGE	MEAN INFECTION VALUE (± SE)
No Change				
Glucose	99 ± 19 mg/dl	7/15	0	0
Ca	9.0 ± 1.6 mg/dl	14/16	0	0
K	4.1 ± 0.5 mEq/L	20/31	0	0
SGPT	16 ± 9 IU/ml	27/32	0	0
Decrease				
AkP	26 ± 8 K.B.R. U	27/32	6	7 ± 1 K.B.R. U
Pi	4.6 ± 1.2 mg/dl	27/32	10	1.5 ± 0.1 mg/dl
Cl	100 ± 5 mEq/L	20/31	9	83 ± 1 mEq/L
Na	149 ± 7 mEq/L	20/31	10	127 ± 2 mEq/L
Total Protein	8.0 ± 0.7 gm/dl	34/47	30	6 ± 0.4 gm/dl
Amylase	399 ± 107 U/dl	20/31	19	128 ± 30 U/dl
Increase				
Bilirubin				
Total	0.39 ± 0.17 mg/dl	34/47	21	1.05 ± 0.06 mg/dl
Direct	0.17 ± 0.13 mg/dl	34/47	10	0.57 ± 0.02 mg/dl
LDH	83 ± 39 IU/ml	20/31	20	180 ± 34 IU/ml
BUN	17 ± 4 mg/dl	34/47	30	40 ± 2 mg/dl
SGOT	16 ± 5 IU/L	34/47	30	49 ± 11 IU/L

a. Measured over 4-6 days for each monkey.

the monkeys to challenge are shown in Table III. Considering only undiluted vaccines, one monkey in the DEC, 4 monkeys in the CEC group and 6 monkeys in the commercial vaccine group showed fever sometime after challenge. Onset of fever was earliest in the commercial group and latest in the DEC group. Fever continued longer in the group receiving commercial vaccines than in the group receiving DEC or CEC vaccines. Table III shows that the least number of days with rickettsemia and lowest mean peak rickettsemia titers were found in monkeys receiving the DEC vaccine.

TABLE III. COMPARISON OF DISEASE AFTER CHALLENGE OF MONKEYS VACCINATED WITH VARIOUS RMSF VACCINES

		ILLNESS			RICKETTSEMIA			DAYS
GROUP	NO.	No. Febrile	Fever Onset Day	Days	No.	Days	PFU/ml	OF DEATHS
DEC								
Undiluted	6	1	8.5	5	0	0	0	-
1:10	5	2	4.0	6.5	1	3	50	-
1:100	6	5	5.8	5.4	3	7	30	-
CEC								
Undiluted	6	4	3.9	4.5	1	1	30	-
1:10	6	5	3.4	8.8	4	20	360	-
1:100	6	5	3.5	5.8	3	11	1900	6
Yolk-sac								
Undiluted	6	6	3.2	9.4	3	16	310	11
1:10	6	4	3.2	6.8	3	16	510	10
1:100	6	6	4.1	4.7	3	15	3600	6,8
Controls								
Immune	4	0	-	-	0	-	-	-
Nonimmune	4	4	3.5	6.2	4	25	6000	7,7,8,10

All nonimmune controls died within 10 days after inoculation of challenge organisms. None of the test monkeys immunized with undiluted or the 1:10 dilution of CEC or DEC vaccines died. At the 1:100 dilution none of the monkeys vaccinated with DEC vaccine and only 1 vaccinated with CEC died after challenge. However, with the commercial vaccine, 1 monkey in both undiluted and 1:10 groups and 2 monkeys in the 1:100 group died after challenge.

Hematologic data did not reflect significant differences between groups. There were negligible changes in hematocrit determinations. White blood cell counts reflected normal to slightly higher than normal levels during infection. Neutrophils began to rise within 2-3 days after challenge in all groups (except undiluted DEC) including immune controls. Lymphopenia was evident 3-9 days after challenge in all but the immune control group.

This vaccine study indicates that in rhesus monkeys the cell-culture vaccines are more efficacious than the yolk sac vaccine.

In order to assess the lethality of R. rickettsii and the ability of the organism to cause RMSF, a study was designed using 4 groups (4 monkeys per group) to accurately determine the LD_{50} for subsequent challenge studies. Each group received 1 ml SC injections containing serial 10-fold dilutions of viable rickettsiae. Temperatures were recorded daily for indication of illness and deaths were recorded. The LD_{50} from this experiment was determined to 1.35 $\log_{10}$ PFU/ml. A study was then designed to examine the immune response elicited by varying vaccination schedules using CEC vaccines. Five groups of monkeys (4 per group) received SC vaccinations according to the following schedule:

TABLE IV. VACCINATION SCHEDULE

GROUP	NO. VACCINATIONS	DAY OF VACCINATION		
		Initial	2nd	3rd
I	3	1	15	45
II	2	1	30	
III	2	1	15	
IV	3	1	8	15
V	1	1		
VI	0			

Group VI served as nonimmune controls. Prior to each vaccination and 4 weeks after the last vaccination, monkeys were bled for antibody levels. Microagglutination (MA) data indicate seroconversion of monkeys with one vaccination.

Booster inoculations did not appear to increase MA levels in each monkey; however, the antibody titer in all vaccinated monkeys was 2-4 fold greater than the prevaccination titers.

Monkeys were challenged SC with 10^3 LD_{50} of R. rickettsii and were monitored for temperature response, rickettsemia (Table V) and hematologic parameters. The WBC counts were similar except for the control groups VI

TABLE V. MULTIPLY VACCINATED MONKEYS' RESPONSE TO R. RICKETTSII CHALLENGE

GROUP	NO. ILL / TOTAL	NO. WITH RASH	NO. WITH RICKETTSEMIA	NO. DEAD
I	3/4	0	0	0
II	0/4	0	0	0
III	0/4	0	0	0
IV	1/4	0	0	0
V	2/4	0	0	0
VI	4/4	3	3	3

which showed an early leukocytosis. Increased neutrophils accounted for the early rise in WBC in the control groups; the control groups also exhibited lymphopenia. Group I had a normal WBC pattern but lymphocytes decreased while neutrophils increased. Groups II, III and IV showed no lymphopenia analogous to the immune control group in the 1st vaccine study. The remainder of the blood cell results were unremarkable. This study suggests that multiple vaccinations with CEC derived R. rickettsii are beneficial in protecting from RMSF.

One male rhesus monkey weighing 8 kg was infected SC in the thigh area with 10^5 PFU/ml R. rickettsii in order to examine infected monkey tissue by indirect immunofluorescence. The monkey was monitored until its rectal temperature decreased substantially on day 6 denoting the terminal phase of the disease. Blood was then drawn from the femoral vein into a heparin-coated syringe and the monkey was killed by IV injection of concentrated KCl solution. Tissue was obtained for histopathology and indirect immunofluorescent procedures. Results are summarized under Work Unit 834 02 301.

Publications:

None.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6422	75 07 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
75 03 24	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	62760A	3A762760A834	01	401
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Induced metabolic sequelae of infectious illnesses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
62 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)
FISCAL YEAR	PRECEDING 75	1.75	167.0
	CURRENT 76	1.0	135.7

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Physical Sciences Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Powanda, M. C.

TELEPHONE: 301 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Tularemia; (U) Zinc; (U) Klebsiella pneumoniae; (U) Pneumococcal sepsis; (U) Mice; (U) Clofibrate; (U) Endogenous mediator(s); (U) Induced metabolic sequelae (U) Rats

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

23 (U) Delineate those changes in host metabolism which occur as a direct consequence of infection and the mechanism(s) by which they occur, as aids in diagnosis, prognosis or evaluation of prophylaxis and/or therapy. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Use changes in host zinc and protein metabolism coupled with histopathology, microbiology and enzymology to delineate the pathogenesis of infection and evaluate therapeutic regimens.

25 (U) 74 07 - 75 06 - Zinc deficiency does not prevent rats from increasing their uptake of zinc by the liver or from synthesizing acute-phase globulins in increased amounts, additional evidence of the essential nature of these host responses to infection. Zinc-deficient rats synthesize more specific antibody and IgG than normals in response to immunization.

Increases in plasma lysozyme alpha-2 macrofetoprotein are found in rats which have bacteremias following intranasal exposure to Klebsiella pneumoniae. These changes appear predictive of death.

Clofibrate not only protects rats against pneumococcal sepsis but also diminishes the growth of Pichinde and herpes simplex virus (HSV) in tissue culture. In the case of HSV it appears that the drug interferes with maturation.

Publications: Biochem. J. 144:173-176, 1974.
Clin. Res. 23:310A, 1975 (abstr.).
Fed. Proc. 34:840, 882, 1975 (abstr.)
Acta Vitamin. Enzymol., In press, 1975.
Am. J. Physiol., In press, 1975.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 401: Induced Metabolic Sequelae of Infectious Illnesses

Background:

Induced metabolic sequelae (IMS) are alterations in host metabolism subsequent to the exposure of animals and man to a microorganism or a toxin and consequent upon phagocytosis of the microorganism, toxin or tissue damage by these agents. The onset of metabolic changes are relatively rapid, within hours or days. Thus IMS can be considered amplifiers of the presence of the microorganism and hence may be of value for diagnostic purposes. But even more important, IMS are indicative of host-microorganism interaction and can aid in elucidating pathogenesis and in developing and assessing therapeutic and perhaps prophylactic measures for infectious illness.

It is possible that these host responses are vestigial in nature, predating and perhaps being totally replaced by immunological defenses. It is more likely that these are delaying actions to give the host time to mount an immunologic attack. The fact that they appear stereotyped and that they occur in protein deficient animals[1] and humans[2] and in Zn-deficient rats[3] argues that they are primitive and most likely fundamental or essential reflexes.

Amino acid, Zn and Fe distributions as well as described serum albumin concentrations and increased acute-phase globulin synthesis during infection and inflammation have been attributed to a substance (or substances) excreted by phagocytes subsequent to and consequent upon phagocytosis.[4,5]

Progress:

I. A decrease in serum Zn occurs during a variety of infectious illness in both men and animals. Experiments with rats indicate that the metal is taken up by the liver. Evidence is accumulating that Zn participates in wound healing and is a cofactor for DNA and RNA synthesis and at least for some aspects of amino acid and protein metabolism. Zn accumulates in the livers of patients dying from malignant diseases. Supplementation of Zn has been shown to protect against the hepatotoxic effects of CCl_4. In earlier studies on turpentine-induced inflammation we observed that the augmented amino acid

movement from muscle to liver, the depression in serum Zn and the uptake of Zn by the liver antedated increased serum protein synthesis. It was postulated that Zn and amino acid redistribution might be causal, or at least a necessary condition for, increased synthesis of acute-phase globulins by the liver rather than merely concomitant phenomena. More recently we have demonstrated in rats infected with Francisella tularensis that decreased serum Zn and increased hepatic Zn concentration, but no increased flux of amino acids into liver, were associated with increased plasma seromucoid content. These last data led us to the present study of the effects of experimentally induced Zn deficiency on various aspects of serum protein synthesis in rats exposed to F. tularensis.

Weanling rats were assigned to 3 dietary groups, Zn deficient, pair-fed and ad libitum food and maintained on their respective dietary regimens for 5 wk. Prolonged Zn deficiency led to lessened growth and a decreased hepatic Zn content yet all dietary groups responded to infection with significant fever and enhanced uptake of the ion by liver. The deficiency of itself elicited decreased seromucoid and ceruloplasmin concentrations yet deficient animals responded to infection with significant increments in these proteins compared to ad lib and pair-fed rats. All dietary groups displayed significant increases in α_2-macrofetoprotein, indicative of de novo protein synthesis. Infected rats in all dietary groups showed significant decrements in ^{3}H-leucine incorporation into muscle, but it appeared that the extent of the decrease was exacerbated by decreased protein intake coupled with Zn deficiency.

The humoral response was examined in another set of rats on the respective diets for 4 wk at which time these rats were immunized with the live vaccine of F. tularensis; 10 days later their plasma was tested for specific agglutinating antibodies and for IgG levels. All 3 dietary groups produced specific antibodies in response to immunization, the Zn-deficient rats displaying the highest titer. Similar results were observed in regard to IgG. This unexpectedly enhanced production of antibodies and IgG may have relevance to the problem of why children with kwashiorkor often have elevated immunoglobulin levels; they may be Zn-deficient.

II. The following study is part of an ongoing collaborative effort with Dr. Berendt in Aerobiology Division (Work Unit No. 834 02 109). Klebsiella pneumoniae is an encapsulated short gram negative rod and causes 10-20% of the bacterial pneumonias in man. Significant necrosis of the lung with frequent abscess formation and lung collapse are observed; death may occur as late as 3-4 wk after onset. Fatality in untreated cases is 70-85% and 20-55% in treated cases.

Development of a rat model was begun. IN inoculation of 10^7 K. pneumoniae into Fisher-Dunning rats engendered a moderate to severe multifocal

bronchopneumoniae and splenitis in 24 hr, evidence of resolution within 3-6 days and an overall mortality of 20-30% in 1 mon. On day 1 postexposure > 4 logs of Klebsiella were detectable in lung with a slight increase on day 2. Considerable numbers could still be cultured from 90% of lungs at 4 wk. There appeared to be a reciprocal relationship between lung titer and plasma Zn concentration, which was significantly decreased on day 1 and maximally depressed on day 2. As the number of organisms in lung was diminished there was a gradual return of plasma Zn toward normal. Moreover, plasma Zn remained somewhat below control values at 21 and 28 days, seemingly reflecting the fact that Klebsiella could be grown out of a high percentage of lungs at these times. Consistent with the presence of bacteria in the spleen, there was a significant increase in mean spleen weight early as day 1, with a striking increase at 21 days.

Cellulose acetate strip electrophoresis revealed a biphasic response in serum albumin, the 1st decrease occurring at the time of maximum lung titer, the 2nd at the time of maximal spleen enlargement. There was no significant change in the α_1 fraction and only a transient slight increase in the α_2 on day 2. There was a biphasic increase in the β region while the γ fraction did not show a significant increase until day 13. The seromucoid fraction which is comprised of many of the serum proteins considered to be acute-phase globulins also displayed a biphasic increase.

Serum lysozyme (LZM) concentration, considered to be an index of phagocytosis, and α_2-MFP, the appearance of which in plasma indicates trauma, inflammation and/or infection, were measured. Significant increases in both became evident when the lung bacterial count reached 7 logs. Since alterations in these two variables appeared to correlate with the severity of the illness as judged by the number of organisms in the lung, we bled control and infected rats from the orbital sinuses at various times. In every instance that LZM increased or α_2-MFP appeared, there was evidence of bacteremia. If these variables continued to increase, death was imminent. It remains to be determined whether these 2 variables or any other metabolic sequelae can be used to monitor therapy.

III. Last year we reported that clofibrate (an antihyperlipidemic agent) appeared to protect some rats from a uniformly lethal infection produced by Streptococcus pneumoniae. Repeating the study we found that rats fed clofibrate as 1.25% of diet for 1 wk had decreased bacteremia and enhanced survival (17/20) while untreated rats died. Survivors were rechallenged at 4 wk, along with new control rats; 11/17 survived, while all controls died. Plasma from clofibrate-treated rats appeared not to have a bactericidal or bacteriostatic effect when incubated with S. pneumoniae. Collaborative study with Dr. Canonico, Bacteriology Division, Work Unit No. 834 01 803.

Since others had reported that clofibrate had some antiviral activity we carried out a preliminary study of our own. We found that 100 µg/ml in Vero cell culture resulted in a 3 log reduction in PFU of Pichinde virus (related to Machupo virus which causes Bolivian Hemorrhagic fever) but had no effect on yellow fever or VEE viruses. Collaborative study LTC Eddy, Virology Division, Work Unit No. 834 03 405.

In collaboration with Dr. William L. Steinhart (Pennsylvania State University College of Medicine, Hershey, PA) the effect of clofibric acid on herpes simplex virus, type I, in human epithelial cell culture was evaluated. A dose of 200 µg/ml of the drug led to a 2-log reduction in PFU; there was a linear log reduction PFU-log dose response from 100-1000 µg/ml. No cytopathic effects of the drug on the cells were noted. The drug could be added as late as 7 hr after the virus and still decrease virus production suggesting that the drug affects the maturation process of the virus.

IV. Alterations in trace metal and amino acid distribution and metabolism and in protein metabolism which occur during infection have been attributed to the action of LEM. Certainly there is evidence of a mediator or mediators in the plasma of infected animals and men. There are also numerous studies indicating that LEM produced by rabbit PMN when injected into healthy rats simulates many of the metabolic alterations associated with infection. But there remains the question of what does an animal's own endogenous mediator(s) do. Since one can prepare LEM from peritoneal PMN after the IP injection of glycogen and apparently phagocytosis is a requirement for production, it is likely that repeated IP injections of a heat-killed organism following an initial injection of glycogen should cause LEM release but this time into the rat itself. Preliminary studies indicate that twice-daily injections of heat-killed S. aureus for 3 days following glycogen administration results in a 40-50% decrease in Zn, a rise in Cu, a decrease in glucose and the appearance of large amounts of α_2-MFP in the plasma. Hepatic glycogen is diminished to < 20% of control values. There is also a decrease in serum albumin to 1/3 of control values and an increase in the α_2 fraction. The model will be explored further to see if repeated injections of glycogen alone will produce the same results. This model will allow us to evaluate to what extent the alterations in host metabolism contribute to the pathophysiology of infection apart from the complications attributable to a proliferating microorganism and the complexities of cellular and humoral immunity. Since repeated injections of heat-killed microorganisms do not result in death yet effect pronounced metabolic alterations, this system may allow us to simulate the metabolic changes observed in chronic infections.

V. To aid in detecting endogenous mediator during infection and to lessen the amount of material needed for assaying LEM during purification, we have turned our attention to mice as a bioassay system. On a volume per weight basis, mice and rats appear equally sensitive in regard to amino acid flux into liver, 50 µg/dg body wt produces a detectable increase in both species; 10-20 µl LEM is sufficient to induce an increase in % PMN in both species. The serum Zn depression in rat and mouse are not strictly comparable in that rat has a response range of 140-40 µg/dl while that of the mouse is 90-40% µg/dl.

Presentations:

1. Pekarek, R. S., and M. C. Powanda. Protein synthesis in zinc deficient rats during tularemia. Presented, Annual Meeting FASEB, Atlantic City, N. J., 14-18 Apr 1975. (Fed. Proc. 34:882, 1975).

2. Powanda, M. C., G. G. Long, P. G. Canonico, F. B. Abeles, J. S. Walker, and R. F. Berendt. Indices of respiratory Klebsiella infection in the rat. Presented, Annual Meeting FASEB, Atlantic City, N. J., 14-18 Apr 1975. (Fed. Proc. 34:840, 1975).

3. Powanda, M. C., G. A. Eddy, W. L. Steinhart, and P. G. Canonico. Antibacterial and antiviral activities of clofibrate. Presented, Annual Meeting, American Federation for Clinical Research 3-5 May 1975. (Clin. Res. 23:310A, 1975).

Publications:

1. Powanda, M. C., R. E. Dinterman, R. W. Wannemacher, Jr., and G. D. Herbrandson. 1974. Distribution and metabolism of phenylalanine and tyrosine during tularemia in the rat. Biochem. J. 144:173-176.

2. Powanda, M. C., R. E. Dinterman, R. W. Wannemacher, Jr., and W. R. Beisel. 1975. Tryptophan metabolism in relation to amino acid alterations during typhoid fever. Acta Vitamin. Enzymol. In press.

3. Powanda, M. C., G. L. Cockerell, J. B. Moe, F. B. Abeles, R. S. Pekarek, and P. G. Canonico. 1975. Induced metabolic sequelae of tularemia in the rat: correlation with tissue damage. Am. J. Physiol. In press.

LITERATURE CITED

1. Powanda, M. C., R. W. Wannemacher, Jr., and G. L. Cockerell. 1972. Nitrogen metabolism and protein synthesis during pneumococcal sepsis in rats. Infect. Immunity 6:266-271.

2. Patwardhan, V. N., R. H. Maghrabi, W. Mousa, M. K. Gabr, and S. el Maraghy. 1971. Serum glycoproteins in protein-calorie deficiency disease. Am. J. Clin. Nutr. 24:906-912.

3. Pekarek, R. S., and M. C. Powanda. 1975. Protein synthesis in zinc deficient rats during tularemia. Fed. Proc. 34:882 (abstr.).

4. Kampschmidt, R. F., H. F. Upchurch, C. L. Eddington, and L. A. Pulliam. 1973. Multiple biological activities of a partially purified leukocytic endogenous mediator. Am. J. Physiol. 224:530-533.

5. Pekarek, R., R. Wannemacher, Jr., M. Powanda, F. Abeles, D. Mosher, R. Dinterman, and W. Beisel. 1974. Further evidence that leukocytic endogenous mediator (LEM) is not endotoxin. Life Sci. 14:1765-1766.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6424	75 01 03	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
74 07 01	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A834	01	404
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Pathogenesis of arthropod-borne encephalitis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 12	74 12	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 75	0.5	42.0
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Pathology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Gorelkin, L.

TELEPHONE: 301 663-7211

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Encephalomyelitis, equine (VEE); (U) Pathogenesis; (U) Molecular biology; (U) Nervous system; (U) Military medicine

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

23 (U) Determine pathogenesis by defining sequential ultrastructural relations between arbovirus-infected organ systems in various animal hosts. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Hamsters infected with VEE are killed at regular time intervals and numerous organs studied by light, electron microscopy and other methods.

25 (U) 74 06 - 74 12 - The acute death of hamsters following virulent VEE inoculation was studied. It was demonstrated that these animals develop extensive transileal necrosis in Peyer's patches and lymphatic and hematopoietic necrosis. Positive blood cultures along with depressed reticuloendothelial system clearance lead to the conclusion that an important factor in the demise of these animals is gram negative septic shock.

Ultrastructural and functional studies were completed. Marked variation was seen in large and small plaque VEE variants interaction with the liver. Large numbers of small plaque variants tend to congregate in the liver following intraportal inoculation. These findings may relate to the virulence of the 2 variants since the large plaque is clinically more virulent and is, as illustrated above, less efficiently removed from the circulation by the important blood-clearing organ the liver. The investigator left the Institute. No further work is planned. Studies are complete.

Publication: Lab. Invest. 32:78-85, 1975.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 404: Pathogenesis of Arthropod-Borne Encephalitis

Background:

Efforts were directed toward defining ultrastructural relations between arbovirus-infected organ systems in various laboratory animal hosts.

Progress and Summary:

The acute death of hamsters following virulent VEE inoculation was studied. It was demonstrated that these animals develop extensive transileal necrosis in Peyer's patches and lymphatic and hematopoietic necrosis. Positive blood cultures along with depressed reticuloendothelial system clearance lead to the conclusion that an important factor in the demise of these animals is gram-negative septic shock.

Ultrastructural and functional studies were completed. Marked variation was seen in large and small plaque VEE variants interaction with the liver. Large numbers of small plaque variants tend to congregate in the liver following intraportal inoculation. These findings may relate to the virulence of the 2 variants since the large plaque is clinically more virulent and is, as illustrated above, less efficiently removed from the circuiation by the important blood-clearing organ the liver.

Publication:

Gorelkin, L., and P. B. Jahrling. 1975. Virus initiated septic shock. Acute death of Venezuelan encephalitis virus-infected hamsters. Lab. Invest. 32:78-85.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC6428	75 07 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
74 07 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	62760A	3A762760A834	01	406
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)
(U) Morphometric analysis of subcellular structures in viral infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 002600 Biology

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: 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 75	1.0	120.6
	CURRENT 76	1.0	83.7

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Pathology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: White, J. D.
TELEPHONE: 301 663-7211
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede each with Security Classification Code)
(U) Electron microscopy; (U) Yellow fever; (U) Encephalitis, equine (VEE); (U) Tularemia; (U) Rocky Mountain spotted fever; (U) Ultrastructure

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 procedures for studying topographic changes in cells interacting with microbial organisms and toxins of military importance. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Standard procedures for preparing biological material for scanning material were modified and adopted for examination of specimens in the scanning electron microscope (SEM). New methodology is developed where required to elucidate the pathogenic changes expressed by alterations to surface structures. Studies are correlated with conventional transmission microscopy.

25 (U) 74 07 - 75 06 - The SEM was used to study vesicular stomatitis virus (VSV) infection of cells grown in culture. First attempts to resolve virions on the surface of cells grown on glass were unsuccessful because of electron charging. The use of aluminum foil as a support substrate for the growth of cell cultures eliminated surface charging. Five cell cultures were infected and viewed in the SEM at high magnifications. The first virus-like structures were solitary cylindrical protuberances from the cell surface. With further incubation, the virions were arranged in tightly packed bundles on the cells. Characteristic morphology of virions from purified suspensions of VSV and an arbovirus, Venezuelan equine encephalomyelitis virus were resolved at a magnification of 100,000 in the SEM. VSV was bullet-shaped and the typical arbovirus structure of a core enclosed in an envelope was observed in preparations of VEE.

Publication: In Scanning Electron Microscopy/1975, p. 411-416, 1975.

*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

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 406: Morphometric Analysis of Subcellular Structures in Viral Infections

Background:

The formation of a secondary electron image of adequate quality to permit visualization and resolution of viral structure is dependent upon a number of factors. Specimen preparation and design of the scanning electron microscope (SEM) are primary considerations. The feasibility of studying virus-cell interactions was demonstrated using a high resolution transmission electron microscope (TEM) equipped with a scanning accessory.

Progress:

At magnifications > 20,000 times it was impossible to use cell cultures grown on glass because of surface charging. Modifications to the coating procedure did not eliminate this problem which was ultimately resolved by using cell cultures grown on aluminum foil. Five cell cultures were used: 2 established mammalian cell lines (BHK-21 and LLC-MK_2) a diploid rhesus monkey cell line (FRhL-2) a primary mammalian cell culture (peritoneal macrophage), and a primary avian duck cell (DEC) culture.

To obtain virus-infected cells for SEM, cell cultures on foil were infected with vesicular stomatitis virus (VSV) at a dose of $\sim$ 10 PFU/cell.

With the exception of the DEC which appeared to be refractory to infection, the cellular changes following inoculation with VSV were similar in each of the remaining cultures. For that reason, all subsequent descriptions pertain to FRhL-2 cell. An uninfected cell is flat and elongated with a relatively smooth surface that contains some microvilli. The cells, grown on aluminum foil and viewed at low magnifications, are of the same size and general conformation as glass-grown cells; however, in the latter cell cultures, the cell surface is smoother and contains very few microvilli, there are more interruptions in the continuity of cell processes; and there are numerous cells with folds of cytoplasm peeling from the glass. By 7.5 hr, marked alterations are seen in the morphology of cells in infected cultures. Some of the cells are contracted and the membrane, thrown into folds, has large numbers of microvilli; some cells are spindle-shaped. At this time some of the cells have cylindrical protuberances which are 100 nm in diameter and 100-200 nm long. These structures are smaller than villi and have a smoother surface. By 12 hr the majority of the cells are lysed

and the remaining cells are round, or rounded with attenuated cytoplasmic strands. The surface of the cell body which surrounds the nucleus contains numerous long microvilli in contrast to the relatively smooth surface of the attenuated strands of cytoplasm. These areas on the surface of cytoplasmic strands are covered with bundles of tightly packed cylinders. Some cylinders are lying on the surface, but the majority appear to protrude from the surface. Examination of these cylinders at higher magnification shows that they have a uniform shape and diameter of 100 nm. The end distal to the cell is consistently rounded and the maximum length of the cylinders is 200 nm.

The morphology of the cylinders seen on the surface of infected cells was compared to that of virions seen in cell-free suspensions of VSV on formvar grids. The morphology of these virions is distinctly bullet-shaped. The rounded end and curved sides of the virion appear brighter than the flattened end and upper surface or background. The virion appeared slightly tapered along its length from a diameter of 120 nm at the rounded end to 100 nm at the flat end. Overall length of the virion in the scanning image is 220 nm.

VSV was selected for this study because it is a large virus, 170 x 70 nm with a unique shape.[1] It was possible to recognize characteristic virions in cell-free suspensions of VSV that were examined in the TEM. At a magnification of 100,000, the image accurately resolved the bullet-shape of the virion. Although the size of the virion in the scanning image was approximately 30 nm larger than the accepted value, it is felt that the thickness of the metal coat accounts for this discrepancy. In the examination of infected cells, size and shape were used as basic criteria for identification of surface structures as virions. At 7.5 hr, it was possible to recognize individual virions emerging from the smooth surface of infected cells. There were no modifications to the cell surface which could be associated with the appearance of virions. Birdwell and Strauss[2] in a TEM study of surface replicas have also shown that virions are released as single units from infected BHK cells by 6 hr. In addition, they reported that villous projections associated with virion production of other cells were not a significant feature in the release of VSV from BHK cells. We have noted that in later stages of infection virions were aggregated into tightly packed bundles, agreeing with the observations of Holmes[3] with respect to VSV infection of L cells.

In an attempt to further assess our scanning equipment we examined VEE. According to Klimenko et al., VEE is an envelope virus with an internal spherical nucleoid; particle-diameter ranges from 60-75 nm.

At a magnification of 100,000 the image of this virion is a spherical body consisting of a central mass surrounded by a narrow, darker area which is ultimately enclosed within a thin, bright band. The diameter of this particle is 100 nm.

The SEM image of the virion we obtained shows an inner core surrounded by an envelope. This configuration is compatible with Klimenko's description of this virion; the larger scanning image is probably due to the coating material.

It is apparent from these observations that a high resolution TEM, with appropriate modifications, can be used to obtain high quality secondary images of viruses.

Presentation:

White, J. D. SEM of vesicular stomatitis virus infection in cell cultures grown on aluminum foil. Presented, Scanning Electron Microscopy Meeting, St. Louis, Mo., 7-11 Apr 1975.

Publication:

White, J. D., and A. T. McManus. 1975. SEM of vesicular stomatitis virus infection in cell cultures grown on aluminum foil. In Scanning Electron Microscopy/1975 (ed. by O. Johari and I. Corwin) p. 411-416, IIT Research Institute, Chicago, Illinois.

LITERATURE CITED

1. Fenner, F., B. R. McAuslan, C. A. Mims, J. F. Sambrook, and D. O. White. 1974. The Biology of Animal Viruses, 2nd ed., p. 127. Academic Press, New York.

2. Birdwell, C. R., and J. H. Strauss. 1974. Maturation of vesicular stomatitis virus: electron microscopy of surface replicas of infected cells. Virology 59:587-590.

3. Holmes, K. V. 1975. Scanning electron microscopic studies of virus-infected cells. I. Cytopathic effects and maturation of vesicular stomatitis virus in L2 cells. J. Virol. 15:355-362.

4. Klimenko, S. M., F. I. Yershov, Y. P. Gofman, A. P. Nabatnikov, and V. M. Zhdanov. 1965. Architecture of Venezuelan equine encephalomyelitis virus. Virology 27:125-128.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6416	75 07 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
74 07 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	62760A	3A762760A834	01	407
b. CONTRIBUTING				
c. CONTRIB/TING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code): (U) Comparative pathogenesis of VEE virus for rodents in relation to host defense mechanisms

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 08	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 75	1.0	108.4
FISCAL YEAR CURRENT 76	1.0	110.0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Virology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Jahrling, P. B.
TELEPHONE: 301 663-7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede each with Security Classification Code)
(U) Virulence; (U) Encephalitis, equine (VEE); Interferon; (U) Military medicine; (U) Reticuloendothelial system

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

23 (U) Characterize the balance between viral invasiveness for target tissues and immunological defense mechanisms and identify procedures which enhance the protective response to viral infections. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Virulent and benign strains of VEE are compared with respect to induction of host defense mechanisms of the hamster, and sensitivity to these mechanisms. Virus to cell interactions identified as important to pathogenesis are isolated in cell culture.

25 (U) 74 07 - 75 06 - The interaction of benign VEE viruses with hepatic RES cells was identified as a critical correlate to the rapid clearance of these viruses from blood of infected hamsters. The role of interferon in moderating infection was demonstrated by correlating preexisting interferon levels in tissues of hamsters with exclusion of challenge virus from those tissues. Benign VEE strains also induced interferon more efficiently than virulent strains, and were more sensitive to it.

VEE-infected hamsters treated with antibiotics survived significantly longer, developed no endotoxemia, and failed to develop lesions in lymphoid tissues. While endotoxin may cause the acute death of VEE-infected hamsters, an endotoxin-like activity is also associated with purified VEE virus.

Publications: Infect. Immunity 9:924-930, 1974.
Arthropod-borne Information Exchange 27:100-101, 1974.
Lab. Invest. 32:78-85, 1975.
J. Gen. Virol. 28, In press, 1975.

*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 65 AND 1498-1, 1 MAR 65 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 407: Comparative Pathogenesis of VEE Virus for Rodents in Relation to Host Defense Mechanisms

Background:

The virulence of VEE viruses for hamsters has been correlated with destruction of lymphoid and hematopoietic tissues.[1,2] Attenuated VEE strains appear to interact more efficiently than virulent strains with host defense mechanisms, and some forms of immunosuppression turn infections with attenuated strains into lethal infections.[3] However, it is not clear what combination of the following factors is responsible for VEE-viral induced destruction of target tissues: (1) growth of virus in target tissues, (2) virus-associated toxins, (3) secondary metabolic changes, or (4) endotoxemia associated with an intestinal lesion. Further, the specific interactions of virus with host defense systems have not been compared among virus strains which differ in virulence. The intermediate range goal of these studies is to identify virus/cell interactions critical to virulence. Once identified, these interactions can be compared in more detail using virulent and benign VEE viruses, for the purpose of defining at the biochemical level the determinants of VEE viral virulence.

Viral Interaction with Hepatic RES Cells. We have reported that benign strains of VEE virus are rapidly cleared from hamster plasma, while virulent strains are not.[1] In order to identify the virus/cell interactions responsible for this selective clearance of benign VEE viruses, the distributions of ^{32}P-labeled viruses from 2 clones of VEE in tissues of hamsters, inoculated via the intracardiac route, were compared. The benign small plaque (SP) virus inoculum was almost completely cleared from the circulation within 30 min; 47.6% of the SP virus inoculum was contained in the liver (Table I). In contrast, the virulent large plaque (LP) virus clone was not cleared from plasma. The concentrations of ^{32}P-labeled virus in organs, relative to blood reflected the degrees to which the organs concentrated the virus (Table II). For SP virus, liver contained 321 times as much virus per gram of tissue as plasma. In contrast, the concentration of LP virus in liver was only 0.19 times that in plasma, probably the result of blood contamination.

The affinity of SP virus for liver was confirmed by electron microscopy (collaborative study with Work Unit 834 01 404). Inoculum SP virions were visualized in phagocytic vacuoles of hepatic endothelial and Kupffer cells, where they appeared to be undergoing degradation. In contrast, LP virus was not concentrated or visualized in the liver. Efficient adsorption of VEE virus to cells of the hepatic RES may be an important factor in the low virulence of some VEE virus strains for hamsters.

TABLE I. DISTRIBUTION OF ^{32}P-LABELED SMALL AND LARGE PLAQUE VEE VIRUSES[a] IN ORGANS OF HAMSTERS 30 MIN AFTER INTRACARDIAC INOCULATION

ORGAN	SMALL PLAQUE		LARGE PLAQUE	
	CPM/Organ[c]	% of Inoculum ± S.E.	CPM/Organ	% of Inoculum ± S.E.
Plasma	4,800	0.71 ± 0.06	126,580	99.2 ± 0.04
Liver	308,520	47.6 ± 2.19	25,310	19.8 ± 0.05
Kidneys	25,689	4.0 ± 0.21	11,140	8.7 ± 0.43
Spleen	7,160	1.1 ± 0.23	3,790	3.0 ± 0.54
Lungs	2,880	0.4 ± 0.05	7,590	5.9 ± 0.68
Brain	23	<0.1 ± 0.01	1,210	0.9 ± 0.31

a. The small plaque inoculum contained 10.2 $\log_{10}$ PFU/ml and 647,133 CPM/ml. The large plaque inoculum contained 9.5 $\log_{10}$ PFU/ml and 127,565 CPM/ml. 1.0 ml of each virus was inoculated directly into the hearts of hamsters anesthetized with nembutol.

b. Each determination is an arithmetic mean, based on 10 hamsters.

c. Virus present in organs of hamsters inoculated with large plaque VEE represents only virus in the contained blood. Since some blood-borne virus was thus counted twice, these percentages add to more than 100%. Concentrations of virus in organs relative to plasma are compared in Table II.

TABLE II. CONCENTRATION OF ^{32}P-LABELED SMALL AND LARGE PLAQUE VEE VIRUSES IN ORGANS RELATIVE TO PLASMA, 30 MIN AFTER INOCULATION[a]

ORGAN	MULTIPLE OF PLASMA CONCENTRATION	
	Small Plaque	Large Plaque
Liver	321.4	0.19
Kidney	26.7	0.22
Spleen	7.5	0.50
Lung	3.0	0.15
Brain	0.22	0.04

a. Calculations are based on the same data as Table I.

In vivo Interferon Studies. Studies were completed which help to define the role of interferon in determining the virulence of VEE virus strains for adult hamsters. These data are detailed in a manuscript being submitted for publication; the general conclusions are outlined below.

Four VEE virus strains, which differ in virulence for adult hamsters, were compared with respect to induction of interferon (IF) in plasma and target tissues (spleen, bone marrow, and brain) following SC inoculation, and with respect to sensitivity to hamster interferon in vivo and in vitro. Two benign VEE strains (Pixuna and TC-83 vaccine) induced less interferon in target tissues than 2 virulent strains (Trinidad donkey and 68U201). However, the Pixuna strain, the ratios of IF: infectious virus in tissues were higher than for the other strains tested, suggesting efficient interferon induction. Pixuna strain also appeared to be relatively more sensitive to hamster IF than the other strains, as tested in 2 ways: (1) In HaK cell culture and in primary hamster bone marrow cell cultures, hamster interferon inhibited the replication of BeAr 35645 more completely than the other strains, although the replication of all strains was significantly inhibited. (2) BeAr 35645 failed to infect 68% of hamsters inoculated 24 hr previously with 200 μg of poly I: poly C, compared to 36, 13, and 20% of similarly treated hamsters which failed to become infected following inoculation of strains TC-83, Trinidad, and 68U201 respectively. Splenectomy, which increased the lethality of TC-83 for adult hamsters[3] significantly depressed the plasma IF response to TC-83 vaccine. Interferon thus appeared to be a significant factor in virulence of VEE viruses in hamsters.

Induction of Early Host Defense Mechanisms. The results of studies were reported in last year's annual report, in which hamsters, inoculated with the TC-83 vaccine strain of VEE were protected against a normally lethal challenge of virulent strain 68U201, inoculated 30 hr after vaccination. These studies were extended, will be published soon, and are summarized here. In hamsters vaccinated 30 hr prior to challenge, significantly decreased levels of challenge virus were produced in target tissues (spleen and bone marrow). Inhibition of challenge virus replication was correlated more closely with the interferon concentrations in spleen and bone marrow than with the vaccine virus levels in these tissues at the time of challenge. The induction of early defense mechanisms involved the efficient interaction of virus with spleen; although this interaction was more efficient for TC-83 vaccine, virulent VEE strains were sensitive to the induced protective mechanism, which was mediated, in part, by interferon.

Effect of Antibiotic Therapy on VEE Infection of Hamsters. We have consistently observed that a terminal event of virulent VEE infection of hamsters is the development of bacteremia due to gram negative intestinal flora, which probably results from the transmural necrosis of the ileum around Peyer's patches. Recent data from several lines of investigation suggest that the bacterial sepsis and endotoxemia may be more than simply terminal events, and may be important factors in the development of lesions previously ascribed to the growth of VEE virus in target lymphoid and hematopoietic tissues.

Bacterial sepsis develops between the days 2 and 3 following VEE inoculation. At 44 hr after inoculation of VEE strain Trinidad donkey, 0 of 10 blood specimens

were positive for aerobic enteric bacteria. However, by 56 hr, 3 of 10 were positive, and by 74 hr 9 of 10 specimens were positive for Escherichia coli, Proteus vulgaris, or Streptococcus faecalis. Endotoxemia appears to have preceded overt sepsis, since 5 of 5 bloods obtained 36 hr after infection contained high levels of endotoxin as detected by the experimental Limulus lysate procedure.

Antibiotic treatment of VEE-infected hamsters dramatically affected the disease course. Hamsters were inoculated daily via the IP route with 1 ml of a solution containing 2.5 mg tetracycline and 2.5 mg neomycin, for 3 days prior to SC inoculation of VEE. Inoculation of antibiotics was continued at daily intervals after virus inoculation. Although all of 20 hamsters treated with antibiotics eventually died, survival was prolonged from a mean of 3.2 days for untreated hamsters to 7.6 days; 2 treated hamsters lived for 10 and 11 days. Treated hamsters died precipitously, and were not detectably sick even several hours before death. Histologically, the most severe lesions detected in the tissues of 2 hamsters dying on days 7 and 8 were massive areas of hemorrhage along the olfactory tracts and in the pyriform lobe of the brain. Lymphoid tissues in the spleen, lymph nodes, and intestine were histologically normal, as was the bone marrow except for several areas of minimal necrosis and focal hemorrhage. Antibiotic-treated hamsters did not develop detectable endotoxemia. Virus grew as efficiently in target tissues of antibiotic-treated hamsters as in controls, but in the absence of endotoxemia, no lymphoid cell lesions developed. The development of an intestinal lesion, leading to endotoxemia, appears to be a critical event in the acute death of VEE-infected hamsters.

Endotoxin-like Activity Associated with Purified VEE Virus. In the intact hamster, high viremia levels are always associated with endotoxemia, as detected by the Limulus lysate procedure. To control the remote possibility that VEE virus itself could produce a positive reaction, gradient-purified VEE, strain Trinidad donkey, was tested for endotoxin-like activity (Table III). Both virus preparations tested produced strongly positive results within 4 hr. However, the specific activity of the virus preparations was only about 1% of the specific activity of E. coli lipopolysaccharide. To control the possibility that the apparent endotoxin-like activity associated with VEE resulted from a low level of endotoxin contamination, all reagents used in the purification procedure were tested and found to be negative for endotoxin. Likewise, no endotoxin activity was found in the fractions of sucrose gradients where virus would be expected to band, when either water-lysed BHK-21 cells or endotoxin were applied to gradients and centrifuged in parallel with virus. More rigorous efforts are needed to determine if the endotoxin-like activity of VEE is truly associated with the virus or to a contaminant.

Presentation:

Jahrling, P. B., G. A. Eddy, and W. H. Dietz. Interferon induction and sensitivity as correlates to virulence in experimental VEE virus infections. Presented, Meeting American Society of Tropical Medicine and Hygiene, in Honolulu, Hawaii, 2-6 Nov 1974.

TABLE III. ACTIVITY OF VEE CONCENTRATES IN THE *LIMULUS* LYSATE TEST

VIRUS PREPARATION	PROTEIN CONC. µg/ml	LOG_{10} PFU	DEGREE OF AGGLUTINATION 1 hr	4 hr
1	140	10.5	+++	+++
	14	9.5	++	+++
	1	8.5	0	+
2	78	10.2	++	+++
	7	9.2	0	++
	0.7	8.2	0	0
Control[a]	<1 (1:10)	0	0	+
	(1:100)	0	0	0
	(1:1000)	0	0	0
E. coli LPS	1.0	0	+++	+++
	0.1	0	++	+++
	0.01	0	0	++
Water	-	0	0	0

a. Water-lysed BHK-21 cells.

Publications:

1. Jahrling, P. B. 1974. Effect of antibiotic therapy on VEE infection of hamsters. Arthropod-borne Virus Information Exchange 27:100-101.

2. Gorelkin, L., and P. B. Jahrling. 1975. Virus-initiated septic shock. Acute death of Venezuelan encephalitis virus-infected hamsters. Lab. Invest. 32:78-85.

3. Jahrling, P. B. 1975. Interference between virulent and vaccine strains of VEE virus in mixed infections of hamsters. J. Gen. Virol. 28, In press.

LITERATURE CITED

1. Jahrling, P. B., and W. F. Scherer. 1973. Growth curves and clearance rates of virulent and benign Venezuelan encephalitis viruses in hamsters. Infect. Immunity 8:456-462.

2. Jahrling, P. B., and W. F. Scherer. 1973. Histopathology and distribution of viral antigens in hamsters infected with virulent and benign Venezuelan encephalitis viruses. Am. J. Path. 72:25-38.

3. Jahrling, P. B., E. Dendy, and G. A. Eddy. 1974. Correlates to increased lethality of attenuated Venezuelan encephalitis virus vaccine for immunosuppressed hamsters. Infect. Immunity 9:924-930.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6420	75 07 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
74 07 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	62760A	3A762760A834	01	408
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Pathology of Bolivian hemorrhagic fever in the rhesus monkey

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 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)
FISCAL YEAR PRECEDING 75	1.0	47 5
FISCAL YEAR CURRENT 76	1 0	70.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Pathology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: McLeod, C.
TELEPHONE: 301 662-7211
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pathology; (U) Hemorrhagic fever; (U) Machupo virus; (U) Arenaviruses

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

23 (U) To characterize the gross and microscopic pathologic changes in rhesus monkeys infected with Machupo virus, the etiological agent of Bolivian hemorrhagic fever (BHF). This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Perform complete necropsies on rhesus monkeys dying after inoculation with Machupo virus. Record salient gross lesions; examine tissues histologically.

25 (U) 74 07 - 75 06 - Monkeys that survive acute BHF infection often develop a wasting neurovascular disease characterized by lymphoreticular vasculitis and perivasculitis of the central nervous system (CNS), peripheral nerves and ganglia. Similar vascular lesions and diffuse lymphocytic infiltrations are seen in many other organs.

Similar lesions were seen in the CNS of BHF-infected, immune-serum treated, and clinically normal rhesus monkeys. In most cases it has not been possible to correlate clinical signs with degree of severity of the microscopic lesions.

These unexpected findings in chronic fatal cases and in "successfully" treated monkeys raise the question as to whether or not BHF infection exists in humans as an unrecognized neurovascular disease. The predominance of lymphocytes in the chronic BHF infection in rhesus monkeys suggests an immunological pathogenesis.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 408: Pathology of Bolivian Hemorrhagic Fever in the Rhesus Monkey

Background:

BHF is endemic to parts of northern Bolivia. Machupo virus, a member of the Tacaribe group of the arenaviruses is the etiologic agent. In humans, the disease is seen as a hemorrhagic shock syndrome with high mortality. Pathology of the disease in humans has been reported by Child et al.[1] Characteristic lesions in human cases are widespread hemorrhages and congestion, hepatic necrosis, and activation of the RES with erythrophagocytosis. The rhesus monkey (Macaca mulatta) has been found to be an excellent model for the study of BHF, and a description of the acute infection was reported earlier.[2] Emphasis is presently being given to chronic disease in rhesus monkeys.

Necropsies and microscopic examinations were performed on rhesus monkeys that died or were killed after inoculation with Machupo virus. Chronic disease developed most often in monkeys that were treated with immune serum after signs of illness appeared. Tremors, paresis, incoordination and muscle atrophy have been common signs in chronic BHF.

Progress:

The study of chronic BHF has revealed a wasting neurovascular disease characterized by lymphoreticular vasculitis and perivasculitis of the central nervous system, peripheral nerves and ganglia. Many other organs were affected by similar vascular lesions, as well as diffuse lymphocytic inflammation. These lesions have been seen in serum-treated as well as untreated rhesus monkeys. Similar but less severe lesions have been seen in the CNS of BHF-infected, monkey immune-serum treated, clinically normal rhesus monkeys. These unexpected findings in chronic fatal cases and in recovered monkeys raise the question as to whether or not chronic BHF infection exists in humans as an unrecognized neurovascular disease.

Publications:

None.

LITERATURE CITED

1. Child, P. L., R. B. MacKenzie, L. R. Valverde, and K. M. Johnson. 1967. Bolivian hemorrhagic fever. A pathologic description. Arch. Pathol. 83:434-445.

2. Terrell, T. G., J. L. Stookey, G. A. Eddy, and M. D. Kastello. 1973. Pathology of Bolivian hemorrhagic fever in the rhesus monkey. Am. J. Pathol. 73:477-494.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6426	75 07 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
74 07 01	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A834	01	800
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)
(U) Mechanisms of staphylococcal enterotoxin B-induced gastrointestinal changes

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 002300 Biochemistry; 016800 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 10	75 06	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 75	1.0	123.7
FISCAL YEAR CURRENT 75	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Animal Assessment Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Elwell, M. R.
TELEPHONE: 301 663-7244
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Liu, C. T.
NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Staphylococcus; (U) Enterotoxin (SEB): (U) Gastric emptying; (U) Fructose tolerance; (U) Pharmacologic protection; (U) Monkeys; (U) Rabbits; (U) Military medicine

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

23 (U) Develop a gastrointestinal model for studying the changes and mechanisms of these changes induced by oral staphylococcal enterotoxin B (SEB). This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) The effects of microbial toxins on animal hosts are determined by measuring various physiologic parameters.

25 (U) 74 07 - 75 06 - The oral fructose tolerance test was found to be an index for assessing the rate of gastric emptying in the monkey. When monkey were given oral SEB, the rate of gastric emptying of a fructose meal was found to be increased over control values. These changes occurred 10-30 min following oral SEB, prior to any clinical symptoms of SEB ingestion. Preliminary experiments with intraportal SEB infusion indicated that lethal effects of the toxin may be diminished by this route of infection, but further experiments are required. IV SEB studies in the rabbit have shown the presence of endotoxin as indicated by the Limulus lysate assay. Further controls are required before definite conclusions can be made. Various pharmacologic agents that have been found to have protective effects in endotoxic shock were employed in SEB-challenged rabbits. Pretreatment with prednisilone acetate provided complete protection from lethal doses of SEB. Trasylol, a proteolytic enzyme inhibitor, provided little protection. Using oral antibiotics to lower gut endotoxin levels did not enhance survival rates, although the time to death was increased.

Publications: Proc. Soc. Exp. Biol. Med. 148:424-427, 1975.
Infect. Immunity 11:724-726, 1975

The investigator has been transferred. Studies are complete.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 800: Mechanisms of Staphylococcal Enterotoxin B-Induced Gastrointestinal Changes

Background:

Although vomiting and diarrhea are symptoms commonly associated with SEB intoxication, the mechanisms by which the toxin elicits its effects as well as many of the physiological changes have not been studied extensively. A major objective was to study the effects of oral SEB on gastric emptying rates as reflected by the oral fructose tolerance test in the chair-restrained monkey.

Work was initiated to study the effects of intraportally infused SEB as compared to the results from the IV SEB model of Stiles and Denniston.[1] This project was designed to study further the emetic receptor site for SEB and the role of the liver in SEB detoxification.

Another area of study has been concerned with investigating the role of endotoxin in the IV SEB-induced shock syndrome in rabbits. It has been postulated that SEB-induced shock may be the result of its action on gut capillary endothelium and the subsequent absorption of endotoxin, resulting in shock and death.[2] Considering the hypothesis of endotoxin participation in SEB death, we employed several pharmacologic agents effective in endotoxic shock, for examining the survival rate in the SEB-treated rabbits.

Progress:

I. Studies completed this year were designed to evaluate the oral fructose tolerance test (FTT) as an index of gastric emptying in the chair-restrained monkey and to determine the effect of oral SEB on the rate of gastric emptying. Following an overnight fast, monkeys were administered a 2 gm/kg fructose bolus (635 mosm) via nasogastric tube. Arterial plasma fructose (PF) concentrations were measured at intervals for a period of 90 min. PF concentration was observed to increase along an exponential time course. Because the stomach has been observed to empty along a similar time course, it was hypothesized that the plasma exponential increase may be a result of the stomach emptying, and other factors. It was necessary to determine that PF concentration reflected the input rate of the sugar into the plasma. IV fructose (0.5 gm/kg/hr) was infused from 0-60 min and plasma was sampled from the abdominal aorta. The fructose concentration increased rapidly and fell quickly after infusion was stopped. The equation for the line that fits the falling phase fits the rising phase as well. A half-life of < 2 min lends support to the assumption that PF concentration closely reflects the concurrent input rate of fructose into the plasma from the intestine.

Additional experiments using George's technique[3] for measuring gastric emptying time were conducted. The intragastric (IG) fructose bolus was administered; the actual volumes remaining in the stomach were determined at time intervals through 40 min and expressed as percent of original volume instilled ($n = 4$). Fructose test meals were administered using experimental variables to enhance or delay gastric emptying. As was expected, atropinized monkeys (0.1 mg/kg) had the slowest emptying rate, with 49.5% of the original volume remaining at 40 min. Fructose with citrate was the most rapid meal to empty (13% at 40 min) and fructose alone was intermediate (29.9% at 40 min). The volumes remaining in the stomach by 40 min were significantly different ($p < 0.01$) between atropine and citrate groups. Gastric emptying curves from the dye dilution technique were compared with respect to time with arterial PF concentrations taken during the dye dilution experiments. PF values were normalized by calculating them as percent maximum concentration reached in each animal. Intragastric volume data were expressed as percent maximum volume administered. In each of the 3 test meals, PF increased and IG volume decreased along exponential time courses. In all 3 groups the rate of decrease of gastric volume approached 0 at a time when the PF concentration ceased to increase.

The time constants for the exponential increases of plasma fructose were determined for the 3 groups ($n = 4$), the larger time constant indicating a slower rate of gastric emptying. Atropinized animals had the largest time constant, 17.5 min, while fructose with citrate and fructose alone were 11.5 and 12 min, respectively. When fructose was combined with protein and fat, and given in the same quantity (2 gm/kg) and concentration, a similar comparison could be made with the atropinized group. The inhibitors of gastric emptying (fat and protein) of the fructose meal were reflected by the plasma fructose concentration curves. When SEB (1 mg) was administered orally with the fructose, fat and protein test meal, maximum concentrations were higher and were reached sooner when compared to controls (no SEB), and began to decrease at an earlier time period. This observation was more evident when fructose alone was administered with SEB ($n = 11$) and compared to controls receiving only fructose ($n = 12$). Plasma fructose concentration from SEB recipients reached a higher maximum level sooner than controls and fell more rapidly to baseline levels.

II. An experimental monkey model has been established to study the role of the liver in SEB detoxification and to elucidate further the emetic site for SEB. Previous work at this Institute[1] has shown increased lethal effects of SEB when administered at a low-dose, constant IV infusion (30-60 hr). When infused through a peripheral vein, SEB (15 μg/kg) produced emesis, diarrhea, leukopenia, and death (6 of 8) when infused over a 30-hr time period (0.5 μg/kg/hr).

Although the liver would be accessible by either IV or oral SEB challenge, if toxin were absorbed all molecules must pass through the liver vascular bed before entering the general circulation. The liver may be capable of altering the lethal effects of this toxin which are observed when it is given IV. With oral absorption of toxin or portal infusion, all toxin molecules have initial

exposure to the liver prior to contact with other body tissues.

In our experiments, monkeys were chair-adapted 1 wk prior to surgery in which femoral arterial and venous and portal venous catheters were implanted. The monkeys were allowed to recover from surgery and were clinically normal when the experiment began. We found that in 2 monkeys given 15 μg/kg for 30 hr (0.5 μg/kg/hr) via the portal vein, no vomiting, diarrhea, or deaths occurred. One monkey showed a slight increase in heart rate but no change in blood pressure. Both monkeys were leukopenic, as were the IV infused monkeys of Stiles and Denniston; however, by 30 hr the WBC counts had returned nearly to the prechallenge levels. The significant fall in hematocrit observed in IV monkeys were not present in portal-vein-infused monkeys although 1 of the 2 did show a significant decrease at 24 hr. One monkey showed no change in total protein while the other had a small decrease similar to that observed in IV infused monkeys. One monkey showed no signs of illness; the other seemed somewhat depressed, but continued eating.

It was decided to increase the dose to 30 μg/kg (1.0 μg/kg/hr) and this was shown to be lethal in 1 monkey infused IV for 30 hr. This monkey developed tachycardia, leukopenia, a fall in systolic and diastolic pressure, and a decrease in hematocrit and total protein following IV infusion. Death occurred at 42-46 hr following a cumulative dose of 30 μg/kg.

To date, 1 portal-infused monkey has been tested at this dose (30 μg/kg). The animal showed no signs of illness (depression, anorexia, vomiting, diarrhea) but died 24 hr following termination of infusion. Blood pressure remained constant although tachycardia and transient leukopenia occurred during infusion. It is difficult to explain why this monkey died without showing any signs associated with oral or IV SEB. Assuming it was due to SEB enterotoxemic shock, it is interesting that no emesis or diarrhea occurred in this or the other 2 intraportally infused monkeys.

III. The relationship of endotoxin to IV SEB-induced shock and death in the rabbit has been examined. This required the application of the Limulus lysate technique[4] to detect endotoxin presence in rabbit plasma and liver. Samples of blood were taken prior to IV SEB injection in 2 groups of rabbits. In Group I (n = 4), rabbits were sacrificed at 11 hr and plasma and liver samples were taken. In Group II (n = 4), rabbits were sacrificed at 6 hr. In 7 of 8 rabbits, prechallenge plasma samples were negative for endotoxin, while all were positive when sacrificed post-SEB-challenge. The positive test in the 1 pre-SEB plasma sample may have been a result of contamination as the post-SEB sample developed a positive reaction before this sampling, indicating a greater endotoxin concentration. Group I, which was sacrificed later than Group II, had higher plasma endotoxin levels based upon faster gel times for greater concentration. Tenfold dilutions of liver samples were also positive, but may be a result of the plasma endotoxin in the tissue. Although it appears that endotoxin may be present in SEB-challenged rabbits, further controls must be included before definite conclusions can be made.

IV. Studies were conducted to investigate the role of endogenous endotoxin in the lethal, IV, SEB-induced shock syndrome. Sugiyama[2] postulated that IV SEB may exert an influence on the gut to permit the endogenous endotoxin to enter the circulation and elicit its toxic effect. To test this hypothesis in rabbits, an attempt was made to reduce the concentration of endotoxin available in the gut by treating rabbits prior to SEB challenge with antibiotics to decrease the gram negative gut flora. Kanamycin sulfate (5 gm) was administered at -24 hr and -4 hr directly into the cecum and stomach through a laparotomy. Results were inconclusive, since all controls (n = 4) survived at the dose of toxin administered (400 μg/kg), deaths occurred in the sham surgery group (n = 4) receiving saline (25% mortality), and 1 of 4 antibiotic-treated rabbits survived. Typically, in enterotoxin-challenged rabbits (800 μg/kg) death occurs within 24 hr, while antibiotic-treated rabbits die 2-7 days postchallenge.

This study was repeated with rabbits given kanamycin via nasogastric tube. Five of 6 rabbits in the control group which received 800 μg/kg IV SEB died within 12-18 hr postchallenge. The kanamycin group received antibiotic at -48, -24, and -6 hr challenge; 3 of 4 rabbits died in this group 24-72 hr post-SEB. Although there is no difference in survival between groups, a delayed time-to-death was observed in both antibiotic recipient groups. These deaths may be related to gut flora reestablishment following antibiotic therapy. It will be necessary to include an antibiotic control group to test this hypothesis.

Means of protecting rabbits from the lethal effects of IV SEB through the use of various pharmacologic agents were studied. Assuming that IV SEB induces endotoxin release into the circulation from the gut, or release of a mediator similar to endotoxin in activity, drugs were selected that have been demonstrated to be effective in endotoxic shock. Prednisilone acetate was administered IM to Group I (n = 13) 6 hr prior to a lethal IV SEB challenge (800 μg/kg). Group II (n = 18) received only IV SEB (800 μg/kg). Complete protection was afforded to Group I, while Group II had only 17% survival. Diarrhea occurred in most Group II rabbits but was not observed in Group I. All deaths occurred within 24 hr of challenge except for 1 at 36 hr. Trasylol, a proteolytic enzyme inhibitor, was administered IV to Group III (n = 7) at the time of SEB challenge and again at 30 min. Three rabbits survived (43%), which may indicate some protective effect of this drug, particularly if other methods (pretreatment) of administration were applied.

The pyrogenic response to IV SEB was observed in these same rabbits. All rabbits (Groups I-III) responded to IV SEB with an increase in temperature over base line at 30-60 min postinjection. Fever was maintained until death in Group II, while Group I began to decrease by 12-16 hr. It is interesting to note that, although there is no significant difference between groups in the maximum temperature attained, the reported biphasic pattern observed in Group II was less obvious in Group I and not observed in Group III. Furthermore, the prednisilone-treated rabbits (Group I) reached their maximum temperatures before Group II, and at 2 hr were significantly higher than Group II. The reason for this difference is not known.

Presentation:

1. Pettit, G. W., K. A. Bostian, and M. R. Elwell. Use of intragastric fructose tolerance test in assessment of gastric emptying. Presented, Annual Meeting, FASEB, Atlantic City, NJ, 13-18 April 1975. (Fed. Proc. 34:910, 1975).

Publications:

1. Elwell, M. R., C. T. Liu, R. O. Spertzel, and W. R. Beisel. 1975. Mechanisms of oral staphylococcal enterotoxin B-induced emesis in the monkey. Proc. Soc. Exp. Biol. Med. 148:424-427.

2. Elwell, M. R., M. L. Sammons, C. T. Liu, and W. R. Beisel. 1975. Changes in blood pH in the rat after infection with Streptococcus pneumoniae. Infect. Immun. 11:724-726.

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1. Stiles, J. S., and J. C. Denniston. 1971. Response of the rhesus monkey, Macaca mulatta, to continuously infused staphylococcal enterotoxin B. Lab. Invest. 25:617-625.

2. Sugiyama, H. 1966. Endotoxin-like responses induced by staphylococcal enterotoxin. J. Infect. Dis. 116:162-170.

3. George, J. D. 1968. New clinical method for measuring the rate of gastric emptying: the double sampling test meal. Gut 9:237-242.

4. Levin, J., T. E. Poore, N. P. Zauber, and R. S. Oser. 1970. Detection of endotoxin in the blood of patients with sepsis due to gram-negative bacteria. N. Engl. J. Med. 283:1313-1316.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6427	75 07 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
74 07 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	62760A	3A762760A834	01	801
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)
(U) Radioimmunoassay techniques and their use in infectious disease research

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
	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 75	1.0	168 2
FISCAL YEAR CURRENT 76	1.0	193.8

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Physical Sciences Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: George, D. T.
TELEPHONE: 301 663-7181
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede each with Security Classification Code)
(U) Glucagon; (U) Cortisol; (U) Infection; (U) Carbohydrate metabolism; (U) Insulin; (U) Protein binding radioassay; (U) Leukocytic endogenous mediator

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

23 (U) Provide precise methods for measurement of biologically important compounds and elucidate their roles in maintenance of homeostasis. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Development of hormone assay techniques for the measurement of glucoregulatory hormones. Monitor their alterations throughout the course of various infectious diseases in man and animals. Interpretation of these data and their relationship to host response to infection.

25 (U) 74 07 - 75 06 - Animal studies are in progress to define further the effects of leukocytic endogenous mediator (LEM) upon the glucoregulatory hormones. Preliminary studies indicate that many of the metabolic changes may be associated with the LEM-induced alterations of the glucoregulatory hormones, which result in a significant increase in the liver second messenger system cyclic AMP.

The L-DOPA studies have resulted in findings which indicate that the monoamines may play an important physiologic role in the regulation of the endocrine pancreas. Rat studies are still in progress to more completely define this observation.

Publications: J. Clin. Endocrinol. Metab. 39:618-621, 1974.
Diabetes 23:544-549, 1974.
Clin. Res. 23:320A, 1975 (abstr.).

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 801: Radioimmunoassay Techniques and Their Use in Infectious Disease Research

Background:

Many hormones play important roles in the homeostatic regulation of carbohydrate (CHO), protein and lipid metabolism. The primary glucoregulatory hormones are insulin, growth hormone, glucagon, adrenal-glucocorticoids and catecholamines. Beisel[1] reviewed the alterations in CHO, amino acid, and free fatty acid (FFA) components of blood during various infections in man.

The alterations are not completely explained by the changes in plasma insulin and growth hormone, therefore, studies have been carried out in man,[2] monkeys[3] and rats[4] during various infections. The results of these studies have documented the existence of hyperglucagonemia, relative hyperinsulinemia and alterations in the hosts metabolism of glucose and amino acids. Furthermore, we have also been able to substantiate an elevation of adrenocorticoid levels in man[2] and monkeys during infection.

Since our observation that L-dopa stimulated release of glucagon from monkeys, this phenomenon has been actively investigated in man and rats.

Progress:

Although we have been able to document alterations of endocrine pancreas function associated with infection, the causal events leading to elevated levels of plasma glucagon, hypersecretion of insulin and glucose intolerance are poorly understood. Since our observation of elevated levels of glucagon following L-dopa treatment in the monkey, we have documented a similar response in man.

During the past year we have been involved with rat studies in collaboration with Work Unit No. 834 01 805 to determine the mode of action of L-dopa in stimulating the release of glucagon. Studies in the rat indicate that like monkey and man, the rat has a massive release of glucagon following the IV administration of L-dopa and/or dopamine, which results in elevations of plasma glucose and no changes in plasma insulin. Evaluation of the sympathetic nervous system by use of α and β adrenergic blocking agents have revealed that α blockers

can suppress the L-dopa response but the latter were ineffective, as were blockers of the parasympathetic nervous system. These results are more meaningful when considered in conjunction with studies examining the insulin monoamine relationships, where it has been shown that there are alterations of glucose tolerance following blockade of monoamine synthesis[5] and suppression of insulin secretion by L-dopa and serotonin, in isolated pancreatic islet cell preparations.[6] Furthermore, Dr. Bailey's studies (Work Unit No. 834 01 805) with various agents which block the metabolism of L-dopa, and the dopaminergic receptors indicate that dopamine probably is the agent responsible for the release of glucagon. If we consider our data indicating that dopamine is responsible for the release of glucagon and those of others which indicate that dopamine causes the suppression of insulin,[6] it is possible to postulate that the monoamines may play an important role in the regulation of insulin and glucagon release from the pancreatic islet cells. These data will enable us to explain the stimulus responsible for the hyperglucogonemia of infection.

Furthermore, it may provide us with a means to assess the pancreas ability to secrete glucagon during illness and health.

As a result of a recent publication[7] reporting the similarities between the metabolic sequelae occurring concurrently with infection and (LEM) treatment, studies were initiated to determine the effects of LEM upon the glucoregulatory hormones, insulin and glucagon in the fasted male rat, (collaborative work with Work Unit No. 834 01 020). The initial studies revealed striking elevations of both insulin and glucagon occurring at the same time as the amino acid flux into liver[6] and a significant depression of plasma glucose levels. Further studies done to ascertain the meaning of these findings, have revealed that there is, in fact a log-dose relationship between the dose of LEM and the response of the endocrine pancreas, plasma glucose, amino acid flux into liver, serum Zn, PMN and the liver glycogen concentrations, 5 hr after the administration of LEM. It appeared as if there might be some correlation between the elevated glucagon levels and the associated metabolic changes which were most probably mediated by liver cyclic AMP, as reported by Zenser[4] during infection. Preliminary results indicate that there is a significant elevation of liver cyclic AMP detectable 2 hr after the administration of 0.5 ml/100 gm body wt dose of LEM in fasted male rats. Therefore, it appears as if LEM may be the factor elaborated by the infected host, which is responsible for the metabolic and hormonal alterations observed during sepsis. Furthermore, we have an hypothesis on the probable mode of action of LEM, since the following actions occur: (1) increased endocrine pancreas function resulting in stimulation of the hepatic second messenger system cyclic AMP; (2) the second messenger system may than be responsible for changes in the enzymatic machinery which results in amino acid uptake and glycogen breakdown; and (3) thereafter, the amino acids are utilized for protein synthesis resulting in the documented changes in serum proteins such as α_2 macrofetoproteins, and the glycoproteins associated with infection and LEM treatment. The glucose

resulting from glycogenolysis is probably utilized as an energy source in protein synthesis and incorporated into the acute phase glycoproteins.

In conclusion we are investigating a new working hypothesis for the metabolic actions of LEM. The formulation of this hypothesis was hastened by the collaborative efforts of Work Unit Nos., 834 01 020, 834 01 401, 834 03 011, and 834 01 003.

Publications:

1. George, D. T., F. B. Abeles, and M. C. Powanda. 1975. Alterations in plasma glucose, insulin and glucagon induced by a leukocyte derived factor(s). Clin. Res. 23:320A.

2. George, D. T., and E. J. Rayfield. 1974. L-dopa induced plasma glucagon release. J. Clin. Endocrinol. Metab. 39:618-621.

3. George, D. T., E. J. Rayfield, and R. W. Wannemacher, Jr. 1974. Altered glucoregulatory hormones during acute pneumococcal sepsis in the rhesus monkey. Diabetes 23:544-549.

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1. Beisel, W. R. 1972. Interrelated changes in host metabolism during generalized infectious illness. Am. J. Clin. Nutr. 25:1254-1260.

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3. Curnow, R. T., E. J. Rayfield, D. T. George, T. V. Zenser, and F. R. DeRubertis. 1975. Control of hepatic glycogen metabolism in the rhesus monkey: effect of glucose, insulin and glucagon administration. Am. J. Physiol. 228:80-87.

4. Zenser, T. V., F. R. DeRubertis, D. T. George, and E. J. Rayfield. 1974. Infection-induced hyperglucagonemia and altered hepatic response to glucagon in the rat. Am. J. Physiol. 227:1299-1305.

5. Burr, I. M., A. Jackson, S. Culbert, R. Sharp, P. Felts, and W. Olson. 1974. Glucose intolerance and impaired insulin release following 6-hydroxy-dopamine administration to intact rats. Endocrinology 94:1072-1076.

6. Wilson, J. P., R. W. Downs, Jr., J. M. Feldman, and H. E. Lebovitz. 1974. Beta cell monoamines:further evidence for their role in modulating insulin secretion. Am. J. Physiol. 227:305-312.

7. Wannemacher, Jr., R. W., R. S. Pekarek, W. L. Thompson, R. T. Curnow, F. A. Beall, T. V. Zenser, F. R. DeRubertis, and W. R. Beisel. 1975. A protein from polymorphonuclear leukocytes (LEM) which affects the rate of hepatic amino acid transport and synthesis of acute-phase globulins. Endocrinology 96:651-661.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6428	75 07 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
74 07 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	62760A	3A762760A834	01	802
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Microbial toxins and their role in the pathogenesis of disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
66 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)
FISCAL YEAR	PRECEDING 75	1.0	120.6
	CURRENT 76	1.0	84.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Pathology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Metzger, J. F.

TELEPHONE: 301 663-7211

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Johnson, A. D.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Enterotoxin; (U) Staphylococcus; (U) Escherichia coli; (U) Military medicine; (U) Isotopic tracers; (U) Antigen; (U) Antibody

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

23 (U) Study production and purification and characterize microbial toxins. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Purify toxins of microbial origin in order to study the pharmacologic effects.

25 (U) 74 07 - 75 06 - Optimal medium for production of E. coli enterotoxin was investigated and defined. Fermentation methods for the production of Staphylococcus aureus exfoliative toxin were developed. A purification method for this toxin was developed. Amino acid analysis of this toxin as well as the C terminal amino acid were defined.

Publications: Infect. Immunity 10:503-509, 1974. 12: in press, 1975.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 802: Microbial Toxins and Their Role in the Pathogenesis of Disease

Background:

Microbial toxins play an important role in the pathogenesis of many militarily important infectious diseases. Enterotoxins may be an important factor in causing diarrhea,[1] especially in Asian countries. The Staphylococcus aureus enterotoxins have been studied widely, but only recently has the possible importance of other enterotoxins been investigated.[2] There is now building a quantity of scientific data concerning the Escherichia coli enterotoxins.[3,4] In addition there are a number of other toxins which manifest their effects in ways other than through the gastrointestinal tract. These include the exotoxin of Pseudomonas[5] and the exfoliant toxins of S. aureus.[6]

To better understand the role of microbial toxins, investigations have been centered on: (1) development of fermentation procedures for production, and (2) purification. To date, only crude preparations have been available in sufficient quantity for physiological studies. In order to interpret the data, more purified products must be used, otherwise contaminating substances could be responsible for the changes observed.

Progress:

E. coli enterotoxin. One human strain (408-3) and one porcine strain (P 307) of enterotoxigenic E. coli were utilized to produce fermentor lots of toxins for physiological and purification studies. Three culture media were studied: (1) Syncase (Difco Casaminoacids), (2) Syncase (Sheffield Casaminoacids), and (3) Evan's Medium (Casaminoacids with yeast extract). Syncase (Difco) was superior to the Evan's Medium and toxin production was markedly inhibited by the Sheffield product. This inhibition by the Sheffield product appeared to be due to its high Fe content. Syncase preparations concentrated with either XM-50 or XM-100A Amicon membranes had a dry weight activity between 50-100 µg in the rabbit loop assay and 0.1-0.01 µg in the adrenal cell assay of Donta et al.[7]

Filtrate of an XM-50 crude concentrated culture through an XM-300 was assayed for enterotoxicity. The activity of this filtrate was demonstrated in both rabbit loops and adrenal cells. This confirmed that the enterotoxin is present in 2 MW forms in 24-hr culture filtrate. The largest amount of

the activity is retained on an XM-300 membrane and is of extremely high MW.

Kinetics of E. coli enterotoxin release in the culture supernatant was established utilizing standard fermentation conditions (400 rpm agitation and 24 L/min of aeration) in Syncase medium with an inoculum of strain 408-3. Hourly samples were assayed in both adrenal cells and the rabbit ileal loop.

TABLE I. KINETICS OF E. COLI ENTEROTOXIN RELEASE DURING FERMENTATION.

HR	RESPONSE (TITER)	
	Adrenal Cells	Rabbit Loop
0	-	-
1	-	-
2	-	-
3	+ (1:10)	-
4	+ (1:20)	-
5	+ (1:20)	-
6	+ (1:80)	+ (1:2)
7	+ (1:80)	+ (1:2)
8	+ (1:100)	+ (1:2)
23	+ (> 1:150)	+ (> 1:8)

Exfoliative toxin. An exoprotein of certain strains of Staphylococcus has been described which is responsible for the exfoliation of newborn mice and human infants.[8,9]

S. aureus strains SA and TA were tested for production of the exfoliative toxin. Cultures were propagated in shake flasks of trypticase-yeast medium and in dialysis sacs containing medium 199 in rabbit peritoneum. In vitro cultivation was chosen as the most applicable method of large scale production. Optimal fermentor conditions were established for growth in a 50-L vessel from Fermentation Design. Trypticase-yeast medium was used (pH 7.1) at 37 C; air sparging at a rate of 10 L/min of a gas mixture of 95% air and 5% CO_2; and an agitation rate of 400 rpm. After 20 hr of fermentation, the culture was centrifuged. The supernatant was concentrated 10-fold using an Amicon TC3E system with UM-10 membranes. After concentration, the product was dialyzed and lyophilized.

Ion-exchange chromatography columns were used to purify the toxin. Carboxy methyl cellulose was equilibrated in 0.01 M phosphate buffer at pH 6.0. The toxin was eluted with phosphate buffer at 0.07 M, pH 6.8. Active fractions were combined and dialyzed.

Hydroxypapatite was equilibrated in 0.03 M phosphate buffer, pH 5.7. The toxin was selectively removed from the column by a linear gradient from 0.2 M, pH 5.7 to 0.4 M, pH 5.7. Two distinct peaks were obtained. The first peak contained the exfoliative toxin and the second, smaller peak was found to contain α-hemolysin.

Testing in newborn mice proved that 0.5 µg of the pure toxin caused the mice to lose skin upon gentle rubbing (Nikolsky's sign). The toxin is somewhat resistant to heat inactivation (approximately 50% of the activity remains after heating at 56 C for 30 min).

Isoelectric focusing was employed as an analytical tool. This procedure revealed that the pure toxin is a single component with an isoelectric point of 7.6 (4 C).

Presentation:

Metzger, J. F. Escherichia coli enterotoxin. Presented at 2nd Gordon Research Conference on Microbial Toxins, 22-26 July 1974, Wolfeboro, NH.

Publications:

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9. Kapral, F. A., and M. M. Miller. 1971. Product of Staphylococcus aureus responsible for the scalded-skin syndrome. Infect. Immunity 4:541-545.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6429	75 07 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
74 07 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	62760A	3A762760A834	01	803
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114 (e) (f)			

11. TITLE (Precede with security classification code)

(U) Subcellular biological effects of microbial disease and toxins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 016800 Toxicology; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
60 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 75	1.0	100.0
	CURRENT 76	1.0	217.3

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Canonico, P. J.

TELEPHONE: 301 663-7341

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATOR(s)

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede each with security classification code)

(U) Lysosomes; (U) Peroxisomes; (U) Hepatocytes; (U) Streptococcus pneumoniae; (U) Francisella tularensis; (U) Opsonins; (U) Amino acid transport

23. TECHNICAL OBJECTIVE, 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with security classification code.)

23 (U) Study the effects of infection and/or intoxication upon distribution and integrity of subcellular organelles and to determine the role of tissue enzyme changes in infectious disease. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) A variety of techniques, e.g., fractionation, electron microscopy are used to study subcellular action of toxins and microbial diseases.

25 (U) 74 07 - 75 06 - Autoradiographic and biochemical studies confirmed that, in contrast to mononuclear phagocytes, polymorphonuclear leukocytes from humans, monkeys and rats ingest F. tularensis only in the presence of specific immune serum. This observation led to the development of a sensitive in vitro radiometabolic assay for detection of specific opsonizing antibody to this organism.

The biochemical lesions of hepatic peroxisomes which accompany bacterial infection has been shown to be a nonspecific host response to inflammation, which is tissue specific to liver, and not kidney or lung.

