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US ARMY MEDICAL RESEARCH INSTITUTE
OF INFECTIOUS DISEASES

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U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FREDERICK, MARYLAND 21701

ANNUAL PROGRESS REPORT

FISCAL YEAR 1979

RCS-MEDDH-288(R1)

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Projects 3M162770A841
3M161102BS03
3A161101A91C

1 October 1979

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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 1979 is presented.

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EDITOR's NOTE

This FY 1979 Annual Progress report is a general review of research activities of the U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, conducted on Medical Defense Against Biological Warfare Agents (U) under Projects 3M162776A841 and 3M161102BS03 and In-House Laboratory Independent Research, Project 3A161101A91C.

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 Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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FOREWORD

I. USAMRIID's MISSION

The formal mission tasking USAMRIID reads as follows:

Perform studies on the pathogenesis, diagnosis, prophylaxis, treatment, and epidemiology of naturally occurring infectious diseases of military importance with emphasis on problems associated with the medical defense against biological agents and on those microorganisms which require special containment facilities.

By DOD directive and further Army guidance, USAMRIID performs its Biological Agent Medical Defense research in support of the needs of the three services. This mission, and all work done at USAMRIID, is in keeping with the spirit and letter of both President Nixon's 1969 and 1970 Executive Orders renouncing the use of biological and toxin weapons, and the U.N. Convention (Against) . . . Bacteriological (Biological) and Toxin Weapons . . . of 1972.

II. DISSEMINATION OF INFORMATION:

All work conducted at USAMRIID is unclassified. Results are published in peer-reviewed scientific literature, when accepted, as well as in anuual reports. Results of value to organizations outside the U.S. Department of Defense are shared willingly, often in the hope that such sharing will result in additional information on the validity of scientific results or the efficacy of new products, such as vaccines or other biologicals or drugs. Numerous intra-U.S. and international collaborations exist and are encouraged to expand. USAMRIID prints a cumulative bibliography of published articles, which may be obtained by a request to the Editor, USAMRIID, Fort Detrick, Maryland 21701.

III. THE STRATEGY OF THE PROGRAM:

A. The program rests on the judgment that both natural infectious diseases and potential biological warfare threats exist which could seriously interfere with the functions of U.S. forces. The first requirement for constructing the USAMRIID program is to arrive at an assessment as to which microbial and toxin agents are the highest priority threats. Those agents for which existing medical defenses are adequate are set aside. Those agents being addressed by other agencies within the U.S. or elsewhere are likewise set aside. From the refined list the available resources are applied in priority derived from considerations of the severity of their threat and the scientific feasibility of developing improved medical defenses against the agent.

B. The agents being addressed during the period of this report were:

Bacterial

B. anthracis
F. tularensis
L. pneumoniae
S. pneumoniae
S. typhimurium
P. pseudomallei

Viral

Lassa fever virus
Ebola fever virus
Korean hemorrhagic fever virus
Rift Valley fever virus
Bolivian hemorrhagic fever virus (Machupo)
Argentinian hemorrhagic fever virus (Junin)
Dengue fever virus
Congo/Crimean hemorrhagic fever virus
Sandfly fever virus
Eastern encephalitis virus
Western encephalitis virus
Venezuelan fever virus
Japanese B fever virus
Chikungunya virus
Tacaribe virus
Pichinde virus
Yellow fever virus
Influenza virus

Rickettsial

C. burnetii
R. rickettsiae

Parasitic

P. falciparum
P. viral

Toxins

Pseudomonas A
Diptheria
Botulinum A-G
Anthrax toxins
Staphylococcal enterotoxin

IV. Goals

For each of the agents being addressed, the goals were:

A. Pathogenesis: Sufficient knowledge of the biology of the agent and the responses of the infected or intoxicated host (man, as well as available animal models) to provide a basis for progress in the applied goals which are listed below. Useful cell cultures, organ cultures, and a variety of laboratory animal models must be developed and exploited for the insight they can provide on the pathogenic processes in man, since information from human cases for many of the diseases of concern is limited.

B. Improved Diagnosis: Since the choice of medical interventions for either the prevention or the treatment of infectious/toxic disease can only be optimized when the precise infecting/intoxicating agent is known, the ability to make a rapid and specific identification of the causative agent is an important component of a system for medical defense. Ideally, there should be the capability to confirm the identity of agents isolated from the environment, to detect antigen in appropriate clinical samples taken early in the course of disease, and also to detect antibodies from later clinical cases or convalescents. The technology used should be suitable for use throughout the military medical system, including field facilities operating with austere resources. Not only must the agents of major concern be identifiable, but those more common agents which must be considered in a full differential diagnosis must also be identifiable.

C. Prevention: Prevention of infectious disease by immunization is the most effective, convenient, and economical means to reduce the impact from disease on military forces. This goal, then, commands nearly half of USAMRIID's resources. Vaccine development is expected to continue as a major USAMRIID theme, since technological advances often allow the improvement of vaccines which were once state-of-the-art accomplishments. The application of modern biology to vaccine development is presenting opportunities and challenges not foreseen a few years earlier. Passive immunization, active immunization using killed or living attenuated whole agent, or immunization with sub-unit antigens achieved by older or newer methods are options which must be comparatively evaluated for each agent, to arrive at the optimum immunizing method for military forces in various scenarios.

D. Treatment: The unexpected natural disease outbreak can preempt the opportunity to use prevention, and leave treatment as the major medical means to limit damage to the individual and to maintain military force effectiveness. For many of the diseases of concern to USAMRIID, specific treatments which will reverse pathology have not yet been developed. Therefore, treatment strategy must consist of optimal supportive care to give the host defenses sufficient time to respond and overwhelm the disease insult. For these reasons research on improving treatments at USAMRIID has emphasis on developing new specific treatments and on maximizing the effectiveness of supportive care of the infected patient.

V. SUMMARY OF TRENDS IN FY 79

A. Since many programs at USAMRIID are multidisciplinary and carried out by multiple investigators in loose and shifting consortia over several years, the material covered by this annual report may give only a fragmentary insight of the overall program and its progress. In the following sections trends and accomplishments are highlighted.

B. General Progress Highlights during FY 79: In FY 1979, the research programs of USAMRIID continued to emphasize studies of some of the most virulent and pathogenic microorganisms known. Priority I studies continued on many high-hazard viruses which require special P-4 containment facilities including viruses that cause Lassa fever, Congo/Crimean hemorrhagic fever, Bolivian hemorrhagic fever, Argentine hemorrhagic fever, and Korean hemorrhagic fever (KHF). Most recently, another high-hazard P-4

agent, Ebola virus, was successfully introduced into the program. Other Priority I studies were concerned with Rift Valley fever, Legionnaires' disease, anthrax and botulism.

All Priority I studies at USAMRIID are concerned with microorganisms which are lethal for man, present enormous safety problems and at the same time possess significant BW potential. USAMRIID is one of the few laboratories in the free world where any such agents can be studied with minimum risk to laboratory personnel and no risk to the surrounding environment. It is the only laboratory in the free world where so many P-4 Class Agents can be studied at the same time. The goal of the research is to develop safe and effective vaccines or toxoids to prevent these highly dangerous, but poorly understood diseases and to discover methods by which they can be treated successfully should they occur. These laboratory studies provide a base of information which can be used to scale-up vaccine or toxoid production to industrial-sized operations, which can then be defined and described by standard operating procedures (SOP). These SOP's represent a unique national resource in emergencies, significantly reducing the time required to produce adequate quantitites of a critically neded new vaccine or toxoid. Pathogenesis and immunogenesis studies continue to support the development of vaccines, toxoids and therapeutic measures.

Second-order priority studies included work on Japanese B encephalitis, Chikungunya, and Venezuelan equine encephalomyelitis (VEE). Toxin studies continued with bacterial exotoxins and enterotoxins. New diagnostic capabilities were developed as were new treatment methods for other viruses, bacteria and bacterial toxins. All studies on the rickettsial diseases were transferred to Walter Reed Army Institute of Research with the exception of Q fever, a rickettsial disease with significant BW potential. A national workship to discuss all aspects of Q fever immunization research was held at USAMRIID in August 1979; the leading experts on this topic were invited to this workshop in order to sharpen the thrust of the USAMRIID research program for this disease.

Microorganisms or toxins in Priority II are also highly dangerous for man, possess significant BW potential and pose special problems of safety; however, at an intermediate order of magnitude. Priority III studies, the lowest order of priority at USAMRIID, included work on Western and Eastern equine encephalitis, melioidosis and tularemia.

A review of a few of the more important program achievements during 1979 are briefly summarized. The Institute acquired fixed and transportable P-4 containment plastic human isolators (Vicker's) for the hospital care and safe transport of patients suffering from highly contagious, often lethal infectious diseases. In cooperation with the US Air Force, these units were tested with volunteers under long-flight conditions to simulate the evacuation of a contagious patient from Panama to USAMRIID. The test was most successful and established this unique mode of medical evacuation as an achievable reality for future patients. In conjunction with the Institute's in-house P-4 isolation suite, and the recently upgraded clinical diagnostic laboratory, USAMRIID now has the capability to go anywhere in the world to pick-up patients suspected of having a high-hazard infection, to safely transport the patient to the Institute, and to provide "state of the art" medical care for the patient while insuring maximum protection to the medical and laboratory staff personnel.

Ebola hemorrhagic fever virus was introduced into the program during FY 1979 and has been successfully cultured in vitro. The guinea pig was demonstrated to be an effective model for studying this lethal and poorly understood disease. Preliminary data indicate that the virus can be "plaqued" and, therefore, can be detected and assayed in a relatively simple, straightforward manner during future research investigations. This work has increased in importance as the result of September 1979, information that another outbreak of this disease may have occurred in Southern Sudan with 25 deaths among 61 cases. Efforts to confirm the outbreak's cause as Ebola virus are in progress at the Center for Disease Control (CDC), Atlanta.

Lethal animal models were developed for studying Lassa fever in rhesus and cynomolgus monkeys and in inbred (Strain 13) guinea pigs. Moreover, it was found that cynomolgus monkeys could be treated with partial success using either the antiviral durg, ribavirin, or immune serum. A combination of ribavirin plus immune serum was more effective than either treatment alone. Using this combination, all monkeys could be saved even when treatment was started late in the course of the disease. Application of this new information to human patients being studied by CDC physicians in Sierra Leone is expected early in FY 80.

A new generation of Rift Valley fever (RFV) vaccine has been produced in industrial sized quantitites and tested in humans. The vaccine was shown to be both safe and immunogenic. USAMRIID is the only source of this human RVF vaccine. This vaccine is contributing to a control of the spread of RVF from Egypt to the Sinai, Israel, and other parts of the Middle East. It has been used to protect troops of US, Sweden, and Canada serving with U.N. Forces in Sinai, and is used to protect laboratory workers and field veterinary diagnositician staffs in Egypt,Israel, South Africa, and Rhodesia.

Finally, 700 liter of human botulism multivalent immune plasma have been collected and stockpiled: This product is also a unique resource, inasmuch as the previously available antibotulinum serum was produced in horses, caused severe side effects in many recipients, and is no longer marketed by American firms.

C. Research areas given added emphasis during FY 79: The attenuated strain of Junin virus (virulent strains cause Argentine hemorrhagic fever) was found to protect monkeys and laboratory rodents against both the Argentine and Bolivian forms of hemorrhagic fever. Since the attenuated Junin virus, XJ Clone 3, has already been used in 600 human recipients in Argentina, this potential vaccine strain and a closely related one were both emphasized during FY 1979.

Studies were initiated on another high hazard arenovirus, Ebola virus. Cultural methodology has been developed to grow the virus consistently and successfully. A guinea pig model is in the process of being standardized in order to test crude, first generation vaccines.

Major areas of clinical research were emphasized using volunteers and included: (a) the evalauation of an experimental dengue-2 vaccine developed by WRAIR: (b) the evaluation of antimalarial drugs against either Plasmodium vivax or Plasmodium falciparium in volunteers. These two studies were

sponsored by WRAIR but were performed at USAMRIID because of its unique experimental hospital ward. (c) The evaluation of the immunologic response of volunteers to booster administration of botulinum toxid. Also, the evaluation of additional lots of botulinum toxoid. (d) The evaluation of a new generation of RVF vaccine expoliting recent advances in technology. (e) The evaluation of the physical performance capabilities of volunteers infected with sandfly fever virus in a study conducted as a collaborative project with USARIEM.

Another key program initiated during FY 79 was to collect high titer human antibotulinum plasma from individuals who previously had been immunized repeatedly with the existing polyvalent botulinum toxoid. At this time 700 liter of human immune plasma have been collected and processed under contract. The Army's as well as, the Food and Drug Administration's approval was obtained for using this human hyperimmune botulinum plasma for the therapy of acute botulism. In addition,contract arrangements are underway to convert large quantities of this plasma into hyperimmune botulinum immunoglobulin, a procedure that should improve the storage, transport, and administration aspects of antibotulinum therapy.

Finally, during FY 1979, USAMRIID successfully developed suitable model infections in laboratory animals for Legionnaires' disease, KHF, Lassa fever, Congo/Crimean hemorrhagic fever, RVF, and infections with arenaviruses less dangerous than Lassa virus, i.e., Pichinde and Tacaribe. In searching for a representative model, especially for such a difficult disease to study as KHF, a large variety of both common and little used laboratory animal species were tested, including some, such as cotton rats, vesper mice, and voles.

D. Research Areas completed or for which efforts are diminishing in FY 79: Human testing of the Rocky Mountain spotted fever vaccine has continued and the vaccine has consistently been demonstrated to be safe and immunogenic. The USAMRIID vaccine constitutes a limited but unique resource, because in comparative testing with the old commercial vaccine, it was shown to be significantly better. Moreover, the commercial vaccine was recently withdrawn from the market. Further testing of the USAMRIID vaccine will be done at the NIH. Attempts to develop a potent inactivated BHF vaccine in a certifiable substrate were discontinued due to low yields of virus antigen and difficulties in obtaining consistent viral inactivation.

E. List of significant accomplishments for FY 79.

1. The USAMRIID ILIR Program was judged Number one/A among 39 competing laboratories in FY 1978. USAMRIID also won "Laboratory of the Year Honors" in FY 1978. These outstanding achievements became known to the Institute during FY 1979 and are reported here rather than in the FY 1978 report. Dr. Percy A. Pierre, Assistant Secretary of the Army presented the awards to COL Richard F. Barquist, Commander of USAMRIID, in a ceremony held at Fort Detrick on 12 December 1979.

2. Ebolavirus, a high-hazard P-4 virus was successfully introduced into the program.

3. USAMRIID acquired fixed and transportable P-4 containment plastic human isolaters for the hospital care and safe transport of patients suffering

from highly contagious diseases. The first cooperative test with the US Air Force were conducted under long flight conditions.

4. Lethal animal models were developed for studying Lassa fever in monkeys and inbred guinea pigs.

5. Monkey studies revealed that Lassa fever could be treated succussfully by the antiviral drug, ribavirin, or immune serum, or a combination of both.

6. A new generation of Rift Valley fever vaccine was produced in industrial size quantitites and tested in humans.

7. Seven hundred liters of human botulinum multivalent immune plasma were collected and stockpiled.

8. The first human evaluation was conducted of an experimental Dengue-2 vaccine developed by WRAIR.

9. Antimalarial drugs were evaluated against P. vivax and P. falciparum in volunteers as part of the WRAIR program.

10. Long-term booster doses of botulinum toxoid were evaluated in volunteers.

11. Additional lots of botulinum toxoid were evaluated in volunteers.

12. Physical performance capabilities of volunteers infected with sandfly fever virus were evaluated in a collaborative project with USARIEM. additional studies were conducted in volunteers to evaluate the ability of transfer factor to induce protection against tularemia.