Primary monolayer cultures of rat hepatocytes show characteristics of active transport for amino acids, i.e., alpha-amino-isobutyric acid (AIB). The uptake of AIB by cultured hepatocytes is enhanced by glucagon and unaffected by leukocytic endogenous mediator.

Publications: Infect. Immunity 11:146-151, 466-469, 12: In press, 1975.

* Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 803: Subcellular Biological Effects of Microbial Disease and Toxins

Background:

Complex enzymatic and metabolic events in the host are required for efficient defense against infection by microorganisms. However, certain bacteria can mitigate host defense mechanisms by altering the normal physiology of cells and their organelles, promoting cellular dysfunction and enhancing host susceptibility to infectious disease.[1] Characterization of the morphology and function of cells and their organelles in normal and diseased animals is, therefore, essential for delineating host defense mechanisms to infection and inflammation, differentiating between specific and nonspecific host responses, and clarifying mechanisms of microorganism-induced pathophysiology.

Progress:

I. We previously reported that Francisella tularensis, a facultative intracellular parasite, failed to activate granulocyte hexose monophosphate shunt (HMP) activity in the absence of specific immune serum.[2] Since HMP activation accompanies phagocytosis, these data suggested that F. tularensis is not phagocytized by monkey peripheral PMN. The interaction of F. tularensis with rhesus monkey PMN was, therefore, further assessed by autoradiographic and biochemical techniques.

Autoradiography was employed because the pleomorphism and extremely small size (0.2 - 0.7 μm) of this organism precluded detection by light microscopy of its presence within PMN. When F. tularensis, living vaccine strain (LVS), radiolabeled with ^{3}H-uridine, was cultured with PMN in the presence of normal serum, no intracellular localization of bacteria was observed; in contrast, when specific immune serum was employed, characteristic silver grain development in autoradiographs appeared over the cytoplasm of most PMN.

Circulating PMN from humans and rats reacted similarly to those of monkeys, i.e. immune serum was required for phagocytosis of F. tularensis. In contrast to PMN, oil-induced monkey peritoneal mononuclear (MN) phagocytes ingested F. tularensis in the absence of specific opsonins.

The burst in oxidative metabolism mediated through activation of the HMP shunt, which accompanies particle ingestion by PMN, was used as an indicator for phagocytosis. Release of $^{14}CO_2$ from the oxidation of radiolabeled glucose was increased significantly when monkey PMN and Salmonella typhimurium, Escherichia coli, or latex beads were incubated at a particle-to-cell ratio of 100:1. In contrast, activation failed to occur when PMN were incubated with F. tularensis (LVS or SCHU S4) at the same bacteria-to-cell ratio, or at a ratio of 1000:1, although heat-killed LVS organisms stimulated a small but significant increase in HMP shunt activity. In contrast, marked activation of the shunt occurred when specific immune serum was used as opsonin. Enhanced stimulation was obtained by addition of guinea pig complement.

Exploratory studies were conducted to investigate the possibility that LVS organisms contained an antiphagocytic substance. Neither living nor heat-killed LVS organisms were capable of modifying the bactericidal capacity of PMN for E. coli in the reaction mixtures, implying that phagocytosis of E. coli was unaffected by LVS organisms. Likewise, addition of LVS cell lysates or polysaccharide did not inhibit HMP shunt activation of PMN during phagocytosis of latex spheres or E. coli (Table I).

TABLE I. EFFECT OF LVS LYSATE AND POLYSACCHARIDE ON HMP SHUNT ACTIVITY OF PHAGOCYTIZING MONKEY PMN AS MEASURED BY OXIDATION OF [1-^{14}C]GLUCOSE.

CULTURE COMPONENTS[a]	NO. EXPERIMENTS IN TRIPLICATE	MEAN CPM/10^6 PMN/30 MIN ± SD
PMN + NS	20	175 ± 26
PMN + NS + PS	6	305 ± 71
PMN + NS + LYS	4	301 ± 85
PMN + IM + PS	2	202 ± 34
PMN + IM + LYS	4	695 ± 110**
PMN + LTX	3	1,700 ± 175**
PMN + LTX + PS	4	1,620 ± 52**
PMN + LTX + LYS	3	1,685 ± 41
PMN + E. coli	3	455 ± 81**
PMN + NS + E. coli	6	1,420 ± 252**
PMN + NS + E. coli + PS	7	2,940 ± 250**
PMN + NS + E. coli + LYS	3	2,900 ± 210**

a. NS, normal serum; PS, polysaccharide; LYS, bacterial lysates; IM, immune serum; LTX, latex beads.

** P < 0.01, relative to PMN + NS.

A significant, small increase in HMP shunt activity occurred when immune sera plus bacterial lysates were added to PMN control mixtures, suggesting that cell-wall fragments were phagocytized in much the same manner as non-disrupted LVS cells.

These results clearly illustrate that PMN *in vitro* in contrast to mononuclear phagocytes, are unable to phagocytize *F. tularensis* in the absence of immune serum. This inability deletes one of the natural barriers to its dissemination to target organs since limitation of an infection during the first 18 hr is primarily dependent upon rapidly migrating PMN. The later migration of MN phagocytes to inflammatory lesions obviates their ability to control the early stages of infection. Hence, it is only in the immune host that PMN can play a significant role by limiting or preventing the early dissemination of *F. tularensis* to intracellular sites of specific target tissues.

The demonstration that monkey PMN phagocytize *F. tularensis* only in the presence of immune serum led to development of an *in vitro* radiometabolic assay for detection of specific opsonizing antibody to this organism. The burst in oxidative metabolism that accompanies ingestion of opsonized organisms by PMN and results in release of $^{14}CO_2$ from radiolabeled glucose was used as an indicator for detection of specific opsonizing antibodies.

The radiometabolic phagocytic assay for opsonizing antibody was performed by adding 0.025 ml of test serum, 0.1 ml of LVS suspension, and 0.2 ml of Hanks balanced salt solution (HBSS) containing 4 mM KCN and 0.5 μCi of [1-^{14}C]glucose to a nonsiliconized plastic test tube fitted with a rubber cap and hanging plastic center well containing filter paper saturated with 0.1 ml of hyamine-OH. After a 15-min opsonization period, 0.1 ml of leukocyte suspension was added and the reaction mixture was incubated for 30 min at 37 C with vigorous shaking. The reaction was stopped by injecting 0.5 ml of trichloracetic acid; released $^{14}CO_2$ was collected during an additional 45-min incubation. The plastic center well was then transferred to a scintillation vial containing 10 ml of Scintolute and radioactivity was counted in a liquid scintillation counter. Opsonizing activity was equated to HMP shunt activation and reported as CPM of $^{14}CO_2$ released from the oxidation of [1-^{14}C]glucose by 10^6 PMN/30 min.

The temporal appearance of opsonizing antibody to *F. tularensis* was determined in 2 groups of 4 monkeys following vaccination with either 10^8 or 10^4 LVS. Opsonizing antibody development in these monkeys was compared with geometric mean microagglutinating (MA), and CF antibody titers.

Serum opsonizing activity in monkeys vaccinated with 10^8 organisms reflected a bimodal response (Table II). Opsonizing activity, observed as early as day 3, was significantly elevated over prevaccination values by day 4 and peaked by day 11. Opsonizing activity declined moderately through day 30 but rebounded to a 2nd peak at day 44. HA titers in this group of monkeys were significantly elevated by day 4; maximal titers were achieved by days 9 and 11 and persisted through day 30 but, in contrast to serum opsonizing

activity, decreased thereafter. MA titers generally followed the same course as HA titers but were substantially lower and essentially negative by day 60. In contrast to HA and MA titers, CF activity did not appear until the 3rd week. Maximum CF activity was observed on day 30 and paralleled the appearance of the 2nd phase of opsonic activity.

TABLE II. SEROLOGICAL ANTIBODY ACTIVITY IN 4 MONKEYS VACCINATED WITH 10^8 F. TULARENSIS, LVS.

DAY RELATIVE TO VACCINATION	OPSONIC TITER	RECIPROCAL TITER		
	Mean CPM/30 min/10^6 PMN	MA	HA	CF
-5	117	0	10	4
-2	134	0	8	4
0	174	0	8	4
1	106	0	8	4
2	201	0	6	4
3	466	4	8	4
4	813	70	40	4
5	1346	226	450	5
6	1271	224	1240	6
7	1068	220	1060	6
9	1655	185	1390	7
11	1722	218	1395	8
13	1295	216	1045	23
15	1170	230	910	38
19	1104	255	1230	101
23	1183	185	1060	152
26	1011	150	1070	181
30	862	120	1210	215
37	1446	118	720	128
44	1718	82	490	107
51	1547	50	480	51
58	1419	4	380	64

One of 4 monkeys vaccinated with 10^4 organisms failed to develop opsonic activity or HA, MA or CF titers. In the remaining monkeys (Table III), serum opsonins were first detected on day 5, were significantly elevated by day 13, reached maximum by day 37, and remained elevated through day 60. Maximum opsonic activity in this experimental group was not appreciably different from maximum values observed in monkeys vaccinated with 10^8 organisms. Other antibody responses were markedly different from that of the group immunized with 10^8 LVS; titers developed slowly and failed to achieve the magnitude of the previous response. Likewise, MA titers developed slowly, with moderate elevation observed from days 26 to 45. CF titers were only slightly elevated during the later phase of the experimental period.

TABLE III. SEROLOGICAL ANTIBODY ACTIVITY IN 4 MONKEYS VACCINATED WITH 10^4 F. TULARENSIS, LVS.

DAY RELATIVE TO VACCINATION	OPSONIC TITER	RECIPROCAL TITER		
	Mean CPM/30 min/10^6 PMN	MA	HA	CF
-5	134	0	4	5
-2	143	0	0	5
0	197	0	4	5
1	148	0	6	5
2	182	0	2	5
3	187	0	0	4
4	116	0	6	4
5	249	0	8	4
6	205	0	4	4
7	228	0	4	4
9	246	4	12	5
11	196	2	15	5
13	321	6	10	5
15	802	8	8	5
19	1123	20	75	8
23	1218	26	110	6
26	1239	48	210	6
30	1025	46	206	16
37	1708	75	310	10
44	1543	48	270	8
51	1655	27	290	8
58	1472	0	80	8

The MA technique has been routinely used as a serological screen for F. tularensis-specific antibodies in humans. Our data, however, suggested that this technique was relatively insensitive. In fact, 4 of 7 monkeys with positive serum opsonic activity had no MA activity at 60 days. Consequently, sera from individuals vaccinated against F. tularensis were obtained from a serum bank and evaluated for opsonizing and MA activities. Results shown in Table IV confirm the insensitivity of the MA test. With the exception of 1 subject, sera from vaccinated individuals having nondiagnostic MA titers (< 1:20) demonstrated opsonic activity as long as 13 yr.

TABLE IV. COMPARISON OF MA AND OPSONIC ACTIVITIES IN F. TULARENSIS-VACCINATED AND NONVACCINATED SUBJECTS.

SUBJECT	STATUS OF IMMUNIZATION	ELAPSED TIME SINCE IMMUNIZATION (~ year)	RECIPROCAL MA TITER	OPSONIC ACTIVITY[a] (CPM)
1	-		0	105
2	-		0	210
3	-		0	355
4	-		0	230
5	-		0	200
6	-		0	210
7	-		0	260
	Group Mean ± SD			224 ± 75
8	+	0.1	0	760[a]
9	+	0.1	0	2130[a]
10	+	0.2	0	2450[a]
11	+	0.7	0	2880[a]
12	+	13	0	510[a]
13	+	13	0	270[a]
14	+	0.7	20	430[a]
15	+	0.7	20	700[a]
16	+	5	20	2170[a]
17	+	5	20	1170[a]
18	+	13	20	1820[a]
19	+	13	20	1200[a]
20	+	4	40	1160[a]
21	+	0.1	80	2460[a]
22	+	13	80	2430[a]
23	+	14	160	2080[a]

a. > 2 SD from controls.

The sensitivity for detecting interactions between microorganisms and immune serum is affected by the complex nature of the reactants involved. Conventional serological techniques are dependent upon detection of an antibody-induced change in the physical characteristics of either soluble or particulate antigens, i.e. agglutination or precipitation. In contrast, the radiometabolic assay for opsonic activity is a measure of the enzymatic response of PMN to phagocytized antigen-antibody complexes. This technique proved highly sensitive for detection of antibody against F. tularensis, which is phagocytized only in the presence of immune serum.

Antibodies involved in *in vitro* reactions are not necessarily a measure of host resistance to infection. For example, no correlation exists between agglutinin titers and protection in rats immunized against *F. tularensis*. Opsonic activity, by measuring the capacity of serum to enhance phagocytosis, may provide a better estimate of host defense capability to limit dissemination of infecting microorganisms.

II. Studies to characterize the effects of infection on the functional and morphological properties of peroxisomes, subcellular organelles of animal cells,[3] were continued.

Assays were performed to determine the activity of the peroxisomal enzymes catalase (CAT), amino acid oxidase (AAO) and urate oxidase (UO) in rat liver, kidney and lung following SC inoculation with 10^4 *Streptococcus pneumoniae* (Table V).

TABLE V. PEROXISOMAL ENZYME ACTIVITY IN RAT TISSUES FOLLOWING SC INOCULATION WITH 10^4 *S. PNEUMONIAE*.

TIME (Hr)	Liver	Kidney	Lung
	CATALASE (μM/MIN/mg PROTEIN) ± SE		
0	170 ± 9	75 ± 5	33 ± 6
24	135 ± 11	70 ± 14	41 ± 8
48	99 ± 17	57 ± 7	27 ± 6
72	69 ± 7	68 ± 2	25 ± 3
	URATE OXIDASE (nM/MIN/mg PROTEIN) ± SE		
0	4.08 ± 0.21	0.85 ± 0.06	1.11 ± 0.06
24	3.30 ± 0.17	---	---
48	2.73 ± 0.14	0.93 ± 0.13	1.44 ± 0.12
72	2.83 ± 0.27	0.86 ± 0.04	1.18 ± 0.02
	D-AMINO ACID OXIDASE (nM/MIN/mg PROTEIN) ± SE		
0	10.0 ± 0.9	42.3 ± 5.6	5.6 ± 0.6
48	8.5 ± 0.9	38.9 ± 3.2	7.5 ± 0.7
72	9.7 ± 1.5	39.7 ± 4.8	5.2 ± 0.1

CAT and UO activity were significantly depressed in liver but not in kidney and lung. AAO activity, assayed at 48 and 72 hr was not altered in any of the 3 tissues. To determine whether the observed changes in liver CAT and UO activities were specific to infection or, instead, a general response to inflammation, enzymatic activities were determined following injection of a sterile talcum suspension (Table VI). CAT and UO, but not AAO, were maximally depressed in liver 48 hr after the inflammatory challenge. The activity of these enzymes in kidney and lung was not altered.

TABLE VI. PEROXISOMAL ENZYME ACTIVITY IN RAT TISSUES FOLLOWING INOCULATION WITH STERILE TALC.

TIME (Hr)	Liver	Kidney	Lung
	CATALASE (μM/MIN/mg PROTEIN) ± SE		
0	163 ± 7	57 ± 3	35 ± 6
24	127 ± 1	52 ± 33	26 ± 4
48	118 ± 8	67 ± 77	23 ± 5
72	130 ± 4	46 ± 2	20 ± 5
	URATE OXIDASE (nM/MIN/mg PROTEIN) ± SE		
0	4.60 ± 0.16	0.72 ± 0.11	0.90 ± 0.10
48	2.53 ± 0.56	0.72 ± 0.07	0.87 ± 0.10
	D-AMINO ACID OXIDASE (nM/MIN/mg PROTEIN) ± SE		
0	9.0 ± 1.1	41.4 ± 2.7	5.2 ± 1.4
48	7.8 ± 0.7	47.4 ± 5.4	5.3 ± 1.6

These studies demonstrate that the reported decrease in peroxisomal activity during infection is in fact a nonspecific response to inflammation. This response is tissue-specific since it occurs in liver but not in kidney or lung. Finally not all peroxisomal enzymatic activities, e.g. AAO, are depressed during infection. This observation is consistent with previous morphologic data demonstrating that during pneumococcal infection the number of liver peroxisomes does not decrease by more than 50%. Since CAT and UO were shown to decrease by as much as 90% it follows that some peroxisomal components must not be adversely affected during infection.

It is anticipated that knowledge of which enzymes are depleted and which are spared during inflammation will assist in evaluating the merit of proposed function of these organelles in regulating gluconeogenesis and fatty acid and cholesterol metabolism.[4]

To determine whether the reduction in CAT and UO activity is due to decreased synthesis or an increased catabolic rate monospecific antibodies to these enzymes are being prepared. To date ~10 mg of rat liver CAT has been purified. Antibodies to this antigen will be produced in rabbits and goats.

III. The liver plays an important role in host defense mechanisms against infection. Accordingly, many studies have been reported to define hepatic functions during infectious illness. Such studies, however, employing in vivo, isolated perfusion and liver-slice techniques cannot often differentiate between primary hepatic responses and secondary responses, or secondary responses mediated by extrahepatic factors. Furthermore, metabolic and functional interactions between different cell types of the liver cannot be evaluated by the methods cited above. For these reasons, many workers have attempted to isolate and purify viable hepatic parenchymal cells (HPC).

Freshly isolated suspensions of rat liver cells, however, exhibit a number of altered metabolic and morphologic properties. To circumvent these problems, methods for establishing primary nonproliferating monolayer cultures of isolated hepatocytes have been described. These systems permit the separation of irreversibly damaged cells from undamaged or minimally damaged cells and allow for repair of minor cellular injuries incurred during isolation. Such cultures demonstrate a number of metabolic functions characteristic of liver in vivo.

Employing primary monolayer cultures of rat hepatocytes we have examined the effects of leukocytic endogenous mediator (LEM) and glucagon on the transport of the nonmetabolizable amino acid analogue, α-amino-isobutyric acid (AIB).

In contrast to fresh suspension, cultured HPC were not freely permeable to AIB and showed a nearly linear rate of uptake during the first 2 hr when incubated at 37 C. The uptake of AIB appeared to be energy dependent since (1) it was inhibited by 2 mM KCN and 10 mM iodoacetate and (2) failed to occur when cells were incubated at 4 C. The addition of 100 nM glucagon stimulated the uptake of AIB by ~23%. Both crude or partially purified $(NH_4)_2SO_4$ preparations of LEM failed to stimulate amino acid transport in these cells.

The apparent Km for AIB uptake by monolayer cultures of HPC was calculated to be ~19 mM. Glucagon did not alter the apparent Km but did increase V_{max} by 20%. Neither Km nor V_{max} were affected by the addition of LEM.

Results of other investigations employing in vivo or isolated perfused liver techniques suggest that LEM directly enhances amino acid uptake by the liver.[5] In contrast, the present study which evaluated amino acid transport in homogeneous populations of cultured, hormone-sensitive, adult parenchymal liver cells suggests that LEM does not have a direct effect on amino acid transport in liver.

Publications:

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2. Proctor, R. A., J. D. White, E. Ayala, and P. G. Canonico. 1975. Phagocytosis of Francisella tularensis by rhesus monkey peripheral leukocytes. Infect. Immunity 11:146-151.

3. Canonico, P. G., A. T. McManus, J. A. Mangiafico, L. S. Sammons, V. G. McGann, and H. G. Dangerfield. 1975. Temporal appearance of opsonizing antibody to Francisella tularersis: detection by a radiometabolic assay. Infect. Immunity 11:466-469.

4. Canonico, P. G., M. C. Powanda, G. L. Cockerell, and J. B. Moe. Relationship of β-glucuronidase and lysozyme to pathogenesis to tularemia in immune and nonimmune rats. Infect. Immunity 12: In press.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6429	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. SMSN MNTH'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 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	62760A	3A762760A834	01	804
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Physiology of membrane alterations in staphylococcal enterotoxin production

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. STARY DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 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 75	0.5	23.8
FISCAL YEAR CURRENT 76	0.5	43.3

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Pathology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Altenbern, R. A.

TELEPHONE: 301 663-7211

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Metzger, J. F.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Physiology; (U) Enterotoxin, Staphylococcus; (U) Bacterial genetics

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 the physiological and genetic basis of the secretion of enterotoxin from selected strains and mutants of Staphylococcus aureus. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Study of the amounts of enterotoxin produced under a variety of experimental conditions by certain specific mutations induced in the parent strains.

25 (U) 74 07 - 75 06 - A procedure has been developed for greatly enhancing enterotoxin B production of S. aureus by isolating carbohydrate-negative mutants from previously isolated membrane mutants. This regimen works well for 2 strains and has increased enterotoxin production in one strain 100-fold. Success has also been attained by applying this method to increasing production of type D enterotoxin.

Publication: Can. J. Microbiol. 21:275-280, 1975.

* Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 01: Pathogenesis of Infection of Military Importance

Work Unit No. 834 01 804: Physiology of Membrane Alterations in Staphylococcal Enterotoxin Production

Background:

It has been demonstrated that some mutagen-induced membrane alterations increase enterotoxin B production by a factor of 2 to 3. These membrane mutants have radically altered membrane structure and/or composition by virtue of their osmotic stability and response to dye inhibition and enterotoxin production at different growth temperatures.

Progress:

Attempts to isolate carbohydrate-negative (car) mutants from higher SEB-producing membrane mutants from both strain 14458 and 778 has succeeded. A few of these double mutants are strikingly enhanced in SEB production by a factor of up to 100. In addition, SED production by S. aureus strain 494 has been increased 12-fold by isolating, in sequence, membrane mutants and their car mutants from selected membrane mutants. It is felt, at present, that this procedure is a general method for enhancing enterotoxin production starting with any wild type of S. aureus.

Further investigation has shown that some single sugar fermentation mutants also show increased enterotoxin production. By suitable genetic techniques, strong indications have been obtained that the mutation leading to a fermentation defect also leads to greatly altered enterotoxin formation. Some of the enzymes of sugar fermentation are bound to the S. aureus cell membrane and, it is assumed currently, that mutated fermentation enzymes distort the cell membrane sufficiently to increase secretion of enterotoxin.

This technique will be applied to other extracellular proteins produced by S. aureus to determine if elaboration of other staphylococcal toxins can be controlled by similar genetic manipulations.

Publication:

Altenbern, R. A. 1975. Membrane mutations and production of enterotoxin B and alpha hemolysin in Staphylococcus aureus. Can. J. Microbiol. 21:275-280.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6411	75 07 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
4 07 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	3A061101A91C	00	132
b. CONTRIBUTING	62760A	3A762760A834	01/805	
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Catecholamines and serotonin response studies to bacterial toxins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 012600 Pharmacology; 002300 Biochemistry

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: 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 75	1.0	30.0
FISCAL YEAR CURRENT 76	1.0	43.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Physical Sciences Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Bailey, P. T.

TELEPHONE: 301 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Catecholamines; (U) Serotonin; (U) Tryptophan; (U) Toxins; (U) Nervous system;

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

23 (U) Determine effects of bacterial toxins on catecholamines and serotonin in the peripheral and central synaptic areas of the body. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Study effects of Escherichia coli lipopolysaccharide on levels of dopamine, norepinephrine and serotonin in selected brain areas in rats with attempts to correlate them with other clinical findings. Monitor their alterations throughout the course of various infectious diseases after treatment with pharmacological agents.

25 (U) 74 07 - 75 06 - Studies were completed of endotoxin-tryptophan poisoned rats. There was an increase in serotonin and dopamine levels in all the brain areas studies during endotoxemia; while norepinephrine levels decreased. Preliminary studies indicated that the increased lethality of endotoxin-tryptophan poisoned rats was the result of hypoglycemia.

* 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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 3A061101A91C: In-House Laboratory Independent Research (U)
(3A762760A834):

Task No. 3A061101A91C 00:
(3A762760A834 01): (Pathogenesis of Infection of Military Importance)

Work Unit No. 91C 00 132: Catecholamines and Serotonin Response Studies to
(834 01 805): Bacterial Toxins

Background:

Many investigators have assigned a major role to catecholamines and serotonin in mediating the effects of endotoxin. Recently Moon and Berry[1] postulated that a primary metabolic effect of endotoxin is to impair enzymatic regulation, thereby upsetting metabolic homeostasis in the host. Their studies showed that mice treated with tryptophan 4 hr after endotoxin died convulsively and in larger numbers than those given endotoxin alone. Morris and Moon[2] stated that the excess tryptophan was being shifted from the kynurenine to the serotonin pathway in the endotoxin-poisoned mice. The observation that cyprohepatadine (a serotonin antagonist) protected the endotoxin-poisoned mice against a delayed injection of tryptophan indicated the possible involvement of serotonin as the cause of the convulsions. This observation disagrees with the majority of the available information which states that serotonin is not the putative neurotransmitter in convulsions.

Progress:

To elucidate the involvement of serotonin in convulsions as observed by Moon and Berry,[1] studies have been conducted with pharmacological agents that interfere with serotonin synthesis and metabolism in mice and rats. In order to block the conversion of tryptophan to serotonin, P-chlorophenylalanine (tryptophan hydroxylase inhibitor) was administered. This drug was found to significantly protect the rats during endotoxemia but not during the administration of endotoxin + tryptophan. The same results were also observed with α-methyl-dopa (decarboxylase inhibitor), 5,6-dihydroxytryptamine (neurotoxic agent capable of destroying serotonergic pathways), and probenecid (an agent which prevents serotonin metabolites from crossing the blood brain barrier).

Catecholamines have been shown to be elevated during convulsive states in laboratory animals. To eliminate catecholamine involvement, α-methyl-p-tyrosine (a tyrosine hydroxylase inhibitor) was administered and found to afford a greater degree of protection than serotonin antagonist. However, 6-hydroxydopamine (a drug which produces a chemical sympathectomy) was unable to afford any degree of protection.

Biochemical estimation of the biogenic amines in different brain areas (e.g., hypothalamus, diencephalon, brain stem, and cerebral cortex) indicated that an LD_{50} of endotoxin causes a significant increase in the levels of serotonin and dopamine, while norepinephrine decreases. Endotoxin + tryptophan-treated rats revealed significantly higher serotonin levels than seen in the endotoxin alone groups, suggesting that greater quantities of tryptophan are being converted to serotonin during endotoxemia. It is well known that animals given lethal doses of endotoxin die convulsively from hypoglycemia. In our studies severe hypoglycemia was noted in rats injected with tryptophan 4 hr after endotoxin, which was greater than endotoxin alone.

Based on these findings, the observed convulsions noted during endotoxin + tryptophan administration is most likely the results of hypoglycemia rather than serotonin.

Collaborative studies with Work Unit No. 834 01 801 have revealed a possible mechanism for the noted glucagon response after L-dopa treatment in rats. Pretreatment with RO44602 or α-methyl-dopa, 2 potent dopa decarboxylase inhibitors, abolished the secretion of glucagon observed after L-dopa administration, pointing out that L-dopa has to be converted to dopamine in order to stimulate the release of glucagon. Dopamine was found to elevate plasma glucagon to a greater degree than L-dopa. Inhibition of dopamine-β-hydroxylase by disulfiram or fusaric acid caused a very significant increase in plasma glucagon alone. The administration of haloperidol (an agent that blocks the dopaminergic receptor) blocked the L-dopa release of glucagon. Therefore, dopamine is considered to be one possible neuro-transmitter responsible for the release of glucagon.

Apomorphine, a dopamine receptor stimulation, failed to elevate plasma glucagon levels. This lack of effect of apomorphine may be due to the location of the receptor (intra- or extracellular).

Presentation:

Bailey, P. T. The role of serotonin during endotoxemia. Presented, Department of Pharmacology, Howard University School of Medicine, Washington, D. C. 12 Mar 1975.

LITERATURE CITED

1. Moon, R. J., and L. J. Berry. 1968. Role of tryptophan pyrrolase in endotoxin poisoning. J. Bacteriol. 95:1247-1253.

2. Morris, K. M., and R. J. Moon. 1974. Quantitative analysis of serotonin biosynthesis in endotoxemia. Infect. Immunity 10:340-346.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6410	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'D	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
74 07 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	62760A	3A762760A834	02	002
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Evaluation of experimental vaccines in man in BW defense research

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 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)
FISCAL YEAR PRECEDING 75	0	0
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Medical Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME: Edelman, R.
TELEPHONE: 301 663-7281
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Burke, D. S.
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Vaccines; (U) Immunization; (U) Military medicine; (U) Human volunteers; (U) Tularemia; (U) Skin test

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

23 (U) Evaluate experimental vaccines developed by various contractors, organizations or other governmental agencies. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Test vaccines are given to experimental animals, and when considered safe, to volunteers. Vaccinees are followed to evaluate the clinical and laboratory responses to vaccination and to assess the vaccine's protective efficacy in preventing laboratory-acquired infections.

25 (U) 74 07 - 75 06 - A review of preexisting data had indirectly confirmed the protective efficacy of VEE vaccine as determined from crude epidemiologic data from Fort Detrick, from the low overall incidence of reported breakthrough infections, and from evidence derived from attempts at revaccination. No cases of breakthrough infections occurred with VEE subtypes I-A, I-B or I-C. All 6 breakthroughs occurred with more distantly related virulent VEE subtypes. Cross-reactivity of serologic responses between WEE and VEE vaccines has been confirmed in prospective studies in humans. The fourth culture-proved breakthough of the LVS tularemia vaccine was diagnosed and treated. Three cases of aerogenic Rocky Mountain spotted fever in laboratory workers was diagnosed and treated.

Army Investigational Drug Review Board applications were submitted for (1) Venezuelan Equine Encephalomyelitis Vaccine, Inactivated, Dried, MNLBR 109; (2) Western Equine Encephalomyelitis Virus Vaccine, Inactivated, Dried, Lot 2-1974; and (3) Rocky Mountain Spotted Fever Vaccine, Inactivated SS Strain, Chick Embryo Cell Origin.

Publication: Ann. Intern. Med. 81:594-600, 1974.

* 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

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 002: Evaluation of Experimental Vaccines in Man in BW Defense Research

Background:

As a result of the termination of the draft, the availability of volunteers through Project Whitecoat has been severely limited. Research under this work unit has been confined to review and reassessment of preexisting information; observations made on at-risk laboratory workers; limited trials using volunteers drawn from the professional staff of USAMRIID; and submission of clinical research protocols to the Army Investigational Drug Review Board for the future testing of new vaccines.

Progress:

I. Attenuated VEE Vaccine. Data collection for a comprehensive review of the affects of attenuated VEE vaccines in man continues. To date, 7 different vaccine products have been used to immunize man. Table I presents the vaccine designation, the vaccine producer, and the total number of human vaccinees for each product. These data were obtained by combining all reports obtained from laboratories that used these vaccines.

In 1959, direct challenge of TC-80 vaccinees with virulent Trinidad VEE virus confirmed the protective efficacy of that vaccine. However, no direct challenge studies have been performed to test the immunity induced by the subsequent, more attenuated, vaccines. Nonetheless, a substantial body of indirect evidence attests to the probable efficacy of the products.

TABLE I. SUMMARY OF THE VACCINE SOURCE AND NUMBER OF VACCINEES FOR EACH OF THE SEVEN ATTENUATED VEE VACCINES

DESIGNATION	SOURCE	NO. OF VACCINEES
TC-50	Fort Detrick (Berge et al)	18
TC-80	Fort Detrick (Berge et al)	80
TC-81/2-4	Fort Detrick (McKinney et al)	63
TC-82/2-9	Fort Detrick (McKinney et al)	1,487
TC-93 NDBR-102	National Drug Co.	80
TC-83/3-2	Fort Detrick (McKinney et al)	∿ 2,000
TC-83 NDBR-102[a]	National Drug Co.	1,473

a. Vaccine currently in use.

First, since the adoption of routine vaccination of at-risk laboratory workers with the attenuated VEE vaccines, laboratory-acquired infections have virtually disappeared from Fort Detrick (Table II). Only one case of symptomatic laboratory infection with VEE has occurred since routine vaccination procedures were adopted in 1961.

TABLE II. OCCURRENCE OF SYMPTOMATIC INFECTIONS WITH VIRULENT VEE VIRUS AT FORT DETRICK, 1953-1974, BY 2-YEAR PERIODS

YEARS	NO. CASES/2-YEAR PERIOD
1953-54	2
1955-56	5
1957-58	5
1959-60	5
1961-62[a]	2
1963-64	0
1965-66	0
1967-68	0
1969-70	0
1971-72	0
1973-74	1

a. Routine use of attenuated VEE vaccines instituted.

Collection of all available data on approximately 5,000 persons vaccinated because of a high risk of development of virulent infections has disclosed only 10 persons who have contracted VEE despite prior immunization with an attenuated VEE vaccine. The breakthrough cases were detected in several laboratories (See Table III).

Analysis of these 10 infections disclosed that 4 vaccinees did not develop an antibody response to vaccination, and should therefore not be regarded as "breakthrough" infections, but instead as vaccination failures. Despite the fact that the vast majority of at-risk personnel were exposed only to the Trinidad or the Mexican epizootic (subtypes 1A and 1B) strains of virulent virus, no cases of breakthrough are known to have occurred with these subtypes. Of the 6 true breakthroughs of the immunity afforded by the attenuated VEE vaccines, all have occurred with virulent VEE subtypes more distantly related antigenically to the vaccine (1A) subtype. This information is summarized in Table III.

TABLE III. SUMMARY OF VEE BREAKTHROUGH INFECTIONS

ORGANIZATION,[a] YEAR (IDENTIFICATION) IMMUNIZING PREP.	YR TO INFECTION	VEE STRAIN (TYPE)	SEVERITY	SEROLOGY (NEUT OR HI)
MARU, 1961 (AC) TC-80	0.25	3880 (I-D)	2+	+
INV, 1962 (JDM) "Berge vaccine"	1	BeAn8007 Mucambo (III)	3+	+
USAMRIID, 1965 (PP) Killed + TC-82	1.8	Trinidad?	±	-
(GA)	2.4	Trinidad?	±	-
MARU, 1965 (LG) TC-83	1.0	3880 (I-D)	2+	+
Cornell, 1965 (MM) TC-83	1.75	63A216	2+	+
USAMRIID, 1967 (WD) Killed + TC-82	4.0	Trinidad?	±	-
MARU, 1968 (PF) TC-83	1.5	Mena II (I-E)	+	+
CDC (Mexico), 1972 (Ent.) TC-83	0.16	P1437 (I-B)	2+	-
USAMRIID, 1973 (NL) TC-83	4.0	?	2+	+

a. MARU: Middle America Research Unit, Panama
INV: National Virology Institute, Mexico
CDC: Center for Disease Control, Atlanta

Revaccination data further substantiates the protective efficacy of the attenuated VEE vaccines. Table IV depicts the plaque reduction neutralizing (PRN) antibody response to revaccination in 16 persons revaccinated because of a low or absent HI antibody level. The presence of detectable PRN antibody in recently vaccinated persons appears to correlate well with protection against reinfection with the attenuated vaccine virus. In persons vaccinated more than 4 yr previously, the presence of neutralizing antibody in the serum no longer appears to correlate with protection.

TABLE IV. REIMMUNIZATION WITH TC-83 VEE VACCINE: EFFECT OF TIME ELAPSED SINCE PREVIOUS TC-83 VEE VACCINATION AND EFFECT OF "PRE-REIMMUNIZATION" SERUM PRN ANTIBODY TITER

YEARS SINCE 1ST TC-83 VACCINATION	PRE-REIMMUNIZATION TITER RANGE	N	AVERAGE INCREASE IN TITER AFTER REVACCINATION	% INCREASED
< 1 yr	< 1:10	5	2.20	60
< 1 yr	≥ 1:10	7	0	14
≥ 4 yr	< 1:10, 1:10, 1:80, 1:160	4	2.75	75

In the last annual report, a boosting of VEE HI titers by WEE vaccination was reported. This cross-reactivity of HI antibody response to vaccination among group A arbovirus vaccines has been confirmed prospectively, and has been extended to suggest that a similar, but lesser, cross-reactivity between PRN antibody responses may also exist (Table V). At present no information exists to assess what effect, if any, this cross-reactivity has on the immunity of USAMRIID laboratory workers to VEE infections.

TABLE V. "BOOSTING" OF VEE ANTIBODY TITERS 18 PAIRED SPECIMENS BY SIMULTANEOUS "BOOSTING" IMMUNIZATIONS WITH INACTIVATED WEE AND EEE VACCINES

TEST	TITER (GMT) Before	After	P
HI	1:22	1:49	0.01
PRN	1:97	1:185	>0.05<0.10

II. Inactivated VEE Vaccine. An application to the Army Investigational Drug Review Board for the clinical use of Venezuelan Equine Encephalomyelitis Vaccine, Inactivated, Dried, MNLBR 109 (formalin-inactivated TC-83) has been submitted.

III. LVS Tularemia Vaccine. A 27 year old white male (S.L.), a laboratory technician, developed a paronychia of the left thumb with fever, lymphangitis, and axillary lymphadenitis. Cultures of the lesion yielded a moderate growth of Francisella tularensis; blood cultures were negative. Oral treatment with 500 mg tetracycline, 4 times/day for 10 days, resulted in the complete clearing of symptoms.

A review of the immunization history revealed that he had been inoculated with the LVS tularemia vaccine 2.5 yr previously and had developed a satisfactory microagglutination response to vaccination. This case is only the fourth culture-proven case of tularemia infection in a human vaccinated with this vaccine.

IV. Inactivated WEE Vaccine. (Protocol Med FY 75-1). During the period covered by this report, supplies of WEE vaccine, Lot 1-1967, became exhausted and acceptability trials of a Merrell-National Laboratories product, WEE MNLBR-106, were undertaken to evaluate the safety of this new vaccine. Volunteers were selected from the professional staff of USAMRIID who had received prior WEE immunization. Three subjects received 0.1 ml of the test vaccine ID, and 3 subjects were administered 0.5 ml of the test vaccine SC. No adverse reactions to vaccination were noted, either by clinical or laboratory assessment. Table VI illustrates the serologic responses to vaccination. On the basis of these data plus the absence of local responses to vaccination, the subject vaccine was deemed to have marginal antigenic potency in man.

TABLE VI. SEROLOGIC RESPONSES TO VACCINATION WITH WEE MNLBR-106

		RECIPROCAL TITER			
		HI		PRN	
VOLUNTEER	DOSE (ml)	Before	After	Before	After
A	0.1 ID	40	40	40	80
B	0.1 ID	40	80	160	1280
C	0.1 ID	320	320	1280	640
D	0.5 SC	40	40	160	160
E	0.5 SC	40	40	80	160
F	0.5 SC	20	40	160	320
GMT		40	50	160	284

However, as the results of the safety testing were acceptable, the WEE vaccine MNLBR-106 was considered suitable for further evaluation in at-risk laboratory personnel. The requirement that all vaccinees with WEE vaccine must demonstrate an antibody response to vaccination before laboratory exposure to virulent WEE is permitted, continues to be applied.

In the period covered by this report, an application for clinical use of an investigational drug was submitted to the U.S. Army Investigational Drug Review Board for an additional WEE vaccine, designated Western Equine Encephalomyelitis Virus Vaccine, Inactivated, Dried, Lot 2-1974, which had been produced at USAMRIID.

V. Rocky Mountain Spotted Fever. During the period covered by this report, 3 unrelated cases of RMSF were diagnosed among laboratory personnel. The diagnosis was confirmed by isolation of Richettsia rickettsii from acute blood specimens of all 3. The degree of illness ranged from a mild, low-grade, febrile illness with headache to a moderately severe, prostrating disease with shaking chills, confusion, and oral temperatures 104 F. All cases were treated with tetracycline and recovered without sequelae. Two had known exposure to aerosols of RMSF; in one case the route of infection is unknown and is presumed to be aerogenic.

An application to the Army Investigational Drug Review Board has been submitted for the clinical use of Rocky Mountain Spotted Fever vaccine, Inactivated SS Strain, Chick Embryo Cell Origin.

Publications

White, III, C. S., W. H. Adler, and V. G. McGann. 1974. Repeated immunization: possible adverse effects. Reevaluation of human subjects at 25 years. Ann. Intern. Med. 81:594-600.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6411	75 07 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
74 07 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	62760A	3A762760A834	02	003
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Evaluation of prophylaxis and therapy of infectious diseases in man

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 003200 BW, CW, RW

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
	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 75	0	0
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Medical Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME: Edelman, R.
TELEPHONE: 301 663-7281
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Burke, D. S.
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Prophylaxis; (U) Therapy; (U) Infectious diseases; (U) Volunteers; (U) Military medicine;

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

23 (U) Assess the effect of microbials and various drug regimens in various infectious diseases. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Various drugs are tested in volunteers under strict protocol conditions.

25 (U) 74 07 - 75 06 - As a concomitant of termination of the draft, the availability of human volunteers through Project Whitecoat has been severely limited. As such, no studies have been done in this work unit during the fiscal year.

* 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

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Agsinst Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 003: Evaluation of Prophylaxis and Therapy of Infectious Diseases in Man

Background:

The work unit was established to assess the effect of antimicrobials and various drug regimens for various diseases in man.

Progress:

As a concomitant of termination of the draft, the availability of human volunteers through Project Whitecoat has been severely limited. As such, no studies have been done in this work unit during the fiscal year.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB6416	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DSED'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
75 03 26	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A834	02	009
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Studies with diploid cell cultures for production of military vaccines

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 01	74 08	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 75	0.5	40.3
	CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Virology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: McManus, A. T.
TELEPHONE: 301 663-7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede each with Security Classification Code)

(U) Diploid cell culture; (U) WI-38; (U) Immunization; (U) Military medicine; (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) Evaluate human diploid cell cultures for use as substrate for preparation of viral and rickettsial vaccines. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Diploid cells are studied to establish the technical requirements to produce and quality-control cell strain substrates for human vaccine.

25 (U) 73 07 - 74 08 - A diploid cell line from fetal rhesus lung (DBS-FRhL-2) has been found to support the growth of dengue 2 virus to levels comparable to primary African green monkey tissue culture. A previously reported dengue 2 attenuated strain has been physically and biologically purified.

Large quantities of prequality-controlled fetal rhesus lung cells have been prepared. This should allow work at the same passage level and maintain a passage history for vaccine needs in certified cells.

The investigator has been transferred from the Institute. Any further experimental studies needed on diploid cells will be carried out by Dr. Cole under Work Unit 02 407, Accession No. DA OB6420.

Diploid cells for vaccine substrates will be provided by the tissue culture section of the division.

Publication: J. Biol. Standardization 2:329-332, 1974.
Trans. Roy. Soc. Trop. Med. Hyg. 69:179, 1975.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 009: Studies with Diploid Cell Culture for Production of Military Vaccines

Background:

Virus vaccine production is dependent upon the availability of primary animal tissue explants or avian eggs. The major disadvantages of these are logistical and biological. The supply of primary animal tissues, e.g., monkey kidneys, to be used as vaccine substrates may be limited in time of global emergency. This problem could be avoided by maintenance of large animal colonies in CONUS, although the cost of such colonies would be great.

The biological liability of using primary animal tissues for vaccine production is the possibility of contamination with adventitious agents, the presence of which must be determined for cell cultures prepared from each primary tissue. The cost and time factors involved in quality control measures can be reduced if a well characterized, contamination-free cultured diploid cell is used.

There are 2 major advantages to cultured diploid cells: (1) cells can be grown to large numbers quickly and stored indefinitely, and (2) cells from the stored seed can be tested exhaustively and well characterized.

Progress and Summary:

A diploid cell line from fetal rhesus lung (DBS-FRhL-2) was found to support the growth of dengue 2 virus to levels comparable to primary African green monkey tissue culture. A previously reported dengue 2 attenuated strain was physically and biologically purified.

Large quantities of prequality-controlled FRhL-2 cells were prepared, thus allowing work at the same passage level in certified cells.

The investigator has been transferred from the Institute. Any further experimental studies needed on diploid cells will be carried out by Dr. Cole under work unit 834 02 407.

Diploid cells for vaccine substrates will be provided by the tissue culture section.

Publication:

1. McManus, A. T., D. R. Parker, R. H. Kenyon, J. P. Kondig, and G. A. Eddy. 1974. Use of frozen chick and duck embryo cells for plaque assays of arboviruses and rickettsiae. J. Biol. Standardization 2:329-322.

2. McManus, A. T., and G. A. Eddy. 1975. Increased potency of killed VEE (TC-83) vaccine in mice previously infected with EEE virus. Trans. Roy. Soc. Trop. Med. Hyg. 69:179.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
LA OE6413	74 11 11	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REG |OING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 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	62760A	3A762760A834	02	012
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Characterization of the intestinal immune response

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 01	74 08	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 75	1.0	100
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Mathis, R. K.

TELEPHONE: 301 663-7341

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Dangerfield, H. G.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immunology; (U) Germ-free mice; (U) Peyer's patches; (U) Lymphoid tissue; (U) Military medicine

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

23 (U) Define the mechanisms involved in development of local intestinal immune responses. This is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Immunize mice orally with horseradish peroxidase. Transfer gut-associated-lymphoid tissue suspensions to normal and germ-free irradiated C-3-H mice. Measure IgA response by immunodiffusion and corroborate antigen presence by a variety of techniques. After the initial studies use the data to assess the mechanisms in orally induced immunity and responses to enteric infection and enterointoxication.

25 (U) 73 07 - 74 07 - A colony of Bittner-free, gnotobiotic C-3-H mice was established and maintained to time of breeding. When progeny attained similar maturity, mono-contamination with Bacillus sp. occurred. Studies with conventional C-3-H mice were in progress while gnotobiotic facilities were being reestablished. However, a variety of problems were encountered, including contamination of the gnotobiotic animals.

Therefore, the study was terminated, so that the investigator could be assigned to work of greater priority.

*Available to contractors upon ord(thator's approval)

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 012: Characterization of the Intestinal Immune Response

Background:

The response of localized gut-associated lymphoid tissue (GALT), Peyer's patches, is critical to the development of a normal intestinal immune barrier. Gnotobiotic animals, which can be exposed to a single antigen via the intestinal route, afford a means to evaluate the primary responses of GALT to antigenic stimuli.

Progress and Summary:

A colony of Bittner-free, gnotobiotic C-3-H mice was established and maintained to time of breeding. When progeny attained similar maturity, monocontamination with Bacillus sp. occurred. Studies with conventional C-3-H mice were in progress while gnotobiotic facilities were being reestablished. However, a variety of problems were encountered, including contamination of the gnotobiotic animals.

Therefore, the study was terminated, so that the investigator could be assinged to work of greater priority.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6415	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. SUBM'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 01 06	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	62760A	3A762760A834	02	013
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Cellular phenomena in lymphatic tissues during immune responses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 12	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 75	0.5	84.0
FISCAL YEAR — CURRENT 76	1.0	137.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Physical Sciences Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Anderson, A. O.

TELEPHONE: 301 663-7211

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immunology; (U) Lymphocytes; (U) Vaccines; (U) High endothelial venules (HEV)

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

23 (U) Define regulatory mechanisms of lymphocyte migration and cellular interaction in tissues during the in vivo immune response and develop methods for manipulating them for immunization. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Use morphological and radiolabel indicators of lymphocyte kinetics to study factors influencing lymphocyte traffic into lymph nodes. Success will permit testing of materials for adjuvant activity.

25 (U) 74 12 - 75 06 - A morphological marker which indicates the rate of lymphocyte traffic into lymph nodes has been identified and correlated with conventional radiolabeling cellular kinetics. Increased lymphocyte traffic was seen in lymph nodes draining skin grafts, and sites of immunization with pertussis and tetanus toxoid. Treatment of experimental animals with heparin reduced migration of lymphocytes into lymphatic tissues as well as causing tissue depletion of lymphocytes by mobilization. Heparin also prevented lymphocyte trapping in lymph nodes stimulated by antigens which have adjuvant activity. The lymph node microvasculature is specialized because of the presence of arteriovenous communications and venous sphincters located proximal and distal to HEV. Alterations in the contractile state of these structures has been shown to influence vascular permeability and lymphocyte traffic in lymph nodes.

Publications: Fed. Proc. 34:842, 862, 993, 1975.
Lab. Invest. 32:441, 1975.
Amer. J. Pathol., in press, 1975.
Lab. Invest., in press, 1975.

*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 65 AND 1418-1, 1 MAR 65 (FOR ARMY USE) ARE OBSOLETE.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 013: Cellular Phenomena in Lymphatic Tissues During Immune Responses

Background:

The phenomenon of lymphocyte recirculation by long-lived lymphocytes was first described and characterized within this decade. Constant traffic of immunocompetent cells through lymphatic tissues exposes a heterogeneous population of lymphocytes to sites of antigen concentration. In the rat, 10^9 lymphocytes pass through lymphatic tissues every 24 hr. This traffic provides for clonal selection which is necessary for induction of immunity. However, this response can no longer be regarded simply as the result of interactions between individual lymphocytes and the appropriate antigen. Recent studies have established that successful immunization depends upon a complex sequence of antigen-binding, antigen-presentation and cellular collaboration between thymus-derived lymphocytes, marrow-derived lymphocytes and macrophages. It is likely that these interactions take place in or near high endothelial (HE) venules (HEV), since lymphocytes arrive in lymphatic tissues by selectively migrating across the walls of HEV, and immunoblasts are first seen in the immediate perimeter of HEV after immunization.

Progress:

Studies were conducted in collaboration with Contract DAMD17-74-C-4095. In vivo and in vitro techniques were used to study the basic mechanisms of lymphocyte recirculation and the biological phenomena associated with alterations of lymphocyte traffic into lymphatic tissues.

Tissue lymphocyte traffic. It was important to develop a morphological indicator of lymphocyte traffic into lymphatic tissues since autoradiographic studies require extended period of time before analysis. The morphological lymphocyte migration index (LMI) provided a rough estimate of the rate of lymphocyte emigration into lymphatic tissues. The LMI was defined as the number of interendothelial (or migrating) lymphocytes divided by the number of endothelial cells in that HEV at the instant of fixation. An LMI for a given node is the mean of $\geq$ 100 determinations. It was necessary to sample a large number of HEV cross-sections since the intranodal variability of individual LMI was great (range 0.05-2.0, in a given node). However, when

sufficient HEV were sampled the internodal variability of mean LMI (± SE) was slight for control nodes (LMI = 0.69 ± 0.05) and other nodes studied under identical experimental conditions (e.g. 24 hr after pertussis antigen stimulation plus 1 hr dose of 2.5 μ/gm body wt of heparin, LMI = 0.32 ± 0.02). The intranodal variability is undoubtedly related to the heterogeneity of waxing and waning background antigenic stimulation which is always present in lymph nodes. Since background stimulation tends to be present focally in a given node, wide sampling minimized error.

To justify the LMI as an important marker of lymphocyte traffic we conducted morphological and autoradiographic kinetic studies in parallel. Thoracic duct lymphocytes (TDL) were collected from syngeneic Lewis rats, labeled with ^{3}H-uridine, and infused into the femoral veins of 30 rats at concentrations of 4 x 10^{8} cells/0.5 ml. Axillary lymph nodes were excised and fixed at 10, 30, 90, 180, and 360 min after infusion. LMI were calculated for both labeled and total migrating lymphocytes in autoradiographs of lymph nodes; 0.5 μm Epon sections were used. In addition, redistribution of labeled cells across vessel walls was recorded by counting labeled cells in the lumen, the wall, and the immediate vicinity (1 HPF) of each HEV. The data produced by these experiments are shown in Table I and II.

TABLE I. EFFECT OF ADDING 4 x 10^{8} ^{3}H-URIDINE LABELED LYMPHOCYTES TO THE CIRCULATING POOL.

MINUTES	LABELED CELLS			TOTAL LMI
	Luminal lymphocytes/HE	1 HPF surrounding HEV	Labeled LMI	
0	-	0	0	0.69
10	0.33	7.7	0.23	1.04
30	0.16	29.4	0.27	1.38
90	0	96.0	0.21	1.14
180	0.09	98.2	0.24	1.00
360	-	55.0	-	0.68
720	-	73.0	-	-
1,440	-	119.0	-	-

TABLE II. REDISTRIBUTION OF LABELED LYMPHOCYTES DURING EMIGRATION INTO LYMPHATIC TISSUES. (DIFFERENTIAL LOCALIZATION OF LABELED LYMPHOCYTES).

MINUTES	PERCENT			
	Luminal	Endothelial	Perivenular	Parenchymal (1 HPF)
0	<u>100</u>	0	0	0
10	22	<u>41</u>	30	7
30	7	20	<u>37</u>	36
90	0	3	20	<u>77</u>
180	6	8	19	<u>67</u>
360	9	7	13	<u>71</u>
720	14	9	23	<u>54</u>
1,440	10	10	14	<u>66</u>

These data indicated that there was a linear uptake of syngeneic lymphocytes into the lymphatic tissue surrounding HEV < 90 min after IV infusion. Lymphocytes began migrating into tissue immediately after infusion so that 78.4% of these labeled cells were enroute in 10 min. The reappearance of labeled cells in HEV lumens between 90 and 180 min was indicative of recirculation. One labeled lymphocyte (or 4 total lymphocytes) migrated across the wall of each 6-μm segment of HEV each minute after infusion and the mean transit time from the blood to the efferent lymphatic was between 3 and 6 hr. Loading the circulating pool of lymphocytes by adding 4×10^8 TDL doubled the total LMI by 30 min, suggesting that the rate of migration can be influenced by increasing the numbers of cells available to migrate. The converse experiment was performed in animals depleted of small lymphocytes by chronic thoracic duct drainage. LMI remained at normal levels until the 4th day of drainage. LMI gradually fell from 0.70-0.02 by the 9th day.

<u>Skin graft model</u>. (Lymph node microvasculature and lymphocyte traffic.) This study described histologic, ultrastructural and radiolabeling characteristics of the microvasculature in regional nodes draining skin allografts. From 6-48 hr postgrafting, these nodes showed increased vascular permeability and altered lymphocyte traffic (Table III).

TABLE III. SEQUENTIAL CHANGES IN REGIONAL NODES DRAINING SKIN ALLOGRAFTS

DAYS AFTER GRAFTING	MICROVASCULAR ALTERATIONS	HISTOLOGIC FINDINGS
1-2	Contracted venous sphincters. Dilated segmental veins. Altered vascular permeability.	RBC and PMN in sinuses. Mast cell degranulation. Lymphocytic plugging of cortical sinuses.
3-7	Dilated cortical capillaries. Disappearance of germinal centers. Lengthening and arborization of HEV.	Proliferation of lymphocytes in paracortex. Formation of primary follicles. Mitotic figures in HEV.
14-28	Long, arborized HEV persist. Avascular nodules in cortex. Prominent medullary capillaries.	Numerous small lymphocytes in paracortex. Large germinal centers. Prominent medullary cords.

The rapid rise in LMI from 0.69-1.28 and the apparent plugging of intermediate sinuses by lymphocytes suggested that both increased entry and decreased egress of recirculating cells contributed to "lymphocyte trapping" (Table IV).

TABLE IV. HISTOLOGIC SIGNS OF ALTERED LYMPHOCYTE TRAFFIC IN LYMPH NODES DRAINING SKIN ALLOGRAFT SITES

DAYS AFTER GRAFTING	HISTOLOGIC FINDINGS IN NODES			
	Regional		Contralateral	
	LMI	Lymphocyte plugs[a/]	LMI	Lymphocyte plugs
0.5	1.28	++	0.75	0
1	1.14	+++	0.69	0
2	1.20	+	0.81	0
3	0.84	0	0.68	0
4	0.97	0	0.74	0
7	0.82	0	0.71	0
14	0.81	0	0.68	0
28	0.68	0	0.70	0

a. Lymphocyte plugs + = tight aggregates of lymphocytes in cortical sinuses.

0 = individual lymphocytes dispersed in cortical sinuses.

This was followed by redistribution of cortical capillary arcades as existing germinal centers dissolved and proliferating lymphocytes infiltrated the cortex. Normal microvascular patterns reappeared at 7-14 days as primary and secondary nodules formed in the enlarged nodes. Increased length and arborization of HEV resulted from focal proliferation of endothelial cells in transition zones from high to low endothelium (Tables V and VI).

TABLE V. HEV STRUCTURAL CHANGES IN REGIONAL NODES DRAINING SKIN ALLOGRAFTS

DAYS AFTER GRAFT	HEV LENGTH μm	RATIO side branches: main trunks	% side branches lined by HE	NODE WEIGHT mg
0	408	2.1	38	18.0
1	512	1.8	38	21.0
2	627	3.6	52	25.5
3	621	5.0	72	28.5
4	660	7.4	76	29.8
6	744	6.1	81	43.5
10	650	6.0	96	-
14	537	3.1	66	38.5
28	566	2.4	40	27.8

TABLE VI. RADIOAUTOGRAPHIC STUDIES OF ^{3}H-THYMIDINE INCORPORATION[a] BY HE CELLS IN LYMPH NODES DRAINING SKIN ALLOGRAFTS

PARAMETER	MEAN + SD		p
	Regional Axillary Nodes	Contralateral Inguinal Nodes	
% labeled HE cells	21.7 ± 4.5	6.3 ± 3.3	0.001
No. consecutively ^{3}H-HE cells at transition zones	32.1 ± 4.9	30.4 ± 3.0	0.20
% transition zones containing ^{3}H-HE cells	73.9 ± 8.2	12.4 ± 7.4	0.0025

a. Rats continuously infused with ^{3}H-thymidine from day 3-6 after skin grafting.

The large number of proliferating HEV endothelial cells in regional nodes suggested that this process may be related to the distribution, intensity and duration of antigenic stimulation provided by allogeneic skin grafts. Proliferation of HEV provided an expanded surface area for increased total lymphocyte traffic into the node, and may have facilitated selection of antigen-recognizing effect or lymphocytes from the peripheral circulation.

The skin graft model is useful to demonstrate these alloantigen-induced vascular and lymphocyte traffic changes. Since degenerating grafts undoubtedly release a heterogeneity of antigens which include particles capable of producing adjuvant effects, it was necessary to repeat these studies with more purified antigens. Soluble tetanus toxoid and pertussis vaccine were selected for these studies since both are adequate antigens. Tetanus toxoid fails to produce "trapping" or adjuvant effects, and pertussis can be used as an adjuvant in other immunizations.

Injection ID of tetanus toxoid into rats produced a transient increase in the LMI in regional nodes. The LMI rose from 0.69 to 1.44 by 15 min, but this was short-lived and returned to normal by 24 hr. Alterations in the cortical medullary microvasculature occurred over the following 3-4 days. Arteriovenous communications and capillary arcades in the subcapsular region, were passively displaced by developing germinal centers between 4 and 6 days. Capillary beds in the medullary cords enlarged and dilated as the number of plasma cells in those areas increased. There were no changes in the length and arborization of cortical HEV.

The vascular studies in pertussis-injected rats are still incomplete; however, lymphocyte traffic into nodes increased from 0.70 ± 0.16 to 1.29 ± 0.16 in 24 hr and remained elevated for 48 hr more. Increased influx of lymphocytes was associated with decreased efflux which is consistent with the trapping phenomenon.

Pharmacological regulation of lymphocyte traffic. Heparin and other polyanions have been shown to produce an acute lymphocytosis in the peripheral blood of cattle, rodents, and humans. The site of action of heparin in producing this effect has never been identified; it is not clear whether heparin produces lymphocytosis by mobilization of tissue lymphocytes or blockade of lymphocyte recirculation. Recent studies have answered some of these questions.

In this study single IV injections of heparin at dosages of 0.15-0.30 units/gm body wt failed to alter the blood lymphocyte count in Wistar rats. Higher doses (1.0-2.5 units/gm) caused a doubling of the peripheral lymphocyte count within 30-60 min and this lymphocytosis could be sustained for several hours by repeated hourly injections of heparin. Histologic studies

demonstrated progressive depletion of small lymphocytes in lymph nodes. No changes in the ratio of small lymphocytes to other cells could be detected in spleens.

Similar heparin injections in cannulated rats caused a 50-80% increase in TDL output, and external drainage of these cells prevented the peripheral blood lymphocytosis. When histological sections were used to estimate the rate of lymphocyte entry into lymph nodes by counting the number of lymphocytes attached to the surface or migrating between endothelial cells in 100 HEV/node from at least 4 rats. This ratio fell from 0.69 $\pm$ 0.06 to 0.05 $\pm$ 0.01 after 6 hr of heparin therapy.

These findings suggested that heparin altered lymphocyte traffic by mobilizing cells from lymph node sinuses and blocking lymphocyte recirculation. Tissue depletion and blocked lymphocyte recirculation were also produced in peripheral inflammatory lesions and in lymph nodes involved in the process of lymphocyte trapping. Chronic respiratory disease (CRD) lesions, which are endemic in laboratory rats, were progressively depleted of small lymphocytes by 6 hourly injections of (2.5 U/gm body wt) heparin. During this same interval, the HEV in the CRD lesions showed a fall in the LMI to 0.04 by 6 hr. A neutralizing dose of protamine permitted the LMI to rise to normal levels within 1 hr in all experiments. Lymphocyte trapping induced by pertussis vaccine was prevented by heparin administration; however, the reduced LMI was still double the LMI in heparin treated normal lymph nodes.

Site of action of heparin. ^{35}S-heparin was used to show that heparin bound irreversibly to lymphocyte surfaces. After 15 min incubation of TDL with ^{35}S-heparin, 90% of the radioactivity remained with the cell pellet after 4 buffer washes. Binding of labeled heparin to lymphocyte surfaces could be blocked by prior addition of cold heparin and reduced by mixture of cold with ^{35}S-heparin. Cationic Alcian blue dye was used in electron microscopic studies of the surface charge distribution of normal and heparin-treated lymphocytes. TDL collected without heparin, and incubated with Alcian blue, bound this dye in discrete patches separated by nonstaining microvilli. Prior incubation of TDL or collection of TDL from heparinized rats caused a redistribution of surface charges as indicated by dye binding; which was linear to all of the lymphocytes' surface; 29% of these cells had additional "caps" of dye at one pole.

Since heparin binds to lymphocyte surfaces and alters their surface characteristics, it is likely that heparin interferes with the lymphocyte-endothelial cell interactions necessary for emigration at HEV. Further studies are necessary to elucidate whether heparin is also acting on the lymphocyte's surface metabolic activity (cAMP or cGMP) or on its ability to migrate (microtubules and microfilaments).

Lymphocyte chemotaxis. Preliminary studies of lymphocyte chemotaxis in modified Boyden chambers were unsuccessful. However, the probable sources of error have been identified and work will be restarted.

Presentations:

1. Anderson, A. O. Structure and function of the lymph node vasculature. Presented, Seminar Vet. Path. Dept., 4 Oct 1974 and Departmental Seminar, Dept. of Pathology, 8 Oct 1974, Johns Hopkins University, Baltimore, MD.

2. Anderson, A. O. Regulation of lymphocyte recirculation in the lymph node. Presented, Cellular Immunity Seminar, University of Wisconsin School of Medicine, Madison, WI, 24 Jan 1975.

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1. Bell, W. R., N. D. Anderson, A. O. Anderson, and R. G. Wyllie. 1975. Heparin-induced coagulopathy in rats. Fed. Proc. 34:862.

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19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Eigelsbach, H. T.
TELEPHONE: 301 663-7341
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Hunter, D. H.
NAME: Janssen, W. A.

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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) Evaluate potential for enhancement of nonspecific and/or specific host resistance and define the role of humoral and cell-mediated immunity in faculative intracellular infections as part of a comprehensive program for medical defense against BW agents.
24 (U) Quantitate differences in specific and nonspecific antibacterial resistance in mice and explore feasibility of regulating immune response. Using passive transfer of immune serum and/or lymphoid cells to syngeneic recipients with fully competent or selectively depressed immune mechanisms, determine resistance attained by active and passive immunization by Francisella tularensis challenge of graded virulence.
25 (U) 74 07 75 06 - Under appropriate conditions passively transferred spleen cells from mice immunized with live tularemia vaccine will ensure high-grade protection (survival approaching 100%) to nonimmune recipients against IV, IP, or SC challenge with fully virulent F. tularensis. This model affords an opportunity for separation of the 2 limbs of acquired immunity, i.e., cell-mediated and humoral, with evaluation in terms of survival. It will permit definitive studies on mechanisms involved in effective control of infections caused by highly virulent facultative intracellular bacteria.

Nonspecific resistance against Listeria monocytogenes and Salmonella typhimurium was demonstrated in the AKR-J mouse after immunization with live tularemia vaccine suggesting potential for nonspecific induction of effective host resistance to infectious diseases of military significance by live vaccines currently in medical use.

Publications: In: Manual of Clinical Microbiology, pp. 316-319, 1974.
J. Reticuloendothel. Soc. 16:36a, 1974.

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BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 103: Host Resistance to Facultative Bacteria

Background:

Within the past decade significant contributions have been made toward elaborating the roles of cell-mediated (CMI) and humoral immunity to resistance to infections caused by facultative intracellular bacteria. In these infections, cellular factors have been allotted the predominating role to the virtual exclusion of the humoral arm of the defense system. Evidence is based primarily on reduction in growth of the infecting microorganism in the spleen and liver or by a delay in time of death of laboratory animals which at the time of challenge had simultaneously received dissociated spleen cells and/or macrophages from immune donors (whose serum was found inert in passive protection tests). The problem is complex; improved model systems are required to determine more precisely the specific and nonspecific elements involved in defense against facultative intracellular bacteria, including the apparent subtle interplay between humoral and cellular factors in the expression of immunity. We postulated that the availability of a highly effective live tularemia vaccine, LVS, containment facilities to work safely with fully virulent strains, and the marked susceptibility of mice to Francisella tularensis by various inoculation routes should make it possible to obtain more definitive evidence concerning the roles of CMI and humoral immunity in defense against a facultative intracellular bacterial infection.

Progress:

I. Our initial goal was to provide a model that would afford syngeneic recipients of spleen cells from immunized donors (immune spleen cells) survival against infection with a fully virulent strain of F. tularensis. The selected strain, SCHU S5, is streptomycin-resistant and capable of causing severe, potentially fatal, disease in humans or death in various laboratory animals infected with $\leq$ 1-10 organisms. In our previously described experiments,[1] spleen cells obtained from LVS-immunized AKR-J mice were transferred IP to syngeneic recipients which were simultaneously challenged SC with SCHU S5. The rate of growth of SCHU S5 in spleens and livers of streptomycin-treated recipients was significantly delayed, findings comparable to those reported by Mackaness[2] in studies with Listeria monocytogenes. However, with an identical group of mice that were not sacrificed, none survived challenge. We postulated that a virulent strain might prove more effective than the vaccine strain for stimulation of immunogenically competent cells for passive transfer. The present report

describes an experimental model in which passive transfer of nonfiltered, primarily mononuclear spleen (lymphoid) cells protected ~100% of syngeneic recipients against fatal infection with SCHU S5; these findings suggest the necessity for continued specific antigenic stimulation for immune spleen cells to transfer immunocompetence.

Comparison of protective activity of immune spleen cells from LVS-boostered and SCHU S4-boostered donors. AKR-J mice were immunized SC with 161 ± 32 (SE) viable LVS and 30-day survivors were boostered by the IP route with either 10^6 viable LVS or 10^3 viable SCHU S4. Usually 5-15% of the animals succumbed after the initial LVS injection, 2-3% after the LVS booster, and 10-30% after SCHU S4. In general, an increasingly greater number of vaccinees died when the time of the booster dose was extended beyond 30 days. Immune spleen cell transfers were made 3, 6, 12 and 19 days after the booster and recipient mice were challenged 18 hr later with 21-25 MLD SCHU S5. Recipients were treated with streptomycin 2 X daily for 5 days. All recipients of 3.9×10^7 spleen cells obtained 12 days after the SCHU S4 booster dose survived challenge; this protective activity was eliminated when the inoculum was reduced to 3.9×10^7 cells. No significant protection was conferred with cells harvested at 3, 6 or 19 days after the SCHU S4 booster or at any time after LVS (Table I).

TABLE I. TRANSFER OF PROTECTION TO AKR-J RECIPIENTS OF SYNGENEIC SPLEEN CELLS FROM LVS-VACCINEE DONORS ADMINISTERED A SCHU S4 OR LVS BOOSTER.

BOOSTER DOSE[a]	DAY POST-BOOSTER	DONOR CELLS TRANSFERRED		RECIPIENT SURVIVAL AFTER SCHU S5 CHALLENGE[b] (%)
		Spleen cells ($\times 10^7$)	Spleen cell-associated live *F. tularensis*	
10^3 SCHU S4	3	15	490,000	0
	6	13	10,000	0
	12	39	560	100
	12	3.9[c]	56	0
	19	17	42	17
10^6 LVS	3	20	0	0
	6	23	0	0
	12	26	0	0
	19	20	0	0

a. Booster administered IP 30 days after vaccination.

b. Groups of 6 AKR-J recipients challenged IP with 21-25 SCHU S5 and administered 400 µg streptomycin 2 X daily for 5 days.

c. A 1:10 dilution of the 12-day 39×10^7 cell pool.

Examination of spleen cell suspensions indicated that preparations from immune spleens contained an appreciably higher proportion of medium to large lymphocytes and macrophages than corresponding preparations from normal spleens. Lymphoid cell distribution in suspensions from normal spleens consisted of 65% small lymphocytes, 28% medium to large lymphocytes, 3% neutrophils and 4% macrophages. In contrast, cell suspensions from immune spleens that were 2-4 times normal size consisted of 85-90% lymphocytes, evenly distributed between small and medium-to-large cells, ~4% neutrophils and 9-12% macrophages. Lymphoid cells > 15 μ in diameter having distinctly indented oval nuclei and vacuolated cytoplasm were counted as macrophages. There were no appreciable morphological differences between suspensions from LVS- and SCHU S4-boostered mice. Therefore, effective induction of immune spleen cells by 10^3 SCHU S4 but not by 10^6 LVS organisms cannot be readily explained by qualitative and/or quantitative differences in post-booster spleen cell populations. Postulates based upon antigenic differences between the strains are weakened by the knowledge that vaccination with strain LVS affords high-grade immunity to the AKR-J mouse and other hosts against challenge with SCHU S4. Since cell-associated viable LVS organisms were never cultured from suspensions of spleen cells from LVS-boostered mice, it is possible that a lack of sufficient antigenic stimulus to immunologically committed T-cells might not provide macrophage activation at a level sufficiently high to result in expression of immunity.

Immunization schedule and streptomycin therapy of donors. A series of 4 experiments were conducted to expand and confirm these findings and to determine the effect of various schedules for immunization of donors and for streptomycin therapy of recipients. Again, a high degree of protection (approaching 100%) was achieved by transfer of immune spleen cells from syngeneic donors at 12-13 days post-booster but no protection was observed at 19 days (Table II). Although increasing numbers of live spleen cell-associated bacteria (SCHU S4) were transferred as the interval between primary immunization with LVS and the booster with SCHU S4 was prolonged, there was no apparent effect on survival of challenged recipients of immune spleen cells. Likewise, administration of streptomycin to recipients 1, 2 or 3 X daily for 5 days after receipt of immune spleen cells had no influence on the immunocompetence of the passively transferred cells. All recipients that received no streptomycin therapy, however, died $\leq$ 5 days. In contrast to results in Table I, transfer of 6-day post-booster spleen cells provided significant survival (67% of 6 mice). All mice in control groups died $\leq$ 10 days after challenge; mean time-to-death varied from 4.3 $\pm$ 0.5 to 6.2 $\pm$ 2.3 days. These challenged control groups included (1) untreated mice, (2) streptomycin-treated mice, (3) streptomycin-treated normal spleen cell recipients, (4) streptomycin-treated recipients of heat-inactivated immune spleen cells, and (5) streptomycin-treated recipients of freeze-thawed immune spleen cells.

TABLE II. INFLUENCE OF SPLEEN CELL DONOR IMMUNIZATION SCHEDULE, SPLEEN CELL-ASSOCIATED ORGANISMS, AND STREPTOMYCIN TREATMENT OF RECIPIENTS ON SURVIVAL OF RECIPIENTS AFTER CHALLENGE WITH SCHU S5.

IMMUNIZATION OF DONORS TIME IN DAYS		DONOR CELL TRANSFER		SCHU S5 CHALLENGE DOSE (No. organisms)	STREPTOMYCIN THERAPY SCHEDULE (hr)[a]	SURVIVAL[b]
Primary to booster	Booster to cell transfer	Spleen cells ($\times 10^8$)	Cell-associated live SCHU S4			
66	12	1.7	8,200	125	12	100
43	6	1.0	14,000	53	8	67
	12	1.4	3,500	57	8	100
	19	2.8	1	52	8	0
34	13	1.4	540	33	24	100
		1.4	540	33	12	100
31	12	1.4	162	26	12	97[c]

a. Recipients administered 400 µg streptomycin (0.1 ml SC) every 8, 12 or 24 hr for 5 days.

b. 20 days after challenge.

c. 35 mice/group; all others 6 mice/group.

Replication of spleen cell-associated streptomycin-sensitive SCHU S4 in the recipient and potential induction of active immunity was investigated. Unchallenged groups of mice that had received 13-day spleen cells from the 34-day vaccinee-booster group (Table II) were sacrificed 1, 3 and 8 days after cell transfer. Spleens from recipients that were treated with streptomycin at 12- or 24-hr intervals contained comparable numbers of organisms; ~ 10^2, 10^4 and < 10 viable SCHU S4 were recovered at 1, 3 and 8 days after transfer, respectively. When unchallenged recipients and previously challenged survivors were inoculated SC 35 days after transfer of immune spleen cells with 100 MLD of strain SCHU S4, all mice died of tularemia $\leq$8 days, indicating a lack of active immunity.

Since we have shown that SCHU S4 was capable of limited multiplication despite the streptomycin therapy administered, it is possible that the relatively large SCHU S4 populations associated with immune spleen cells harvested prior to 12 days after booster constituted an overwhelming burden that could not be effectively handled by the streptomycin treatment given recipients and thus precluded early expression of the potency of the immune spleen cells. The failure of immune recipients to survive backchallenge indicates that the antigenic mass resulting from multiplication of SCHU S4 or SCHU S5 in recipient mice was insufficient for active immunization. Moreover, if, despite streptomycin treatment of the recipients, spleen cell-associated virulent SCHU S4 organisms were capable of effective replication within the 1st few days after transfer, it is probable that death from tularemia infection rather than active (but transient) immunity, would have occurred.

Effect of challenge route on protective activity of immune spleen cells. Since utilization of the IP route for both cell transfer and challenge might favor the expression of resistance in recipient mice, the protective activity of immune spleen cells was compared to syngeneic recipients challenged IV, SC, or IP (Table III). Immune spleen cells (3.0×10^8) from LVS-vaccinees that were boostered on day 30 with SCHU S4 were transferred IP to groups of 6-8 recipient mice 12 days later. Recipients were treated SC with streptomycin twice daily for 5 days. All mice, including normal spleen cell recipients and challenge controls, were inoculated with 26 MLD of strain SCHU S5 at 18 hr after spleen cell transfer. Survival ratios indicated that the route of challenge had no significant effect on expression of protective activity conferred by immune spleen cells.

TABLE III. ADOPTIVE TRANSFER OF RESISTANCE TO CHALLENGE BY VARIOUS ROUTES WITH SCHU S5 IN AKR-J RECIPIENTS OF IMMUNE OR NORMAL SYNGENEIC SPLEEN CELLS.

CHALLENGE ROUTE (26 SCHU S5)	NO. SURVIVORS/NO. CHALLENGED[a] (TTD ± SD)	
	Immune spleen cells[b]	Normal spleen cells
IP	8/8	0/8 (4.8 ± 0.6)[c]
IV	7/8 (6)	0/8 (5.3 ± 0.4)
SC	8/8	0/8 (6.1 ± 0.7)

a. Survival recorded 20 days after SCHU S5 challenge of streptomycin-treated recipients of spleen cells.

b. Immune spleen cells (3×10^8) contained 760 cell-associated live SCHU S4.

c. Time-to-death. All normal cell recipients died < 7 days.

Some investigators in studies on the relative importance of humoral vs. cell-mediated defenses against infectious diseases routinely use the IP route of challenge[3] while others recommend the IV[4] or the SC route.[5] In the pioneering study on passive protection of mice against tularemia by transfer of immune lymphoid cells, Allen[6] observed that increased resistance, discernible primarily by delay in time of death, was demonstrated in animals challenged IP but not by the SC route. He postulated that localization of transferred immune cells in the peritoneal cavity or its efferent lymphatics was responsible for their effectiveness against IP- but not SC-injected organisms. With our experimental model, high-grade transient immunity is afforded equally well against all 3 challenge routes. Two possible explanations are: (1) the strains and/or method for immunization of donor mice provided a more effective spleen cell population or (2) the AKR-J mouse was more immunocompetent for F. tularensis than the inbred strain employed by Allen.

Immune spleen cell-associated SCHU S4 and potential requirement for continued presence of antigen for expression of immunity. To study further the possibility of early development of active immunity by the transfer of live spleen cell-associated organisms, spleen cells containing intracellular SCHU S4 were obtained by inoculating the tail vein of normal AKR-J mice with 10^9 live SCHU S4 organisms. Spleens were removed 3 hr later from 2 mice; the cells were dissociated, pooled and suspended in RPMI 1640 + 100 units/ml penicillin and 100 mg/ml streptomycin for 1 hr. The suspension was then

washed 3 X in RPMI without antibiotics and diluted to obtain ~ 500 and 5,000 spleen cell-associated SCHU S4 organisms/ml, comparable to or greater than concentrations found in immunocompetent spleen cells. Transfer of either 500 or 5,000 spleen cell-associated live SCHU S4 organisms resulted in death of all recipients despite streptomycin therapy. These results suggest that induction of early active immunity under conditions described for the model is unlikely. However, to exclude this possibility consideration was given to streptomycin treatment of donors at various times after the SCHU S4 booster. Data presented in Table IV suggest that treatment of the donor to eliminate or reduce spleen cell-associated organisms resulted in an appreciable reduction in adoptive immunity conferred on recipients by spleen cells from streptomycin-treated immunized-boostered syngeneic donors.