13. P-4 microbiological containment facilities were constructed on the Clinical Ward and Clinical Laboratory.

14. An attenuated vaccine strain of Junin virus was found to protect monkeys and laboratory rodents against both the Bolivian and Argentine forms of hemorrhagic fever.

15. Adult guinea pigs were shown to be as suitable as the less available primate models for comparative neurovirulence testing of the tenuated Junin virus vaccine.

16. The effectiveness of a single dose of human Rift Valley fever vaccine was demonstrated in sheep.

17. The Rocky Mountain spotted fever vaccine was shown to be safe and immunogenic in volunteers.

18. Work on a new attenuated Chikungunya vaccine was initiated.

19. A soluble nontoxic antigen from Legionella organisms was found to protect against all six serotypes.

20. Programs were initiated to permit compliance with the specific provisions of the new Good Laboratory Practices Regulation for studies related to vaccine certification.

21. Tests of new adjuvants for enhancing the immunogenic response to viral vaccines were begun; these included tests with liposomal vaccines in collaboration with WRAIR.

22. Animal models were developed for study of airborne Japanese B encephalitis virus in monkeys and mice and for P. pseudomallei infections in mice and hamster. Methods were devised to quantitate dermal hypersensitivity reactions and to detect the magnitude of suppressor and helper cell lymphocyte functions in mice with either live or killed tularemia vaccines. Model infections in laboratory animals were also developed for Legionaires' disease, Korean hemorrhagic fever, Lassa fever, Congo/Crimean hemorrhagic fever, Rift Valley fever and less dangerous arenavirus including Pichinde and Tacaribe.

23. Studies using nude mice revealed that the lethal encephalitis caused by Tacaribe virus was immune-mediated and required the presence of functioning T.-lymphocytes.

24. An effective in vitro model was established for studying macrophage interrelationships with F. talarensis and C. burnetii organisms.

25. Computerized technology was initiated at the USAMRIID for collecting physiological response data in laboatory animals during various infections, diseases and toxemias.

26. Biochemical studies defined in greater detail the mechanisms used at the cellular level for providing metabolizable energy in the infected host, and for the hepatic production of acute phase reactant serum glycoproteins and metallothionines.

27. Diagnostic developmental studies included new RIA, ELISA, and CLIA techniques, as well as the preparation of spot slide for fluorescent antibody identification of 30 different viruses.

VI. EXTRAMURAL RESEARCH:

While this report deals principally with USAMRIID's in-house effort, total program progress is the result of the combination of the in-house effort augmented and supplemented by efforts by contractors from macademia and industry. Individual contractor's reseach is synopsized in reports which are filed with the Defense Technical Information Center (DTIC). A list of contracts in place during FY 79 is included as Appendix C. Readers desiring specific contract report should make request to DTIC.

VII. QUESTIONS:

Questions or comments about this report are welcomed and may be addressed to:

Commander
USAMRIID
Fort Detrick, Frederick, MD 21701

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1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6410	79 10 01	DD-DR&E(AR)636

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

10. NO 'CODES'	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	001
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Procede with Security Classification Code)

(U) Evaluation of experimental vaccines in man for BW defense

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 — PREVIOUS 79	1.0	156.7
FISCAL YEAR — CURRENT 80	2.0	89.0

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Ostroy, P. R.

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Vaccines; (U) Prophylaxis; (U) Therapy; (U) Infectious diseases; (U) Human volunteers

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 USAMRIID, various contractors, organizations or other governmental agencies. Assess effect of antimicrobials, various drug regimens and immune plasma in treatment of militarily important infectious diseases. This work unit is an essential element and a comprehensive program for medical defense against BW agents and other infections of unique military importance by allowing testing in man of newly developed experimental vaccines, new drugs for chemoprophylaxis or therapy and immune plasma or globulins.

24 (U) Test vaccines, experimental drugs and newly developed hyperimmune plasma and/or globulins are given to human volunteers after both full safety testing in animal models and approval under strict protocol conditions which have undergone evaluation by scientific review and medical ethics review.

25 (U) 78 10 - 79 09 - Clinical and serological observation of professional staff, laboratory workers and support personnel continued with administration of vaccines to at-risk laboratory personnel. Human clinical trials were conducted with 3 vaccines: Rocky Mountain spotted fever, Rift Valley fever and a live attenuated dengue-2. A major clinical study conducted in cooperation with NIH on transfer factor was completed Evaluation of a new antimalarial drug was conducted in human volunteers with both falciparum and vivax malaria. Comprehensive studies were conducted on a new botulinum toxoid and collection and evaluation of botulinum immune plasma (human origin). Evaluation of prophylaxis utilizing hyperimmune plasma was conducted with admission to the special isolation unit of 2 individuals exposed to Lassa fever and one exposed to Korean hemorrhagic fever. In addition, individuals were admitted for evaluation of possible yellow fever and an exposure to dengue-1. The Vickers isolation equipment was tested with 2 trials of hospitalization of a patient at USAMRIID and a stimulated evacuation from Panama.

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U).

Work Unit No. A841 00 001: Evaluation of Experimental Vaccines, Prophylaxis and Therapy in Man for BW Defense Against Infectious Diseases of Special Military Importance

Background:

This work unit is a comprehensive research effort incorporating all areas of human volunteer testing and evaluation, as well as utilization of experimental vaccines, antimicrobial drugs, hyperimmune plasma, and special medical isolation procedures in man. This work unit incorporates studies of prophylaxis and therapy against both potential biological warfare threats, as well as infectious diseases of special military importance. The Medical Research Volunteer Subjects (MRVS) Program has enabled USAMRIID to actively conduct clinical studies in man.

Progress:

Rocky Mountain Spotted Fever (RMSF).

Medical Division Protocol FY 78-4, "Proposal for the Clinical Evaluation of a Two Dose Schedule of Inactivated Rocky Mountain Spotted Fever Vaccine, Undiluted (IND 862)" was completed. Thirteen volunteers, including professional staff, non-commissioned officers, and MRVS were picked, and after giving full informed consent, received a preliminary medical and serologic examination. Each volunteer was in good health and serologically negative by microagglutination (MA) and immunofluorescent antibody (FA) titers and lymphocyte transformation (LT) for RMSF. Each volunteer received two 0.5 ml dose of the vaccine SC 28 days apart. Two of the 13 volunteers experienced minor local reactions consisting of pain at the injection site. No systemic reactions were noted.

As measured by the MA test, all 13 volunteers had at least an 8-fold rise in titer (mean = 16) 8 weeks after the first vaccine dose. Seven volunteers tested by MA one year after immunization maintained an elevated titer of at least 4-fold (mean = 13); 70% had an MA titer at least one year that was identical to their 8-week titer. The remaining 2 had a 2-4-fold decrease.

Twelve of the 13 (92%) volunteers had at least a 16-fold FA titer rise (mean = 59) 8 weeks after the initial dose of vaccine. At 6 months, 11 of 12 (90%) had at least an 8-fold titer rise (mean = 25). At one year, only 2 of 7 (30%) maintained at least a 4-fold titer rise (mean = 6). Four of 7 (70%) had no IFA titer at the end of one year.

LT tests at 8 weeks showed a stimulation index (SI), CPM (RMSF)/CPM (controls), range of 7-50. In comparison, the ranges of the 1:10 and 1:3 dilutions were 5 of 15 and 7 of 10, respectively. However, only 3 of 5 that had lymphocyte transformations performed at 8 weeks had a SI greater than 5 and only one was greater than 20. No LT studies were conducted at 1 yr.

An Addendum to Protocol FY 78-4, "Proposal for the Clinical Evaluation of a Two Dose Schedule of Inactivated Rocky Mountain Spotted Fever Vaccine, Undiluted (IND 862) to "Assess the Efficacy and Safety of a Booster Dose of Undiluted, Inactivated Rocky Mountain Spotted Fever Vaccine (IND 862)," has been accepted by the Division Chief's and Human Use Committees. Volunteers from trial number 2, Project FY 78-4, remaining on active duty at Fort Detrick, will be requested to volunteer to receive a 3rd "booster" dose of 0.5 ml of the undiluted RMSF. LT, MA, and FA antibody titers will be measured 1, 2 and 3 weeks prior to inoculation to provide an adequate baseline and at 1, 2, 4 and 24 weeks afterwards. Commencement of this amended protocol is pending further review.

Because of CDC's persistent requests to protect at-risk laboratory workers against RMSF and the scarcity of the existing laboratory-prepared vaccine, the USAMRIID Immunization Committee proposed that the existing vaccine prepared by Merrill-National (MN) IND 862, (Lots 1 and 2) be qualified for human use. Accordingly, addendum to Protocols FY 76-1 and FY 78-4 was submitted and approved by the Division Chiefs' and Human Use Committees.

Two immune volunteers (by virtue of a previously documented naturally or laboratory-acquired infection) as evidenced by elevated FA and MA antibody titers will receive one SC inoculation of 0.1 ml of a 1:10 dilution of MN Lot 1 of the vaccine. These immune volunteers will be recruited from USAMRIID's professional staff.

Four nonimmune USAMRIID volunteers will receive two SC injections, on day 0 and 28, of 0.5 ml of the undiluted MN Lot 1 of the RMSF vaccine.

All laboratory evaluations, including immunologic parameters will be performed accordingly to the original protocols. Multiple dilutions of various preparations of killed Rickettsia rickettsii antigen have been standardized for the LT test, in preparation for evaluation of the cell-mediated immune (CMI) response to booster infections of inactivated RMSF vaccine IND 862 in previously immunized individuals.

Venezuelan Equine Encephalomyelitis Vaccine, Inactivated (VEE).

Protocol No. 77-1, Addendum 1, "Acceptability Study of Venezuelan Equine Encephalomelitis Vaccine, Inactivated, Dried, MNLR 109, Lot No. C-84-1, IND 914," was outlined in last year's annual report. Addendum 2 was completed to study the effect of a 3rd booster immunization to 4 previously boosted individuals and 4 not previously boosted. The additional immunization at 6 mon was sought to determine the potential to induce titers to VEE. Seven individuals gave consent to an additional booster and one of the original control (nonimmunized) MRVS consented to continue as a control. Serologic testing has now been completed and complete results are shown for this booster with selected titers (80% PRN) from previous immunizations shown for reference:

TABLE I. PRN_{80} TITERS

	RECIPROCAL PRN_{80} TITER						
	16 Jun 77	14 Jul 77	28 Jul 77	19 Apr 78	8 May 78	22 May 78	18 Jun 78
Previously Boosted (Immunization dates 16 Jun 77, 14 Jul 77Jul, 18 Apr 78)							
CA	< 10	10	640	40	10,000	10,000	10,000
GG	< 10	< 10	80	10	NS	NS	1,280
JR	< 10	10	160	10	≥ 10,000	5,120	2,560
Not Previously Boosted (Immunization dates 16 Jun 77, 18 Apr 78							
GM	< 10	40	20	< 10	2,560	5,120	5,120
RD	< 10	10	40	10	5,120	5,120	2,560
RL	< 10	NS	40	10	≥ 10,000	≥ 10,000	NS
RB	< 10	NS	< 10	< 10	≥ 10,000	≥ 10,000	5,120
Control (No Immunizations)							
TA	< 10	< 10	< 10	< 10	< 10	< 10	< 10

Rift Valley Fever (RVF).

Protocol FY 78-1, "Evaluation of the Human Response to the Administration of Rift Valley Fever Vaccine, Formalin-Inactivated, Tissue Culture Origin, NDBR 103, Lot 1-6 (IND 365)" and FY 79-5, " Evaluation of the Human Response to the Administration of Rift Valley Fever Vaccine, Inactivated, Dried TSI-GSD-200." The serologic studies relating to the project were completed during this time period. Lots 1-5 were found, as far as possible given the number of subjects involved, to be identical to Lot 6, Run 1 with which so much previous experience had been gathered. An addendum to protocol FY 78-1 was submitted involving the investigation of the response to two doses of 0.5 ml of Lot 6, Run 1 given on 2 occasions, day 0 and day 28. The immunological response to this regime was found to be variable and only those people who received concurrent vaccinations with VEE were found to have a dependably high response. Of the 5 who received RVF vaccine only, 2 failed to respond adequately, one responded marginally, and 2 responded with sufficient level of antibodies to be considered protected. A second protocol (FY 79-5) was developed during this time. The first part of this protocol dealt with the safety of the vaccine when administered to humans; 7 subjects were involved, and as no adverse effect was found, initiation of the

efficacy testing procedures. Volunteers who had never been exposed to the vaccine or RVF virus were injected with Lot 1 in doses of 1.0, 0.3 and 0.1 ml of the vaccine on days 0, 10 and 28. Immunologic response was measured by means of plaque-reduction reducing neutralizing antibody (PRN); 1 ml was uniformly efficacious in inducing immunity; 0.3 ml induced measurable PRN antibodies, although at a slightly lower level; 0.1 ml resulted in only about a 50% response rate. Subsequently, Lots 2, 3, and 5 were tested in doses of 0.3 ml given on days 0, 10 and 28. All lots produced satisfactory levels of measurable PRN antibodies. At the end of this time period a further lot, Lot 4 was begun through the testing phase but to date the study is incomplete. Safety testing was done throughout this period on all recipients; volunteers experienced any local or systemic reaction that could be ascribed to the vaccine.

Dengue Fever, Type 2 (DEN-2).

Protocol FY 78-2, "Evaluation of Human Response to the Administration of Dengue Virus Vaccine (Type 2) Live, Attenuated" (IND-1257) developed by WRAIR was reported in the previous annual report. Results were evaluated by multiple scientific review sessions which suggested that the next phase should be a repetition of the first study, using groups of volunteers given serial dilutions of the vaccine. It was felt that using 10-fold dilutions of the vaccine in 5 groups might reveal a dose in which immunogenicity was equivalent to the undiluted vaccine, but with less reactogenicity. It was also suggested that attempts be made to evaluate the potential transmission of the vaccine virus by mosquitoes. A new protocol was written, reviewed, and approved. Protocol 79-1, "Immunization with Live Attenuated Dengue Virus Vaccines, Study No. 2 Response to Varied Doses of DEN-2 (PR 159/S-1) Vaccine in Adult Volunteers" (IND-1257), was designed for 36 volunteers, however, only 11 of MRVS volunteered. The vaccine was given in double-blind fashion and utilized 10-fold dilutions of the vaccine with 5 volunteers receiving an estimated dose of 10^4 PFU, 5 volunteers, 10^3 PFU, and one, 10^2 PFU. The study was conducted on an outpatient basis with the volunteers restricted to the Frederick valley area, in which mosquito surveys revealed no genus capable of transmitting the virus. In addition, 7 of the volunteers participated in a live mosquito-feeding study, designed to assess the risk of transmission of the vaccine virus. Of this group, one of the volunteers had a febrile episode 5 days following administration of the vaccine, the remaining volunteers all tolerated immunization without significant reactions, although one developed a faint peripheral rash.

In an effort to complete Project FY 79-1, two additional groups of volunteers were recruited to receive this DEN-2 vaccine. In this group, 6 individuals were immunized in a double-blind fashion with 5 receiving estimated doses of 3×10^5 PFU and one volunteer, a placebo. A third group was begun several weeks later with 4 volunteers who were immunized with dilutions of the vaccine from group 2.

All of the volunteers selected had received no prior immunization to yellow fever (YF). The first 6 individuals from Protocol FY 78-2, had had multiple YF immunizations; there was concern that this might have played some role in the immune response. The second 2 groups of study FY 79-1 were conducted on a outpatient basis with the volunteers restricted to the greater Washington-Baltimore-Frederick area. This modification was made because of the snow and freezing temperatures at the time of the study, which eliminated possible risks of introduction of virus to local mosquito populations.