TABLE IV. EFFECT OF STREPTOMYCIN THERAPY OF LVS VACCINATED SCHU S4-BOOSTERED DONOR AKR-J MICE ON IMMUNITY PROVIDED BY PASSIVE TRANSFER OF SPLEEN CELLS 12 DAYS POST-BOOSTER.

THERAPY[a]		SPLEEN CELL-ASSOCIATED SCHU S4 ORGANISMS	NO. SURVIVING/TOTAL OF SPLEEN CELL RECIPIENTS[b]
Donor	Recipient		
3-5 Incl.	None	0	1/6
6-8	None	6	0/6
6-11	None	3	1/6
6-11	0-4	3	0/6
9-11	None	14	1/6
None	None	500	0/6
None	0-4	500	4/6

a. Streptomycin (400 µg in 0.1 ml) SC every 8 hr on days indicated (Donors: Days post-booster; recipients: Days after spleen cell transfer).

b. 20 days post-IP challenge with 25 viable SCHU S5.

Regardless of the treatment schedule employed for donors, only 3 of 30 recipients survived whereas in the group treated as described for the model, 4 of 6 survived. These data suggested that streptomycin treatment of donors might reduce the quality and/or quantity of immunocompetent spleen

cells and/or that the presence of adequate specific antigen may be necessary for continued mitotic activity of spleen cells and the resultant production and liberation of mediators of cellular immunity. To examine these possibilities, comparable groups of recipients were inoculated with immune spleen cells or with immune cells and 10^7 killed SCHU S4 organisms (Table V).

TABLE V. EFFECT OF ADDED KILLED ANTIGEN ON ADOPTIVE IMMUNITY TRANSFERRED BY SPLEEN CELLS ADMINISTERED AT VARIOUS TIMES POST-BOOSTER[a]

THERAPY[b] (Days post-booster of donors)	DAYS AFTER BOOSTER SPLEEN CELLS TRANSFERRED	SPLEEN CELL-ASSOCIATED VIABLE SCHU S4 TRANSFERRED	NO. SURVIVING/TOTAL SPLEEN CELL RECIPIENTS[c]	
			Alone	+ 10^7 killed SCHU S4
None	19	4	7/10	10/10
None	26	0	3/5	5/5
None	40	0	3/5	5/5
3-5 Incl.	12	10	3/6	6/6
None	12	1ŧ5	7/8	

a. AKR-J donors vaccinated SC with 10^3 LVS and boostered IP 30 days later with 10^9 SCHU S4.

b. Streptomycin (400 µg in 0.1 ml) administered SC to donors every 8 hr on days indicated and to recipients every 12 hr on days 0-4 after spleen cell transfer.

c. 20 days post-challenge; all control animals including those that received normal spleen cells alone or with 10^7 killed SCHU S4 died in < 7 days.

When immune spleen cells were transferred 19, 26 or 40 days after the booster or 12 days after booster when donors had been treated with streptomycin, 40-50% of the challenged recipients died. Although no immune spleen cell-associated SCHU S4 were recovered from suspensions transferred 26 or 40 days post-booster, the majority of the mice, 3 of 5 in each case, survived challenge indicating that active immunity did not play a role in the system.

However, 100% of comparable groups injected IP with 10^7 phenol killed dialyzed SCHU S4 organisms at the time of immune spleen cell transfer survived challenge. These results support the hypothesis that presence of adequate specific antigen is required for continued expression of maximal CMI. It should be noted that adoptive immunity persisted in these animals well beyond 12 days post-SCHU S4 booster in contrast to data presented in Table II. This discrepancy may be accounted for by the use of male AKR-J mice to obtain data presented in Tables I and II whereas female mice were used in latter experiments. We have observed consistently that female AKR-J mice are more resistant to LVS and retain adoptive immunity for a longer period than male AKR-J mice.

Date presented in this report demonstrate that under appropriate conditions passively transferred spleen cells from immunized mice will ensure high-grade protection (survival approaching 100%) for nonimmune recipients against IV, IP or SC challenge with a fully virulent strain of F. tularensis. Procedures critical to the experimental model are: (1) primary immunization of 11-14-wk-old AKR-J mice with live tularemia vaccine; (2) secondary immunization on day 30 with virulent strain SCHU S4; (3) 12 days following booster, immune spleen cell transfer to syngeneic recipients following 18 hr later by challenge of recipient mice by the IP, SC or IV route with 25-50 MLD of streptomycin-resistant strain SCHU S5, and (4) administration of streptomycin therapy to all recipient mice for 5 days from time of spleen cell transfer. Evidence was presented that active immunity failed to develop in recipients of immune spleen cells despite the presence of spleen cell-associated live organisms. Therefore this model affords an opportunity for separation of the 2 limbs of acquired immunity, cell-mediated and humoral, with evaluation in terms of survival. More importantly, it will permit definitive studies on mechanisms involved in effective control of infections caused by highly virulent facultative intracellular bacteria.

Preliminary data were also obtained which indicate that the continued presence of adequate specific antigen may be necessary to stimulate continued mitotic activity of immune spleen cells thereby ensuring uninterrupted production and liberation of mediators of cellular immunity.

II. Efforts to induce and quantitate the phenomenon of nonspecific resistance (NSR) against bacterial infections have continued. We reported previously that in vivo induction of nonspecific bactericidal activity of peritoneal macrophages against Vibrio parahemolyticus paralleled mobilization of neutrophils and macrophages in spleens of mice following vaccination with F. tularensis, strain LVS.[1] However, evaluation of host resistance to heterologous bacterial infection was dependent upon acquisition of strains (L. monocytogenes, Salmonella typhimurium) with known virulence for mice.

Having determined that representative ATCC strains of L. monocytogenes and of S. typhimurium were essentially avirulent for mice, L. monocytogenes strain Mack and S. typhimurium strain Keller var. Copenhagen (obtained from Dr. S. I. Vas, McGill University) were employed for virulence titrations in AKR-J mice (Table VI). The minimal lethal dose was 1-3 organisms for S. typhimurium and 2 x 10^5 for L. monocytogenes. Comparison of responses of 8 inbred strains of mice indicated that AKR-J (male and female) mice were among the most susceptible to those microorganisms and highly satisfactory for the proposed studies.

TABLE VI. LETHAL DOSE RESPONSE OF AKR-J MICE TO IP CHALLENGE WITH S. TYPHIMURIUM, STRAIN KELLER, OR L. MONOCYTOGENES, STRAIN MACK.

S. TYPHIMURIUM		L. MONOCYTOGENES	
Dose	Dead/Total	Dose	Dead/Total
85	6/6	260,000	5/6
28	6/6	90,000	4/6
9	6/6	30,000	3/6
3	6/6	10,000	1/6
1	1/6	3,000	4/6
0.3	2/6	1,000	0/6

Investigation of temporal changes in spleen cell population, bactericidal activity of peritoneal macrophages (PM) and delayed type hypersensitivity (DTH) were continued and expanded for correlation with host resistance to heterologous challenge. Parameters of responses of AKR-J mice following SC vaccination with ~100 LVS organisms are presented in Table VII. A marked increase in bactericidal PM and splenic neutrophils and macrophages was observed by days 3-9 postvaccination corresponding to the time at which significant numbers of LVS were recovered from the spleen. During this interval spleen increased in weight and splenic lymphocytes increased in number with a population shift from small to large. By day 9 specific immunity, DTH, as measured by localized swelling in antigen injected footpads, was noted.

TABLE VII. PARAMETERS OF RESPONSE OF AKR-J MICE FOLLOWING SC VACCINATION WITH F. TULARENSIS, STRAIN LVS.

PARAMETER	MEAN OF 6 MICE BY DAYS POST-VACCINATION						
	0	1	3	6	9	12	20
Bactericidal peritoneal macrophages (%)[a]	63	61	84	93	94	62	58
Splenic leukocytes (total x 10^7)	4.9	4.1	6.8	7.5	14.7	17.7	11.4
Neutrophils (%)	3	4	20	12	10	2	2
Macrophages (%)	6	6	12	13	21	17	8
Lymphocytes (%)							
Small, <10	58	67	41	20	23	19	32
Medium, 10-15μ	29	21	23	41	28	36	41
Large, > 5 μ	4	2	4	14	18	26	17
Spleen weight (mg)	53	60	70	153	220	207	140
Intracellular LVS (viable count x 10^3/spleen)	0	0	1.1	57	0.2	0.003	0
Footpad volume change (%)[b]	-0.1	5.6	-1.3	2.6	22	32	18

a. Means for 3 mice.

b. Specific DTH reaction to tularemia antigen (Foshay).

To examine the postulate that host NSR can be correlated with increased microbicidal activity of mononuclear phagocytic cells, groups of LVS-vaccinated mice and appropriate controls were challenged IP with lethal doses of L. monocytogenes or S. typhimurium at various intervals (1-90 days) postvaccination. Other modalities with potential for induction of NSR were also explored. Results of Listeria challenges are shown in Table VIII. These data indicate that NSR against L. monocytogenes in LVS vaccinees appeared as early as day 3, was maximal on days 6-12, persisted in ~50% of mice through day 30 but was no longer detectable on days 60-90. Similar effects were observed for groups immunized simultaneously with LVS and diptheria and tetanus toxoids and pertussis vaccine, adsorbed (DPT),

Merrell-National Laboratories, Cincinnati, Ohio, or 7.6% Na-caseinate. Either DPT or Na-caseinate alone, however, were capable of inducing NSR within 3 days but this response, in contrast to that observed after LVS, was relatively short-lived (days 9-12).

TABLE VIII. NONSPECIFIC RESISTANCE IN AKR-J MICE INDUCED BY VARIOUS TREATMENTS AGAINST IP LISTERIA MONOCYTOGENES (MACK)

TREATMENT ON DAY 0 (Dose, route)	NO. SURVIVORS/TOTAL BY POST-TREATMENT DAY								
	1	3	6	9	12	20	30	60	90
None	1/12	0/18	0/12	2/12	1/18	1/12	0/24	1/12	0/12
LVS vaccine (10^2 SC)	0/12	6/18	10/12	11/12	12/12	6/12	7/18	0/6	2/6
DPT vaccine (0.1 ml IM)		5/6			6/6		0/6	1/6	
LVS + DPT (10^2 SC) (0.1 ml IM)		6/6			6/6		4/6	0/6	
7.6% Na-Caseinate (2 ml IP)	0/6	6/6	6/6	5/6		0/6	2/6		
7.6% Na-Caseinate + LVS (2 ml IP) (10^2 SC)	0/6	5/6	6/6	6/6		4/6	5/6		

The survival pattern for LVS-vaccinated mice following challenge with S. typhimurium was essentially the same as that following challenge with L. monocytogenes (Table IX); investigation of the effects of other treatments is in progress. Although the time of appearance of NSR showed good correlation with mobilization of activated phagocytic cells, its persistence through day 30 is unlikely to be related to the same phenomenon. Neither can these data be explained by the hypothesis of Mackaness[2] that NSR against heterologous organisms appears at the same time as the specific DTH reaction. However, the results suggest that effective host resistance to infectious diseases of military significance may be induced temporarily and non-specifically by vaccines currently in medical use.

TABLE IX. NONSPECIFIC RESISTANCE IN AKR-J MICE INDUCED BY VARIOUS TREATMENTS AGAINST IP S. TYPHIMURIUM (KELLER).

TREATMENT ON DAY 0 (Dose, route)	NO. SURVIVORS/TOTAL BY POST-TREATMENT DAY					
	3	9	12	30	60	90
None	2/12	2/6	6/24	0/12	4/12	1/12
LVS vaccine (10^2 SC)	9/12	6/6	15/18	5/12	2/6	2/6
DPT vaccine (0.1 ml IM)			2/6			
LVS + DPT (10^2 SC) (0.1 ml IM)			4/6			
7.6% Na-Caseinate (2 ml IP)			2/6			

Presentation:

Eigelsbach, H. T., D. H. Hunter, and W. A. Janssen. Mechanisms of host resistance to tularemia. Annual Meeting Reticuloendothelial Society, 2-5 Dec 1974, Seattle, Wash. (J. Reticuloendothel. Soc. 16:36a, 1974).

Publication:

Eigelsbach, H. T. 1974. Francisella tularensis. Chapter 28, pp. 316-319. In Manual of Clinical Microbiology, 2nd ed. (E. H. Lennette, E. H. Spaulding, J. P. Traunt), American Society for Microbiology, Washington, D. C.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6426	75 07 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
74 07 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	62760A	3A762760A834	02	104
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Mechanisms of complement activation in infectious diseases

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 10	75 08	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 75	1.0	75.7
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Fine, D. P.
TELEPHONE: 301 663-7341
SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE

Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Complement; (U) Alternate pathway; (U) Diplococcus pneumoniae; (U) Serotypes; (U) Endotoxin

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

(U) Define pathways and conditions for interaction of complement with bacterial endotoxins and evaluate in terms of host resistance to infectious diseases. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Determine the ability of endotoxin and pneumococci of various serotypes to activate complement in chelated serum.

25 (U) 74 07 - 75 06 - Pneumococcal serotypes varied in their ability to activate complement via the alternate pathway. Those types which do not activate this pathway (and thus could not be opsonized prior to the development of specific antibody) are generally more virulent than those which do, implying significant pathogenetic importance for the alternate pathway. Studies to isolate the cellular component reactive with the alternate pathway have been unsuccessful.

The contribution of the complement system to the pathogenesis of RMSF has been investigated by measuring serial complement changes during infection of rhesus monkeys and by infecting guinea pigs deficient in C4 (genetically) or C3-9 (cobra-factor induced). Serial complement changes were also determined in a human infected with RMSF in a laboratory accident. The tentative conclusion was that RMSF does not represent an immune complex disease and that complement is not critical to development of full-blown disease, a conclusion at odds with the generally accepted hypothesis.

Publication: Proc., 14th Interscience Conf. Antimicrobial Agents and Chemotherapy, Abstract 145, 1974.

*Available to contractors upon originator's approval.

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A (NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 104: Mechanisms of Complement Activation in Infectious Diseases

Background:

An important objective of these investigations is to determine the contribution of the alternate complement pathway[1] to the pathophysiology of various infectious diseases. This pathway, also known as the properdin system, can produce in the absence of antibody almost all the well known effects of complement activation: vasodilatation, phagocyte attraction, opsonization and bacteriolysis. The biologic potential of such a system, especially in pre-antibody phases of infections, is obvious. We have reported previously[2] that endotoxin activates both the classic and alternate pathways. Subsequent studies have involved primarily Streptococcus pneumoniae[1] and Rickettsia rickettsii. Attempts have been made to determine whether a given organism could activate the alternate complement pathway and whether that reaction was biologically important.

Many experiments reported herein were performed using differential cation chelation to block selectively the classic complement pathway.[3] Ethyleneglycoltetraacetic acid (EGTA) chelates Ca^{2+} avidly but Mg^{2+} poorly. The classic complement pathway, which is Ca^{2+}-dependent, is inhibited in serum chelated with this agent. The alternate pathway, being Mg^{2+}-dependent and Ca^{2+}-dependent, remains functional in such serum. Thus, by measuring serum complement levels before and after challenge by a given substance, one can selectively determine the pathway activated, e.g., CF which is not inhibited by EGTA represents alternate pathway activation; fixation inhibited by EGTA represents classic pathway activation.

Progress:

It was reported previously that pneumococcal serotypes varied in their ability to activate complement in EGTA-chelated serum, i.e., by the alternate pathway.[1] Over a range of ~ 10^5 - 10^9 organisms/ml, serotypes III, IV, VII, VIII, XII, XIV and XXV consumed complement via the alternate pathway. In contrast, type I, even in doses exceeding 10^9 organisms/ml, failed to activate the alternate pathway. This difference was seen in sera from numerous individuals, including one with documented antibodies to serotype I.

The ability of types I, IV or VIII to activate the alternate pathway was eliminated by absorption of the serum 3 times at 0 C for 30 min with organisms of the same type. Conditions of the absorptions were such that type-specific antibody, but not complement, was removed. Reactivity of serotypes VII, XII, XIV and XXV was unaffected by the absence of antibody.

Thus, it appeared that these pneumococcal serotypes tested could be separated into 3 groups. Type I did not activate the alternate complement pathway even in the presence of type-specific antibody; types III, IV and VIII required antibody to activate the alternate pathway and types VII, XII, XIV and XXV possessed intrinsic, antibody-independent reactivity with the alternate pathway. Such an interpretation implies that types VII, XII, XIV and XXV could be opsonized (and thus ingested and killed by phagocytes) early in infection prior to the production of antibody by the host. In contrast, types I, III, IV and VIII would not be opsonized until the host had begun antibody production, several days after infection. One would thus expect types I, III, IV and VIII to be more virulent, an expectation generally borne out by epidemiologic studies of pneumococcal types in bacteremia.[4]

These results indicated that some pneumococcal serotypes could activate the alternate complement pathway and suggested a relationship between pathogenicity and inability to activate the alternate pathway. Consequently, studies were initiated to demonstrate what component of the organism activated the alternate pathway. Although one would expect a priori that the capsular polysaccharide, classically correlated with virulence, would be the reactive subcellular fraction, investigations to date have militated against that conclusion: (1) repeated washings of the organisms failed to affect the results and (2) rough strains of types I and XXV (developed by serial passage of organisms on blood agar plates) behaved exactly like their parent smooth strains.

Capsular polysaccharide from type XXV pneumococcus, a type capable of activating the alternate pathway was isolated as described by Kabat and Mayer.[5] In experiments to date, this preparation, in concentrations of 0.5 to 6 mg/ml, has yielded equivocal results; complement in EGTA-chelated serum was fixed on one occasion but not in 2 other experiments; further studies are planned. In another series of investigations, type XXV pneumococci were washed, disrupted by freezing and thawing, and separated by differential sedimentation into polysaccharide, cell wall and intracellular contents. Each fraction was then tested for CF ability in EGTA-chelated serum. Only the initial washings activated the alternate pathway. These experiments will be repeated using more definitive methods of separation.

Sera from patients with sickle-cell disease have been reported to have decreased opsonic activity due to a defect in their alternate complement pathway.[6,7] This defect is assumed to be the result of functional hyposplenism commonly observed in this disease. Based on these observations and our findings with pneumococcal serotypes, we postulated that serum from hyposplenic individuals would not react via the alternate pathway with pneumococcal

serotypes that readily activate this pathway in normal serum. Because sera from hyposplenic humans were not immediately available, we elected to test sera from hyposplenic rhesus monkeys. Surprisingly, sera from hyposplenic and normosplenic monkeys reacted exactly like sera from normal humans, i.e., complement was consumed over a wide range of organism concentrations. This observation casts serious doubt on the assumption that hyposplenism is a primary factor in the opsonic deficit in sickle-cell disease. Nevertheless, should sera from hyposplenic humans become available, the appropriate studies will be performed.

In studies with Rickettsia rickettsii, interrelationships of the clotting and complement systems and their contributions to the pathogenesis of RMSF are under investigation (in collaboration with Work Units 834 01 007 and 834 01 300). It has been widely assumed that RMSF represents an immune complex disease analogous to serum sickness because: (1) the diffuse hemorrhagic rash resembles the rash of some immune complex diseases; (2) thrombocytopenia and disseminated intravascular coagulopathy (DIC) are frequent complications that, in other situations, have been related to complement activation, and (3) hypocomplementemia of varying degrees has been observed in some RMSF patients. Confirmation of this assumption would be of considerable importance and have obvious therapeutic implications. Therefore, experiments were initiated to test the underlying hypothesis that the rash and severe illness of RMSF would be closely associated with the onset of antibody production, i.e., soluble immune complex formation, decreased complement and platelet levels, and accelerated clotting.

Rhesus monkeys were infected IV with R. rickettsii, and hemolytic titrations of the C2 and C3 components of complement were measured in samples of serum obtained at various intervals. C3 is central to both the classic and alternate pathways and would be depressed with activation of either pathway, whereas C2 would be depressed only if the classic pathway were activated. Thus, comparison of these 2 values would allow some inference regarding the pathway involved. It should be noted, however, that complement is an acute-phase reactant; a postinfection rise in synthesis might obscure any decrease in values secondary to complement consumption. Nonetheless, investigators have occasionally noted very low levels of complement during RMSF in man, and in other conditions, e.g., where complement activation is known to be pathogenetically significant, important consumption of complement measurably overrides increased synthesis.

In rhesus monkeys, there were some complement changes in individuals which might suggest activation by immune complexes. An example is seen in Table I. This monkey was inoculated IV with 10 EID. Vasculitis and antibody both appeared about day 7 at which time C3 levels decreased from the acute-phase type of elevation (154% of baseline) to baseline (97%) before rising again (162%) during the convalescent period. C2 levels paralleled C3 levels, implying that any activation was by the classic pathway.

TABLE I. SERIAL C3 LEVELS IN A RHESUS MONKEY FOLLOWING IV INOCULATION ON DAY 0 WITH 10 EID R. RICKETTSII.

C3 LEVELS BY DAYS AS % OF DAY 0									
-12	-5	0	2	4	6	7	11	14	22
136	130	100	136	143	154	120	97	105	162

Between days 8 and 11, platelets decreased to subnormal levels (97,000) and fibrin split products appeared in the serum. From such results, one could conclude that circulating immune complexes were activating complement, coincident with a vasculitis and mild DIC. But these changes were neither impressive nor even statistically significant. One would be hard put either to prove that complement was activated or to conclude that any complement consumption was pathogenetically important either to the rash or to the DIC. Conclusions become even more difficult when data from all monkeys are combined; no statistically significant changes in complement occurred at any time. And when higher doses of organisms (10^5) were administered, animals developed rash, coagulopathy and death within 48-72 hr when complement levels were well above baseline.

These data raised questions about the role of complement, and indeed of immune complexes, in the pathogenesis of RMSF. Therefore, guinea pigs genetically deficient in C4, i.e., lacking classic pathway activity, were inoculated IP with 10^7 EID R. rickettsii. Signs of disease, including vasculitis and coagulopathy, were indistinguishable from those in normo-complement animals, implying that complement, at least the classic pathway, is not critical to the clinical disease. To extend this idea further, normal guinea pigs were depleted of the later acting complement components (C3-9) by injection of cobra factor prior to infection. These studies are in progress but preliminary evaluation suggests that complement depletion did not affect the vasculitis and coagulopathy.

Recently a USAMRIID technician acquired RMSF in a laboratory accident and subsequently developed rash and thrombocytopenia. Serial complement levels were measured throughout his illness (Table II), with no depression of complement detected at any time. Evaluation of our data from these studies supports the concept that immune complexes and complement activation do not contribute significantly to the pathogenesis of RMSF, even though they may often be demonstrable during some phases of the illness.

TABLE II. SERIAL COMPLEMENT LEVELS IN A PATIENT WITH RMSF.

CH_{50} UNITS/ml BY DAYS AFTER FEVER ONSET						
2	3	4	5	7	10	21
43	35	36	51	41	41	41

Presentations:

1. Fine, D. P. Pneumococcal type-associated variability in alternate complement pathway activation. Presented, 14th Interscience Conference of Antimicrobial Agents and Chemotherapy, San Francisco, CA, 12 Sep 1974. (Proc., Abstract 145).

2. Fine, D. P. The complement system. Presented, Symposium on Immunology in Medicine, American College of Physicians Regional Meeting, Galveston, TE, 5 Dec 1974.

Publications:

None.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 74. p. 235-239. Fort Detrick, Maryland.

2. Fine, D. P. 1974. Activation of the classic and alternate complement pathways by endotoxin. J. Immunol. 112:763-769.

3. Fine, D. P., S. R. Marney, Jr., D. G. Colley, J. S. Sergent, and R. M. Des Prez. 1972. C3 shunt activation in human serum chelated with EGTA. J. Immunol. 109:807-809.

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5. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunochemistry. 2nd ed. (Charles C. Thomas), Springfield, Ill., pp. 838-850.

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7. Johnston, Jr., R. B., S. L. Newman, and A. G. Struth. 1973. An abnormality of the alternate pathway of complement activation in sickle-cell disease. N. Engl. J. Med. 288:803-808.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6415	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. SUM'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM'RY
74 07 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	62760A	3A762760A834	02	105
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Procede with Security Classification Code)

(U) Immune responses of peripheral leukocytes

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 03	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 75	1.0	70.2
	CURRENT 76	1.0	120.6

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: McGann, V. G.

TELEPHONE: 301 663-7341

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Eigelsbach, H. T. POC:DA

NAME:

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Immunity; (U) Tularemia; (U) Humoral antibody; (U) Military medicine; (U) Lymphocytes

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

23 (U) Evaluate factors affecting development, persistence and expression of in vitro reactivity to antigenic stimulation of peripheral lymphocytes from immunized subjects and relate cellular response to host resistance. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Isolate antigens from Francisella tularensis that can induce a specific proliferative response in sensitized lymphocytes. Employ selected preparations to examine the temporal course of development and duration of lymphocyte responsiveness as related to resistance in immunized and/or convalescent monkeys.

25 (U) 74 07 - 75 06 - Procedures for culture and processing of late log-phase filtrates of F. tularensis, SCHU S4, were developed and standardized. Ten 200-mg lots of concentrated, dialyzed filtrate were chromatographed on G-200 Sephadex columns and 5 eluate pools (F-I - F-V) were characterized by immunodiffusion and electrophoresis. Specific transformation of sensitized human or monkey peripheral lymphocytes (PL) was employed to compare these fractions with classical tularemia antigens, i.e., Foshay, ether-extractable (EEA) and polysaccharide (CHO) antigens. All antigens evoked specific transformation of sensitized human PL, but reactions were suppressed by antibody-positive autologous serum. With PL from groups of control and tularemia-survivor monkeys, only Foshay and EEA reactions clearly identified all the survivors; CHO reactions showed no between-group differences; and while F-reactions could differentiate between groups, there was wide gradation of responsiveness. Investigation of the relationship of PL transformation to host resistance is in progress.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 105: Immune Responses of Peripheral Leukocytes

Background:

In recent years an extensive body of literature has accumulated regarding involvement of cellular elements in host defense against microbial infections. Although a variety of in vitro systems for detecting specific responsiveness of sensitized lymphocytes appear to involve potential mediators of cellular immunity, definitive evaluation of their correlation with specific resistance has not been accomplished. The present investigations were designed to assess the hypothesis that antigen-specific transformation of lymphocytes from experimental animals vaccinated with live tularemia vaccine was indicative of the resistant state of the lymphocyte donor. Selection of Francisella tularensis antigens for in vitro studies was influenced by reports that resistance of mice to tularemia infection was unrelated to delayed type hypersensitivity responses with classical tularemia antigens, i.e., Foshay-type antigen (phenol-killed bacteria), ether-soluble cell wall antigen (EEA) or phenol-extracted polysaccharide (CHO).[1,2] Exploratory investigations[3] suggested the feasibility of employing antigenic components from F. tularensis culture filtrates because crude filtrates induced blastogenesis of sensitized lymphocytes, lacked lymphotoxic and mitogenic activity and contained a variety of antigens, some of which identified with components in classical tularemia antigens. Partial separation of serological reactive fractions could be effected by column chromatography with G-100 or DEAE-Sephadex.

Progress:

Antigen preparations. During exploratory studies, it became apparent that several lots of virulent F. tularensis, strain SCHU S4 culture would be required for preparation of adequate amounts of candidate antigens. Cultural conditions and procedures for harvesting and processing bacterial cells and culture filtrates have been standardized. An aliquot of seed culture (stored at -70 C) was employed to initiate 3 serial passages in MCPH medium, each of which was incubated on a reciprocal shaker for 16 hr. Cultures from the 3rd passage were chilled, pooled and treated with antibiotics (100 µg kanamycin, 1000 µg streptomycin and 1000 units penicillin G/ml culture). Subsequent processing was performed at 4 C: after the culture pool was centrifuged for 1.5 hr at 19,600 x g, the bacteria were washed twice with cold 0.15 M NaCl supplemented with antibiotics

and twice with 0.15 M NaCl, precipitated with acetone, dried and stored under vacuum; culture supernatant was recentrifuged to remove residual bacteria, sterilized by filtration through a 0.45-μm membrane and stored at -20 C. After completion of safety tests in mice, thawed crude filtrate was concentrated with Bio-Fiber 50 and exhaustively dialyzed against water; non-dialyzable material was lyophilized and stored under vacuum.

Partial separation of components in concentrated filtrate was achieved by gel filtration on G-200 Sephadex columns; separation was markedly superior to that previously reported for G-100 Sephadex.[3] Although serologic activity of eluate fractions with rabbit antiserum against crude filtrate or EEA and with 4-6-wk convalescent monkey serum served as a guide for preparation of filtrate pools, it did not correspond to spectrophotometric activity. Five antigenically dissimilar pools (F-I to F-V) were prepared by combining comparable fractions of eluate from ten 200-mg lots of concentrate. Characteristics of eluate pools from filtrate and from similarly processed uninoculated medium were compared (Table I).

TABLE I. CHARACTERISTICS OF ELUATE POOLS FROM G-200 SEPHADEX COLUMN CHROMATOGRAPHY ON SCHU S4 CULTURE FILTRATE OR UNINOCULATED MEDIUM.

TEST MATERIAL	POOL[a]	MW[b] (x 10^5)	CHROMOGEN	% ELUATE[c]	ANTIGENIC COMPONENTS, pH 8.3 (No. of reactions)
Culture filtrate	F-I	10	0	10	Anionic (1) No charge (1)
	F-II	1-3	0	12	Anionic (3) Cationic (1)
	F-III	0.5 - 1.0	0	7	Anionic (1) No charge (1) Cationic (1)
	F-IV	0.1 - 0.5	+++	54	Cationic (1)
	F-V	< 0.1	+	18	NR
Medium	M-I	0.1 - 0.2	+++	13	NR
	M-II	< 0.1	++++	87	NR

a. No measurable amounts of filtrate or medium recovered in eluates beyond F-V or M-II, respectively.

b. Approximate molecular size; G-200 column calibrated with Blue Dextran, bovine serum albumin, chymotrypsinogen A and myoglobin.

c. Calculated from dry weight of dialyzed eluate pools.

Medium components were chromogenic, serologically nonreactive and apparently localized in eluate fractions associated with components $\leq 10^4$ molecular weight; they were probably responsible for the high proportion of serologically nonreactive material in fractions F-IV and F-V. Analysis by immunodiffusion and immunoelectrophoresis indicated that pools F-I and F-II were antigenically unique but that pools F-III and F-IV shared a cationic component, with the major portion localized in F-III. Two minor antigens, one in F-III and one in F-IV, that were not detectable by immunoelectrophoresis are not included in Table I. Lyophilized eluates F-I to F-III were less readily soluble than the chromogenic pools. For lymphocyte stimulation studies, all preparations were redissolved in medium RPMI 1640, sterilized by filtration through a 0.45-μm membrane and stored at -20 C.

Ether-extractable antigen prepared from acetone-dried bacteria was purified by the $(NH_4)_2SO_4$ precipitation procedure of Ormsbee and Larson.[4] Redissolved dialyzed precipitates formed colloidal solutions that could be filtered through a 0.45-μm membrane and remained in suspension during storage at 4 C. After lyophilization, however, particle size in reconstituted preparations was considerably larger and filtered material contained little antigenic activity. Consequently, lyophilized EEA resuspended to a concentration of 1.0 mg/ml in medium RPMI 1640 was sterilized by exposure to irradiation with ^{60}Co for 24 hr and stored at 4 C. Over the course of several weeks, the sediment gradually disappeared and at the time lymphocyte stimulation studies were initiated, the irradiated preparation appeared to be colloidal.

Lymphocyte stimulation. Log_{10} dilutions of pools F-I - F-V, Foshay-type antigen, purified EEA and tularemia CHO were compared to determine the capability of these antigens to evoke specific transformation of lymphocyte microcultures. Initial studies were performed with peripheral lymphocytes from vaccinated, skin-test positive humans. Six replicate cultures of 10^5 lymphocytes in RPMI 1640 supplemented with nonimmune monkey serum or with autologous serum were exposed to each antigen concentration; cultures were pulsed with 0.02 μCi ^{14}C-thymidine for 4-6 hr before harvest. Although no significant stimulatory activity was observed in 48-hr cultures, indicating that the antigen preparations were not mitogenic, all antigens evoked stimulation of ^{14}C incorporation in 5-day cultures suggesting induction of specific responses (Table II). In the absence of antigen, cultures with negative monkey serum reacted like corresponding cultures with autologous serum; lymphocytes remained viable but there was no evidence of lymphocyte transformation. In the presence of antigen, however, ^{14}C incorporation was suppressed in cultures with the antibody-positive autologous serum. These findings imply that successful demonstration of specific stimulation of lymphocytes in whole blood cultures will be limited by serum antibody content of the lymphocyte donor.

TABLE II. ^{14}C-THYMIDINE INCORPORATION BY SENSITIZED HUMAN LYMPHOCYTES CULTURED FOR 5 DAYS WITH F. TULARENSIS ANTIGENS IN MEDIUM WITH NEGATIVE MONKEY OR AUTOLOGOUS SERUM.

STIMULATING ANTIGEN		MONKEY SERUM CULTURE		AUTOLOGOUS SERUM CULTURE	
Type	Dose[a]	Geom. Mean ^{14}C CPM	SI[b]	Geom. Mean ^{14}C CPM	SI
None	0	36	1.0	30	1.0
Foshay	40,000	1,632	45	112	3.7
	4,000	549	15	69	2.3
	400	89[c]	2.5	32[c]	1.1
EEA	25.0	1,316	36	458	15
	2.5	2,314	64	399	13
	0.25	2,306	64	376	12
	0.025	2,070	58	345	12
CHO	25	1,244	12.4	67	2.2
	2.5	40[c]	1.1	53[c]	1.8
F-I	25	1,409	39	147	4.9
	2.5	625	17	209	7.0
	0.25	125	3.5	158	5.3
F-II	25	1,480	41		
	2.5	154	4.3		
	0.25	117	3.2		
F-III	25	888	25		
	2.5	161	4.5		
	0.25	41[c]	1.1		
F-IV	25	467	13		
	2.5	110	3.0		
	0.25	50[c]	1.4		
F-V	25	526	15		
	2.5	975	27		
	0.25	60[c]	1.7		

a. Foshay antigen, bacteria/culture; others, µg/culture.

b. Stimulation Index = $\frac{\text{Mean CPM with antigen}}{\text{Mean CPM without antigen}}$.

c. No significant stimulation; value < 2 SD different from "no antigen."

The EEA preparation had the greatest stimulatory effect; responses to 0.025 - 2.5 µg EEA were essentially equivalent while the response to 25 µg was somewhat reduced. Transformation with Foshay-type antigen increased linearly from doses of 10^2 - 10^4 bacteria/culture, as did stimulation with F-I, F-III and F-IV at doses from 0.25 - 25 µg/culture. The CHO preparation was active only at 25 µg.

Lymphocytes from a monkey that had survived a persistent 35-day infection with SCHU S4 two years previously and separated lymphocytes from a monkey with no known experience with F. tularensis or its antigens were tested for ^{14}C incorporation after 3, 4, 5 and 7 days culture with $\log_{10}$ dilutions of antigen preparations. Values for cultures from the nonimmune monkey were not significantly different from background counts whereas stimulation of ^{14}C incorporation was observed with cultures from the immune monkey. Responses to Foshay-type or EEA antigens were essentially the same at 3, 4 and 5 days, but significantly diminished at 7 days. Since maximum response to most other antigens occurred at 4 days, subsequent cultures were pulsed with ^{14}C and harvested on day 4. Lymphocytes were obtained from 7 monkeys that survived SCHU S4 infection 2 yr previously and from 7 nonimmune animals. In each series of tests, $\log_{10}$ dilutions of antigen and 6 replicates were employed for paired cultures from an immune and a nonimmune monkey. The stimulation index (SI) for each set of replicates, i.e., the geometric mean ^{14}C CPM for antigen-stimulated cultures divided by the mean CPM for corresponding antigen-free cultures, was calculated to allow comparison of responses between individuals and between groups (Table III). Lymphocytes from all SCHU S4 survivors exhibited significantly better responses to Foshay and EEA antigens than lymphocytes from any of the control monkeys. There was no difference between groups when CHO or F-IV were stimulating antigens. Individual responses of survivors to all other antigens were highly variable; CPM and SI for lymphocytes from 5 immune monkeys were similar to those reported for sensitized human lymphocytes, but values for 2 immune animals were consistently low. Their mean SI (2.2) with antigens F-I, F-II, F-III and F-V, was somewhat higher than the corresponding mean for 6 control monkeys but significantly lower ($P < 0.001$) than the mean (8.2) for 1 control animal. Lymphocyte responsiveness was unrelated to severity of the previous SCHU S4 infection or to agglutinin or precipitin titers of the donors.

These findings indicate that specific lymphocyte responsiveness of recovered monkeys persists for at least 2 yr and is the same order of magnitude as that of lymphocytes from skin-test positive immunized humans. Responsiveness, however, may reflect only prior exposure to a single antigen or group of antigens, rather than persistence of host resistance to infection. Evaluation of the relationship of antigen-induced transformation to resistance of the individual will require a larger group of animals. Preliminary assessment will be performed with mice, a species for which vaccine-induced resistance is of relatively brief duration.

TABLE III. ANTIGEN-INDUCED TRANSFORMATION OF LYMPHOCYTES FROM 7 NONIMMUNE MONKEYS AND FROM 7 MONKEYS THAT SURVIVED TULAREMIA INFECTION.

TEST ANTIGEN	DOSE/10^5 LYMPHOCYTES	MEAN SI ± SD		SIGNIFICANT RESPONSES[b]	
		Nonimmune	Survivor	Nonimmune	Survivor
Foshay	$4x10^4$ bacteria	2.0 ± 0.6	24 ± 15**	0	7
EEA	0.25 µg	1.9 ± 0.8	19 ± 14**	0	7
CHO	25.0 µg	4.8 ± 4.4	10 ± 4.9	0	2
F-I	25.0 µg	2.4 ± 0.8	26 ± 18**	1	5
F-II	25.0 µg	1.8 ± 1.8	16 ± 13*	1	5
F-III	25.0 µg	2.1 ± 2.6	8.4 ± 7.5	1	4
F-IV	25.0 µg	2.8 ± 3.0	5.8 ± 4.6	1	3
F-V	25.0 µg	2.3 ± 3.3	8.3 ± 6.2*	1	3

a. Stimulation Index (SI) = Geometric mean ^{14}C CPM for 6 replicate antigen-stimulated cultures divided by corresponding value for 6 replicate antigen-free cultures; each value in the table represents the arithmetic mean SI for the group of 7 individuals.

b. Individual donors with SI > mean ± 2 SD for nonimmune groups.

* $P < 0.05$, ** $P < 0.02$ (paired for unpaired t-test).

Publications:

None.

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3. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974. pp. 241-246. Fort Detrick, Maryland.

4. Ormsbee, R. A., and C. L. Larson. 1955. Studies on Bacterium tularense antigens. II. Chemical and physical characteristics of protective antigen preparations. J. Immunol. 74:359-370.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0E6424	75 07 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
74 07 01	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A834	02	106
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Immunoprotective characterization of RNA-rich fractions

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	75 07	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 75	1.0	106.2
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Physical Sciences Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Andron, II, L. A.

TELEPHONE: 201 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immunology; (U) RNA-rich fractions; (U) Bacterial pathogens; (U) Military medicine

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

23 (U) Isolate and then characterize specific RNA fractions of pathogens and host spleen cells for immunoprotective methods. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Inoculate selected strains of mice with RNA fractions with and without adjuvants; subsequently challenge them with virulent or low-virulent organisms for assessment of protective effect.

25 (U) 74 07 - 75 06 - RNA-rich phenol extracts of a broken-cell supernatant from strain LVS, Francisella tularensis protected mice against lethal challenge with strain 425 but not against, strain SCHU-S4. The protective extracts were efficient inducers of specific opsonins, demonstrating one mechanism by which such extracts confer protection. Back-challenge studies of mice vaccinated with RNA-rich extracts suggest the potential usefulness of a 2-step procedure for the induction of high grade immunity.

Three primary objectives were established; (1) the definition of an infectious model system with which to study the potential protective effects of RNA-rich extracts, (2) development of suitable extraction and characterization methods for active extracts, and (3) determination of immunoprotective characteristics of active extracts. These objectives have been accomplished. The work unit is completed.

Publication: Infect. Immunity 12: in press, 1975.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 106: Immunoprotective Characterization of RNA-Rich Fractions

Background:

Ribosome-derived and RNA-rich extracts protect mice against a variety of bacteria and protozoa. Protection has been shown with submicrogram amounts of such vaccines against Mycobacterium tuberculosis, Salmonella typhimurium, and Vibrio cholerae. In addition, cross-protection between serotypes has been shown with preparations from Streptococcus pneumoniae, V. cholerae, and Neisseria meningitidis.

We have developed and tested 3 procedures for the isolation of RNA-rich extracts from Francisella tularensis. RNA-rich phenol extracts of a broken-cell supernatant from strain LVS protected mice against lethal challenge with F. tularensis strain 425 but not against strain SCHU S4. This protective activity is not a general property of bacterial RNA since extracts prepared from Staphylococcus aureus had no protective effect against tularemia. The protective activity was sensitive to RNAse but not pronase, suggesting an important role for RNA in the activity of these extracts. However, protective activity is not confined to a specific form of "immunogenic" RNA as shown by fractionation experiments with 5-20% density gradients. Details of these experiments have been reported.[1]

Progress:

One important aspect of the mechanism by which RNA-rich bacterial extracts confer protection may be the induction of agglutinating or opsonizing antibodies. To test this postulate, pooled sera from 4 groups of 50 mice each were assayed for HA titer and opsonizing activity in collaboration with Dr. Canonico and Mr. Mangiafico. Group 1 consisted of untreated controls; group 2, mice immunized SC with 10^2 LVS, boosted IP 30 days later with 10^2 SCHU S4, and bled after 15 days; group 3, mice vaccinated with 50 µg of S. aureus RNA-rich extract 15 days prior to serum collection; and group 4, mice vaccinated on an identical schedule with 50 µg of F. tularensis RNA-rich extract. HA

assays were performed using monkey RBC sensitized with F. tularensis lipopolysaccharide. Since phagocytosis of microorganisms in the presence of opsonizing antibody will result in an accelerated rate of conversion of glucose to CO_2 via the hexose monophosphate shunt, we determined the capability of each serum pool to accelerate conversion of glucose-1-^{14}C to $^{14}CO_2$ during phagocytosis of killed tularemia cells by monkey leukocytes.[2] The results, shown in Table I, indicated that tularemia RNA-rich extracts and live F. tularensis were highly effective for induction of specific opsonins. HA antibody was detected only in sera which showed opsonic activity.

TABLE I. OPSONIZING AND HA ANTIBODIES IN SERA OF MICE VACCINATED 15 DAYS PRIOR TO BLEEDING

IMMUNIZATION OF SERUM DONORS	SERUM ANTIBODY	
	Opsonic activity[a] (cpm ± SD)	HA Titer
None	800 ± 77	<1:10
Extract B - S. aureus (50 µg)	675 ± 156	<1:10
Extract B - LVS (50 µg)	2,400 ± 370	1:20
Live LVS + booster of live SCHU S4	1,300 ± 136	1:40

a. $^{14}CO_2$ cpm

Back-challenge studies of mice vaccinated with either heat-killed tularemia or RNA-rich extracts of tularemia suggested the potential usefulness of a 2-step procedure for the induction of high-grade immunity. For example, tularemia (strain 425) which is too virulent to be used as a live vaccine strain,[3] may be acceptable as an immunogen when used in conjunction with prior vaccination with RNA-rich extracts or other killed preparations. This procedure would involve administration of a low dose of the virulent strain 15 days after a dose of RNA-rich extract. Such extracts have the advantage of being prepared free of endo- or exotoxin contamination.

Presentation:

Andron, L. A., and H. T. Eigelsbach. Characterization of protective effects in mice of RNA-rich extracts from Francisella tularensis. Presented, D.C.-MD. Chapter, American Society for Microbiology, Fort Detrick, MD. 27 Apr 1974.

Publication:

Andron, II, L. A., and H. T. Eigelsbach. 1975. Biochemical and immunodogical properties of ribonucleic acid-rich extracts from Francisella tularensis. Infect. Immunity 12: ir press.

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974, p. 247-250, Fort Detrick, MD.

2. Proctor, R. A., J. D. White, E. Ayala, and P. G. Canonico. 1975. Phagocytosis of Francisella tularensis by rhesus monkey peripheral leukocytes. Infect. Immunity 11:146-151.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6427	75 07 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
74 07 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	62760A	3A762760A834	02	107
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Procede with Security Classification Code)

(U) Cell-mediated immunity to pulmonary infection

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 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: 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 75	1.4	143.0
FISCAL YEAR CURRENT 76	0.1	7.8

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Hetsko, C. M.

TELEPHONE: 301 663-7341

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Dangerfield, H. G.

NAME: Walker, J. S. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Influenza virus; (U) Migration inhibition factor (MIF); (U) Infectious disease; (U) Immunity; (U) Lungs

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

23 (U) Characterize the cell-mediated immune response of the lung and evaluate its role in defense against infectious diseases of military significance. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) After adapting an assay for macrophage inhibition factor (MIF), measure cell-mediated immunity in lungs from experimental animals exposed to disease by parenteral or aerosol routes.

25 (U) 74 07 - 75 06 - Further improvements have been achieved in the agarose micro-droplet assay for MIF; PPD-stimulated cells from BCG-immune guinea pigs showed good MIF activity in this system. When influenza investigations were discontinued, other infectious antigens were evaluated for possible study. Respiratory Mycoplasma pneumoniae studies in the hamster have begun, but MIF results are equivocal at present.

Significant findings have been obtained in immune studies with the mouse tularemia model. Time of appearance and disappearance of MIF correlates closely with the time at which spleen cells can passively transfer protection against lethal challenge to non-immune recipients. Both MIF activity and successful passive cell transfer of immunity require an active and ongoing immune response in the donor cells for the mouse tularemia model.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 107: Cell-Mediated Immunity to Pulmonary Infection

Background:

The lung is the portal of entry and site of disease for many microorganisms which infect man. Although considerable information has been acquired regarding antibody mediated defense mechanisms in the lung, relatively little is known about the role of cell-mediated immunity (CMI) in pulmonary infection.[1,2]

In CMI responses, specific antigen stimulates specifically sensitized lymphocytes of thymic origin (T cells) to release various mediators which are responsible for the development of the characteristic mononuclear inflammatory reaction associated with delayed type hypersensitivity. It is postulated that, under the influence of mediators released by specifically stimulated lymphocytes, macrophages accumulate and are stimulated to counteract the invading organism.[3]

Assays for one mediator released in the CMI reaction, migration inhibition factor (MIF), have become well established as an *in vitro* correlate of the CMI response *in vivo*. MIF acts to decrease the random migration of normal macrophages. Although techniques for MIF measurement are rather complex, inhibitory effects can be observed on macrophages migrating out of capillary tubes, or out of microdroplets of agarose.

Investigations to be reported have been directed toward development and utilization of the agarose microdroplet MIF assay for evaluation of cellular aspects of the immune response in the lungs of experimental animals.

Progress:

In the agarose MIF assay system,[4] macrophages or a mixture of immune lymphocytes and macrophages are incorporated into microdroplets of a special agarose which has a melting point of 37 C. After the cell-containing droplets are deposited into 96-well flat bottom microtiter dishes, tissue culture media is used to bathe the cells, and migration of macrophages from the droplets is observed microscopically after 18 and 24 hr incubation. Migration distances are measured in 4 quadrants of each droplet with a microscopic grid; a number of droplets can be evaluated for each experimental situation. When MIF is present, migration distances are diminished and inhibition can be quantitated with the following formula:

$$\% \text{ Inhibition} = \left(1 - \frac{\text{Mean migration distance with antigen}}{\text{Mean migration distance without antigen}}\right) \times 100$$

Two agarose droplet techniques for detecting MIF are available: (1) the 2-step assay in which immune lymphocytes are cultured with antigen and the culture supernatant is added to medium surrounding droplets of macrophages, or (2) the 1-step assay in which immune lymphocytes and macrophages are contained in the same droplet and antigen is added to the bathing medium. The 1-step assay is generally less complex and more sensitive.

Improvements have been achieved in the agarose microdroplet assay by decreasing the size of the agarose microdroplets from 2 to 1 µl, the volume of bathing medium from 0.2 to 0.1 ml, and by eliminating the need for a cementing precoat of agarose. This has been accomplished without comprising the sensitivity of the bioassay system, and has further diminished the quantity of cells required and increased the number of replicate determinations which can be made on each sample.

Agarose microdroplets containing guinea pig oil-induced peritoneal exudate cells yield very consistent control migration values. Likewise, consistent inhibition of migration is observed in the 1-step assay for MIF when peritoneal exudate cells from PPD-skin-test positive, BCG-immunized guinea pigs are observed in the presence of PPD (Table I). These MIF results are comparable to those reported by other investigators.[3,4]

TABLE I. ONE-STEP MIF ASSAY WITH PERITONEAL EXUDATE CELLS FROM PPD-SENSITIZED AND CONTROL GUINEA PIGS.

ANTIGEN DOSE (µg/ml PPD)	% INHIBITION	
	Control (Negative PPD Skin Test)	BCG-Immune (Positive PPD Skin Test)
1	7	16
10	10	23[a]
25	7	20[a]
100	3	47[a]

a. Significant inhibition, > 20%.

When MIF work at the Institute with influenza[5] in the mouse was discontinued other possible infectious organisms with potential for use as CMI models in respiratory disease were evaluated. The choice of microorganisms

was restricted to those under consideration for study in the Aerobiology Division. As a result, model systems antigens evaluated for possible collaborative studies included tularemia and parainfluenza type I in mice and Mycoplasma pneumoniae in hamsters.

Attempts to detect systemic MIF with the 1- and 2-step assays in peritoneal exudate cells of mice exposed to aerosols of parainfluenza type I were unsuccessful. Likewise, MIF activity could not be detected with spleen cell suspensions from mice immunized against parainfluenza type 1, BCG or tularemia.

In collaboration with the Laboratory of Infectious Diseases, National Institutes of Health, and the Aerobiology Division, a number of studies were initiated to evaluate immunity against M. pneumoniae in the hamster. A description of this model and associated findings are reported under project 834 02 416. To date, MIF activity has been equivocal, and work is continuing on development of an improved antigen preparation for MIF stimulation.

Significant progress has been achieved with tularemia-immune mice in collaborative studies with Work Unit 834 02 103. Following parenteral immunization of AKR-J mice with the living vaccine strain of tularemia (LVS) and IP exposure 30 days later to the virulent strain, SCHU S4, passive transfer of immune spleen cells protected nonimmune mice against lethal challenge. Donor spleen cells were immunocompetent from 6-19 days after SCHU S4 booster, and conferred protection to all recipients when transfer was effected at 12 days.

Oil-induced peritoneal exudate cells from similarly treated mice were evaluated with the 1-step MIF assay; stimulation was effected with several dilutions of dialyzed, phenol-killed tularemia antigen (Table II). Minimum MIF activity was observed at 16 days only in preparations stimulated with the highest antigen concentration. Although these LVS-treated mice were resistant at 30 days post-LVS to challenge with 10^3 minimum lethal doses (MLD) of SCHU S4, their stimulated cells failed to produce MIF and spleen cells were incapable of passively transferring immunity to nonimmune animals. Marked MIF responses were noted at 7 and 14 days following SCHU S4 booster, some activity at 21 days and no MIF response at 28 and 31 days.

The presence of MIF in exudate cells from tularemia-immune mice correlated very closely with the ability of immune spleen cells to protect nonimmune recipients against lethal challenge. These findings provide the first evidence that the presence of this in vitro indicator of CMI closely correlates with the in vivo ability of cells to passively protect against lethal challenge and also provides further substantiation of the use of MIF as an in vitro indicator of CMI.

TABLE II. MIF RESPONSES OF PERITONEAL EXUDATE CELLS FROM AKR-J MICE IMMUNIZED WITH F. TULARENSIS LVS STRAIN AND INOCULATED 30 DAYS LATER WITH STRAIN SCHU S4.

DAY POST TREATMENT		% INHIBITION[a] BY DOSE KILLED SCHU S4/ml of test antigen		
LVS	SCHU S4	10^9	10^8	10^7
0	--	-20	- 3	- 4
		-24	- 8	- 7
		-16	- 6	- 4
16	--	16	6	4
		16	5	3
		21[b]	10	8
30	0	-10	- 2	- 9
		- 2	2	10
		- 1	5	11
		- 8	- 9	2
37	7	61[b]	50[b]	45[b]
		70[b]	57[b]	40[b]
		68[b]	62[b]	38[b]
		61[b]	51[b]	35[b]
44	14	53[b]	25[b]	9
		40[b]	38[b]	
		50[b]	26[b]	31[b]
51	21	30[b]	3	4
58	28	18	10	8
		- 3	- 2	-10
61	31	6	- 1	- 5

a. Values represent means for 9 replicate droplets prepared with cells pooled from 5 mice. Negative value = enhancement.

b. Significant inhibition, > 20%.

The data also suggest that the appearance of MIF in this system, and the ability of spleen cells to confer passive protection, are dependent upon an ongoing and active CMI response in the animal, and not upon the potential ability of the animal to mount a CMI response in an otherwise unstimulated animal. A similar hypothesis has been expressed[6] recently; the mouse tularemia data provide some substantiation. Confirmatory studies are being completed, and 2-step MIF assays with tularemia immune spleen cells are being evaluated.

The agarose microdroplet assay with the tularemia antigen system provides very consistent results and permits us to observe a dose-response relationship to stimulatory antigen. There is a decided advantage over other MIF assay systems, as it may now be possible to quantitate the MIF response, in addition to reporting MIF activity qualitatively.

Publications:

None.

LITERATURE CITED

1. Mackaness, G. B. 1971. The J. Burns Amberson lecture -- The induction and expression of cell-mediated hypersensitivity in the lung. Am. Rev. Resp. Dis. 104:813-828.

2. National Heart and Lung Institute, Respiratory Diseases Task Force Report on Problems, Research Approaches, Needs. DHEW Publication No. (NIH) 73-732. p. 6, 7, 10, 13, 121, 125. 1972.

3. David, J. R. 1973. Lymphocyte mediators and cellular hypersensitivity. N. Engl. J. Med. 288:143-149.

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5. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Program Progress Report, FY 1974. p. 251-256. Fort Detrick, Md.

6. Lefford, M. L. 1975. Delayed hypersensitivity and immunity in tuberculosis. Am. Rev. Resp. Dis. 111:243-246.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0E6428	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 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	62760A	3A762760A834	02	108
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Characterization of host protease inhibitor responses in infectious disease

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 03	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 75	1.0	111.0
FISCAL YEAR CURRENT 76	0.3	37.6

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Berninger, R. W.
TELEPHONE: 301 663-7341
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Mathis, R. K.
NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Alpha-1-antitrypsin; (U) Protease inhibitor; (U) Francisella tularensis; (U) Salmonella typhimurium; (U) Rickettsia rickettsii

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

23 (U) Evaluate the role of alpha-1-antitrypsin in host responses to infection, especially molecular changes with emphasis on carbohydrates. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Infect rhesus monkeys with various organisms and obtain sequential blood samples for quantitation, purification and charcterization of alpha-1-antitrypsin glycoprotein.

25 (U) 74 07 - 75 06 - Increased serum protease inhibitory activity during infection of rhesus monkeys with R. rickettsii was associated with increased serum alpha-1-antitrypsin and absolute neutrophil count but was unrelated to severity of clinical signs or serum alpha-2-macroglobulin.

Rabbit antimonkey alpha-1-antitrypsin was produced and electrophoretic techniques (acid starch gel, Laurell, and rocket) were developed and standardized for analysis of alpha-1-antitrypsin in serum or in purified preparations. Procedures for purification of serum alpha-1-antitrypsin were modified and a highly purified preparation was produced in quantity.

Standardization of monosaccharides with gas liquid partition chromatography is in progress. Preliminary data from an uninfected monkey suggest similar carbohydrate composition for monkey and human alpha-1-antitrypsin.

Publications: Clin. Res. 22:707A, 716A, 1974.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 108: Characterization of Host Protease Inhibitor Responses in Infectious Diseases

Background:

Although the role of proteolytic enzyme inhibitors in host responses during infectious diseases in humans is largely uncharacterized, marked increases in serum levels of α-1-antitrypsin (α-1-AT) are seen during several human infectious diseases.[1] In vitro studies indicate important functional properties for the human glycoprotein protease inhibitor, α-1-AT, in maintenance of tissue integrity.[2] It is the major serum inhibitor of several protease enzymes which are active during the inhibitory process. Other serum protease inhibitors, including α-2-macroglobulin (α-2-MG), may contribute additional protection during infection.

Evidence was reported previously that the rhesus monkey was an excellent model for investigation of protease inhibitor responses during infection.[3] The range of values for trypsin inhibitory capacity (TIC) of normal monkey sera was comparable to that of normal human sera; infection-induced increases in serum TIC of monkey sera occurred and preliminary observations indicated that TIC activity was retained in purified preparations of rhesus monkey α-1-AT. In the present studies investigations of α-1-AT and TIC responses following infection were continued and efforts were directed toward improving procedures for purification and adapting or developing techniques to assay qualitative as well as quantitative changes in the molecular structure of α-1-AT induced by infection.

Progress:

Evaluation of the TIC assay procedure was continued with 40 samples of human sera; the mean value for the group was 1.16 ± 0.21 (SD). This is comparable to report in the literature of 1.07 ± 0.12[4] and 1.15 ± 0.10.[5] Reproducibility was within 5% for replicate assays and for assays performed at various times on individual sera.

Assays of blood specimens obtained from 13 patients with influenza-like illness failed to show the expected increased TIC activity during illness; in fact, all values were abnormally low. Inquiry revealed that blood specimens were processed for plasma. Since serum, rather than plasma, was employed for all previous work, a study was initiated to determine the effect of

anticoagulants upon the TIC assay. The effect of heparin, EDTA, Na-citrate or Na-oxalate on TIC activity was examined by assaying mixtures of anti-coagulant with sera from 3 normal humans (Table I). Although 15 units of heparin/ml of serum or 0.15% EDTA appeared to have no effect on TIC activity, 0.38% Na-citrate decreased TIC values; 0.15% oxalate also decreased values for 2 of 3 samples. Therefore, in all subsequent studies only sera were employed. The mechanism involved with citrate and oxalate suppression is unknown.

TABLE I. EFFECTS OF 15 UNITS OF HEPARIN/ml, 0.15% EDTA, 0.38% Na-CITRATE OR 0.15% OXALATE ON TIC ACTIVITY IN SERA FROM 3 NORMAL HUMANS.

ANTI-COAGULANT	TIC (mg/ml)		
	RM	KM	GC
None	0.88	1.35	0.93
Heparin	0.90	1.38	0.88
EDTA	0.91	1.32	0.99
Citrate	0.57	0.74	0.74
Oxalate	0.82	1.06	0.68

Throughout the past year, samples of rhesus monkey serum or plasma were obtained from several colleagues who were examining various parameters of host response in a variety of microbial infections. In one series of studies, sequential samples of sera were obtained from 24 monkeys prior to and following IV injection with Rickettsia rickettsii, Sheila Smith strain. Groups of monkeys were inoculated IV with challenge doses ranging from 10 - 10^6 chick embryo median lethal doses ($CELD_{50}$).

Serum TIC activity was compared with levels of α-1-AT and α-2-MG, leukocyte counts and clinical manifestations. A wide spectrum of clinical signs was observed ranging from no apparent illness to mild to severe febrile reaction to disseminated intravascular coagulopathy (DIC) and death. Incidence and time of death (Table II) indicate there was an erratic dose response. As expected, mean serum TIC values increased following inoculation with rickettsiae (Table III), but the increase was unrelated to severity of clinical signs.

TABLE II. INCIDENCE AND DAY OF DEATH IN RHESUS MONKEYS CHALLENGED IV WITH R. RICKETTSII.

CHALLENGE DOSE ($CELD_{50}$)	NO. DEATHS / NO. CHALLENGED	DAY OF DEATH
10^1	1/4	8
$10^{1.5}$	5/6	7, 7, 7, 8, 8
10^2	1/4	9
10^6	8/10	3, 4, 4, 4, 4, 4, 5, 5

TABLE III. SERUM TRYPSIN INHIBITORY CAPACITY (TIC) MEAN VALUES (mg/ml) FOR RHESUS MONKEYS INOCULATED IV WITH 10^1 - 10^6 $CELD_{50}$ R. RICKETTSII.

TIME (Days)	TIC mg/ml ± SD				
	0[a] (n = 2)	10^1 (n = 4)	$10^{1.5}$ (n = 6)	10^2 (n = 4)	10^6 (n = 10)
Control	1.40 ± 0.19	1.38 ± 0.16	1.42 ± 0.24	1.50 ± 0.25	1.39 ± 0.21
1	1.75		1.41 ± 0.46		1.74 ± 0.24[b]
2		1.59 ± 0.10		1.53 ± 0.24	2.04 ± 0.47[b]
3	1.44		1.37 ± 0.28		2.02 ± 0.36[b]
4		1.66 ± 0.18		1.86 ± 0.56	1.99 ± 0.48[b]
5			1.99 ± 0.26[b]		1.82[c]
6	1.50	1.90 ± 0.46		2.22 ± 0.66	2.10[c]
7			2.24 ± 0.26[b]		1.46
8	1.74	2.48 ± 0.58[b]	2.85[c]	2.34 ± 0.47[b]	
9			2.46[c]		1.58
11	1.46	2.42 ± 0.71[b]	3.16[c]	2.19 ± 0.43[b]	2.00[c]
14	1.36	2.30[c]		1.85 ± 0.30	1.86[c]
15			1.96[c]		2.40[c]
20			1.93[c]		2.45[c]
22	1.62	2.81[c]		1.77 ± 0.23	1.73
27			1.68		1.25

a. Animals inoculated with noninfected yolk sac at a concentration and amount equivalent to that of the 10^6 $CELD_{50}$ inoculum.

b. Value exceeds corresponding prechallenge control mean + 2 SE.

c. Value exceeds corresponding prechallenge control mean + 2 SD.

In Tables III - VI, values without a recorded SD were derived from 1 or 2 samples. Although magnitude and duration of TIC responses were unaffected by challenge dose, onset of response appeared to be dose-related. However, the response of control monkeys was equivocal; 1 of the 2 monkeys inoculated with uninfected yolk sac, equivalent in concentration and amount to that employed with the maximum challenge, exhibited significantly increased TIC activity as compared with its prechallenge mean value. Studies with similarly treated control monkeys are in progress to evaluate this finding.

TABLE IV. SERUM α-1-ANTITRYPSIN (α-1-AT) IN RHESUS MONKEYS FOLLOWING IV CHALLENGE WITH 10^1 - 10^6 R. RICKETTSII AS ESTIMATED BY RADIAL IMMUNODIFFUSION (RID) AGAINST ANTIHUMAN α-1-AT.

TIME (Days)	mg/100 ml ESTIMATE OF α-1-AT ± SD				
	0[a] (n = 2)	10^1 (n = 3)	$10^{1.5}$ (n = 3)	10^2 (n = 3)	10^6 (n = 2)
Control	528 ± 34	524 ± 45	513 ± 56	544 ± 65	511 ± 48
1	570		567 ± 58		560
2		550		627 ± 110	640[c]
3	540		597 ± 145		620[c]
4		547 ± 68		627 ± 143	590
5			667 ± 144		600
6	570	660[c]		667 ± 144	700[c]
7			710 ± 115[b]		700[c]
8	555	707 ± 101[b]	750[c]	727 ± 91[b]	
9			570		720[c]
11	555	665[c]	860[c]	720[c]	750[c]
14	520	675[c]		745[c]	
15			830[c]		720[c]
20			630[c]		750[c]
22	520	900[c]		630	
27			670[c]		570

a. Animals inoculated with noninfected yolk sac at a concentration and amount equivalent to that of the 10^6 $CELD_{50}$ inoculum.

b. Value exceeds corresponding prechallenge control mean + 2 SE.

c. Value exceeds corresponding prechallenge control mean + 2 SD.

TABLE V. SERUM α-2-MACROGLOBULIN (α-2-MG) IN RHESUS MONKEYS FOLLOWING IV CHALLENGE WITH 10^1 - 10^6 R. RICKETTSII-RELATIVE MEAN AMOUNTS ESTIMATED IN mg % BY RADIAL IMMUNODIFFUSION (RID) AGAINST ANTIKUMAN α-2-MG.

TIME (Days)	mg/100 ml ESTIMATE OF α-2-MG ± SD				
	0[a] (n = 2)	10^1 (n = 3)	$10^{1.5}$ (n = 3)	10^2 (n = 3)	10^6 (n = 2)
Control	385 ± 71	351± 54	394± 68	399± 32	295± 39
1	340		360± 66		275
2		355		377± 40	240
3	340		373± 57		270
4		317± 50		377± 12	220
5			367± 46		290
6	340	310		383± 40	270
7			400± 79		250
8	355	300 ± 104	370	310± 60	
9			270		270
11	340	255	460	205	250
14	300	285		340	
15			290		230
20			290		340
22	350	270		395	
27			340		250

a. Animals inoculated with noninfected yolk sac at a concentration and amount equivalent to that of the 10^6 $CELD_{50}$ inoculum.

In an attempt to correlate serum TIC activity with the amount of α-1-AT in serum, the technique of radial immunodiffusion (RID) was employed. Estimated quantities were derived from reactions with albumin α-1-AT serum because single factor antimonkey sera were not available (Table IV). It is important to note that the data represent relative values for monkey α-1-AT because there is only partial identity between the monkey and human molecular species; nonetheless, values for monkeys inoculated with rickettsiae follow the same trends as serum TIC activity.

Since ~ 10% of human TIC activity has been attributed to the α-2-MG fraction,[6] similar estimates for rhesus monkey α-2-MG were derived from reactions with antihuman α-2-MG serum (Table V). In contrast to the findings with α-1-AT, there were no infection-induced changes in α-2-MG levels.

Typical neutrophilic responses occurred following rickettsial challenge (Table VI). Values for each animal were normalized with respect to the corresponding prechallenge mean of absolute neutrophil counts; group means were derived from these normalized values. It can be seen that neutrophil responses to the lower doses of rickettsial challenge preceded enhanced serum TIC activity.

TABLE VI. GROUP MEANS OF NORMALIZED ABSOLUTE NEUTROPHIL COUNTS FOR RHESUS MONKEYS INOCULATED IV WITH 10^1 - 10^6 $CELD_{50}$ OF R. RICKETTSII.

TIME (Days)	NORMALIZED NEUTROPHIL COUNTS ± SD				
	0[a] (n = 2)	10^1 (n = 4)	$10^{1.5}$ (n = 6)	10^2 (n = 4)	10^6 (n = 10)
Control	100	100 ± 0	100 ± 0	100 ± 0	100 ± 0
1	99		126 ± 36		179 ± 90[b]
2		143 ± 30[b]		116 ± 10[b]	276 ± 38[b]
3	84		368 ±100[b]		296 ±150[b]
4		322 ± 48[b]		218 ± 70[b]	219 ±103[b]
5			438 ±179[b]		121
6	134	444 ± 92[b]		262 ± 79[b]	106
7			203 ± 76[b]		188
8	159	342 ±142[b]	148	225 ± 46[b]	
9			148		288
11	148	343 ±161[b]	443	277 ± 31[b]	152
14	88	261 ±150		127 ± 47	61
15			474		314
20			522		140
22	78	292 ± 241		80 ± 32	72
27			374		244

a. Animals inoculated with noninfected yolk sac at a concentration and amount equivalent to that of the 10^6 $CELD_{50}$ inoculum.

b. Values exceed corresponding prechallenge control mean + 2 SE.

In previous studies, we reported elevation of TIC activity in rhesus monkeys infected with Francisella tularensis, SCHU S4, or Salmonella typhimurium.[3] These microorganisms, as well as rickettsiae, are either known or suspected to possess endo- and/or exotoxins. Therefore, a preliminary study was performed to evaluate TIC activity in rhesus monkeys infected with Streptococcus pneumoniae, type I (ATCC #6301), a toxin-free microorganism. Three monkeys were inoculated IV with 10^8 S. pneumoniae; sequential bleedings were obtained for TIC assay and neutrophil determinations. Prechallenge baseline and maximum postchallenge values are presented in Table VII.

TABLE VII. SERUM TRYPSIN INHIBITORY CAPACITY AND NEUTROPHIL COUNTS OF MONKEYS (n = 3) CHALLENGED IV WITH S. PNEUMONIAE.

INOCULUM	MEAN TIC mg/ml		MEAN NEUTROPHILS/mm^3 x 10^3	
	Baseline	Postchallenge Maximum (Day)	Baseline	Postchallenge Maximum (Day)
10^8	1.04 ± 0.02[a]	2.64 (2)	1.78	17.7 (1)
	1.10 ± 0.02	2.38 (2)	2.66	17.7 (1)
	1.16 ± 0.17	2.10 (2)	2.77	18.2 (1)
0.85% Saline	1.12 ± 0.02	1.47 (4)	3.11	8.05

a. Mean ± SD.

Although maximum responses occurred early (within 1-2 days) the course of response for both parameters resembled that observed in rickettsial infection.

Efforts were successful in developing the acid starch gel electrophoresis (ASGE) technique to determine if rhesus monkey α-1-AT exhibits molecular heterogeneity similar to that observed in humans. Normal rhesus monkey serum had 2 dark staining bands similar in mobility to the normal human genetic bands, MM; in addition, 2 slower dark staining bands were noted in the region that corresponded to α-1-AT.

During this past year, α-1-AT was isolated in quantity. The 50-75% $(NH_4)_2SO_4$ precipitate from pooled rhesus monkey sera was dissolved in 0.025 M phosphate buffer, pH 7.6, with 1 mM β-mercaptoethanol and applied to a DEAE cellulose column. Active fractions, determined by RID, were further purified by passage through a concanavalin A-sepharose column. Dialysis against distilled water followed by lyophilization at 0 C yielded a white solid. This isolated α-1-AT exhibited the same ASGE pattern as serum α-1-AT. In addition, SDS polyacrylamide gel electrophoresis revealed a single protein band. Immunoelectrophoresis indicated that the isolated α-1-AT contained a

small amount of albumin contaminant. Rabbits were immunized with this material in complete Freund's adjuvant to obtain antisera for immunoanalysis of monkey serum α-1-AT by Laurell's 2-directional electrophoresis[7] and for quantitation with the rocket technique.[8] In subsequent preparations the albumin contaminant was removed by rechromatographing on the concanavalin A-sepharose column. Molecular weight of this purified material is estimated by SDS polyacrylamide electrophoresis to be ~ 60,000.

This material will be employed to investigate infection-induced alteration in carbohydrate (CHO) or amino acid portions of the α-1-AT molecule. It is postulated that the increased rate of synthesis and release of α-1-AT from the liver during infection may be associated with altered CHO component(s) of the α-1-AT molecule; possibly, late addition of carbohydrate residues, including sialic acid, may govern the rate of transport of α-1-AT from the liver.[9] Gas liquid partition chromatography (GLPC) is a precise method for analysis of CHO portions of molecules. Standardization of this technique with D-mannose, sialic acid, N-acetyl-D-galactosamine, α-L-glucose, N-acetyl-D-glucosamine, D-fucose and α-D-galactose against mannitol is in progress. Retention time with GLPC identifies CHO moieties and their absolute amounts are derived from calculation of response factor (RF):

$$RF = \frac{\left[\frac{\text{Monosaccharide area count}}{\text{Mannitol area count}}\right]}{\left[\frac{\text{mMole monosaccharide}}{\text{mMole mannitol}}\right]}$$

Preliminary results are shown in Table VIII. Following completion of standardization studies, purified α-1-AT from normal and infected monkeys will be analyzed. Preliminary data from an uninfected monkey suggest that the CHO composition of its α-1-AT is similar to that of human α-1-AT.

TABLE VIII. STANDARDIZATION OF GAS LIQUID PARTITION CHROMATOGRAPHY TECHNIQUE WITH MONOSACCHARIDES (MANNITOL INTERNAL STANDARD).

MONOSACCHARIDE	RETENTION TIMES (min)	RF	
		Experimental	Literature
Mannitol	76.45	1.0	1.0
D-Mannose	50.15, 54.46	1.04	1.0
Sialic Acid	153.13	0.52	0.48
N-acetyl-D-galactosamine	84.13, 89.95	a	0.63
α-L-glucose	63.77, 68.05	a	0.91
N-acetyl-D-glucosamine	88.21, 96.53, 100.66, 107.18	a	0.77
D-Fucose	24.31, 25.87, 28.25	a	0.71
α-D-galactose	52.07, 56.06, 61.17	a	1.0

a. In progress.

Presentations:

1. Mathis, R. K., and R. W. Berninger. Alpha-1-antitrypsin (α-1-AT) during infections in the rhesus monkey. Presented, Eastern Section, American Federation of Clinical Research, Boston, Mass., 10-11 Jan 1975. (Clin. Res. 22:707A).

2. Berninger, R. W., R. K. Mathis, and R. M. Leese. Isolation and characterization of rhesus monkey alpha-1-antitrypsin (α-1-AT). Presented, Eastern Section, American Federation of Clinical Research, Boston, Mass., 10-11 Jan 1975. (Clin. Res. 22:716A).

Publications:

None.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6416	75 07 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
75 02 19	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	4. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A834	02	109
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Aerosol therapy of respiratory bacterial infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense;

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
75 02	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 75	0.5	107.0
	CURRENT 76	0.5	131.6

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Aerobiology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Berendt, R. F.

TELEPHONE: 301 663-2439

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Walker, J. S.

NAME: Hetsko, C. M. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Bacterial diseases; (U) Antibiotics; (U) Aerosols; (U) Respiratory tract

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

23 (U) Determine effects of antibiotic aerosols on respiratory bacterial infections in selected animal models. This is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) After obtaining data on feasibility of aerosol therapy, determine pharmacodynamics, toxicity, and efficacy.

25 (U) 75 02 - 75 06 - Aerosols of antibiotics have proven to be more effective in treatment of Klebsiella pneumoniae infection than intramuscular inoculation. Increased effectiveness was most marked early in the disease process, before dissemination from the lungs had become extensive. Further investigation indicated that increasing inhaled or intramuscular dosage from 10 to 20 mg/kg resulted in lower survival of infected mice. The percent survival again increased at levels of 40 mg/kg and above. Studies of kanamycin clearance showed that the antibiotic persists at high concentration in the lungs for at least 48 hr after aerosol administration; very little antibiotic was detected in blood or kidneys. Initial experiments indicate that physical factors such as solute concentration and particle size may play an important role in the efficacy of aerosol treatment.

Publication: Proc., 14th Interscience Conf. Antimicrobial Agents and Chemotherapy, Abstract 176, 1974.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 109: Aerosol Therapy of Respiratory Bacterial Infections

Background:

Aerosol antibiotic therapy has had varying popularity with the medical profession. After World War II the ready availability of penicillin resulted in the publication of hundreds of reports dealing with aerosol therapy of numerous infectious diseases. Very few of these reports are of value today except for those of Barach and co-workers[1] who recognized the importance of such factors as particle size, breathing pattern, configuration of the respiratory tract, and site of particle deposition.

In the early 1950's the use of aerosols for administration of antibiotics declined, primarily because of the discovery of broad-spectrum antibiotics that were effective orally and because of increasing concern with adverse reactions such as bronchospasm and hypersensitivity.[2]

In recent years interest in aerosol antibiotic therapy has revived, primarily because certain aminoglycoside antibiotics such as kanamycin, gentamicin and polymyxin B are poorly absorbed from the lung.[3]

The model system chosen for preliminary work has been the Swiss-ICR mouse infected with an LD_{95} of Klebsiella pneumoniae. In some studies, F-344 rats were substituted for mice. Kanamycin aerosol therapy was used.

Progress:

The results of preliminary experiments investigating the feasibility of aerosol therapy were previously reported.[4] These experiments, summarized in Table I, established the superiority of aerosol over IM kanamycin therapy at various times after infection. When multiple doses of antibiotic were given

TABLE I. EFFECT OF TIME OF ADMINISTRATION ON RESPONSE TO 5 MG/KG BODY WT OF KANAMYCIN

TIME OF TREATMENT (hr postexposure)	ROUTE OF TREATMENT	RESPONSE (Survivors/25)	% SURVIVORS	P
0	None	3	12	
6	IM	18	72	0.05
	Aerosol	25	100	
24	IM	9	36	0.005
	Aerosol	23	92	
30	IM	8	32	0.005
	Aerosol	21	84	

at 24, 48 and 72 hr, aerosol therapy resulted in 92% survival in contrast to 70% for IM-treated mice (Table II). If therapy was delayed until 48 hr, however, only half the mice survived following aerosol treatment, whereas 23% survived after IM inoculation. Combined aerosol and IM therapy did not provide a significant advantage over the aerosol alone.

TABLE II. EFFECT OF MULTIPLE-DOSE THERAPY

TREATMENT[a]	TIME OF THERAPY (hr post-exposure)	RESPONSE (Survivors/ total)	% SURVIVORS	
None	24,48,72	3/50	6	P<0.005
Aerosol		46/50	92	P<0.025
IM		35/50	70	P<0.005
None	48,72,96	84/91	8	P<0.005
Aerosol		38/76	50	P<0.01
IM		59/77	23	P<0.005
Combined		10/24	58	

a. Treatment consisted of doses of 3-10 mg/kg; total = 30 mg/kg.

Because low doses of antibiotic often led to better survival than high doses, the effect of selected doses of kanamycin administered by aerosol or IM routes has been determined. Results (Table III) suggest that as the dose of antibiotic was increased (20-30 mg/kg-aerosol, 20 mg/kg-IM), survival may have decreased. Doses above these levels were more effective. This observation confirms previous work with 5 mg/kg body wt. Although very high doses were effective in this experiment, these dose levels are those normally considered toxic for this antibiotic. However, no deaths nor obvious signs of toxicity were observed in uninfected mice in this experiment. This experiment will be repeated to determine whether the observed differences are statistically significant.

TABLE III. EFFECT OF KANAMYCIN DOSE ON RESPONSE TO K. PNEUMONIAE INFECTION

DOSE OF ANTIBIOTIC (mg/kg)	% SURVIVAL	
	Aerosol	IM
10	60	60
20	55	40
30	30	60
40	60	70
100	95	90
Untreated control	5	

Because rats have larger tidal volumes than mice, they have been substituted for mice in pharmacodynamic experiments. Rats were given 20 mg/kg body wt of kanamycin by aerosol or IM injection. Five animals from each group were killed at 0, 24 and 48 hr. High levels of antibiotic were found in the lungs for 48 hr following aerosol administration (Table IV).

TABLE IV. CLEARANCE OF KANAMYCIN FROM RATS FOLLOWING IM OR AEROSOL TREATMENT

TIME AFTER TREATMENT (hr)	ANTIBIOTIC CONCENTRATION (μg/gm or ml)					
	Blood		Lung		Kidney	
	Aerosol	IM	Aerosol	IM	Aerosol	IM
0	0.73	8.75	13.4	1.80	0.32	3.38
24	0.10	0	3.32	0.68	0.34	1.85
48	0.13	0	2.98	0.73	0.29	1.71

Less antibiotic was found in the lungs after IM injection, but clearance of antibiotic was slow. Although the blood level following IM treatment was initially high, no antibiotic was detected $\geq$24 hr. This evidence suggests that the therapeutic levels of antibiotic will persist in the lungs for at

least 48 hr after a single aerosol dose, whereas multiple doses will be necessary to maintain high blood levels by IM inoculation. The minimum inhibitory concentration (in vitro) was calculated to be 1.0 µg/ml. Thus, inhibition in the lungs following aerosol administration could be expected for a much longer period than after IM inoculation.

Recently, a great deal of variation in response to aerosol therapy has been observed. Investigation of this variation has shown that the cause was variation in atomizer output (Table V) resulting from solute concentration used to provide selected dose levels.

TABLE V. EFFECT OF INITIAL CONCENTRATION ON AMOUNT DISSEMINATED AND RECOVERY OF KANAMYCIN

ANTIBIOTIC CONCENTRATION (mg/ml)	ML DISSEMINATED/ MIN	SAMPLER RECOVERY (mg/ml)	% RECOVERY
50	0.35	0.194	25.0
100	0.20	0.620	70.1
150	0.15	1.140	114.6
200	0.10	0.770	77.4

Experiments to correlate the stage of disease in K. pneumoniae-infected rats with induced metabolic sequelae (IMS) in rats (Work Unit No. 834 01 401) have continued. Preliminary results indicate that increases in serum lysozyme and α_2-macrofetoprotein (α_2-MFP) levels correlate with the presence and severity of bacteremia. Significant changes in these 2 components did not appear until a certain "threshold" concentration of bacteria was present in the lungs ($>10^{4.2}$/gm). Continuous increase of these components was pathognomonic of a fatal outcome. It is anticipated that certain induced biochemical sequelae can be employed to monitor the course of infection and efficacy of therapy.

Future studies for this work unit will include determination of effect of combined aerosol and IM therapy with K. pneumoniae-infected rats and mice, utilizing the observed changes in IMS in addition to survival and bacterial levels. Additional studies of pharmacodynamics in normal and infected rats are planned as well as initiation of long-term studies involving toxicity and hypersensitivity reaction experiments following aerosol and IM administration of kanamycin.

Presentations:

Berendt, R. F., G. G. Long, and J. S. Walker. Kanamycin-aerosol therapy of respiratory Klebsiella pneumoniae infection in mice. Presented, 14th Interscience Conference on Antimicrobial Agents and Chemotherapy. San Francisco, CA. 11-13 Sep 74 (Proc., Abstract 176).

Publications:

None.

LITERATURE CITED

1. Barach, A. L., G. Garthwaite, and G. J. Beck. 1952. Antibiotic therapy in infections of the respiratory tract. Arch. Intern. Med. 90:808-849.

2. Bopp, K. 1973. Possibilities and limitations of antibiotic therapy by means of aerosols. Proc. First International Congress on Aerosols in Medicine. p. 254-257. Vienna, Austria.

3. Miller, W. F. 1973. Aerosol therapy in acute and chronic respiratory disease. Arch. Intern. Med. 131:148-155.

4. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974, pp. 47-56, Fort Detrick, Md.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OB6419	75 07 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
74 10 24	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	62760A	3A762760A834	02	300
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Procede with Security Classification Code)

(U) Immunologic studies with rickettsiae of the spotted fever group

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 06	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 75	1.0	66.2
FISCAL YEAR CURRENT 76	1.0	146.3

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Rickettsiology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Kenyon, R. H.

TELEPHONE: 301 663-7465

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Williams, R. G.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Immunology; (U) Vaccines; (U) Rocky Mountain spotted fever; (U) Military medicine; (U) Rickettsia rickettsii

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

23 (U) Develop vaccines of low reactogenicity for immunoprophylaxis against specific rickettsial diseases. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Propagate representative strains in tissue culture systems. Assess the feasibility of producing rickettsial suspensions of quality and quantity suitable for vaccines for human use.

25 (U) 74 07 - 75 06 - Five thousand human doses of a cell culture grown Rocky Mountain spotted fever (RMSF) vaccine were prepared, tested, and a proposal for human trials has been submitted to the Army Investigational Drug Review Board. Research on a potential vaccine to protect against all spotted fever members is promising, but studies were hampered by lack of suitable animal models.

A study correlating the available methods of RMSF antibody measurement has been completed and a new radiometabolic method evaluated. Studies to measure lymphocyte stimulation and methods for early detection of RMSF are in progress.

Publication: J. Clin. Microbiol., in press, 1975.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 300: Immunologic Studies with Rickettsiae of the Spotted Fever Group

Background:

RMSF is the most severe of the spotted fever group of rickettsiae and is the only one for which a vaccine has been developed. Although these diseases can be controlled with tetracyclines, treatment presupposes that the physician is fully acquainted with the diseases and administers appropriate treatment promptly. Early diagnosis of spotted fever is difficult; most cases are diagnosed after the illness.

The only RMSF vaccine currently available is the Cox-type vaccine prepared from rickettsiae grown in chick egg yolk sacs and killed with formaldehyde.[1] Dupont et al.[2] studied this vaccine in prisoners and concluded it offered little or no immunity. Our purpose was to produce an improved vaccine which offered substantial protection against RMSF. In addition, there are plans to study the feasibility of a vaccine protecting against all spotted fever members producing disease in man.

Measurement of immunity to RMSF continues to be a problem. Although several methods of antibody measurement are available, their correlation with immunity is disputed. An indication of immunity other than challenge is highly desirable for human vaccine study.

Progress:

All final container testing on the RMSF CEC-grown vaccine was completed and a request for approval of this vaccine for human immunization trials was submitted to the Army Investigational Drug Review Board (AIDRB).

The efficacy of this CEC-grown vaccine was tested in rhesus monkeys and compared with the commercial chick yolk sac (CYS) vaccine. Characteristics of these vaccines are shown in Table I. Monkeys were immunized with a single injection of serial 10-fold dilutions of vaccine and challenged 30 days later. Antibody responses, rickettsemia levels, and deaths after challenge are shown in Table II.

TABLE I. CHARACTERISTICS OF THE CEC AND CYS VACCINES

VACCINE	NO. RICKETTSIAE/ML	PROTEIN CONTENT mg/dl
Commercial	None observed	472
CEC-grown	1.3×10^8	68

TABLE II. EFFICACY OF RMSF VACCINES IN MONKEYS

VACCINE	DILUTION ($\log_{10}$)	NO.	RECIPROCAL GMT	NO. WITH RICKETTSEMIA	MEAN PEAK RICKETTSEMIA (PFU/ml)	DEATHS
CEC	0	6	37	1	30	0
	-1	6	14	4	360	0
	-2	6	7	3	1,900	1
Commercial	0	6	2	3	310	1
	-1	6	<.2	3	510	1
	-2	6	<.2	3	3,600	2
Immune controls		5	320	0	-	0
Nonimmune controls		4	0	4	6,000	4

In our laboratory antibody can be first demonstrated and the highest titer detected using MA test or fluorescence microscopy. These studies indicate that in rhesus monkeys the CEC vaccine is more efficacious and contains considerably less extraneous protein than the commercial vaccine. Commercial production of a RMSF CEC-grown vaccine is practical and highly desirable.

To evaluate a potential human RMSF vaccine, an adequate immunological test is necessary. A comparison of currently available methods of antibody

detection was made in both rhesus monkeys and in 2 cases of human laboratory-acquired RMSF. A radiometabolic assay for opsonizing antibody (in cooperation with Dr. Canonico) was also used. A brief summary of the results of these tests is presented in Table III. The methods routinely used in clinical

TABLE III. COMPARISON OF METHODS OF RMSF ANTIBODY DETECTION IN RHESUS MONKEYS AND HUMANS

ANTIBODY TEST	APPEARANCE (days)	TYPICAL RECIPROCAL TITER ON PEAK DAY
Fluorescence	6-9	4,096
MA	6-12	2,048
CF	9-12	64
Weil-Felix(OX-19)	12	32
Opsonizing	12-17	Not applicable

laboratories (CF and Weil-Felix) appeared late in the disease and demonstrated low antibody titers. While the radiometabolic technique is not as sensitive as other methods, its appeal lies in the fact that it measures opsonizing antibody which has been suggested to be a major mode of in vivo defense against rickettsial disease.[4] However, using this method no significant antibody could be detected in monkeys vaccinated with either of the vaccines despite the fact that the monkeys were protected against challenge.

This study indicates that a reliable measurement of cellular immunity against RMSF is needed. Although preliminary lymphocyte stimulation studies with monkeys were promising, results were inconsistent. Cooperative studies (Work Unit 834 02 417) showed that humans convalescent from RMSF (D.L and J.M.) and one individual working with, but having had no symptoms of, RMSF demonstrated significant lymphocyte stimulation. Lymphocyte stimulation can be detected in convalescent guinea pigs; tests of vaccinated guinea pigs are incomplete. At this point the value of lymphocyte stimulation testing in a vaccine study is yet undetermined.

After preparation of a suitable RMSF vaccine, our goal is preparation of a vaccine which will protect against all or at least the most important spotted fever members. We have studied some basic characteristics of these organisms in our laboratory and the results of these studies are shown in

TABLE IV. BASIC CHARACTERISTICS OF THE SPOTTED FEVER GROUP OF RICKETTSIAE

RICKETTSIA	PFU IN CYS	TOTAL PARTICLE COUNT IN CEC ($\times 10^7$)	"TOXIN" PRESENT		NO. DEATHS OR +,-				
							Mice		
			CYS[a]	CEC	Guinea pigs n = 6	Hamsters n = 5	Suckling	Adult n = 5	Monkeys
siberica	2.0×10^6	12	+[b]	-	0	0	±	3	
australis	2.5×10^7	12	-	-	0	0	+	0	0/1
akari	9.0×10^6	66	-	-	0	0	+	5	
conori	2.0×10^6	74	+	+	0	0	+	0	0/1
parkerii	3.0×10^6	20	+	-	0	0	-	0	
rickettsii (Sheila Smith)	2.0×10^7	20	+	+	5	0		0	9/10

a. Lackman et al.[5]

b. + death; - no death.

Table IV. The problem in studying vaccine efficacy for these members is lack of suitable animal models. R. rickettsii kills guinea pigs and Rickettsia akari kills mice, but the other members cause only mild disease in animals tested. Our only available tool is fever response of guinea pigs which is probably inadequate. Guinea pigs response to infection with these organisms is only 3-4 days of moderate fever. With cross-protection studies where immunity is most likely marginal, significant differences are difficult to measure. Despite these shortcomings using guinea pig fever responses, a combined vaccine of gradient-purified R. rickettsii and Rickettsia australis offers significant protection against Rickettsia conori and R. siberica as well as R. rickettsii and R. australis, but protection against R. akari is questionable. Plans to continue these studies involve immunization of guinea pigs and mice with a series of 2 or 3 vaccinations, followed by attempts to measure cross-reactive MA antibody titer; after challenge, we will assess mouse deaths for R. akari and monitor fever responses for the others.

Since we were measuring rickettsemia levels in RMSF-infected animals, it was desirable to determine whether rickettsiae were found in plasma, RBC or WBC. Whole blood from infected guinea pigs was separated on Ficoll-Hypaque gradients and in hematocrit tubes. The results are shown in Table V.

TABLE V. LOCALIZATION OF RICKETTSIAE IN GUINEA PIG BLOOD DURING RMSF INFECTION

	PFU BY DAYS					
SITE	1	2	3	4	5	6
Whole blood	0	280	820	140	4,200	750
WBC	0	0	10	60	400	90
RBC	0	40	340	30	350	90
Plasma	0	200	370	45	310	10

These data show that early in infection rickettsiae are predominantly found in the plasma fraction, however as infection progresses, the organisms appear in WBC. The significance of rickettsiae found with RBC is not known. It seems reasonable that early in infection rickettsiae replicate in susceptible cells somewhere in the body, are distributed by the circulatory system, and are later engulfed by WBC. With typhus it has been demonstrated that the rickettsiae

replicate within WBC of nonimmune hosts.[3] However, this has not yet been shown for RMSF.

Work has been initiated to investigate the use of fluorescent antibody (FA) in the early detection and diagnosis of RMSF. Preliminary work with Mr. Bagley has led us to believe that direct FA staining of the buffy coat of infected symptomatic animals might be promising. The first few preparations have been made using centrifuged whole blood in Wintrobe tubes to obtain a pledget of WBC (buffy coat). Direct stain with Gimenez was not promising; however, direct FA stains appear to be positive for rickettsiae. Another method, that of separating the platelets from WBC by differential centrifugation, might be a better approach. This is currently being investigated.

Presentation:

1. Kenyon, R. H. Preliminary studies of a killed Rocky Mountain spotted fever vaccine derived from chick embryo cell cultures. Workshop on Rocky Mountain Spotted Fever, Center for Disease Control, Atlanta, GA, 5-6 December 1974.

Publication:

1. Kenyon, R. H., and C. E. Pedersen, Jr. 1975. Preparation of Rocky Mountain spotted fever vaccine suitable for human immunization. J. Clin. Microbiol. 1:In press.

LITERATURE CITED

1. Cox, H. R. 1939. Rocky Mountain spotted fever. Protective value for guinea pigs of vaccine prepared from rickettsiae cultivated in embryonic chick tissues. Pub. Health Rep. 54:1070-1077.

2. DuPont, H. L., R. B. Hornick, A. T. Dawkins, G. G. Heiner, I. B. Fabrikant, C. L. Wisseman, Jr., and T. E. Woodward. 1973. Rocky Mountain spotted fever: a comparative study of the active immunity induced by inactivated and viable pathogenic Rickettsia rickettsii. J. Infect. Dis. 128:340-344.

3. Gambrill, M. R., and C. L. Wisseman, Jr. 1973. Mechanisms of immunity in typhus infections. II. Multiplication of typhus rickettsiae in human macrophage cell cultures in the nonimmune system: influence of virulence of rickettsial strains and of chloramphenicol. Infect. Immunity 8:519-527.

4. Gambrill, M. R., and C. L. Wisseman, Jr. 1973. Mechanisms of immunity in typhus infections. III. Influence of human immune serum and complement on the fate of Rickettsia mooseri within human macrophages. Infect. Immunity 8:631-640.

5. Lackman, D. B., E. J. Bell, H. G. Stoenner, and E. G. Pickens. 1965. The Rocky Mountain spotted fever group of rickettsias. Health Lab. Sci. 2:135-141.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6421	75 07 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
74 10 24	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	3A061101A91C	00	134
b. CONTRIBUTING	62760A	3A762760A834	02/301	
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Physicochemical and biological characterization of rickettsial proteins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 09	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 75	0.7	26.0
FISCAL YEAR CURRENT 76	1.0	30.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Rickettsiology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Wachter, R. F.

TELEPHONE: 301 663-7465

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Pedersen, Jr., C. E.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Vaccines; (U) Q fever; (U) Rickettsia; (U) Military medicine

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

23 (U) Develop a vaccine composed of immunogenic proteins which would be protective against a spectrum of rickettsial diseases. This is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Purify, isolate and compare proteins of rickettsiae; determine antigenic, immunogenic, allergenic and other properties.

25 (U) 74 07 - 75 06 - Sodium sulfite precipitation of the soluble Phase I antigen of C. burnetii from trichloracetic acid extracts concentrated and partially purified the antigen, eliminating the need for dialysis. The antigen complex did not dissociate in water or buffer but was soluble in 0.001 M EDTA. Czechoslovakian workers have demonstrated in humans the potential of Phase I antigen as a chemovaccine. We found that the sulfite-antigen complex is also immunogenic. Guinea pigs were completely protected by one dose (12 micrograms) against 10,000 median infectious doses of C. burnetii either the soluble or particulate form of the complex and partially protected by 1.2 and 0.12 micrograms of the particulate form. Postchallenge sera contained agglutinating and CF antibodies.

The indrect immunofluorescence technique was successfully utilized for the reliable detection of R. rickettsii in the tissues of an experimentally infected rhesus monkey. The procedure is rapid and looks promising due to its relative simplicity.

Purified R. rickettsii examined by transmission and scanning beam electron microscopy revealed characteristic surface topography.

Publications: Infect. Immunity 12, in press, 1975.

J. Clin. Microbiol. 1, in press, 1975.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 301: Physicochemical and Biological Characterization of Rickettsial Proteins

Background:

During this report period the following activities occurred: (1) a new research plan of the above title was developed and approved; (2) results of earlier investigations on different cell types of phase I Coxiella burnetii were confirmed and expanded by additional experiments and submitted for publication; (3) research was continued on the soluble phase I antigen of C. burnetii.

The potential of the phase I antigen as a chemovaccine for human use was demonstrated recently by Brezina et al.;[1] in a study with 46 volunteers 2 doses of 1 ml of a trichloracetic (TCA) extract of the antigen were effective in producing phase I and II antibodies and in protecting the vaccinees without untoward local or systemic effects.

Progress:

I. Preparation of purified samples of C. burnetii for protein characterization. We have purified samples of the Henzerling and Nine Mile strains of C. burnetii, phases I and II. The purification procedure consists of sedimentation from 30% sucrose homogenates, Celite adsorption, centrifugation through sucrose cushions (45% sucrose for phase I rickettsiae, 30% for phase II) and banding in continuous sucrose gradients. From yolk-sac homogenates we have obtained purified preparations containing 0.8-3.8 mg of rickettsiae/ml and 1.4×10^{10} - 1.2×10^{11} rickettsiae/ml. Initial results of CF tests indicated an absence of host yolk-sac material in these samples. Results to date suggest that a higher yield of Q fever rickettsiae can be obtained more readily from yolk-sac preparations than from cell-culture preparations.

Preliminary gel electrophoretic analyses indicate differences in the polypeptide profiles of Rickettsia rickettsii and C. burnetii and between phases I and II of C. burnetii.

Concentration of phase I antigen by Na_2SO_3 precipitation; immunogenicity of resultant sulfite-antigen complex. The soluble phase I antigen of C. burnetii has been evaluated by Brezina et al.[1] by study in man. In our

experience the phase I antigen is present in TCA extracts in relatively low concentrations. Use of these extracts as vaccines requires the removal of TCA, which generally has been accomplished by dialysis. We have investigated the use of Na_2SO_3 as a specific precipitant to concentrate the phase I antigen, and at the same time, to circumvent the need for dialysis.

Sodium sulfite, which selectively precipitates serum albumins of high carbohydrate content,[2] was employed by Cohen et al.[3] to precipitate the so-called soluble fraction of typhus vaccines, presumably by specific interaction with free aldehyde groups in the carbohydrate portion of the antigen. We found that Na_2SO_3 (17.5-20%) caused rapid precipitate formation in both dialyzed and undialyzed TCA extracts of phase I antigen. Precipitation, followed by low speed centrifugation, eliminated all measurable CF antigenicity from the supernatant. Recovery of antigenicity may depend upon the physical state of the precipitated antigen: the sulfite-antigen complex did not dissociate, or dissociated only partially, when suspended in water or phosphate buffer and recovery was 10%; suspension in 10^{-3} M disodium EDTA resulted in disappearance of particles, a decrease in optical density and 50-100% recovery of antigenicity. Following precipitation, 40-50% of the protein and about 30% of the carbohydrate of the original TCA extract remained in the supernatant, indicating that this precipitation had effected partial purification as well as concentration of the antigen.

Samples of the complex, both as a suspension in phosphate buffer and as a soluble product in EDTA, were compared for immunogenicity with the dialyzed TCA extract from which they were derived. Complex samples were resuspended to original volumes and, therefore, contained less protein than the TCA extract (12 vs 30 µg/ml). Protection tests were performed as follows: guinea pigs, in groups of 6, were given single SC injections of 1 ml amounts of 1:1, 1:10, or 1:100 dilutions of the samples; 26 days later the animals were bled intracardially and challenged 1 day later with 10^4 ID_{50} of the Henzerling strain of C. burnetii, 4th yolk-sac passage, injected IP. Daily rectal temperatures were recorded for 10 days following challenge. Animals were again bled by heart puncture on day 15. Guinea pigs whose temperatures were < 104 F were considered to be protected. Postvaccination and postchallenge sera were tested for phase I and II antibodies by both the CF and MA reactions (Table 1).

Undilute TCA extract and sulfite-antigen samples protected all animals; partial protection was given by the 1:10 and 1:100 dilutions of the extract and of the sulfite complex suspended in buffer. Neither phase I nor phase II antibodies were detected in postvaccination sera by either the CF or MA test. Postchallenge sera, however, contained both phase I and II antibodies. By the MA reaction 93% of the serum samples were positive for phase I antibodies and 87% for phase II. By the CF test 87% of these samples contained phase II antibodies but only 8% had phase I antibodies and these were of a low level.

TABLE 1. PROTECTION OF GUINEA PIGS AGAINST CHALLENGE WITH C. BURNETII BY PHASE I ANTIGEN PREPARATIONS.

PREPARATION	DILUTION	NO. FEBRILE/ TOTAL	RECIPROCAL GEOMETRIC MEAN TITER			
			CF-I	CF-II	MA-I	MA-II
TCA extract	1:1	0/6	0	81	64	64
EDTA soluble		0/6	5	161	32	57
Buffer suspension		0/6	2	102	32	32
TCA extract	1:10	3/6	0	18	10	13
EDTA soluble		5/5	0	13	81	100
Buffer suspension		4/6	0	57	45	45
TCA extract	1:100	4/6	0	6	36	64
EDTA soluble		5/5	0	37	7	37
Buffer suspension		4/5	0	11	28	24
Controls		0/6	0	4	3	5

For individual animals no obvious correlation between number of fever days and antibody levels was noted, e.g., some animals with no CF and MA antibody titers and no fever days.

In addition to its potential usefulness in concentrating and purifying the phase I antigen, the sulfite reaction may provide a mechanism for comparing the immunogenic effectiveness of soluble and insoluble forms of the same antigen.

II. The indirect immunofluorescence (IIF) technique was successfully utilized for the reliable detection of R. rickettsii using a heterologous antibody coupled with fluorescein isothiocyanate. The fluorescence was specific for the presence of rickettsiae on the basis of control tissues from normal uninfected monkeys. Titrations of tissues using cell culture techniques confirmed the presence of rickettsiae in sections which clearly demonstrated fluorescence by microscopic examination. Those sections which showed the most conclusive evidence of R. rickettsii included: skin at the inoculation site, skeletal muscle of the thigh, skin of the scrotum, testicles, anterior nares, heart, kidney, liver, brain, spleen, pancreas, and larynx. Control sections from a normal monkey were negative.

High intensity nonspecific staining precluded absolute determination of the presence or absence of R. rickettsii in the bladder, sternum, regional and mesenteric lymph nodes and adrenal and salivary glands. Sections from the uninfected control monkey also stained nonspecifically. No rickettsiae or rickettsial antigens were detected by IIF in sections of the lung, stomach, small intestine or ear. Titrations of the various tissues show conclusively that rickettsiae were disseminated throughout the monkey. Sections which clearly demonstrated rickettsiae by IIF correlated very well with the presence of infectious organisms. Histologic lesions consisted of vasculitis and thrombosis in vessels of nares, larynx, tongue, testicle, epididymis, skeletal muscle, ear and skin. Other microscopic lesions included adrenitis, myocarditis, splenitis, lymphadenitis, interstitial pneumonia and multifocal hepatic necrosis. Phosphotungstic acid hematoxylin staining confirmed the presence of thrombi in vessels. In most tissues histologic evidence of vasculitis correlated well with presence of rickettsiae by IIF. However, blood vessels had essentially normal morphologic features in brain, kidney and pancreas, even though these tissues contained rickettsiae. Inflammatory and necrotic lesions in other tissues and organs appeared to be referable to rickettsial growth or obstruction of blood vessels.

We concluded that the IIF technique is capable of demonstrating intact rickettsiae as well as rickettsial antigens in primate tissues. Those specimens which elicited positive IIF reactions and also showed high numbers of rickettsiae, would be the material of choice for studies involving IIF screening of tissue samples. The IIF technique is more rapid than conventional cell culture assays and due to its relative simplicity, would be applicable to the examination of multiple specimens of biopsy tissues suspected of containing rickettsiae.

Purified R. rickettsii have been examined by transmission (TEM) and scanning beam (SEM) electron microscopy. Using the agar pseudoreplica technique the air dried rickettsiae appear to have an electron translucent limiting membrane (cell wall) and an electron dense central core containing cytoplasmic constituents. In contrast, critical point dried, carbon replica specimens show the organism with a tightly bound cell wall. Using SEM, R. rickettsii were found to vary in size and shape. While predominantly fusiform, many coccobacillary and elongated forms were found. In addition, rickettsiae were found apparently in the process of binary fusion.

We believe that this study demonstrates the value of using multiple techniques for examination of the surface topography of microorganisms. The agar pseudoreplica technique is a relatively simple and rapid method for examination of specimens by the high resolution obtainable with the TEM. However, organisms are subjected to air drying procedures which may induce shrinkage, surface distortion and collapse. Use of critical point drying seems to induce fewer artifacts in specimens than conventional air drying

techniques. Although TEM offers higher resolution than SEM, one can perceive 3-dimensional perspective by the use of SEM. For these reasons critical point dried specimens were examined by both SEM (for perspective) and TEM (for resolution). Therefore, each method offers an appeal for use; however, to describe specimens fairly, one should employ multiple procedures for the preparation and examination of microorganisms.

Different seed strains of R. rickettsii (Sheila Smith and Iowa) as well as the Rickettsia species siberica, conorii, parkeri, akari, australis and montana have been prepared in duck embryo tissue culture and chick yolk sac. Preliminary concentration procedures have been performed to assess the feasibility of disc electrophoretic analysis of constituent polypeptides of the spotted fever rickettsiae. The prototype organism R. rickettsii (SS strain) has been shown to contain multiple proteins when examined by the discontinuous system. In addition, this strain has been shown to react by gel diffusion with human and monkey antisera.

Publications:

1. Wachter, R. F., G. P. Briggs, J. D. Gangemi, and C. E. Pedersen, Jr. 1975. Changes in buoyant density relationships of two cell types of Coxiella burnetii phase I. Infect. Immunity 12:In press.

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2. Moore, D. H., L. Levin, and G. K. Smelser. 1945. Electrophoretic and salt fractionation of the serum proteins of normal and hypothyroid rats. J. Biol. Chem. 157:723-730.

3. Cohen, S. S., L. A. Chambers, and J. R. Clawson. 1950. Studies on commercial typhus vaccines. III. The concentration and isolation of the rickettsia-specific soluble antigen of commercial typhus vaccine. J. Immunol. 65:465-473.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OD6424	75 07 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
74 10 24	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A834	02	302
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Studies of Coxiella burnetii, Strain M-44

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 10	75 06	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 75	0.5	71.6
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Rickettsiology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Johnson, J. W.
TELEPHONE: 301 663-7465
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pathology; (U) Immunology; (U) Vaccine; (U) Q fever; (U) Coxiella burnetii; (U) Military medicine

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

23 (U) Study the biological properties of Strain M-44 of C. burnetii in laboratory animals, including pathology, survival and recrudescence, virulence and phase, assay methods and immunology. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Examine laboratory animals and other biological systems infected with the M-44 strain using a variety of laboratory techniques.

25 (U) 74 07 - 75 06 - A study of the pathology of the M-44 strain of C. burnetii in guinea pigs indicated that lesions observed in the principal target organs, liver, spleen and heart, were hepatitis sometimes developing into necrosis, splenitis and myocarditis. Lesions were generally minimal with very few severe lesions found. Evidence is presented for the reactivation of the M-44 infection in recovered guinea pigs. Stresses causing the reactivation were pregnancy and treatment with the drugs cyclophosphamide and methyl prednisolone acetate.

The aims of the study have been met. The work unit is completed.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 302: Studies of Coxiella burnetii, Strain M-44

Background:

The M-44 strain of Coxiella burnetii, which is markedly attenuated for guinea pigs, has been developed by Genig[1] and others[2,3] as a live vaccine for the immunization of individuals in areas where the incidence of Q fever is high. While the vaccine provided good protection and a high seroconversion rate, it still caused a low incidence of mild clinical symptoms in recipients. Robinson and Hasty[4] further reduced the virulence of this strain for guinea pigs by heating a suspension of the rickettsiae to 56 C and isolating surviving microorganisms. They prepared a live vaccine from the isolate and subsequently reported on its immunogenicity and virulence properties in guinea pigs. To further develop this vaccine, the present work unit was established to investigate other biological properties of the strain. With this report on the survival of C. burnetii in guinea pigs and an additional pathology study, the objectives of the program have been completed and this represents the final report for the work unit.

Progress:

I. In the previous annual report,[5] a pathology study of the M-44 strain of C. burnetii in guinea pigs was presented. Because of the rather high rate of nonspecific lesions in various organs of these animals, it was considered desirable to repeat the study. The experiment was conducted using the same protocol as reported previously, but the major target organs of the infection, which were now identified, were given more thorough study.

Results essentially confirmed the previous findings. Table I shows that the principal lesions were found in the liver, spleen and heart. The lung was excluded from this study as the high rate of pneumonia-pneumonitis in both control and infected animals made the recognition of disease-specific lesions very unreliable.

As before, most lesions observed were minimal, with few sections showing severe tissue alterations except in the lung. The livers of infected animals exhibited a moderate amount of hepatitis which in some cases had progressed to necrosis. No granulomas were observed. In the spleen about 1/3 of the infected

animals showed splenitis which apparently did not progress during the experiment. The major lesion of the heart was a myocarditis, found in ~38% of the infected guinea pigs. A few lesions in the epicardium and endocardium were also found, but were observed in both control and infected animals at about the same rate. Other abnormalities found such as peritonitis, adrenalitis or serositis were also about equally distributed between the two groups.

TABLE I. PATHOLOGIC LESIONS IN GUINEA PIGS INOCULATED WITH THE M-44 STRAIN OF C. BURNETII

		INCIDENCE %	
ORGAN	LESION	Infected (n=21)	Control (n=14)
Liver	Hepatitis	33	14
	Necrosis	8	14
	Granuloma	0	0
Spleen	Splenitis	33	7
	Necrosis	0	0
	Granuloma	0	0
Heart	Epicarditis	0	14
	Endocarditis	14	14
	Myocarditis	38	0

II. Guinea pigs inoculated with the M-44 strain harbored living organisms in their tissues for ~10-12 wk; beyond this time, rickettsiae could not be detected in untreated animals (Table II). To determine whether the organism survived for longer periods of time, experiments were set up to stress recovered guinea pigs at various intervals after infection and attempt to isolate the organism from tissue samples or find other evidence for a reactivation of the infection. The stresses used were pregnancy, a condition known to reactivate virulent Q fever, and treatment with the cortisone derivative methyl prednisolone or the immunosuppresive drug cyclophosphamide.

For the pregnancy study, female guinea pigs were inoculated with the M-44 strain and, at 2-wk intervals following infection, 1 or 2 animals were mated with normal males. Pregnant animals were allowed to come nearly to term, usually between 60 and 65 days, and were then killed. Spleen, liver, kidneys,

TABLE II. DETECTION OF C. BURNETII, STRAIN M-44, DURING INFECTION IN GUINEA PIG

				ORGAN HOMOGENATE		
				Guinea Pig	Embryonated Egg	
WEEKS	SEX	MA TITER[a]	SPLEEN SLIDE[b]	MA≥1:8	Yolk-sac smear[b]	Guinea Pig MA≥1:8
2	M	8	+	+	+	+
	F	8	+	+		+
4	M	256	+	+	+	+
	F	64	+	+		+
6	M	128	-	+	+	+
	F	256	+	+		+
8	M	512	-	+	+	+
	F	64	-	+		+
10	M	256	-	+	+	+
	F	128	+/-	-		+
12	M	32	-	-	-	-
	F	16	-	-		+
14	M	64	-	-	-	-
	F	128	-	-		-

a. Reciprocal, phase II antigen.

b. Impression slide, Gimenez stain.

placenta and amnion were removed aseptically, homogenized in saline A and inoculated IP into normal guinea pigs and embryonated eggs. Guinea pigs were bled 28 days later, reinoculated with the M-44 strain and bled again in 7 days to detect any anamnestic response. Sera were tested for Q fever antibodies by the MA test using a phase II C. burnetii antigen. Embryonated eggs were examined for the presence of rickettsiae by preparing smears of yolk sacs and

staining by the Gimenez procedure.[6] Negative yolk sacs were given one additional egg passage and examined by stained smear and by guinea pig inoculation. Guinea pigs were bled and reinoculated.

TABLE III. DETECTION OF C. BURNETII STRAIN M-44 IN PREGNANT GUINEA PIGS

			ORGAN HOMOGENATE		
			Guinea Pig	Embryonated Egg	
WEEKS POSTINFECTION	MA TITER[a]	SPLEEN SLIDE[b]	MA≥1:8	Yolk-sac Smear[b]	Guinea Pig MA≥1:8
12	512	+	+	+	+
14	128	+	+	+	+
22	64	+/-	+	-	+
26	128	-	-	-	-
28	32	-	-	-	-
30	64	+/-	-	-	+
30	N.D.	-	-	-	-
34	16	-	+	+	+
38	8	-	-	-	-
40	<8	-	-	-	-
42	<8	-	-	-	-
44	<8	-	-	-	-
44	<8	-	+	-	+
46	<8	-	-	-	+
46	<8	-	-	-	-

a. Reciprocal microagglutination titer phase II antigen.

b. Impression slide, Gimenez stain.

Fifteen pregnant guinea pigs were killed between 12 and 46 wk postinfection (p.i.) and their organs tested for the presence of rickettsiae. Table III shows that organ suspensions from 5 animals, those sacrificed at 12, 14, 22, 24 and 44 wk p.i., induced C. burnetii antibodies in subinoculated guinea pigs, and 3 of these, at 12, 14 and 34 wk, produced positive yolk sac smears in embryonated eggs. These were the only rickettsial isolates obtained. No attempt was made to isolate organisms from guinea pigs. Two additional animals, one sacrificed at 30 wk and the other at 46 wk, may have had a reactivated infection. Guinea pigs inoculated with 2nd egg passage of organs from these animals developed an anamnestic response when reinoculated with the M-44 strain, indicating a priming of these animals by the yolk-sac material. However, no other evidence was obtained to substantiate this observation.

For the drug treatment study, groups of guinea pigs infected 1-2 yr previously with the M-44 strain were given either daily doses of 20 mg of 5-methyl prednisolone acetate (Depo-Medrol) for 7 consecutive days or twice weekly doses of 50 µg of cyclophosphamide (Cytoxan) for 4 wk. Following treatment, they were sacrificed, and homogenates of spleen, liver and kidneys were inoculated into embryonated eggs and guinea pigs as indicated in the pregnancy study. A summary of the results of these experiments is shown in Table IV.

TABLE IV. DETECTION OF C. BURNETII STRAIN M-44 AFTER DRUG TREATMENT OF RECOVERED GUINEA PIGS

TREATMENT	NO. ANIMALS	INFECTED	WEEK KILLED	REACTIVATION RATE[a]	
				No. Pos.	%
Prednisolone	5	Yes	56	4	80
Cyclophosphamide	3	Yes	56	2	67
	3	Yes	96	2	67
Saline	4	Yes	14 + 24	0	0
Prednisolone	4[b]	No		0	0
Cyclophosphamide	3[b]	No		1	33
Vaccine(killed)	3	No	8 + 12	1	33

a. Measured by specific immune response in guinea pigs receiving organ suspensions of treated animals.

b. Drug treated normal animals caged in room with infected animals.

The prednisolone-treated animals were all killed 56 wk after the original infection. Antibody responses of guinea pigs inoculated with homogenates of these organs indicated that 4 of 5 had developed a Q fever infection. Smears of embryonated eggs were negative for C. burnetii following 2 serial passages, and none of the animls inoculated with yolk-sac homogenates produced a measurable MA antibody response to C. burnetii antigen.

Two groups of guinea pigs previously injected with the M-44 strain were treated with cyclophosphamide, one at 56 wk, the other at 96 wk. Responses of subinoculated animals receiving organ material from these guinea pigs indicated that 2 of 3 animals in each group harbored rickettsiae. Again, none of the embryonated eggs injected with these organ homogenates showed evidence of rickettsial infection either by smear or guinea pig inoculation after 2 serial passages.

Because isolation of rickettsiae from apparently reactivated infections proved difficult, 3 control studies were included. These were (1) treatment of recovered animals with saline in place of drugs, (2) treatment of normal guinea pigs with drugs after being caged in the same room with infected animals, and (3) injection of large doses of killed C. burnetii to determine whether sufficient antigen could be carried over in organ homogenates to elicit an antibody response in subinoculated animals.

Infected guinea pigs taken from the same groups used for drug treatment experiments were inoculated with a saline solution instead of drugs using the same injection and sacrifice schedules. Organ homogenates from these animals were also treated as indicated above. None of the subinoculated animals gave a positive serological response to C. burnetii antigen and none of the eggs were shown to be infected either by smear or guinea pig inoculation.

Normal guinea pigs housed for 1-3 mon in the same room as infected animals were treated with prednisolone or cyclophosphamide and sacrificed. Serum from these animals gave no evidence of Q fever infection. When their organs were subinoculated into additional guinea pigs, the serum from one animal in the cyclophosphamide group reacted positively to Q fever by the MA.

To determine whether enough C. burnetii antigen could be carried over from the organs of one animal to produce a serological response in another, the following experiment was conducted: normal guinea pigs were each inoculated with the sediment from 20 ml of killed Q fever vaccine (Lot No. FP2315, WRAIR) and 2 or 3 mon later were sacrificed and their organs subinoculated into new animals. Among these animals, one showed a positive serological response to Q fever antigen. However, the antibody titer in this animal was much higher than in animals receiving vaccine, which suggests a cross-infection rather than a carry-over of antigen.

Since virulent Q fever is known to be highly contagious and since isolation cages were not used in these studies, the possibility of cross-infection with the attenuated M-44 strain was considered. A group of 9 normal guinea pigs were caged for 35 days with an equal number of newly infected animals. Their sera were tested for C. burnetii antibody both before and after the experiment. All guinea pigs were found to be serologically negative at the beginning, but one of the 9 normal animals gave a positive response to Q fever antigen at the end. This brief study suggested a cross-infection rate of perhaps 10-15%, which is well below the response rate of infected guinea pigs subjected to the various stresses discussed in this report.

From the evidence presented here, it would appear that an infection of the M-44 strain of C. burnetii can be reactivated in recovered guinea pigs, as long as 2 yr after the initial infection, by the application of physiological or chemical stresses such as pregnancy or treatment with methyl prednisolone or cyclophosphamide.

Publications:

None.

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5. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1973. Annual Progress Report, FY 1973. pp. 277-280. Fort Detrick, Md.

6. Giménez, D. F. 1964. Staining rickettsiae in yolk-sac cultures. Stain Technol. 39:135-140.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6414	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. SUBJ'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 12 20	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	62760A	3A762760A834	02	303
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Immunopathogenesis of Rocky Mountain spotted fever

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 12	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. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

19. RESOURCES ESTIMATE

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING 75	1.0	66.2
CURRENT 76	1.0	118.8

20. PERFORMING ORGANIZATION

NAME: Rickettsiology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Oster, C. N.

TELEPHONE: 301 663-7465

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Rocky Mountain spotted fever; (U) Antigen; (U) Immunology; (U) Radioimmunoassay; (U) Rickettsia; (U) Military medicine

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

23 (U) Develop a radioimmunoassay for detection of rickettsial antibodies and antigen; modify the assay to detect soluble antigen-antibody complexes. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Modify an indirect radioimmunoassay technique for use with rickettsiae to test the time relationship during experimental infections in guinea pigs and rabbits.

25 (U) 74 12 - 75 06 - Trials are underway to develop radioimmunoassay techniques to detect antibody to RMSF, antigens of R. rickettsii, and soluble immune complexes. The technique in 3-ml vials failed to work in the microagglutination test. Attempts are underway to apply the system to microtiter plates.

*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 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 303: Immunopathogenesis of Rocky Mountain Spotted Fever

Background:

There have been no major developments in the diagnosis or treatment of RMSF in 25 yr. The case fatality rate has remained in the range 7-10%, and the incidence seems to be significantly increasing.

Of the many reasons advanced for the continued high case fatality rate, two seem plausible: (1) delayed or missed diagnosis, leading to inadequate treatment, and (2) occasional induction of acute immune-complex disease with fulminant arteritis which progresses, despite antimicrobial therapy.

The problem with delayed diagnosis lies partially in the fact that there is no currently available test that can confirm the diagnosis accurately; most are serologic tests that need to demonstrate rising titers in paired acute and convalescent sera. Therefore, trials are underway to develop a radioimmunoassay (RIA) to detect antibodies to RMSF, and antigens of Rickettsia rickettsii during the course of experimental disease. Modification of this RIA might permit detection of soluble immune complexes and, therefore, serve as a tool to investigate the roles played by these complexes in the pathogenesis of RMSF.

Progress:

Development of a radioimmunoassay for the detection of antibody to R. rickettsii in serum specimens is in progress.

Initial studies used the following procedure: particulate R. rickettsii antigen (Ag), Sheila Smith strain, partially purified from CEC cultures by rate zonal centrifugation, is incubated with test serum (Ab_1) in phosphate buffer. Species-specific second antibody (Ab_2), labeled with ^{125}I by the lactoperoxidase method, is then added and incubation continued. The particulate immune complex, $Ag\text{-}Ab_1\text{-}Ab_2\text{-}^{125}I$, is pelleted by centrifugation and counted in a γ-counter. Results are computed as percentage of the total CPM added which are retained in the pellet.

Initial studies using this procedure have been unsuccessful in detecting the presence of anti-R. rickettsii Ab in known MA positive sera, despite manipulations in reagents, equipment and techniques.

Due to lack of success with the preliminary technique, other approaches are being investigated. An attempt was made to grow R. rickettsii in DEC monolayers in 3-ml vials to use as solid-state Ag for the assay for Ab_1. Unfortunately, we were unable to propagate rickettsiae in these vials because it proved nearly impossible to prevent contamination with bacteria in the antibiotic-free medium needed for growth of rickettsiae. This study will continue in an attempt to overcome this obstacle.

Another approach to use solid-state Ag, by fixing Ag to microtiter wells, is currently being investigated. Several techniques will be applied: using these Ag-coated wells, a direct test, using a human globulin preparation with high anti-R. rickettsii titer labeled with ^{125}I, will be used to define the system; an inhibition assay of Ab employing preincubation of Ag-coated wells with test serum, and secondary incubation with labeled anti-R. rickettsii Ab; and an indirect test in which test sera will be incubated in Ag-coated wells, and bound Ab detected by incubation with labeled second antibody.

Additionally, rabbits have been immunized with the live Sheila Smith strain in an effort to stimulate production of high titer anti-R. rickettsii Ab. If this Ab is shown to be specific and of high affinity, it will be used in the assays described above to detect Ab and Ag.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION* DA 0F6418	2. DATE OF SUMMARY* 75 07 01	REPORT CONTROL SYMBOL DD-DR&E(AF)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
75 03 25	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	62760A	3A762760A834	02	304
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code) (U) Strain characteristics of Rickettsia rickettsii and other members of the spotted fever group

12. SCIENTIFIC AND TECHNOLOGICAL AREAS* 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
75 03	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 75	0.5	57.0
	CURRENT 76	1.0	146.3

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME:* Rickettsiology Division USAMRIID
ADDRESS:* Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Johnson, J. W.
TELEPHONE: 301 663-7465
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Rickettsial diseases; (U) Spotted fevers; (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) Define and characterize markers in Rickettsia rickettsii strains of differing virulence as well as other members of the spotted fever group, in order to asses value as candidates for live vaccines. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Grow strains of rickettsiae in embryonated eggs and cell culture, select clones and compare with the parent strain. Test clones in a variety of ways in laboratory animals. Determine protective efficacy of candidate strains.

25 (U) 75 03 - 75 06 - Two strains of R. rickettsii, the virulent Sheila Smith (SS) and the low virulent Iowa, have been obtained and stock cultures prepared from them. Growth and assay systems for each strain have been established in chick fibroblast and L-cell cultures, and the growth and plaquing potential of each has been investigated, in a preliminary way, in duck fibroblast, BS-C-1, LLC-MK-2, FRhL-103 and Vero cell cultures. Several substrains isolated from limiting dilutions of assays of the SS strain were very similar in guinea pig virulence to the parent strain. Preliminary experiments suggest that an interferon-like inhibitor may be formed in chicken fibroblast and L-cell cultures of the SS strain.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 304: Strain Characteristics of Rickettsia rickettsii and Other Members of the Spotted Fever Group

Background:

Among strains of R. rickettsii, virulence is the only substantial differentiating characteristic. Variations in this property have been observed subjectively in human disease and have been quantitated fairly reliably in guinea pigs by Price.[1] Marker studies using other properties have been only moderately successful due to either lack of adequate differentiation or instability of the marker. More recently, methods of cell and tissue culture have been applied to rickettsial studies with some success in propagation, assaying and cloning these organisms. The present work unit has been established to explore the capabilities of methods for the identification and development of additional strain characteristics which may be useful in the preparation of vaccines against these organisms. As one approach to this investigation, we are currently studying R. rickettsii in an attempt to find one or more strains which may prove useful as a live vaccine.

Progress:

Two strains of R. rickettsii have been obtained and are being characterized in this laboratory. One is the Sheila Smith (SS) strain,[2] which is highly virulent for guinea pigs causing severe illness and high mortality; it grows well in chick fibroblast cells. The other is the Iowa strain[3] which has a very low virulence for guinea pigs, causing only a low grade fever for a few days; it also grows well in embryonated eggs and chick fibroblasts. Three properties of these strains are presently being investigated: (1) growth of the rickettsiae in various tissue culture cell lines; (2) virulence of clones isolated from cell lines and from embryonated eggs; and (3) production of and sensitivity to interferon or interferon-like substances by the 2 rickettsiae.

Stock cultures of both have been prepared, and a plaque assay for each has been established in chick fibroblast tissue cultures. Various parameters of the assay have been investigated such as volume of inoculum, diluent, time and temperature of adsorption, most efficient overlay and time for optimum plaque formation. From the data, a plaquing procedure was adopted to include an

adsorption step using 0.2 ml of inoculum on the cell sheet in a T_{30} flask for 1 hr at 33 C. Infected monolayer plates were overlaid with Earle's minimal essential medium (EMEM) containing 0.5% agarose, and were incubated for 7 days at 33 C and stained with 0.02% neutral red.

A number of cell cultures were investigated to determine whether they would support rickettsial growth or form plaques. Among these were chick and duck fibroblasts, BS-C-1, Vero, FRhL-103, LLC-MK_2 and L-cells. Rickettsial growth and plaque formation were analogous in duck and chick fibroblasts. Under the same conditions BS-C-1, Vero and FRhL-103 cell lines did not develop very well characterized plaques and the growth of rickettsiae appeared lower. The LLC-MK_2 and especially the L-cell lines were much more effective in producing plaques. In L-cells, the SS strain gave round, clear, sharply defined plaques about 1-2 mm in diameter. They were easily counted, even when close together, and were usually 2-3 times more numerous than in corresponding chick fibroblast cultures. The Iowa strain in this cell line usually formed very small, pinpoint plaques which were often difficult to see or count, but also appeared to be in greater numbers than were found in chick fibroblasts. The LLC-MK_2 line formed clear, well-defined plaques also, but quantitative comparisons of plaque numbers have not yet been completed.

Cloned isolates of the SS strain are being collected to compare virulence in guinea pigs with that of the parent strain. Isolates are being derived from 2 sources, the yolk sacs of embryonated eggs from limiting dilutions and isolated plaques in monolayers of chick fibroblasts and L-cells. Stock cultures of 4 such substrains from eggs have been prepared and tested for virulence; all have shown the virulence level of the parent strain.

Experiments were initiated to determine whether the infected L-cell or chick fibroblast cultures produced growth inhibiting substances, such as interferon. Fluids from infected cell monolayers were collected at daily intervals after infection, centrifuged to remove rickettsiae and overlaid onto fresh cell monolayers for 24 hr. Cultures were infected with a dilution of R. rickettsii that would give about 100 PFU/flask, overlaid with EMEM containing agarose, and incubated until plaques developed. In 2 experiments with the SS strain in L-cell cultures, supernatants from infected cultures appeared to markedly inhibit rickettsial plaque formation when compared with control cultures. In one experiment with chick fibroblast cultures, no inhibition was found. These experiments are being repeated with both cell cultures and with the Iowa as well as the SS strains.

Publications:

None.

LITERATURE CITED

1. Price, W. H. 1953. The epidemiology of Rocky Mountain spotted fever. 1. The characterization of strain virulence of Rickettsia rickettsii. Am. J. Hyg. 58:248-268.

2. Kenyon, R. H., W. M. Acree, G. G. Wright, and F. W. Melchior. 1972. Preparation of vaccines for Rocky Mountain spotted fever from rickettsiae propagated in cell culture. J. Infect. Dis. 125:146-152.

3. Weiss, E. 1960. Some aspects of variation in rickettsial virulence. Ann. N. Y. Acad. Sci. 88:1287-1297.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6420	75 07 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
74 07 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	62760A	3A762760A834	02	407
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e)(f)			

11. TITLE (Precede with Security Classification Code)

(U) Development of arbovirus vaccines for diseases of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
64 06	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 75	1.0	134.8
	CURRENT 76	1.0	124.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Virology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Cole, Jr., F. E.

TELEPHONE: 301 663-7241

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) Cell culture; (U) Langat virus; (U)Arboviruses; (U) Vaccines; (U) Western equine encephalitis

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

23 (U) Produce inactivated or attenuated arbovirus vaccines which may be combined or administered sequentially for prophylaxis in geographically oriented ways. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Arboviruses are propagated in primary cell culture and inactivated with formalin or selected for attenuation. Products are tested for safety and potency in animals. Efficacy is determined by subsequent challenge, or by determination of serological conversion.

25 (U) 74 07 - 75 06 - Nineteen plaque-isolated Langat virus clones were tested for markers of decreased neurovirulence. From these, 10 clones with the lowest peripheral intraperitoneal (IP) virulence for mice were selected. These clones were inoculated IP into mice and the virus content of the brains assayed daily for 10 days. Five clones which elicited the lowest levels of virus in the brain were thus selected on the basis of apparently decreased neurotropism. Growth curve studies revealed little difference between clones. Ten rapid passages in chick embryo cell (CEC) culture did not further adapt the clones to CEC. However, repeated CEC passage resulted in decreased peripheral virulence and increased neurotropism in mice. Immunogenicity for hamsters was increased by CEC passage. Studies will continue using a newly isolated Langat strain. Due to an urgent requirement for additional quantities of WEE vaccine for use in at-risk personnel, Langat studies were held in abeyance for 3 mon to permit production and evaluation of 2 lots of WEE vaccine. In-process and final product tests were completed in Mar 75; an AIDRB submission was prepared and forwarded to HQDA for approval to use the WEE vaccine in man.

Publication: In: Internat. Conf. Vaccines Against Equine Encephalitis. Panamerican Zoonosis Center, Buenos Aires, Argentina, 1975.

*Available to contractors upon originator approval

DD FORM 1 MAR 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 407: Development of arbovirus vaccines for diseases of military importance

Background:

Product improvement and development of new or alternate procedures is basic to all vaccine research programs. Thus, studies are continually performed in an effort to improve existing products. Such is the case with attenuated Langat (LGT) vaccine, a tick-borne group B arbovirus in the Russian spring-summer encephalitis (RSSE) subgroup. As reported previously, vaccine prepared at USAMRIID using the vaccine of Dr. Price of Johns Hopkins University (Contract No. DA-49-193-MD-2398) as starting seed did not pass the monkey neurovirulence tests we employed.[1] Because of these untoward reactions in monkeys, a decision was reached to attempt to obtain a more attenuated candidate LGT vaccine virus. Since the Price vaccine strain (TP-21) was the only strain available with any documentation of altered virulence, attempts have been made to select clones from the vaccine and parental strains of Langat virus which, hopefully, might provide us with an even more attenuated vaccine virus.

In an ancillary project which required stoppage of all Langat studies, 2 lots of WEE vaccine were prepared and tested during a 3-mon period to meet a pressing need for additional amounts of this product for immunization of at-risk personnel.

Progress:

I. As reported last year studies were initiated to obtain a more attenuated plaque isolate of LGT virus for study as a candidate vaccine.[1] A total of 19 clones (plaques) were selected from the USAMRIID Master Seed (attenuated) and from the parent (nonattenuated) strain. Each plaque was transferred directly to CEC flask cultures and propagated at 35 C for 5 days to produce working seed virus for further evaluation. The original 19 clones were first titrated intracerebrally (IC) and IP in 12- to 14-day-old mice to select the strains exhibiting the greatest difference in titer by the 2 routes of inoculation; 10 clones were thus selected for further study on the basis of their low IP peripheral virulence.

These 10 clones were next inoculated via the IP route into 21- to 24-day-old; daily for 10 days thereafter, brains were removed, triturated and the virus content determined by the plaque technique in LLC-MK_2 cell cultures. Results of these titrations are shown in Table I. Clones L-1, -2, -5, -10 and -18 exhibited the lowest virus titers and were examined further on the basis of this evidence of decreased neurotropism in mice.

TABLE I. VIRUS CONTENT OF BRAINS OF MICE AFTER IP INOCULATION WITH CLONES OF LGT VIRUS

CLONE NO. L-	PFU/0.2 ML BRAIN SUSPENSION[a/] BY DAYS									
	1	2	3	4	5	6	7	8	9	10
1	[b/]	-	-	-	-	-	7	-	-	-
2	-	-	-	-	-	-	110	-	-	2
3	-	-	-	-	-	-	-	230	2	13
5	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	8	110
10	-	-	-	-	-	10	-	3	-	-
13	-	-	-	-	2	-	5	230	30	37
14	-	-	-	-	65	10	420	15	850	590
18	-	-	-	-	-	-	-	8	3	5
19	-	-	-	-	100	255	40	70	347	298

a. Brains of 3 mice/clone/day were pooled prior to testing.
b. No virus detected.

To determine whether the 5 selected clones were practical selections for a vaccine to be produced in CEC culture, growth curves were next determined for each. Flask cultures of CEC were inoculated with 10^{-1} - 10^{-4} dilutions of the original working seed of each clone. After viral adsorption at 35 C for 1 hr, maintenance medium (MM; medium 199 containing 0.5% human serum albumin (HSA) and antibiotics) was added and the cultures incubated at 35 C. Samples were withdrawn and stored at -70 C daily for 7 days, after which virus content was determined by plaquing in LLC-MK_2 cell cultures. As shown in Table II no marked differences were noted among the strains in terms of peak titer or the temporal nature of growth. Peak titers were generally obtained by day 3 postinoculation at all levels of virus input (not shown) and were generally maintained through day 7. Peak titers ranged from 5×10^6 to 1×10^7 PFU/ml.

The 5 selected clones were subsequently subjected to 10 serial passages in CEC cultures at 72-hr intervals using a high viral input, viz. a 10^{-1} dilution of a given harvest was used as inoculum for the next CEC culture passage. This rapid passage technique was employed to determine: (1) whether such passage would further adapt the virus to these cells and (2) the effect of such cell culture passage on known viral markers.

The data gave no evidence of further adaption to CEC culture (Table III). There was no increase in titer with passage as measured by the plaque technique in LLC-MK_2 cultures. However, strong evidence of the importance of "heavy" or fully-sheeted monolayers of CEC to ultimate virus yield is seen in the titers of the 4th, 6th and 10th passage harvests. These cell cultures were practically confluent, but not nearly as tightly packed with cells as is generally obtained at USAMRIID. It should be noted that little or no CPE was associated with the virus yields shown.

TABLE II. GROWTH OF LGT VIRUS CLONES IN CEC CULTURES, PLAQUED ON LLC-MK_2 CELL CULTURES

CLONE NO.	RANGE[a] OF PFU/ML BY DAYS x 10^5						
	1	2	3	4	5	6	7
L-1	0[b]	0.1-0.6	10-100	10-50	10-50	10	10-40
L-2	0	0.1-0.6	20	10-100	10-50	20-60	10
L-5	0	0.6-1.0	20-40	10-80	9-100	20-100	10-40
L-10	0	0.3-1.0	20-100	10-100	10-50	10-40	8-40
L-18	0	0.1-0.4	10-20	10-50	10-40	8-40	8-20

a. Highest and lowest PFU given for 4 virus inputs of 10^{-1} - 10^{-4}; 0 = $\leq$ 0.009

TABLE III. EFFECT OF RAPID, SERIAL PASSAGE ON VIRUS YIELD OF LGT VIRUS CLONES GROWN IN CEC CULTURE

CEC PASSAGE NO.	PFU/ML BY CLONE x 10^6				
	L-1	L-2	L-5	L-10	L-18
1	3.0	0.8	2.0	2.0	0.9
2	20.0	10.0	20.0	10.0	2.0
3	3.0	0.6	3.0	2.0	0.6
4	0.7[a]	0.4	0.4	0.7	0.5
5	2.0	0.7	0.4	1.0	0.7
6	0.6	0.6	0.7	0.7	1.0
7	2.0	1.0	2.0	2.0	3.0
8	2.0	2.0	2.0	1.0	10.0
9	2.0	1.0	2.0	2.0	0.04
10	0.5	0.2	0.4	0.5	0.2

a. Underlined data indicates that CEC cultures were not as "heavy" as usual at time of inoculation.

To determine the effect of CEC culture passage on peripheral and neurovirulence, the 10th-passage harvests (see Table III) were titered in 21- to 24-day-old mice by the IP and IC routes. Shown in Table IV are the results of these titrations and, for comparative purposes, the results of similar titrations performed previously on the 5 clones prior to CEC passage.[1]

TABLE IV. EFFECT OF RAPID, SERIAL PASSAGE IN CEC CULTURE ON PERIPHERAL- AND NEUROVIRULENCE OF IGT VIRUS CLONES

CLONE NO.	LOG_{10} TITERS PER ML BY:			
	IP ROUTE		IC ROUTE	
	Pre-CEC Pass. [a]	10th CEC Pass. [b]	Pre-CEC Pass. [a]	10th CEC Pass. [b]
L-1	5.0	2.5	5.4	5.4
L-2	4.9	<1.0	5.3	5.3
L-5	≥1.5	1.0	5.8	5.3
L-10	≥4.0	1.3	5.7	5.9
L-18	≥2.8	1.2	6.2	6.9

a. Titrated in 11-15-day-old mice.
b. Titrated in 20-24-day-old mice.

All 5 clones exhibited lower IP titers after 10 CEC passages, suggesting that a decrease in peripheral virulence had resulted from the cell culture passages. Titers obtained by the IC route with 10th CEC passage viruses were similar to those seen with the original, unpassaged clones (Pre-CEC), suggesting that no increase in neurovirulence had occurred as a result of the 10 culture passages. These results taken alone were encouraging in terms of using the "seed" system normally employed in vaccine production, wherein several passages are required to produce "clean" Master and Production Seeds and final products.

However to assess the effect of CEC passage on neurotropism, groups of 21- to 24-day-old mice were inoculated IP with - 10^4 PFU of a given clone from the 10th CEC passage. Daily for 10 days thereafter brains were removed from 2 to 3 mice in each clone group and frozen for subsequent assay as pools by the plaque technique. In comparison to the unpassaged clones (see Table I), the CEC-passaged clones not only elicited higher virus content in the brains (Table V), but with one exception, clone L-18, virus was found in the brains over a longer period of time. Thus neurotropism seems to have been increased by passage in CEC culture. Since 10 passages in culture were chosen as the cut-off point because it was far beyond that normally required for use of the "seed" system, clones with lower CEC passage history would have to be examined to determine if increased neurotropism was a slowly acquired characteristic (via selection) or was more spontaneous (via mutation) in nature.

TABLE V. VIRUS CONTENT OF BRAINS OF MICE AFTER IP INOCULATION OF 10TH CEC-PASSAGED CLONES OF LGT VIRUS

CLONE NO.	PFU/0.2 ML BRAIN SUSPENSION[a] BY DAY POSTINOCULATION: 1	2	3	4	5	6	7	8	9	10
L-1	—[b]	-	-	-	-	700	1700	3300	450	300
L-2	-	-	-	-	-	-	2300	3300	50	10
L-5	-	-	-	-	-	800	1650	-	-	400
L-10	-	-	-	-	3000	450	-	2050	-	NT[c]
L-18	-	-	-	-	-	-	-	400	-	-

a. Brains of 2 to 3 mice/clone/day were pooled prior to assay.
b. No virus detected.
c. Not tested; no sample available on day 10.

In addition to the above evaluations, the immunogenicity of the 5 clones before and after the 10 CEC passages was determined by seroconversion tests in hamsters. Groups of 5 hamsters were each inoculated IP with 0.5 ml decimal dilutions of the clones. Sera were obtained at 28 days and tested for antibody in an PR_{80} serum dilution neutralization test (PRNT). In this test sera that could be diluted at least 1:10 and still reduce the plaque count by 80% were considered positive for LGT antibody. In this way data as + or - was obtained for each of the animals originally given decimal dilutions of the clones and the median immunizing dose could therefore be determined by the method of Reed and Muench.[2] The data shown in Table VI indicate a marked increase in hamster immunogenicity after 10 CEC passages, with the exception of clone L-18. Since the pre- and post-CEC passage harvests had approximately the same titers in terms of IC LD_{50} for adult mice (not shown in Table), the increased immunogenicity can only be attributed to a change in the viruses ability to benignly infect the hamster.

Future studies on the development of an attenuated LGT vaccine will involve intense evaluation of a newly isolated strain which may be available with either a short well-documented history, or perhaps, as zero-passage infected, tick suspension (COL P. Russell, personal communication). The advantages of such a strain, heretofore unavailable, cannot be overstated in view of the increasingly more stringent Federal regulations on biologicals.

During the fall of 1975 an urgent need developed for an additional supply of inactivated WEE vaccine to be used for vaccination of at-risk personnel. For a period of approximately 3 mon other studies were held in abeyance to permit production and evaluation of 2 lots of vaccine.

TABLE VI. EFFECT OF RAPID, SERIAL PASSAGE IN CEC CULTURE ON HAMSTER IMMUNOGENICITY OF LANGAT VIRUS CLONES

CLONE NO.	LOG_{10} MEDIAN IMMUNIZING DOSE OF VIRUS:	
	Pre-CEC	Post-10th CEC
L-1	Negligible	1.4
L-2	1.5	4.2
L-5	1.0	3.5
L-10	2.5	4.5
L-18	4.4	3.7

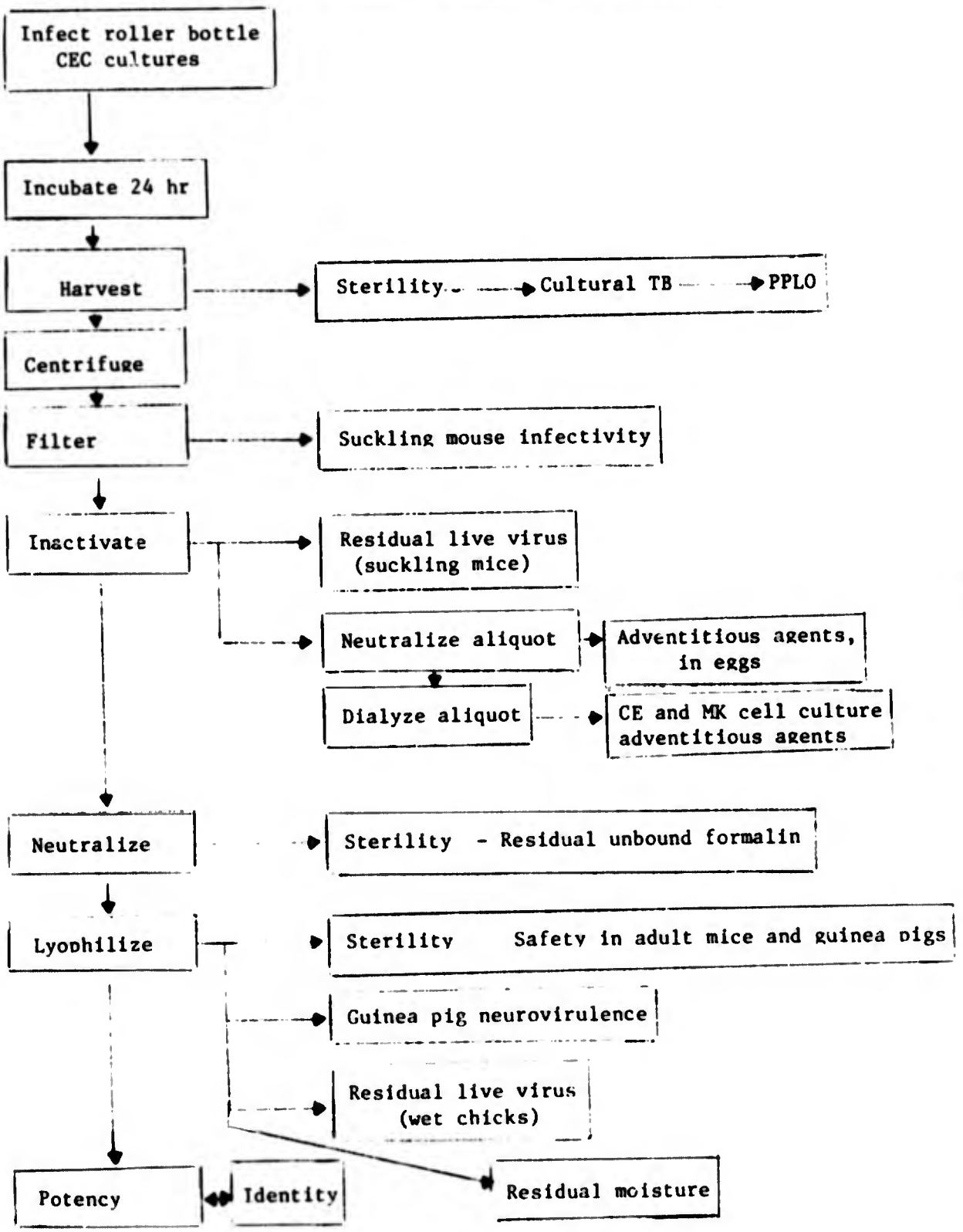
Basic procedures used in both of these production runs are outlined in the Summary Flow Chart. B-11 strain WEE virus, previously used to prepare approved vaccines[3] was obtained from Merrell-National Laboratories as Production Seed.

Roller bottle cultures of CEC were prepared from specific pathogen-free (COFAL) embryonated chicken eggs as previously described.[4] After incubation at 37 C for 18 - 24 hr the original growth medium was removed and replaced with MM (Medium 199 containing EBSS, 0.25% (v/v) HSA, USP, and 50 µg/ml each of neomycin and streptomycin). The cultures were then incubated for an additional 18 - 24 hr.

Prior to infection maintenance medium was removed from all cultures; 90% of the cultures were then inoculated with 10 ml of a 10^{-4} dilution of Production Seed virus made in MM without HSA. The remaining 10% of the cultures served as controls and received 10 ml of the diluting medium. Following an adsorption period of 1 hr at 37 C the inocula were removed and the cultures were washed twice with 100 ml of HBSS. Fresh MM (150 ml/ bottle) was then added to each culture; all cultures were incubated at 37 C.

Approximately 24 hr later, when the infected cultures evidenced 25 to 50% CPE, the virus fluids were harvested and pooled. Samples were removed for sterility testing, including Mycobacterium tuberculosis, Mycoplasma testing and infectivity titrations.[5] The bulk virus was then clarified by centrifugation at 1000 x g for 15 min and passed through a Millipore 0.45-µm filter. For inactivation the filtered fluid was brought to 37 C and 10% formaldehyde (reagent grade) solution was added to give a final concentration of 0.05% formaldehyde (0.13% formalin). This bulk material was incubated

SUMMARY FLOW CHART FOR WEE VACCINE PRODUCTION



for 48 hr at 37 C with constant stirring. Samples were periodically taken to ensure that the virus had been inactivated within 16 hr. Bulk inactivated virus was next placed at 4 C for 6 days with constant stirring. Samples were then removed for subsequent testing. These tests included:[5] (1) assays for residual live virus using 3 serial passages in suckling mice; (2) tests for adventitious agents in which embryonated eggs were inoculated with samples of Bulk Inactivated virus after the residual formalin had been neutralized; and (3) tests for adventitious agents in which monkey kidney and CEC cultures were inoculated with control fluid or neutralized, Bulk Inactivated virus which had been dialyzed.

After the holding period of 6 days at 4 C the formalin in the Bulk Vaccine was neutralized with $Na_2 SO_3$. Samples were taken and tested[5] for sterility and for residual unbound formalin. The Bulk Vaccine was then dispensed as 5.4-ml portions into 15-ml vaccine vials, freeze-dried (lyophilized) and subsequently stored at -20 C. Tests on the Final Product included:[5] (1) sterility; (2) general safety in adult mice and guinea pigs;(3) guinea pig neurovirulence in which the animals were observed for signs of disease and finally necropsied to permit histopathological examination of the brain; (4) residual live virus assay in chicks ≤12-hr old; (5) residual moisture; (6) potency in guinea pigs and hamsters,[4] and (7) identity, in which case the potency test served a dual purpose since a known strain of WEE virus was used as challenge material for the immunized animals.

After the successful completion of these required tests an Army Investigational Drug Review Board submission was prepared and forwarded for approval to use the product in man.

Publication:

Eddy, G. A., F. E. Cole, Jr., C. E. Pedersen, Jr., and R. O. Spertzel. 1975. Vacunas atenuadas de arbovirus del grupo A: ventajas e inconvenientes de su uso en equinos. In International Conference on Vaccines Against Equine Encephalitis. Panamerican Zoonosis Center, Buenos Aires, Argentina. p.35-41.

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2. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. Amer. J. Hyg. 27:493-497.

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5. Food and Drug Administration. 1973. Biologics. Code of Federal Regulations, Title 21, subchapter F. U. S. Government Printing Office, Washington, D. C.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6423	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. GRP'G INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
75 04 22	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	62760A	3A762760A834	02	411
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code): (U) Evaluation of promising compounds for antiviral use against diseases of medical importance to the military

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 12	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 75	2.0	198.7
FISCAL YEAR CURRENT 76	2.0	254.0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Animal Assessment Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME: Stephen, E. L.
TELEPHONE: 301 663-7244
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Sammons, M. L.
NAME: Kuehne, R. W. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Laboratory animals; (U) Model virus disease; (U) Encephalitis, equine (VEE); (U) Chemotherapy; (U) Interferon; (U) Influenza; (U) Military medicine

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

23 (U) Evaluate compounds that have previously demonstrated both in vitro and in vivo viral inhibitory activity for the prophylaxis and treatment of virus infections of importance to the military. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Test candidate compounds in tissue culture and laboratory animals against selected viruses.

25 (U) 74 07 - 75 06 - Tilorone and its analogs are effective antiviral agents against yellow fever (YF) of mice, but not of monkeys. A lysine-stabilized poly I:poly C preparation has been shown to be highly effective both in the prophylaxis and treatment of YF in the monkey and mouse. In addition, this preparation has shown significant activity against VEE of mice. Very significant progress has been made in the chemotherapy of influenza in mice. These studies have been extended to characterize completely the effectiveness of rimantadine and have been extended to describe equal therapeutic activity for ribavirin. Two additional compounds, 11,567 and kethoxal, were not effective.

Publications: Fed. Proc. 34:799, 960, 1975.
Antimicrob. Agents Chemother. 8, In press, 1975.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 411: Evaluation of Promising Compounds for Antiviral Use Against Diseases of Medical Importance to the Military

Background:

The prophylactic efficacy of Tilorone® and 3 of its analogs 11,877, 11,567 and 11,002 against VEE virus disease in mice was reported.[1] Recent studies have characterized the therapeutic limitation to use of these compounds in the VEE-mouse model. In addition, the antiviral activity of these compounds has been evaluated against yellow fever (YF) virus disease in mice and monkeys. The indirect YF-mouse model was used to assess the antiviral activity of several compounds. The theory of the indirect model depends on the assumption that mice will not be immunized by a live virus if an antiviral compound prevents replication to critical antigenic mass; thus, successfully treated mice would be susceptible to backchallenge. In order to test the validity of the indirect concept, an indirect mouse model using TC-83 strain of VEE virus was designed and evaluated.

Theoretical considerations initially indicated that poly I:poly C might play a key role in the therapy of viral diseases through induction and release of interferon. Experience has proven, however, that poly I:poly C is much less effective in man than in rodents. Man, subhuman primates and some animals (other than rodents) have an enzyme that hydrolyzes poly I:poly C rapidly to an inactive state, thus accounting for its unsuccessful use. A lysine-stabilized poly I:poly C (PI:Cl), more resistant to enzymatic degradation, is now available. Recent studies using the PI:Cl have shown it to be a highly effective prophylactic and therapeutic antiviral agent against Asibi strain YF of rhesus monkeys.

The successful treatment of influenza virus disease in mice using rimantadine-HCl has now been developed fully. Other compounds have also been evaluated in this model disease; for example, ribavirin was found to be extremely effective in treatment of influenza. Recent technological developments now permit continuous administration of the antiviral compounds by SPA (< 5 μm). This method of treatment, especially when using ribavirin, is superior to either IP or intermittent SPA methods. Since numerous compounds have now been shown to have antiviral activity against one or more viruses, the tissue culture studies have been directed toward defining mechanisms. An assay system is operating to detect interferon (IF) in monkey serum or to perform direct tissue culture inducement studies.

Progress:

I. Since Tilorone and its analogs successfully protected mice challenged with VEE virus, their usefulness in the prophylaxis of Asibi strain YF of mice was also evaluated; this strain given SC to mice immunizes them against subsequent intracerebral (IC) challenge and is not lethal. The antiviral compounds were given at the time of the immunizing inoculation of virus. Tilorone and its analogs are effective antiviral agents against YF, since all mice treated, regardless of dosage of compound, were susceptible to backchallenge.

Several antiviral compounds have been evaluated in the indirect YF-mouse model and the direct VEE-mouse model. Since all compounds evaluated in the indirect YF-mouse model have shown potent antiviral effects (Table I) and only

TABLE I. ANTIVIRAL EFFECTS OF VARIOUS COMPOUNDS IN MICE CHALLENGED WITH EITHER VEE VIRUS (TRINIDAD) OR YF VIRUS (ASIBI)

COMPOUND	VEE		YF
	Indirect	Direct	Indirect
Tilorone		+[a]	+
11,002		+	+
11,567	+	+	+
11,877		+	+
Kethoxal	-	-	+
Mepacrine	-	-	+
PI:Cl	+	+	+
Rimantadine		-	+
Amantadine		-	+
WR2721		-	+
SM1213	-	-	
Levamisole	-	-	
Bis-benzimidazole			+
Ribavirin		-	

[a] $p < 0.005$, compared to untreated control group by χ^2 analysis.

the IF releasers have been active in the direct VEE mouse model, an indirect VEE-mouse model was chosen, using the vaccine strain (TC-83) of VEE virus as the immunizing challenge. It was necessary to use TC-83 instead of the Trinidad strain since the virulent strain is lethal to mice by all routes of inoculation. The concept of an indirect mouse model for use in chemotherapy studies appears to be valid since no discrepancies occurred between the direct and indirect VEE models.

II. A significant delay in onset of viremia occurred in VEE-infected monkeys treated with PI:C1 (3 mg/kg, IV) 8 hr prior to challenge and repeated on days 1-4, 7, 9, 11, 15 and 17. The mean duration of viremia for treated monkeys was 2.5 days vs. 4 days for untreated monkeys. There was no apparent reduction in peak titer, although onset was delayed and duration shortened. Perhaps the most significant observation in this study was that 3 of 4 treated and infected monkeys may have died of a virus disease not normally fatal in rhesus monkeys. While it is not possible at this time to attribute these deaths to PI:C1 potentiation of VEE virus disease, it is clear that neither PI:C1 nor VEE virus alone killed monkeys. The possibility of potentiating an otherwise nonfatal disease raises significant questions relative to the widespread use of PI:C1 as an antiviral drug.