Although 3 of the volunteers were admitted to Ward 200 for unrelated injuries or illnesses, there was no demonstrable illness correlated with DEN viremia. In summary, a total of 21 volunteers (2 given placebo) have been inoculated to date. Eight of the 19 vaccine recipients seroconverted and 5 had detectable viremia. The isolated DEN virus had growth characteristics identical to the vaccine virus; antibody levels detected on the 30th day postinoculation were consistent with DEN infection. The frequency of seroconversion at each vaccine dilution did not correlate with the dosage, so that it is suggested that immunization is not dose-dependent, at least in the range covered by these experiments.

While none of the volunteers developed local reactions, a variety of symptomatic illnesses were reported by 11 of the 21 volunteers in the first 3 weeks following vaccination. The majority of these complaints could be attributed to incidential infections and trauma. Two of the 8 people who seroconverted were symptomatic; one developed headache, myalgia, eye pain and rash consistent with clinical DEN. The other had symptoms consistent with low back strain. Leukopenia occurred in 2 people who seroconverted and in one who did not. In total, one of the 8 individuals who seroconverted developed a DEN-like illness, while 3 others had mild clinical symptoms only.

In the mosquito-feeding experiments, virus was isolated from 2 of 72 mosquitoes feeding on one viremic volunteer. This provides evidence that individuals receiving the vaccine who become viremic are capable of infecting the natural DEN vectors.

A second study conducted under Protocol FY 79-1 was also begun. Eleven volunteers who had previously been inoculated with the DEN-2, live, attenuated virus vaccine were reimmunized with undiluted vaccine. This group included both those who had seroconverted previously and those who had not. The frequency of seroconversion at each of the previous vaccine dilutions did not correlate with the virus dosage, so it was suggested that the development of neutralizing antibody was not dependent upon the dose received. None of the volunteers receiving the second immunization developed local reactions at the injection site, and there were no indications of symptomatic illness reported by any volunteers in the time period since vaccination. Serologic data will be obtained and reported later.

Active recruiting for volunteers for additional DEN studies under Protocol FY 79-1 was conducted during the last quarter of FY 79. One very key study will be the immunization of individuals who are seronegative for DEN, but have demonstrable YF titers. Previous studies suggest that YF-immune individuals may have better responses to DEN-2 vaccine. Another study to be conducted will use volunteers who are both DEN and YF virgins and will evaluate the immunogenicity and reactogenicity of the vaccine given ID rather than SC.

Botulism

Botulinum Toxoid. Protocol FY 79-4 entitled, "Evaluation of the Human Response to the Administration of Botulinum Toxoid, Adsorbed, Pentavalent (ABCDE), MDPH (IND 161)," was completed. This study was an evaluation and comparison of the clinical and immunologic responses of human volunteers to each of 3 lots[1 old (Parke-Davis) and 2 new (MDPH #1 and MDPH #2)] of pentavalent botulinum toxoid. Three groups of volunteers (total = 54) were immunized with 1 of 3 lots of toxoid according to 1 of 3 dosage schedules. Two weeks after completion of the initial toxoid series of 3 injections, the 2 new lots of toxoid had stimulated immune responses (geometric mean, 0.30 and 0.36 for type A; 0.08 and 0.10, type B) which were equal to or greater

than those elicited by the Parke-Davis toxoid (geometric mean, 0.14 for type A and 0.02, type B). Titers to type C, D, and E botulinal toxins are currently being determined. There appeared to be no detectable difference in the clinical reactogenicity of the 2 new lots compared to the old product. This lack of difference in reactogenicity is important, in that one of the new MDPH lots contained significantly less formalin and had been anticipated to be less reactogenic.

Protocol FY 79-3, "Evaluation of Immunologic Response to Booster Administration of Botulinum Toxoid, Adsorbed, Pentavalent (ABCDE) (IND 161)," was conducted at the Pine Bluff Arsenal in Arkansas. This study was carried out in conjunction with a contract for the collection and delivery of hyperimmune plasma detailed in this report below.

The anamnestic immune response of 24 of these immune plasma donors to a booster immunization in Feb 1979 with botulinum toxoid, adsorbed pentavalent (Parke-Davis toxoid) was evaluated by mouse neutralization test. These studies were performed by Major George Lewis of Pathology Division, USAMRIID. The geometric mean serum titer for the donor group to type A toxin was 0.82 units/ml at the time of boosting, and was 8.98 units/ml 28 days later. The group prebooster geometric mean titer to type B toxin was 0.06 units/ml, while the 28-day postbooster titer was 0.52 units/ml. The type E geometric mean was 0.16 units/ml before boosting and 2.3 units/ml afterwards. During the 1960's the individuals in this group received the initial toxoid series of 3 immunizations with 4-7 subsequent boosters. The last booster prior to Feb 1979 was given during the period 1969-1970. The approximate 10-fold increase recorded for both types A and B neutralizing antibody and even greater for type E, in response to a single booster after a 9-year lapse of immunization, is highly significant (P 0.001) and represents an excellent anamnestic response.

Botulism Immune Plasma (Human) (IND 1332). Protocol FY 79-3 was also conducted with an objective of creating a pool of donors for a plasmapheresis program to obtain pentavalent antitoxin of human origin against botulinum toxins. The project involved a potential donor pool of approximately 120 federal employees in Pine Bluff, Arkansas area. During this reporting period, 2 groups of individuals were identified and entered into the plasmapheresis program. In the initial group, approximately 35 individuals were given screening examinations; 28 of them received 0.5 ml of botulinum toxoid, adsorbed, pentavalent (ABCDE) distributed by CDC. Each individual received a preimmunization serologic evaluation; one week following immunization each volunteer began a program of plasmapheresis in which it was anticipated the volunteers would be plasmapheresed twice weekly for 16 weeks. Twenty-six of the 28 individuals had either no reaction or only mild erythema or slight induration following immunization. One individual experienced a moderate reaction with erythema and induration, and one individual experienced a severe reaction, with erythema, induration and edema of the arm. All 28 individuals completed the entire 16-week period of plasmapheresis with no major problems. This initial group resulted in the acquisition by USAMRIID of approximately 500 L of botulism immune plasma of human origin. In the second group selected for plasmapheresis from Pine Bluff, 26 individuals received a booster immunization; 24 had either no reaction or only mild erythema or slight induration following immunization, and 2 had moderate reactions with erythema and induration. Twenty-four individuals completed the 16-week course of biweekly plasmapheresis. The volunteers in this group yielded an additional 650 L of botulism immune plasma of human origin. As in the previous group, all the plasma was collected as single donor unit material.

A pilot lot of botulism immune plasma of human origin of approximately 52 L was shipped to the Bureau of Laboratories, Michigan Department of Health, Lansing, MI, and for custom fractionating to produce botulism immune globulin of human origin. Approximately 1 L of botulism immune globulin was recovered; it is being held in bulk until identity, sterility and pyrogenicity testing is completed.

"Determination in Humans of the Effective Half-Life of Botulism Immune Plasma (Human) (IND 1332) Administered Intravenously," USAMRIID Protocol FY 79-7, was initiated during the period. The objective was to determine the effective half-life of specific antibodies in volunteers given plasma IV. In addition to studying the disappearance of botulinum antibodies, several antibodies are also being tested. Following determination of blood volume by the Nuclear Medicine Service at WRAMC, the one volunteer in this study thus far received 340 ml of blood group type-specific immune plasma collected at USAMRIID. Approximately 2,965 IU of type A neutralizing activity and 595 U of type B activity were diluted in an isotopically determined volunteer plasma volume of 2,503 ml during the 1-1½ hr administration period. The circulating neutralization activity (titer) to types A and B toxins were determined at various time intervals and are shown in Table II. Serum titers to types C, D and E botulinal toxins are currently being determined.

TABLE II. NEUTRALIZATION ACTIVITY (TITER) TO TYPE A AND TYPE B BOTULINAL TOXINS OF SERA COLLECTED FROM A VOLUNTEER ADMINISTERED BOTULISM IMMUNE PLASMA (HUMAN) IND-1332

	TITER (10/ml)	
	A	B
0	0	0
2	1.92	0.49
8	1.92	0.62
24	2.43	0.49
48	1.50	0.38

Q Fever Vaccine.

A new protocol entitled "Clinical Evaluation of Formalin-Inactivated, Dried Henzerling strain, Phase I, Q Fever Vaccine, NDBR 105 (IND 610), for Safety and Immunogenicity," was developed and submitted to a Blue-Ribbon panel of prominent rickettsiologists from the University of Maryland, Rocky Mountain Laboratory, Hamilton, WRAIR and other places throughout the United States at a Q fever immunization discussion held at USAMRIID.

This protocol was developed for testing the safety and immunogenicity of the phase I Q fever vaccine, NDBR 105. After lengthy in-depth discussions, it was decided that the protocol should be amended to divide the subjects into 3 groups. Each group was to be initially tested with a dose of 0.2 ug/ml of the phase I Q fever antigen, followed 1 week later by 6, 30 or 60 ug/0.5 ml SC in those volunteers have negative skin tests.

The humoral immune response will be measured by the MA and CF tests; CMT will be measured by LT test. Six months after the single dose of vaccine, a follow-up skin test will be performed, using 0.2 ug/ml of the phase I antigen. LT tests will be correlated with skin test results to determine the vaccine dose providing the highest rate of conversion. If satisfactory conversion rates are not found with a single dose of the vaccine, a 2-dose protocol will be submitted. Animal testing of this and other lots of this phase I vaccine should proceed according to the original protocol.

Establishment of the microimmunofluorescent test to measure IgG and IgM levels to Coxiella burnetii was recommended.

In addition to the preparation and presentation of the above protocol, standdardization of phase I, phase II and insoluble burnetii antigen for the LT test has been carried out in preparation for the vaccine trial.

A joint Protocol (FY 78-3) was started between USAMRIID and NIAID, "Transfer of Cell-Mediated Immunity to Microbial Antigens with Dialysable Leukocyte Extracts (Transfer Factor)." Six MRVS were recruited; they participated in a series of skin tests, collection of transfer factor, and subsequent reevaluation of skin test response to a multiple battery of immunogens. Although a complete statistical analysis of the response is still being evaluated, there did not appear to be significant demonstration of transferable CMI as had been hypothesized.

Malaria. During the last year phase II studies were performed on Protocol 79-2, entitled, "Evaluation of WR 171,669 in the Treatment of Plasmodium vivax (Chesson Strain) Malaria." This experimental drug, phenanthrenemethanol, has shown promising antimalarial activity in preclinical testing and in a small number of human subjects. Six volunteers were inoculated with the Chesson strain of vivax; only 2 became patent. The others were treated with a standard course of chloroquine phosphate when patency failed to develop.

In a continuation of Protocol 77-3 "Evaluation of WR 171,669 in the Treatment of Multi-Drug Resistant P. falciparum Malaria," 8 volunteers were inoculated with the Smith strain of P. falciparum. This strain is resistant to chloroquine. All the volunteers became patent. Important treatment parameters for both protocols are summarized in TABLE III. To date, WR 171,669 has resulted in cure in all cases. Cure is defined as elimination of parasites from the blood, disappearance of signs and symptoms of malaria, and lack of recrudescence during a 60-day follow-up period.

TABLE III. PHASE II STUDIES WITH WR 171,669. FOLLOW-UP AT 60 DAYS

PATIENT NUMBER	DRUG DOSE	TOXICITY	PARASITE COUNT/mm^3 AT TREATMENT	PARASITE COUNT/mm^3 HIGHEST	PARASITE CLEARANCE TIME (h)	DEFERVESCENCE (h)
P. vivax (Chesson)						
1	250 mg Q6H x 12	None	850	1040	64	50
2		Mild nausea, diarrhea	700	1010	69	62
P. falciparum (Smith)						
1	250 mg Q6H x 12	None	220	222	28	90
2	"	"	1200	1200	70	108
3	"	"	1620	1620	71	81
4	250 mg Q6H x 8	Mild diarrhea	230	230	27	54
5	"	None	190	600	40	98
6	"	"	150	430	63	104
7	250 mg Q6H x 4	"	270	280	63	104
8	"	"	230	420	69	104

Sandfly Fever. "An Evaluation of Physical Performance Capabilities During Sandfly Fever Infection." Protocol No. 79-6, was conducted to measure anticipated decrements in physical performance capabilities in comparison to various biochemical changes in muscle before, during and after experimentally induced sandfly fever in volunteers. The purpose was to evaluate the capacity of soldiers to perform light physical exercises during periods of clinical fever and early convalescence from a representative infectious illness. The evaluation of physical performance as well as the biochemical and histologic examinations of the muscle biopsies (Work Unit 841 051) are still being analyzed and will be reported in the future.

Endocrine and metabolic aspects of the infection were conducted in conjunction with the muscle evaluation (Work Unit 841 052). The effect of acute illness produced by sandfly fever virus (SFV) on insulin secretion, carbohydrate metabolism and insulin receptor physiology was evaluated in 3 males who granted fully informed consent to oral glucose (1.75 g/kg) tolerance tests (OGTT) and insulin receptor studies both prior to SFV inoculation and at onset of fever. Fasting glucose (106 ± 4 mg/dl was elevated ($P < 0.01$) above control (93 ± 4 mg/dl) at least at onset of fever, but prior to changes in dietary carbohydrate intake. Fasting immunoreactive insulin (IRI) values were also increased (22 ± 4 compared to 12 ± uU/ml, $P < 0.05$). Glucose values during OGTT were elevated at all points compared to preinoculation controls and failed to return to fasting by 120 min. Insulin response was elevated (50-130%, $P < 0.05$) at each time point. Increased insulin: glucose ratios during illness suggest insulin resistance as an explanation for the carbohydrate intolerance. Insulin receptor studies on peripheral mononuclear cells demonstrated a 7.5-30% decrease in binding at onset of illness, compared to preinoculation values. The decreased binding was associated with a 25-75% loss of insulin receptors during infection ($P < 0.05$). Receptor affinity remained the same in 2 volunteers, but was markedly increased in one. In conclusion, the onset of mild viral illness in man is associated with hyperinsulinism, glucose intolerance, insulin resistance and decreased binding of insulin to peripheral mononuclear leukocytes, attributed to decreased receptor numbers.

Hospitalized Exposures

Lassa Fever. Two individuals were hospitalized this year because of possible exposure to Lassa (LAS) virus.

One male military laboratory technician was admitted to the isolation suite following a potential LAS exposure. The individual noted a tear in his plastic suit while getting ready to leave the LAS suite. He was unable to establish the time of the tear and during his duties in the suite had handled concentrated virus, done procedures involving centrifugation and had direct contact with infected animals. It was determined by close examination of the suit that this represented the most minimal chance of exposure, and it was the decision of the attending physician not to use hyperimmune plasma. During his hospitalization no symptoms or signs of Lassa fever developed. There were no laboratory findings consistent with any illness. A decision was made after 14 days of hospitalization to release the patient based upon the extremely low probability of exposure and high probability that infection, were it to occur, would have manifested itself by that time.

The second admission to the isolation suite occurred when a senior male medical technician was admitted following an accident in which he stuck a stainless steel pin into his finger while working with a partially anesthetized guinea pig which had been exposed to an aerosol of LAS 3 days before. Because of strong evidence of the presence of virus in the salivary glands of the pig, and because the animal's fur had also been exposed to the aerosol challenge, the individual was judged to have had an exposure. The individual was hospitalized and given a complete physical examination; it was normal, with the exception of mildly elevated blood pressure and the presence of fine rales in the lungs compatible with chronic, excessive smoking. The patient was treated with approximately 200 ml of hyperimmune plasma on the day of exposure. He remained clinically well with no clinical or laboratory evidence of infection. There was no rise in antibody titer following administration of the passive antibody, and no virus was recovered from the patient. After 21 days in strict isolation, the patient was discharged and was seen on a periodic basis as an outpatient.

Korean Hemorrhagic Fever (KHF). A senior male civilian microbiologist was bitten by a squirrel monkey which had been inoculated with the agent of KHF approximately 21 days earlier. The virus was taken from another squirrel monkey, which had died of the disease, and inoculated into the monkey involved in the bite. This monkey at the time of exposure was clinically ill with fever and appeared to be entering a preterminal period of hypothermia. Because the pathophysiology of KHF is not well understood it was decided that in the best interest of the patient and his contacts to hospitalize him in both normal and reverse isolation, as well as to initiate treatment with hyperimmune plasma. The individual received one unit of high titered, hyperimmune plasma on the day of exposure. There was no evidence during the 21 days of hospitalization of the appearance of any clinical symptoms or laboratory evidence of infection with KHF. The patient was discharged and was seen on a periodic basis as an outpatient.

Yellow Fever (YF). One enlisted male surveillance technician was admitted to the isolation unit for diagnosis of a febrile illness characterized by nausea, malaise, and temperatures to 105 F. The individual was one of several persons identified as being present and potentially exposed during a failure in the air handling system of Virology Suite #3, which resulted in possible positive pressure flow from the suite. Although the suite contained YF, there was no known virus open during the time of the air failure. While in isolation, the patient responded to symptomatic treatment and did not develop YF. Serology for YF was negative at the time of admission and remained so.

Dengue-1 (DEN-1). One female technician was hospitalized on the open ward following a needle stick in which the needle had previously been used to obtain a blood sample from a primate which had been given DEN-1 virus. The patient was admitted to the open ward and confined to the inside of Bldg. 1425 for the period of hospitalization, in order to avoid exposure to the native mosquito population if the patient became viremic. The patient demonstrated no clinical evidence of active infection and was discharged following a period of observation equivalent to the incubation period of the disease.

Isolation Facilities and Equipment

Vickers Bed Isolator. USAMRIID acquired 2 of the Vickers bed isolators for P4 level containment of high hazard disease. Although no actual illnesses or exposures were confined to the Vickers unit during this period there were 2 separate

trials of the unit with a hospital corpsman volunteering to spend 5 days in the isolator with a full simulation of all procedures. One male and one female participated as volunteers; during the simulated isolation, there were no problems encountered with patient care or morale. No equipment failures occurred nor were there personnel errors during the hospitalization. These trials contributed significantly to both the training of the staff and the development of a Vickers unit manual for use during actual hospitalization.

Vickers Isolator Transport Equipment. During this past year, there was a joint Air Force and Army evaluation of the aircraft transit isolator.

The isolator was developed by Vickers Ltd, Medical Engineering of Basingstroke, England, and the Royal Veterinary College of Great Britain. The aircraft isolator encloses the patient in a negatively pressurized plastic envelope; exhaust air is filtered using HEPA filters. This transit isolator is compatible with both the bed containment isolator and the stretcher isolator, thus enabling the patient to be directly transferred without breaking microbiological barriers.

The aircraft isolator was tested on both C-130 and C-141 aircraft. The procedures tested and evaluated on both aircraft included loading and securing the isolator to the aircraft floor and unloading it. On a C-130 flight from San Antonio, TX, to Charleston, SC, physical characteristics (e.g., positioning, power supply, etc.) of the isolator were evaluated, and a brief simulated patient trial was accomplished. A simulated aeromedical evacuation from Charleston, SC, to Panama on a C-141 was conducted. Patient care techniques were developed, evaluated and practiced on this flight. The isolator was also connected to the C-141 electrical and oxygen systems. Liaison between the Air Force and USAMRIID was established and determination of the functions of the 2 groups involved with transportation of highly infectious diseases was accomplished.

Publications:

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OD6419	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	003
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2;2			

11. TITLE (Precede with Security Classification Code)

(U) Mechanisms of immunoprophylaxis against aerosol-disseminated respiratory 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 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 79	1.0	174.8
FISCAL YEAR CURRENT 80	1.0	238.5

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Respiratory pathogens; (U) Airborne infections; (U) Franciscella tularensis; (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) Characterize host immunity induced against respiratory infections by administration of microbial antigen by aerosol, intranasal and parenteral routes to appropriate animal models. Determination of optimal methods of immunoprophylaxis is an essential element in military medicine as a major determinant of resistance against potential BW respiratory pathogens.

24 (U) Immunogenic determinants for resistance against aerosol disseminated respiratory disease are being studied in the Fischer 344 inbred rat/tularensis model system. The rats vaccinated by different routes (aerosol and parenteral) are then treated with an immunosuppressant drug before and after vaccination to evaluate the contribution of the various effector arms of immunity to the host's defense against respiratory tularemia.

25 (U) 78 10 - 79 09 - Fischer 344 inbred rats were vaccinated with either the live vaccine strain (LVS) or with an inactivated Foshay-type vaccine of F. tularensis. When they were treated with cyclophosphamide, an immunosuppressant that totally or severely depressed the production of humoral antibodies, they survived aerosol and intraperitoneal challenge (100,000 cells) with virulent F. tularensis. All nonvaccinated control animals died. This is the first direct demonstration that serum antibody is not a critical component of the immune system for total protection against lethal tularemia. Corollary studies also showed that LVS vaccine administered by aerosol, intranasal instillation or parenteral routes provided practically equal protection to the rat against respiratory tularemia.

* Available to contractors upon originator's approval

* U.S. GPO: 1974-543-843/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 003: Mechanisms of Immunoprophylaxis Against Aerosol-Disseminated Respiratory Diseases

Background:

Francisella tularensis has long been recognized as a respiratory pathogen which poses a significant threat as a BW agent (1). Protection against airborne tularemia ideally would be via immunoprophylaxis. In understanding immunity to aerosol-disseminated tularemia, however, one must consider the role of cellular immunity, specific humoral antibodies and local secretory antibodies which may well be dependent on the method used to vaccinate the host. Cell-mediated immunity is now recognized as a primary immune arm for protection against lethal tularemia, while the role of another immune arm, humoral antibodies has not yet been fully delineated. However, it also is recognized that humoral antibodies are not predictive of the immune state of tularemia. One may then ask the questions: (A) to what extent, if any, do humoral antibodies contribute to the hosts' defense; (B) can a vaccinated host, in the absence of detectable serum antibodies, still be considered protected against challenge with virulent F. tularensis; and (C) does the route of vaccination influence the resistance of the host against respiratory tularemia? The research reported here was directed toward answering some of these questions.

Progress:

The usefulness of the Fischer 344 inbred rat to study immunoresponsiveness to attenuated and virulent strains of F. tularensis has been described (USAMRIID Annual Report, 1978). Utilizing the Fischer rat, comparative studies were pursued to determine the relationship between host response and protective immunity against respiratory tularemia in vaccinated and nonvaccinated control rats treated with cyclophosphamide (CY), an immunosuppressant. CY is a cytotoxic drug that severely depresses the production of humoral antibodies, and depending on dose, may potentiate the cellular immune response (2). Because of the toxicity of CY, reference studies were conducted to establish a dose of drug which would cause a definitive leukopenia (an indicator of immunosuppression) in the rat, but not a significant number of mortalities. CY was administered IP to male rats at dosages ranging from 25-250 mg/kg body weight. All rats injected with > 100 mg/kg died within 3-5 days. The 100-mg/kg treated rats appeared ill and had acute diarrhea lasting 2-6 days; these rats fully recovered. At levels of ≤ 50 mg/kg no physical signs of toxicity were observed. Leukopenia ($2,750/mm^3$) persisted for 6 days followed by rebound leukocytosis ($16,500/mm^3$). Based on these results, a CY dose of 50 mg/kg for a 250-gm rat was selected for subsequent use. Rats weighing < 250 gm were much more susceptible to the toxic effects of CY.

Initial experiments studied the effect of a single dose of CY on altering humoral and cellular immunity against respiratory tularemia. The rats received one IP dose of CY (50 mg/kg) 3 days before vaccination with either the live vaccine strain (LVS) or with an inactivated Foshay-type (FO) vaccine prepared from F. tularensis. LVS vaccination promotes both cellular immunity and humoral

agglutinins against F. tularensis while the FO vaccine induces primarily serum agglutinins. The 1-dose CY regimen failed to maintain suppression of humoral antibodies through day 21, the time of aerosol challenge with 1 x 10^3 cells of the virulent SCHU S4 strain of F. tularensis. Also in these studies, assays for macrophage migration inhibition factor (MIF), an indicator of cell-mediated immunity, were negative for most vaccinated rats. A few rats showed marginally positive MIF assays. However, all vaccinated rats, whether treated with CY or not, survived the aerosol challenge indicating that the 1-dose CY treatment had no effect on host susceptibility.

In subsequent experiments, Fischer rats were given CY 3 times during a 27-day period to maintain total or significant suppression of humoral antibodies. The rats were pretreated first with CY given IP (53 mg/kg) 3 days before IM vaccination with either LVS ($10^{3.9}$ cells) or with the inactivated vaccine ($10^{9.0}$ cells). Subsequent injections of CY were given on days 10 and 21 after vaccination. The CY treated-vaccinated rats as well as other groups of control rats that were given only LVS, or FO vaccine, and control rats that received only CY all were challenged on day 24. Two types of challenge were administered by aerosol exposure (inhaled dose of $10^{4.95}$ cells) or by IP injection ($10^{5.4}$ cells) with virulent F. tularensis, SCHU S4. The IP challenge was included to provide a severe route of challenge. The pertinent response data of the vaccinated and CY treated rats are summarized in Table I.

TABLE I. RESPONSE OF RATS TO VACCINE (DAY 10) AND CY TREATMENT (DAY 21)

	POSTVACCINATED			CY-TREATED		
TREATMENT	WBC	Titer[a]	MIF[b]	WBC	Titer	MIF
CY-FO	1,619	23	0/5	1,681	3	2/5
CY-LVS	1,533	25	2/5	1,882	2	1/5
CY	1,948	0	0/3	1,255	0	3/3
FO	7,266	160	0/5	6,277	30	0/5
LVS	9,197	1,082	0/5	6,346	280	1/5
Controls	7,714	0	0/3	5,235	0	0/3

[a] Reciprocal agglutinin titer
[b] No. that showed MIF activity/No. tested

Leukopenia was observed in the CY-vaccinated and CY-control rats after the 2nd (day 10) and 3rd injection (day 21) of CY. Based on earlier studies, the leukopenia would persist until time of challenge on day 24. In addition, the negligible serum titers of 1:3 and 1:2 in the 2 CY-vaccinated groups indicated an effective, if not total, suppression of humoral antibody production. Serum agglutinin titers for the non-CY treated FO- and LVS-vaccinated rats were at titers usually seen 25-28 days after vaccination. Results of MIF assays on peripheral leukocytes from the day 10 and day 21 vaccinated rats appeared marginal. None of the CY-FO rats showed MIF on day 10, but on day 21, 2 of 5 did. This may be the result of mild stimulation of cellular immunity by CY. Of the CY-LVS vaccinated rats, 40% showed MIF on day 10 and only 20% were positive on day 21. MIF activity also was detected in only 20% of the LVS-vaccinated rats on

day 21. Why more LVS-vaccinated rats did not have measurable MIF activity with or without CY treatment, is obscure. It is known that the effects of CY are markedly dependent on time of administration relative to vaccination, on CY dose and on the time chosen for measuring the immune responses (3). It also is possible that MIF activity may not be a consistent nor perhaps the appropriate marker for cell-mediated immunity in Fischer 344 rats. This inbred rat appears to be very susceptible to the toxic effects of CY (50 mg/kg is LD_{10-20}) and the 50-mg dose administered to the rats may have been too low to induce detectable MIF activity. Administration of CY to other rat strains and to mice are at 200-300 mg/kg doses.

The survival response data of the vaccinated and control rats, challenged with virulent F. tularensis SCHU S4 on day 24 after LVS vaccination are shown in Table II. As in previous experiments, practically all of the nonvaccinated rats died within 8 days after either aerosol or IP challenge with F. tularensis, SCHU S4. Of the CY-treated but nonvaccinated rats, 33 and 20% survived the aerosol and the more severe IP challenge, respectively. This suggests that some non-specific stimulation of cellular immunity was induced. In fact, MIF activity was detected in 3/3 of these rats prior to challenge.

TABLE II. SURVIVAL RESPONSE OF IP CHALLENGE RATS

	NO. SURVIVAL/NO EXPOSED (% survival)			
	Aerosol		IP	
VACCINE	($10^{4.95}$)		($10^{5.11}$)	
CY-FO	9/9	(100)	5/8	(63)
CY-LVS	15/15	(100)	9/9	(100)
CY	3/9	(33)	1/5	(20)
FO	10/10	(100)	10/10	(100)
LVS	10/10	(100)	10/10	(100)
Control	1/6	(17)	0/4	(0)

All LVS- and FO-vaccinated control rats survived both types of challenge. Humoral antibody titers were in the range usually obtained at 25-30 days. Of interest is the equal protection afforded the FO-vaccinated rats against a lethal challenge despite the much lower antibody titer (1:30) than that measured (1:280) for the LVS-vaccinated rats. Of greater interest was that none of the CY-LVS vaccinated rats succumbed to any challenge despite practically nonexistent serum antibody titers. All of the CY-FO-vaccinated rats also survived the aerosol exposure and 63% survived the IP challenge in the absence of any significant humoral antibody. The protection afforded these CY-FO-vaccinated rats must be credited to the potentiation of some degree of cellular immunity by the CYtreatment. FO vaccine, per se, induces primarily humoral antibody, which in this situation was suppressed by CY. These results, therefore, are the first direct demonstration of protection against a leghal tularemia challenge in a vaccinated host model in which the humoral arm of immunity was effectively suppressed by experimental manipulation. These data also confirm that serum antibody is not a critical component of the immune system in providing full protection to a

lethal respiratory challenge. These findings add further support to the general consideration that primary protection against tularemia resides in the cell-mediated component of the host's immune defense mechanism.

However the still unanswered question is, what contribution to humoral antibodies make, if any, to a host's resistance to respiratory tularemia? Other investigators have reported that passive transfer of specific immune serum to tularemia dos not protect against mortality but does modify the disease (4). It now becomes pertinent to determine how the disease is modified by serum antibody. Will vaccinated rats with serum antibodies suppressed by CY treatment and then challenged with F. tularensis still be protected against bacteremia and be resistant to a systemic infection, as occurs in rats with demonstrated circulating antibodies? In addition, is the lack of systemic dissemination a result of the serum antibodies opsonizing tularemia cells enabling phagocytosis by PMN? PMN cells are unable to phagocytize tularensis organisms in the absence of immune serum. Investigative efforts to consider these questions will be continued.

In corollary studies, it also was determined that LVS vaccine, administered by IP, SC, or IM route, by IN instillation or exposure to aerosols, provided practically equal protection against challenge with aerosols of virulent F. tularensis.

Publications: None

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1. Crozier, D. 1963. The threat of biological weapons attack. Mil. Med 128:81-85.

2. Ascher, M. S., D. Parker, and J. L. Turk. 1977. Modulation of delayed-type hypersensitivity and cellular immunity to microbial vaccines: effects of cyclophosphamide in the immune response to tularemia vaccine. Infect. Immun. 18:318-323.

3. LaGrange, P. H., G. B. Mackaness, and T. E. Miller. 1974. Potentiation of T-cell-mediated immunity by selective suppression of antibody formation with cyclophosphamide. J. Exp. Med. 139:1529-1539.

4. Thorpe, B. D., and S. Marcus. 1965. Phagocytosis and intracellular fate of Pasteurella tularensis. III. In vivo studies with passively transferred cells and sera. J. Immunol. 94:578-585.