The delay in onset of viremia in treated monkeys produced a similar delay in the antibody response. There was no potentiation of the antibody response in treated monkeys and their antibody levels were not excessively high prior to death. The IF released in serum in response to injections of PI:C1 was sporadic and less than expected. IF was detected in the sera of the virus control monkeys. While PI:C1 treatment and consequent IF did not alter peak viremia, IF apparently delayed time of onset.

The same regimen of PI:C1 given to rhesus monkeys altered significantly the otherwise uniformly fatal course of YF virus infection, since all survived with no overt signs of disease. Viremias of treated monkeys were 10,000- to 100,000-fold lower than the peak viremia of the untreated monkey, and the time to onset was delayed (Table II). Treated monkeys all resisted backchallenge at 42 days. This finding is very significant since most antiviral agents do not permit sufficient viral replication for stimulation of host immunity.

TABLE II. RESPONSE OF INDIVIDUAL MONKEYS GIVEN PI:C1 AND SUBSEQUENTLY CHALLENGED WITH ASIBI STRAIN YF VIRUS

	LOG_{10} PFU/ml OF VIRUS							
	Drug Control		Treated				Virus	Room
DAY	1	2	1	2	3	4	Control	Control
-1 to 1	-[a]	-	-	-	-	-	-	-
2	-	-	-	-	-	-	3.1	-
3	-	-	-	-	-	-	6.7	-
4	-	-	-	-	-	-	7 7	-
5	-	-	-	-	-	-	8.7	-
6	-	-	-	-	-	3.5	died	-
7	-	-	1.5	2.8	-	4.8		-
8	-	-	3.5	3.1	-	2.3		-
9	-	-	4.8	2.3	2.1	-		-
10	-	-	4.4	-	3.4	-		-
11	-	-	2.6	-	4.5	-		-
12	-	-	-	-	3.3	-		-
13-17	-	-	-	-	-	-		-

[a]Negative

All treated monkeys developed significant YF-neutralizing antibody titers (Table III). No comparison can be made to control monkeys regarding time of

TABLE III. EFFECT OF PI:C1 IN INDIVIDUAL MONKEYS CHALLENGED WITH YF (ASIBI STRAIN) VIRUS

	RECIPROCAL DILUTION GIVING PR_{80}					
	Drug Control		Treated/Challenged (n=4)		Virus	Room
DAY	1	2	$\bar{M}$	(95% CL)	Control	Control
-1 to 5	-[a]	-	-	-	-	-
6	-	-	-	-	died	-
7	-	-	20	-		-
8	-	-	25	(20-32)		-
9	-	-	40	(27-60)		-
10	-	-	40	(25-65)		-
11	-	-	40	(30-53)		-
12	-	-	95	(53-172)		-
13	-	-	80	(60-106)		-
14	-	-	113	(72-177)		-
15	-	-	80	(59-109)		-
16	-	-	53	(37-75)		-
17	-	-	67	(37-122)		-
42	-	-	905	(741-1106)		-

[a]Negative

appearance of antibody or peak titers since no inoculated control monkeys have survived long enough to develop detectable antibody. As with VEE, YF stimulated the release of IF in the virus control monkeys (Table IV). The importance of the IF system is emphasized since all treated monkeys survived. In addition, it is clear that time of initial IF stimulation is important since the virus control monkeys in the VEE and YF studies demonstrated significant levels of IF.

TABLE IV. SERUM IF RESPONSE OF INDIVIDUAL MONKEYS GIVEN PI:Cl (3.0 mg/kg) IV AND SUBSEQUENTLY CHALLENGED WITH ASIBI STRAIN YF

	IF RESPONSE (I.U./ml)							
	Drug Control		Treated				Virus	Room
DAY	1	2	1	2	3	4	Control	Control
- 1	-[a]	-	-	-	-	-	-	-
0[b]	32	-	50	125	16	-	-	-
1[b]	16	200	500	125	80	100	-	-
2[b]	-	-	-	-	16	25	-	-
3[b]	-	-	-	-	-	20	16	10
4[b]	-	-	-	-	-	-	400	-
5	-	-	-	-	-	-	400	-
6	-	-	-	-	-	-	died	-
7[b]	400	-	-	-	-	125		-
8	-	-	-	-	-	125		-
9[b]	400	-	25	32	-	16		-
10	-	-	-	-	-	-		-
11[b]	-	-	32	-	-	-		-
12	-	-	16	-	25	-		-
13	-	-	-	-	-	-		-
14	-	-	-	-	-	-		-
15[b]	-	-	-	-	-	-		-
16	-	-	-	-	-	-		-
17[b]	-	-	25	-	-	-		-

[a]Negative = < 10 units/ml.

[b]Days PI:Cl was given.

Since the IF response to PI:Cl has been less than anticipated, a study was designed to evaluate the IF-inducing capacity of poly I:poly C stabilized with either poly-l-lysine or poly-d-lysine (PI:Cd) and Tilorone analog 11,567 in monkeys (Table V). Both PI:Cl and PI:Cd stimulated significant amounts of IF. Further, PI:Cd caused significantly greater IF titers than PI:Cl at 8 and 30 hr. The problems of antigenicity and toxicity with PI:Cd probably outweigh the increased ability to induce IF. Both PI:Cl and PI:Cd caused significant neutrophilia that persisted until 24 hr after the 1st injection of complexed poly I:C. These changes were transient and inapparent at either 6 or 24 hr after the 2nd treatment.

TABLE V. SERUM IF RESPONSE OF RHESUS MONKEYS GIVEN VARIOUS IF INDUCERS

GROUP	IF RESPONSE (I.U./ml) BY HR					
	0[a]	2	8[a]	24	30	48
Saline control (n=1)	<5	<5	20	<5	<5	<5
Analog 11,567 75 mg/kg, oral (n=3)						
Mean (95% CL)	<5	<5	7 (4-13)	<5	<5	<5
PI:C1 3 mg/kg, IV (n=4)						
Mean (95% CL)	<5	11 (7-16)	30 (26-35)	32 (27-37)	149 (133-167)	12 (6-22)
PI:Cd 3 mg/kg, IV (n=4)						
Mean (95% CL)	<5	79 (35-177)	631 (387-1028)**	106 (57-200)	841 (545-1299)*	60 (39-92)

[a]Hour of treatment. *P <0.01 and **P <0.005 compared to PI:C1 (Student's t test).

In order to evaluate whether antibody is produced against PI:C1 given repeatedly, a group of monkeys previously given a protracted course of PI:C1 therapy were given a 2nd series of injections (Table VI). The IF responses were greater after the 2nd injection and confirmed previous observations. The IF responses of the monkeys previously treated with PI:C1 were consistently higher than the IF responses of monkeys with no previous treatment, but not significantly. Since it was hypothesized that monkeys with anti-PI:C1 antibodies would respond to subsequent PI:C1 injections with less IF, the tentative conclusion is that little antibody against PI:C1 was formed.

The results of a 2nd study of the prophylactic efficacy of PI:C1 against YF in rhesus monkeys were in general agreement. The monkeys treated 8 hr after infection responded with lower peak titers, and 2 of 4 monkeys were not detectably viremic. There was no delay in onset of viremia in this group. When therapy was initiated 24 hr after infection, the onset of viremia was 24 hr earlier than for control monkeys and longer in duration. Peak titers were not different from those of controls and both monkeys died. Unlike the previous study, 2 of 4 monkeys in the -8 hr group died, and 1 of 4 in the +8 hr group. The time to death (TTD) of the monkeys is similar to that of 2 of 4 dead monkeys in the previous VEE study. For this reason, possible toxicity of the

TABLE VI. EFFECT OF A PRIOR COURSE OF THERAPY ON SERUM IF RESPONSE OF INDIVIDUAL MONKEYS GIVEN 3 mg/kg PI:C1 IV

GROUP	IF RESPONSE (I.U./ml) + BY HR						
	0[a]	2	8	24[a]	26	32	48
Saline control (n=1)	<5	16	<5	5	<5	<5	<5
No prior PI:C1 (n=2)							
Mean	<5	50	25	7	224	70	25
(95% CL)		(20-125)			(200-250)	(10-500)	
Prior treatment 10 injections (n=6)							
Mean	<5	136	261	56	1214	709	11
(95% CL)		(69-267)	(127-537)	(37-84)	(849-1735)	(394-1275)	(6-19)

[a]Hr of PI:C1 treatment.

inducer is presently being investigated since 2 of 3 dead monkeys in this study were not viremic at the time of death and histopathological results do not reveal liver pathologic changes similar in degree to other monkeys dying of YF virus disease. Monkeys treated initially 8 hr after infection had earlier antibody responses than pretreated monkeys, but this probably reflected the difference in time of onset of viremia rather than an accelerated immunological response. All of the monkeys that died in the -8 hr and +8 hr groups had neutralizing antibodies prior to death. The presence of neutralizing antibody in these monkeys is another indication that death was not primarily the result of the YF. The IF data agree with the 1st study.

III. In order to clarify the questions of whether the aerosol or IP route of administration of antiviral drugs is superior, the virus challenge dose was increased from $10^{4.2}$ EID_{50} in study 3 to $10^{4.9}$ EID_{50} in studies 1 and 2. Survival rates (Table VII) of controls were 10 and 11% in studies 1 and 2, respectively, and 40% in study 3 from a previously reported experiment. The challenge dose of virus is a critical determinant of survival and becomes increasingly important when one realizes that the survival curves are not parallel in treated vs. nontreated animals. In general, antiviral drugs are most effective when the challenge dose of virus is relatively small. As the challenge dose of virus is increased, the curves depicting survival for treated vs. nontreated animals approach one another. It is clear that when initial treatment is delayed as long as 6 days postinfection, no beneficial effect is apparent. The difference in results between experiments 2 and 3 is largely related to the challenge dose of virus. That survival was significantly improved in the group treated by continuous aerosol is of particular interest.

TABLE VII. EFFECT OF RIMANTADINE ON SURVIVAL OF MICE CHALLENGED WITH INFLUENZA BY THE AEROSOL ROUTE

STUDY	DURATION TREATMENT (days)	ROUTE AND REGIMEN	SURVIVAL No./Total	%	χ^2	P
1--Challenged with 4.9 $\log_{10}$ EID_{50}						
	Virus control	-	4/41	10		
	6-10	IP, 1/day	8/36	22		
		IP, 3/day	3/35	8		
		SPA, continuous	2/26	8		
2--Challenged with 4.9 $\log_{10}$ EID_{50}						
	Virus control	-	5/45	11		
	3-6	IP, 1/day	12/45	27		
		IP, 3/day	10/45	22		
		SPA, 1/day	8/41	20		
		SPA, continuous	11/30	37		<0.05
3--Challenged with 4.2 $\log_{10}$ EID_{50}						
	Virus control	-	12/30	40		
	3-6	IP, 1/day	28/29	97		<0.001
		SPA, 1/day	27/29	93		<0.001

Preliminary experiments were completed to contrast the effectiveness of rimantadine with 2 other antiviral compounds, Tilorone analog 11,567 and kethoxal. Kethoxal had no significant antiviral activity, except that mean TTD was increased. Analog 11,567 given as SPA had no beneficial effect. The Tilorone analogs tend to be unstable in solution. It is possible that the compound was inactivated during the dissemination process, since significant antiviral activity was demonstrated by the IP route or that the cells of the lung were insensitive to IF inducement by these compounds. The increase in percent survival and mean TTD is significant. In addition, a significant reduction in lung titer was evident.

In order to compare the potency and efficacy of amantadine and rimantadine in the treatment of mouse influenza, equivalent dosages of amantadine and rimantadine were given to mice. Rimantadine was more efficacious than amantadine in treating influenza of mice since significant increase in survival occurred only in the mice given amantadine in a continuous SPA beginning at 6 hr postinfection. Neither drug appeared to decrease the lung virus titer of treated mice.

Additional experiments to characterize potentially useful antiviral compounds for treating influenza principally involved the use of ribavirin.

Ribavirin (Table VIII) was highly effective in the treatment of influenza by both aerosol and IP routes and all regimens. In addition, treatment by the aerosol route beginning at 6 hr was significantly better than at 3 days. Continuous aerosols did not appear to be better than single administration except that the lung virus titers were lower at 7 days.

TABLE VIII. EFFECT OF RIBAVIRIN ON SURVIVAL, TTD AND LUNG TITERS OF MICE CHALLENGED WITH INFLUENZA BY THE AEROSOL ROUTE

STUDY Route, Regimen	SURVIVAL No./Total	SURVIVAL %	TTD days	GEOM. MEAN LUNG TITER BY DAYS 4	GEOM. MEAN LUNG TITER BY DAYS 7
1--Challenged with 4.5 $\log_{10}$ EID_{50}/mouse					
None	19/60	31	7.3	7.8	5.3
IP, 6 hr-4 days, 1/day	38/60	63***	8.8**	7.4	5.4
SPA, 6 hr-4 days, 1/day	56/60	93***	10.3	7.2	5.2
SPA, 6 hr-4 days, continuous	39/40	97***	9.0	6.2***	4.9
2--Challenged with 4.6 $\log_{10}$ EID_{50}/mouse					
None	14/60	23	7.4		5.6
IP, 3-7 days, 1/day	29/60	48**	9.5***		6.1
SPA, 3-7 days, 1/day	39/60	65***	11.1		5.5
SPA, 3-7 days, continuous	24/40	60***	12.2***		4.3***

P < 0.01, *<0.005, χ^2 analysis of survival and t test.

The results of a 2nd study were similar. Unlike rimantadine, ribavirin treatment decreased lung virus titers (Table IX) in the mice treated with SPA beginning at 6 hr. This effect was not apparent for mice treated initially at 72 hr, except in mice given ribavirin by continuous aerosols.

TABLE IX. EFFECT OF RIBAVIRIN TREATMENT ON LUNG TITERS IN MICE INFECTED WITH INFLUENZA BY SPA

TIME POST-INFECTION (hr)	GEOM. MEAN LOG_{10} LUNG VIRUS TITER ± SE			
	Virus Control	IP 1/day	Aerosol 1/day	Aerosol continuous
		6-hr - 4 days		
6	3.95 ± 0.15			
24	8.35 ± 0.14	7.93 ± 0.04*	7.79 ± 0.09*	7.31 ± 0.69
48	7.97 ± 0.13	8.17 ± 0.08	8.16 ± 0.03	6.80 ± 0.39*
72	7.93 ± 0.23			
96	7.99 ± 0.16	8.05 ± 0.15	6.91 ± 0.25**	6.29 ± 0.22***
144	7.18 ± 0.08	6.93 ± 0.18	6.03 ± 0.18***	5.42 ± 0.10***
168	6.29 ± 0.31	6.25 ± 0.05	5.00 ± 0.38	5.22 ± 0.19*
216	2.56 ± 0.79	1.67 ± 0.67	1.0 ± 0	1.67 ± 0.67
		3-7 days		
72	8.00 ± 0.13			
96	7.28 ± 0.14	7.57 ± 0.14	7.67 ± 0.18	7.40 ± 0.20
144	6.98 ± 0.12	7.27 ± 0.13	6.43 ± 0.44	6.28 ± 0.09*
168	5.71 ± 0.31	5.78 ± 0.21	5.41 ± 0.62	4.31 ± 0.36*
216	2.13 ± 0.61	3.32 ± 1.16	1.24 ± 0.24	3.05 ± 1.27

*P <0.05, **P <0.025, ***P<0.005.

IV. Mouse L-929 tissue culture cells were used to establish an in vitro system to assess IF induction. Tilorone and its 3 analogs did not induce detectable amounts of IF; however, they did elicit antiviral activity evidenced by VEE virus plaque reduction (PR) (a similar observation was made in the previous report[1] in Vero cell culture) in direct proportion to the concentration of the compounds up to toxic levels or where extensive CPE occurred. These observations are of interest since they suggest a possible antiviral mechanism unrelated to inducement of IF. Since mixtures of poly I:C and DEAE-dextran reportedly have a synergistic effect with Tilorone, these compounds were evaluated in the mouse L-929 cell culture system. IF was detected in response to the poly I:C alone in concentrations comparable to those reported; however,

tests did not demonstrate a synergistic effect between Tilorone or its analogs and poly I:C or DEAE-dextran. IF synthesis was less than that observed with poly I:C and about the same as with DEAE-dextran alone. As expected, IF synthesis was not demonstrated in Vero cell tissue culture in concurrent tests utilizing the same test mixtures; therefore, it was concluded that Tilorone and its analogs do not induce or cause the synthesis of IF in mouse L-929 and Vero tissue culture cell lines.

The relative toxicity CPE and antiviral (PR) properties of the various compounds against VEE virus in Vero cell tissue culture are shown in Table X.

TABLE X. ANTIVIRAL ACTIVITY OF VARIOUS COMPOUNDS AGAINST VEE VIRUS IN VERO CELL TISSUE CULTURE

TEST COMPOUND	CONCENTRATION (μg/ml)	PLAQUE REDUCTION 80%	PLAQUE REDUCTION 50%	CPE
Rimantadine	100	-	+	-
	80	-	+	-
	60	-	-	-
	40	-	-	-
	20	-	-	-
SM-1213	100	-	+	-
	80	-	+	-
	60	-	-	-
	40	-	-	-
	20	-	-	-
Kethoxal	100	+	+	+
	80	+	+	+
	60	+	+	±
	40	+	+	±
	20	+	+	-
PI:Cl	100	-	+	-
	80	-	-	-
	60	-	-	-
	40	-	-	-
	20	-	-	-

Two compounds, WR2721 and ribavirin (not shown), showed no toxicity or antiviral activity against VEE virus in tissue culture or in mice. Kethoxal demonstrated the highest degree of toxicity to the tissue culture cells and the greatest antiviral activity. Kethoxal was not active against VEE in the mouse model, but showed significant activity against YF in the indirect mouse model. Rimantadine and SM-1213 both showed moderate antiviral activity in high concentration, but inactivity in the VEE mouse model. PI:Cl showed marginally positive antiviral activity at the highest concentration. PI:Cl was active against YF in the indirect mouse model and VEE in both the direct and

indirect mouse model. The disparity between tissue culture and animal studies is demonstrated by the 3 compounds that showed positive activity in cell culture but were inactive in the animal system.

IF inducement tests were completed using PI:C1, PI:Cd and NIH Reference Reagent poly I:C (Ref PIC) on mouse L-929 tissue culture cells. No detectable IF was induced by any of these compounds in the absence of DEAE-dextran. When DEAE-dextran was combined with Ref PIC, significant amounts of IF were detected. The most potent ratio of Ref PIC:DEAE-dextran was 50:200 μg/ml. With this ratio, > 1,000 units of IF/ml were detected. No IF could be detected in response to various combinations of PI:C1 and DEAE-dextran.

Vesicular stomatitis virus (VSV) was evaluated for possible substituion for VEE virus currently being used in the IF plaque reduction assay to increase the assay sensitivity. Since various investigators have reported on the similarities between human and monkey IF, it is apparent that either Vero or LLC-MK_2 cell cultures may be used with VSV for tissue culture plaque reduction assay for IF. Although the LLC-MK_2 cell line appears to be more sensitive than the Vero cell line, the shorter incubation period required for development of VSV plaques on the latter may be an advantage in IF assays. The use of VSV virus for IF assays will more closely relate the IF data from our laboratory with that provided by NIH, since it is the same virus. VEE virus appears to be approximately 5-fold less sensitive to IF than VSV in both Vero and LLC-MK_2 cell culture. In the L-929 tissue culture, VSV is 10-fold more sensitive to mouse IF than VEE virus. Future IF induction studies in tissue culture are planned with human foreskin fibroblasts and the various poly I:C preparations.

Presentations:

1. Stephen, E. L., W. L. Pannier, M. L. Sammons, S. Baron, H. B. Levy, and R. O. Spertzel. Prophylaxis of yellow fever in rhesus monkeys by a nuclease-resistant derivative of poly I:poly C. Presented, 59th Annual Meeting, FASEB, Atlantic City, N. J., 13-18 Apr 75. (Fed. Proc. 34:960, 1975).

2. Walker, J. S., J. W. Dominik, J. B. Moe, R. O. Spertzel, and E. L. Stephen. Treatment of influenza infection of mice using rimantadine hydrochloride by the aerosol and intraperitoneal routes. Presented, 59th Annual Meeting, FASEB, Atlantic City, N. J., 13-18 Apr 75. (Fed. Proc. 34:799, 1975).

Publication:

Stephen, E. L., J. W. Dominik, J. B. Moe, R. O. Spertzel, and J. S. Walker. 1975. Treatment of influenza infection of mice using rimantadine HCl by the aerosol and intraperitoneal routes. Antimicrob. Agents Chemother. 8: In press.

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2. Levy, H. B., G. Baer, S. Baron, C. F. Gibbs, M. Ladarola, W. London, and J. M. Rice. 1974. Induction of interferon in nonhuman primates by a nuclease-resistant polyriboinosinic polyribocytidylic acid complex. IRCS Med. Sci. 2:1643.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6415	75 07 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
74 07 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	62760A	3A762760A834	02	415
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)
(U) Studies in respiratory immunization

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 010100 Microbiology

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: 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 75	1.0	103.0
FISCAL YEAR CURRENT 76	1.0	173.8

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Aerobiology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Scott, G. H.
TELEPHONE: 301 663-2439
SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Immunization; (U) Influenza; (U) Particle size; (U) Immunoprophylaxis; (U) Aerosols; (U) Military medicine

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

23 (U) Characterize and evaluate basic mechanisms of aerogenic immunization against respiratory infections. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Respiratory pathogen vaccines attenuated by adaptation to growth at suboptimal temperatures are used as model systems to study the genesis of immunity for protection against respiratory infections.

25 (U) 74 07 - 75 06 - The antibody responses and resistance to reinfection of parenterally immunized mice were examined following booster doses of live influenza virus administered either in aerosols or parenterally. The booster doses did not prevent reinfection or virus replication in the lungs of mice following respiratory challenge with virulent influenza virus. Aerosol-immunized mice were totally immune to rechallenge. Infectious virus-antibody complexes appeared in lung fluids of aerosol-infected mice within 4 days indicating the early appearance of antibody in the respiratory tract. The number of lymphoid cells bearing surface immunoglobulins of the IgA and IgG classes, as well as nonimmunoglobulin-bearing cells, increased in the lungs of aerosol-infected mice to a peak level within 14 days postinfection, but decreased to near baseline values by 28 days.

Several strains of mice and 2 of guinea pigs were screened for infectivity of Q fever infection by several routes of challenge. Further studies will be carried out on pathologic changes in both guinea pig strains and DBA/2 mice.

*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 65 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 415: Studies in Respiratory Immunization

Background:

Previous reports[1,2] have discussed antibody responses in serum and respiratory secretions, and their role in defense against respiratory infections in mice vaccinated with a model respiratory pathogen, influenza virus, by respiratory and parenteral routes. Following parenteral vaccination, antibody was found early in the sera, but only small amounts were detected in lung fluids after 2-3 wk. Following aerosol infection, antibody was found in sera and lung fluids. Measurable amounts of IgA and IgG, and trace amounts of IgM, all of which were demonstrated to bind specifically with the infecting virus, appeared in lung washings of aerosol-infected mice. Mice immunized by the aerosol route resisted rechallenge with 100 respiratory (R) LD_{50} of virulent virus. Parenteral vaccination modified the course of disease in challenged mice and reduced mortality rates, but did not prevent reinfection of the respiratory tract. This work has been expanded to examine: (1) appearance of infectious virus-antibody complexes in lung fluids of aerosol-infected mice; (2) influence of influenza infections on the distribution of immunoglobulin-producing cells of each type within the lung; and (3) effects of booster doses of live virus administered by the IP or aerosol routes to mice that had previously been immunized by the IP route. In preparation for studies on the immunogenesis of Q fever infections, studies were initiated to screen several inbred strains of mice as well as Hartley and Moen-Chase strains of guinea pigs for susceptibility to Coxiella burnetii administered by the IP, IN, intratracheal (IT), and aerosol routes.

Progress:

I. Booster inoculations. Swiss-ICR mice were inoculated IP with a primary dose of $10^{5.7}$ egg ID_{50} of virulent influenza virus; 7 days later mice from this group were given booster doses of either 80 EID_{50} in SPA, or $10^{5.7}$ EID_{50} by the IP route. Two weeks after administering the booster doses, both groups of mice were challenged by the respiratory route with 80 RLD_{50} of aerosolized virulent virus.

Serum HI responses in mice given booster doses by either the respiratory or IP routes were not different from mice which received only a primary IP injection (Table I). Antibody was not detected in concentrated (10 x) bronchoalveolar washings (BAW) from mice that had received only a single IP

dose; however, increased HI activity was measured in BAW of mice that received a secondary dose by either route.

TABLE I. HI RESPONSES IN IP IMMUNIZED MICE (n=10) FOLLOWING ADMINISTRATION OF BOOSTER DOSES BY 2 ROUTES

DAYS AFTER BOOSTER	RECIPROCAL HI TITERS[a] BY TREATMENT					
	Primary IP Only		Primary IP, Boosted by Aerosol		Primary IP, Boosted IP	
	Serum	BAW	Serum	BAW	Serum	BAW
0	20	<2				
7	34	<2	35	8	26	4
14	41	2	52	32	45	11

a. Geometric mean titers of sera; 10-mouse pool of BAW concentrated 10-fold prior to assay.

Two weeks after receiving booster doses, the immunized mice and 25 uninfected control mice were challenged with 80 RLD_{50} of virulent virus by the respiratory route. Virus isolations and assays of lung tissues taken from these mice 2 and 5 days postchallenge are recorded in Table II.

TABLE II. PROTECTIVE[a] EFFECTS OF IP- AND AEROSOL-BOOSTER DOSES OF INFLUENZA VIRUS ON IP IMMUNIZED MICE

ROUTE OF BOOSTER DOSE	DAYS POST-CHALL.	NO. INFECTED/ TOTAL	GEOM. MEAN[b] VIRUS TITERS (EID_{50}/LUNG)	SURVIVORS	
				No./Total	Percent
IP (n=30)	2	5/5	5.6	20/20	100
	5	2/5	2.0		
Aerosol (n=30)	2	3/5	3.5	20/20	100
	5	0/5	0		
Uninfected Controls (n=25)	2	5/5	7.7		
	5	2/2	5.1	0/18	0

a. Mice were challenged by SPA with 8C RLD_{50} of virulent influenza virus.
b. Includes titers from infected lungs only.

Lung infections were detected in 60% of the aerosol-boosted mice at 2 days, but lung virus titers were low (3.5 EID_{50}/lung) and short-lived; no virus could be detected at 5 days. The lungs of all of the IP-boosted mice were infected as indicated by significant lung virus titers at 2 days, which were not completely cleared by 5 days. All of the immunized mice survived the respiratory challenge while none of the nonimmune control mice survived. These data confirm our earlier observations that although immunization of mice by the IP route may result in protection against death, infection of the respiratory tract does occur following respiratory challenge with virulent influenza virus. Virus recovered from IP immunized mice was found to be fully infectious. IN instillation in normal mice of infected lung suspensions prepared 24, 48 and 96 hr after aerosol challenge of IP immunized mice resulted in lung infections with virus replication in all inoculated mice to titers $>10^5$ EID_{50}/lung.

II. Infectious virus-antibody complexes. Antibody appeared later in the respiratory tract of mice following respiratory infection than it did in sera following parenteral inoculation. This suggested the possibility that antibody was produced early during respiratory infection but was not detectable because it was complexed with antigen (live virus) present in the lungs. This possibility was examined using the procedures of Zalan.[3] Samples of BAW obtained from mice at 4 and 7 days following respiratory infection were diluted to contain approximately 10^4 EID_{50} of virus when mixed with an equal volume of goat-antimouse immunoglobulin. The mixtures were held at room temperature for 30 min and then titrated for infectivity in embryonated eggs. Controls included normal goat serum and normal mouse serum mixed with infected BAW, and goat-antimouse serum added to virus suspensions in allantoic fluid (Table III).

TABLE III. EFFECT OF HOST SPECIFIC ANTIGLOBULIN ON INFECTIVITY OF INFLUENZA VIRUS IN BAW

INFECTED SPECIMEN	SERUM ADDED	INFECTIVE TITER (Log_{10} EID_{50}/ml)
BAW (day 4)	Goat-antimouse	3.0
(day 7)	Goat-antimouse	2.0
Controls		
BAW	Normal goat	>4.0
BAW	Normal goat	>4.0
BAW	None	>4.0
Allantoic fluid	Goat-antimouse	>4.0

None of the control treatments resulted in reduced infectivity of BAW. The infectious titers of BAW samples obtained at 4 days were reduced at ≥10-fold, and those of 7-day BAW ≥100-fold after incubation in the presence of goat-antimouse immunoglobulins. The presence of virus-antibody complexes in BAW samples as early as 4 days suggests that specific antibody production was initiated earlier during respiratory infection than previously realized, based on HI titrations of BAW. Although antibody appeared in serum within 3-4 days after IP inoculations, its appearance was delayed following even severe lung infections and was not detected until a week postinfection. The presence of antibody in the respiratory tract prior to its appearance in the blood suggests that much of the early respiratory antibody was synthesized and secreted locally.

III. Immunoglobulin-bearing cells in mouse lungs. The distribution of immunoglobulin (Ig)-bearing cells in the lungs of mice immunized with sublethal doses of influenza virus was examined and compared with those from uninfected mice. Using a modification of procedures described by Noble et al.,[4] lymphoid cells from mechanically disrupted lungs were concentrated by low speed centrifugation on a layer of Ficoll-Hypaque solution. These lymphoid cell enriched suspensions were prepared from lungs taken from mice at various intervals during and after infections initiated by exposure to SPA of influenza virus. Cells in the suspensions were examined for the presence of surface Ig by staining with fluorescein conjugates of polyvalent antiglobulin containing antibodies to IgA, IgM, and IgG as well as with conjugates containing monospecific antibodies to IgA and IgG. The average number of nucleated cells recovered by these procedures, and the numbers staining positively with each of the conjugated antisera, are recorded in Table IV.

TABLE IV. DISTRIBUTION OF LYMPHOID CELLS BEARING IMMUNOGLOBULIN IN MOUSE LUNGS INFECTED WITH INFLUENZA VIRUS

CELLS	MEANS x10^4 BY DAYS OF 2-3 REPLICATES			
	0	8	14	28
Total recovered/lung	81	285 (3.5)[a]	457 (5.6)	200 (2.5)
Lymphoid/lung	11	211 (19)	223 (20)	32 (2.9)
Stained with:				
Polyvalent anti-IgA, IgM and IgG	4.5	46 (10)	114 (25)	6.0 (1.3)
Monospecific anti-IgA	1.2	17 (14)	45 (37)	4.0 (3.3)
Monospecific anti-IgG	4.0	26 (6.5)	78 (19)	2.0 (-2)

a. Fold increase over baseline.

Cell suspensions from uninfected lungs contained only 10-15% lymphocytes, some erythrocytes, macrophages, foamy cells, epithelial cells, etc. More than 95% of the cells retained viability as indicated by the trypan blue exclusion. About 50% of the live lymphoid cells from uninfected lungs stained positive with the polyvalent conjugates; most of these cells were also stained with monospecific IgG while only ~10% stained with monospecific IgA conjugates. The total number of lymphoid cells present in the lungs increased dramatically by day 8 and were 20-fold higher than baseline values at 14 days, but decreased to near baseline by 28 days. Immunoglobulin-bearing cells increased at a rate similar to that of the lymphoid cells and were 25-fold higher than baseline values by 14 days. However, they still accounted for only 50% of the lymphoid cells present, indicating that in addition to the increased numbers of Ig-bearing cells the respiratory infections stimulated accumulation of non-Ig-bearing cells, possibly T-cells, in the lungs. This is consistent with the findings of MAJ Hetsko, Bacteriology Division, that macrophage inhibition factor (MIF) levels in the lung reach their peak at 14 days. The numbers of IgA- and IgG-bearing cells both increased substantially following infection, but the greatest relative increase occurred with IgA-bearing cells.

The demonstrated increase in lymphoid cells, IgA- and IgG-bearing cells in immune lungs is consistent with observed increases in IgA and IgG class antibodies found in bronchoalveolar fluids of mice during and after a sublethal influenza infection. The findings are interpreted as supporting evidence that respiratory infections stimulate local production of antibody of both the IgA and IgG classes.

IV. Host susceptibility screening, Q fever. Several inbred strains of mice as well as Hartley and Moen-Chase strain guinea pigs have been screened for susceptibility to infection with C. burnetii by the IP, IN, IT, or aerosol routes. Groups of animals were given doses ranging from $10^{4.2}$ - $10^{8.7}$ $MIPID_{50}$ of the Phase I, Nine-mile strain of the rickettsia and observed for overt signs of illness and mortality for 28 days. Serum samples were collected from surviving animals and tested at a 1:50 dilution for anti-C. burnetii antibodies using the indirect immunofluorescent antibody (IFA) technique described by Bozeman and Elisberg.[5] Mortality and morbidity data for mice are recorded in Table V. Data for guinea pigs are in Table VI. By comparison with outbred Swiss mice, all of the inbred strains were more susceptible in terms of both parameters. None of the Swiss-ICR mice died, and illness was observed only in the IP group that received the highest dose ($10^{8.2}$ $MIPID_{50}$).

TABLE V. TITRATION OF MORBIDITY AND MORTALITY IN MICE CHALLENGED BY 3 ROUTES WITH C. BURNETII

	% MORTALITY AT VARIOUS LOG_{10} DOSES						
	8.2	7.2	6.2		4.2		4.7
STRAIN	IP	IN	IP	IN	IP	IN	Aerosol
DBA/1	80	10	0	0	0	0	-
DBA/2	50	46	0	30	0	8	0
AKR/J	50	5	15	0	5	0	-
C57BL/6	70	0	15	5	0	5	-
BALB/C	80	5	10	0	0	0	-
Swiss-ICR	0	0	0	0	0	0	0
	DAYS OF OVERT CLINICAL ILLNESS						
DBA/1	15	2	5	0	0	0	
DBA/2	10	8	5	5	0	5	0
AKR/J	14	0	5	0	0	0	
C57BL/6	10	4	8	0	0	0	
BALB/C	17	0	7	0	0	0	
Swiss-ICR	7	0	0	0	0	0	0

TABLE VI. TITRATION OF MORBIDITY AND MORTALITY IN GUINEA PIGS INOCULATED WITH C. BURNETII

	% MORTALITY AT VARIOUS LOG_{10} DOSES							
	8.2		7.2	6.2		4.2		5.3
STRAIN	IT	IP	IN	IN	IP	IN	IP	Aerosol
Hartley	83	90	10	0	10	0	10	100[a]
Moen-Chase	37							100
	DAYS OF OVERT CLINICAL ILLNESS							
Hartley	8	4	0	0	20	0	0	4
Moen-Chase	5							3

a. Died 8-12 days.

Generally, less illness and fewer deaths resulted from IN administration of rickettsiae than from IP inoculations. Although sporadic deaths occurred with AKR/J, C57BL/6, and BALB/C strain mice following IN administration, a significant number of mortalities occurred only among DBA/2 mice when inoculated by this route. Mice did not succumb to $10^{4.7}$ $MIPID_{50}$ of rickettsiae given by SPA (2 μm in diameter). This was the highest dose that could be practically administered to mice in aerosols with the equipment available. By contrast 37% of Moen-Chase and 83% of Hartley strain guinea pigs died following IT administration of $10^{8.2}$ $MIPID_{50}$, and all guinea pigs of both strains died 8-12 days after exposure to doses of only $10^{5.3}$ $MIPID_{50}$ in SPA. These studies are being extended by examining histological changes in the DBA/2 strain of mice as well as Hartley and Moen-Chase guinea pigs infected with Q fever by the respiratory route.

Presentations:

Scott, G. H. Respiratory immunization against influenza infection in mice. Presented, Maryland Branch American Society for Microbiology, University of Maryland Dental School, Baltimore, Md. 21 Nov 1974.

Publications:

None.

LITERATURE CITED

1. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1973. Annual Progress Report, FY 1973, pp. 325-328, Fort Detrick, Md.

2. U.S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974, pp. 303-313, Fort Detrick, Md.

3. Zalan, E., and N. A. Labzoffsky. 1973. In vivo studies of infectious influenza virus-antibody complex. Acta Virol. 17:215-219.

4. Noble, P. B., J. H. Cutts, and K. K. Carroll. 1968. Ficoll flotation for the separation of blood leukocyte types. Blood 31:66-73.

5. Bozeman, M. F., and B. L. Elisberg. 1963. Serological diagnosis of scrub typhus by indirect immunofluorescence. Proc. Soc. Exp. Biol. Med. 112:568-573.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OD6419	75 07 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
74 07 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	62760A	3A762760A834	02	416
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Mechanisms of immunoprophylaxis against airborne infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 08	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 75	1.0	118.0
FISCAL YEAR CURRENT 76	1.0	173.8

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Aerobiology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Jemski, J. V.
TELEPHONE: 301 663-2439
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) Viral diseases; (U) Airborne infections; (U) Immunoprophylaxis

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

23 (U) Develop and evaluate methods for immunization against respiratory infections. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Using influenza vaccines (killed and attenuated) study the efficacy of different methods of immunization including large and small particle aerosols against a respiratory challenge with virulent virus.

25 (U) 74 07 - 75 06 - Mice were vaccinated intranasally (IN) with small (SPA) or large (LPA) particle aerosols with an attenuated temperature-sensitive, recombinant A2/68 influenza (H3N2) virus--ts-1(E). Circulating virus neutralizing and hemagglutination-inhibition antibodies were obtained for all vaccinates. Increased immunoglobulin (IgG, IgA) levels in bronchoalveolar washes also were elicited in the IN group. Upon challenge with aerosols of a mouse virulent H2N2 influenza virus, 100% protection was obtained for the SPA and IN-mice while 86% of the LPA-group survived. The strong protection afforded the aerosol-vaccinated group appears to implicate the involvement of local immunity. Preliminary studies also were implemented using a Mycoplasma pneumoniae-hamster model system to study host immune defense mechanisms against upper and lower respiratory infections in collaboration with the National Institute of Allergy and Infectious Diseases.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 416: Mechanisms of Immunoprophylaxis Against Airborne Infections

Background:

Previous reports have indicated our unsuccessful attempts to immunize mice with a cold-adapted variant (CAV) of A2/Aichi/2/68 influenza virus. At best, only minimal seroconversions were obtained in CAV-vaccinated mice regardless of the method of vaccination, i.e., small- (SPA) or large-particle aerosols (LPA) or IN. Prior to terminating the influenza-mouse model phase of the project, an additional effort involved the evaluation of a temperature-sensitive, recombinant mutant strain derived from an H3N2 and an H3N2 influenza A2 virus.[1] The mutant virus, labelled ts-1(E) has a temperature-sensitive genetic defect obtained from the H2N2 virus with a shut-off temperature of 38 C, but contains the contemporary influenza H3N2 surface antigens. It was of pertinent interest, therefore, to study the immunogenicity of the ts-1(E) recombinant when deposited as aerosols in the upper respiratory tract or in the lungs of mice in which basal temperatures are 32-34 C and 37 C, respectively. Investigations also were initiated to determine the feasibility of infecting hamsters with aerosols of Mycoplasma pneumoniae. The mycoplasma-hamster system then would replace the influenza-mouse model for studying the pathogenesis and host immune defense mechanisms involved in upper and lower respiratory infections.

Progress:

The ts-1(E) recombinant virus was obtained from Dr. R. Chanock's laboratory at NIAID. It had been produced in primary bovine-kidney cells.[1] We passed the mutant 2 times in 10-12-day-old embryonated eggs incubated at 32 C for 48 hr/passage. The harvested allantoic fluid from the second pass was stored at -60 C in vials containing penicillin-streptomycin, but no serum stabilizers. The EID_{50} titer was calculated at 7.3 logs with a HA titer of 1:32.

Six-week-old ICR-derived mice were vaccinated with the ts-1(E) mutant administered by aerosol inhalation or IN. For the SPA, the MMD was $\sim$2.2 µm, the mice were exposed in the Henderson aerosol tube. For LPA vaccination, the spinning top apparatus was employed which yields an MMD of $\sim$8 µm.[2] IN vaccination involved the instillation of 0.05 ml of the vaccine suspension in the anterior nares of lightly anesthetized mice. The rate of infection and the degree of replication of the ts virus in the respiratory tract of the 3 groups of vaccinated mice were determined for 27 days following exposure (Table I).

TABLE I. REPLICATION OF TS-1(E) RECOMBINANT VIRUS IN RESPIRATORY TRACT OF MICE VACCINATED[a] BY DIFFERENT ROUTES

DAY AFTER VACCINATION	LUNG			URT		
	SPA	IN	LPA	SPA	IN	LPA
Mouse response by respiratory tissue. (No. positive/5)						
1	5	5	0	5	5	3
3	5	4	2	4	5	4
7	4	2	0	4	5	2
10	0	0	2	1	0	2
14	0	1	0	0	1	0
27	0	0	0	0	0	0
Geometric mean $\log_{10}$ tissue titers of the positive mice.						
1	3.9	>4.0	-	3.9	>4.0	2.3
3	>4.0	3.4	>4.0	3.6	>4.0	3.5
7	3.4	3.6	-	3.2	3.3	4.0
10	-	-	2.0	3.2	-	2.5
14	-	2.2	-	-	2.2	-

a. Vaccination dose (EID_{50}) = SPA, $10^{4.1}$; LPA, $10^{4.1}$; IN, $10^{4.7}$.

The ts-1(E) mutant replicated to a moderate level in the vaccinated mice and maximal titers appeared to be reached by day 3. In contrast, wild-type virus peaks at 4-6 $\log_{10}$/lung on day 1 and steadily declines to nondetectable levels by 10 days. The lower titers obtained with the ts-1(E) strain resulted from the 38 C shut-off temperature. For ts mutants of influenza virus, temperature sensitivity produces a modified in vivo growth pattern.[3] Another result of the 38 C shut-off temperature was that the upper respiratory tract (URT) titers equaled those observed in the lung. For LPA-vaccinated mice, URT virus titers were at expected levels. Lung virus, however, was detected at 3 and 10 days but in only 4 of 10 of the LPA animals. This suggested that the lung infections occurred as a result of viral infection of the URT. The lack of pulmonary pathology in any of the mice of the 3 vaccinated groups evidenced an acceptable level of attenuation for the ts-1 mutant.

Antibody titers and immunoglobulin (Ig) levels were determined for serum and bronchoalveolar wash (BAW) fluids obtained from the vaccinated and non-vaccinated control mice on days 7, 14 and 27. The serum samples from 5 individual mice were titered at each sampling period. The BAW fluids from 10 mice were separated into 2 pools of 5 mice each to yield 2 replicate samples for antibody and Ig measurements. The BAW samples were concentrated 15-fold. Immunoglobulin (IgG, IgA, IgM) levels in the serum and BAW samples were estimated by the Mancini radial immunodiffusion assay procedures.[4] Serum

virus neutralizing (SN) antibody titers were assayed in eggs while HI titers for serum and BAW were based on standard microtiter HI tests with 4 antigen units. The data are shown in Tables II and III.

TABLE II. SERUM ANTIBODY RESPONSE OF MICE VACCINATED WITH TS-1(E) MUTANT OF INFLUENZA A VIRUS (H3N2)

VACCINATION ROUTE	NO. POSITIVE/5 ASSAYED (MEAN TITER) Days After Vaccination 7 SN[a]	7 HI	14 SN	14 HI	27 SN	27 HI
Nonvaccinated	0	0	0	0	0	0
IN	5	2 (1:20)	5	4 (1:40)	5	5 (1:80)
SPA	5	4 (1:15)	5	5 (1:44)	5	5 (1:52)
LPA	0	0	5	3 (1:17)	5	4 (1:55)

a. ≥4-fold increase.

SN antibodies against the ts-1(E) virus were demonstrable for all IN- and SPA-mice as early as 7 days. SN activity for the LPA mice was delayed until 14 days. The same temporal pattern was obtained for serum HI antibody response among the 3 groups of mice. Serum IgA, IgG and IgM values for all groups of mice showed no increase over baseline levels (data are not shown).

TABLE III. BRONCHOALVEOLAR WASH ANTIBODY RESPONSE OF VACCINATED MICE

PARAMETER	GEOMETRIC MEAN (2 REPLICATES OF 5 EACH) BY DAYS Nonvaccinated 7	Nonvaccinated 14	Nonvaccinated 27	SPA 7	SPA 14	SPA 27	LPA 7	LPA 14	LPA 27	IN 7	IN 14	IN 27
IgA (100 mg/ml)	0	0	0	0	0	0	0	0	0	0	20[a]	0
IgG (100 mg/ml)	27	32	34	<15	25	36	0	<15	32	35	63[b]	51
HI titer (reciprocal)	0	0	0	0	0	15	0	0	0	0	40	40

a. P<0.001 (T test) compared to zero values.
b. P<0.05 (T test) compared to other group values.

The only significant increase in BAW immunoglobulin levels was elicited for IgA and IgG at 14 days for the IN mice. At 27 days all Ig concentrations had returned to baseline values. Although the IN-IgG value still appeared elevated at that time, it was not statistically distinguishable from the other groups. Positive HI titers for BAW fluids were obtained at 14 and 27 days for the IN group, but only at 27 days for the SPA vaccinated mice. BAW fluids from the LPA animals were negative for all antibody measurements.

To correlate the observed antibody responses to protection of the host, the ts-1(E) vaccinated and control mice were challenged 28 days later. Results of the SPA-challenge with a mouse-adapted, virulent influenza A/68 (H3N2) virus at a presented dose of $10^{3.1}$ EID_{50} are shown in Table IV.

TABLE IV. RESPONSE OF TS-1(E) VACCINATED MICE CHALLENGED WITH SPA OF VIRULENT INFLUENZA A VIRUS (H3N2)

	TITERS OF CHALLENGE VIRUS AT 2 DAYS					
	Lung		URT			
VACCINATION ROUTE	Pos./5	Titer[a] (EID_{50})	Pos./5	Titer (EID_{50})	SURVIVAL No.	%
Nonvaccinated	5	>4.0	5	>4.0	0/50	0
IN	1	3.5	0	0	50/50	100
SPA	0	0	0	0	50/50	100
LPA	2	4.0	2	4.0	43/50	86[b]

a. Reciprocal of mean $\log_{10}$ titer for positive animals.

b. Significant at $\chi^2 = <0.025$ from the other survival rates.

All nonvaccinated mice typically responded with high concentrations of challenge virus in both regions of the respiratory tract at 2 days after challenge. By 10 days the entire group had died. In contrast, the IN- and SPA-mice were protected; challenge virus replication in the lung was totally inhibited for the SPA-mice and markedly restricted in the IN-vaccinated mice. Upper respiratory tract virus proliferation was completely repressed in these 2 groups of mice. For the LPA-vaccinated mice, the challenge virus was moderately inhibited in the upper and lower respiratory tract. The survival rate of 86% obtained for this group was statistically significant (χ^2 analysis) from the other 2 vaccinated groups and nonvaccinated controls. This partial protection may have been afforded by the serum HI and neutralizing antibodies obtained for these animals (Table II).

Preliminary studies also were implemented in collaboration with NIAID for determining the feasibility of utilizing hamsters exposed to aerosols of

M. pneumoniae as a model system for studying immune response to respiratory infections. Hamsters were infected with SPA or LPA of a virulent strain of M. pneumoniae as described for the influenza studies. The response parameters of aerosol-infected hamsters were compared to the known response performance of hamsters infected with M. pneumoniae by the conventional IN route. The responses were evaluated by titration of respiratory tissues for M. pneumoniae concentration, CF antibodies and pulmonary pathology. Histological lesions were scored on a 1 to 8 basis. Assay procedures were performed at intervals from 0-48 days after initial infection. Some preliminary results are shown in Table V.

TABLE V. COMPARISON OF HAMSTER RESPONSE TO INITIAL INFECTION WITH M. PNEUMONIAE ADMINISTERED BY VARIOUS ROUTES

ROUTE OF ADMINISTRATION (Log_{10} CFU/Animal)	NO. WITH MEAN LUNG LESION SCORE ≥ 3 BY DAYS				AT 14 DAYS	
	0	14	22	40	Lung[a] CFU/gm ± SD	Log_2 CF Titer
IN-Sham (0)	0/9	0/9	0/5	0/4	<2.0	NEG
IN (6.4)	0/9	9/9	0/5	1/3	5.1±0.93	3.6
IN (3.3)	0/4	2/4	ND[b]	0/4	5.4±0.5	ND
SPA (4.1)	0/9	2/9	0/5	2/4	6.6±0.44	NEG
LPA (3.6)	0/9	0/9	0/5	0/4	<2.0	NEG

a. Log_{10} CFU/animal.
b. No data.

No overt clinical illness was exhibited by the hamsters regardless of exposure route or dose level. Hamsters initially infected IN with $\sim 10^6$ CFU showed proportionally more animals with more severe lung pathology at the 14-day peak response time than the SPA-infected group. However, IN hamsters which received $10^{3.3}$ CFU had lung lesion scores equivalent to the SPA group and not to the IN high-dose animals. The difference in lung pathology between the IN and SPA hamsters may actually be an infecting dose-dependent phenomenon or a factor involving deposition site of the inoculum; it will be investigated further. The similar levels of recoverable organisms obtained at 14 days from the lungs of the 3 groups of hamsters also were of interest primarily because only the IN high-dose animals had positive CF titers. The LPA-infected group had negative responses in all aspects. The various groups of hamsters, therefore, will be challenged at 40-70 days to determine whether protection is affected by the different methods used for initial infection.

Publications:

None.

LITERATURE CITED

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4. Mancini, G., A. D. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 2:235-254.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6412	75 07 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
74 07 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	62760A	3A762760A834	02	417
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Basic properties and clinical application studies on transfer factor

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
72 08	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

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
PRECEDING 75	1.9	75.7
CURRENT 76	1.0	106.8

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Ascher, M. S.

TELEPHONE: 301 663-7341

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Marker, S. C.

NAME:

POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Infectious diseases; (U) Transfer factor; (U) Immunity; (U) Cell-mediated immunity; (U) Lymphocyte transformation; (U) Delayed type hypersensitivity

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

23 (U) Study lymphocyte transformation and an in vitro method for the assay of transfer factor. Assess the importance of transfer factor in infectious diseases of military medical importance. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Develop a microtechnique for assay of transfer factor using lymphocyte transformation of cells from monkeys and humans known to be sensitized to tularemia, Venezuelan equine encephalomyelitis, Q fever, or Rocky Mountain spotted fever. Transfer factor will be prepared from the same donors. Various biochemical techniques will be utilized as appropriate.

25 (U) 74 07 - 75 06 - Cell-mediated immunity as measured by the in vitro correlate lymphocyte transformation has been demonstrated and characterized in humans in response to antigens derived from VEE virus, parainfluenza virus, RMSF rickettsiae and tularemia antigen. Using the end-point of enhancement of such lymphocyte transformation in the presence of dialysable transfer factor (TF), an in vitro assay was developed for TF and the optimal dose of antigen, culture time, and dose of TF were characterized. Column chromatography of TF preparations has been performed with in vitro assay of the eluate fractions. Components(s) responsible for in vitro activity in fractionated extracts were retarded on beds of Sephadex G-25, Biogel P-2 and P-6, and DEAE cellulose Purification in the sense of elution of activity in a discrete band has been accomplished only with DEAE.

Publication: Clin. Res. 23:287, 1975.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 417: Basic Properties and Clinical Application Studies on Transfer Factor

Background:

The importance of cell-mediated immunity (CMI) in protection against or recovery from infectious diseases has recently been emphasized by several clinical "experiments of nature." In the laboratory there are numerous in vitro tests available to assess CMI and its induction or modification by immunization or the transfer of cells or cell products. Such transfers have led to dramatic clinical and immunologic responses in congenital and acquired immunodeficiency diseases, infectious diseases and cancer. The active moiety responsible for the transfer of CMI has been termed transfer factor (TF). The studies reported herein are concerned with developing systems for in vitro assessment of CMI to infectious diseases in man and experimental animals. With the in vitro assay for TF developed in this laboratory,[1] these antigen systems allow relatively direct experiments on the nature and mechanism of action of TF.

Progress:

For development of in vitro correlates of CMI microbial and synthetic antigens have been employed in lymphocyte transformation tests. We have extended observations reported with VEE virus and tularemia[2] to include studies with parainfluenza antigen, dinitrochlorobenzene (DNCB), and RMSF vaccine antigen. Additional recent experiments with the VEE systems have consisted of culturing lymphocytes from 23 people immunized with the live vaccine and from 10 normal controls without a history of exposure to the antigen. As can be seen in Table I, there is a 9-fold difference in reactivity to purified viral antigen between the 2 groups. This difference and overall response of the vaccinees is comparable to other well characterized soluble antigen systems and the clearest demonstration to date of lymphocyte transformation to a viral antigen. In addition, as illustrated in Table II, serial prospective observations have shown acquisition of lymphocyte reactivity to VEE and tularemia during the 4th wk after immunization. The subsequent decline in response to VEE shown by the data was due to deterioration of the antigen preparation during storage at 4 C and does not necessarily reflect a true decrease in immunologic reactivity.

TABLE I. LYMPHOCYTE TRANSFORMATION RESPONSES OF VACCINES AND CONTROLS TO PURIFIED VEE ANTIGEN (1 µg/ml), AS MEASURED BY ^{14}C-THYMIDINE INCORPORATION.

CELL DONORS	GEOM. MEAN CPM	95% CONF. LIMITS
Controls (n = 10)	94	49 - 180
Vaccinees (n = 23)	833	533 - 1,302

TABLE II. DEVELOPMENT OF LYMPHOCYTE TRANSFORMATION TO VEE AND TULAREMIA ANTIGENS AFTER VEE IMMUNIZATION, AS MEASURED BY ^{14}C-THYMIDINE INCORPORATION.

ANTIGEN	GEOM. MEAN CPM BY WK[a]									
	0	1	2[b]	4	6	8	10	12	15	18
None	24	28	57	344	37	38	32	31	49	58
VEE 1 µg/ml	66	40	174	4,985	3,516	1,949	927	1,138	471	394
Tularemia 10^6/ml	41	45	265	31	4,664	2,686	3,775	4,217	4,396	4,165
Positive Control (SK-SD[c] 20 /ml)	5,413	5,398	-	3,190	3,451	5,220	2,706	2,950	5,341	4,525

a. 6 replicate cultures.

b. Tularemia vaccine given.

c. Streptokinase-streptodornase.

Collaborative studies (Work Unit 834 02 300) on lymphocyte transformation induced with rickettsial antigens were initiated. A candidate formalin-killed RMSF vaccine, Sheila Smith strain, grown in DEC cultures, purified by sucrose gradient centrifugation and lyophilized, was emplo ed. Lymphocytes were obtained from 9 individuals with diverse RMSF antigenic experience (Table III). Markedly enhanced transformation was demonstrated only with lymphocyte cultures from 2 individuals with a history of clinical illness and from the individual who prepared the RMSF-DEC antigen. Lymphocytes from all other individuals, regardless of their antigenic history, exhibited a minimal response. To examine the significance of these findings, experiments with an experimental animal model are required and evaluation of the RMSF antigen in a guinea pig whole blood lymphocyte transformation assay is in progress.

TABLE III. LYMPHOCYTE TRANSFORMATION TO RMSF VACCINE ANTIGEN, AS MEASURED BY ^{14}C-THYMIDINE INCORPORATION.

GROUP EXPERIENCE (Identification[a])	GEOM. MEAN CPM BY ANTIGEN DILUTION				
	0[b]	10^{-4}	10^{-3}	10^{-2}	10^{-1}
Controls					
No exposure					
(DF)	58	62	95	79	636
(MA)	27	77	83	54	293
Laboratory workers					
(RW)	69	53	46	175	368
(PK)	57	118	99	519	155
(DM)	24	197	125	112	131
Clinical illness					
(DL)	66	212	1,018	2,864	3,138
Vaccinees					
(Lederle yolk-sac)					
Laboratory workers					
(HS)	58	27	43	45	114
(RK)	28	958	2,116	4,073	5,547
Clinical illness					
(JM)	30	267	2,271	3,617	3,321

a. DM, DL and JM laboratory accidents.

b. Undiluted = 3 x 10^8 rickettsiae/ml.

Laboratory accidental exposure; DM, treated with tetracycline; no clinical illness.

RK prepared DEC vaccine employed as antigen.

Dr. Hilary Koprowski, Wistar Institute, Philadelphia, has obtained evidence on the basis of recovering parainfluenza I virus from the brain of a multiple sclerosis patient, that this disease is the result of an aberrant immune response to the viral antigen in individuals with an excess of HL-A7 tissue histocompatibility antigen. At his request preliminary titration with purified parainfluenza I viral antigen was performed to determine its capability to effect human lymphocyte transformation. The wide spectrum of responses obtained with lymphocytes from 10 normal individuals was similar to that observed with other ubiquitous antigens. There was no correlation with tissue type in this small sample.

Preliminary studies have been conducted in preparation for a cooperative study with Dr. William Levis, National Cancer Institute, to examine in vitro transfer of contact sensitivity. Upon receipt from Dr. Levis of TF from DNCB positive donors, we will evaluate the capability of the material to effect specific transformation of nonsensitized lymphocytes in the presence of DNCB.

Work on the in vitro assay of TF has continued and we have established that concentrations of leukocyte dialysate capable of 10-fold enhancement of lymphocyte proliferation in the presence of antigen fail to stimulate lymphocytes in the absence of antigen. Time-course experiments suggest that previously described "maxima" or "toxicity"[1] with large doses of TF may be explained in part by earlier peaking of lymphocyte transformation and subsequent decline. Leukocyte dialysate appeared to have a threshold effect, in that enhancement of lymphocyte transformation was obtained at that dose of antigen which stimulated some reactivity alone (Table IV). Cells that were unable to respond to a given dose of tuberculin purified protein derivative (PPD), 1 µg/ml, were not enhanced by TF in vitro, whereas cultures of the same cells that responded to a larger dose of antigen, 10 µg/ml, were enhanced by TF. This observation is in contrast to the in vitro effects of most nonspecific adjuvants that enhacce transformation only at suboptimal doses of mitogen or antigen, e.g., low dose enhancement demonstrated in our laboratory with folic acid.

TABLE IV. INTERACTION BETWEEN ANTIGEN (PPD) AND TRANSFER FACTOR (TF) IN INDUCING TRANSFORMATION OF NONSENSITIZED LYMPHOCYTES, AS MEASURED BY ^{14}C-THYMIDINE INCORPORATION.

TF (µl)	GEOM. MEAN CPM (n = 6), BY PPD DOSE		
	None	1 µg/ml	10 µg/ml
None	43	62	360
50	38	106	396
100	34	92	617
150	41	91	1,103

This *in vitro* assay has been employed to identify active fractions in chromatographic eluates of crude leukocyte dialysates during our efforts to purify and characterize TF. In gel filtration chromatography with Sephadex G-25, *in vitro* activity was recovered from a broad region of eluate that centered on ~ 3 column volumes. The transformation activity of these fractions did not correspond to their spectrophotometric activity at 260 nm (Table V). This lack of correlation is in contrast with reports by Zuckerman *et al.*,[3] that *in vitro* activity was associated mainly with the region at 5/4 column volumes of eluate and high absorbance at 260 nm, i.e., peak 3 in Table V. Evidence was obtained with ion exchange chromatography that the material responsible for *in vitro* activity resides in molecules with affinity for the resin DEAE cellulose in 0.01 M salt and can be eluted with a higher salt concentration. This latter procedure may therefore be applicable to large scale purification of TF for *in vitro* and eventually *in vivo* testing. Fractions with active material have been exchanged with Dr. Novelli at Oak Ridge for comparative assays of lymphocyte transformation and cytotoxicity in the system described by Byers *et al.* for tumor immunity.[4]

TABLE V. SPECTROPHOTOMETRIC ACTIVITY AND LYMPHOCYTE TRANSFORMATION WITH ELUATE FRACTIONS FROM SEPHADEX G-25, AS MEASURED BY ^{14}C-THYMIDINE INCORPORATION.

ELUATE FRACTION	OPTICAL DENSITY (260 nm)	GEOM. MEAM CPM (n = 6)	
		Without AG	With AG
Buffer blank	0	30	1,101
1	0.55	64	70
2	1.81	133	1,179
3	0.84	82	3,949
4	0.18	75	3,904
5	0.04	83	3,738
6	0	57	3,077
7	0	111	3,485
8	0	95	3,867
9	0	88	3,249
10	0	99	3,334
11	0	139	2,931

Presentation:

Marker, S. C., and M. S. Ascher. Specific lymphocyte transformation to Venezuelan equine encephalitis virus (VEE). Presented, 14th Interscience Conference on Antibiotic Agents and Chemotherapy, San Francisco, Calif., Sep 1974.

Publication:

Ascher, M. S., and L. A. Andron. 1975. In vitro properties of leukocyte dialysates containing transfer factor: micro method and recent findings. Clin. Res. 23:287 (abstract).

LITERATURE CITED

1. Ascher, M. S., W. J. Schneider, F. T. Valentine, and H. S. Lawrence. 1974. In vitro properties of leukocyte dialysates containing transfer factor. Proc. Nat. Acad. Sci. 71:1178-1182.

2. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974. p. 327-332. Fort Detrick, Maryland.

3. Zuckerman, K. S., J. A. Neidhart, S. P. Balcerzak, and A. F. LoBuglio. 1974. Immunologic specificity of transfer factor. J. Clin. Invest. 54: 997-1000.

4. Byers, V. S., A. S. Levin, A. J. Hackett, and H. H. Fudenberg. 1975. Tumor-specific cell-mediated immunity in household contacts of cancer patients. J. Clin. Invest. 55:500-513.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OE6414	75 07 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
74 07 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	62760A	3A762760A834	02	418
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)*

(U) Molecular structure of Group C, Bunyamwera supergroup and ungrouped arboviruses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 01	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 75	1.0	71.6
	CURRENT 76	1.0	78.8

19. RESPONSIBLE DOD ORGANIZATION

NAME:* USA Medical Research Institute of Infectious Diseases

ADDRESS:* Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME:* Virology Division USAMRIID

ADDRESS:* Fort Detrick, MD 21701

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

NAME:* Rosato, R. R.

TELEPHONE: 301 663-7241

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Arboviruses; (U) Proteins; (U) Bunyamwera; (U) Military medicine; (U) Arenaviruses

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

23 (U) Identify and characterize the structural components of selected arboviruses and arena viruses. This is a basic element in a comprehensive program for medical defense against BW agents.

24 (U) Employing radiolabeled purified virions, examine said virions, detergent-released subvirion components (and possibly normal and infected host cells) for structural components as to numbers, molecular weights, composition and location or possible function within the virions.

25 (U) 74 07 - 75 06 - The structural proteins of Tacaribe virus have been tentatively identified and molecular weights assigned to each class. Seven proteins are resolved in virions obtained from a single-cycle of rate-zonal density gradient centrifugation whereas only 4 are detected after subsequent isopycnic centrifugation. The loss of structural components may be related to the significant change observed in the ratio of plaque forming unit:total particle count following isopycnic centrifugation. Attempts to determine the location and/or possible function of the observed components in the virion have for the most part been unsuccessful. We have reached the point where subsequent treatments of purified virions have almost reduced the levels of incorporated isotope to such low levels as to be neither reproducible nor interpretable.

Publication: Abstracts, Am. Soc. Microbiol. Meeting, p. 217, 1975.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Preventation and Treatment of Biological Agent Casualties

Work Unit No. 834 02 418: Molecular Structure of Group C, Bunyamwera Supergroup and Ungrouped Arboviruses

Background:

As initially proposed this work unit was to characterize as to number, molecular weights (MW), and location within virions the structural proteins of arboviruses, specifically group C, Bunyamwera supergroup and ungrouped viruses within the arbovirus classification. Oriboca virus was fully characterized as to these parameters.[1] Additional data have been obtained on the characterization of Pacui virus and is presented. Pacui, previously classified as an ungrouped arbovirus has now been included as a member of the Phlebotomus Fever group of arboviruses, all members of which are Bunyavirus-like in morphology. The investigations concerning the structural composition of Tacaribe virus, an arenavirus, have been less than satisfying in that although the basic number and MW of proteins contained within the virus have been tentatively characterized, attempts to determine the location of the components within the virion have been unsuccessful. The basic difficulty is that sufficiently stable, highly labeled, high titered virus preparations, which will retain sufficient radiolabel throughout the numerous manipulations to which they must be subjected, have been impossible to obtain. Such difficulties with arenaviruses have probably accounted for the limited number of published reports[2,3] on their structural protein composition. The data from studies relating to the identification of the structural components of Tacaribe virus are summarized.

Progress:

I. A limited number of additional Pacui virus preparations and polyacrylamide gel electrophoresis analyses have been examined. The additional data as to the percent distribution of the polypeptides in control and NP-40 detergent-degraded virions have not changed greatly and add support to previously reported results. The distributions of polypeptides doubly labeled are given in Table I.

TABLE I. PERCENT DISTRIBUTION OF PACUI VIRUS POLYPEPTIDES

	% TOTAL DPM IN PEAK						
	^{3}H-uridine			^{14}C-amino acids			
Top[a/]	V-3	V-2	V-1	Top	V-3	V-2	V-1
60	13	< 5	< 5	<10	30	15	18
93	< 5	0	0	<10	23	20	21
53	<10	< 5	< 5	12	30	12	17
47	<10	< 5	< 5	15	33	14	20
43	18	< 5	<10	<10	38	14	18

a. Activity that did not enter the gel more than 1-2 slices

Approximately 60% of the ^{3}H-uridine remains on top of the gel column; the ^{14}C-amino acid label is distributed approximately 30, 15, 20% between V-3, V-2, and V-1 respectively. The uridine found in the areas under the polypeptede peaks is normally less than 5-10% and results from background and spillover from the carbon to tritium channel when carbon counts are disproportionally high. Similarly, the carbon label found at the top of the gel in the region of maximum tritium label, and the amount of low MW material with either label that runs with the dye front is also less than 10% of the total radioactivity present.

An additional Pacui virus preparation has been degraded and analyzed; the data concerning the distributions of uridine and amino acid labeled components for control and detergent released top and peak components support those data previously reported. The actual assignment of MW to each of the components and molar ratios between polypeptides should constitute the last studies with this virus. Investigations on the structural polypeptides of Pacui virus have been and are a very minor portion of this work, in that virions are prepared and analyzed when materials, resources and time permit.

II. Evaluation of additional data obtained on 8% SDS-phosphate polyacrylamide gels (system referred to as PAGE) using Tacaribe virions, obtained by a single-cycle of rate-zonal density gradient centrifugation, indicates that virions labeled with ^{3}H-amino acids are found in 5 regions of the gels whereas virions labeled with ^{3}H-glucosamine and treated identically are resolved into 4 regions. Coelectrophoresis of the labeled Tacaribe virion

preparations with standard virion markers of Sindbis, Oriboca and/or Japanese encephalitis virions allowed tentative MW ranges to be assigned to all components. The amino-acid labeled components have MW ranges of >100,000, 88-99,000, 61-65,000, 30-35,000 and 10-15,000, whereas the glucosamine-labeled components have ranges of >100,000, 82-88,000, 53-56,000 and 44-46,000. These differ somewhat from those previously reported but are based on the incorporation of additional data to the estimates. The proteins and glycoproteins of MW 61-65,000, 53-56,000, 44-46,000 and 30-35,000 were not coincident when resolved by PAGE and must therefore be considered as separate entities at this time. Coincidence did occur between both the amino-acid and glucosamine-labeled components of >100,000 and often between the 88-99,000 amino acid labeled and 82-88,000 glucosamine labeled components. On the basis of these data it is suggested that Tacaribe virus prepared by a single cycle of rate-zonal sucrose density gradient centrifugation may contain up to 7 classes of protein. Of the 7 possible MW classes 4 (>100,000, 82-99,000, 53-56,000 and 44-46,000) are glycoproteins and the remaining 3 (61-65,000, 30-35,000 and 10-15,000) are not glycosylated. It should be noted that all components with MW >100,000 may be composed of multiple units that do not enter the gel column. When ^{3}H-uridine was incorporated into the RNA of the virions, subsequent PAGE analysis indicated that with a single exception essentially all of the radioactivity remained on top of the gels as would be expected for the large molecular weight RNA moieties. The exception was the continued presence of a minor uridine-labeled moiety of 85-95,000. Its weight is too high to be the 4S (29,000) and too low to be the 18S (700,000) RNA moieties reported to be present in arenaviruses.[4,5] The uridine-labeled components were not studied further.

When preparations of the amino acid labeled virions used in the previous experiments were subjected to isopycnic centrifugation in sucrose for 24 hr the virions appeared to lose structural components. Electropherograms showed the presence of only 3 major components of estimated MW of >100,000, 56-66,000, and 30-40,000. Preparations have lost the 88-99,000 and 10-15,000 moieties. Changes in the estimated MW determinations also occurred when glucosamine-labeled virions were treated identically, in that only 3 moieties of >100,000, 70-75,000 and 50-55,000 were resolved, the 44-46,000 moiety being missing.

We believe that this was an actual loss of structural components rather than an indication of further purification since the ratio of PFU: total virions of Tacaribe virions changed from ~1:12 - ~1:750 when examined after one cycle of rate-zonal and a single cycle of isopycnic centrifugation respectively. When examined under similar conditions Pichinde virions had ratios of 1:17 and 1:250 indicating the greater stability of Pichinde virus. The lack of stability of Tacaribe virus has been a major problem throughout these studies and hence the loss of structural components and infectivity through a single cycle of isopycnic centrifugation was not unexpected.

The isopycnic data indicated that labeled Tacaribe virions had a density in sucrose of 1.16 - 1.18 gm/cm^{3}. Degradation of ^{3}H-amino acid labeled virions, purified by rate-zonal centrifugation, with the nonionic detergent NP-40 followed by isopycnic banding gave the presence of 2 components of different densities. One component slightly less dense than the intact virion

appeared to retain all of the proteins associated with the amino-acid labeled isopycnically prepared virion (>100,000, 56-66,000, 30-40,000) while another component with a density $\geq$1.24 gm/cm^3 appeared to lack the 30-40,000 MW moiety. The density of 1.24 is maximal in our sucrose system; therefore, this component has, in fact, been pelleted. ^{3}H-glucosamine-labeled virions treated in a similar manner gave comparable results as to presence of less dense and more dense components. There was however, a new component at the top of the gradient that contained the bulk of the radioactivity. The less dense component was found to be incompletely degraded virus that could be removed by further detergent treatment. The dense component contained MW moieties of >100,000, 67-70,000 and 53-59,000. We have been unable to characterize the glucosamine-labeled component at the top of the gradient but the electropherograms indicate that the 70-75,000 MW moiety predominates. A possible correlation may exist between the association of glucosamine with a component of increased density in our system and that of a glucosamine association with nucleic acid reported to occur in Pichinde virus.[2]

A decision has been reached to terminate this work unit. Our decision is based not so much on the change of direction from arboviruses as originally proposed to arenaviruses as addended, but on the realization of the current state of the art concerning arenaviruses. The data being obtained has begun to suffer from the lack of sufficiently highly-labeled, purified stable virions that are the essential starting material for studies of this nature. Finite determination of the morphogenesis of structural proteins and their location and function within the intact virion must await the methods necessary for the production of virions in quantity which in turn will allow the incorporation of the greater levels of the required isotopes necessary for studies of this nature. We have reached the point where subsequent treatments of purified virions have almost reduced the levels to such values as to be neither reproducible nor interpretable. Any further studies of this nature will be conducted in cooperation with other on-going work units within the Institute.

III. The preliminary results obtained from the feasibility study conducted in cooperation with Work Unit 02 411 to develop a suitable mouse model as an indicator of the efficacy of antiviral chemotherapeutic agents against arenaviruses are most encouraging. An indirect mouse model in which 21-23 day-old mice were given a single SC dose of Tacaribe virus 13 days prior to challenge by the intracerebral route appeared suitable based on the preliminary results of the initial study. The single SC dose completely protected all mice from subsequent chllenge while all unprotected controls died. The effect of various antiviral agents against this apparent single protective SC dose are to be evaluated under the primary work unit.

Presentations:

1. Rosato, R. R. Structural components of Oriboca virus. Invited participant, National Institute of Allergy and Infectious Diseases sponsored Bunyavirus Workshop, Washington, D. C., Dec 2-3, 1974.

2. Rosato, R. R., M. D. Douglas, and G. A. Eddy. Structural characterization of Tacaribe virus. Presented, Annual Meeting American Society for Microbiol, New York, N. Y., 27 Apr - 2 May 1975 (Abstracts, p. 217).

LITERATURE CITED

1. Rosato, R. R., M. L. Robbins, and G. A. Eddy. 1974. Structural components of Oriboca virus. J. Virol. 13:780-787.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6416	75 07 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
75 04 16	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	62760A	3A762760A834	02	419
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)
(U) Effect of complexed antigen on the immune responses of nonhuman primates to vaccine

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 04	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 75	2.0	257.0
	CURRENT 76	3.0	282.0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Animal Assessment Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Houston, W. E.
TELEPHONE: 301 663-7244
SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME: Harrington, D. G.
NAME: Heard, C. D. POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) DEAE-dextran; (U) Complex; (U) Immunology; (U) Antigen; (U) Monkeys; (U) Mice; (U) Military medicine; (U) Encephalitis, equine (VEE); (U) Antibody

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

23 (U) Study immune response of rhesus monkeys to formalinized vaccine complexed with hyperimmune serum in order to improve weakly antigenic vaccines. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) A complex at equivalence is used to immunize rhesus monkeys and Swiss mice. Antibody production and protection against challenge are determined. Effect of adjuvants in eliciting primary humoral antibody responses are determined.

25 (U) 74 07 - 75 06 - Correlation was shown between the humoral antibody response in monkeys and protection in mice. Compared to antigen controls, significantly higher titers were seen in monkeys immunized with antigen-IgG complexes at equivalence. Titers persisted for 1-2 mon longer than those from antigen controls. Greater production was demonstrated in mice with complexes at equivalence compared to vaccine alone. DEAE-dextran was shown to be a potent adjuvant for VEE virus vaccine in rhesus monkeys. Titers persisted at high levels more than 4 mon. Adjuvant effect of DEAE-dextran was not observed in mice, guinea pigs or hamsters. A biphasic (IgM-IgG) antibody pattern was shown in rhesus monkeys immunized with killed VEE vaccine (KVEE). KVEE-IgG, complexed at equivalance, stimulates early, persistent IgG production. Methodology has been adapted for quantitation of JE virus and its neutralizing antibody. Labeled JE virus and concentrated, specific IgG are now ready for equivalence determinations. Animal studies are anticipated in the near future. Studies are in progress to investigate cellular mechanisms involved in complex enhancement.

Publications: Abstracts, Am. Soc. Microbiol., p. 80, 1975.
Infect. Immunity 10:437-442, 1974.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 419: Effect of Complexed Antigen on the Immune Response of Nonhuman Primates to Vaccine

Background:

Because of the inability to produce certain viral agents in quantity, effective vaccines cannot be prepared. Intrinsically, some viruses produce better immunity in the host than do others. Inactivation of live vaccines usually reduces toxicity to the host, but also reduces efficacy. Combining soluble antigens with specific antibody at equivalence and antigen excess produces enhanced antibody responses in mice.[1,2] The exact mechanism of enhancement is unknown; however, it appears that stimulated antigen-processing is a factor. Similar enhancement of the primary antibody response in rhesus monkeys and protection in mice has been reported previously[3] if killed VEE vaccine (KVEE) was complexed with specific immune γ-globulin (IgG) at equivalence.

To investigate further the rhesus monkey model for enhancing primary immune responses to marginal vaccines, formalin-inactivated JE virus was chosen for the following reasons: (1) It has weakly immunogenic properties in man; (2) JE virus is immunologically distinct from VEE; (3) studies at USAMRIID indicate that following immunization with formalin-inactivated JE virus, rhesus monkeys developed very low levels of JE virus-neutralizing antibody, and received partial protection when challenged with virulent JE virus (MAJ Wear, CPT Scott, personal communication); and (4) a pool of rhesus monkey JE virus immune sera for IgG fractionization and equivalence determination was available.

Progress:

I. We reported last year that booster doses given 3 days following immunization with KVEE-IgG complexes at equivalence stimulated an anamnestic response similar to boosters given 7 days following immunization with KVEE vaccine alone. Booster doses 3 days after immunization with KVEE failed to elicit a persistent secondary response. The ability to initiate a secondary-type response early in the immune reaction with 1 immunization would be a highly beneficial tool in the field of vaccine utilization.

Initial antigen processing results primarily in the synthesis of 19S γ-globulin (IgM)[4] followed by 7S (IgG). The mechanism for this change in class of antibody is not understood, but it has been suggested that the initial IgM antibody may be the response obtained to antigen alone, while the IgG antibody may be the response to antigen-antibody complexes.[5] Considering that <u>in vivo</u>

complexing of antigen and actively produced antibody occurs, equivalent proportions of these reactants might contribute to the conversion of IgM to IgG. Using this as a working hypothesis, antisera obtained from temporal bleedings of rhesus monkeys immunized with the KVEE-IgG complex and KVEE alone were separated into IgG and IgM components by ultracentrifugation (195,000 x g for 16 hr) on a 10-40% sucrose gradient. By reacting 0.5-ml fractions from the gradient with radial immunodiffusion agar plates containing anti-IgG or anti-IgM, one can determine which fractions contain total antibody class of each. These fractions are then pooled and used in VEE PRNT for antibody determination. Table I shows that the initial antibody type is predominantly IgM.