5. Proctor, R. A., J. D. White, E. Ayala, and P. G. Canonico. 1975. Phagocytosis of Francisella tularensis by rhesus monkey peripheral leukocytes. Infect. Immun. 11:146-151.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6420	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ORG'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 08 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	62776A	3M162776A841	00	006
b. CONTRIBUTING				
c. [illegible]	STOG 79-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Immunochemical studies with the nonindigenous tick-borne rickettsiae

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
76 10	79 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 79	1.4	83.6
	CURRENT 80	0	0

19. RESPONSIBLE DOD ORGANIZATION

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NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Immunology; (U) Vaccines; (U) Tick-borne rickettsiae; (U) Laboratory animals; (U) Biochemistry

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 of military importance. Demonstrate the potential of components of tick-borne rickettsiae to protect against disease caused by members of this group. This program will encompass studies vital for military defense against the biological warfare potential of tick-borne rickettsiae.

24 (U) Study pathogenesis of the tick-borne typhus group rickettsiae and define suitable animal models for the evaluation of vaccine efficacy. Produce tissue-culture grown rickettsiae; isolate, purify and define various immunogenic components for the production of vaccines. Study cell-mediated and humoral immune responses to vaccines prepared from inactivated or subunit vaccines in suitable animal models of disease.

25 (U) 78 10 - 79 07 - Studies on the mechanism of cross-immunity within the spotted fever group of rickettsiae was examined in inbred mice. Results suggested that cell-mediated immunity confers such cross-protection. A cynomolgus monkey model for Rocky Mountain spotted fever was defined. Using this model our USAMRIID-produced RMSF vaccine protected against an aerosol challenge as well as a subcutaneous challenge; protection was still demonstrable at one year. Studies were completed on the genetic basis of susceptibility to R. akari infection in inbred mice; a manuscript has been prepared for publication. Most rickettsial research has been transferred to WRAIR.

Publications: Infect. Immun. 22:411-417, 1978; 25:580-582, 1979.
J. Clin. Microbiol. 10:719-723, 1979.

*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

* U.S. GPO: 1974-546 943/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 006: Immunochemical Studies with the Nonindigenous Tick-Borne Rickettsiae

Background:

Included among the tick-borne rickettsial diseases are Rocky Mountain spotted fever (RMSF), the tick-borne rickettsiosis of the eastern hemisphere, and rickettsial pox (1). RMSF, caused by Rickettsia rickettsii, found in the United States, is the most severe of these diseases. Rickettsia conorii, the etiological agent of fievre boutonneuse, South African tick bite fever and Indian and Kenyan tick typhus, causes a severe nonfatal infection, and is prevalent throughout parts of Europs, Africa and the Mediterranean basin. North Asian tick-borne typhus of Russia and Mongolia (Rickettsia sibirica) also causes a severe nonfatal infection. Queensland tick typhus (Rickettsia australis) is a moderately severe tick-borne infection found in Australia, and rickettsial pox (Rickettsia akari) is a relativly benign mite-borne infection found in the United States and Russia. Due to the possibility of potential military traffic in selected geographical areas, R. rickettsii, R. conorii, and R. sibirica should be considered of military concern. Neither R. australis, due to its geographical isolation, nor R. akari, due to low virulence and incidence, is of military significance.

A chick embryo cell culture-grown vaccine against R. rickettsii was prepared (2), and is under test in man. A prototype vaccine prepared from R. rickettsii and R. conorii was evaluated in guinea pigs and found to offer protection against the militarily significant spotted fever members. In concert with vaccine preparation, evaluation of immune responses to vaccination and infection is under investigation. Such studies in mice are presently focused on R. akari infection, which is lethal without "toxic action" (3).

Elucidating the genetic basis for varying degrees of natural resistance to infectious diseases is an area of increasing interest. Such resistance is important not only when animal models are developed to test the presumptive pathogenesis of a disease in man, but as a key to why an animal is susceptible to a particular disease. There appears to be a relationship between susceptibility of mice to a lethal rickettsial challenge and their genetic background. Studies have been initiated in mice using R. akari to explore such a genetic basis for susceptibility.

Progress:

Both athymic and euthymic BALB/C mice survived R. akari infection after antibiotic treatment. Preliminary studies showed that treatment on days 3 and 4 was sufficient for euthymic mice, but prolonged treatment was necessary for survival of athymic mice. If treatment was continued through day 11, these mice survived. If treatment was discontinued before day 11, mice died from infection within 5 or 6 days. Both athymic and euthymic mice developed IFA antibodies by day 14 (athymic = 1:2048, euthymic = 1:4096). However, as shown in Table I, when rechallenged, athymic mice were subject to reinfection and deaths occurred, while euthymic mice were completely resistant on rechallenge. Immunization with inactivated rickettsiae completely protected euthymic mice while similarly treated immunized athymic mice showed no protection against challenge.

TABLE I. EFFECT OF VARIOUS TREATMENTS ON PROTECTION OF BALB/C MICE AGAINST R. AKARI (10^4 PFU)

TREATMENT	NUMBER SURVIVORS/TOTAL	
	Euthymic	Athymic
Killed rickettsiae	5/5	0/5
None	0/5	0/5
Infected, antibiotic-treated; rechallenged 14 days later	4/4	0/4
Noninfected, antibiotic-treated; challenged 14 days later	0/4	0/4

The effect of passive transfer of R. akari immunity by antiserum is shown in Table II. Antiserum from euthymic mice protected euthymic mice from a fatal R. akari infection. However, the same antiserum did not protect athymic mice; conversely, antiserum from athymic mice completely protected euthymic mice. This suggests that antibody production against R. akari is T-cell independent.

TABLE II. EFFECT OF PASSIVE TRANSFER OF IMMUNITY BY ANTISERUM ON PROTECTION AGAINST R. AKARI CHALLENGED AT 4 HR

MOUSE SERUM (IFA titer)	RECIPIENT MICE	NUMBER SURVIVORS/TOTAL
Euthymic, convalescent (1:4096)	Euthymic	4/4
	Athymic	0/4
Athymic convalescent (1:2048)	Euthymic	4/4
Euthymic, normal (1:4)	Athymic	0/4

The effect of transfer of immune spleen cells to syngeneic recipients is shown in Table III. When spleen cells from euthymic convalescent mice were transferred to syngeneic euthymic mice, 90% of the recipients survived challenge. When aliquots of the same spleen cell suspension were transferred to syngeneic athymic mice, 67% of the recipients survived challenge. Spleen cells from normal, uninfected euthymic mice offered no protection against challenge.

TABLE III. EFFECT OF SPLEEN CELL TRANSFER ON IMMUNITY TO R. AKARI 16 HR AFTER CHALLENGE WITH 10^4 RICKETTSIAE

MOUSE SPLEEN CELL DONOR	SPLEEN CELL RECIPIENT MOUSE	NO. SURVIVORS/TOTAL
Convalescent euthymic	Euthymic	9/10
	Athymic	10/15
Normal euthymic	Euthymic	1/5
	Athymic	0/5

Since passive transfer of functional R. akari antibody did not protect athymic mice, the competency of macrophages from athymic mice was examined. Table IV shows the effect of antibody coating on the ultimate fate of R. akari exposed to peritoneal macrophages grown in vitro. Since a low rickettsia:cell ratio was used, no rickettsiae were observed in any cells 5 hr after infection, but by 48 and 72 hr a difference between cells infected with normal- or antibody-treated rickettsiae was evident. When rickettsiae were pretreated with specific antiserum, few or no rickettsiae were observed in the macrophages at 5, 48 or 72 hr, while rickettsial multiplication occurred in the presence of normal serum. No difference in handling rickettsiae could be detected with macrophages from euthymic or athymic mice. In vitro, macrophages from athymic mice can completely destroy R. akari in the presence of antibody, but clearing of infection in vivo in the presence of antibody apparently does not occur. One possible explanation for this is that macrophages recruited in vivo in response to mineral oil stimulation are activated and, therefore, more capable of killing antibody-coated rickettsiae. Successful response to rickettsial infection in vivo may require a T-cell dependent macrophage activation step which cannot occur in athymic mice. However, when immunocompetent splenic cells from convalescent syngeneic donors were introduced, the athymic recipient became refractory to challenge. It seems reasonable that once competent T-cells are available in vivo, potentially competent macrophages in athymic mice can somehow receive the message to activate and destroy invading rickettsiae.

TABLE IV. EFFECT OF ANTIBODY COATING OF R. AKARI ON INFECTION OF PERITONEAL MACROPHAGES GROWN IN VITRO

SERUM TREATMENT	TIME (hr)	% CELLS INFECTED[a]	
		Euthymic mice	Athymic mice
Normal	5	2	1
	48	75	85
	96	99	97
Immune	5	0	0
	48	1	1
	96	1	1
None	5	2	2
	48	80	72
	99	72	99

[a]Cells examined for rickettsiae by direct fluorescent antibody; 4 fields of 100 cells counted to calculate % infected.

R. akari causes a fatal infection of both athymic and euthymic BALB/C mice. In athymic mice, a lack of functional T-cells in concert with the production of functional antibody make this an excellent model for the study of acquired immunity to rickettsial infection.

We have shown that mice convalescent from any spotted fever group rickettsial infection resist subsequent challenge with R. akari. Immune mechanisms responsible for this cross-protection have been elusive. Passive transfer of heterologous antiserum offers no protection. Initial attempts to transfer immunity with spleen cells alone were unsuccessful, but occasional survivors were encountered when both spleen cells and antiserum were transferred In the experiments summarized here, mice were infected with sublethal doses of rickettsiae, boosted on day 12 with 10^6 viable rickettsiae, spleens removed and spleen cells transferred on day 21 (about 1.5 donor spleens/recipient) and the recipients challenged at 5 hr with R. akari. Results are shown in Table V. It appears that antiserum alone is without effect on cross protection, but with R. australis and R. sibirica, spleen cells alone can ameliorate challenge with R. akari. The fact that spleen cells can, and immune serum cannot offer some degree of protection suggests a role for cell-mediated immunity.

TABLE V. TRANSFER OF SPOTTED FEVER IMMUNITY TO R. AKARI INFECTION IN C3H/HeJ MICE

	NO. SURVIVORS/4		
RICKETTSIA	Spleen Cells alone	Spleen Cells + antibody	In vivo controls[a]
akari	4	ND	4
conorii	0	0	4
rickettsii	1	0	4
australis	4	4	4
sibirica	4	4	4
controls	-	-	0

[a]Mice convalescent from spotted fever, then challenged with R. akari.

Preliminary studies showed that C3H/HeJ mice were susceptible to R. conorii, R. sibirica, and R. australis, as well as to R. akari. We expanded those studies to determine if other inbred mice with different genetic defects demonstrated susceptibility to spotted fever group rickettsiae. Results are presented in Table VI. None is susceptible to R. rickettsii; this is noteworthy since it is the member of the spotted fever group with the greatest "toxic" action for mice. R. sibirica is lethal for C3H/HeN and C3H/HeJ mice. R. australis is somewhat lethal for C3H/HeJ, C57/BL10, and C57/BL6 mice. R. conorii is lethal at high doses for C3H/HeN and C3H/HeJ mice. From these data one can gather that not one single genetic locus is responsible for resistance/susceptibility to spotted fever rickettsiae as a group. Time permitting, the genetics of resistance/susceptibility of each member could be examined and defined.

TABLE VI. SUSCEPTIBILITY OF INBRED MICE TO SPOTTED FEVER GROUP RICKETTSIAE

RICKETTSIA	MIPLD$_{50}$ C3H/HeN	C3H/HeJ	A/J	C57/BL10	C57/BL6
australis	$>10^{7.0}$	$10^{6.0}$	$>10^{7.0}$	$10^{6.2}$	$10^{6.0}$
conorii	$10^{5.5}$	$10^{4.6}$	$>10^{6.0}$	$>10^{6.0}$	$>10^{6.0}$
sibirica	$10^{2.5}$	$10^{1.0}$	$>10^{6.0}$	$>10^{6.0}$	$>10^{6.0}$
rickettsii	$>10^{7.0}$	$>10^{7.0}$	$>10^{7.0}$	$>10^{7.0}$	$>10^{7.0}$

Using the C3H/JH mouse model, preliminary experiments showed that as with R. akari, mice convalescent from any 1 member of the spotted fever group resist challenge with R. conorii, R. sibirica, or R. australis. An experiment was performed to determine the role of antibody in such heterologous protection. Results are presented in Table VII. In contrast to R. akari infection, considerable cross protection appears to be conferred by heterologous antiserum. For example, antiserum against R. australis, R. rickettsii, or R. sibirica appears to protect against R. conorii. Similar cross protection by antiserum was observed with R. sibirica infection. Further studies are under way to characterize cross protection in the spotted fever group using the C3H/HeJ mouse model.

TABLE VII. EFFECT OF PASSIVE TRANSFER OF SPOTTED FEVER ANTISERUM ON HETEROLOGOUS AND HOMOLOGOUS PROTECTION

CHALLENGE Rickettsia	NO. SURVIVORS/TOTAL BY ANTISERUM[a] akari	australis	conorii	rickettsii	sibirica	none
akari	3/3	1/4	0/4	0/3	0/3	0/4
conorii	0/3	3/3	3/3	3/3	3/3	0/3
sibirica	0/2	3/3	1/3	3/3	3/3	0/3

[a] R. australis not done.

Since outbred guinea pigs often show an inconsistent response to Rocky Mountain spotted fever (RMSF), studies were performed on the feasibility of an inbred guinea pig model for this diseases. Twenty-four strain 2 guinea pigs were injected with 10^3 R. rickettsii and temperatures and deaths monitored. Eight guinea pigs (12%) died; all showed a fever response. Neither magnitude of fever, duration of fever, nor death appeared to be significantly more consistent than with outbred Hartley strain guinea pigs. Significant lymphocyte transformation and macrophage migration inhibition were measured in these animals. Microagglutinatine (MA) antibody first appeared on day 7, about 4 days after onset of fever. Spleens were removed from strain 2 guinea pigs from convalescent or vaccinated donors and transferred to syngeneic recipients. After challenge, recipients of spleens from convalescent guinea pigs showed no signs of disease while recipients of spleens from vaccinated guinea pigs were offered no protection (Table VIII).

TABLE VIII. TRANSFER OF RMSF IMMUNITY BY SPLEEN CELL TRANSFER USING INBRED STRAIN 2 GUINEA PIGS

SPLEEN CELLS FROM[a] GUINEA PIGS	RECIPIENT RESPONSE FOLLOWING CHALLENGE (Mean days)	Deaths/Total
Convalescent	0.4	0/5
Vaccinated	4.0	4/4
Normal	7.1	5/6

[a]Cells from approximately 1 spleen transferred to each recipient.

Studies on the genetic susceptibility of inbred,outbred and inbred-hybrid mice to the virulent Kaplan, and avirulent Hartford strains of R. akari have been terminated. The $MIPLD_{50}$ has now been determined for 23 inbred (Table IX), 4 outbred (Table X), and 4 inbred-hybrids (Table XI). The strain survey is a comprehensive list, since it represents most of the known H-2 haplotypes and strains having diverse genealogies. The response patterns were defined as susceptible, intermediate or resistant. Susceptible mice were characterized by a $\log_{10}$ $MLD_{50} < 1$; intermediate mice by a $\log_{10}$ $MLD_{50} \geq 1$ but < 3; and resistant mice evidenced a $\log_{10}$ $MLD_{50} > 3$.