TABLE I. ANTIBODY SPECIES ELICITED IN RHESUS MONKEYS BY KVEE VACCINE.

DAY AFTER IMMUNIZATION	GEOM. MEAN NEUTRALIZING ANTIBODY TITERS		
	Whole Serum	IgG	IgM
1	0	-	-
5	0	-	-
6	4.7	2	4.0
7	6.7	2	4.0
10	53.8	2	21.3
11	53.8	16.6	10.7
14	38.1	8.7	6.0
17	26.9	9.3	4.0
18	19.0	10.7	4.7
23	9.5	8.0	2.0
25	8.0	6.0	2.0
28	8.0	8.0	0

Conversion from IgM to IgG takes place between 10 and 14 days postimmunization. In contrast, those monkeys immunized with equivalent complexes (Table II) exhibited a different pattern. IgG was the predominant species formed initially and throughout the response period. The titers were also significantly enhanced when compared to antigen controls. The suggestion is that these complexes are either priming the animals so that IgG can be formed early, or that immunization with complexes elicits antibody by a different route than antigen immunization.

TABLE II. ANTIBODY SPECIES ELICITED IN RHESUS MONKEYS BY KVEE-IgG EQUIVALENT COMPLEXES

DAYS AFTER IMMUNIZATION COMPLEX	GEOM. MEAN NEUTRALIZING ANTIBODY TITERS		
	Whole Serum	IgG	IgM
1	0	-	-
4	0	-	-
5	9.5	6.4	2.5
6	11.3	8.0	2.5
7	16.0	10.1	2.5
10	27.0	16.0	2.5
11	90.5	40.3	3.2
14	512.0	128.0	4.0
17	512.0	128.0	4.0
18	512.0	80.6	3.2
23	76.1	40.3	4.0
28	64.0	32.0	3.2
39	76.1	25.3	3.2
72	76.0	32.0	2.5

The 1st phase of a cooperative study within the division concerning clearance of TC-83 VEE, KVEE, and KVEE-IgG by the RES has been completed in rhesus monkeys (Work Unit 834 01 010).

Temporal protection studies are now being concluded using KVEE vaccine and KVEE-IgG complexes at equivalence. Two separate groups of mice were immunized (0.3 ml, SC), 1 with KVEE and 1 with KVEE complexes, along with appropriate IgG and challenge controls. Sufficient mice were incorporated into each group to challenge 40 mice/day for 14 days with the Trinidad strain. A separate, smaller group (XRT) received 500 R total-body x-irradiation 24 hr prior to immunization with complexes at equivalence to determine whether a general immunosuppressant would effect protection. Table III shows that 79-95% protection was afforded mice by KVEE complex, whereas 7-8 days were required to attain an equivalent level of protection if KVEE vaccine alone was the immunogen

TABLE III. TEMPORAL PROTECTION AFFORDED MICE BY IMMUNIZATION WITH KVEE AND KVEE COMPLEXES

CHALLENGED DAY POSTIMMUNIZATION	% PROTECTION[a]			
	KVEE	KVEE Complex	IgG Controls	XRT + Complex
1	12	88	90	0
2	39	86	98	13
3	57	88	93	13
4	50	95	100	25
5	59	88	90	13
6	57	88	79	0
7	69	86	81	0
8	78	85	66	25
9	79	86	43	38
10	71	80	50	13
11	76	73	36	0
12	74	79	42	38
13	86	81	14	0
14	80	85		

[a]Challenge controls (n=6/time) died at all times.

Considerable protection was found in the IgG control group, indicating that protection with complex is probably of a passive nature in the early response period. Protection with immune complex appears to be of a specific nature (as opposed to interferon or other nonspecific substances) since heterologous challenge with Semliki Forest virus, on a daily basis, resulted in 90-100% lethality throughout the challenge period data (not shown). Immune suppression with x-irradiation prior to immunization with complexes resulted in somewhat variable but drastically reduced protection, indicating that the protective nature of the complex was not entirely of a passive nature.

Finally, the adjuvant effect of DEAE-dextran, an anion exchange resin on the primary immune response of rhesus monkeys to KVEE, was reported last year. For comparison purposes, neutralizing antibody titers shown in the last report, showing a stimulatory effect on antibody production by both concentrations of DEAE-dextran, are included in Table IV.

TABLE IV. NEUTRALIZING ANTIBODY RESPONSES IN RHESUS MONKEYS (n=4) IMMUNIZED WITH KVEE[a] AND KVEE[a] COMPLEXED WITH DEAE-DEXTRAN

DAYS AFTER IMMUNIZATION	GEOM. MEAN NEUTRALIZING ANTIBODY TITER		
	KVEE alone	KVEE + 1 mg/kg DEAE-dextran	KVEE + 5 mg/kg DEAE-dextran
0, 3	0	0	0
5	0	16.0	22.6
7	6.7	32.0	64.0
10	53.8	256.0	430.5
11	53.8	215.3	430.5
14	38.1	256.0	362.0
18	19.0	256.0	362.0
23	9.5	152.0	181.0
28	8.0	76.1	215.3
39	6.7	64.0	181.0
50	5.6	38.0	90.5
72	0	53.8	304.0
120	0	76.1	256.0

[a]Diluted 1:2.

Evaluation of this compound in rodent species is now completed. Enhancement of the antibody response by DEAE-dextran, seen in monkeys, was not found in mice, guinea pigs and hamsters. While KVEE vaccine affected the 3 rodent species in different manners (Table V), no significant differences within species could be detected if DEAE-dextran was used as an adjuvant. In addition, correlation of antibody response and protection among rodents is poor, as guinea pigs responded with respectable antibody to the vaccine preparations and were only partially protected, whereas mice responded with much lower

TABLE V. ANTIBODY RESPONSES AND PROTECTION STUDIES IN GUINEA PIGS, MICE AND HAMSTERS IMMUNIZED WITH KVEE AND KVEE COMPLEXED WITH DEAE-DEXTRAN

DAYS AFTER IMMUNIZATION	GEOM. MEAN NEUTRALIZING ANTIBODY TITER		
	KVEE	KVEE + 1 mg/kg DEAE-dextran	KVEE + 5 mg/kg DEAE-dextran
Guinea pigs (n=10)			
5	22.6	13.9	36.7
7	45.2	43.6	91.2
10	50.7	32.0	103.8
14	96.8	58.9	146.9
21	138.0	74.1	137.1
35	297.9	214.8	297.2
(Protection Study)			
No. surviving/Total	2/8	1/5	2/6
%	25	20	33
Mice (n=40)			
5	27.5	20.0	20.0
7	9.0	22.6	16.0
10	19.0	45.2	32.0
14	22.6	25.4	32.0
21	38.0	32.0	45.2
35	26.8	19.0	32.0
(Protection Study)			
No. surviving/Total	25/26	18/21	11/11
%	96	88	100
Hamsters (n=12)			
5	204.2	102.3	251.2
7	128.0	128.0	204.2
10	80.4	80.4	100.0
14	128.0	256.0	256.0
21	161.1	322.1	322.1
35	4.0	512.0	512.0
(Protection Study)			
No. surviving/Total	14/15	18/18	18/18
%	93	100	100

antibody titers but exhibited excellent protection. Titers are geometrical mean values derived from individual animal bleedings. All animals were challenged with 10^3 $MICLD_{50}$ Trinidad VEE on day 35 postimmunization.

II. In preliminary experiments we have developed a reliable tissue culture plaque assay procedure for quantitating JE virus, and for titrating its neutralizing antibody, employing a plaque-reduction, serum-dilution, virus-neutralization test. Furthermore, a sufficient quantity of JE virus has been propagated in tissue culture and characterized to serve as the inactivated immunizing antigen and virulent challenge virus for the forthcoming in vivo studies.

To determine in vitro antigen-antibody equivalence by the radioisotope precipitation (RIP) technique,[6] concentrated, purified antigen-antibody components have been prepared and characterized. JE virus was endogenously radiolabeled with ^{3}H-amino acids, concentrated by differential centrifugation and sucrose density gradient separation, titrated, and the radiolabel count determined prior to formalin inactivation. The sera from monkeys previously immunized with JE virus were pooled, and 2 lots of specific IgG were separated from pools of JE immune monkey sera by Sephadex treatment, concentrated, and characterized for protein content and JE-neutralizing antibody levels. All preliminary experiments have been, or are being, finalized with the radiolabeled JE virus and the concentrated IgG prior to determining in vitro virus-antibody equivalences. Thereafter, in vivo immune response studies in monkeys and mice will be initiated.

Presentation:

1. Houston, W. E., R. J. Kremer, C. L. Crabbs, and R. O. Spertzel. Alterations in the primary immune response in rhesus monkeys immunized with antigen-antibody complexes. Presented, Annual Meeting, American Society for Microbiology, New York, N. Y., 27 Apr-2 May 75. (Abstracts, p. 80).

Publication:

1. Houston, W. E., C. E. Pedersen, Jr., F. E. Cole, Jr., and R. O. Spertzel. 1974. Effects of antigen-antibody complexes on the primary immune response in rhesus monkeys. Infect. Immunity 10:437-442.

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6. Gerloff, R. K., B. H. Hoyer, and L. C. McLaren. 1962. Precipitation of radiolabeled poliovirus with specific antibody and antiglobulin. J. Immunol. 89:559-570.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OE6417	75 07 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
74 07 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	62760A	3A762760A834	02	420
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Procede with Security Classification Code)*

(U) Evaluation of sequential immunization against selected attenuated group B arboviruses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	75 09	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 75	2.5	237.0
	CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME:* Animal Assessment Division USAMRIID
ADDRESS:* Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME:* Wear, D. J.
TELEPHONE: 301 663-7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Scott, S. K.
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Vaccines; (U) Arboviruses; (U) Yellow fever; (U) Langat virus; (U) Volunteers; (U) Military medicine; (U) Dengue-1

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

23 (U) Confirm and test further in rhesus monkeys the immunization procedure for group B arboviruses developed and tested in spider monkeys under contract with Johns Hopkins University. If satisfactory, test the sequence in volunteers. This procedure, when perfected, will provide a means for protecting military personnel against these serious viral diseases. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Repeat procedures developed in a new monkey species. Following immunization, rhesus monkeys will be challenged with other group B arboviruses.

25 (U) 74 07 - 75 06 - A dose of 6 logs of live Japanese encephalitis (JE) virus was given intracerebrally (IC) to 30 rhesus monkeys in order to test the group B sequential immunization procedure as a priming mechanism; it proved to be a rigorous challenge. When data on the clinical responses of the different monkey groups were enumerated and analyzed statistically, there was a significant difference in responses between only sequentially immunized and live JE (subcutaneous) as compared with nonimmune controls.

Nine sequential group and 5 control monkeys were given 6 logs of West Nile virus IC. Eighty-nine percent of the sequentially immunized monkeys exhibited clinical signs of encephalitis as opposed to 100 percent of the controls, however the severity of tremors was much less in the experimental group; thus, some protection was afforded.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Aspects of Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 420: Evaluation of Sequential Immunization Against Selected Attenuated Group B Arboviruses

Background:

Price et al.[1] have shown that vaccinating spider monkeys sequentially with live, attenuated yellow fever, Langat and dengue-2 vaccines, induces broad immunity to SC challenge with high doses (5-6 $\log_{10}$) of a variety of group B arboviruses including JE, SLE, (West Nile) and dengue-1, -3 and -4. The rationale behind this concept stems from the work of Casals and Brown[2] which demonstrated the cross-reactivity of HI antigens in the group B arboviruses as well as selected cases of cross-protection within subgroup complexes[3] such as dengue types 1-4.

Although recently Price (Contract DA-49-193-2398) found some exceptions to cross-protection,[4] Casals (personal communication) found that a low challenge dose (2 $\log_{10}$) of dengue-4 in sequentially immunized spider monkeys provided resistance to challenge, whereas, similarly immunized monkeys receiving a high challenge dose (6 $\log_{10}$) showed much less resistance.

Dengue-1 viremia data following challenge with 4 $\log_{10}$ virus[4] tended to support Price's findings in that 6 control monkeys showed viremia, while only 2 of 6 rhesus monkeys immunized with the sequential vaccination scheme were viremic.

Our study is designed to confirm or modify, in rhesus (Macaca mulatta) monkeys, Winston Price's sequential immunization scheme.

Progress:

I. Rhesus monkeys were obtained from Prime Labs, Inc. Each monkey's serum was screened for HI antibodies against selected group B arboviruses. HI-antibody free serum was further screened for neutralizing antibody to selected group B arboviruses.

Thirty monkeys were divided into 5 groups of 6 each: group 1, immunized with yellow fever, Langat and dengue 2 viruses; 2, immunized as in group 1 + 2 SC inoculations of killed JE virus; 3, receiving only the 2 killed JE virus vaccine inoculations; 4, receiving one SC inoculation of virulent live JE virus (by the SC route, JE virus produced a subclinical infection in these monkeys), and 5, control monkeys given appropriate culture media in parellel with group 1. Each of these 5 groups of monkeys were subsequently challenged intracerebrally (IC) with 100 rhesus monkey LD_{100} (5 $\log_{10}$ PFU) of JE virus.

All monkeys of group 5 died following onset of weakness and paralysis and developed no detectable antibodies. Immunization with live JE virus (group 4) protected all but 1 of 6 monkeys from death or serious illness. This immunization 35 days before IC challenge resulted in serum antibody levels capable of serum neutralizing 80% (SN_{80}) of the JE virus when 1:40 - 1:80 dilution of the serum was used. The one monkey which died had no detectable antibody level on day of challenge through death.

Sequential immunization + 2 vaccinations with killed JE vaccine (group 2) protected 5 of the 6 monkeys from death. Antibody response by the day of challenge was 1:10 - 1:20 SN_{80} in all 6 monkeys. Four of the 5 surviving monkeys became paralyzed from the neck downward and required help to eat and drink.

Killed JE immunization only (group 3) and sequential immunization (group 1) protected 4 of 6 of the monkeys from death in each group. Most of these surviving monkeys became paralyzed caudal to the cervical area. Only 3 monkeys of group 3 had detectable (1:10 SN_{80}) antibody on the day of IC challenge. These levels did not rise by postchallenge day 7, yet all 3 monkeys survived.

Increasing signs of morbidity within each group were assigned values of 1 through 6 (minimum neurological signs to death). Friedman's rank sums test of days 11-21 postchallenge demonstrated significant differences between monkeys of group 5 (control) and group 2 or group 4.

The above assigned numbers were also listed in Table I with the percent of observation time each monkey group demonstrated those signs of disease. The immunized (other than live JE) monkeys were free of clinical disease signs, 25% compared to 11%, of the observation time for the nonimmune controls. The nonimmunized monkeys were severely ill (4-6 clinical category) more of the observed time than the immunized monkeys, with the group 2 monkeys severely ill the least time of the first 3 groups.

Although protection (low mortality, somewhat lower morbidity than controls) was afforded to JE-challenged monkeys by previous priming with heterologous group B viruses and immunization with killed homologous virus (groups 2), this immunization was not as effective as immunization with live homologous virus alone.

II. Fourteen rhesus monkeys were divided into 2 groups: 1, nine immunized with yellow fever, Langat and dengue-2 viruses and 2, five control monkeys. Each of these 2 groups of monkeys were challenged IC with 6 $\log_{10}$ WN virus, with group 1 18 mon after immunization. All monkeys of group 2 had elevated temperatures ($\geq$ 104.8 F), depression, tremors, paresis, 4 had incoordination and 2 had convulsions. Five of 9 monkeys in group 1 had elevated temperatures, 8 had tremors, 4 had paresis, and 2 had incoordination, but of less severity than those in group 2.

TABLE I. SEVERITY OF CLINICAL SIGNS IN RHESUS MONKEYS CHALLENGED IC WITH JE VIRUS 7 MON AFTER SEQUENTIAL IMMUNIZATION COMPARED TO KILLED JE VIRUS BOOSTERED, KILLED AND LIVE JE VIRUS IMMUNIZATION

IMMUNIZATION GROUPS	% OBSERVATION DAYS[a] BY CLINICAL CATEGORY[b]						
	0	1	2	3	4	5	6
Sequential immunization (SI) only	23	1	4	1	42	16	13
SI + killed JE	26	0	9	2	45	13	5
Killed JE only	26	1	1	0	49	10	13
Live JE only	53	2	18	3	11	0	13
Nonimmunized	11	0	4	0	16	13	56

a. Number of days that 6 monkeys were classified at above clinical evaluation divided by 96 possible (16 days x 6 monkeys) x 100.

b. Clinical categories:

0 - No clinical signs of disease
1 - No neurological signs, but ill
2 - Intention tremors
3 - Paresis or paralysis in 1 limb only
4 - Paresis or paralysis in more than 1 limb but eats and drinks
5 - Moribund
6 - Dead

TABLE II. SEVERITY OF CLINICAL SIGNS IN RHESUS MONKEYS CHALLENGED IC 18 MON AFTER SEQUENTIAL IMMUNIZATION

IMMUNIZATION GROUPS	% OBSERVATION DAYS[a] BY CLINICAL CATEGORY[b]						
	0	1	2	3	4	5	6
Sequential immunization (SI)	30	11	5	22	25	7	0
Nonimmune	19	4	3	25	11	32	6

a. Number of days that 9 monkeys in SI group and 5 monkeys in control group were classified at above clinical evaluation divided respectively by 162 possible (18 days x 9 monkeys) or 90 possible (18 days x 5 monkeys) x 100.

b. Clinical categories:

0 - No clinical signs of disease.
1 - Fever (104.0-104.7 F) or barely detectable depression; slight change in behavior; mildly reduced activity.
2 - Fever ($\geq$ 104.8 F) or obvious depression; reduced response to stimuli; slow reaction; barely detectable paresis.
3 - Slight neurologic signs: paresis, mild impaired use of limbs, head tilt and/or barely detectable intention tremors.
4 - Moderate neurologic signs: paresis, unable to support weight on 1 or 2 limbs with impaired mobility and/or obvious intention tremors in more than 1 limb.
5 - Severe neurologic signs: ataxia and/or gross intention tremors which involve the whole body.
6 - Convulsions.

The severity of clinical signs exhibited by the 14 monkeys were assigned values 1 through 6 (barely detectable to convulsions). The results are shown in Table II with the percent of observation time each monkey group demonstrated those signs of disease. The sequentially immunized group was not only free of signs a greater percent of the observation time but demonstrated severe (5-6 clinical category) signs for a shorter period of time.

All 14 monkeys exhibited viremia and had a rise in serum neutralization antibody titers to West Nile virus.

III. Nonimmunized rhesus monkeys were challenged SC and/or IV with JE, Murray Valley encephalitis (MVE), SLE and WN viruses. Neither consistent temperature elevations nor neural signs of CNS disease were noted. Zymosan, a sterile yeast extract, was found to potentiate IV- and some SC-virus challenged monkeys as shown by temperature elevations and CNS signs. However Zymosan alone was found to produce histologic CNS lesions indistinguishable from those caused by viral encephalitis and in some cases late overt neural symptoms. For example: 12 rhesus monkeys were given 0.25 ml Zymosan IC and then divided into 4 groups and given SC 0.5 ml WN virus of (1) 7 $\log_{10}$ PFU, (2) 5 $\log_{10}$, (3) 3 $\log_{10}$ and (4) no virus. Gross intention tremors were noted in one monkey each from groups 1 and 2 and all 3 monkeys of group 3. Although the group 4 monkeys were without tremors 2 monkeys were killed on day 30 and histopathologic lesions of lymphocytic meningitis were found in the brain and spinal cord meninges.

West Nile virus was passed 6 times in LLC-MK_2 cell cultures and twice in FRhL cells. Instead of becoming more virulent for the rhesus monkey the virus became attenuated and caused no symptoms when injected with Zymosan.

Publications:

None

LITERATURE CITED

1. Price, W. H., J. Casals, I. Thind, and W. O'Leary. 1973. Sequential immunization procedure against group B arboviruses using living attenuated 17D yellow fever virus, living attenuated Langat E5 virus, and living attenuated dengue 2 virus (New Guinea C isolate). Amer. J. Trop. Med. Hyg. 22:509-523.

2. Casals, J., and L. V. Brown. 1954. Hemagglutination with arthropod-borne viruses. J. Exp. Med. 99:429-449.

3. Clarke, D. H., and J. Casals. 1965. Arboviruses; Group B. In Viral and Rickettsial Infections of Man, 4th ed. (ed. by F. L. Horsfall and I. Tamm), J. B. Lippincott, Philadelphia, p. 606-658.

4. Price, W. H. May 1, 1974. Final Report. Contract DA-49-193-MD-2398. The Johns Hopkins University School of Hygiene and Public Health, Baltimore, Md.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6418	75 07 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
74 07 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	62760A	3A762760A834	02	421
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code): (U) Chemical structures and mechanisms involved in virulence of Venezuelan equine encephalomyelitis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	75 09	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 75	1.0	114.0
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME:* USA Medical Research Institute of Infectious Diseases
ADDRESS:* Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME:* Virology Division USAMRIID
ADDRESS:* Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME:* Marker, S. C.
TELEPHONE: 301 663-7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Jahrling, P. B.
NAME: Pedersen, Jr., C. E. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Encephalitis, equine (VEE); (U) Vaccines; (U) Arboviruses; (U) Therapy

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

23 (U) Study purified VEE virus for characterization of protein and carbohydrate contents in terms of biological function, i.e., virulence, immunogenicity and other surface phenomena. This characterization will provide a sound basis for development of improved vaccines and modalities of treatment for VEE and related viral diseases in military personnel. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Purify further a 90% pure VEE virus, characterize its protein and carbohydrate components by tryptic mapping, gas chromatography and amino acid analyses. Apply the techniques developed to other arboviruses.

25 (U) 74 07 - 75 06 - A unique nonimmunological procedure for the differentiation of group A arboviruses was developed. Studies on the adsorption mechanism showed a dependence of the adsorption process on pH and salt concentration. This dependence was a reproducible and reliable phenomenon that readily detected differences between virus strains. The nucleocapside protein of Western equine encephalitis virus was found to have repetitive sequences, or be a protein dimer of 18,000 molecular weight.

Due to the departure of the investigator from the Institute, no further work will be done. This work unit is terminated.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 421: Chemical Structures and Mechanisms Involved in Virulence of Venezuelan Equine Encephalomyelitis

Background:

Our objectives are to determine the chemical structures on group A arboviruses responsible for binding neutralizing antibody and for the initial adsorbtion of virus to cell receptors. Efforts have been concentrated on the absorbtion structures. Since adsorbtion represents the initial attachment of virus to cell, it seemed necessary to first determine if the adsorbtion process, as studied, would lead to productive infection, or merely to the ineffectual binding of virus to cell. Another major problem was to find some aspect of adsorbtion that could be determined reliably and quantitatively in order to allow us to distinguish between viruses. Preliminary experiments showed the time course of adsorbtion to be a very unreliable measure. Further investigation, showed the dependence of adsorbtion on salt concentration and pH to be the reliable parameters needed.

Structural studies on the viral proteins were continued.

Progress:

I. Studies of Viral Attachment Performed at 37 C.

Some of the preliminary studies of the viral attachment mechanism resulted in a nonimmunological procedure that readily differentiated among group A arboviruses. The procedure does not require specific immune sera or specialized equipment for biochemical studies of the virions. The method consisted of plaquing a fixed number of viruses on chick embryo fibroblast monolayers in an array of defined phosphate buffered solutions. After allowing 1 hr for virus attachment at 37 C the inoculum is removed, and the monolayers overlayed with agar. The number of plaques formed depends on a complex interaction among these many variables: cellular and viral sensitivity to the buffered solutions, the effect of the buffer components and pH on the adsorbtion sites of both cell and virus, and the effect of the buffered solutions on other events of early infection, such as penetration and uncoating.

Although we made no attempt to sort out the relative importance of the various contributing factors in these early experiments, it was apparent that the method was of value in differentiating between closely related viruses.

The method potentially could provide an objective marker for vaccine strain viruses and other mutant viruses. Depending on the mechanism involved in producing the plaque count seen with each buffered solution, the procedure might provide a method for mutant selection.

The results obtained by using this method on 3 representative group A arboviruses, are shown in Table I. Although difficult to visualize in tabular form, the plaque count for each virus has a distinct pattern that depends on the type of buffered solution used to apply the inoculum to the monolayers.

II. Studies of Viral Adsorbtion Performed at 0 C.

Adsorbtion is the initial step in viral infection, the temperature being independent binding of virus to cell. The process may be of importance in organ tropism and the relative virulence of viruses. By limiting our study to this single rigorously defined event, perhaps the adsobtion process can be understood at the molecular level; perhaps the chemical structure of both host cell and viral adsorbtion sites can eventually be resolved and understood in terms of their role in the biology of infection.

The method used in these studies was to adsorb approximately 200 PFU of virus in 0.2 ml buffered solution (0.15 M NaCl, 0.05 M Na phosphate buffer, pH 5.5-8.0) to chick cell monolayers for 1 hr, remove the inoculum, and overlay the infected monolayers with agar. All studies were performed at 0 C to eliminate the energy requiring process of penetration. The plaque count after 48 hr incubation shows a marked dependence of the adsorbtion process on the Ph of the adsorbtion solutions, (column A, Table II).

The number of plaques formed changes if the inoculum is not removed (columns B and C). Once the inoculum is removed the plaque count does not change when the monolayers are washed (column D) with Hanks balanced salt solution (HBSS). The adsorbtion buffers have no effect on the sensitivity of the chick cell monolayers to infection (column E) or on the viability of the virus in the inoculum (column F).

These studies with plaqued virus are the ground work for rational selection and identification of mutants based on adsorbtion properties. By their very nature, these studies assure that the process under study is important in infection.

Structural Studies of the Group A Arbovirus. During our chemical studies of the group A arbovirus proteins, we looked at the nucleocapsid protein of WEE virus in greater detail than previously. In our analysis of this protein we observed only half the number of peptides in tryptic digests that would be expected on the basis of amino acid analysis and MW of 18,000, one-half the previous estimates.

TABLE I. RELATIVE PLAQUING EFFICIENCY OF 3 GROUP A ARBOVIRUSES WHEN THE FIRST HOUR OF INFECTION WAS PERFORMED IN AN ARRAY OF 18 BUFFERS[a/]

	PLAQUE COUNT/0.2 ML								
pH	WEE 1			EEE Cambridge			VEE Trinidad		
	A	B	C	A	B	C	A	B	C
5.5	150	110	220	10	5	0	160	140	155
6.0	50	20	130	140	50	120	170	200	210
6.5	55	55	80	50	20	120	25	25	200
7.0	50	75	80	45	25	120	20	25	180
7.5	45	40	60	40	25	120	15	20	160
8.0	50	35	50	35	5	115	10	15	150

a. 0.05 M phosphate buffer and A - 0.15 M NaCl
B - 0.15 M NaCl and 0.025% human serum albumin
C - 0.025% human serum albumin

TABLE II. STUDIES ON THE ADSORBTION OF EEE VIRUS, ARTH 167, AT 0 C TO CHICK EMBRYO FIBROBLASTS

pH	PLAQUE COUNT/0.2 ML					
	A	B	C	D	E	F
5.5	6	25	17	22	65	70
6.0	48	100	53	58	82	77
6.5	69	111	78	82	78	68
7.0	21	90	18	20	90	78
7.5	16	87	12	18	85	50
8.0	21	71	12	10	82	68

A. EEE virus inoculum in 0.15 M NaCl. Buffer removed after 1 hr adsorbtion.
B. Inoculum not removed before agar overlay.
C. Inoculum removed.
D. Inoculum removed and the monolayer washed once with HBSS before the agar overlay.

E. Chick embryo fibroblast monolayers incubated with buffered solutions for 1 hr at 0 C, solution was removed, and sensitivity of the monolayers to infection tested by infection with a constant amount of virus in HBSS.

F. Lability of inoculum tested by incubating the virus in buffered solutions for 1 hr at 0 C then, after a 100-fold dilution into HBSS, the ability of these virions to infect a chick cell monolayer was tested.

The nucleocapsid protein was isolated by preparative SDS polyacrylamide gel electrophoresis. Table III presents the integer amino acid composition of purified nucleocapsid protein calculated from 9 amino acid analyses. The integer values shown were calculated assuming 2 cysteine and 2 histidine residues/molecule. The sum of the integer values for lysine and arginine predict 25 tryptic peptides per molecule of 18,000. In our tryptic digests, only 23 peptides could be identified. This number is one-half the number predicted for a protein of 35,000 MW. Barring an unusually large number of trypsin-resistant bonds in the protein or an unusually specific loss of peptides during preparation, finding only 23 tryptic peptides suggests that either the nucleocapsid protein is a 35,000 MW protein with repetitive sequences, or a dimer of an 18,000 MW protein.

TABLE III. AMINO ACID COMPOSITION OF WEE VIRUS NUCLEOCAPSID PROTEIN

AMINO ACIDS	INTEGER VALUES
Lysine	15
Histidine	2
Arginine	9
Aspartic Acid	15
Threonine	8
Serine	7
Glutamic Acid	14
Proline	17
Glycine	13
Half-cystine	2
Valine	14
Methionine	4
Isoleucine	5
Leucine	10
Tyrosine	5
Phenylalanine	5
Tryptophan	5

When SDS polyacrylamide gel electrophoresis was performed using a purified nucleocapsid protein sampler, 2 specific aggregations of the nucleocapsid protein were seen. One aggregation had a MW of 110,000, and probably represents a hexamer of the hypothetical 18,000 MW protein. The other aggregation had a MW of 230,000 and probably represents the dodecamer. If these preferred subunit combinations seen in SDS gels, are indicative of the quarternary structure of the native protein, these specific combinations may occur in the capsomere structure. If each of the 92 capsomerers present in the icosahedron were hexamers of the 18,000 MW protein the calculated nucleocapsid diameter would be 36 nm. This diameter correlates well with the diameter of the nucleocapsid of Sindbis virus as measured by electron microscopy.

Presentation:

Marker, S. C., and M. S. Ascher. Specific lymphocyte transformation to Venezuelan equine encephalitis virus (VEE). Presented, 14th Interscience Conf. Antimicrobial Agents and Chemotherapy, San Francisco, CA. 11-13 Sep 1974 (Program, Abstract 13).

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6419	74 10 24	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
74 07 01	K. COMPLETION	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	3A061101A91C	00	134
b. CONTRIBUTING	62760A	3A762760A834	02/422	
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)
(U) Immunochemical studies of antigenic components of arboviruses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS
003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 07	74 09	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 75	0.3	9
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Rickettsiology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Pedersen, Jr., C. E.
TELEPHONE: 301 663-7465
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Immunology; (U) Vaccines; (U) Arboviruses; (U) Electrophoresis, protein

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

23 (U) Develop methods for optimal enhancement of antigenicity of arbovirus subunit vaccines. This is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Vaccinate selected laboratory animals with fractions of purified virus pools. Challenge them at appropriate intervals for determination of efficacy.

25 (U) 73 08 - 74 09 - We have separated and recovered the 3 virion proteins of TC-83 vaccine strain VEE virus. All proteins were capable of stimulating precipitating antibody but the largest possess antigenic determinants for the production of neutralizing antibody. Comparative electrophoresis of different alphaviruses shows strain to strain and virus to virus differences in molecular weights of the virion envelope components. Examination of VEE complex virions has shown 4 different envelope patterns into which virus strains can be classified. Implication of biochemical analyses coupled with serologic classification are discussed. The primary objective of the study has been met. No further work will be carried out since the investigator has been reassigned.

Publications: Arch. Ges. Virusforsch. 41:28-39, 1973.
Infect. Immunity 8:901-906, 1973.
Virology 60:312-314, 1974.
Arthropod-Borne Virus Exchange 27:97-99, 1974.
J. Virol. 14:740-744, 1974.
Am. J. Epidemiol. 101:245-252, 1975.

*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

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 422: Immunochemical Studies of Antigenic Components of Arboviruses

Background:

Develop methods for optimal enhancement of antigenicity of arbovirus subunit vaccines.

Progress and Summary:

The 3 virion proteins of TC-83 vaccine strain VEE virus have been separated and recovered. All proteins were capable of stimulating precipitating antibody but the largest possesses antigenic determinants for the production of neutralizing antibody. Comparative electrophoresis of different α-viruses shows strain to strain and virus to virus differences in molecular weights of the virion envelope components. Examination of VEE complex virions has shown 4 different envelope patterns into which virus strains can be classified. Implication of biochemical analyses coupled with serologic classification are discussed. The primary objective of the study has been met.

Presentation:

Pedersen, Jr., C. E. Comparative studies of VEE complex viruses. Presented, American Society of Tropical Medicine and Hygiene Meeting, Honolulu, Nov 2-6, 1974.

Publications:

1. Pedersen, Jr., C. E., S. M. Marker, and G. A. Eddy. 1974. Comparative electrophoretic studies on the structural proteins of selected group A arboviruses. Virology 60:312-314.

2. Pedersen, Jr., C. E., and G. A. Eddy. 1974. Separation, isolation and immunological studies on the structural proteins of Venezuelan equine encephalomyelitis virus. J. Virol. 14:740-744.

3. Pedersen, Jr., C. E. 1974. Electrophoretic studies of Venezuelan equine encephalomyelitis viruses. Arthropod-Borne Virus Information Exchange 27:97-99.

4. Pedersen, Jr., C. E., and G. A. Eddy. 1975. Comparative analyses of members of the Venezuelan equine encephalomyelitis virus complex. Am. J. Epidemiol. 101:245-252.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6426	75 07 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
74 07 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	62760A	3A762760A834	02	423
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) In vitro studies of human immune response

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 01	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 75	1.0	114.0
	CURRENT 76	1.0	127.2

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Virology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Edelman, R.
TELEPHONE: 301 663-7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Felsburg, P. J.
NAME: Eddy, G. A. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Cell-mediated immunity; (U) Virus vaccine; (U) Typhoid fever; (U) Mitogens; (U) Lymphocyte transformation; (U) Estrogens

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

23 (U) Establish and standardize in vitro techniques for studying cell-mediated and humoral components of the human immune system in viral infections. This is an essential element in a comprehensive program for medical defense against BW agents.
24 (U) Examine in a very detailed way blood specimens collected from at-risk personnel receiving routine immunizations at the Institute. Included will be hematology, antibodies, electrophoresis, B and T cell kinetics, and interferon levels.
25 (U) 74 07 - 75 06 - Several assays have been developed for measuring changes in human and primate lymphocyte subpopulations (T and B cells) during viral and bacterial infections. A fall in absolute numbers of lymphocytes occurs in volunteers ill with typhoid fever; changes in percentages of these cells were associated with Salmonella typhi challenge, but not illness, and thus do not provide laboratory markers for resistance or susceptibility to S. typhi. The percentage of active -E rosette cells (A-RFC) rose significantly in persons having positive tuberculin and tularemia skin test responses. Skin test negative persons did not show a rise in their A-RFC. The rise in A-RFC also occurred after antigen was added to cultured lymphocytes from sensitized donors; it did not rise in lymphocytes cultured from nonsensitized persons. These observations measuring A-RFC in vivo and in vitro suggests that the A-RFC test may be a new and sensitive assay for cell-mediated immunity and a rapid diagnostic test for sensitization to microbial antigens.

Estrogens in pharmacological and physiological doses significantly depressed the lymphocyte blastogenic response to plant mitogens.

*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 65 AND 1498-1 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 423: *In vitro* Studies of Human Immune Response

Background:

Classical medical virology has dealt primarily with the study of viral pathogenesis and host immune response to viral infection. Relatively less has been reported concerning the effects of virus infection on the function of the immune system, particularly in humans.

Certain viruses have been shown to alter the immune response to other noninfectious antigens in laboratory animals. Depending upon the virus, the humoral and cellular immune responses may be enhanced or depressed. There are relatively few data concerning the effect of virus infections on the immune response to other infectious agents, including viruses themselves.

Although evaluation of vaccines is primarily based upon a generalized humoral response to the immunizing antigen, little is known concerning the response of the cell-mediated immune system.

We have adopted and developed lymphocyte assays to examine the interaction of cellular and humoral immune responses in viral, rickettsial and bacterial infections.

Progress:

I. Work with the cytofluorograph, summarized in the 1974 Annual Report, was postponed. Instead we devoted most of our efforts towards developing assays of lymphocyte subpopulation, and in exploring the effect of estrogens on lymphocyte transformation. These other research efforts are described.

II. Standardization of the various techniques for quantitation of lymphocyte subpopulations were conducted on 56 healthy adults, who contributed a total of 110 normal blood samples. The mean percentage and standard deviation for the various assays were: active E rosette cells = 27.0 $\pm$ 3.5; total -E rosette cells = 65 $\pm$ 6; B cells determined by surface immunoglobulin fluorescence = 28 $\pm$ 4; B cells determined by detection of cells bearing complement receptors = 20 $\pm$ 3.

We observed that the active E-lymphocyte (A-RFC) rosette formation is strongly dependent upon many technical variables, such as the ratio of numbers of lymphocytes to sheep erythocytes, centrifugation speed, method and time

used for resuspending of the cell pellets, and the lot of fetal calf serum used in the culture medium, as well as the pH of the culture medium itself.

In collaboration with LTC Hilmas, we examined B and T cell subpopulations in rhesus monkeys following irradiation and vaccination with VEE, strain TC-83. The results of the study are summarized in Work Unit 834.01010. The normal values for 8 monkeys closely resemble the human values.

In the Fort Detrick clinic, 5 personnel were inoculated with TC-83 VEE vaccine, while 3, were given 17-D yellow fever vaccine. The effect of these vaccines on their T and B lymphocyte kinetics was studied. All subjects showed a decrease in the A-RFC and T-RFC during the first week after vaccination and a slight rise in their circulating B cells, measured by Ig membrane fluorescence. However, these were pilot studies in "at risk" persons, and suitable baseline values for B and T cells were not obtained; we were thus unable to draw valid statistical conclusions regarding these changes in lymphocyte subpopulations. The in vitro response of peripheral lymphocytes to various plant mitogens was depressed during the first 2 wk post-vaccination but again interpretation of the data was hampered by the absence of required bleedings.

In order to correct the inherent deficiency of experimental design using "at risk" volunteers, we attempted to study more completely prison inmate volunteers at the Maryland House of Correction, Jessup, Md. This study was done in collaboration with the University of Maryland (Contract DA 49-193-MD-2867). Seven volunteers were given TC-83 vaccine and 4 control volunteers were given vaccine diluent only. We attempted to measure B and T cell kinetics before and after vaccination. Unfortunately, during the baseline period, another viral illness appeared in many of the volunteers, invalidating most of our results.

At the request of the University of Maryland, we collaborated in a typhoid fever study performed at the Maryland House of Correction. Prison inmate volunteers were orally challenged with 10^5 Salmonella typhi as part of a larger study designed to evaluate a new oral typhoid vaccine. Twenty-one volunteers were bled repeatedly 6 days before and 30 days after challenge, and changes in their lymphocyte subpopulations were measured. Total WBC fell significantly in volunteers who developed typhoid fever, but not in volunteers who resisted S. typhi challenge (well persons). In contrast to changes in total numbers of peripheral lymphocytes in sick persons, the percent of T-RFC fell significantly in both sick and well persons between 8 and 15 days after challenge; the percent of A-RFC and complement receptor-bearing lymphocytes fell significantly between 3 and 17 days after challenge in both sick and well volunteers. In contrast to the fall in T-cells, percent of Ig-bearing B lymphocytes rose significantly in both volunteer groups between 12 and 17 days. These results indicate that changes in the absolute number of circulating lymphocytes are associated with S. typhi illness, but that changes in percentages of lymphocyte subpopulations are not.

Rather, subpopulation changes are associated with S. typhi challenge. The alterations thus do not provide laboratory markers for resistance or susceptibility to S. typhi. Because the A-RFC assay probably measures immunocompetence, the rather profound fall in total numbers of A-RFC in typhoid patients (determined by multiplying the total numbers of lymphocytes by the percent A-RFC) is compatible with observations made in Europe and Asia that skin test anergy exists in typhoid fever patients. The rise of Ig-bearing cells and fall in complement receptor cells after challenge provides indirect evidence in man that the 2 receptor sites may be located on functionally different B cells. The phytohemagglutinin-induced response of peripheral blood lymphocytes taken from S. typhi-challenged volunteers was analyzed. The response to PHA stimulation was essentially the same in sick and well volunteers, and 4 USAMRIID control individuals.

With Dr. Canonico we measured serum lysozyme levels in these typhoid project volunteers. The lysozyme levels rose significantly in the group ill with typhoid, but not in the well persons. It thus appears that typhoid fever, in addition to regional enteritis, should be considered in the differential diagnosis of elevated lysozyme levels in inflammatory diseases of the bowel.

During the baseline period of the TC-83 vaccine study done with the University of Maryland at Jessup, we tested all volunteers with tuberculin (PPD), streptococcal and Monilia commercial skin test antigens. We did this to ascertain their immunological competence. All of the persons responded to at least one skin test antigen and all of them had a significant rise in their A-RFC assay for several days after the skin test. No change occurred in the T-RFC assay. Two additional volunteers were tested with intermediate strength PPD, and both had positive skin tests and rised in their A-RFC.

In an attempt to find and evaluate skin test negative persons, 6 volunteers from USAMRIID were inoculated ID with intermediate - strength PPD. Three persons had a strongly positive skin response (>10 mm induration), one had a negative or borderline reaction ($\leq$4 mm induration) and 2 had no responses. A striking and significant rise of A-RFC occurred in all 6 volunteers one day after the skin test. The rise peaked at 3 days and fell to normal levels by 6 days. No change occurred in the T-RFC assay. Tests of the 3 tuberculin-negative persons later with more concentrated PPD antigen (2nd strength) produced a positive skin reaction, suggesting the A-RFC was more sensitive than intermediate strength PPD for detecting prior sensitization to mycobacterial antigen.

The effect of PPD skin test antigens were then tested in 9 USAMRIID volunteers, all negative by history to intermediate PPD. They were given 2nd strength PPD, and 6 volunteers responded with a positive skin test and rise in the A-RFC. By contrast, 3 volunteers did not develop a delayed cutaneous hypersensitivity response, and did not show a rise in their A-RFC. The apparent immunological specificity of the A-RFC was confirmed in 9 USAMRIID staff who volunteered to receive the Foshay tularemia skin test antigen. Four persons previously vaccinated with LVS tularemia vaccine gave a positive skin test

response and developed a rise in their A-RFC, while 5 nonvaccinated persons did not respond locally to tularemia skin test antigen and did not develop a positive A-RFC. These studies, taken together, strongly suggest that the A-RFC is a highly sensitive and immunospecific index of prior sensitization to the microbial antigens.

Working under the premise that the skin test antigen alters the lymphocyte in some manner to increase the percentage of A-RFC circulating in positive individuals, we attempted to develop a completely in vitro technique which would negate the necessity of applying a skin test. Purified lymphocytes were obtained from tularemia positive and negative volunteers. The lymphocyte populations were divided in half; one-half was diluted in culture medium, and the other in culture medium plus 100 mg/ml of tularemia skin test antigen. These were incubated at room temperature for 4 hr after which the A-RFC assay was performed. The untreated or control cultures remained in the normal range for both the negative and positive individuals, whereas the cultures with tularemia antigen showed a significant (>2 SD from control) increase in A-RFC only in the positive individuals. The treated cultures from the negative patients exhibited no rise in A-RFC.

Kinetic studies were conducted in a similar manner at 37 C with 2 PPD-positive individuals. The A-RFC were examined at 0, 1, 2, 4, and 6 hr. The untreated cultures remained normal, whereas the PPD-treated cultures showed a significant increase in A-RFC at 2 hr and a peak at 4 hr. The specificity and many variables of this in vitro test are being examined. Its potential importance would be the ability to assess delayed hypersensitivity in an individual quickly and without the necessity of exposing the patient to a potentially harmful skin test.

The effects of therapeutic levels of estrogens on the response of peripheral lymphocytes to plant mitogens were examined. Estrogen doses of 100, 50, 25, and 10 μg/ml produced 90, 70, 60, and 33% reductions, respectively, in the response to the 3 mitogens tested PHA, concanaballin A (con A) and Poke-weed mitogen (PWM). The cultures with no mitogens revealed no significant difference at any concentration of estrogen. All cultures were harvested 5 days after the addition of mitogen which is the optimum time of harvest for normal cultures. To test whether the estrogens were producing a true depression or an accelerated or delayed response, we conducted a kinetic study with 100 μg/ml estrogen and harvested the cultures daily for 8 days. The results revealed that the estrogen effect was one of true depression with the response to PWM more depressed than that of PHA and con A. Again, no significant differences were noted in the unstimulated cultures. The next question was, when does the effect of the estrogen occur in relation to the addition of the mitogens? Mitogens were added at 0 time, and estrogens were added at 0, 1, 2, 4, 6, 12, 24, 48, 72, and 96 hr. The cultures were harvested on day 5 (120 hr). The purpose of this experiment was to determine whether the estrogens competed with the mitogens for receptor sites on the lymphocyte, or whether the estrogens induced a metabolic change in the lymphocyte. Maximum depression occurred when the estrogens were added 2 hr after the

mitogens; however, even when the estrogens were added at 96 hr, a 50% reduction in the response to the mitogens was observed. The question as to whether the estrogens are competing for receptor sites is still unanswered, since it is remotely possible that at the later time intervals we may be seeing competition for sites on new progeny cells which themselves can be stimulated by the mitogens. Further studies are in progress to define the mechanism of action.

Physiologic levels of estrogens (100 - 1000 pg/ml) in addition to therapeutic levels, have been tested in 7 individuals. Significant depression in the response to conA has been observed, although the depression is only 10 - 15%. No significant changes were observed in the unstimulated cultures at any of the concentrations.

In some preliminary work, 100 µg/ml of estrogen also appears to cause an in vitro reduction in the number of A-RFC. This appears to occur within the first 10 min of exposure to the estrogen. Dose-response curves mimic those observed in the lymphocyte transformation studies. These studies showing in vitro immuno·epression by estrogen may be relevant to reports of depressed delayed cutaneous hypersensitivity during pregnancy.

Publications:

None

LITERATURE CITED

1. U. S. Army Medical Research Institute of Infectious Diseases. July 1974. Annual Progress Report, FY 1974. pp. 367-371, Fort Detrick, MD.

2. Wybran, S. and H. H. Fudenberg. 1973. Thymus-derived rosette-forming cells in various human disease states: cancer, lymphoma, bacterial and viral infections, and other diseases. J. Clin. Invest. 52:1026-1032.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6410	74 10 07	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
74 07 01	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A834	02	424
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Procede with Security Classification Code)

(U) Defining vaccine efficacy by electron microscopy

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 05	74 10	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 75	0.7	70.0
	CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Virology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Gangemi, J. D.

TELEPHONE: 301 663-7241

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Electron microscopy; (U) Arboviruses, group A and B; (U) Arenaviruses; (U) Vaccines

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

23 (U) Establish techniques for physical particle enumeration with the aid of the electron microscope of four different groups of organisms: spotted fever group of rickettsia, groups A and B arboviruses and the arenaviruses. Quantitate and define the composition of the vaccine preparations with respect to antigenic mass and purity.

24 (U) Quantitate rickettsiae, groups A and B arboviruses and arenaviruses by an agar sedimentation procedure. Independent particle counting method and infectivity measurements will be employed to test the validity of this procedure. If the sedimentation technique proves to be a valid method of quantitation, an operating procedure will be established and the quantitation of vaccines and other virus suspensions will be done on a routine, technical support basis.

25 (U) 74 06 - 74 10 - Effects of aggregation of virions of formalin-inactivated VEE virus resulting from lyophilization were examined. Prior to freeze-drying the virions were well dispersed; aggregation was severe afterwards and the vaccine was 5-7 times more effective before lyophilization. Purification and concentration techniques, such as sucrose banding and polyethylene glycol precipitation, also produced virion aggregation. These resulted in lower titers of plaque forming units (PFU) as counted by the electron microscope. PFU:particle ratios were obtained for rickettsiae and viruses of Groups A, B and arena. The most noteworthy of these is the 1:20 ratio for arenaviruses (1:2 for group A). It is theorized that there may be some defective particles in the arenavirus; if so, it may be possible to select defective clones for a vaccine source.

A routine procedure for analysis and physical quantitation by electron microscopy has been established. Objectives have been obtained. Further studies will be conducted under a technical support plan.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 424: Defining Vaccine Efficacy by Electron Microscopy

Background:

If all particles from a known volume of suspension are sedimented upon a flat film, their 3-dimensional configuration should produce a 2-dimensional picture from which both the total particle count and the state of aggregation can be derived. Such a sedimentation procedure was first demonstrated for influenza virus in 1949. This procedure is characterized by its sensitivity, giving useful particle count data from suspensions of concentrations as low as 10^8 virus particles/ml. The sensitivity of the procedure is 4 or 5 $\log_{10}$ greater than other counting procedures and is capable of various sorts of adaptations, in order to satisfy the peculiarities of the particles involved. While difficulties inherent in the sedimentation technique are sometimes encountered when particles do not adhere too firmly to a receiving surface, it is still the most appealing technique for studies dealing in both quantitative and qualitative assessments of particle suspensions.

Progress and Summary:

Effects of aggregation of virions of formalin-inactivated VEE virus resulting from lyophilization were examined. Prior to freeze-drying the virions were well dispersed; aggregation was severe afterwards and the vaccine was 5-7 times more effective before lyophilization. Purification and concentration techniques, such as sucrose banding and polyethylene glycol precipitation, also produced virion aggregation. These resulted in lower titers of plaque forming units (PFU) as counted by the electron microscope. PFU-particle ratios were obtained for rickettsiae and viruses of groups A, B and arena. The most noteworthy of these is the 1:20 ratio for arenaviruses (1:2 for group A). It is theorized that there may be some defective particles in the arenavirus; if so, it may be possible to select defective clones for a vaccine source.

A routine procedure for analysis and physical quantitation by electron microscopy has been established. Objectives have been obtained. Further studies will be conducted under a technical support plan.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6417	75 07 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
75 03 19	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	62760A	3A762760A834	02	425
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code): (U) Biophysical and biochemical characterization of arenaviruses and their structural components

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
75 03	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 75	0.3	30.0
FISCAL YEAR CURRENT 76	1.0	65.0

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Virology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME: Gangemi, J. D.
TELEPHONE: 301 663-7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Arenaviruses; (U) Biophysics; (U) Biochemistry; (U) Electron microscopy; (U) RNA viruses

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

23 (U) Isolate and characterize by electron microscope virion subpopulations of arenaviruses in order to understand their infectivity and composition. This is an essential element in a comprehensive program for defense against BW agents.

24 (U) Prepare large quantities of selected viruses; concentrate and study them by a variety of biophysical and biochemical means. Infectivity of subpopulations will be assayed by appropriate techniques.

25 (U) 75 03 - 75 06 - Concentrated stocks of arenaviruses containing 14 logs of purified particles have been prepared from infected cell culture supernatants by polyethylene glycol precipitation and sucrose gradient centrifugation. Transmission and scanning electron microscopy has been used to characterize external components of purified virions while freeze-fracturing techniques have been used to characterize morphologically internal components. Polyacrylamide gel electrophoresis has been used to characterize biochemically virion structural components.

Fractionation of highly concentrated virion populations into 3 major subpopulations containing particles 70-90, 110-130 and 150-180 nm has been accomplished in large volume zonal rotors.

Virion nucleic acid and host-cell, incorporated ribosomes have been isolated from whole (unfractionated) virion concentrates and examined with the electron microscope.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 425: Biophysical and Biochemical Characterization of Arenaviruses and Their Structural Components

Background:

Thin-sections of infected cells and negative stains of pelleted virions have revealed morphological differences between virions of arenavirus populations. Most apparent in these differences is the variation in particle size.[1] The significance of these size differences with respect to infectivity and virulence is unknown; however, since both multiple pieces of RNA and host cell ribosomes are involved in virion maturation, it appears likely that some differences may result from the nonuniform incorporation of these components.

Biochemical and biophysical studies on arenavirus subpopulations which contain particles of a particular size have been unsuccessful due to virion concentration and fractionation difficulties encountered in conventional small-volume ultracentrifuge rotors.[2] Similar difficulties encountered with hepatitis-associated particles, which like arenaviruses also differ in size, were resolved with the aid of large volume zonal rotors.[3] We have applied the advantages of large volume zonal rotors to the fractionation of arenaviruses and have found that several distinct virion subpopulations can be isolated in quantities which will make both biochemical and biophysical studies feasible.

Progress:

Highly concentrated stocks of Pichinde virus containing 14 $\log_{10}$ of purified virions have been prepared from infected cell culture supernatants by polyethylene glycol precipitation and sucrose gradient centrifugation. Transmission and scanning electron microscopy has been used to characterize external components of purified virions while freeze-fracturing techniques have been used to characterize internal components. Results from electron micrographs indicate that virions (1) range in size from 70 - 300 nm, (2) contain spike-like projections which look more like knobs when observed at higher magnifications, and (3) contain a bilaminar membrane covering which surrounds an unstructured interior containing massive numbers of incorporated host cell ribosomes.

Fractionation of highly concentrated virion populations into 3 major subpopulations containing particles of 70-90, 110-130 and 150-180 nm respectively has been accomplished in a Sorvall reorienting zonal rotor. Infectivity (particle: PFU ratios) and virion morphological differences were observed between these subpopulations. Attempts to isolate virion clones which produce progeny virus with the characteristics of the subpopulation

from which they were isolated are now underway.

Polyacrylamide gel electrophoresis in continuous and discontinuous systems has been used to characterize virion structural components from whole (unfractionated) virion populations. Results of these structural studies have indicated the presence of at least 3 distinct polypeptides (70,000, 35,000 and 12,000 daltons) which are believed to be specified by the viral genome, and approximately 23 other polypeptides believed to be structural components of the ribosome units incorporated into virions during maturation. Studies are now in progress to characterize virion and ribosomal structural components from fractionated viral subpopulations which have different infectivity and morphological characteristics.

Virion nucleic acid and ribosomal components have been isolated in CsCl gradients and examined with the electron microscope. Polysome-like structures were observed in CsCl fractions containing either viral RNA or ribosomal components. Experiments are now in progress to determine the relationship, if any, between viral nucleic acid and incorporated host cell ribosomes.

Publications:

None

LITERATURE CITED

1. Murphy, F. A., P. A. Webb, K. M. Johnson, S. G. Whitfield, and W. A. Chappell. 1970. Arenoviruses in Vero cells: ultrastructural studies. J. Virol. 6:507-518.

2. Pfau, C. J. 1974. Biochemical and biophysical properties of the arenaviruses. Progr. Med. Virol. 18:64-80.

3. Neurath, A. R., L. Cosio, A. M. Prince, and A. Lippin. 1973. Purification of hepatitis B antigen associated particles: use of a reorienting gradienc rotor. Proc. Soc. Exp. Biol. Med. 144:384-390.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6429	75 07 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
74 07 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	62760A	3A762760A834	02	804
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Controlled enzymatic and chemical alteration of microbial proteins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 08	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

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING 75	1.0	120.6
CURRENT 76	1.0	231.5

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Pathology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME: Spero, L.
TELEPHONE: 301 663-7211
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Metzger, J. F.
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Bacterial toxins; (U) Amino acids; (U) Enzymes; (U) Toxoids; (U) Vaccines; (U) Staphylococcus

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

23 (U) Modify proteins of microbial origin that play roles in their biological effects in order to prepare more effective immunogens against militarily important diseases. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Staphylococcal enterotoxin C (SEC) undergoes a rapid, limited digestion by trypsin. Studies are concerned with characterization of the fragments and their role in the serological, emetic, and mitogenic activity of the whole molecule.

25 (U) 74 07 - 75 06 - SEC undergoes a limited digestion with trypsin in which 2 peptide bonds are hydrolyzed, the more rapid one in the disulfide loop between lysine and valine and the second in the amino terminal region between lysine and aspartic acid. The product remains as a single molecular species that is fully active serologically and has strong mitogenic activity. The amino terminal polypeptide, molecular weight 6,500, can be separated from the remainder of the molecule, after unfolding in strong denaturant. Serological activity is demonstrable on the larger 21,900 molecular weight polypeptide. The 6,500 fragment has no serological activity and no emetic activity. It is, however, a potent mitogen for mouse spleen cells, thus identifying the region of the enterotoxin that contains the mitogenic binding site.

Publication: Fed. Proc. 34:824, 1975
J. Biol. Chem. 250: in press, 1975.

*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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 02: Prevention and Treatment of Biological Agent Casualties

Work Unit No. 834 02 804: Controlled Enzymatic and Chemical Alteration of Microbial Proteins

Background:

The staphylococcal enterotoxins are simple proteins elaborated by Staphylococcus aureus which cause emesis and diarrhea in a limited number of mammalian species. Several antigenically defined forms SEA, SEB, and SEC have been isolated in very pure form; all consist of a single polypeptide chain with one disulfide bridge and have a MW of ~ 28,000. We have been investigating partial enzymatic breakdown as a means of separating and identifying antigenic and toxic sites. SEB undergoes a limited digestion by trypsin yielding a product (SEB-T) with a single cleavage in the -S-S- region of the molecule. SEB-T retains full serological and emetic activity. After reduction and carboxamidomethylation the 2 polypeptide fragments of SEB-T could be separated on CM-cellulose in 8 M urea. Although the fragments were unstable after removal of denaturant it was possible to demonstrate antigenic determinants on both; neither possessed emetic activity.

SEA appears to be completely resistant to trypsin but SEC like SEB undergoes rapid hydrolysis of a single bond in the -S-S- region. SEC contains, in addition, a second susceptible bond outside of this loop.

Progress:

The more alkaline variant of SEC, called SEC_1, has been employed. On treatment with trypsin at a ratio of substrate: enzyme of 40:1 to 50:1 in 0.05 M Tris-HCl buffer at pH 9.0 at 30 C 2 peptide bonds were hydrolyzed, one very rapidly in the disulfide loop and the second outside the loop at about 1/5 this rate. The first was a Lys-Val bond and second a Lys-Asp bond. The new amino terminal residues were identified by the dinitrophenylation technique and the Lys residues by treatment with carboxypeptidase B. After a 3-hr digestion period no unreacted SEC_1 remained and only about 2% of the singly-cleaved material, SEC_1-T_1. The remainder, the doubly cleaved material SEC_1-T_2, behaves like a single molecular species: only a single component with an elution volume equivalent to untreated SEC_1 is observed on gel filtration; only a single component with a sedimentation coefficient of 2.85 S at 5.4 mg/ml is observed in the analytical ultracentrifuge; and a MW

of 29,000 is found by sedimentation equilibrium with no curvature of the ln c vs. r^2 plot. When examined by polyacrylamide gel electrophoresis in the presence of SDS 2 components are seen. The larger has by this technique a MW of 22,000 ± 600 and the smaller < 10,000. (The estimation of molecular weights in the latter range is extremely hazardous as there is a sharp break in the standard curve.) An excellent separation of these 2 components is achieved on a Sepharose 6B column in 6 M guanidine·HCl.[1] Molecular weights were calculated from the elution position of the components from these columns by Ackers' method[2] and the values obtained were 23,000 and 5,000. The best estimate of these MW was obtained by the application of the method of Delaage[3] to an amino acid analysis of the fragments. Values of 21,900 and 6,500 were obtained. The amino acid values for these fragments, their sum and the values for SEC_1 are shown in Table I. It is apparent that there is satisfactory agreement in most instances.

TABLE I. AMINO ACID ANALYSIS OF SEC_1 AND ITS DERIVED TRYPTIC PEPTIDES.

AMINO ACID	RESIDUES/MOLECULE			
	6,500 Peptide	21,900 Peptide	Σ of Columns 1 & 2	SEC_1 a/
Lys	8	25	33	31
His	3	4	7	6
Arg	0	3	3	3
Asp	10	31	41	43
Thr	3	13	16	15
Ser	4	10	14	15
Glu	4	17	21	19
Pro	3	4	7	6
Gly	1	14	15	15
Ala	4	5	9	7
Cys/2	0	-b/	-b/	2
Val	4	14	18	18
Met	1	3	4	7
Ile	2	9	11	10
Leu	5	12	17	16
Tyr	3	14	17	17
Phe	2	9	11	10
Trp	-b/	-b/		1

a. Recalculated from the data of Huang et al.[4] by the method of Delaage[3].

b. Not determined.

The NH_2-terminal amino acids of the 2 fragments of SEC_1-T_2 were found to be glutamic acid for the 6,500 material and Asp for the 21,900 material. Since SEC_1 has an NH_2-terminal glutamic acid, the 6,500 fragment must comprise the amino terminal end of the molecule. It is noteworthy that positions 54 and 55 in the SEB sequence are occupied by Lys-Asp and that there is considerable similarity in amino acid composition between SEB 1-54 and the 6,500 fragment which contains 57 amino acid residues (Table II).

TABLE II. COMPARISON OF THE AMINO ACID COMPOSITION 6,500 FRAGMENT FROM SEC_1-T_2 AND SEB 1-54

AMINO ACID	RESIDUES/MOLECULE	
	6,500 Peptide	SEB 1-54
Lys	8	6
His	3	2
Arg	0	0
Asp	10	9
Thr	3	1
Ser	4	6
Glu	4	5
Pro	3	3
Gly	1	1
Ala	4	1
Cys/2	0	0
Val	4	3
Met	1	2
Ile	2	4
Leu	5	5
Tyr	3	3
Phe	2	3
Trp	- a/	0

a. Not determined.

SEC_1-T_1, the singly cleaved product has been successfully separated from SEC_1-T_2 by chromatography on Sepharose 6B in 6 M guanidine·HCl. After a digestion of 45 min with trypsin, a mixture of approximately equal amounts of SEC_1-T_1 and SEC_1-T_2 is obtained containing a small percentage of unreacted SEC_1. In concentrated denaturant the SEC_1-T_2 is degraded to its 2 peptides and SEC_1-T_1 appears as a leading shoulder on the large fragment peak. Rechromatography of this fraction 2 times yields a preparation of SEC_1-T_1 devoid of SEC_1-T_2 but containing 7% SEC_1. This contamination does not impose a significant restraint of the testing of biological parameters.

In gel immunodiffusion SEC_1, SEC_1-T_1, and SEC_1-T_2 give reactions of full identity with each other. Quantitative precipitin curves against rabbit anti-SEC_1 are virtually identical for the native enterotoxin and the 2 derivatives. SEC_1-T_1 and SEC_1-T_2 are potent mitogens for mouse spleen lymphoid cells. They produce indistinguishable dose-response curves but are somewhat less active than native enterotoxin.

When the polypeptide fragments of SEC_1-T_2 are dialyzed to remove the guanidine employed in their separation, the 6,500 material stays in solution but the 21,900 material precipitates rapidly. This fragment is soluble in 2 M urea, 0.1% SDS, and 0.1% cetyl pyridinium chloride. Some success has been achieved in determining serological properties in the last solvent. At a concentration of 700 µg/ml positive precipitin reactions are obtained in Ouchterlony and Mancini gel diffusion tests. The Ouchterlony pattern indicates a reaction of full identity while the Mancini rings are equivalent to those obtained with 1/14 the quantity of SEC_1. It has not been possible to demonstrate precipitating activity in solution. Attempts to demonstrate inhibition of the precipitation of serologically equivalent amounts of SEC_1 and rabbit anti-SEC_1 by the 21,900 fragment were uninterpretable because of the large amounts of protein precipitated by the detergent. Cetyl pyridinium chloride is also very toxic for mouse spleen cells so that it has not yet been possible to test the 21,900 material for mitogenic activity.

The 6,500 polypeptide gives no precipitation in gel diffusion with rabbit anti-SEC_1. Similarly it does not form a precipitate in solution with antibody nor does it inhibit SEC_1-anti-SEC_1 precipitation. It thus appears to be devoid of antigenic determinants. This fragment was not emetic for rhesus monkeys at a dose as high as 10 µg/kg body weight. In this experiment the IV median effective dose (ED_{50}) for SEC_1 was determined for the first time; a value of 0.02 µg/kg was obtained. This is significantly more active than SEB whose ED_{50} has been estimated to be 0.1 µg/kg.

The 6,500 fragment is mitogenic for mouse spleen lymphoid cells. A typical dose-response curve is obtained with stimulation increasing to a maximum at $\sim$ 20 µg/250 µl of cell suspension and then declining at higher levels. At maximum stimulation at 16.4-fold increase over the control is achieved. This stimulation of ^{3}H-thymidine uptake is considerably less than that obtained with SEC_1 but is nevertheless highly significant. These results appear to define the region of the molecule containing the binding site for mitogenic activity.

Three antigenic variants, SEA, SEB, and SEC, have been shown to be equally potent polyclonal mitogens, demonstrating that the binding site to splenic lymphocytes represents a conformational feature that does not correspond to the major antigenic determinants. However, it was unknown whether there is a relation between emesis and mitogenic activity. Since formaldehyde destroys toxicity completely, the effect of this treatment upon mitogenicity was investigated. Toxoids of SEB were prepared at the 3 pH values,

5.0, 7.5, and 9.5[5] and were filtered through 0.2-μm filters to remove large aggregates. All 3 toxoids are mitogenically active with an order of potency 7.5-fSEB > 5.0-fSEB > 9.5-fSEB. Viability and conversion to blast forms for the 7.5-fSEB are equivalent to values obtained with native SEB. It requires about 10 times the amount of the toxoid to induce a maximum response and in most trials this is lower than that obtained with the native enterotoxin.

The efficacy of the toxoids prepared at different pH values has been suggested to be related to the molecular size of the toxoid aggregate.[5] It was surprising, therefore, to find that the filtered 7.5-fSEB preparation also passed completely through an XM-300 membrane indicating that all the molecules were < 300,000 MW. When the 7.5-fSEB is fractionated on Sephadex G-100, 3 fractions are obtained corresponding to a monomeric, a dimeric, and a polymeric form with a MW range of 100,000-300,000. All are mitogenic but the polymeric material is by far the most potent far surpassing the parent toxoid and approaching unaltered SEB in activity. The reduced mitogenicity of the monomeric and dimeric forms is accompanied by an absence of serological activity in these fractions. It is proposed that the intramolecular crosslinks that predominate in these forms are highly disruptive of surface structure. Finally, it appears clear that the mitogenic binding site represents a surface element of the enterotoxin distinct from the conformational feature that is responsible for its primary biological activity, emesis and diarrhea.

Publications:

1. Warren, J. R., L. Spero, W. H. Adler, and J. F. Metzger. 1975. Lymphocyte activation by staphylococcal enterotoxin. Fed. Proc. 34:824 (abstr.).

2. Spero, L., J. F. Metzger, J. R. Warren, and B. Y. Griffin. 1975. Biological activity and complementation of the two peptides of staphylococcal enterotoxin B formed by limited tryptic hydrolysis. J. Biol. Chem. 250: In press.

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1. Fish, W. W., K. G. Mann, and C. Tanford. 1969. The estimation of polypeptide chain molecular weights by gel filtration in 6 M guanidine hydrochloride. J. Biol. Chem. 244:4989-4994.

2. Ackers, G. K. 1967. A new calibration procedure for gel filtration columns. J. Biol. Chem. 242:3237-3238.

3. Delaage, M. 1968. Sur la recherche du poids moléculaire le plus cohérent avec l'analyse des acides amines d'une protéine. Biochim. Biophys. Acta 168:573-575.

4. Huang, I.-Y., T. Shih, C. R. Borja, R. M. Avena, and M. S. Bergdoll. 1967. Amino acid composition and terminal amino acids of staphylococcal enterotoxin C. Biochemistry 6:1480-1484.

5. Warren, J. R., L. Spero, and J. F. Metzger. 1973. Antigenicity of formaldehyde-inactivated staphylococcal enterotoxin B. J. Immunol. 111:885-892.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC6411	75 07 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
74 10 08	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	62760A	3A762760A834	03	008
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Procede with Security Classification Code)

(U) Mathematical and computer applications in infectious disease research

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 009700 Mathematics and statistics

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 11	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 75	1.0	30.0
	CURRENT 76	0.5	32.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Physical Sciences Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Higbee, G. A.

TELEPHONE: 301 663-2640

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Powers, T. J.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Diagnosis; (U) Computers; (U) Medicine; (U) Military medicine

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

23 (U) Develop and apply techniques for utilization of computers, statistics and methematics to process and interpret scientific data. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Current installation of a high-speed computer will enhance the effectiveness of this work unit. Theories and disciplines of numerical analysis, differential equations, statistical tests of hypotheses, experimental design, information storage and retrieval, and pattern recognition are utilized to analyze and interpret data gathered by investigators.

25 (U) 74 07 - 75 06 - The work begun in previous years has been completed or continued either under this work unit or its associated Technical Support Plan (TSP-04). New projects have been added in early diagnosis of infection and in consultation.

Research efforts have been concentrated in the areas of biochemical indices of infection, influence of infection on taste testing, and mathematical modeling and analysis of physiological systems.

Publication: Fed Proc. 34:465, 1975.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 008: Mathematical and Computer Applications in Infectious Disease Research

Background:

The objective of effective utilization of computers, statistics, and mathematics in handling data generated within the Institute is pursued in 3 main areas: early diagnosis of infection, mathematical modeling and analysis of physiological systems, and consultation. Work is generally performed in conjunction with many other work units within the Institute. This often allows a single technique or solution to be applied to many similar problems.

During the past year the CDC 3300 computer at Fort Detrick was moved to Fort Sam Houston, Texas. For the last 6 months, computer support was provided to USAMRIID by WRAIR's CDC 3500, with programs and data transported to WRAIR by courier service. The resulting 2-7 day turnaround time for computer jobs caused delays in research progress of some investigators who relied on operational programs for their data analysis. This delay in turnaround also decreased the effectiveness of the Computer Science Office in responding to new programming requests by investigators. Limited automatic data processing support has retarded progress in this work unit during the past year.

A request for Specified Automatic Data Processing Equipment (AR 18-1, Appendix I) was prepared and sent to OTSG requesting the rental of a high-speed terminal consisting of a card reader, paper tape reader, high-speed printer, magnetic tape unit, and CRT operator station. A Data 100 terminal is now installed in the Institute, connected to WRAIR's CDC 3500 computer, and some programs are being processed through the terminal. All of the terminal equipment is not yet fully operational. More information concerning the computer support will be found under Technical Support Plan (TSP)-04.

Progress:

Progress in the collection and analysis of data to determine possible biochemical indices of infection has consisted of developing a computer data base and writing program to determine correlations between clinical observations and serum analyses.[1] The current data base consists of 272 samples from West Virginia University, USAMRIID analyses on 167 of these serum samples, and 317 samples from the University of Maryland. Computer programs have been

written to sort the data on one or any combination of the 17 patient history and diagnosis code parameters. Work is continuing on the design of programs to do statistical analyses of the sorted biochemical data.

In a typhoid fever study, volunteers were tested to determine if the changes in serum trace metals (Zn, Fe, Cu) during infection were reflected in changes in ability to detect and/or distinguish salt, sweet, sour, and bitter tastes. Data on 24 males are being analyzed to detect differences in taste before and after infection. Possible changes in taste will also be investigated before and after fever. Programs for statistical analysis of the data have been written and final computer results are being compiled.

One of the objectives of the Institute is to develop animal models for physiological studies, with the purpose of defining therapeutic measures of diseases. Since rhesus monkeys are commonly used as primate models in these studies, it is necessary to have a method of determining the body surface area (BSA) of living rhesus monkeys so that cardiac index, oxygen consumption, and dosages in fluid therapy can be calculated. Because limited information was available for calculating monkey BSA, a project was initiated to develop a method of determining the body surface area of a living rhesus monkey.

Based on measurements made by the Animal Assessment Division of BSA (1,940-3,020 cm^2), body weight (3.2-5.3 kg) and body length from head to anus (41.9-53.7 cm) for 31 rhesus monkeys, the following equation was determined for calculating the BSA of a rhesus monkey as a function of weight and length:

$$BSA\ (cm^2) = W(kg)^{0.6046} \times L(cm)^{0.1862} \times 514.$$

Using this equation, the average difference between measured and calculated BSA for the 31 monkeys was $0.59 \pm 1.56\%$. A paired-t comparison of calculated and measured areas showed no significant differences between measured and calculated BSA.

In order to demonstrate the validity of the least-squares procedure used to determine the best values of the exponents and multiplicative constant in the above monkey equation, the same procedure was used on DuBois' human data[2] ($n = 43$) to develop an accurate equation for human BSA. DuBois' measured and our calculated human BSA values did not differ significantly, using a paired-t test. Thus, the least-squares procedure used to develop the monkey BSA equation was verified by using the procedure to develop an accurate human BSA equation.

For rapid determination of BSA from weight and length, the principle of alignment charts was used to construct a nomogram for the BSA of rhesus monkeys, based on the above equation.

Four different measures of body length were taken on each monkey: (1) head to heel, (2) head to anus, (3) nose to heel, and (4) nose to anus. The distance from head to nose was found to be constant, so only 2 measures of body length, head to heel and head to anus, were evaluated further. The length from head to anus was determined to be the most suitable measure of body length.

An equation for calculating the BSA of a rhesus monkey, from weight only, was determined to be:

$$BSA(cm^2) = 969\ W(kg)^{2/3}$$

The average difference between measured and calculated BSA for this equation was 0.73 + 1.58%, slightly larger than for BSA as a function of weight and length. The measured and calculated areas did not differ significantly using a paired-t test.

DuBois' equation for calculating human BSA can be used to calculate BSA of rhesus monkeys if the equation is multiplied by a factor computed to be 1.147. When the monkey data were processed through DuBois' human equation multiplied by 1.147 ($BSA\ (cm^2) = W(kg)^{0.425} \times L(cm)^{0.725} \times 82.4$), the average difference between measured and calculated BSA was $0.76 \pm 1.64\%$. There was no significant differences between the measured and calculated BSA, by paired-t test.

The above 3 equations for determining the BSA of a living rhesus monkey have average differences between measured and calculated BSA of <1%, with ranges of differences from -14 to +23%.

Consultations were held with investigators of the Physical Sciences, Pathology, Animal Assessment, Rickettsiology, Aerobiology, Virology, and Administrative Divisions of the Institute to develop solutions to specific short-term computational or statistical problems. Consultation was also provided to the Frederick County Comprehensive Health Planning Council relative to the analysis of a psychiatric study at the Frederick Memorial Hospital.

Presentation:

Higbee, G. A., M. L. Sammons, and C. T. Liu. Determination of body surface area in the rhesus monkey. Presented, 59th Annual Meeting, FASEB, Atlantic City, N. J. 13-18 Apr 1975. (Fed. Proc. 34:465).

Publication:

None.

LITERATURE CITED

1. Wannemacher, Jr., R. W., and G. A. Higbee. 1975. Attempts to develop a diagnostic profile of biochemical values in man. p 67-80. In 1974 USAMRIID Planning Session, Fort Detrick, MD.

2. DuBois, D., and E. F. DuBois. 1916. A formula to estimate the approximate surface area if height and weight be known. Arch. Intern. Med. 17:863-871.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6412	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8. ENTRY	9. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 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	62760A	3A762760A834	03	009
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Procede with Security Classification Code)

(U) Biophysical parameters of host response to infection and/or immunization

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
69 11	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 75	1.0	64.8
FISCAL YEAR CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Hughes, F. L.

TELEPHONE: 301 663-7341

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Zenser, T. V.

NAME: Seburn, D. W. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Procede EACH with Security Classification Code)

(U) Electron paramagnet resonance (EPR); (U) Spin labels; (U) Biological membranes; (U) Phagocytosis; (U) Encephalitis, equine (VEE)

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

23 (U) Apply biophysical principles to investigations of mechanism(s) of host response to infection. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Apply EPR techniques to study concentration changes in transition metal proteins of serum. Use spin labels incorporated into biomembranes to probe membrane response to stress. Optically measure immunological reactions on the surface of a metal film.

25 (U) 74 06 - 75 07 - EPR observations were made on paramagnetic nitroxyl radicals bound to the vaccine strain (TC-83) of VEE virus. The results showed that viral lipid is organized in a bilayer form and permitted presentation of a general structural model for membranes of the group A arboviruses. Nitroxyl spin labels at concentrations of the order of 0.000001 to 0.0000001 M were found to reduce dramatically the osmotic fragility of human erythrocytes. Parallel EPR measurements of the order parameter indicated that the increased stability of membranes was not a function of increased rigidity in the lipid bilayer. It is proposed that stabilization results from nitroxyl interactions with intrinsic membrane proteins. The proposal is in accord with contemporary membrane models presented by other investigators.

Publication: Biochim. Biophys. Acta.394:102-110, 1975.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 009: Biophysical Parameters of Host Response to Infection and/or Immunization

Background:

In the last 5 yr, methods have been developed for preparation and purification of the electron paramagnetic nitroxide free radicals. The use of these radicals as probes has permitted the extension of electron paramagnetic resonance (EPR) into previously inaccessible areas of biological research. This extension has been most fruitful in the study of structure of biological membranes where the label acts as an inert or relatively weakly-interacting probe. An example of this use is our recent study[1] of the membrane of VEE virus. Recently it has been realized that the nitroxides could be used under conditions of stronger biological interaction where the probe serves as an enzyme substrate or as a protein conformation modifier. The interpretation of such experiments is inherently more difficult than that for the case of the inert probe but the information obtainable appears to be not only valuable but of a kind unobtainable by other methods. This latter approach has appeared so productive that most of the effort during this reporting period has been devoted to such studies.

Progress:

An initial phase of experiments with VEE virus[1] was completed. This consisted of (1) demonstrating that the label did not appreciably affect virus structure as indicated by the fact that plaque-forming capability was unchanged with virus exposed to label at concentrations up to 10 times the level used for the EPR experiments and (2) determining that the label 1-oxyl, 2,2,6,6-tetramethyl piperidine (TEMPO)[1] did not detectably bind to the virus. The latter finding confirmed our previous conclusion regarding viral lipid membrane rigidity.