TABLE IX. RESPONSE OF INBRED MOUSE STRAINS TO R. AKARI (KAPLAN STRAIN)

INBRED STRAIN	LOG_{10} MLD_{50}	H-2 KAPLOTYPE	RESPONSE PATTERN
A/HeJ	1.7	a	Intermediate
A/J	2.2	a	Intermediate
A/WySn	1.9	a	Intermediate
AKR/J	3.8	k	Resistant
AL/N	5.0	a	Resistant
BALB/cAnN	4.3	d	Resistant
BALB/cDub	3.0	d	Intermediate
BALB/cJ	2.5	d	Intermediate
BALB/cNCr1BR	4.2	d	Resistant
C3H/HeJ	0.0	k	Susceptible
C3H/HeDub	4.9	k	Resistant
CeHeB/FeJ	5.0	k	Resistant
C3H/HeN	5.1	k	Reistant
C3H7B/HeN	4.5	k	Resistant
C3H/StCr1BR	5.1	k	Resistant
CeH/fMai	4.4	k	Resistant
CeH/BiSim	4.2	k	Resistant
C57B1/6J	3.2	b	Resistant
C57L/J	3.7	b	Resistant
CBA/J	4.6	b	Resistant
DBA/2J	4.2	d	Resistant
SJL/J	3.0	s	Intermediate
SWR/J	4.1	q	Resistant

TABLE X. RESPONSE OF OUTBRED MOUSE STOCKS TO R. AKARI KAPLAN

OUTBRED STOCK	LOG_{10} MLD_{50} (PFU + SD)	RESPONSE PATTERN
Swiss		
Mai: (s)	3.8 ± 0.2	Resistant
Albino		
Caw:CF_1	4.2 ± 0.2	Resistant
ICR		
Dub:(ICR)	4.7 ± 0.2	Resistant
Wrc:(ICR)	5.0 ± 0.2	Resistant

TABLE XI. RESPONSE OF F_1 INBRED HYBRID MICE TO KAPLAN INFECTION.

F_1 HYBRID	SEX	LOG_{10} MLD_{50} (PFU ± SD)	RESPONSE PATTERN
$AKD2F_1$/J (AKR/J x DBA/2J)	M	4.6 ± 0.2	Resistant
	F	5.1 ± 0.2	Resistant
CAF_1/J (BALB/cJ x A/J)	M	3.2 ± 0.2	Resistant
	F	3.6 ± 0.5	Resistant
$CB6F_1$/J (BALB/cJ x C57BL/6J)	M	4.6 ± 0.1	Resistant
	F	4.4 ± 0.2	Resistant
$C3D2F_1$/J (C3H/HeJ x DBA/2J)	M	4.1 ± 0.2	Resistant
	F	4.9 ± 0.2	Resistant

These studies were carried out with the Kaplan strain of R. akari, since the Hartford strain proved avirulent. The differences in lethality between the two strains probably resulted from selection of an avirulent organism during plaque purification of the original Hartford material, since Custer and Fuller in 1952 (4) could not distinguish the two strains by lethality for albino mice.

A study was performed to test the protective capacity of one dose of Hartford strain against 1000 LD_{50} of the Kaplan strain (based on C3H/HeJ strain) challenged on day 28. Four inbred strains were used: 1 susceptible (C3H/HeJ), 2 intermediate (A/HeJ, A/J) and 1 resistant (CAF/1J). The results in Table XII show that 10^2 PFU of the Hartford strain afford 100% protection for all mouse strains against 1000 LD_{50} of the Kaplan strain.

The F_1 progeny (inbred-hydrid) derived from cross inbred parents with different susceptibilities to the Kaplan strain were uniformly resistant to Kaplan challenge, regardless of sex, indicating that resistance was a dominant character. Testing of the F_2 and parental backcross generations of C3H/HeJ

and DBA/2J hybrids yielded ratios of resistant to susceptible animals that suggested resistance was under multigeneic control (Table XIII). However, the ability of Kaplan strain rickettsiae to produce plaques in C3D2F1/J mouse embryo cell cultures suggested that resistance was not due to the inability of these rickettsiae to enter and proliferate in the cells of resistant animals.

TABLE XII. EFFECT OF PRIOR IMMUNIZATION WITH THE AVIRULENT HARTFORD STRAIN ON SUBSEQUENT CHALLENGE WITH THE VIRULENT KAPLAN STRAIN

	NO. DEATHS/5 AS FUNCTION OF PFU					
STRAIN	10^4	10^3	10^2	10^1	10^0	None
C3H/HeJ	0	0	0	2	0	5
A/HeJ	0	0	0	0	3	5
A/J	0	0	0	0	1	1
CAF1/J	0	0	0	0	0	0

TABLE XIII. RESPONSE OF F_2 AND PARENT BACKCROSS GENERATIONS OF C3H/HeJ AND DBA/2J MICE TO KAPLAN INFECTION (100 PFU)

HYBRID	NO. SURVIVORS/ TOTAL
Parental backcross	
DBA/2J x $C3D2F_1/J$	174/174
C3H/HeJ x $C3D2F_1/J$	197/229
F_2	
$C3D2F_1/J$ x $C3D2F_1/J$	92/94

A chronological investigation was undertaken to examine the closely related C3H sublines of inbred mice for mutational events in their history. The results from Table XIV suggest at least one mutation occurred after 1950. Further investigation would require subline derived from the C3H/HeJ strain to closer approximate the time the mutation happened and to determine if more than one mutation occurred.

We have described a rhesus monkey model for RMSF and have evaluated test vaccines using this model. Due to decreasing availability of rhesus monkeys, a cynomolgus monkey model was evaluated. Clinical responses of 21 monkeys exposed by aerosol or subcutaneous routes are summarized in Table XV. Such responses are similar to those seen by RMSF in rhesus monkeys and in humans.

TABLE XIV. C3H SUBLINE MLD_{50} AND RESPONSIVENESS.

GENEALOGY				LD_{50}	RESPONSE PATTERN
			C3H/BiSim (1959)	4.2	Resistant
		C3H/Bi (1931)			
			C3H/fmai (1959)	4.4	Resistant
C3H/st (1920)	C3H/An		C3H/StCrlBR (1969)	5.1	Resistant
			C3HfB/HeN (1952)	4.5	Resistant
			C3H/HeN (1952)	5.1	Resistant
	C3H/He (1941)		C3H/HeJ (1947)	0	Susceptible
			C3HeB/FeJ	5.0	Resistant
			C3H/HeDub (1968)	4.9	Resistant

TABLE XV. CLINICAL RESPONSES OF CYNOMOLGUS MONKEYS TO R. RICKETTSII INFECTION

CHALLENGE (N)	NO. Ill	NO. Rickettsemia	NO. Rash	FEVER Time to onset (Range)	FEVER Duration (Range)	FEVER No. Dead	Mean Days to death (range)
10^1							
SC (3)	3	3	0	6(5-7)	5.7(4-7)	3	11(10-11)
Aerosol (4)	3	2	0	7.3(7-8)	2.7(2-4)	2	11.5 (11-12)
10^3							
SC (3)	3	3	0	3(2-4)	7.3(5-9)	2	10
Aerosol (4)	4	4	1	5.7(5-6)	4.0(3-5)	2	12(11-13)
10^5							
SC (3)	3	3	0	4	5	2	8
Aerosol (4)	4	3	1	3	6.7(5-9)	2	10

Splenomegaly was noted on gross pathological examination. The most common lesions microscopically were vasculitis and thrombosis of the capillaries, arterioles and venules of the facial skin, ears and nares. The lesions were characterized primarily by perivascular infiltration of lymphocytes and plasma cells. Vasculitis was also seen in the palpebral conjunctiva, testes, uterus, thymus, heart, skeletal muscle and abdominal mesentery. Varying degrees of interstitial pneumonia (no difference was seen in monkeys infected by either route) and minimal adrenitis were noted. Two monkeys infected SC had fibrin thrombi in glomerular tufts, suggesting disseminated intravascular coagulation.

The cynomolgus monkey model was used to evaluate our tissue culture-derived RMSF vaccine. Since most laboratory exposures to RMSF have been by aerosol, protective efficacy of our vaccine against aerosol challenge was also determined. Monkeys were vaccinated with 2 doses of vaccine 1 month apart, and challenged 1 month later. Clinical responses after challenge are shown in Table XVI. Undiluted vaccine totally protected monkeys against both challenges, even at extremely high doses.

TABLE XVI. CLINICAL RESPONSES OF VACCINATED AND UNVACCINATED CYNOMOLGUS MONKEYS (N=2/GROUP) AFTER CHALLENGE WITH R. RICKETTSII

ROUTE AND VACCINE	CHALLENGE DOSE (PFU)	NO. WITH Rickettsemia	NO. WITH Fever	FEVER Time to onset (range)	FEVER Duration	NO. DEAD
SC	10^5	2	2	3	0[a]	2
None	10^3	2	2	5.5(5-6)	9	1
1:250	10^3	0	1	6	8	0
1:100		0	1	6	6	0
1:10		0	0			0
Undiluted	10^3	0	0			0
Aerosol						
None	10^5	2	2	4.5(4-5)	0[a]	2
	10^3	2	2	6	0[a]	2
Undiluted	10^5	0	1	2	2	0
	10^3	0	0			0

[a]Died

To determine the duration of protection, 3 monkeys were vaccinated, 2 challenged at 6 months, and 1 challenged at 1 year by the SC route. Both vaccinated monkeys were completely protected at 6 months. At 1 year, the vaccinated monkey showed 1 day of fever and anorexia, but no other symptoms of disease. Nonvaccinated control monkeys at both times died.

The cynomolgus monkey appears to be a suitable substitute for the rhesus monkey for the study of RMSF in nonhuman primates. The clinical and physiological responses and pathological changes are similar in the two species. RMSF resulting from SC infection is indistinguishable from the disease acquired by aerosol exposure.

The work unit was terminated due to transfer of rickettsiology functions to WRAIR.

Presentations:

1. Kenyon, R. H. Cross-reactions with spotted fever group of rickettsiae Presented, Annual Meeting, American Society for Microbiology, Los Angeles, CA, May 1979 (Abstracts, p. 72).

2. Anderson, G. W., Jr. Susceptibility of mouse strains to Rickettsia akari. Presented, Maryland Branch, American Society for Microbiology, Fort Detrick, MD,27 Apr 1979; Annual Meeting, American Society for Microbiology, Los Angeles, CA, 4-8 May 1979; Rickettsiology Conference, Donaldson Brown Center of University of Maryland, Port Deposit, MD, 22-25 Mar 1979.

3. Anderson, G. W., Jr. Development and use of genetically defined mouse models for viral and rickettsial research. Presented, Radar Clinic, Ft. Myers, VA, 6 Jun 1979.

4. Kenyon, R.H. Current status of CEC-grown RMSF vaccine, Presented, NIAID Conference on RMSF, Bethesda, MD, Nov 1979.

Publications:

1. Gonder, J. C., R. H. Kenyon, and C. E. Pedersen, Jr. 1979. Evaluation of a killed Rocky Mountain spotted fever vaccine in cynomolgus monkeys. J. Clin. Microbiol. 10:719-723.

2. Kenyon, R. H., R. A. Kishimoto, and W. C. Hall. 1979. Exposure of guinea pigs to Rickettsia rickettsii by aerosol, nasal, conjunctival, gastric, and subcutaneous routes and protection offered by an experimental vaccine. Infect. Immun. 25:580-582.

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3. Bell, E. J. and E. G. Pickens. 1953. A toxic substance associated with the rickettsias of the spotted fever group. J. Immunol. 70:461-472.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6419	79 06 15	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	007
b. CONTRIBUTING				
c. [illegible]	STC 79-7.2.2			

11. TITLE (Precede with Security Classification Code): (U) Immunoprophylactic role of macrophages in airborne, rickettsial respiratory 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
75 07	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	1.0	82.5
FISCAL YEAR CURRENT 80	0	0

19. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Respiratory diseases; (U) Macrophages; (U) Coxiella burnetii; (U) Phagocytosis; (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) Determine the role of macrophages as a host defense mechanism against infectious disease and investigate means of enhancing host defenses by stimulation of the macrophage system or modification of cellular and humoral immune responses. This research is essential for developing approaches to successful immunoprophylaxis against this recognized BW threat and the treatment of casualties in BW operations.

24 (U) Study the ability of peritoneal macrophages from normal and immune guinea pigs to phagocytize and destroy Coxiella burnetii in vitro; then conduct similar studies with alveolar macrophages. To study the role of cellular and humoral immunity in improving host response against respiratory disease.

25 (U) 78 10 - 79 06 - The fate of phases I and II C. burnetii in guinea pig peritoneal macrophages was examined. Guinea pigs were vaccinated with killed phase I C. burnetii, then challenged with tritiated phase I or II organisms. The macrophages were removed, homogenized and fractionated in linear sucrose gradients. It was shown that both phases were in the same fraction as the lysosomal enzymes.

To assist other studies the interaction between guinea pig macrophages and Legionella pneumophila was examined. Results will be reported elsewhere. The work unit is terminated due to the transfer of the principal investigator.

Publications: Infect. Immun. 22:68-71, 1978
J. Infect. Dis. 139:191-196, 1979
Milit. Med. 144:183-187, 1979

*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

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0D6416	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	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	62776A	3M162776A841	00	009
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Determinants for virulence and attenuation of arbo- and arenavirus vaccine candidates

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 79	1.0	234.8
FISCAL YEAR CURRENT 80	1.0	220.5

19. RESPONSIBLE DOD ORGANIZATION
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NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Arenaviruses; (U) Lassa virus; (U) Vaccine therapy; (U) Chemotherapy; (U) Laboratory animals

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

23 (U) Models for lethal arenavirus infections in primates and rodents are developed for use in testing the protective efficacies of antiviral drugs and immune serum therapy regimens. Experimental vaccines are developed and tested in animal models. Attenuated virus strains are characterized using biological and biochemical markers of virulence. These investigations will contribute to development of more effective vaccines to protect military personnel.

24 (U) Direct and indirect effects of virus replication in animal tissues and cell cultures are assessed. Humoral and cellular immune responses are measured and correlated with altered disease patterns.

25 (U) 78 10 - 79 09 - Five primate species were tested for susceptibility to Lassa virus infection. The disease course in rhesus and cynomolgus monkeys and inbred guinea pigs as measured by virus replication, histopathology, immunofluorescence, and enzyme fluctuations were similar to human disease. Treatment with ribavirin or immune serum was effective when initiated soon after infection; if treatment was delayed until after the onset of clinical signs, only the combination of ribavirin plus immune serum was effective. Antibodies measured by immunofluorescence were detected at 10 days, simultaneously with viremia; neutralizing antibodies in survivors were not detected before day 45. Virus growth in MRC-5 cells and inactivation studies with formalin or psoralen suggest that development of an inactivated Lassa vaccine is feasible.

Publication: Arch. Virol. 62:53-62, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 009: Determinants for Virulence and Attenuation of Arbo- and Arenavirus Vaccine Candidates

Background:

Lassa fever is a severe, often fatal disease of man caused by Lassa (LAS) virus. The disease was originally described in 1969 in Nigeria. Since then additional outbreaks have been reported in Nigeria, Liberia and Sierra Leone (1-3). High fatality rates (ranging from 20-40%) have been reported in hospitalized patients, although serologic data suggest that subclinical infections may occur. Until now, management of LAS has been largely symptomatic and supportive; specific treatment, using immune serum, has been attempted in a small number of patients (4). The success of this procedure has been equivocal; no virologic data for treated patients has been reported. Management of LAS would be facilitated if an effective antiviral drug were available; moreover, the number of patients might be substantially reduced if an effective vaccine were available to protect hospital and laboratory workers who are at high risk to severe infection. The primary purpose of this work unit is to develop and test vaccines, antiviral drugs, and immunotherapy regimens for prevention and treatment of human Lassa virus infections.