A logical extension of this study was investigation of the role of lipids in cellular attachment and morphogenesis, and the capability of the lipid-containing viruses for secondary infection. A primary consideration was the control exercised by the viral genome on viral lipid composition. Although older reports indicated that the lipid composition of group A arboviruses was host-cell dependent, more recent experiments have demonstrated

that the lipid composition of purified Sindbis and VEE viruses has little or no dependence upon the type of host cell. In our recent experiments with TC-83 VEE virus, the bilayer not only bound a number of nitroxide-labeled lipids but also bound unlabeled stearic acid and a synthetic phosphotidylethanolamine. Although these findings suggested that the viral genome cannot absolutely specify the lipid composition, there was a strongly implied requirement for secondary genome control.

Investigations of the secondary aspect of control of the lipid composition do not require knowledge of the basic mechanisms of that control. In our structural model,[1] however, primary-to-quarternary structures on the exterior surface of the virus capsid and the interior surface of the envelope protein sheath are directly under viral genome control. The stearic influence of these surfaces can provide the secondary control for types and concentrations of lipids that will best fit the bilayer between these 2 surfaces. Control of lipid would be reflected by viral yield and infectivity. A virus-host-cell combination that can be induced to produce the proper distribution of lipids could be expected to yield virions with a structure capable of surviving purification treatments while maintaining the capability for secondary infectivity.

In earlier experiments, virus yields of strain TC-83, i.e., plaque-forming ability in PFU, were found to be 10 times greater for baby hamster kidney (BHK) than for chick embryo (CE) cells. More recent studies by others suggest that many CE cell cultures yield a comparable number of virions as indicated by electron microscope (EM) count or total virus protein. Thus, it appears that the low PFU yield from CE cultures could have resulted from the production of many virions which are defective in replication or infective ability.

A program has been initiated to investigate the role of viral lipids and lipid composition in VEE virus infectivity. Modification of the plaque-forming ability of defective VEE virus by incubation with various lipids known to be associated with viral envelope has been attempted. Virus was grown on CE cells, partially purified by centrifugation and Genetron 113 extraction, treated with lipids and assayed for particle count by EM and PFU on duck embryo cells (DEC). Twenty different single and binary-mixed fatty acids and phospholipids were employed. Although initial results were encouraging because the lipid treatment was nondestructive, and the reproducibility of PFU performed in 2 separate laboratories was $10^{\pm 0.3}$, they were disappointing in the low quantity of defective virus produced. Future efforts will concentrate on reproducing the exact growth and assay conditions necessary to attain a particle count: PFU ratio of ~ 10.

Although previous efforts have been directed toward development of a paramagnetic spin-label assay for detecting changes in host cell membranes as a consequence of infection or pharmacological treatment, it became apparent

that results of EPR-derived membrane rigidity required correlation with another experimental measure of membrane structural strength; it was proposed that observation of erythrocyte properties could provide the necessary data for such a correlation.

In exploratory work, methods similar to those reported by Seeman[2] were employed. Briefly, a stock suspension of human RBC obtained from heparinized whole blood was prepared daily by washing in phosphate buffered saline (PBS), 0.154 M NaCl in 0.01 M Na-phosphate, pH 7.0, and resuspending to ~ 4 x 10^8 RBC/ml in the same buffer. RBC prepared in this manner were maintained at 0 C until use. Cellular resistance to osmotic shock (osmotic fragility) was tested by incubating 100 µl of RBC suspension in a "shock" solution consisting of 1.5 ml of hypotonic saline and 50 µl of absolute ethanol with or without spin-label additive. Hypotonic saline (0.074 - 0.079 M NaCl) was adjusted for each batch of cells to produce 50-70% hemolysis. After 10 min at room temperature the test mixtures were centrifuged at 1500 x g for 45 sec and optical absorbance of the supernatant was measured at 543 nm. The final concentration of cells (~ 10^7 cells/ml) was such that there was a linear relationship between hemoglobin absorbance and number of lysed cells. The dose-response of the effect of additive upon osmotic fragility was determined by addition of various concentrations to the 50-µl ethanol component of the "shock" solution. Data were expressed as hemolysis ratios, i.e., the ratio of absorbance with additive to absorbance without additive.

Studies were conducted with the following additives: etiocholanolone, pregnanolone, the stearic acid derivatives I(5,10), I(12,3) and I(2,14) and a spin-labeled analogue of androstane. In the 3 spin-labeled stearic acids, the paramagnetic radical label, N-oxyl-oxazolidine, was located 10, 3 or 14 carbon units from the carboxyl group of the acid. These labeled molecules intercalate into the lipid bilayers of biomembranes and the depth of penetration of the label radical into the membrane depends upon this radical -- carboxyl group separation.[1] Hemolysis ratios for RBC exposed to etiocholanolone, pregnanolone and the spin label stearic acid derivatives I(5,10) and I(12,3) are presented in Table I. Values for etiocholanolone and pregnanolone are in accord with corresponding published data,[4] and those for unlabeled stearic acid are values reported by Seeman.[2] Stabilizing effects of the spin-labeled stearic acid derivatives on the RBC membrane were dependent not only upon the presence and concentration of the derivative but also upon the position of the label with respect to the stearic acid carboxyl group. Data for the derivative I(2,14) and labeled androstane were similar with minimum hemolysis ratios at concentrations of 1.14 x 10^{-5} M and 1.99 x 10^{-5} respectively. Thus, these results indicate that the capacity of an oxazolidine-carrying molecule for strengthening the membrane depends upon its penetration depth.

TABLE I. CONCENTRATION EFFECTS OF SPIN-LABEL ADDITIVES ON OSMOTIC FRAGILITY OF RBC.

ADDITIVE CONCENTRATION (M)	HEMOLYSIS RATIO[a]				
	Etiocholanolone	Pregnanolone	Stearic acid[2]	I(5,10)	I(12,3)
8×10^{-4}	1.00				
6×10^{-4}	0.94				
4×10^{-4}	0.58				
2×10^{-4}	0.43				
8×10^{-5}	0.79	0.39			
6×10^{-5}	0.84	0.34			
4×10^{-5}	0.87	0.34			
2×10^{-5}	0.91	0.51	0.30	0.02	
8×10^{-6}	0.94	0.78	0.49	0.16	0.42
6×10^{-6}	0.95	0.82	0.55	0.25	0.06
4×10^{-6}	0.96	0.85	0.62	0.40	0.04
2×10^{-6}	0.98	0.92	0.75	0.56	0.14
8×10^{-7}		0.96	0.89	0.73	0.41
6×10^{-7}		0.97	0.93	0.77	0.53
4×10^{-7}		0.98	0.99	0.83	0.64
2×10^{-7}		0.99		0.89	0.76
8×10^{-8}				0.93	0.83
6×10^{-8}				0.94	0.84

a. $\frac{\text{Absorbance at 543 nm of supernatant of lysed RBC with additive}}{\text{Absorbance at 543 nm of supernatant of lysed RBC without additive}}$.

At higher reagent concentrations the reagents usually produced lysis above control values.

Singer's[3] concept of a membrane pictures proteins floating in a "sea" of bilayer-formed lipid. It is implied that the proteins have influence over closely adjacent areas of lipid and, by conformational changes, are able to regulate not only gross aspects of the membrane such as osmotic fragility but also its more subtle properties such as those of ion transport. Present information in terms of Singer's concept suggests that stearic acid and its spin-label analogues should, in contrast to our results, weaken the lipid bilayer. This contradiction leads us to the conclusion that the oxazolidine radical has its effect through interaction with proteins which control membrane properties rather than through direct influence on lipid bilayer structure.

Since the labeled molecules bind to RBC, it was necessary to develop an experimental procedure which would permit direct comparison of the "older parameter," S, for bilayer rigidity with the estimate for osmotic fragility. Two major problems had to be considered: (1) osmotic shock experiments involve nonequilibrium solution conditions, whereas EPR experiments require steady-state conditions with RBC suspensions which are stable for the time required to derive a reagent dose response curve, and (2) the sensitivity of the EPR spectrometer is such that the magnetic resonance sample must contain $\sim 10^8$ cells/ml, i.e., and order of magnitude greater than the final RBC concentration in osmotic studies, while at this higher RBC concentration the hemoglobin-lysed cell relationship is nonlinear.

The following modified procedure proved to be satisfactory. Cells were prepared as previously described with the exception that the stock RBC suspension was adjusted to a concentration of $\sim 4 \times 10^9$ cells/ml and stored at 0 C until used. A test mixture containing 50 µl of stock RBC suspension, 1.5 ml of PBS and 50 µl of ethanolic spin-label solution was incubated for 20 min at room temperature. The hemolytic ratio was determined by mixing a 200-µl portion of this suspension with the "shock" solution (1.5 ml hypotonic saline + 50 µl of ethanol) and processing the mixture as previously described. The remaining suspension was centrifuged for 45 sec at 1500 x g, resuspended to a volume of 225 µl with supernatant and employed for EPR studies. EPR observations indicated that the 20-min incubation treatment allowed thermodynamic equilibrium to be established for partition of label between cell and aqueous phases. A thermodynamic calculation using very conservative approximations leads to the conclusion that: if an EPR sample was concentrated by centrifugation and resuspended in a portion of the supernatant after phase equilibrium was achieved, the bound-label:cell ratio remained unchanged and equal to that of cells used for osmotic shock experiments. Experiments were generally performed in duplicate; deviation range for the hemolysis ratio was 4-8% and for the EPR parameter, 3-5%.

The order parameter, S, was compared with the hemolysis ratio for RBC incubated with various concentrations of the stearic acid derivative I(12,3) (Table II). These data indicate that stiffness of the bilayer, as measured

by S, did not change throughout the range of concentrations where significantly decreased osmotic fragility occurred. Values in this experiment cannot be compared with data in Table I because these RBC were exposed to I(12,3) prior to addition of hypotonic solution rather than in the hypotonic solution.

TABLE II. HEMOLYSIS RATIO AND S[a] VALUE FOR RBC EXPOSED TO VARIOUS CONCENTRATIONS OF SPIN-LABELED ADDITIVES.

CONCENTRATION (M)	S	HEMOLYSIS RATIO
2×10^{-5}		0.60
8×10^{-6}		0.25
6×10^{-6}	0.745	0.30
4×10^{-6}	0.743	0.42
2×10^{-6}	0.740	0.75
8×10^{-7}	0.735	0.89
6×10^{-7}	0.733	0.91
4×10^{-7}	0.731	0.95
2×10^{-7}	0.731	0.97

a. S is the EPR order parameter. Hemolysis ratio measured as indicated in text for EPR samples.

Our data reveal that spin labels have strong biological interaction with RBC membranes. This interaction provides dramatically decreased osmotic fragility at certain critical label concentrations and the effects are similar in character and magnitude to those produced by biologically active steroids.

Our investigations demonstrate: (1) over a wide range of perturbing stress, the phospholipid bilayer shows little change in mechanical properties as monitored by S values; and (2) if the nitroxide moiety is placed at the proper depth in the bilayer, the protein-protein interactions responsible for membrane stability are strengthened. These findings provide additional information and confirm the recent proposal for a RBC membrane model.[4]

These findings have stimulated us to reevaluate the relationship between the older concept of membrane stability and more recent ideas[5] of membrane fluidity-rigidity which have been derived from EPR observation of labels in membrane lipid bilayers. Contemporary membrane models suggest that membrane proteins float in a "sea" of lipid arranged in a bilayer form. Current spin-label investigations suggest that this lipid bilayer can be divided into 2 relatively distinct forms: a "non-fluid" portion closely associated with certain proteins and a relatively "fluid" portion in which phospholipids have high lateral mobility. The rigidity (S value) of the "non-fluid" portion is expected to be significantly greater than that of the "fluid" portion. Butler has presented evidence that a given spin label monitors the average bilayer rigidity, characteristic of both portions, biased by relative label solubility in the 2 portions.[6] Application of these concepts to RBC suggests that their membranes have a high proportion of "non-fluid" and a fairly rigid "fluid" portion of bilayer.

In another collaborative program, the effects of perturbants such as toxins or mitogens on alterations of outer cell membrane fluidity-rigidity are being investigated. The experimental system consists of a suspension, with or without cholera toxin, of C57 B mouse thymus cells tagged with a nitroxide-labeled stearic acid probe for EPR measurements of the order parameter, S. In exploratory studies, variables included: (1) cell concentration, (2) presence or absence of toxin and/or the phosphodiesterase inhibitor, RO-20, (3) temperature and time of toxin exposure; and (4) elapsed time for EPR measurements. The adenylate cyclase system was assayed to determine c-AMP accumulation (Work Unit 834 01 003). Preliminary results indicated that: (1) under certain conditions, spin label can interact with the adenylate cyclase system depending strongly on the position of the nitroxide label on the stearic acid chain and hence on the depth of penetration of the nitroxide into the lipid bilayer of the membrane; (2) the thymus cell contains a "reductor" which can chemically reduce the spin label, with tentative evidence that the reductor site is close to the RO-20 action locus; and (3) stability and reproducibility of thymus cell preparations are such that S values have a precision of $\pm$ 2%. Preliminary data indicate that the label does not act as an inert probe but is chemically active. This property has been found with most previously observed viable membranes, as contrasted with biosynthetic bilayers. Such systems are intrinsically more difficult to analyze but, as we have found for the RBC label system, much information can be derived from them under the proper experimental conditions.

Publication:

Hughes, F., and C. E. Pedersen, Jr. 1975. Paramagnetic spin label interactions with the envelope of a group A arbovirus. Lipid organization. Biochim. Biophys. Acta 394:102-110, 1975.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC6413	75 06 02	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
74 07 01	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62760A	3A762760A834	03	010
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Trace metal metabolism during infectious disease of military medical importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
67 01	75 05	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 74	0.5	100.0
FISCAL YEAR CURRENT 75	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Pekarek, R. S.

TELEPHONE: 301 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Hauer, E. C.

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Bacterial and viral diseases; (U) Leukocytic endogenous mediator (LEM); (U) Trace metals; (U) Toxemia; (U) Volunteers; (U) Animal models

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

23 (U) Assess trace metal changes during infectious illness as a possible aid in early identification of disease etiology. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Measure serum and tissue trace metals during infectious illness of laboratory animals and man and determine the mechanisms responsible for the observed changes.

25 (U) 74 07 - 75 05 - Serum trace metal profiles are being determined on patients with complicated and uncomplicated bacterial and viral infections and other inflammatory stresses for computer analysis as part of an ongoing program for the early identification of disease etiology. Significant alterations in both serum chromium and glucose utilization were observed in volunteers infected with sandfly fever virus. When these volunteers were given preexposure and postexposure intravenous glucose tolerance tests significant decreases in both serum chromium disappearance rates and glucose disappearance rates were observed. A highly significant correlation was found. Studies on the effects of infection and inflammation on mortality and metabolism of zinc, amino acid and protein are currently being done on zinc-deficient and pair-fed rats. (Work Unit 834 01 401, Accession No. DA OA6422, where they will be reported.)

This work unit was held active most of the year, for possible assignment to a new investigator. The study will not be continued as a separate work unit. Trace metal profiles will continue to be performed as part of Technical Support Plan, TSP-14.

Publications: Proc. IX Nutr. Congr., Mexico, Vol. 2:193-198, 1975.
Clin. Chem. 21:528-532, 1975.
Diabetes 24:350-353, 1975.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 010: Trace Metal Metabolism During Infectious Disease of Military Medical Importance

Background:

The initiation of acute bacterial or viral infections in man or laboratory animals results in significant alterations in serum, urine and tissue trace metal concentrations. Trace metal profiles observed in volunteers experimentally infected with typhoid fever differed from those observed in volunteers infected with sandfly fever virus or receiving live attenuated VEE virus vaccine. Studies in animal models have shown that these alterations, which represent a redistribution independent of gastrointestinal absorption and increased excretion, may be mediated by leukocytic endogenous mediator (LEM) (Work Unit 834 03 011) during infection and endotoxin intoxication. However, relatively little is known concerning the functional role of these metals during infection or inflammation.

Chromium has been shown to be an essential micronutrient with its deficiency being characterized by impaired glucose tolerance. Recent studies in healthy individuals demonstrated that serum Cr concentrations fell precipitously following IV administration of 30-gm glucose. In individuals experimentally infected with sandfly fever virus a significant decrease in glucose disappearance rates (k) was observed during the height of illness following an IV glucose load even in the presence of elevated insulin levels. Corresponding to these changes in glucose disappearance rates, significant decreases in serum Cr concentrations were also observed. Further, a direct correlation appeared evident between k values for glucose disappearance and preloading serum Cr concentrations in both the normal and infected state of the individual volunteers.

Progress:

Serum trace metal profiles were determined on patients with complicated and uncomplicated bacterial and viral infections and other inflammatory stresses for computer analysis as part of an ongoing program for the early identification of disease etiology. Significant alterations in both serum Cr and glucose utilization were observed in volunteers infected with sandfly fever virus. When these volunteers were given preexposure and postexposure

intravenous glucose tolerance tests significant decreases in both serum Cr disappearance rates and glucose disappearance rates were observed. A highly significant correlation was found.

This work unit was held active most of the year, for possible assignment to a new investigator. The study will not be continued as a separate work unit. Trace metal profiles will continue to be performed as part of Technical Support Plan, TSP-14.

Publications:

1. Pekarek, R. S., and W. R. Beisel. 1975. Redistribution and sequestering of essential trace elements during acute infection. p. 193-198. In Prognosis for the Undernourished Surviving Child. Proc. Ninth Congr. Nutr., Mexico, 1972, Vol. 2 S. Karger, Basel.

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3. Pekarek, R. S., E. C. Hauer, E. J. Rayfield, R. W. Wannemacher, Jr., and W. R. Beisel. 1975. Relationship between serum chromium concentrations and glucose utilization in normal and infected subjects. Diabetes 24:350-353.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6414	75 07 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUM'RY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
75 03 26	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	62760A	3A762760A834	03	011
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Chemical mediators of infection of military medical importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 02	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 75	0.5	112.2
CURRENT FISCAL YEAR 76	1.0	163.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Physical Sciences Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Mapes, C. A.

TELEPHONE: 301 663-7181

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Leukocytic endogenous mediator (LEM); (U) Military medicine; (U) Phagocytosis; (U) Bioassay; (U) Pyrogen; (U) Host metabolism

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

23 (U) Purify LEM to determine whether LEM and endogenous pyrogen are the same molecule; prepare sufficient quantities of active LEM for investigational progress; and develop a pyrogen assay. This is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Study the effects of endogenous mediator(s) on various aspects of host metabolism in order to elucidate the mechanisms involved during infectious illness.

25 (U) 74 07 - 75 06 - Despite striking similarities between LEM and endogenous pyrogen, previous studies within the Institute have attempted to neither correlate nor separate the biological responses attributed to these molecules. Initial projects have included production of LEM, development of a pyrogen assay, studies to delineate differences between LEM and endogenous pyrogen, attempted confirmation of previous reports concerning LEM, and evaluation of butanol-methanol fractionation and SE-Sephadex chromatography as a first step in LEM purification. Currently, efforts are being directed toward purification of LEM and/or endogenous pyrogen.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 011: Chemical Mediators of Infection of Military Medical Importance

Background:

Crude exudates obtained from stimulated PMN alter several biological parameters when administered to laboratory animals. The metabolic alterations observed in rats include: decreased serum Zn and Fe concentrations concomitant with increased liver concentrations; increased concentrations of serum Cu, ceruloplasmin, α_1- and α_2-acute-phase globulins; increased release of neutrophils from bone marrow; a flux of amino acids into the liver; increased RNA synthesis; and decreased portal vein glucose concentrations accompanied by increased concentrations of glucagon and insulin.[1-4] These exudates also contain a substance, classically referred to as endogenous pyrogen (EP), which causes an elevation in temperature when administered to rabbits.[5] The above alterations in metabolism are characteristic host responses during many infectious and inflammatory processes. Thus, it has been postulated that leukocytic endogenous mediator (LEM) may be an intermediate in stimulation and regulation of host responses during some infections, endotoxemia, and acute inflammatory stresses.

Progress:

Since initiating study, primary emphasis has been on production of LEM. Approximately 4 L of LEM have been prepared from the peritoneal exudate cells of 600 rabbits. Dose responses for each preparation were determined with respect to depression of plasma Zn concentrations, release of neutrophils from bone marrow, and a flux of [1-^{14}C]α-aminoisobutyric acid (AIB) into liver. Approximately one-half of the LEM prepared had substandard biological activity.

The following factors were studied in an attempt to obtain consistently active LEM preparations: effect of leukocyte concentration on the release of LEM; stimulation of exudate cells by addition of S. aureus; cell viability; ability of cells to phagocytize; cell lysis during the incubation; weight and presumably the age of rabbits; DNAase treatment of crude LEM; and freezing and thawing of LEM preparations. The results of these studies can be summarized as follows: (1) The problem of obtaining inactive LEM preparations apparently can be circumvented by making the contractor meet specifications for rabbits

weighing 4-6 lb. This statement is based on the observation that active LEM has been obtained from all groups of rabbits weighing >4 lb; while no active preparations were obtained from smaller rabbits. Cell viability was the only demonstrable difference in preparations obtained from the 2 weight groups. Peritoneal exudate cells from the smaller rabbits showed as little as 30% viability; whereas, a minimum viability of 65% was characteristic of cells obtained from larger rabbits. (2) The optimal leukocyte concentration for release of LEM is 10^8 cells/ml. (3) No enhancement of activity was obtained by addition of heat-killed S. aureus to the incubation during release of LEM. (4) No correlation has been made between cell lysis and LEM activity. (5) Neither freezing and thawing nor DNAase treatment were detrimental to LEM.

Pyrogen assay. A colony of 48 healthy, temperature-stable rabbits, intended for long term usage, has been selected by the following criteria: (1) rabbits receive 1 wk of veterinary care, after which, those showing signs of clinical illness are culled; (2) healthy rabbits are monitored 4 consecutive days for temperature stability. Rabbits are acceptable for pyrogen assay if their temperatures are between 101 and 103° F and do not vary more than 0.3° during the last 60 min of a 2-hr selection period. Rabbits not meeting the above criteria on 2 consecutive days are culled from the colony. The health of the rabbits is maintained by oral administration of oxatetracycline on alternate weeks to control respiratory disease and by administration of sulfa drugs as required for control of coccidiosis.

LEM preparations contain a pyrogenic substance that causes optimal fever 45 min after IV injection into the marginal ear vein of rabbits; 50 µl of a typical LEM preparation causes approximately a 2.0° elevation in temperature.

Purification of LEM. Purification has been impeded by a lack of adequate quantities of crude LEM. However, butanol-methanol fractionation and SE-Sephadex chromatography have been evaluated as a first step in the purification. In addition, the possibility of using either glass beads or Concanavalin A-agarose as an affinity adsorbent has been investigated.

Publications:

None.

LITERATURE CITED

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3. Wannemacher, R. W., Jr. Dec. 1972. Current concepts of the regulation of early metabolic changes. p. 113-121. In Commission on Epidemiological Survey, Annual Report FY 1972, to the Armed Forces Epidemiological Board, Wachington, D.C.

4. George, D. T., F. B. Abeles, and M. C. Powanda. 1975. Alterations in plasma glucose, insulin and glucagon induced by a leukocyte derived factor(s). Clin. Res. 23:320A.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6426	75 07 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 HISTY'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
74 07 15	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	62760A	3A762760A834	03	103
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with security Classification Code)

(U) Early recognition of microorganism components or products

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

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: 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 75	1.0	111.0
FISCAL YEAR — CURRENT 76	1.0	120.6

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Bacteriology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic institution)
NAME: Hawley, H. B.
TELEPHONE: 301 663-7341
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Berninger, R. W.
NAME: Dangerfield, H. G. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Counterimmunoelectrophoresis; (U) Streptococcus pneumoniae; (U) Gas chromatography; (U) Rickettsia ricketsii; (U) Bacteroides fragilis; (U) Antigen; (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) Develop reliable procedures for rapid identification of microbial infections. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Use counterimmunoelectrophoresis (CIE) and gas chromatography (GC) to identify infection with organisms of military significance.

25 (U) 74 07 - 75 06 - GC has been used successfully to give characteristic profiles for washed bacteria, including B. fragilis, grown in Brewer's thioglycollate broth. Preliminary studies were initiated to develop an animal model of Bacteroides infection in order to identify this microorganism in serum and abscess fluid.

The GC technique was incapable of distinguishing between R. rickettsii infected duck embryo cell (DEC) cultures and noninfected cell cultures. Therefore, R. rickettsii grown in DEC cultures, inactivated by formalin and purified on a sucrose gradient were obtained from Rickettsiology Division and employed to immunize a rabbit. High titered precipitating antibody appearing within 7 days will permit feasibility studies with the CIE technique.

Studies with CIE are in progress to compare the serum levels of pneumococcal capsular polysaccharide in normal and asplenic rhesus monkeys following IV inoculation with S. pneumoniae and to evaluate the role of this antigen in the pathogenesis of disseminated intravascular coagulopathy reported to occur in asplenic individuals.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 103: Early Recognition of Microorganism Components or Products.

Background:

Counterimmunoelectrophoresis (CIE) and gas chromatography (GC) are techniques which have been shown to be potentially useful for early, specific detection of infectious microorganisms. CIE is an immunoprecipitin technique which utilizes electric current for rapid concentration and combination of negatively charged antigen with positively charged antibody. This technique can provide specific, sensitive identification of anionic antigen when high titer specific antibody is available. GC can detect minute amounts of volatile substances; identification of components in unknown samples may be achieved by comparing their chromatograms with those of known standards. This procedure requires that all samples and standards be chemically treated to form volatile derivatives prior to analysis. Because chromatograms cannot differentiate between a substance intrinsic to an infectious microorganism and the same substance in the milieu of that organism, it is necessary to prepare a chromatogram for each uninfected milieu, e.g., culture medium, serum, etc. Since each milieu presents a different problem in sample preparation, standard chromatograms for the microorganism in every milieu must be prepared.

In the present study application of CIE and GC to development of an early diagnostic capability for Bacteroides fragilis and Rickettsia rickettsii is under investigation. B. fragilis, an anaerobe, causes considerable morbidity and mortality primarily in association with abdominal trauma and wound abscesses. RMSF is the most common rickettsial illness in the United States with a recorded incidence of 774 cases in 1974[1] and a fatality rate of approximately 6%.[2] Antibiotic therapy is effective for both infections if the diagnosis can be made early; however, as yet no laboratory method for early diagnosis is available.

Progress:

In previous studies, immunization of rabbits with B. fragilis antigens prepared by EDTA or phenol extractions produced antibody which was only weakly reactive by CIE with either extract, freeze-thawed organisms, culture supernatants or sonicated organisms.[3] However, efforts were directed towards detection of B. fragilis by GC.

Bacteria were grown in Brewer's thioglycollate broth, harvested, washed twice with saline, once with sterile distilled water and lyophilized. Organisms (1 mg dry weight) were digested with 1 ml 3 N methanolic-HCl for 4 hr at 75-80 C; samples were evaporated to dryness over P_2O_5 and dissolved in 500 µl of pyridine. Volatile silyl derivatives were formed by adding 100 µl of hexamethyldisilazane and 50 µl of trimethylchlorosilane. After mixtures were centrifuged at 1500 rpm for 15 min, ~5 µl of the supernatant was injected into the chromatograph for analysis. The chromatograph was fitted with a 6-ft glass column packed with 3% OV-1 and programmed over time from 120-200 C. In addition to the anaerobic gram negative bacillus, B. fragilis, 4 aerobic gram negative bacteria (Escherichia coli, Pseudomonas stutzgeri, Klebsiella pneumoniae and Serratia marcescens) were studied by GC.

When silyl derivatives of the 5 strains were examined on the same day, 18 chromatographic peaks were identified by characteristic retention time (Table I). Peaks 3, 13, 17 and 18 were unique for B. fragilis, peak 4 for E. coli and peak 16 for S. marcescens.

TABLE I. GAS CHROMATOGRAPHIC RETENTION TIMES.

PEAK NO.	RETENTION TIME CORRELATED (MIN)				
	B. fragilis	E. coli	P. stutzgeri	K. pneumoniae	S. marcescens
1	14.46	14.45	14.42	14.42	14.43
2	14.72	14.92	14.85	14.85	14.86
3	15.35[a]	-	-	-	-
4	-	15.62[a]	-	-	-
5	15.80	-	15.81	15.82	15.82
6	16.45	16.45	16.45	16.45	16.45
7	-	-	-	16.80	16.82
8	17.11	17.12	17.11	17.11	17.10
9	17.51	17.53	17.52	17.49	17.4[illegible]
10	18.02	18.06	18.05	18.05	18.03
11	18.37	18.39	18.39	18.39	18.37
12	-	19.02	-	19.01	18.99
13	19.66[a]	-	-	-	-
14	20.00	20.06	20.03	20.10	20.07
15	-	20.74	20.77	20.73	20.72
16	-	-	-	-	21.12[a]
17	21.43[a]	-	-	-	-
18	22.71[a]	-	-	-	-

a. Peak unique for the organism with respect to others analyzed.

It was postulated that if retention times for peaks in chromatographic profiles for the 5 strains were identical, it might be possible to distinguish strains by differences in relative area associated with each peak. Such a calculation is shown for the peaks common to the 5 strains (Table II). Additional data will be required to determine if this type of analysis would be useful. If an experimental model can be developed, GC analysis of organisms and of biological fluids from infected animals will be initiated.

TABLE II. ANALYSIS OF GAS CHROMATOGRAPHIC PEAKS COMMON TO 5 BACTERIAL STRAINS.

PEAK NO.	AREA (%)				
	B. fragilis	E. coli	P. stutzgeri	K. pneumoniae	S. marcescens
1	4.30	2.58	3.08	2.69	2.84
2	2.63	0.63	0.8€	0.71	0.77
6	53.73	48.69	55.89	56.02	52.47
8	15.95	16.72	19.12	20.28	19.82
9	12.24	18.96	11.36	10.31	12.31
10	2.97	4.39	2.51	1.94	1.60
11	7.47	6.56	6.83	6.48	6.43
14	0.73	1.48	0.36	1.57	3.77
Total area	34326	38048	32941	24198	31223

In a similar analysis performed with R. rickettsii grown in duck embryo cell (DEC) culture, the pattern was not significantly different from that of uninfected DEC culture. Therefore, a different type of sample preparation in which all sugars are converted to oximes prior to formation of the silyl derivatives was tried. The chromatograph column was packed with 3% OV-17 and programmed over time from 140-250 C. However, it still was impossible to differentiate between R. rickettsii-infected and -uninfected cells.

The use of CIE for detecting R. rickettsii has been limited by the lack of high titered precipitating antibody. Recently we obtained from Dr. Kenyon (Rickettsiology Division) a lyophilized preparation of formalin-killed R. rickettsii, Sheila Smith strain, grown in DEC culture and purified by

sucrose gradient centrifugation. This material was reconstituted to contain 10^8 rickettsiae/ml in sterile pyrogen-free water for injection; 0.5-ml of this suspension was emulsified with 0.5-ml CFA; a 0.5-ml dose was inoculated IM into the flank of a rabbit. A 0.5-ml booster without adjuvant was administered SC 10 days later. Serial bleedings were performed at weekly intervals and sera were titrated by the CIE technique for antibody against the R. rickettsii purified antigen (Table III). High titered precipitating antibody developed within 7 days following the initial injection. Based upon these encouraging results, studies are in progress to immunize additional rabbits and to utilize the existing antiserum to evaluate CIE for detection of antigen in sera from R. rickettsii infected animals or humans.

TABLE III. CIE PRECIPITATION REACTION OF PURIFIED R. RICKETTSII AND RABBIT POSTIMMUNIZATION SERA.

DAYS AFTER PRIMARY INOCULATION	RECIPROCAL SERUM DILUTION				
	100	200	400	800	1600
0	-				
7	+	+	+	+	
15	+	+	+	+	
21	+	+	+	+	-

CIE methodology previously used for detection of Streptococcus pneumoniae[4] has been modified to conform to that described by Coonrod and Rytel[5] and is being employed to investigate the pathophysiology of pneumococcal infection in normal and asplenic rhesus monkeys. Asplenic humans are unusually susceptible to severe, and often fatal, pneumococcal illness for reasons which are yet unknown. Rytel et al.[6] have recently presented data from 2 patients (one asplenic) suggesting a causative role for pneumococcal capsular antigens in the development of disseminated intravascular coagulation (DIC) which is frequently the terminal event in pneumococcal sepsis. Pneumococcal capsular polysaccharide (PCP) has been purified from 3 L of S. pneumoniae, type I (ATCC 6301), broth culture with a modification of the Kabat and Mayer procedure[7] and employed as a standard for quantitative CIE determinations. With specific S. pneumoniae type I antibody (Difco) concentrations $\geq$0.25 µg PCP/ml were detected. The purified PCP was also used to detect antibody in the micro-MHA test. Bacteremia, antigenemia and antibody were evaluated in blood from normal rhesus monkeys following IV inoculation with 10^8 S. pneumoniae type I (Table IV). All monkeys recovered after penicillin

therapy; none developed DIC. Asplenic rhesus monkeys inoculated IV with 10^6 S. pneumoniae are currently being evaluated. Preliminary evidence suggests that at least one of the animals has developed DIC.

TABLE IV. DEVELOPMENT OF BACTEREMIA, ANTIGENEMIA AS DETECTED BY CIE AND HA ANTIBODY IN RHESUS MONKEYS FOLLOWING INOCULATION WITH TYPE I S. PNEUMONIAE.

PARAMETER	MONKEY NO.[a]	VALUE BY EXPERIMENTAL DAY							
		-4	-1	0	1	2	3	4	6
Bacteremia (no./ml)	583		-	-	$2x10^3$	$4x10^3$	$1x10^2$	-	-
	591		-	-	$1x10^6$	$2x10^4$	$5x10^4$	-	-
	830		-	-	$1x10^6$	$3x10^3$	$7x10^3$	-	-
	Control		-	-	-	-	-	-	-
Antigenemia (µg PCP/ml serum	583	-	-	-	-	0.25	-	-	-
	591	-	-	-	0.25	0.25	0.5	-	-
	830	-	-	-	-	0.25	-	-	-
	Control	-	-	-	-	-	-	-	-
Reciprocal micro HA titer	583	-	-	-	-	-	-	40	640
	591	-	-	-	-	-	-	80	1280
	830	-	-	-	-	-	-	40	640
	Control	-	-	-	-	-	-	-	-

a. Monkeys 583, 591 and 830 inoculated IV on day 0 with 10^8 S. pneumoniae type I; control (803) inoculated IV with sterile culture medium. All animals treated IM on days 3, 4 and 6 with 300,000 units Procaine Penicillin.

Publications:

None.

LITERATURE CITED

1. Center for Disease Control. 1974. Cases of specified notifiable diseases: United States. Morbidity Mortality 23:441.

2. Center for Disease Control. 1974. Fatal Rocky Mountain spotted fever. Morbidity Mortality 23:301-302.

3. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1974. Annual Progress Report, FY 1974. pp. 425-432. Fort Detrick, Md.

4. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1973. Annual Progress Report, FY 1973. pp. 403-407. Fort Detrick, Md.

5. Coonrod, J. D., and M. W. Rytel. 1973. Detection of type-specific pneumococcal antigens by counterimmunoelectrophoresis. I. Methodology and immunologic properties of pneumococcal antigens. J. Lab. Clin. Med. 81: 770-777.

6. Rytel, M. W., T. H. Dee, J. E. Ferstenfeld, and G. T. Hensley. 1974. Possible pathogenic role of capsular antigens in fulminant pneumococcal disease with disseminated intravascular coagulation (DIC). Am. J. Med. 57: 889-896.

7. Kabat, E. A., and M. M. Mayer. 1961. Experimental Immunochemistry, 2nd ed. Charles C. Thomas, Springfield, Ill., pp. 840-842.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6422	75 07 01	DD-DR&E(AR)634

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
74 07 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	3A061101A91C	00	133
b. CONTRIBUTING	62760A	3A762760A834	03/105	
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Laser beam scattering for rapid identification of bacteria

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
73 01	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 75	0.5	25.0
	CURRENT 76	0.5	25.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Pathology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Altenbern, R. A.

TELEPHONE: 301 663-5371

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Metzger, J. F.

NAME:

POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Pathogens; (U) Identification; (U) Bacterial diseases; (U) Lasers

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

23 (U) Evaluate the usefulness of scattering of a laser beam for rapid identification of bacteria from a variety of sources. This is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Utilizing equipment initially developed by Science Spectrum, Inc., under contract with the U. S. Army Medical Research and Development Command, examine the light scattering characteristics of a variety of bacteria.

25 (U) 74 07 - 75 06 - Technical difficulties with the laser beam apparatus have prevented any meaningful accumulation of data. It is anticipated that, in the coming year, some definitive information will be obtained.

* 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 65 AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3A061101A91C: In-House Laboratory Independent Research (U)
(3A762760A834):

Task No. 3A061101A91C 00:
(3A762760A834 03): (Laboratory Identification of Biological Agents)

Work Unit No. 91C 00 133: Laser Beam Scattering for Rapid Identification
(834 03 105): of Bacteria

Background:

Contract reports from Science Spectrum, Inc. claimed that the ratio of the third maximum to the first minimum obtained in a laser scan of a single bacterial cell was characteristic for each of the bacterial species employed. The objective of the current project was to determine if various genera of the enteric bacteria could be differentiated by this method.

Progress:

There has been no progress in the work due to frequent and extended malfunctioning of the laser beam apparatus.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OC6418	75 07 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
74 07 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	62760A	3A762760A834	03	403
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code): (U) Separation and purification of arbovirus agents and their rapid detection in clinical specimens

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 10100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
61 11	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

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING 75	1.0	121.6
CURRENT 76	1.0	123.6

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL
NAME: Metzger, J. F.
TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION
NAME: Virology Division USAMRIID
ADDRESS: Fort Detrick, MD 21701

PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Levitt, N. H.
TELEPHONE: 301 663-7241
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Cellulose acetate electrophoresis; (U) Immunology; (U) Arboviruses; (U) Serology; (U) Antigen-antibody reactions; (U) Fluorescent antibody; Encephalitis, equine (WEE)

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

23 (U) Develop methodology for the rapid detection and identification of group A arboviruses causing militarily significant disease. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Viruses propagated in tissue culture are identified by the detection of specific antibody complexed to radioactive 125-I labeled gamma globulin.

25 (U) 74 07 - 75 06 - A solid phase radioimmunoassay has been developed for the rapid detection and identification of WEE virus. Primary duck cell monolayers, grown in 1 x 3 cm vials, were inoculated with specimens containing virus in concentrations of 2 logs or more. After overnight incubation, cell monolayers were fixed and exposed to either specific iodine-labeled globulin or unlabeled specific rabbit antiserum and then iodine-labeled goat antirabbit gamma-globulin. After washing to remove unbound isotope, vials were counted in a gamma counter. This technique allows us to detect and identify WEE virus in clinical specimens within 24 hr.

Publications: Life Sci. 15:37-44, 1974.
Am. J. Trop. Med. Hyg. 24:127-130, 1975.

* Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 403: Separation and Purification of Arbovirus Agents and Their Rapid Detection in Clinical Specimens

Background:

There is an urgent need for more rapid and economical methods of arbovirus detection. While available methods allow for detection, quantitation and differentiation of most arboviruses, they require a long period of time. Elimination of the need for animal assays and certain tedious biological procedures would greatly facilitate the detection and identification process.

We previously reported the development of a microprecipitation test (MPT) for the rapid detection of viral antibody and a modification of this MPT employing fluorescein-conjugated γ-globulin for the detection and differentiation of the 3 group A arboviruses: VEE, WEE and EEE.[2] We are presently reporting the development of a solid phase radioimmunoassay (RIA) using ^{125}I-labeled γ-globulin for the rapid identification of selected group A arboviruses.

Progress:

Our laboratory, in continuing to pursue the development of methods for rapid identification of viruses, has developed a solid phase radioimmunoassay (RIA), for the identification of certain group A arboviruses using a procedure similar to the fluorescent antibody technique. Forghani et al.[3] recently described a similar technique using Herpes virus as the test virus.

In brief, monolayers of primary duck embryo cell culture (DEC), were infected with virus, incubated overnight, fixed with acetone, then either exposed directly to ^{125}I-labeled specific γ-globulin or exposed first to unlabeled specific rabbit antiserum and then to ^{125}I-labeled goat antirabbit γ-globulin. The latter indirect method utilizing ^{125}I-labeled goat antirabbit γ-globulin eliminates the requirement for numerous labeled specific antiviral globulins.

DEC monolayers were grown in 1 x 3-cm vials (10^4 cells/vial) which could be placed inside an isotope counting tube after test manipulation. WEE virus was used as the model virus to demonstrate the specificity and sensitivity of the procedure. VEE-virus infected cells served as heterologous virus controls. I-labeling of γ-globulin, prepared by $(NH_4)_2 SO_4$ fractionation, was accomplished by the lactoperoxidase procedure described by Marchalonis.[4] DEC monolayers were inoculated with varying concentrations of WEE virus. Concurrently, replicate cell cultures were inoculated with VEE virus. Additional uninfected vials served as control specimens for use in determining normal values. After overnight incubation at 35 C, cells were acetone fixed and ^{125}I-labeled anti-WEE γ-globulin was added. Vials were incubated 2 hr, rinsed 5 x with PBS, and counted. A ratio was determined from the infected (I) vial count and a corresponding uninfected or control (C) vial count. An I:C ratio of 2 was selected to indicate positive vials.

Results of this experiment are seen in Table I. All vials inoculated with ≥ 2 logs of WEE virus were positive after overnight incubation. The specificity of the procedure was demonstrated by the lack of reactivity of cells infected with VEE virus at all concentrations tested.

An experiment was designed to compare the direct and indirect methods of the RIA. Replicate vials of DEC were inoculated with relatively low concentrations of WEE virus and incubated overnight. After acetone fixation, the vials were assayed either directly by exposure to ^{125}I-labeled anti-WEE γ-globulin or indirectly by first being absorbed with unlabeled rabbit anti-WEE serum and then exposed to ^{125}I-labeled goat antirabbit γ-globulin. Vials were rinsed 5 x with PBS in the direct method to remove unbound isotope. In the indirect method the vials were rinsed 3 x with PBS after 2 hr incubation with specific rabbit antiserum and 5 x after 1-hr incubation at 35 C with ^{125}I-labeled goat antirabbit γ-globulin.

The results of this experiment are found in Table II. As anticipated the indirect method was significantly more sensitive when compared to the direct method. In addition, it eliminated the requirement of numerous labeled, specific antiviral globulins.

A final experiment was performed to examine the feasibility of using this solid-phase RIA for the detection and identification of WEE virus in clinical specimens. Corresponding tissue specimens from infected and non-infected fetuses of rhesus monkeys were examined in parallel for virus content by both a plaque assay and our RIA procedure. The selected tissues were homogenized with tissue grinders, centrifuged at 800 xg for 20 min and the resultant supernatants used as inocula for testing.

It can be seen (Table III) that all tissues tested from the infected fetus contained WEE virus as determined by plaque assay. Each of these tissues when tested by RIA after 23 hr growth, resulted in I:C ratios of >2.0. These ratios were indicative of WEE virus infection.

We have described a solid-phase RIA for the rapid detection and identification of WEE virus. It appears that this technique could be used for selected group A arboviruses and other viruses of clinical significance.

TABLE I. SPECIFICITY OF SOLID-PHASE RADIOIMMUNOASSAY

INPUT VIRUS (Log PFU/ml)	I:C RATIO[a]	
	WEE	VEE
6	5.9	1.2
5	6.0	1.5
4	5.8	1.2
3	4.6	1.3
2	2.2	1.0
1	0.9	1.1

a. Determined by counts of infected vials divided by counts of uninfected vials.

TABLE II. COMPARATIVE SENSITIVITIES OF DIRECT AND INDIRECT METHODS

INPUT VIRUS (Log PFU/ml)	I:C RATIO	
	Indirect	Direct
4	7.1	2.8
3	8.7	2.9
2	8.6	1.5
1	3.9	1.0

TABLE III. RADIOIMMUNUNOASSAY OF WEE VIRUS-INFECTED TISSUES

SPECIMEN[a/]	VIRUS TITER[b/] (PFU/ml)	I:C RATIO[c/]
Optic nerve	1.5×10^2	3.9
Eye	5.0×10^2	3.0
Cerebellum	5.0×10^5	4.2
Choroid plexus	6.5×10^3	4.2
Spinal fluid	8.0×10^3	3.3

a. Fetal rhesus monkey tissues.
b. Determined by plaque assay.
c. Control value (C) was obtained from vials inoculated with corresponding tissue fluids from an uninfected monkey fetus.

Publications:

1. Levitt, N. H., K. R. Amsler, R. W. McKinney, and D. M. Robinson. 1974. Microprecipitation test for the detection of adenovirus antibody. Life Sci. 15:37-44.

2. Levitt, N. H., H. V. Miller, C. E. Pedersen, Jr., and G. A. Eddy. 1975. A microprecipitation test for rapid detection and identification of Venezuelan, eastern and western encephalomyelitis viruses. Am. J. Trop. Med. Hyg. 24:127-130.

LITERATURE CITED

1. Levitt, N. H., K. R. Amsler, and R. W. McKinney. 1971. Rapid detection of viral antibody by cellulose acetate electrophoresis. Appl. Microbiol. 22:143-144.

2. Levitt, N. H., H. V. Miller, C. E. Pedersen, Jr., and G. A. Eddy. 1974. A microprecipitation test for rapid detection and identification of Venezuelan, eastern, and western encephalomyelitis viruses. Am. J. Trop. Med. Hyg. 24:127-130.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6419	75 05 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
75 03 26,	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	62760A	3A762760A834	03	404
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Early immune response in Venezuelan equine encephalitis infection

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
70 12	74 08	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 75	1.0	140.6
	CURRENT 76	0	0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Virology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: McManus, A. T.

TELEPHONE: 301 663-7241

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Arboviruses; (U) Antigen-antibody reactions; (U) Serology; (U) Military medicine; (U) Encephalitis, equine (VEE)

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

23 (U) Investigate antibody classes, the role of the complement system and the physiological and immunological bases for the second febrile response in VEE-infected monkeys. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Infect monkeys with VEE and measure a variety of parameters, including phagocytosis, pathology and competitive antiglobulin neutralization.

25 (U) 73 07 - 74 08 - Ontogeny of the immunoglobulin classes in VEE-infected rhesus monkeys was examined. Sucrose density gradient fractionation of convalescent rhesus monkey sera showed 19S neutralizing antibody present 7 days postinfection. Peak 19S titer was at day 12. Days 15 and 18 showed reducing titers, and specific macroglobulin was barely detectable by day 21. The 7S neutralizing antibody response was detected at day 12 postinfection with infection and plateau at day 18.

The investigator has left the Institute. No further work is planned on this work unit.

Publication: Trans. Royal Soc. Trop. Med. Hyg. 69:172, 1975.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 404: Early Immune Response in Venezuelan Equine Encephalitis Infection

Background:

An understanding of the pathophysiological basis of a clinical infectious disease is of obvious advantage in attempting to prevent or treat that disease. Observations of viral infections in nonhuman primates may offer an experimental model for obtaining information applicable to human disease.

Progress and Summary:

Ontogeny of the immunoglobulin classes in VEE-infected rhesus monkeys was examined. Sucrose density gradient fractionation of convalescent rhesus monkey sera showed 19S neutralizing antibody present 7 days postinfection. Peak 19S titer was at day 12. Days 15 and 18 showed reducing titers, and specific macroglobulin was barely detectable by day 21. The 7S neutralizing antibody response was detected at day 12 postinfection with infection and plateau at day 18.

The investigator has left the Institute. No further work is planned on this work unit.

Publications:

1. McManus, A. T., and G. A. Eddy. 1975. Increased potency of killed VEE (TC-83) vaccine in mice previously infected with EEE virus. Trans. Royal Soc. Trop. Med. Hyg. 69:172.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6420	75 07 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
74 07 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	62760A	3A762760A834	03	405
b. CONTRIBUTING				
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Study Bolivian hemorrhagic fever and other Tacaribe group viruses

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense;

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 06	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 75	2.0	204.7
	CURRENT 76	3.0	306.9

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Virology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Eddy, G. A.

TELEPHONE: 301 663-7241

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Scott, S. K.

NAME: Brand, O. M. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede each with Security Classification Code)

(U) Bolivian hemorrhagic fever; (U) Machupo virus; (U) Arenavirus; (U) Viral immunity; (U) Viral pathogenesis

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

23 (U) Define a rhesus monkey model for Bolivian hemorrhagic fever (BHF), attempt to prepare a killed vaccine against the virus, define the efficacy of prophylaxis and treatment with immune serum, study the pathogenesis of the disease, explore supportive therapy and conduct a joint study of a candidate attenuated vaccine virus with the Middle America Research Unit. This work unit is an essential element in a comprehensive program for defense against BW agents.

24 (U) Evaluate efficacy of human BHF immune globulin, study drug therapy, study pathogenesis and continue efforts to attenuate Machupo virus.

25 (U) 74 07 - 75 06 - The dosage of BHF immune globulin (human origin) which protects monkeys against severe disease from Machupo virus infection has been determined. The phenomenon of late encephalitis which appears to result from a supraoptimal dose of the human product has been investigated. Vaccine development efforts have included a study of cross-protection by other viruses of the Tacaribe complex, and attempts to attenuate Machupo virus by passage in chick embryo cells. Results indicate that significant cross-protection against Machupo virus occurred with both Tacaribe and Tamiami viruses and that a combination of 2 viruses was more protective than one. Attempts to attenuate Machupo virus have suggested some attenuation after 20 passages in chick embryo cells. Other experimental work included interferon sensitivity studies, a stabilized poly-I:poly-C trial, antiviral studies with drug clofibrate and further exploration of viral pathogenesis.

Publications: Proc., Army Sci. Conf., Vol. I:293-302, 1974.
Arthropod-borne Virus Information Exchange 28:135-136, 1975.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A762760A834: Medical Defense Against Biological Agents (U)

Task No. 3A762760A834 03: Laboratory Identification of Biological Agents

Work Unit No. 834 03 405: Study Bolivian Hemorrhagic Fever and Other Tacaribe Group Viruses

Background:

Having shown that monkey immune serum can be successfully employed in both the treatment and prophylaxis of BHF in monkeys, the model was a logical tool for assessment of the human product. Moreover, certain unusual features of the rhesus model, i.e., immunity in the absence of detectable antibody, warranted further exploration.

Progress:

I. Evaluation of BHF Immune Globulin (human origin). A project to plasmapherese 16 immune volunteer donors in Bolivia yielded 220 units of plasma which were fractionated and lyophilized at the Massachusetts State Laboratory. Our in vitro test showed that the product had a PRNT titer of about 1:2000 at the 80% plaque reduction level. A study was undertaken to test the efficacy of the human globulin in protecting monkeys from severe disease if they were treated 4 hr after inoculation of 100 PFU of Machupo virus. The results are shown in Table 1.

These data show that administration of antibody shortly following exposure clearly protected monkeys from the typical clinical disease which usually commences on day 6 or 7 and continues until death. Monkeys in the 2 higher dosage groups showed little or no clinical evidence of illness. However, many of these monkeys developed severe neurological signs of illness on about day 40. This late neurological disease was characterized by acute onset of paresis or paralysis, incoordination, deep depression and coma. Death occurred within a few days after onset of signs and the course was unlike that described for initial clinical signs. There was a strong suggestion that the highest dosage of globulin was more likely to result in late neurological signs than the middle dosage level.

A second and similar study was initiated to further explore this phenomenon. The second study was similar to the first except that cynomolgus rather than rhesus monkeys were used and the dosages of globulin were decreased somewhat.

TABLE I. PRELIMINARY DOSE-RESPONSE STUDY OF PROPHYLACTIC EFFICACY OF BHF IMMUNE GLOBULIN (HUMAN) IN RHESUS MONKEYS

DOSE ml/kg	INITIAL CLINICAL SIGNS			LATE NEUROLOGICAL SIGNS		
	Positive/ Total	Days Duration	Deaths (mean day)	Positive/ Total	Dead/ Total	Mean Day of Onset (death)
1.5	0/3	0	0	3/3	2/3	41 (46)
0.5	0/3	7	0	1/3	1/3	46 (51)
0.15	3/3	≥14	1 (19)	0/2	0/2	-
None	3/3	≥14	3 (19)	-	-	-

TABLE II. DOSE-RESPONSE STUDY OF EFFICACY OF BHF IMMUNE GLOBULIN IN CYNOMOLGUS MONKEYS

DOSE ml/kg	INITIAL CLINICAL ILLNESS			LATE NEUROLOGICAL SIGNS		
	Positive/ Total	Days Duration	Deaths (mean day)	Positive/ Total	Mean day of onset (death)	Dead/ Total
1.0	0/3	-	0	1/3	30 (33)	1/3
0.3	0/3	-	0	1/3	34 (39)	1/3
0.1	3/3	≥14	3 (24)	-	-	-
None	2/2	≥14	2 (23)	-	-	-

A third study is still underway and the preliminary results suggest that the treatment with human BHF globulin prior to virus infection avoids the late neurological problem completely. From the standpoint of laboratory accidents however, this is not feasible, and we are obliged to seek a dosage that optimizes both the avoidance of initial clinical and late neurological disease. Inasmuch as severe neurological signs are not a prominent feature of human illness in BHF, our principal concern is with the initial clinical signs.

II. Cross Protection by Other Viruses of the Tacaribe Complex.

We carried out a series of experiments to measure the degree of cross protection provided by arenaviruses of the Tacaribe complex against Machupo virus infection of rhesus monkeys. Six different arenaviruses listed in Table III were inoculated into rhesus monkeys at the specified dosages. The monkeys were held for various periods of time as indicated and then challenged with Machupo virus. The responses to challenge were measured. Only 2 of the viruses given alone protected well against challenge. Tacaribe virus given at a dose of 6.3 $\log_{10}$ 60 days before Machupo virus challenge protected the 2 recipients against severe disease and detectable viremia (Group I). However, a 10-fold lower dose, 5.3 $\log_{10}$, of the same virus gave less protection when the monkeys were challenged 58 days later, as evidenced by severe illness in the monkeys and the subsequent death of one (Group II). Nevertheless this lower dose of Tacaribe virus protected monkeys in Groups III and IV challenged with Machupo virus 5 or 21 days after Tacaribe virus inoculation. A dose of 8 $\log_{10}$ of Tacaribe virus given at the time of Machupo virus inoculation did not prevent severe disease although 1 of 2 monkeys survived (Group V).

Tamiami virus also gave substantial protection against Machupo virus. Each of 2 monkeys in Group VI which received 6.0 $\log_{10}$ of Tamiami virus were protected against severe disease when challenged with Machupo virus 2 mon later. These 2 monkeys exhibited little or no viremia; their clinical signs were mild to moderate. One of the 2 monkeys in Group VI inoculated with Amapari virus showed no clinical response to Machupo virus challenge 58 days later, whereas the other became severely ill and died relatively early, day 14. All surviving monkeys in the study developed neutralizing antibody to Machupo virus by day 28 except one monkeys in Group IV.

Pichinde, Latino and Parana viruses given alone did not protect the monkeys from severe disease following Machupo virus challenge 2 mon later (Groups VIII, IX and X). Two other monkeys, Group XI, given Parana and Latino viruses simulataneously were partially protected against Machupo virus challenge. They exhibited only moderate clinical signs and little or no viremia.

None of the monkeys showed evidence of clinical disease during the 2 mon following inoculation of Pichinde, Parana, Amapari, Tacaribe or Tamiami virus. One of 2 monkeys inoculated with Latino virus developed facial edema. We do not know whether this was related to Latino virus inoculation.

Although these studies were carried out with small groups of monkeys. they indicate a significant cross-protection of monkeys against Machupo virus by some of the viruses of the Tacaribe complex. The protection may be relatively brief and may be closely associated with the dose of cross-protecting virus. The data indicate that the monkeys responded serologically to the original virus prior to challenge, but the evidence indicates that the protection afforded did not correlate well with either neutralizing or CF antibody against Machupo virus. Moreover, high doses of monkey anti-Tacaribe immune serum did not protect against Machupo virus infection and disease.

TABLE III. CROSS-PROTECTION AGAINST MACHUPO VIRUS IN RHESUS MONKEYS BY OTHER VIRUSES OF THE TACARIBE COMPLEX

GROUP	CROSS-REACTING VIRUS			RESPONSE TO MACHUPO CHALLENGE[a]		
	Virus	Dose Log_{10}	Day Inoculated	Severity of Illness (No.)	Viremia[b]	Dead/ Total
I	Tacaribe	6.3	-60	Mild (2)	None	0/2
II	Tacaribe	5.3	-58	Severe (2)	Intermittent to typical	1/2
III	Tacaribe	5.3	- 5	None to mild (2)	None	0/2
IV	Tacaribe	5.3	-21	Mild (2)	None to intermittent	0/2
V	Tacaribe	8.0	0	Severe (2)	Typical	1/2
VI	Tamiami	6.0	-58	Mild to moderate (2)	None to intermittent	0/2
VII	Amapari	6.0	-58	None (1) Severe (1)	None Typical	1/2
VIII	Pichinde	7.3	-60	Severe (2)	Typical	1/2
IX	Latino	5.4	-60	Severe (2)	Typical	2/2
X	Parana	6.8	-60	Severe (2)	Typical	2/2
XI	Parana + Latino	6.8 5.4	-60	Mild to moderate	None to intermittent	0/2
XII	Controls	None	-	Severe (6)	Typical	6/6

a. All monkeys were challenged with approximately 1000 PFU of Machupo virus on day 0.

b. Viremia was measured on days 7, 10, 14 and 17. Typical viremia consisted of detectable circulating virus on at $\geq$ 3 of the 4 days sampled. Intermittent viremia was defined as detectable circulating virus on at least one but <3 of the 4 days tested.

III. Drug and Interferon Studies. There is in vitro evidence that the drug clofibrate, which is used therapeutically in controlling lipid metabolism, reduced the replication of arenaviruses by a factor of 1000. A preliminary in vivo study with Machupo virus in monkeys gave equivocal results; we currently are carrying out a similar drug trial in guinea pigs. There are some difficult dosage problems with this drug which in humans is given orally 2 to 4 times daily. We are attempting to give the drug IM.

We have also tested a poly l-lysine stabilized form of poly-I:poly-C in Machupo-virus infected monkeys. The drug had no effect. This may have been due in part to the relative insensitivity of Machupo virus to interferon. We tested a series of arenaviruses against primate interferon and found that the viruses tested were approximately 20- to 100-fold less sensitive to interferon than was vesicular stomatitis virus (VSV). The exception was Machupo virus which was 1000-fold less sensitive to interferon than VSV.

IV. Adaptation of Machupo Virus to Chick Embryo Cell Culture. We have used a sample of human spleen material from a fatal case of BHF as a source of virus for vaccine development. Virus was isolated in certified FRhL cells and then carried in chick embryo cell culture in 2 separate passage lines. Both virus lines showed indications of progressive adaptation to chick cells as shown in Table IV.

TABLE IV. EFFECT OF CHICK EMBRYO CELL CULTURE PASSAGE ON TITER OF MACHUPO VIRUS

STRAIN	PASSAGE LEVEL IN CHICK EMBRYO CELLS				
	2	5	10	15	20
1	1.6×10^2	1.6×10^2	1.7×10^3	5.6×10^4	2.7×10^5
2[a]	-	-	1.2×10^4	1.6×10^4	1.2×10^5

a. Strain 2 was derived from strain 1 after passage 5.

Preliminary evidence in guinea pigs indicated that virulence was lost quite rapidly even by intracerebral challenge. We found that passage 7 was still highly virulent for monkeys but no subsequent passages have been tested completely. Currently, passages 18 and 23 of strain 2 are being tested in monkeys and the results will be forthcoming within a few weeks. The marked adaptation to chick cells suggests that the virus has been altered genetically.

V. Pathogenesis in Rhesus Monkeys. We have carried out a few studies intended to shed some light on the pathogenesis of Machupo virus in rhesus

monkeys. All studies were preliminary in nature and may be followed up by more extensive investigations either in monkeys or guinea pigs. In general we observed that immunosuppresion prior to infection did not alter the course of illness although it did delay the onset of viremia. Immunosuppression after inoculation of virus resulted in a rapid fulminating disease course with death in 7 - 10 days. Antigenic stimulation with Salmonella typimurium prior to infection resulted in more rapid onset of viremia and higher viremia titers. It was of interest that Tacaribe virus immune serum offered no protection against Machupo virus, and in fact resulted in a more rapid onset of viremia. These observations remain unexplained as yet.

Publications:

1. Eddy, G. A. Mar 1975. A preliminary report on the cross protection by arenaviruses of the Tacaribe complex against Machupo virus. Arthropod-borne Virus Information Exchange 28:135-136.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6413	75 07 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
74 07 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	3A061101A91C	00	131
b. CONTRIBUTING	62760A	3A762760A834	03/407	
c. CONTRIBUTING	Cards 114(e) (f)			

11. TITLE (Precede with Security Classification Code)

(U) Morphogenesis and morphology of Venezuelan equine encephalomyelitis virus

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002600 Biology (Pathology)

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
74 05	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 75	1.0	30.0
	CURRENT 76	1.0	30.0

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

NAME: Metzger, J. F.

TELEPHONE: 301 663-2833

20. PERFORMING ORGANIZATION

NAME: Pathology Division USAMRIID

ADDRESS: Fort Detrick, MD 21701

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

NAME: Veltri, B. J.

TELEPHONE: 301 663-7211

SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Encephalitis, equine (VEE); (U) Morphogenesis; (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) Study by electron microscopy VEE virus morphology during various stages of morphogenesis. This work unit is an essential element in a comprehensive program for medical defense against BW agents.

24 (U) Examine VEE-infected cells using electron microscopic, stereoscopic, freeze-fracture, freeze-etch, and ultrathin sectioning techniques.

25 (U) 74 07 - 75 06 - Electron microscopy has provided important information on development characteristics of group A arboviruses. Ultrathin sections demonstrated the presence of virus precursors in cells infected with several members of the group, as well as budding at the cell surface. Morphological features found within the first 9 hr were seen at later times in infection, showing that virus multiplication became asynchronous after the first cycle. Formation of virions took place on cell surfaces with the participation of the vacuole membrane; the envelope is formed of the cytoplasmic or vacuole membrane. Studies of freeze-fractured and -etched viruses revealed a fracture plane through the core; several nucleic acid strands were seen in the core. Fracture planes were noted through and on the cell envelope. There was a fracture above the virus projections and another through the projections. A hollow space measured ca. 1 nm in diameter. Projections are 6 nm and virus core and envelope are 50 nm in diameter. The length of the projections measures ca. 10 nm, thus making the VEE virus a 70-nm sphere.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3A061101A91C: (3A762760A834): In-House Laboratory Independent Research (U)

Task No. 3A061101A91C 00: (3A762760A834 03): (Laboratory Identification of Biological Agents)

Work Unit No. 91C 00 131: (834 03 407): Morphogenesis and Morphology of Venezuelan Equine Encephalomyelitis Virus

Background:

VEE virus, a member of the group A arboviruses, propagates in several primary and continuous cell cultures. Although investigators have been studying the pathogenesis of VEE virus, few have worked on the morphogenesis and morphology.[1]

Viruses in the serological group A are known to be lipid-containing RNA[2] viruses which consist of a core particle enveloped by a membrane. This virus is a convenient model for morphological, biological, and biochemical studies because of its short cycle of reproduction. Earlier it had been shown that VEE virions have a diameter of 60-75 nm, and consist of a lipoprotein envelope and a spherical core (nucleoid) with a diameter of 30-40 nm.

Electron microscopy has provided important information on the development characteristics of group A arboviruses. Ultrathin sections have demonstrated the presence of virus precursors in cells infected with several members of the group, and the budding of virus particles at the cell surface.

Progress:

Ultrathin sections of cells infected with VEE virus at input multiplicities of 100-200 PFU/cell were studied at various intervals after infection, 6-18 hr. Morphological features found within the first 9 hr were also observed at later times of infection, thus showing that virus multiplication became asynchronous after the first cycle.

Fibrillar structures which were never observed in noninfected monkey kidney cells (LLC-MK_2) were detected in the cytoplasm 6 hr after infection. Structures composed of fibrillar components, aggregates of ribosomes, and viral nucleoids were also observed in the cytoplasm at later stages of infection. Fibrillar components consisted of cylindrical structures with a diameter of $\sim$ 40 nm almost 2 μm in length.

Nucleoids of VEE virus form in close proximity to the fibrillar component emerging from the hemispheres of the cylindrical structures. When a nucleoid was oriented in the plan of the ultrathin section, an osmiophobic hollow with a diameter of 4-8 nm was revealed, the whole nucleoid being composed of osmiophilic fibrils packed in an organized manner.

Formation of virions took place on the cell surface with the participation of the cell membrane or in cytoplasmic vacuoles with the participation of the vacuole membrane. The envelope of the virions is formed of the cytoplasmic or vacuole membrane.

Freeze-fracture and etching techniques were used in this study to demonstrate fractured surface planes of the virus core, virus membrane, nucleic acid strands, and viral projections. This technique includes double replicas, i.e., one replica is the mirror image of the complementary replica. Double replicas help interpretation by providing positive identification of complementary surfaces. Stereoscopic analysis of replicas provide a better guide to relative elevation through shadowing, and provides more information, better resolution, and identification of replica artifacts. This approach reveals information heretofore unnoticed in a 2-dimensional plane.

Although ultrathin sections confirmed most of the findings of Bykowsky et al.[1] on VEE virus, freeze-fracture[3] preparations have revealed information of the internal and outer structures of the VEE virus.

Virions have been observed within the cytoplasm of LLC-MK_2 cells. Fibrillar components consisting of cylindrical structures demonstrated the virus forming in close proximity and emerging from the hemispheres of these structures. Virions were also observed emerging from vacuoles within the cell vacuole. These findings were not noted by Brown et al.,[4] who observed fractured virus emerging only from chick cell membranes. No virions were noted within the cytoplasm.

Morphological studies of the freeze-fractured and etched viruses revealed a fracture plane through the virus core. Several nucleic acid strands were observed in the virus core. Fracture planes were noted through the cell envelope and on the surface of the virus envelope. There was a fracture above the virus projections and another fracture through the projections. A hollow space was observed in the cross-fracture of projection. This hollow space measured $\sim$ 1 nm in diameter. The projections measure 6 nm in diameter. The virus core and envelope measure 50 nm in diameter. The length of the projections measures $\sim$ 10 nm in length, thus, making the VEE virus 70 nm in diameter. Nucleic acid strands measure approximately 1 nm in diameter and their appearance is similar to a ball of cotton.

These observations have been made from single replicas and very few double replicas. Many preparations have to be made in order to obtain perfect double replicas. Physical factors with specimen preparation and instrument orientation have been interfering with double replica production. The problem has been discussed with Dr. R. Steere, of the U.S. Department of Agriculture, and Dr. McAlear, Catholic University. Morphological and morphogenic studies will be done on all 6- to 18-hr specimens.

Pure VEE virus specimens have also been studied with ultrathin section and freeze-fracture techniques. The observations noted in virus-infected LLC-MK_2 cells have also been revealed in pure virus specimens.

Publications:

None.

LITERATURE CITED

1. Bykovsky, A. F., F. I. Yershov, and M. Zhdanov. 1969. Morphogenesis of Venezuelan equine encephalomyelitis virus. J. Virol. 4:496-504.

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APPENDIX A

TECHNICAL SUPPORT PLANS

TSP-01
AUTOMATED BIOCHEMICAL TECHNIQUES

Karen A. Bostian, B.S.
Physical Sciences Division

Objective:

Develop and utilize reliable methods to quantitate concentrations of various metabolites in biological fluids including blood, urine, and tissue extracts from both man and laboratory animals using the autoanalyzer (Technicon).

Progress and Summary:

All methods not referenced can be found in previous annual reports.[1-3]

Major developments in the past year included:

1. Completion of, receiving clearance for, and submission to the Journal of Laboratory and Clinical Medicine of a paper titled "Sequential Changes in the Concentration of Specific Serum Proteins during Typhoid Fever Infection in Man."

2. Utilization of a fructose assay in the development of a method for measuring gastric emptying time. The research was initiated and developed by Captains Pettit and Elwell, Animal Assessment Division.

3. Utilization of an ortho-toluidine method for glucose analysis to determine glucose concentration and to relate this to glycogen concentration in extracts from livers. The samples were in 2 N HCl; we were able to run them without neutralizing them as would be required by the use of a glucose oxidase procedure for glucose analysis.

4. Use of a gum guaiac-glucose oxidase[4,5] procedure for glucose determination. Excellent correlation was seen between glucose values found using this procedure and those found using the ortho-toluidine procedure on Somogyi filtrates. There are advantages and disadvantages involved with the use of each method and time, number of samples, amount of sample available, and availability of reagents to make it possible to determine which method should be used unless the investigator requests that a specific method for glucose analysis be used.

5. Utilization of equipment to set up a method for running serum hexosamine determinations (personal communication, LTC P. Z. Sobocinski).

6. Utilization of equipment to set up a method for running sialic acid determinations.[6]

7. Work with Mr. Matson to establish methods for utilizing our systems for lipid analysis in conjunction with his separation techniques to determine lipid concentrations in tissue extracts.

8. Completion of specific protein analysis on samples from a project at Jessup on typhoid fever conducted by LTC Robert Edelman, Virology Division.

The tests we have run during the past year are shown in Table I.

TABLE I. TESTS RUN DURING FY 1974

TEST	NO. SAMPLES	INVESTIGATOR
Specific proteins (IgA, IgM, IgG, C_3, Transferrin, α_2-Macroglobulin,	3130	Edelman - Typhoid fever study
Haptoglobin, α_1-Antitrypsin,	4210	Klainer, Beisel
α_1-Acid glycoprotein, Albumin)	190	Mosher
FFA	4664	Kaufmann
Triglycerides	1452	
Cholesterol	743	
Glucose	1534	
Fructose	874	
Triglycerides	827	Matson
Cholesterol	786	
FFA	760	
Phospholipids	437	
Cholesterol, triglycerides, FFA	78	Klainer, Beisel
Fructose	3586	Pettit, Elwell
Lowry protein	1898	Canonico
Glucose	497	
Cholesterol	114	
Triglycerides	108	
LDH	20	
Glucose (glycogen)	381	Sobocinski, Powanda
SGOT	173	
Glucose	94	
Lactate	94	
Pyruvate	94	
Urea	35	
Lowry protein	2945	Powanda
Glucose	1840	
Cholesterol	148	
Triglycerides	148	
T_4	112	

TABLE I. TESTS RUN DURING FY 1974 (Continued)

TEST	NO. SAMPLES	INVESTIGATOR
Glucose	2843	Beall
Urea	1321	
FFA	217	
LDH	94	
SGPT	86	
SGOT	30	
Glucose	482	Wannemacher
Lowry protein	291	
Urea	131	
T_4	80	
Phosphorous	832	Liu
FFA	429	
Glucose	146	
Phosphorous	1211	Neufeld
Lowry protein	29	
Glucose	103	Abeles
FFA	69	
SGOT and SGPT	20	Elwell
Kjeldahl N and Lowry protein	261	Johnson, Spero
Glucose	5198	George, Rayfield
	610	Bailey
	506	Curnow
	275	Mapes
	388	Method comparison
Lowry protein	4285	Mosher
	2178	Proctor
	1341	Rausch
	419	Curnow
	197	Johnson
	112	Zenser
	107	Hughes
	65	Kenyon

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TSP-02

AUTOMATED AMINO ACID DETERMINATIONS

Richard E. Dinterman, B.S.
Physical Sciences Division

Objective:

Amino acid analysis of various biological specimens.

Progress and Summary:

I. Services Rendered:

TYPE OF SAMPLE	NUMBER	SPECIMEN	INVESTIGATOR
Physiologic (33 amino acids)	116	Human serum (myocardial infarct)	Klainer Wannemacher
	111	Human serum (various illnesses)	Klainer Wannemacher
	40	Human serum (controls)	Klainer Wannemacher
	125	Human urine samples	Wannemacher
	52	Monkey serum (yellow fever)	Wannemacher Curnow
	12	Rat plasma	Powanda
Hydrolysate	38	SEB and SEC hydrolysates	Spero
	57	VEE and WEE hydrolysates	Marker
	8	SEB hydrolysate	Metzger
	8	CIG and α-CIG samples	Mosher
	24	Rat tissue hydrolysates	Wannemacher Dinterman
Phenylalanine and tyrosine	150	Rat tissue samples	Powanda Wannemacher Dinterman
	78	Rat serum	
Alanine	24	Rat serum	Dinterman

II. Technical Advancements:

Enzymatic hydrolysis procedures required by investigators raised interest in tryptophan quantitation along with the usual 18 hydrolysate amino acids. Tryptophan has not been quantitated in the past because it is destroyed during normal acid hydrolysis and gives a very poor chromatographic peak with the lithium citrate buffers employed with the Technicon TSM amino acid analyzer. Research on this problem revealed that a mixture of 3 parts Na-citrate, pH 5.26, + 1 part Li-citrate (same pH) yields not only a measurable tryptophan peak but also separates lysine and histidine better than previously used techniques. This reagent mixture is presently being used as the primary buffer for the basic column when quantitation of the 18 hydrolysate amino acids is requested.

Increased interest in the elevated phenylalanine/tyrosine (Phe/Tyr) ratio during infection has established numerous samples to be analyzed for just Tyr and Phe. Recent technique changes now allow for accurate quantitation of them in only 45 min as opposed to an older 90-min procedure.

The Autolab on-line integrator has been coupled to both TSM analyzers. It's memory and programmable features have enabled it to be adapted to the hydrolysate chromatogram, each amino acid giving a distinct separate peak. This automatic integrated area-computing method eliminates the tedious manual peak height measurements and calculations giving an immediate printout of amino acid concentrations. Further work must be done to determine the feasibility of this computing system when dealing with the 33 physiologic amino acids, some of which show overlapping peaks.

TSP-04

AUTOMATIC DATA PROCESSING SUPPORT FOR USAMRIID

Thomas J. Powers, 1LT, MSC
Administrative Division

Objective:

Automatic data processing technical support consists of maintaining the capability of utilizing computers and calculators to process repetitive data generated by work units within USAMRIID.

Progress and Summary:

Loss of the CDC 3300 computer from Fort Detrick has caused a significant degradation in the ability of the Computer Science Section to provide timely and continuous technical support to the Institute. The progress of some of the work units at USAMRIID has definitely been hampered by the loss of timely computer support. To remedy the loss of computer support, a document was prepared to acquire a terminal to provide direct access into the CDC 3500 computer at WRAIR. Interim measures were taken to provide some computer capability using a courier to WRAIR 3 times a week. This system provided an average turnaround of from 2 to 7 days. The computer terminal, a Data 100 Model 78, was installed at the end of March 1975. Since installation, the terminal has experienced a variety of problems that have delayed the return of adequate computer support. Some of these problems include controlware problems with Data 100 emulator package, incompatibility of RIXON TA-201 modem with the Western Electric modem at WRAIR, an unusual amount of downtime, or the system was unavailable during prime working hours at the WRAIR computer center, delay in arrival of paper tape controlware support, terminal hardware problems, and high error rates on some voice grade FX lines to Washington, D.C. The transfer of our operation from the CDC 3300 to the CDC 3500 at WRAIR also involved a great deal of program and data base conversion. In summary, the loss of the CDC 3300 caused both a degradation of computer support and an increased workload due to the conversion effort involved in switching from one system to another.

Current technical support tasks are:

1. Immunization - work is in progress to convert the immunization data base to the CDC 3500. Following this conversion the system will be redesigned to handle both immunizations and the full range of medical screening required for the occupational health and safety program. The current system is still in a nonoperational status.

2. Library - the library system provides an accounting of all volumes of the USAMRIID Medical Library, including volumes on current or temporary loan. The data base, formerly maintained on cards, has been converted to disc. Master lists of all volumes accountable to the library have been generated as required. Updates are continually being made to the file to keep it current.

3. Paper Tape - computer programs are used to read paper tape punched on the β and γ liquid scintillation counters. As data are read they are checked for errors and stored on disc files for retrieval and analysis by subsequent programs. About 302 paper tapes have been processed during the year.

4. Registry of Infectious Organisms - a disc file of all infectious organisms currently used at USAMRIID is continually updated to reflect current usage. The computer file is periodically sorted and printed by organism, division, location and registry number and the listings are provided to any division requesting them.

5. Label Printing - during the past year, nearly 5,200 labels of ≤ 5 lines have been printed for test tubes or other requirements.

6. Calculators - programming support has been provided to divisions requesting solutions to special problems capable of being handled on our programmable calculators.

7. Probit Analysis - probit analyses for determination of LD_{50} are being processed for investigators on an as-required basis.

8. k Values - k values (metabolic or chemical rate constants) for biological/chemical systems have been determined from appropriate disappearance curves.

Current special support tasks are:

1. Automatic Data Acquisition - major efforts in cardiovascular analysis have been centered on the development and time-sequence analysis of a growing data base of over 70 monkey parameters. This system is currently being converted to the CDC 3500 at WRAIR.

2. Differential II - the computer programs provided with this equipment for measuring light diffraction from a bacterium have been converted to the CDC 3500 and further testing has been resumed.

TSP-05
TECHNICAL SUPPORT FOR THE BIOASSAY OF INTERFERONS
Bruno J. Luscri, Ph.D.
Virology Division

Objectives:

Improve yields of human cell culture interferons (IF); study some in vivo and in vitro factors which may aid in the application of IF in experimental virus infections, and collaborate with other divisions to investigate areas of interest.