Progress:

Development of animal models for human LAS infections. Five primate species were tested for susceptibility to LAS, strain Josiah. Viremia levels were also compared (Table I). Squirrel and capuchin monkeys sustained mild infections and never became clinically ill. They developed only low titer viremias following SC inoculation of 10^6 PFU. In contrast, 2 of 3 African green monkeys died, and all developed high-titer viremias, in excess of 5 $\log_{10}$ PFU/ml. Likewise, 6 of 10 rhesus monkeys died, and all developed moderately high viremias (4-5 $\log_{10}$ PFU/ml). Cynomolgus monkeys were uniformly killed, and developed viremias in excess of 6 $\log_{10}$ PFU/ml. Cynomolgus monkeys thus appeared to be the primate of choice for protective efficacy studies, although the African green and rhesus monkey models might more closely approximate the human disease.

TABLE I. VIREMIA AND LETHALITY IN PRIMATES INOCULATED SC[a]

PRIMATE SPECIES	DEAD/TOTAL	MEAN VIREMIA ($\log_{10}$ PFU/ml) BY DAYS						
		3-4	5-6	7	9-10	12-13	16-17	21-24
Squirrel	0/ 4	<[b]	1.8		1.9	1.2	1.2	<
Capuchin	0/ 3	<		2.6	2.6	2.1	1.8	<
Rhesus	6/10	0.9	2.8	3.7	4.2	4.4	3.0	1.7
African green	2/ 3	2.8		4.6	5.6	5.5	3.6	2.9
Cynomolgus	12/12	2.2	4.1		5.2	6.1	4.2	2.6

[a] $10^{6.1}$ $\log_{10}$ PFU LAS, strain Josiah.
[b] < = Viremia < 0.7 $\log_{10}$ PFU/ml, or undetectable.

All infected rhesus monkeys became ill by day 7; they remained huddled in the corners of their cages, developed coughs and often petechial rashes, especially on the face. Rectal temperatures were normal or slightly elevated until shortly before death, when they fell precipitously. The rhesus monkeys which survived recovered slowly over a period of about 3 weeks, and did not develop any obvious sequelae.

Table II shows the development of antibodies in surviving rhesus monkeys in relation to viremia. All monkeys developed FA antibodies by day 10. There is a period between days 10 and 24 when both viremia and circulating antibodies are detectable. This raises the possibility of circulating virus/antibody complexes, and

TABLE II. VIREMIAS AND ANTIBODY RESPONSES IN UNTREATED, SURVIVING RHESUS MONKEYS (n = 4)

DAYS AFTER INOCULATION	VIREMIA (log_{10} PFU/ml)	TITER	
		IFA	LNI[b]
3	0.9	< [a]	
6	2.5	<	
9	3.1	<	
10	3.1	120	
12	3.0		
14	2.9	640	<
17	2.8	1280	
20	1.5		
24	0.9	2560	<
30	<		0.4
45	<	2560	2.1
60	<	3120	2.6
180	<	2560	4.2

[a] < = Viremia < 0.7 log_{10} PFU/ml: IFAT < 10; LNI < 0.3.
[b] LNI = log_{10} neutralization index at a 1:10 dilution of serum.

will be investigated more fully. The neutralizing antibody response was first detected at convincing titers on day 45, as measured in a virus dilution, neutralization test using serum at a 1:10 dilution. Neutralization titers continued to increase between days 60 and 180. The sensitivity of this test must be increased, since even sera with high log LNI had no detectable activity when tested at a 1:100 dilution. Nevertheless, it is clear that neutralizing antibody, which is presumably protective, develops late and continues to increase for 3-6 mon after infection, possibly longer. Rhesus monkeys which died did not develop neutralizing antibodies, but had FA responses similar to the survivors. It is clear that FA titers do not necessarily correlate with neutralizing antibody titers. Selection of human donors of immune plasma for immunotherapy, on the basis of FA titers alone might be hazardous. Taken too early, such sera might even be infectious. Taken a little later, serum with apparently high potential efficacy might in fact contain little or no neutralizing antibody.

To gain some insight into the pathogenesis of LAS in rhesus monkeys, we obtained tissues from those which died and measured virus concentrations. Table III shows that the highest concentrations of virus were recovered from liver (average titer, 7.6 log/gm). However, virus also replicated to titers significantly higher than blood in all visceral tissues tested, including lung, adrenal, pancreas,

TABLE III. VIRUS CONCENTRATIONS IN TISSUES OF RHESUS MONKEYS (n = 6. Mean day of death = 13.6)

TISSUE	LOG_{10} PFU/ml or gm ± SE
Serum	5.6 ± 0.6
Liver	7.6 ± 0.5
Lung	6.9 ± 0.4
Adrenal	6.9 ± 0.5
Pancreas	6.8 ± 0.5
Spleen	6.7 ± 0.3
Kidney	6.5 ± 0.3
Lymph node	6.2 ± 0.5
Brain stem	5.5 ± 0.4
Spinal cord	5.4 ± 0.3
Cerebellum	5.4 ± 0.5
Cerebrum	5.0 ± 0.4
Pleural fluid	7.3 ± 0.7

spleen, kidney and lymph nodes. In contrast, CNS tissues did not appear to be major sites; however, the possibility of virus replication in critical sites, such as vascular endothelium, cannot be excluded.

Histopathologic examination of these tissues is in progress. The lesion found most consistently in rhesus monkeys is necrotizing hepatitis, usually focal and involving few if any inflammatory cells. Another consistent lesion is interstitial pneumonia. There are a few other lesions, including infarctions of the adrenals and mild myocarditis. Using direct immunofluorescence (DFA) LAS is most easily visualized in liver. Individual cells, probably hepatocytes, contain large quantities of virus and are scattered randomly throughout the parenchyma. Lung sections reveal intensely staining individual cells within the thickened alveolar septae with occasional free macrophages in alveolar spaces. Adrenals contain large quantities of virus in the epithelial cells of the zona glomerulosa extending into the zona fasciculata. In most cases, there is no corresponding lesion detected in H & E stained sections.

Spleens contain large infected cells, distributed randomly throughout the red pulp; virus seems to be largely excluded from the white pulp. Although there is a good deal still to learn about pathogenesis of LAS in rhesus monkeys, we believe that the disease process is a reasonable model for the human disease, and that we have enough information to use the model to test the efficacy of a promising antiviral drug, ribavirin.

Use of primate models to test efficacies of antiviral drug and immune serum regimens. Table IV compares viremia titers in rhesus monkeys treated with ribavirin initially on day 0, or on day 5, vs. untreated controls. Ribavirin treatment, initiated on day 0, resulted in delayed onset and diminished viremia. Among monkeys treated initially on day 5, the progression of viremia was halted soon after treatment began, and the viremia concentrations never exceeded 3.5 log_{10} PFU/ml. The monkeys treated initially on day 0 experienced very mild clinical disease; those treated on day 5 had a more severe course, but all eventually survived. One treated monkey developed what appeared to be transient blindness between days 32 and 38. We are not sure what caused this, but we have not seen it again in a large number of treated cynomolgus monkeys. Nevertheless, we are unsure of how to evaluate this

observation, and recognize the possibility that monkeys surviving acute LAS may develop sequelae referable to the CNS. In man, various auditory defects have been described during convalescence following LAS infection.

TABLE IV. VIREMIAS IN RHESUS MONKEYS TREATED INITIALLY ON DAY 0 OR 5 WITH RIBAVIRIN VS. LETHALLY INFECTED, UNTREATED CONTROLS

	MEAN LOG_{10} PFU/ml		
	Ribavirin		
DAYS	Days 0-18	Days 5-18	Controls
3	<[a]	<	1.0
5	<	3.1	2.9
7	1.6	3.2	4.1
10	2.0	3.1	5.1
12	2.1	3.0	5.9
14	1.4	2.3	all dead
17	<	1.8	
21	1.1	1.1	
24	<	<	

[a] < 0.7.

One further observation in rhesus monkeys confirms the impression that ribavirin treatment was beneficial. In Table V, transaminase concentrations in serum of infected monkeys are tabulated. In untreated, control rhesus monkeys, both SGOT and SGPT values increased markedly, peaking on day 10. In contrast, they were elevated only slightly in the monkeys treated on day 5 and did not fluctuate significantly at all in those treated on day 0. A manuscript has been submitted to the Journal of Infectious Diseases on the ribavirin studies.

TABLE V. SERUM TRANSAMINASE CONCENTRATIONS IN INFECTED RHESUS MONKEYS

		MEAN IU/L		
		Ribavirin		
ENZYME	DAYS	Days 0-18	Days 5-18	Controls
SGOT	3	29	29	29
	5	20	53	36
	7	22	56	72
	10	21	51	630
	12	23	37	1170
	14	36	38	all dead
	17	23	31	
	21	19	33	
SGPT	3	14	14	14
	5	16	19	17
	7	14	27	20
	10	11	17	185
	12	11	15	350
	14	NT	11	all dead
	17	11	11	
	21	12	12	

The pathogenesis of LAS for cynomolgus monkeys was very similar, except for uniform susceptibility. Six of 12 cynomolgus monkeys died 10-12 days after inoculation, 4 between days 13-17, and 1 each on days 24 and 42. Viremia titers in all cynomolgus monkeys were remarkably consistent. All infected monkeys uniformly developed viremias > 5 logs. Like the rhesus, cynomolgus all developed IFA antibodies by day 10, but died before mounting a neutralizing antibody response. The distribution of virus in tissues, and the lesions were similar to those described for rhesus. Thus, cynomolgus monkeys are also a reasonable model for human LAS, and are the primate of choice for efficacy testing of vaccines, antiviral drugs and immunotherapy regimens. Table VI summarizes the results of various treatment studies using infected cynomolgus monkeys and ribavirin, immune serum or a combination of them initiated on days 0, 4, or 7 following infection. The immune serum was a high titered pool obtained from rhesus monkeys; it had an FA titer of 10,240, and LNI of 4.1. We infused IV 1 ml serum/kg of body weight and achieved passive FA titers of 20. We reinfused serum on days 3 and 6 after the first dose, but could no longer monitor passive FA titers, since monkeys began making their own antibody. In the day 0 treatment group, one of the 4 serum-treated and none of the 4 ribavirin-treated monkeys died. However, both groups developed moderately high viremias, and became clinically ill. In contrast, monkeys treated with a combination of ribavirin + serum on day 0 never developed detectable viremias or became clinically ill, although they seroconverted. The absence of viremia does not mean that virus infection was averted. In fact, when one of these combined-treatment monkeys was sacrificed at 40 days, virus was easily isolated from a number of visceral tissues, including adrenal, heart and kidney. Other monkeys from this group will be sacrificed at later dates to determine if virus persists for longer periods of time. It is clear that combined treatment was more effective than either treatment alone.

A similar comparison was made in monkeys treated initially on day 4. There was no "serum-only" group, but a comparison of viremias in the ribavirin group with those of the combined group suggests again that combined therapy was more effective than ribavirin alone.

In a third experiment, initial treatment was delayed until day 7. The viremia curves for all 3 groups were practically superimposable. However, 2 of 3 ribavirin-treated and 2 of 3 serum-treated monkeys died, but, none of the 4 monkeys which received the combined treatment died. These preliminary results suggest that combined therapy, initiated late (on day 7), was more effective than either serum or ribavirin alone. The possibility should also be recognized that under certain conditions, treatment with immune serum might be worse than no treatment at all. This problem demands immediate attention. Other possible correlates to protection were also examined. One was the fluctuation of transaminases in treated and control monkeys. Untreated monkeys developed high values of SGOT by day 10 or 12. This did not occur in monkeys treated early. Combined treatment, even delayed until day 7, appeared to reduce the elevations of SGOT. We are developing similar data for another enzyme, creatinine phosphokinase. It appears that clinical intervention with serum + ribavirin can minimize the destruction of tissues, which is reflected by increases in serum enzyme levels.

Similar studies are in progress using inbred strain 2 and strain 13 guinea pigs, in which the pathogenesis of LAS approximates the disease course in primates. Use of the guinea pig model will permit sequential sacrifice studies to define more precisely the mechanisms by which serum, ribavirin or their combination are protective.

TABLE VI. EFFECT OF RIBAVIRIN, IMMUNE SERUM OR COMBINED TREATMENT ON VIREMIA AND MORTALITY IN CYNOMOLGUS MONKEYS

		LOG_{10} PFU/ml			
DAY TREATMENT INITIATED	DAYS AFTER INFECTION	Ribavirin Only (dead/total)	Serum only	Combined	Controls
0		(0/4)	(1/4)	(0/3)	(12/12)
	3-4	<[a]	0.7	<	2.5
	6-7	2.2	3.0	<	4.0
	9-10	4.1	3.4	<	5.5
	13-14	4.2	3.3	<	5.5
	16	3.9	3.0	<	4.1
	19	3.0	2.8	<	3.0
	26	2.1	2.3	<	2.6
	42	<	<	<	<
4		(0/4)	NT	(0/3)	(12/12)
	3-4	2.8		3.3	2.5
	6-7	3.8		3.8	4.0
	9-10	3.7		3.5	5.5
	13-14	3.6		2.5	5.5
	16	2.7		1.6	4.1
	19	2.5		1.3	3.0
	26	2.5		1.9	2.6
	42	<		<	<
7		(2/3)	(2/3)	(0/4)	(12/12)
	3-4	2.4	1.8	2.3	2.5
	6-7	4.3	4.1	4.4	4.0
	9-10	4.2	4.5	5.3	5.5
	13-14	4.1	4.7	4.4	5.5
	16	3.6	2.3	3.2	4.1
	19	3.8	2.5	3.0	3.0
	26	2.9	<	2.7	2.6
	42	<	<	<	<

[a] < 0.7

Preliminary studies toward production of an inactivated LAS vaccine. Following a preliminary screening of certifiable cells, MRC-5 cells were selected as the most probably useful substrate for replication of LAS. Table VII presents the growth kinetics of LAS in these cells maintained in the absence of fetal calf serum, but supplemented with 0.5% human serum albumin. At a multiplicity of 10, peak titer is attained by day 2, and is a little over 7 logs/ml. This material may also contain defective interfering (DI) particles, which may boost the effective antigen yield still higher. At lower multiplicities, somewhat less virus is produced, and no evidence exists that DI particles are produced. Ideally these yields should be increased about 10-fold, although the titers presently attained may be adequate for an effective inactivated vaccine.

Table VIII gives the results of initial studies of inactivation procedures. Formalin (0.1%) reduced the infectivity from 6 to 5 logs almost instantaneously, but then had no effect during 1 hr at room temperature. However, formalin totally

TABLE VII. REPLICATION OF LAS IN MRC-5 CELLS MAINTAINED IN HMEM WITH 0.5% HUMAN SERUM ALBUMIN

	LOG_{10} PFU/ml BY MOI[a]		
DAY	1.0	0.01	0.0001
0	<	<	<
1	5.6[b]	4.7	<
2	7.2	6.1	2.5
4	7.0	6.2	5.1
7	6.9	6.1	6.7

[a]MOI = multiplicity of inoculum (PFU/cell).
[b]Interfering particles present.

TABLE VIII. INACTIVATION OF LAS IN HMEM WITH 0.5% HSA

TIME	LOG_{10} PFU/ml			
(min)	Formalin[a]	Psoralen	γ-Radiation	No treatment
0	6.2	6.2	6.2	6.1
1	5.1	5.5		
5	5.1	3.8	5.5	
10	5.0	2.8	4.3	6.0
20	5.1	1.7	2.8	
60	5.0	<	<	6.1
18 hr	<	<	<	3.8

[a]Concentrations employed: Formalin, 0.1%; psoralen (AMT), 10 μg/ml; γ-radiation, 31,000 R/min.

inactivated the virus when treatment was continued overnight. Gamma radiation totally inactivated the virus within 1 hr. Likewise, psoralen, an intercalating agent in the presence of long wavelength UV light, totally inactivated infectivity within 1 hr. We are presently preparing 1-L quantities of vaccine, using each of these inactivation techniques. We plan to test their efficacy first in guinea pigs, then in rhesus and cynomolgus monkeys, and eventually in those of us at high risk to infection.