Progress and Summary:

In initial studies, the rhesus lung cell cultures (DBS-FRhL-2) did not yield as much IF as the human skin cells (CCL54) when induced by NDV or superinduction procedures. These results prompted the production of most IF preparations from CCL54 cells. The IF produced in FRhL, and previously prepared IF from BS-C-1 cells, conferred greater antiviral activity in human skin cells than in homologous cell monolayers.

Since the human reference standard 69/19A (designated to contain 5,000 IF units) titered 20,000 in CCL54 and 7,080 in the monkey cell line (BS-C-1) with VSV as the challenge virus, 1 unit of IF activity in the human skin cells is equal to 0.25 international reference unit (IRU) and in the BS-C-1 cells is equal to 0.71 IRU.

Using a priming and superinduction procedure described by Billiau et al,[1] Table I shows that it is possible to produce increased yields of CCL54 IF. All IF preparations were collected in 20 ml of maintenance medium with 4% human serum. Four lots of each type of preparation with about 40-120 ml of each lot were produced. Table II shows that human skin cells induced solely with NDV virus to produce the IF, IF yields could be also increased by collecting the IF in a small volume of maintenance medium but in this procedure containing fetal calf serum as the serum source.

IM injection of 10 ml of a pool of the primed superinduced IF (Prep. 1, Table I) into rhesus monkeys revealed that the bulk of the IF appears in their sera from 1-12 hr (Table III). Technical assistance was provided by members of Animal Resources Division. Arenaviruses (Pichinde, Parana, Tacaribe, Tamiami) were sensitive to a representative sample of this IF in BS-C-1 cells but to a lesser extent than VSV. This type of IF was shown to confer 60 times less antiviral activity to Vero cells than to the human skin cells when using VSV as the indicator virus.

In preliminary studies with Aerobiology Division, the IF obtained by priming with the virus-induced IF (Prep. 2, Table I) was used to obtain results which suggested no IF destruction by impinger usage, a 2- to 4-fold loss in IF titer by aerosolization, with an IF recovery of 7% on BS-C-1 cells to 13.4% recovery on CCL54 cells (Table IV).

TABLE I. SUMMARY OF IF TITERS FROM HUMAN SKIN CELLS[a] BY PRIMING AND SUPER-INDUCTION

PRIMING IF		IF TITERS VS. VSV BY $PR_{50} \times 10^3$ 6.5-24 HR HARVEST	
Homologous	Used at dilutions	Human skin (CCL54)	Monkey (BS-C-1)
Virus, NDV induced IF, 18 hr harvest			
Prep 1: Collected in 140 ml MM[b]	1/2, 1/3	100-400	10-20
Prep 2: Collected in 20[c] and 70[d] ml MM	1/10, 1/32	14-50	2-7.08
Primed-superinduced IF, 24-48 hr harvest			
Prep 1: Collected in 20[e] ml MM	1/2, 1/20	50-128	10-28

a. Contained in $\frac{1}{2}$-gal roller bottles at ≈ 14 days of age.
b. MM = maintenance medium and priming IF titered 2,500-5,000 in CCL54 cells.
c. Titered 63,000 in CCL54 cells.
d. Titered 2,000 in monkey cells, BS-C-1.
e. Titered 8-16,000 in monkey cells, BS-C-1.

TABLE II. IF PRODUCTION IN ROLLER BOTTLES OF HUMAN SKIN CELLS BY NDV AND COLLECTED IN A VOLUME OF MAINTENANCE MEDIUM USED IN PRIMING AND SUPERINDUCTION PROCEDURES

AGE OF CELLS USED TO PREPARE THE IF (days)	IF TITERS: PR_{50} OF VSV CELLS USED TO ASSAY IF	
	CCL54 (Human skin fibroblasts)	BS-C-1 (Monkey cells)
14	63,000	7,080
21	35,500	7,080

TABLE III. PASSIVE SERUM IF LEVELS OF RHESUS MONKEYS INOCULATED WITH EXOGENOUS HUMAN CELL CULTURE IF ($\approx 5 \times 10^6$ U/kg)

MONKEY NO.	WEIGHT kg	IF LEVELS[a] BY HR AFTER IM INJECTION						
		0	1	6	12	24	36	48
488	3.2	< 25	56	447	178	56	14	32
539	2.8	28	100	320	178	56	35	40
557	3.0	28	112	447	112	40	32	32
564	3.4	< 20	40	90	56	< 20	< 20	< 20

a. Reciprocal.

TABLE IV. EFFECT OF IMPINGER OPERATION, NEBULIZER REFLUXING, AND AEROSOLIZATION ON RECOVERY OF HUMAN IF.

DESTRUCTION	IF TITERS PR_{50} VSV		
	Aerosolization #1[a]	Aerosolization #2[b]	
	BS-C-1	BS-C-1	CCL54
Impinger			
Prior operation	2,000	2,240	14,000
Postoperation	< 4,200	≈ 1,780	16,000
Nebulizer and aerosol recovery			
Prespray	2,000	1,780	20,000
Postspray	1,131	800	6-11,000
Aerosol sample	6.4	< 2	20-22

a. No antifoam in impinger.
b. Antifoam in impinger.

In <u>in vivo</u> and <u>in vitro</u> studies with Physical Sciences Division, the appearance of an antiviral factor in the serum of mice following LEM injection appeared to be related to dosage (Table V). A 2-hr and 24-hr treatment of CCL54 cells with cholera toxin did not diminish VSV replication extracellularly, nor did

TABLE V. EFFECT OF LEM ON APPEARANCE OF MOUSE SERUM IF

GROUP	NO. MICE	LEM TREATMENT (ml)	IF TITER[a]
I-IV	20	0-0.01, 0.03, 0.1	None at 1/10 and above[b]
V	5	0.3	5
VI	5	1.0	14

a. Reciprocal of dilution of sera reducing VSV plaques linearly to 50% of control plaques in L929 cells. Internal mouse IF standard titered 10,000.
b. Log_{10} dilutions: 1.5, 2.5, 3.5, 4.5.

this treatment alter the amount of IF synthesized by VSV (Table VI). While cyclic 3',5'-adenosine monophosphate (cAMP) has been shown to potentiate induction of the antiviral state by IF in chick cells,[2] these results suggest that cAMP may not potentiate IF synthesis by VSV in human skin cells.

TABLE VI. EFFECT OF CHOLERA TOXIN (cAMP) ON VSV AND IF YIELDS[a] IN HUMAN SKIN CELLS AT 2 TIME INTERVALS

GROUP	CHOLERA TOXIN[a] Day 0	2 HR		24 HR	
		Mean VSV Yield ± SE (n = 3)	IF Titer[b]	Mean VSV Yield ± SE (n = 3)	IF Titer
I	0		< 3		< 3
II	0 + VSV	7.6 ± 0.03	50	7.5 ± 0.18	126
III	200 ng		< 3		< 3
IV	200 ng + VSV	7.9 + 0.06	50	7.9 ± 0.18	56

a. Assayed on CCL54 cells, log_{10}.
b. Per 25-cm^2 flask of CCL54 monolayers.

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TSP-06

STANDARDIZATION AND PERFORMANCE OF PLAQUE REDUCTION SERUM DILUTION, VIRUS NEUTRALIZATION TESTS

Helen H. Ramsburg, A.B.
Virology Division

Objective:

To determine specific virus neutralizing antibody titers in sera of various mammalian species after virus infection or in response to vaccination.

Progress and Summary:

Standard plaque reduction (PR) and serum neutralization (SN) tests[1] were performed to measure the level and duration of homologous and/or heterologous neutralization antibody formed in response to vaccination with VEE given singly or in combination with EEE and/or WEE. New lots of WEE vaccine were evaluated as well as the experimental vaccines for VEE, EEE, and WEE which are currently under investigation. Where applicable, at-risk personnel were tested for the presence of VEE, EEE, WEE, and yellow fever (YF) neutralizing antibodies, Table I. In addition, PR-SN tests were performed on tissue culture fluids to confirm virus identity during the progress of vaccine studies and during the preparation of new lots of vaccines.

Suspensions of the attenuated 17-D strain of YF, the small plaque (SP) mutant attenuated Arth-167 strain of EEE, a SP isolate from the attenuated Clone-15 strain of WEE, and the attenuated TC-83 strain of VEE were prepared in Vero tissue culture (TC) cells for use in PR-SN tests. Results obtained from growth curve studies showed that these viruses required incubation periods comparable to their respective parent "hot" strain for maximum virus yield.

The overlay media routinely used on the Vero TC cells for performing standard PR-SN tests for the group A arboviruses is EBME (2X) containing 4% fetal bovine serum. This was found to lack the necessary nutrients for maintaining the LLC-MK2 TC cells used for performing standard PR-SN tests for the group B arboviruses. However, 1% LAH-HBSS (2X) containing 10% calf serum, pH 7.3, carries the cells through the prolonged incubation period characteristic of the group B arboviruses.

A PR-SN test was standardized for the dengue-1 and West Nile viruses. The test procedure employed for West Nile is comparable to that used for certain other group B arboviruses. However, dengue-1 does not plaque when standard procedures used for certain other group B arboviruses are carried out. It was found necessary to incorporate 0.01 μg/ml of DEAE dextran in only the 2nd overlay, which is used for staining purposes. When the pH of both overlays was adjusted to pH 8.3, tests could be read on the 7th day instead of the 14th day required for pH 7.3. Regardless of pH, the plaques

TABLE I. SUMMARY OF GROUP A AND B ARBOVIRUS PR NEUTRALIZATION TESTS PERFORMED

INVESTIGATOR	SERUM TESTED	NUMBER OF SAMPLES TESTED							
		VEE		EEE	WEE		Langat	Dengue	
		Trinidad	TC-83		B-11	Clone 15		-1	-2
Albrecht[a]	Monkey						16		16
Burke[b]	Human	154		15	18				
Center for Disease Control	Human	36							
Cole	Guinea pig				20	20	250	64	
	Hamster								
	Monkey								
	Rat	80		121	186				
Cornell	Human	95							
Dugway	Human	24	24						
Eddy	Rabbit				12		32		
	Rat	5							
	Ascitic fluid	41							
Edelman	Human	170	43						
	Monkey		75						
Levitt	Rabbit	18							
Massachusetts State Laboratory	Human			44					
McManus	Monkey							56	
Medical Division Protocol FY 69-1	Human	32							
Pedersen	Mouse	113		78	78				
	Rabbit	4		4	4				
Texas A&I University	Human	30							
University California Los Angeles	Human	38							
Wear	Monkey							128	
Woodman	Ascitic fluid	2							

a. 16 monkey sera tested for West Nile.
b. 3 human sera tested for YF.

observed were too small to be clearly visible; plaque size was significantly increased when a 0.5% instead of the standard 1% final concentration of agarose was used for both overlays. The results of studies to determine the incubation period required for maximum virus yield are presented in Table II. Virus was harvested at peak yield and stored at -60 C.

TABLE II. EFFECT OF INCUBATION PERIOD ON YIELD OF VIRUSES (WITH INPUT) IN CELL CULTURES BY NUMBER OF PFU/0.2 ML OF INOCULUM ON HOMOLOGOUS MONOLAYERS

DAYS POST-INOCULATION	LOG_{10} PFU/ML							
	LLC-MK2 TC		Vero TC					
	Dengue 1 3.0[a]	West Nile 6.5	Ampari 4.3[a]	Latino 3.5[a]	Parana 5.5[a]	Pichinde 6.8	Tacaribe 3.6[a]	Tamiami 4.0[a]
	Virus dilution log_{10} plaqued							
	4.0	5.0	5.0	1.0	6.0	7.0	4.0	6.0
1		81		0				
2		51	1	1	91	164	0	5
3		17	4	9	159	174	12	41
4			64	2	145	131	76	97
5	0		87	1	153	175	292	87
6	61		99		145	67	249	64
7	66		52		144			
8	48				11			

a. Low titer of virus seed precluded input of 6.0 logs.

Standard PR-SN tests were performed for detection of homologous and/or heterologous neutralizing antibodies formed in response to sequential immunization with the attenuated 17-D strain of YF, Langat, and dengue-2, followed by challenge with dengue-1 (D-1). Tests were also performed for measuring homologous neutralization antibodies formed in response to immunization with either Langat, dengue-2, or West Nile, followed by challenge with 17-D. Experimental vaccines for Langat and dengue-2 (D-2) were evaluated. The above tests are summarized in Table I.

A PR-SN test, based on the procedures employed for the group A arboviruses, was standardized for 6 viruses in the arena group of arboviruses, using Vero TC cells. However, it was found necessary to make certain modifications in

order to obtain optimal test conditions: (1) the final concentration of agarose was decreased from 1 to 0.5% for Latino in order to increase plaque size; (2) fetal bovine serum used in the overlays was increased from 2-4%; (3) the concentration of neutral red used in the second overlay was decreased from 1:6,300 to 1:9,390 dilution in combination with an increase in volume of overlay from 1.5-2.5 ml/35-mm well. Even though the final concentration of stain (1:18,000 vs. 1:19,800) used for either of these methods is essentially the same, the modified procedure is far superior. Plaques form in 8 days for Tamiami, 6 days for Ampari, Parana and Tacaribe, 5 for Latino and 4 for Pichinde. The results of studies to determine the incubation period required for maximum virus yield are presented in Table II. Virus suspensions were prepared in Vero TC cells for each virus, except Latino, and stored at -60 C. A virus yield of 2.6 $\log_{10}$ for Latino grown in Vero TC cells was inadequate since the minimum acceptable titer for virus preparations used in PR-SN tests is 4.0 logs.

In order to obtain a satisfactory Latino virus preparation, it was necessary to conduct additional studies in other TC cell lines. The BS-C-1 line of continuous green monkey kidney cells and the FRhL line of diploid fetal rhesus monkey lung cells were employed. Studies showed that an incubation period of 3 days for the FRhL cells and 5 days for the BS-C-1 cells was required for maximum virus yield. These optimal incubation periods were used for the serial passage of Latino in both cell lines. A comparison of concurrent titrations carried out in Vero TC cells, showing that maximum yields were reached on the 3rd passage, is depicted in Table III. A virus suspension was subsequently prepared for Latino for use in PR-SN tests.

TABLE III. COMPARISON OF EFFECT OF PASSAGE LEVEL AND CELL LINE ON VIRUS YIELD AT PEAK OF GROWTH CURVE

CELL LINE	$\log_{10}$ VIRUS BY PASSAGE NUMBER			
	1	2	3	4
BS-C-1	4.9	4.3	5.1	4.1
FRhL	3.1	4.0	4.6	4.4

Standard PR-SN tests were performed for detection of homologous and heterologous antibody formed by monkeys in response to infection by one or more of the arena group of viruses, followed by Machupo challenge. Tests performed are summarized in Table IV.

TABLE IV. SUMMARY OF ARENA GROUP ARBOVIRUS PR-SN TESTS PERFORMED

INVESTIGATOR	SPECIMEN TESTED	NO. OF SAMPLES TESTED			
		Latino	Parana	Pichinde	Tacaribe
Eddy	Monkey sera	53	38	30	27

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TSP-07

CELL CULTURE PREPARATION AND SUPPLY LABORATORY

John P. Kondig, B.S.
Virology Division

Objective:

To provide a centralized facility for the preparation or acquisition of cell cultures, media reagents and related materials, including viral vaccine substrates for use by investigators of the Institute.

Progress and Summary:

During the past year the cell culture preparation and supply laboratory has produced approximately 6310 L of tissue culture medium and balanced salt solutions. It has also provided a total of 22 cell lines, strains, or primary cells in the various quantities listed in Table I. Included in the data are the FRhL cells used to prepare a dengue-2 vaccine and the chick embryo fibroblasts to prepare a killed WEE vaccine.

TABLE I. CELL CULTURES PRODUCED

DIVISION AND INVESTIGATOR	ROLLER BOTTLES	T-75 FLASKS[a]	T-25 FLASKS[a]	6-WELL PLATES	TUBES[a]	24-WELL PLATES
Aerobiology						
Kishimoto	50				36	
Scott					110	
Animal Assessment						
Harrington	29	50	5	650		
Houston				6440		750
Stephen		8		2426		638
Bacteriology						
Canonico			824			
Mangiafico				30		
Hughes		82				
Pathology						
Metzger			4982			476
Middlebrook		20				
Mosher		54	132		120	
Pekarek		24				
Zenser			46			

TABLE I. CELL CULTURES PRODUCED (Continued)

DIVISION AND INVESTIGATOR	ROLLER BOTTLES	T-75 FLASKS[a]	T-25 FLASKS[a]	6-WELL PLATES	TUBES[a]	24-WELL PLATES
Rickettsiology						
Bagley					484	
Johnson		90	4980			
Kenyon	494	260	4955			
Oster	71					
Pedersen	1287	495	135			
Sammons			1060			
Williams	25		490			
Virology						
Cole	62	615				
Eddy	342	282	11512	2480		680
Edelman	40	18	72		396	
Gangemi	554	55	320	140		
Jahrling	622	60	1056	9655	288	20
Levitt	4	20	5240	1134	2570	50
Luscri	125	5	6635			
Marker	1452	10	593	1795		
M. Manus	140	2860	60	1460		
Ramsburg		478		9130		
Rosato	182		875	835		15
Wear		262	1555	1475		
Woodman		10	2400	1550		
Seed	1477	8795				
TOTAL - Seed	5479	5758	47927	39202	4004	2629
TOTAL + Seed	6956	14553				

a. Or equivalent.

TSP-8
TECHNICAL SUPPORT OF IMMUNOFLUORESCENT STUDIES
Lauren R. Bagley, Jr.
Rickettsiology Division

Objectives:

The objectives of this technical support program are to (1) label conjugates for direct and indirect immunofluorescent studies, (2) perform direct (DFA) and indirect immunofluorescent (IFA) staining of infected materials provided by investigators, and (3) infect, fix, harvest and stain a variety of cell cultures with infectious agents using established protocols.

Progress and Summary:

Immune sera were prepared in hamsters against Parana and Ampari viruses; in rabbits against mouse and guinea pig γ-globulin and guinea pig whole serum.

Direct fluorescent conjugates were prepared against WEE, Machupo, Ampari, and Parana viruses; indirect conjugates against mice and guinea pig γ-globulin.

Aerobiology Division - Direct and indirect fluorescent stains were made of guinea pig macrophages and WI-38 cells infected with Coxiella burnetii. Positive results were photographed. FA conjugates requested by investigators include anti-C. burnetii, anti-rabbit, anti-human, anti-guinea pig, anti-mouse and anti-monkey.

Pathology Division - Direct and indirect fluorescent staining of frozen sections from guinea pigs infected with RMSF were done. Positive results were photographed. Direct and indirect fluorescent staining of uninfected platelets and WI-38 cell results were photographed. FA conjugates requested by investigators include anti-RMSF and anti-monkey.

Rickettsiology Division - Indirect fluorescent stains were made of frozen sections from a monkey infected with RMSF. Positive results were photographed. Antibody presence and levels were established by indirect FA staining in 15 human and 77 monkey sera. Projects in progress but not completed include: indirect FA staining of bone marrow sections from a RMSF-infected monkey, identifying, by direct or indirect FA, RMSF in guinea pig white blood cells. FA conjugates requested by investigators include guinea pig and monkey anti-RMSF, anti-human, anti-guinea pig, anti-rabbit, normal monkey and anti-monkey.

Virology Division - Indirect fluorescent stains were made of frozen sections from hamsters infected with Latino virus. Positive results were photographed. No positive fluorescent staining was found in monkey sections infected with WEE or VEE or in hamster sections infected with Tamiami, VEE or TC-83. HeLa cells were infected with VEE-T, Vero cells with WEE, MK_2 and Vero cells with Latino virus, Vero and FRhL cells with Tacaribe, Vero and FRhL cells with Pichinde; all infections were positive by direct fluorescent staining. Results were photographed.

Scheduled Projects - FA conjugates will be prepared for monkey anti-RMSF and guinea pig anti-RMSF. Frozen sections will be prepared from an infected monkey. Titration of human sera will be done on infected Pichinde, Tamiami and Tacaribe cells for identification, and conjugate hamster anti-Mayaro.

TSP-9
ANIMAL BLOOD DONOR PROGRAM
Raymond L. Eaton, SFC, NCOIC
Animal Resources Division

Objective:

To provide a source, and supply on request, animal blood or its components as required by investigators.

Progress and Summary:

A total of 18 rhesus monkeys, 34 sheep, 22 goats, 27 burros, 5 geese, 2 chickens, 2 horses, 3 rabbits, and 30 guinea pigs were available as blood donors at the end of the reporting period. Sheep and goat populations were reduced during the year. Excess burros and horses have been reported to the property disposal officer. Additional blood was collected from rhesus monkeys in the holding colony when the demand exceeded the amount available from the donor animals. Only a few chickens and geese are being maintained to fill emergency requests for blood. Commercial sources of chicken and goose blood were evaluated during the year and proved to be satisfactory. The volume of blood collected or purchased by species for each of the using divisions is presented in Table I.

TABLE I. VOLUME OF BLOOD SUPPLIED

SOURCE	VOLUME (ml) BY DIVISION						
	Aero	AA	AR	Bact[a]	Path	PS	Virol
Monkey		5,095	740	4,316 (130)			1,120
Sheep		200		20,435	5	43,750	3,427
Goat				90	400		7
Goose				120 (490)			260
Chicken	400			54 (25)			10
Horse				30	5		
Burro				175	350	1,000	17
Guinea pig	364	34		154 (20)			
Rat				130			
Cow							80

a. (Serology Laboratory).

TSP-10

BIOCHEMICAL, HEMATOLOGIC AND SEROLOGIC SCREENING OF NONHUMAN PRIMATES

Donald G. Harrington, MAJ, VC
Animal Resources Division

Objective:

To survey nonhuman primates for selected biological and hematologic abnormalities or for serological evidence of specific antibodies to allow selection of suitable animals for a particular study.

Progress and Summary:

All nonhuman primates were routinely surveyed following their arrival at USAMRIID for serological evidence of antibody levels to selected organisms of importance in particular studies. Rhesus monkeys were bled for determining detectable HA antibody titers to SEA, SEB, and SEC. All squirrel monkeys (Saimiri sciureus) as well as certain rhesus monkeys were screened for HA titers to Streptococcus pneumoniae, Type I, and influenza A_2/Aichi. The results obtained from 527 monkeys surveyed during the reporting period are presented in Tables I, II, and III.

TABLE I. SEA, SEB, AND SEC HA ANTIBODY TITERS IN RHESUS MONKEYS

RECIPROCAL TITER	SEA	SEB	SEC
< 10	377	381	97
10	10	16	5
20	2	17	15
40	2	3	13
80		7	16
160		5	3
320		5	6
640		1	7
>640		4	14
% POSITIVE	4	13	45

TABLE II. INFLUENZA HI ANTIBODY TITERS IN RHESUS AND SQUIRREL MONKEYS

RECIPROCAL TITER	RHESUS MONKEY	SQUIRREL MONKEY
< 10	191	40
10	18	
20	6	
40	1	
% POSITIVE	12	0

TABLE III. S. PNEUMONIAE HA RECIPROCAL ANTIBODY TITERS IN SQUIRREL MONKEYS

% POSITIVE	NUMBER OF MONKEYS BY TITER		
	<1:10	1:10	1:20
20	32	7	1

TSP-11
LABORATORY ANIMAL SURGERY
Roy T. Faulkner, CPT, VC
Animal Resources Division

Objective:

To establish and provide a surgical capability for laboratory animals. The surgical capabilities should be of such a level as to accomplish common well developed techniques and provide assistance in developing new procedures or techniques needed to support ongoing investigations.

Background Information:

In the past, individual investigators conducted the animal surgery required for their research. Support was provided informally by technicians and veterinarians from AR Division on request. As a result, the record of surgical procedures performed was incomplete, confusion arose regarding the administration of pre- and postsurgical care, and, on occasion, when experience was lacking and assistance was not sought, the operative technique was criticized. To alleviate these problems, a Technical Support Plan (1 Apr 73) and a Standing Operating Procedure (SOP) for animal surgery (10 Dec 74) were established. A controlled, fully coordinated surgical support activity within the division resulted which has alleviated the problems encountered previously and has not encroached upon the investigator's prerogative of determining his requirements or interfering with his desire to conduct those surgical procedures he is qualified to perform.

Progress and Summary:

A well equipped surgical suite was maintained in AR 201 and AR 202 and an additional suite in Building 1412, room 208, became operational on 18 Feb 75. The veterinary staff performed a total of 912 surgical procedures on laboratory animals in support of experimental projects or routine animal care. A summary of the procedures performed is presented in Table I. In many instances, more than one surgical procedure was performed on a laboratory animal at the time of surgery. Table II is a summary of the number of laboratory animals which received surgical procedures either for an investigator or as routine medical care. All animals receiving noninvestigative surgical care are included under AR Division. The large increase in the number of animals used and surgical procedures performed this year was due, in part, to improvement in recording surgical activities. This development resulted from the implementation of the TSP and Animal Surgery SOP.

An evaluation of several methods of catheterizing the portal vein in rhesus monkeys has been completed. It was determined that the right colic and ileocolic vein (both tributaries of the superior mesenteric vein) are the preferred routes of entry into the portal vein. Siliconized (Silastic®) tubing proved to be the ideal catheter material to use. The technique proved

TABLE I. SURGICAL PROCEDURES PERFORMED ON LABORATORY ANIMALS IN AR SURGICAL SUITES

SURGICAL PROCEDURES	BLDG 1425 AR 201	BLDG 1412 AR 208	TOTAL
Catheterizations			
Femoral artery	263	14	277
Femoral vein	316	13	329
Saphenous artery	4		4
Internal jugular	18		18
Carotid artery	12		12
Renal vein	50		50
Portal vein	26	2	28
Left ventricle	48		48
Interior mesenteric vein	10	1	11
Cutaneous skin biopsies	34		34
Thermister implantations	9		9
Cardiac transducer implantations	8		8
Splenectomies	6		6
Gut loop formation for endotoxin production in rabbits	18		18
Experimental techniques			
Thoracic duct catheterization	2		2
Portal vein flow probe implantation	3		3
Heptic vein cathetherization	13	3	16
Brachial artery and vein cathetherization	3		3
Colony care (i.e., fracture repair, hernia repair, lacerations, amputations)	11		11
Dental extractions	25		25
TOTALS	879	33	912

TABLE II. NUMBER OF ANIMALS RECEIVING SURGICAL PROCEDURES IN AR SURGICAL SUITES

DIVISION	SPECIES		
	Rhesus monkeys	Owl monkeys	Rabbits
Aerobiology		6	
Animal Assessment	370		
Animal Resources	45		
Pathology			18
Physical Sciences	16		
Virology	2		
TOTALS	433	6	18

to be safe, was relatively simple to perform, caused minimal periportal liver pathology, and provided a normal healthy monkey postoperatively. The technique is a very efficient means of administering substances intraportally and also in obtaining portal venous blood samples. As of this date, the technique has been used in research projects comparing portal and peripheral venous insulin levels, studying liver utilization of orally administered SEB, and comparing intraportally infused SEB vs. peripheral IV SEB infusion.

Several monkeys were used to evaluate the brachial artery and vein as a route to implant peripheral catheters. Catheters placed in the femoral vessels usually cause edema and thrombosis of the lower extremities in monkeys chaired longer than 5 days. The brachial vessels proved to be too small and fragile to accommodate the catheters utilized by most of the investigators. Catheterization of these vessels was possible if meticulous dissection and smaller catheters were used. However, the procedure took too much time to be utilized as a routine method.

Several other experimental techniques were also evaluated. Two monkeys were utilized for thoracic duct cannulation. Even though both attempts were unsuccessful, it is possible with additional preoperative preparation and instruments that this technique could be easily performed and prove very useful for lymphocyte collection. Flow probes to determine portal blood flow were implanted in 3 rhesus monkeys. The flow probe worked well in one monkey; but, in the other 2 the portal vein became constricted due to the infiltration of fibrous tissue around the probe. This is a common occurrence with the type of probes available. However, portal blood flow was successfully measured by an alternative technique. The portal vein was catheterized as described earlier.

With the addition of a 2nd catheter placed in the inferior mesenteric vein, a dye dilution technique was used to measure the portal blood flow. Catheterization of the hepatic veins was also evaluated. Catheterization would enable one to measure total hepatic blood flow. The technique of catheterization is relatively simple; however, maintenance of a patent and functional catheter in a chaired rhesus monkey was very difficult. Many different approaches and methods were tried; however, none provided reliable results. Hepatic catheters are widely utilized in sheep and dogs; however, the anatomical difference between these 2 species and the rhesus monkey are such that the accepted techniques could not be applied to the monkey.

Publications:

1. Liu, C. T., and R. T. Faulkner. Cardiohepatic responses to staphylococcal enterotoxin B in conscious rhesus monkeys. Fed. Proc. 34:225, 1975.

TSP-12

LABORATORY ANIMAL HEALTH CARE

Robert L. Hickman, MAJ, VC
Donald G. Harrington, MAJ, VC
Roy T. Faulkner, CPT, VC
Bobby R. Collins, CPT, VC

Objective:

To insure a source of healthy laboratory animals for use by the research personnel within USAMRIID and to prevent or curtail outbreaks of disease within the colony.

Progress and Summary:

A total of 115,230 laboratory animals were purchased from 19 commercial and 5 noncommercial sources. Numbers of animal requisitions, animal deliveries, and animals issued to each division are presented in Tables I and II.

TABLE I. ANIMAL REQUISITIONS/DELIVERIES PROCESSED AND RECEIVED

DIVISION	NUMBERS BY QUARTER				
	FY 74-4	FY 75-1	FY75-2	FY 75-3	Total
Aerobiology	43/ 55	51/ 63	27/ 35	37/ 47	158/ 200
Animal Assessment	39/ 40	35/ 43	38/ 39	52/ 53	164/ 175
Animal Resources	7/ 7	7/ 10	6/ 6	5/ 5	25/ 28
Bacteriology	36/ 48	18/ 31	18/ 26	22/ 22	94/ 127
Pathology	13/ 24	14/ 27	24/ 38	16/ 21	67/ 110
Physical Sciences	76/151	49/118	47/ 87	66/143	238/ 499
Rickettsiology	--	--	10/ 15	20/ 20	30/ 35
Virology	40/ 50	29/ 76	25/ 76	28/ 30	122/ 232
Totals	254/375	203/368	195/322	246/341	898/1,406

A total of 208 newly arrived animals from the major contractors were submitted for quality control pathological evaluation. The results are summarized in Table III. One supplier of guinea pigs was temporarily excluded as a vendor source because of a high incidence of severe respiratory disease. Documentation of similar problems with one rabbit and one mouse supplier are being evaluated.

Most of the nonexperimental clinical signs and pathological lesions observed in animals on project correlated with the lesions observed in newly arrived animals. Murine chronic respiratory disease and parasitism were common in both mice and rats. In rabbits, respiratory disease, enteritis and coccidiosis were commonly detected. Pneumonia and streptococcal lymphadenitis continued to be the major guinea pig problems identified.

TABLE II. ANIMALS ISSUED

DIVISION	NUMBER OF SPECIES						
	Mice	Rats	Hamsters	Guinea pigs	Rabbits	Monkeys	Other
Aerobiology	25,070	1,441	862	284	0	128	0
Animal Assessment	25,665	31	158	92	162	682	18
Animal Resources	1,200	0	300	166	0	27	0
Bacteriology	12,920	576	81	406	92	13	0
Pathology	3,265	30	0	634	210	53	0
Physical Sciences	13,790	12,904	70	105	1,327	139	0
Rickettsiology	308	0	25	420	13	43	0
Virology	5,718	118	4,403	1,035	57	63	126
TOTALS	87,936	15,100	5,899	3,142	1,861	1,148	144

TABLE III. PATHOLOGY OF NEWLY RECEIVED ANIMALS

SPECIES	OCCURRENCE OF LESIONS				
	Mice	Rats	Hamsters	Guinea pigs	Rabbits
None observed	65	10	4	4	1
Respiratory disease	21	10	1	24	11
Myoepicarditis	2	1	-	4	2
Hepatitis	2	-	1	7	6
Hepatic fatty metamorphosis	-	-	-	6	-
Nephritis	1	6	-	9	5
Lymphadenitis	-	-	-	5	-
Enterocolitis	1	-	-	-	4
Meningoencephalomyelitis	-	-	-	1	2
Sialoadenitis	-	-	-	2	-
Nematodiasis	21	6	2	-	2
Protozoiasis	10	-	3	-	7
Other	2	-	2	5	2
Total Animals	115	22	10	39	22

Several individual or sporadic disease problems occurred throughout the year. Moist necrotizing perianal dermatitis was observed in a single group of 8-week-old male AKR mice. Bacterial cultures and histologic examination failed to establish etiology. All affected mice were removed and pan populations were reduced to 10-15 animals. No further cases were observed.

An epizootic of pneumonia and a "wasting" syndrome was observed in one group of guinea pigs. Approximately 40% of the animals were affected. A Streptococcus sp. was isolated from several of them. Antibiotic therapy resulted in a satisfactory response initially but failed to prevent reoccurrence when treatment was interrupted. Organisms compatible with Bacillus piliformis were observed in the intestine of one of these animals. A report of possible Tyzzer's disease in a guinea pig is being prepared for publication. Another "wasting" syndrome accompanied by excess salivation was observed in 3 other groups of guinea pigs. At necropsy, splinters of pine shavings were found in the soft tissues of the oral cavity. Use of hardwood bedding for new animals and increased use of false bottoms in the cages appeared to control the problem. No single supplier's animals were implicated. A pathology report of nosematosis in one guinea pig was relayed to the vendor from which it came. Joint housing of guinea pigs and rabbits on the vendor's premises probably was responsible for this rare observation. The vendor indicated he was taking action to maintain separation of the species.

Diarrhea and otitis externa were problems in several groups of rabbits. Heavy infestations of coccidiosis (Eimeria sp.) were treated successfully with oral sulfonamides. Routine use of sulfonamides in newly arrived animals and regular treatment of all rabbits for ear mites was instituted to control these problems. Several sporadic cases of ringworm were also noted in rabbits. Specimens were submitted for dermatophytic identification.

Tyzzer's disease continued to occur sporadically in mice from two sources after they were on project. Lesions compatible with Tyzzer's disease were observed on histologic examination of mice from another vendor. Experiments were conducted to determine if the mice were infected when received or acquired the infection after delivery; to evaluate different routes of disease transmission; to determine the incubation period of the disease; and to assess the effect of chemotherapy. When completed, the results will be compiled for publication.

Nonhuman primates were relatively free of disease with the exception of recurrent enteritis which was successfully treated and a chronic progressive condition in owl monkeys (Aotus trivirgatus) which resulted in a high mortality rate. The etiology of the latter was not determined but transfer of the monkeys from Building 1412 to Building 1425 accompanied by a change in feed has had a beneficial effect.

A total of 3,136 intrapalpebral tuberculin tests were administered to nonhuman primates. Five reactors in one group of 16 rhesus monkeys were

detected. An index case was identified as an anergic monkey with massive central nervous system involvement. All 15 contact monkeys were euthanized. A case report is being prepared jointly with Pathology Division for publication.

No major disease problems occurred in large animals. Intestinal parasites continued to be of some concern but periodic sampling and a regular treatment program has resulted in satisfactory control. The animal population has been reduced with further reductions scheduled in the near future. Two sheep died, one spontaneously and one after exhibiting signs of a central nervous system disorder. Complete necropsies were performed but no definitive etiology was determined.

TSP-13

PACKAGING AND LYOPHILIZATION OF BIOLOGICAL PRODUCTS

Sherman E. Hasty and Charles D. Rapp
Virology Division

Objective:

To provide the Institute with the capacity to process, package and lyophilize biological products (vaccines, antigens, conjugate serums, etc.) needed by the investigators in order to fulfill their missions.

Progress and Summary:

During the period covered by this report, 5 lots of vaccine were freeze-dried.

	Vials
1. RMSF, Kenyon	200
2. Dengue 2, McManus	100
3. WEE (INAC) Lot 1, Cole	400
4. WEE, Lot 2, Cole	400
5. WEE, Clone 15, Coleman, University of Virginia	400
1 lot skin test antigen (TSTA tularemia) Eigelsbach	500
2 lots SEC toxin, Metzger	50
Escherichia coli toxin, Metzger	40
1 lot column chromatography fractions, Zenser	35

Preparations are being made to produce, package and freeze-dry 4,000 1.0-ml fill, 200 15-ml fill, 100 25-ml fill vials of tularemia skin test antigen. The production and part of the packaging should be completed in the period covered by this report.

Potency, sterility and infectivity tests were completed on Lot 2, WEE (INAC) vaccine and are being continued on Lot 1, WEE (INAC) vaccine.

TSP-14
TRACE METAL ANALYSIS
Edward C. Hauer, B.S.
Physical Sciences Division

Objective:

To provide rapid, reliable methods for analyzing trace metals in various biological specimens.

Progress and Summary:

This support plan was implemented on 12 Aug 74 to meet the present and projected research needs of USAMRIID. The operation of this plan is in two parts: (a) analysis and (b) development.

The table lists a summary of analytical work done this year.

SAMPLE	NO. OF ANALYSES						
	Zn	Cu	Fe	Ca	Mg	Other	Total
Serum	1564	513	440	221	221	132[a]	3091
Liver	102	102	--	--	--	--	204
Muscle	24	24	--	--	--	--	48
Brain	5	5	--	5	--	--	15
Urine	--	--	--	132	132	--	264
Other	54[b]	--	26[c]	--	1[c]	12[d]	93
TOTALS	1749	644	466	358	354	144	3715

a. Ceruloplasmin.
b. Dialysate.
c. Medium.
d. Mn-buffer.

Total workload was apportioned as follows:

Physical Sciences	50.7%
Project	21.2%
Animal Assessment	14.6%
Contract	11.5%
Pathology	1.8%
Clinic	0.2%

Development work done this past year has included: (1) Adding normal control serum to each analytical run to improve quality control. (2) Developing methods for determining serum Zn and growth medium iron by flameless atomic absorption. (3) Developing a semimicromethod for the analysis of serum Zn, Cu, or Fe that requires 100 µl of samples instead of the usual 330 µl. (4) Evaluating alternative methods for the analysis of tissue trace metals. The present practice of homogenizing the tissue is not fully satisfactory. Two promising procedures are (1) digesting the tissue with tetramethyl ammonium hydroxide, which gives results for Zn slightly different than the homogenate, and (2) ashing the tissue at low temperature with O_2 plasma. Ashing will be done with an LTA-302 low temperature asher when it is installed.

TSP-15
BACTERIAL CULTURE SERVICE
Wallace G. Fee
Bacteriology Division

Objective:

To provide a centralized facility to maintain a stock culture collection of bacterial strains with complete records on source and cultural history and to supply well-characterized bacterial suspensions for use in approved work units within the Institute.

Progress and Summary:

Bacterial Culture Service was approved as TSP-15 on 26 Sep 74. Services provided USAMRIID investigators are listed in Table I.

TABLE I. INVESTIGATORS FOR WHOM SERVICES WERE PROVIDED DURING FY 1974

INVESTIGATOR	ORGANISM	STRAIN	NO. SAMPLES
Canonico	Streptococcus pneumoniae	Type 1	25
	Salmonella typhimurium	USAMRIID-MIT	1
	Escherichia coli	Type 1	2
	Francisella tularensis	LVS	3
	Staphylococcus aureus	ATCC #14458	2
Fine	S. pneumoniae	ATCC #6301	19
		ATCC #6303	7
		ATCC #6304	7
		ATCC #6307	7
		ATCC #6308	11
		ATCC #6312	8
		ATCC #6314	8
		ATCC #6325	27
	S. aureus	ATCC #14458	1
Dominik	S. pneumoniae	ATCC #6301	15
Powanda	S. pneumoniae	Type 1	3
	F. tularensis	LVS	2
	S. aureus	ATCC #14458	5
Beall	S. pneumoniae	Type 1	23
	S. typhimurium	USAMRIID-MIT	5

TABLE I. INVESTIGATORS FOR WHOM SERVICES WERE PROVIDED DURING FY 1974 (Continued)

INVESTIGATOR	ORGANISM	STRAIN	NO. SAMPLES
Hawley	E. coli	USAMRIID	1
	S. pneumoniae	ATCC #6301	1
	Klebsiella pneumoniae	ATCC #10031	1
	Serratia marcescens	ATCC #13880	1
Pettit	S. pneumoniae	Type 1	4
George	S. pneumoniae	Type 1	3
Beisel	S. typhimurium	USAMRIID-MIT	3
Dinterman	S. pneumoniae	Type 1	26
	F. tularensis	LVS	10
Eigelsbach	Pseudomonas stutzeri	ATCC #17588	3
	S. aureus	ATCC #14458	3
Kishimoto	S. aureus	ATCC #14458	4
	Pseudomonas aeruginosa	USAMRIID - Mouse passed	4
		Fisher, Type I	3
Mahlandt	S. typhimurium	USAMRIID	1
Kaufmann	S. pneumoniae	Type 1	4
	S. typhimurium	USAMRIID-MIT	8
Berendt	S. aureus	ATCC #14458	1
	S. pneumoniae	ATCC #6301	24
	E. coli	USAMRIID	1
	S. pneumoniae	ATCC #6303	1
Neufeld	S. pneumoniae	Type 1	34
	S. typhimurium	USAMRIID-MIT	5
	F. tularensis	LVS	3
Pekarek	S. aureus	ATCC #14458	1
	F. tularensis	LVS	4
Mapes	S. aureus	ATCC #14458	2
Thompson	S. pneumoniae	Type 1	5

TSP-16
RESEARCH SEROLOGY
Joseph A. Mangiafico, M.P.H.
Bacteriology Division

Objective:

To provide a centralized service for the serological evaluation of antigen or antibody content in biological specimens derived from approved work units within USAMRIID, and to conduct basic research for development of new or modified serological procedures in support of specific requirements of these work units.

Progress and Summary:

In the past year a micro-HI test for Sendai virus (Parainfluenza 1) and a solid phase radioimmunoassay (RAI) test for SEB[1] have been added to the procedures performed.

Research was initiated to determine the feasibility of using formalinized gander RBC in the HI test for selected group A arboviruses. At present, fresh gander RBC must be obtained bimonthly and standardized for use in detecting HI antibody to EEE, WEF and VEE viruses. The use of fresh gander RBC has several major short comings: (1) the relatively short shelf-life (2 wk), (2) variation due to uncontrollable changes in physicochemical characteristics during storage, (3) significant animal-to-animal variation in RBC reactivity between donors, and (4) significant seasonal variation in reactivity of RBC from the same gander which may be hormone related. A desirable goal would be to develop procedures for clinical fixation that will stabilize desirable HA properties of gander suspensions.

Gander RBC were formalinized by the method of Feely et al.[2] and stored at 4 C as a 10% suspension in dextrose gelatin veranol solution. Varying suspensions, 0.2 - 0.5% of formalinized RBC in cell-adjusting diluent, were tested for agglutinating ability in the presence of serial 2-fold dilutions of β-propriolactone-inactivated mouse brain antigens of EEE and WEE viruses and the attenuated TC-83 strain of VEE virus. Optimum HA activity with each antigen was obtained with a 0.3% suspension of formalinized gander RBC. Gander RBC 7 and 30 days after formalinization were compared with fresh gander RBC for HA activity in the presence of EEE, WEE and VEE virus antigens as well as normal mouse brain antigen. Comparable titers were obtained with each cell system employed (Table I). Investigations will continue to determine the feasibility of using formalinized cells in the HA test with fresh and frozen sera from animal and human sources.

During the past year, approximately 8,673 serological tests were conducted in support of USAMRIID work projects. Types and numbers of tests performed by division are shown in Table II.

TABLE I. HEMAGGLUTINATION TITERS OF SELECTED GROUP A ARBOVIRUSES WITH FRESH AND FORMALINIZED GANDER RBC

ANTIGEN (STRAIN)	RECIPROCAL TITER WITH CELL SYSTEM		
	Fresh cells	Formalinized cells	
		7 days	30 days
EEE (NJ/60)	1280	1280	1280
WEE (Flemming)	1280	2560	2560
VEE (TC-83)	320	320	320
Normal mouse brain	< 10	< 10	< 10
Diluent control	< 10	< 10	< 10

TABLE II. SEROLOGIC TESTS PERFORMED FOR USAMRIID DIVISION INVESTIGATORS

DIVISION	TYPE AND NUMBER OF TESTS						TOTALS BY DIVISION
	Agglutinin	CF	HA	HI	Oudin	RAI	
Aerobiology	14	60	62	303			439
Animal Assessment			3			112	115
Animal Resources			1127	324			1451
Bacteriology			316				316
Medical	470	531	88	1604			2693
Pathology			172		1148		1320
Physical Sciences	73		24				97
Rickettsiology		450		18			468
Virology	13	249	20	803			1085
Other	49	16	214	320		90	689
TOTALS	619	1306	2026	3372	1148	202	8673

LITERATURE CITED

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2. Feely, J. C., C. P. Sword, C. R. Manclark, and M. J. Pickett. 1958. The use of formalin preserved erythrocytes in the enterobacterial hemagglutination test. Tech. Bull. Reg. Med. Technologists 30:77-82.

TSP-17

AEROSOL SERVICES FOR USE IN EXPERIMENTAL RESPIRATORY DISEASE STUDIES

Joseph W. Dominik, B.S.
Aerobiology Division

Objective:

The objective is to provide aerosol services including quantitative characterization of the aerosols and experimental animal exposures for investigators of USAMRIID (initiated Jan 75). The aerosol facilities, consisting of two 6,200-L environmentally controlled static aerosol vessels, 2 Henderson type aerosol transit tubes with Collison spray devices, and a modified spinning top aerosol system, offer a means for investigators to conduct experiments with respiratory pathogens and simulate natural infection in studying areas such as research in respiratory disease mechanisms, pathogenesis, transmission and therapy.

Progress and Summary:

The aerosol services that were performed for investigators from Animal Assessment, Pathology, Bacteriology and Aerobiology Divisions, and the National Institutes of Health on individual and collaborative experiments are presented in Table I. In addition, 22 aerosol tests were conducted for estimation of concentration, particle size, etc.

TABLE I. AEROSOL SERVICES PERFORMED

INVESTIGATOR	AEROSOL SERVICES			
	No. Exposures	No. Animals Challenged	No. Exposures	No. Animals Treated
Walker--Stephen	20	1484 mice	39	2389 mice[a]
Walker	3	90 mice		
Larson	7	460 mice	2	184 mice[a]
Berendt--Long	10	500 mice	4	200 mice[a]
Berendt	7	385 mice	10	200 mice[a]
	26	26 monkeys[b]	4	42 rats
Jemski, Hetsko--NIH	10	90 hamsters		
Scott	5	265 mice		
	3	32 mice		
		18 guinea pigs		

a. Animals initially challenged were then aerogenically treated on successive days.
b. 18 monkeys used in 2 experiments; 8 challenged twice.

In the category of aerosol tests conducted for concentration or particle size estimate and special testing, the 5 tests performed for Major Walker were to evaluate a laminar flow hood for the Institute in a biological tracer containment study. The 17 tests conducted for Dr. Richard Berendt were as follows: (1) 7 for particle size estimate of drug, (2) 8 for concentration estimates of drug, and (3) 2 for determining the effect of aerosolization on interferon.

In the area of aerosol technology, a continuous aerosol therapy apparatus was designed and developed under the direction of Major Walker and Mr. Harold Young. With this apparatus, animals can be treated aerogenically with drug on a continuous basis for as many hours or days desired.

A total of 3,216 mice, 26 monkeys, 90 hamsters and 18 guinea pigs were aerosol challenged with microorganisms in 91 exposures. The total number of mice and rats aerogenically treated with drug were 2,973 and 42, respectively, in 59 exposures. The number of tests performed in the category of aerosol tests conducted for concentration or particle size estimates and special testing was 22.

APPENDIX B

U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
PROFESSIONAL STAFF MEETINGS

DATE	LECTURER	TITLE OF PRESENTATION
20 Sep 74	Colonel Donald H. Hunter, MSC Dr. Henry T. Eigelsbach Mr. Werner A. Janssen Bacteriology Division	Mechanisms of host resistance in infectious disease.
	Major Paul G. Rausch, MC Bacteriology Division	Lysosomal enzymes in polymorphonuclear leukocytes.
	Major Richard K. Mathis, MC Captain Ronald W. Berninger, MSC Bacteriology Division	Further α_1-antitrypsin studies employing the rhesus monkey model.
	Major Douglas P. Fine, MC Bacteriology Division	Pneumococcal type-associated variability in alternate complement pathway activation.
18 Oct 74	Mr. John P. Kondig Virology Division	Cell culture and cell culture supply.
	Dr. Bruno J. Luscri Mr. Orville M. Brand Virology Division	Production of increased yields of human cell culture interferon and the interferon sensitivity of selected arenaviruses.
	Dr.Robert R. Rosato Captain Joseph D. Gangemi, MSC Virology Division	Biophysical characterization of arenaviruses.
	Captain Peter B. Jahrling, MSC Virology Division	The probable role of endotoxin in the pathogenesis of Venezuelan encephalitis in hamsters.
	Major Stephen C. Marker, MC Virology Division	Effect of adsorption media pH, ionic strength and temperature on group A arbovirus infectivity.
22 Nov 74	Captain Roy T. Faulkner, VC Animal Resources Division	Portal vein catheterization technique for chaired rhesus monkeys.

DATE	LECTURER	TITLE OF PRESENTATION
22 Nov 74	Dr. James W. Johnson Rickettsiology Division	Studies of live Q fever strain M-44 in guinea pigs.
	Major Charles G. McLeod, VC Pathology Division	
	Dr. Richard H. Kenyon Rickettstiology Division	Preparation of an experimental lot of Rocky Mountain spotted fever Vaccine for human immunization.
	Captain Lenora S. Sammons, VC Rickettsiology Division	Experimental Rocky Mountain spotted fever in the vaccinated and unvaccinated rhesus monkey.
	Major Ronald G. Williams, MC Rickettsiology Division	Experimental Rocky Mountain spotted fever in dogs. Description of a possible model.
	Major James B. Moe, VC Pathology Division	Therapy of experimental Rocky Mountain spotted fever in guinea pigs.
17 Jan 75	Captain David T. George, MSC Captain Michael C. Powanda, MSC Lt Colonel Philip Z. Sobocinski, MSC Dr. Frederick B. Abeles Physical Sciences Division Dr. Peter G. Canonico Bacteriology Division	I. Some new observations on leukocyte endogenous mediator(s). 1. Effects on plasma glucose, glucagon, and insulin.
	Dr. Frederick B. Abeles Physical Sciences Division Lt Colonel Gerald A. Eddy, VC Virology Division	2. Abatement of Venezuelan equine encephalomyelitis infectivity in rats.
	Captain Michael C. Powanda, MSC Physical Sciences Division Dr. Richard F. Berendt Aerobiology Division	II. Klebsiella pneumoniae in the rat: A model for respiratory infection.

DATE	LECTURER	TITLE OF PRESENTATION
17 Jan 75	Captain Paul T. Bailey, MSC Physical Sciences Division	III. Effect of exogenous tryptophan on the levels of brain dopamine, norepinephrine, and serotonin during endotoxemia.
	Captain Leo A. Andron, MSC Physical Sciences Division	IV. Preliminary chromatographic studies on transfer factor.
	Major Michael S. Ascher, MC Bacteriology Division	
21 Feb 75	Captain Edward L. Stephen, VC Animal Assessment Division	Prophylaxis of yellow fever in rhesus monkeys by a nuclease-resistant derivative of poly I: poly C.
	Dr. Ching-Tong Liu Animal Assessment Division	Cardiovascular and hepatic responses to SEB in conscious rhesus monkeys.
	Captain George W. Pettit, MSC Animal Assessment Division	Effects of SEB on gastric emptying as reflected by intragastric fructose tolerance test.
	Major William E. Houston, MSC Animal Assessment Division	Alterations in the primary immune response in rhesus monkeys immunized with antigen-antibody complexes.
21 Mar 75	Major Deane F. Mosher, MC Pathology Division	Studies of cold-insoluble globulin, a plasma protein with an affinity for fibrin.
	Major Arthur O. Anderson, MC Pathology Division	Pharmacological regulation of lymphocyte recirculation in lymphatic tissues.
	Dr. John D. White Pathology Division	Scanning electron microscopy of vesicular stomatitis virus infection in cell cultures.
	Major Charles G. McLeod, VC Pathology Division	Virus-like particles associated with inclusions in epithelial cells of Bolivian hemorrhagic fever infected rhesus monkeys.
	Captain Gerald G. Long, VC Pathology Division	Histopathology of experimental respiratory infections in laboratory animals.

DATE	LECTURER	TITLE OF PRESENTATION
25 Apr 75	Major Edward L. Stephen, VC Animal Assessment Division	Aerosol therapy of influenza infection in mice.
	Dr. Richard F. Berendt Aerobiology Division	Aerosol therapy of respiratory bacterial infections.
	Mr. Edgar W. Larson Aerobiology Division	A Sendai virus-mouse model for the study of viral respiratory infection.
	Major Richard A. Kishimoto, MSC Aerobiology Division	Interaction between Coxiella burnetii and guinea pig peritoneal macrophages.

APPENDIX C

U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORMAL PRESENTATIONS AND BRIEFINGS BY VISITORS

Date	Visitor(s)	Subject
24 Sep 74	Lt Colonel Phillip Catanzaro, MC Department of Immunology Walter Reed Army Institute of Research Washington, D.C.	Compartmentalization of the immune system.
22 Oct 74	Captain Kenneth W. Sell, MC, USN LT Commander D. Michael Strong, USN Dr. Aftab Ahmed Ansari Division of Immunology Naval Medical Research Institute Bethesda, Maryland	Immunology of slow virus infections.
12 Nov 74	Dr. Robert A. Prendergast Associate Professor of Ophthalmology and Pathology The Johns Hopkins University School of Medicine Baltimore, Maryland	The sea star and mammalian immune phenomena: Metchnikoff revisited.
10 Dec 74	Dr. David A. Levy Professor of Biochemistry The Johns Hopkins University School of Hygiene and Public Health Baltimore, Maryland	The biology of human IgE.
12 Feb 75	Dr. Arthur Brown Chairman, Department of Microbiology University of Tennessee Knoxville, Tennessee	Cell mediated immunity in cross-protection among group A arboviruses.

Date	Visitor(s)	Subject
18 Mar 75	Dr. Arthur Dannenberg Professor of Environmental Medicine The Johns Hopkins University School of Hygiene and Public Health Baltimore, Maryland	Pathogenesis of TBC: Macrophage activation, enzymes and turnover.
8 Apr 75	Dr. Li-Tsun Chen Assistant Professor Department of Anatomy The Johns Hopkins University School of Medicine Baltimore, Maryland	Histo-physiology of the spleen.
23 Apr 75	Dr. Laszlo Lorand Professor, Department of Biochemistry and Molecular Biology Northwestern University Evanston, Illinois	Molecular analysis of some hemorrhagic disorders.
5 May 75	Dr. Victor M. A. Chambers Radiochemical Centre Amersham, England	Essential criteria in the use of tritium compounds as tracers.
7 May 75	Dr. George C. Parikh Professor, Department of Microbiology South Dakota State University Brookings, South Dakota	Rapid diagnosis and identification of minute quantities of viruses, bacteria, toxins, and antibody in biological fluids.

APPENDIX D

U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES
FORMAL PRESENTATIONS AND BRIEFINGS BY STAFF

Date and Group or Individual	Participating Staff Member(s)	Title
8-11 Jul 74 Second International Conference on Cyclic AMP Vancouver, Canada	Captain Terry V. Zenser, MSC	Infection-induced hyperglucagonemia and altered hepatic response to glucagon in the rat.
10-12 Jul 74 International Symposium on Trace Elements and Human Disease Wayne State University School of Medicine Detroit, Michigan	Dr. William R. Beisel, M.D.	Homeostatic mechanisms affecting plasma zinc levels in acute stress.
14-20 Jul 74 5th International Congress of Radiation Research Seattle, Washington	Lt Colonel Duane E. Hilmas, VC	Quantitative evaluation of tumor vascularity following X-irradiation.
17-21 Jul 74 Second International Congress of Immunology Brighton, England	Lt Colonel Robert Edelman, MC	Participant in workshops on nutritional-immunological interactions and on immunopathology of virus infections.
22-26 Jul 74 Second Gordon Research Conference on Microbial Toxins Brewster Academy, Wolfeboro, New Hampshire	Colonel Joseph F. Metzger, MC	*Escherichia coli* enterotoxin.

Date and Group or Individual	Participating Staff Member(s)	Title
25 Jul 74 Colonel Al Gavazzi, MSC, USAR Mobilization Designee for Commanding General, Medical Service Corps	Colonel Harry G. Dangerfield, MC Lt Colonel Robert A. Massey, MSC	Briefing of USAMRIID mission and tour of facilities.
2 Aug 74 Dr. Roland Möllby The National Bacteriological Laboratory Statens Bakteriologiska Laboratorium Stockholm, Sweden	Colonel Joseph F. Metzger, MC Dr. Leonard Spero, Ph.D.	Briefings of USAMRIID research programs of mutual interest.
11-17 Aug 74 International Conference on Venezuelan and other Equine Encephalitis Vaccines Maracay, Venezuela	Lt Colonel Gerald A. Eddy, VC	Attenuated group A arbovirus vaccines: Advantages and disadvantages of their use in equines.
15 Aug 74 Brigadier General Jack P. Pollock Deputy Commander U.S. Army Health Services Command Fort Sam Houston, Texas	Colonel Joseph F. Metzger, MC and Staff	Briefing of USAMRIID mission and tour of medical facilities.
19-22 Aug 74 Western Hemisphere Nutrition Congress IV Bal Harbour, Florida	Dr. William R. Beisel, M.D.	Cell-mediated and humoral immunity.
30 Aug 74 Division of Metabolism Seminar Walter Reed Army Institute of Research and Walter Reed General Hospital Washington, D.C.	Dr. William R. Beisel, M.D.	Leukocytic endogenous mediator (LEM).

Date and Group or Individual	Participating Staff Member(s)	Title
11 Sep 74 American Association of Laboratory Animal Science National Capital Area Branch Cockeysville, Maryland	Major Robert L. Hickman, VC	The Malaysian silvered leaf monkey, Presbytis cristatus.
2-10 Sep 74 Meeting of the NBC Medical Working Party Headquarters, North Atlantic Treaty Organization (NATO) Brussels, Belgium	Colonel Joseph F. Metzger, MC	Rapid diagnosis of biological agents.
11-13 Sep 74 14th Interscience Conference on Antimicrobial Agents and Chemotherapy San Francisco, California	Major Jerry S. Walker, VC	Kanamycin-aerosol therapy of respiratory Klebsiella pneumoniae infection in mice.
	Mr. Edgar Larson	Virus population dynamics and their mathematical form in influenza infections of mice following respiratory exposure.
	Major Douglas P. Fine, MC	Pneumococcal type-associated variability in alternate complement pathway activation.
	Major Stephen C. Marker, MC	Specific lymphocyte transpormation to VEE virus.
16-20 Sep 74 Pathology of Laboratory Animals Course Armed Forces Institute of Pathology Washington, D.C.	Lt Colonel James L. Stookey, VC	Iatrogenic lesions in laboratory animals.
	Major James B. Moe, VC	Respiratory diseases of rats and mice.
	Major Robert L. Hickman, VC	Tuberculosis in the subhuman primate.

Date and Group or Individual	Participating Staff Member(s)	Title
20 Sep 74 Dr. Marvin E. Lasser, Director of Army Research; Colonel John E. Wagner, Deputy Director; Mr. Mandred Gaile, Asst Director for Laboratory Activities; Dr. V. Garber, Director for Research Programs Office of Chief of Research and Development Command Washington, D.C.	Colonel Harry G. Dangerfield, MC Dr. William R. Beisel, M.D. and Staff	Briefing on USAMRIID mission and research program; tour of facilities.
26 Sep 74 Lt Colonel Marie G. Wister, ANC Nursing Coordinator for U.S. Army Medical Research and Development Command Washington, D.C.	Colonel Joseph F. Metzger, MC Lt Colonel David D. Dryden, MSC Major Dorothy B. Pocklington, ANC	General orientation briefing on the mission of USAMRIID; tour of the medical facilities.
30 Sep 74 Research Training Fellowship Program Group Walter Reed Army Institute of Research Washington, D.C.	Colonel Joseph F. Metzger, MC	Opening remarks.
	Dr. William R. Beisel, M.D.	The USAMRIID research program.
	Lt Colonel Gerald A. Eddy, VC and Staff	Virology Division studies.
	Colonel Harry G. Dangerfield, MC and Staff	Bactericlogy Division studies.
17 Oct 74 10th Meeting of Global Epidemiology Working Group Biomedical Intelligence Sub-committee, Scientific Intelligence Committee Washington, D.C.	Major Jerry S. Walker, VC	New insight on the epidemiology of respiratory diseases.

Date and Group or Individual	Participating Staff Member(s)	Title
30 Oct 74 Maryland Branch, American Society for Microbiology Meeting Goucher College Towson, Maryland	Mr. Edgar W. Larson	Experimental aerobiology and the epidemiological evidence of airborne transmission of infectious diseases.
1 Nov 74 Dr. Bernard T. Tozer Microbiological Research Establishment, Porton Down, Salisbury Wiltshire, England	Colonel Joseph F. Metzger, MC Dr. William R. Beisel, M.D. Dr. Robert A. Altenbern, Ph.D.	Studies of mutual interest on the rapid diagnosis of infectious diseases.
6,12,13 Nov 74 Dr. H. C. Bartleman, M.D. Medical Biological Laboratory of the National Defence Research Organization The Netherlands	Colonel Joseph F. Metzger, MC Dr. William R. Beisel, M.D. and Staff	Briefings on USAMRIID mission and research programs.
3-6 Nov 74 American Society of Tropical Medicine and Hygiene Meeting Honolulu, Hawaii	Lt Colonel Gerald A. Eddy, VC	Extension of mean time to death by antithymocyte serum treatment of VEE virus infected mice.
	Major Carl E. Pedersen, Jr., MSC	Comparative studies of VEE complex viruses.
	Captain Peter B. Jahrling, MSC	Interferon induction and sensitivity as correlates to virulence in experimental VEE virus infection.
7 Nov 74 1974 USAMRIID Planning Session with the Ad Hoc Study Group for Special Infectious Disease Problems Fort Detrick, Maryland	Colonel Joseph F. Metzger, MC	Greetings and discussion of agenda.
	Dr. William R. Beisel, M.D.	Overall status of USAMRIID research and activities.

Date and Group or Individual	Participating Staff Member(s)	Title
7 Nov 74 USAMRIID Planning Session (continued)	Lt Colonel Gerald A. Eddy, VC	Chronic Bolivian hemorrhagic fever in rhesus monkeys.
	Major Charles G. McLeod, VC	Vascular complications of chronic Bolivian hemorrhagic fever in surviving monkeys.
	Captain Albert T. McManus, Jr.MSC	Potentiating effect of attenuated viral vaccine on the immune response to a killed vaccine from the same virus group.
	Major Stephen C. Marker, MC	Purified microbial antigens and lymphocyte transformation.
	Colonel Benjamin J. Veltri, MSC Dr. John D. White,Ph.D.	Part I. Application of new electron. Part II. Microscopy to infectious disease research.
	Captain Donald S. Burke, MC	Fifteen years experience with attenuated Venezuelan equine encephalitis vaccines in man.
	Dr. David J. Hinrichs, Ph.D. Washington State University	Studies on the antigenic composition of Coxiella burneti.
8 Nov 74 USAMRIID Planning Session (continued)		Approaches to the problem of "Early diagnosis"
	Colonel Joseph F. Metzger, MC	Introductory remarks.
	Dr. Neil H. Levitt, Ph.D.	Rapid identification of viruses.
	Dr. Robert W. Wannemacher, Jr.,Ph.D.	Part I: Attempts to develop a diagnostic profile of biochemical values in man.
	Mr. Glen A. Higbee	Part II.
	Dr. Anne Buzzell, Ph.D.	Techniques for the rapid electron microscopic detection (and identification of viruses.

Date and Group or Individual	Participating Staff Member(s)	Title
11 Nov 74 U.S. Department of Agriculture Research Conference in conjunction with the American Society of Agronomy Meeting Chicago, Illinois	Dr. Frederick B. Abeles, Ph.D.	Chemistry of ethylene and its fate in soil.
2-26 Nov 74 Professional Staff Conference John Wesley County Hospital University of Southern California School of Medicine Los Angeles, California	Major Paul G. Rausch, MC	Lysosomal enzymes in polymorphonuclear leukocytes.
21 Nov 74 Maryland Branch, American Society for Microbiology Meeting University of Maryland School of Medicine Baltimore, Maryland	Mr. George H. Scott	Antibody responses of mice to virulent influenza virus administered by aerosols and parenteral routes.
21 Nov 74 U.S. Air Force School of Aerospace Medicine, Medical Aspects of Advanced Warfare Course Brooks Air Force Base, Texas	Colonel Harry G. Dangerfield, MC	Medical defense against biological agents.
22 Nov 74 Bureau of Biological Research Rutgers, The State University New Brunswick, New Jersey	Dr. William R. Beisel, M.D. Dr. Robert W. Wannemacher, Jr.,Ph.D.	Medical aspects of 24-hour rhythms in man. Research aspects of biological clocks.
2-3 Dec 74 National Institute of Allergy and Infectious Diseases Bunyavirus Workshop, National Institutes of Health, Bethesda, Maryland	Dr. Robert R. Rosato, Ph.D.	Structural components of oriboca virus.

Date and Group or Individual	Participating Staff Member(s)	Title
2-5 Dec 74 11th National Reticuloendothelial Society Meeting Seattle Washington	Dr. Henry T. Eigelsbach, Ph.D.	Mechanisms of host resistance to tularemia.
4-6 Dec 74 American College of Veterinary Pathologists Chicago, Illinois	Lt Colonel James L. Stookey, VC	Besnoitia bennetti in two Mexican burros.
	Major James B. Moe, VC	Cerebral tuberculosis in a rhesus monkey.
5-6 Dec 74 Workshop on Rocky Mountain Spotted Fever Center for Disease Control Atlanta, Georgia	Dr. Richard H. Kenyon, Ph.D.	Preliminary studies of a killed Rocky Mountain spotted fever vaccine derived from chick embryo cell cultures.
5-7 Dec 74 American College of Physicians Regional Meeting Galveston, Texas	Major Douglas P. Fine, MC	"Complement" as part of a symposium entitled "Symposium on Immunology in Medicine."
12-13 Dec 74 Workshop on Antivirals in Influenza National Institutes of Health Bethesda, Maryland	Major Jerry S. Walker, VC	1. The efficacy of rimantadine in the treatment of influenza pneumonia in mice. 2. The squirrel monkey as a primate model for clinical influenza.
10-11 Jan 75 Eastern Section, American Federation for Clinical Research Boston, Massachusetts	Captain Ronald W. Berninger, MSC	Isolation and characterization of rhesus monkey alpha-1-antitrypsin (α-1-AT).
	Major Richard K. Mathis, MC	Alpha-1-antitrypsin during infections in the rhesus monkey.

Date and Group or Individual	Participating Staff Member(s)	Title
16-17 Jan 75 U.S. Air Force School of Aerospace Medicine, Medical Aspects of Advanced Warfare Course Brooks Air Force Base, Texas	Colonel Harry G. Dangerfield, MC	Medical defense against biological agents.
21 Jan 75 Seminar at New York University School of Medicine New York, New York	Major Michael S. Ascher, MC Captain Leo A. Andron, MSC	In vitro assay of transfer factor.
22 Jan 75 Brigadier General Robert Bradley, MC, USAR, Mobilization Designee, assigned to Historical Unit Fort Detrick, Maryland	Dr. William R. Beisel, M.D.	Orientation briefing of USAMRIID research activities and a tour of facilities.
28 Jan 75 Symposium "Biochemical and Nutritional Aspects of Trace Elements" 141st Annual Meeting of the American Association for Advancement of Science New York City, New York	Dr. William R. Beisel, M.D.	Trace elements in infectious processes.
30 Jan 75 Surgical Grand Rounds Columbia University New York City, New York	Dr. William R. Beisel, M.D.	Metabolic responses to acute stress.
3 Feb 75 Hematology Division Seminar Tufts University School of Medicine Boston, Massachusetts	Major Paul G. Rausch, MC	Enzymes in granules of polymorphonuclear leukocytes.

Date and Group or Individual	Participating Staff Member(s)	Title
4 Feb 75 Dr. Benjamin L. Harris, Technical Director, and Senior Staff Members Edgewood Arsenal, Maryland	Dr. William R. Beisel, M.D. Mr. William C. Patrick, III Major Isaac Ayala	USAMRIID research management practices.
19-21 Feb 75 19th Annual Meeting of the Biophysical Society Philadelphia, Pennsylvania	Dr. Anne Buzzell, Ph.D.	Electron microscopic assay for virus particles for early diagnosis.
24 Feb 75 Dr. Torkel Wadstrom The National Bacteriological Laboratory Stockholm, Sweden	Colonel Joseph F. Metzger, MC Dr. William R. Beisel, M.D. and Staff members	Participants in discussions and briefings pertaining to research aspects of mutual interest in the USAMRIID program.
10 Mar 75 Dr. P. Hambleton Microbiological Research Establishment Porton Down, Salisbury Wiltshire, England	Dr. William R. Beisel, M.D. and Staff members	USAMRIID studies on the rapid diagnosis of infectious diseases.
12 Mar 75 Department of Pharmacology Lecture Series Howard University Medical School Washington, D.C.	Captain Paul T. Bailey, MSC	The role of serotonin during endotoxemia.
16-17 Mar 75 Conference on Pathobiology of Invertebrate Vectors of Disease New York Academy of Sciences New York City, New York	Major Jerry S. Walker, VC	1. Identification of Rickettsia tsutsugamushi in the life stages of Leptotrombidium fletcheri using isolation and immunofluorescent techniques. 2. Attempts to infect and demonstrate transovarial transmission of R.tsutsugamushi in three species of Leptotrombidium mites.

Date and Group or Individual	Participating Staff Member(s)	Title
18 Mar 75 Seminar at the Medical University of South Carolina Charleston, South Carolina	Major Michael S. Ascher, MC	Transfer factor *in vitro*.
21 Mar 75 Seminar at the University of Virginia School of Medicine Charlottesville, Virginia	Major Robert L. Kaufmann, MC	Lipid changes during bacterial sepsis.
7-11 Apr 75 Scanning Electron Microscopy Meeting St. Louis, Missouri	Dr. John D. White, Ph.D.	SEM of vesicular stomatitis virus infection in cell cultures grown on aluminum foil.
13-18 Apr 75 Annual Meeting, Federation of American Societies for Experimental Biology Atlantic City, New Jersey	Major Arthur O. Anderson, MC	Endothelial proliferation in antigen-stimulated lymph nodes.
	Dr. William R. Beisel, M.D.	Renal depression induced by intravenous staphylococcal enterotoxin B in conscious rhesus monkeys.
	Mr. Glen A. Higbee	Determination of body surface area in the rhesus monkey.
	Dr. Chiang-Tong Liu, Ph.D.	Cardiohepatic responses to staphylococcal enterotoxin B in conscious rhesus monkeys.
	Major Deane F. Mosher, MC	Labeling of cold-insoluble globulin and a fibroblast protein by fibrin-stabilizing factor.
	Captain Michael C. Powanda, MSC	Protein synthesis in zinc-deficient rats during tularemia.

Date and Group or Individual	Participating Staff Member(s)	Title
13-18 Apr 75 Annual Meeting, Federation of American Societies for Experimental Biology (continued)	Captain George W. Pettit, MSC	Use of intragastric fructose tolerance test in assessment of gastric emptying.
	Captain Michael C. Powanda, MSC	Indices of respiratory Klebsiella infection in the rat.
	Major Paul G. Rausch, MC	Granular enzymes of mature polymorphonuclear leukocytes: A phylogenetic comparison.
	Major Edward L. Stephen, VC	Prophylaxis of yellow fever in rhesus monkeys by a nuclease resistant derivative of Poly I:Poly C.
	Mr. William L. Thompson	LEM-induced synthesis of hepatic RNA and acute-phase serum globulins.
	Major Edward L. Stephen, VC	Treatment of influenza infection of mice using rimantadine hydrocholoride by the aerosol and intraperitoneal routes.
	Dr. Robert W. Wannemacher, Jr., Ph.D.	Phenylalanine metabolism in the infected rat.
19 Apr 75 Annual Joint Meeting, Maryland-Washington Branches American Society for Microbiology Fort Detrick, Maryland	Dr. William R. Beisel, M.D.	Welcome and opening remarks.
	Dr. Richard F. Berendt, Ph.D.	Sequential respiratory infection in the squirrel monkey.
	Major Jerry S. Walker, VC	The efficacy of rimantadine in the treatment of influenza pneumonia in mice.
27 Apr-2 May 75 75th Annual Meeting of the American Society for Microbiology New York City, New York	Dr. Robert R. Rosato, Ph.D.	Structural characterization of Tacaribe virus.

Date and Group or Individual	Participating Staff Member(s)	Title
27 Apr-2 May 75 75th Annual Meeting of the American Society for Microbiology (continued)	Major William E. Houston, MSC	Alterations in the primary immune response in rhesus monkeys immunized with antigen-antibody complexes.
28-30 Apr 75 AMA Teaching Program for Practicing Physicians, Nutrition in the Practice of Medicine Boston University Medical Center Boston, Massachusetts	Dr. William R. Beisel, M.D.	Effects of infection on nutritional status.
3-4 May 75 Annual Meeting, American Federation for Clinical Research Atlantic City, New Jersey	Lt Colonel Robert Edelman, MC	The effect of dengue virus infection on the clinical sequelae of Japanese encephalitis: A one year followup study in Thailand.
	Captain Michael C. Powanda, MSC	Antibacterial and antiviral activities of clofibrate.
19 May 75 Dr. Sergei Drozdov Director of Institute of Poliomyelitis and Virus Encephalitides Moscow, Russia	Colonel Joseph F. Metzger, MC Lt Colonel Gerald A. Eddy, VC	Venezuelan equine encephalitis vaccine; tour of facilities.
19-23 May 75 International Workshop on Immune Status in Malnutrition sponsored by the Kroc Foundation and the National Academy of Sciences Santa Ynez, California	Dr. William R. Beisel, M.D.	Malnutrition as a consequence of stress. Nonspecific host defensive factors. Nutritional effects on the responsiveness of plasma acute-phase reactant glycoproteins. Chairman of session, Immunization.

Date and Group or Individual	Participating Staff Member(s)	Title
19-23 May 75 International Workshop on Immune Status in Malnutrition (continued)	Lt Colonel Robert Edelman, MC	Cell-mediated immune response in malnutrition. Defective local leukocyte mobilization in children with kwashiorkor.
8 Jun 75 Graduating Class Governor Thomas Johnson High School Frederick, Maryland	Dr. Harold A. Neufeld, Ph.D.	Address to the graduates of Governor Thomas Johnson High School.

APPENDIX E
PUBLICATIONS OF U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES FISCAL YEAR 1975

1. Altenbern, Robert A. 1975. Membrane mutations and production of enterotoxin B and alpha hemolysin in Staphylococcus aureus. Can. J. Microbiol. 21:275-280.

2. Andron, II, L. A., and H. T. Eigelsbach. 1975. Biochemical and immunological properties of ribonucleic acid-rich extracts from Francisella tularensis. Infect. Immunity 12, In press.

3. Beisel, W. R. 1975. Nutrient wastage during infection. p. 160-167. In Prognosis for the Undernourished Surviving Child, (ed. A. Chavez, H. Bourges, and S. Basta) Proc. Ninth Int. Congr. Nutr, Mexico, 1972, Vol. 2. S. Karger, Basel.

4. Beisel, W. R. 1975. Synergistic effects of maternal malnutrition and infection on the infant. Am. J. Dis. Child. 129:571-574.

5. Beisel, W. R. 1975. Metabolic response to infection. Ann. Rev. Med. 26:9-20.

6. Beisel, W. R. 1975. Nutritional problems in the management of infectious diseases. In Nutritional Support of Medical Practice, Harper and Row, Hagerstown, Md., In press.

7. Beisel, W. R. 1975. Chapter 1: Enterotoxin - mediated disease. In Trace Substances and Health (ed. P. M. Newberne), Marcel Dekker, New York, In press.

8. Beisel, W. R. 1975. The influence of infection or injury on nutritional requirements during adolescence. In Nutrient Requirements in Adolescence, MIT Press, Boston, Mass., In press.

9. Beisel, W. R. 1975. Nonspecific host defensive factors. In Immune Status in Malnutrition. Kroc Foundation Symposia Series, Santa Ynez, Calif., In press.

10. Beisel, W. R. 1975. Malnutrition as a consequence of stress. In Immune Status in Malnutrition. Kroc Foundation Symposia Series, Santa Ynez, Calif., In press.

11. Beisel, W. R., G. L. Cockerell, and W. A. Janssen. 1975. Nutritional effects on the responsiveness of plasma acute-phase reactant glycoproteins. In Immune Status in Malnutrition. Kroc Foundation Symposia Series, San Ynez, Calif., In press.

12. Beisel, W. R., Robert S. Pekarek, and Robert W. Wannemacher, Jr. 1975. Homeostatic mechanisms affecting plasma zinc levels in acute stress. Presentation, International Symposium on Trace Elements and Human Disease, Detroit, Michigan, 10-12 July 1975, Proceedings of the Meeting, In press.

13. Bell, J. C., W. Beckwith, and P. Aylward. 1975. A new, modern research facility: the United States Army Medical Research Institute of Infectious Diseases. J. Am. Med. Technol. 37:35-36.

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