Presentation:

Jahrling, P. B., R. A. Hesse, and J. L. Beall. Radioimmune assay procedures for rapidly identifying alphaviruses and for quantitating antibodies to alphaviruses in mammalian sera. Presented, Annual Meeting, American Society of Tropical Medicine and Hygiene, Chicago, IL, 6-19 Nov 1978.

Publications:

None.

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1. Casals, J., and S. M. Buckley. 1974. Lassa fever. Prog. Med. Virol. 18: 111-126.
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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0F6415	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	010
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Cellular responses in lymphatic tissues following immunization

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 79	1.0	103.4
FISCAL YEAR — CURRENT 80	1.0	133.5

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

ASSOCIATE INVESTIGATORS

NAME:

NAME:

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Vaccines; (U) Lymphocyte recirculation; (U) High endothelial venules (HEV); (U) Immunology

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

23 (U) Define regulatory mechanisms of lymphocyte recirculation and cellular interaction in tissues during the in vivo immune response; use this information to develop new and more potent adjuvants for use with military vaccines; materials developed in this manner should significantly hasten the interval between vaccination and protection.

24 (U) Use morphological and radiolabeled indicators of lymphocyte kinetics to study influence of various factors affecting lymphocyte traffic into lymph nodes during induction of immune responses; assess effector immunity using serological and cellular systems.

25 (U) 78 10 - 79 09 - Lymphocytes respond to chemotactic gradient by orienting toward the stimulus, but the efficiency is reduced, because of special requirements of the lymphocytes or competing stimuli secreted by neighboring cells. Surface receptors are stripped from transmigrating lymphocytes by high-endothelial cells in a process called "scrubbing," which has been further defined using 2 more physiologic markers: FITC and lactoperoxidase-iodination of lymphocyte surfaces following by tracking of the markers in tissues from animals receiving transfusions of these cells. Mechanisms of action of 3 adjuvants, which would have a high probability of being tested in humans were examined; MDP, CP-20 961 and poly(ICLC) all appear to accelerate the emigration of lymphocytes into regional lymph nodes. MDP enhances both influx and egress of cells be degranulating mast cells; CP-20 961 and poly(ICLC) act more like complete Freund's adjuvant by encouraging increased entry while retaining the same rate of exit. CP-20 961 seems to be the most effective and has the least toxicity when used in an emulsion of vaccine and Intralipid.

Publications: Fed. Proc. 38:1429, 1979; In Function/Structure of the Immune System, p. 73-83, 1979; J. Reticuloendothel. Soc., in press, 1979.

*Available to contractors upon originator's approval

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

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 010: Cellular Responses in Lymphatic Tissue Following Immunization

Background:

The induction of immunity requires an intricate series of cooperative and regulatory cellular interactions among classes of T and B cells and macrophages. In vivo immune responses to nonreplicating vaccine antigen most likely begin within the regional lymph nodes (LN) where specialized phagocytic cells bind and display the antigen to recirculating and lodging T and B cells which enter the node by crossing the walls of specialized venules in the paracortex (1, 3). Since migrating lymphocytes comprise a diverse immunoreactive repertoire, the traffic of lymphocytes through lymphatic tissues provides a natural environment for clonal selection (3, 4). The process of coordinating the cellular interactions necessary for induction of immunity is amplified by nonspecific and antigen-specific alterations in lymphocyte traffic through lymph nodes draining antigen inoculation sites (5). Not all antigens are capable of accelerating lymphocyte traffic, and the ability of certain antigens to promote lymphocyte emigration appears to correlate with the immunogenticity and adjuvanticity of the antigen.

Progress:

Lymphocyte Chemotaxis

In Vivo studies (6, 7) suggested that blood-borne lymphocytes might be attracted into LN by chemical gradients diffusing from between venular endothelial cells. This hypothesis was tested in vitro using an agarose chemotaxis system developed by Nelson et al. (8). Directed (D) and random (R) migration after 18-hr incubation was measured for each agent tested and a ratio (D/R) > 2 indicated significant chemotaxis or chemokinesis. Stem-leaf plots of the angles of directional orientation (0-360°) for individual migrating lymphocytes helped to discriminate chemokinesis from chemotaxis (Table I). Clustering of cell polarities about 180° indicated chemotaxis. Random migration in the absence of a chemotactic gradient and chemokinesis induced by stimulatory materials resulted in random orientations or flattened stem-leaf plots (Table II).

TABLE I. STEM-LEAF PLOT DISTRIBUTION OF DIRECTIONAL ORIENTATION OF LYMPHOCYTES MIGRATING TOWARD A GRADIENT OF INFECTED RAT PLASMA

DIRECTION (0-360°)	NO. CELLS
0	10
20	11
40	19
60	10
80	18
100	19
120	24
140	52
160	75
180	59
200	65
220	52
240	28
260	18
280	12
300	9
320	8
340	11

TABLE II. STEM-LEAF PLOT OF DIRECTIONAL ORIENTATION OF LYMPHOCYTES MIGRATING IN THE ABSENCE OF A CHEMOTACTIC GRADIENT (A) AND INTO A GRADIENT OF MATERIAL WHICH IS CHEMOKINETIC (B)

DIRECTION (0-360°)	CHEMOTACTIC GRADIENT ABSENT	CHEMOKINETIC GRADIENT
181-200	15	22
201-220	9	26
221-240	11	21
241-260	7	20
261-280	7	15
281-300	10	23
301-320	13	30
321-340	9	29
341-360	16	32
0-20	13	26
21-40	20	22
41-60	17	25
61-80	12	29
81-100	8	17
101-120	11	21
121-140	9	24
141-160	8	18
161-180	14	20

Thoracic duct lymphocytes (TDL) exhibited positive chemotaxis toward infected rat plasma (D/R 4.3), activated C_3 (D/R 5.6), dialysable leukocyte extracts (DLE) (D/R 5.0), and muramyl dipeptide (MDP) (D/R 3.7). In contrast, lymphocytes failed to show chemotaxis toward normal rat plasma (D/R 1.0), activated C_5 (D/R 1.1) and F-Met-Leu-Phe (D/R 1.2).

The orientations of lymphocytes migrating chemotactically *in vitro* were compared to the orientations of lymphocytes migrating across high endothelial venules (HEV) walls in lymph nodes (Table III).

TABLE III. COMPARISON OF CYTOPLASMIC ORIENTATION OF MIGRATING LYMPHOCYTES *IN VITRO* AND *IN VIVO*

DIRECTION (0-360)	NO. MIGRATING	
	In Vitro	*In Vivo*
0-10	1	
11-30	9	2
31-60	13	2
61-90	10	
91-120	21	12
121-150	26	28
151-180	33	61
181-210	12	19
211-240	14	6
241-280	14	
281-310	11	4
311-340	10	1
341-360		1

Although quite similar, the tighter distribution seen for *in vivo* migration is probably due to the structural limitations of lymphocytes migrating through preformed inter-endothelial channels. While *in vitro*, cells responding chemokinetically are free to orient randomly. This suggests that the differentiation between chemokinesis and chemotaxis for lymphocytes is not relevant to *in vivo* situations. *In vitro* some chemokinesis was seen for all the agents tested but was greatest for MDP and DLE (potential adjuvants). In conclusion, lymphocytes exhibit chemotaxis *in vitro*, but not toward agents putatively attractive for neutrophils. *In vivo* chemotaxis may be partially responsible for the accelerated lymphocyte emigration seen following introduction of antigens or adjuvants.

Lymphocyte scrubbing.

In previous reports "scrubbing" was described ultrastructurally using LN from rats receiving infusions of lymphocytes treated with peroxidase or ferritin-conjugated ligands to cell surface receptors or adsorbed proteins. In the present experiments 2 additional methods of demonstrating scrubbing are described. Fluorescein isothiocyanate (FITC) (1 mg/ml) was added directly to cultures of TDL in minimal essential medium (MEM) without significantly reducing lymphocyte viability. Cells incubated in FITC for 15 min, washed 2X, and reincubated in fresh MEM overnight survived, as

did cells left 18 hr in the FITC solution; 80-90% of these cells were labeled on their surfaces with fluorescein. The 10-20% nonviable cells which took up FITC intracytoplasmically and intranuclearly were also those susceptible to trypan blue disruption. Viability was further assessed by examining for spontaneous locomotion by phase microscopy at 37°C. Normal viable lymphocytes with surface fluorescence also exhibited about 15% spontaneous locomotion. These cells also contained 2-3 apple-green fluorescing granules which seemed to have resulted from nonspecific endocytosis, since capping was not observed unless active locomotion was taking place; this was reversible after locomotion ended. Some of these cells were in the process of dividing, which caused the fluorescent surface molecules to be excluded from the constriction zone. Locomoting lymphocytes had fluorescence over the leading edge, not over the constriction zone or uropod, which suggested that membrane receptors associated with cytoskeletal control either lacked the ability to bind FITC or those that do bind FITC were passively excluded from zones of constriction by molecules that do not bind FITC.

FITC fluorescent TDL were infused IV into syngeneic recipients and the rats were sacrificed at 4-, 18-, and 24-hr postinfusion. Brightly fluorescent material was seen concentrated in Kupffer cells of the liver and sinus macorphages in the spleen at 4 and 18 hr. Lymphocytes bearing surface and granule fluorescence were found in peripheral blood and in the splenic red pulp and on luminal surfaces of LN venules at 4-hr. However, only lymphocytes bearing granule fluorescence were seen in the periarteriolar lymphatic sheath (PALS) or in the paracortex of lymph nodes at 4 and 18 hr. The plump macrophage-like cells of the splenic marginal zone, and the endothelial cells of the LN-HEV contained granules bearing specific fluorescence, suggesting that these cells stripped off FITC-blocked receptors as the cells migrated past. Fluorescence-bearing lymphocytes could be recovered from the thoracic duct when it was cannulated 18 hr postinfusion. Surface-labeled cells could still be recovered from peripheral blood at 24 hr, suggesting that FITC may have influenced the ability of these cells to leave the circulation.

Conclusions which can be drawn from this preliminary study include: Protein receptors directly conjugated with FITC are selectively removed from the surface of recirculating lymphocytes by marginal zone cells and HEV endothelial cells without affecting the viability and ultimate destination of these lymphocytes; and it may be possible to follow lymphocyte traffic quickly by using fluorescence rather than depending upon a radiolabel.

This phenomenon of lymphocyte scrubbing was further studied using lactoperoxidase (^{125}I) iodination of lymphocyte surface proteins and subsequent autoradiography on 0.5-1.0-um plastic sections of lymph nodes from ^{125}I-TDL-infused rats. In these studies autoradiograph grains were found along interendothelial clefts in HEV, in HEV lysosomal granules and in cytoplasmic granules of paracortical lymphocytes. The numbers of cells bearing these grains were much less than were seen for FITC, but this can be attributed to the relative instability of the label over the time course of study.

Rift Valley fever (RVF) host resistance studies. In collaboration with LTC Peters, 70 Lewis rats and 70 Brown Norway rats were vaccinated SC with 0.2 ml of graded dilutions of Lot 2 of the currently available RVF vaccine. Blood was drawn on days 10, 20, and 30; these rats were challenged by SC inoculation with 10^5 Pfu strain Z-501. The mortality data and comparative titers for the 30-day bleeding are shown in TABLE IV.

TABLE IV. COMPARISON FOR DAY 30 OF ANTIBODY TITER AND SURVIVAL OF BROWN NORWAY AND LEWIS RATS VACCINATED WITH DOSES OF RVF VACCINE

VACCINE DOSE (Dilution)	Brown Norway (N=7) PN Titer	Survivors	Lewis (N=7) PN Titer	Survivors
0	0	0	0	7
1:625	1.42	1	0	7
1:125	7.14	1	21.66	7
1:25	22.85	2	31.25	7
1:5	9.28	2	40.00	7
1:1	57.14	7	72.00	7

RVF encephalitis. A CD-1 mouse which had been given a 1/256 dilution of inactivated RVF vaccine at day-14 and subsequent SC inoculation with RVF virus strain (77501), recovered from the acute liver disease but became clinically encephalitic by 8 days. This mouse was perfusion-fixed with warmed Karnovsky's fixative by direct intracardiac infusion after preliminary heparin-dextran wash out. Brain, liver, lymph nodes, spleen, Peyer's patches and thymus were excised and placed in fixative.

Toluidine blue-stained plastic sections of the brain revealed multifocal necrotizing lesions which were associated with local perivascular cuffing. Lymphoid and myeloid cells could be seen in various stages of transvascular diapedesis, and there was formation of a reticular space in the site of the Virchow-Robin perivascular space, which contained the recently emigrated cells. The liver revealed Kupffer cell hyperplasia, centrilobular hepatocellular drop out, spared portal areas and regenerative hepatocellular proliferation in periportal liver lobules. The lymph nodes and spleen showed T-cell region depletion and B-cell region hyperplasia. There were prominent immunoblasts and preplasma cells in the follicular funnel areas of lymph nodes and in the PALS of spleen. In addition, lymphoblastic cells could be found trapped within the blood vascular system and migrating into the cerebral tissue at sites of vascular cuffing. Electron microscopic examination of other peripheral tissues are at present incomplete; however, significant findings were seen in the brain. In the areas of perivascular cuffing occasional neuronal cells were found which had lysed after infection with RVF virus. There were cells within the CNS which did not lyse after infection. These cells were clearly identified as oligodendroglial cells. The viral cores were replicating within the nucleolus, as rods and spheres of virus-like particles could be seen. This type of virus replication is considered unusual for RVF but has been described in human tissues from the Egyptian RVF epidemic. These cells (oligodendrogliocyte) show no evidence of degeneration and the entire sequence of viral core generation in the nucleolus, capsule manufacture in the cytoplasm and incorporation into smooth membrane cysterns were found in each cell.

In other neighboring areas of the brain, lymphoid and myeloid cells were found adhering to myelinated nerve fibers. At these sites focal punched-out demyelination was present, suggesting cellular cytotoxicity of the type seen with antibody-dependent cellular cytotoxicity. This focal demyelination seems to have been caused by local release of digestive enzymes or interaction of membrane-bound enzyme with the myelin.

In other cells lying near the perivascular space intact and defective (coreless) RVF particles could be found.

In conclusion, the observation of chronically infected myelin-forming cells and the evidence of leukocyte-mediated myelin destruction in the brain of a RVF encephalitic mouse, suggests a possible mechanism for the evolution of postviral allergic encephalitis. The fact that oligodendrogliocytes and/or Schwann cells are chronically infected in subacute sclerosing panencephalitis, measles, encephalitis and multiple sclerosis, suggests that heterogenous viruses may be responsible for many chronic neurological diseases.

Adjuvant studies. The need to develop vaccines against viral diseases which affect the health and economy of developing countries has promoted interest in immunostimulatory compounds which may be used to enhance the effectiveness of marginal vaccines. Few viral vaccines are available as living, attenuated strains, so we must rely on formalin-or glutaraldehyde-inactivated vaccines for prophylaxis. Unfortunately, inactivation often reduces immunogenicity, resulting in short-lived protective antibody titers and which may lead to adversely modified disease patterns if natural infection should occur. It is hoped that immunostimulants, such as MDP, poly(ICLC) and CP-20 961, might be useful in overcoming the deficits of inactivated viral vaccines.

Muramyl dipeptide. Female Lewis rats (200 gm) were given single injections of various combinations of the following antigens and adjuvants; complete Freund's adjuvant (CFA), inactivated VEE vaccine virus (C-84), N-acetyl muramyl-L-alanyl-D-isoglutamine (MDP), peanut oil emulsion (A65), and incomplete Freund's adjuvant (IFA). The tissue response at the injection site and in the regional lymph nodes was studied serially in rats sacrificed from each group on days 1, 2, 3, 7, 14, and 21 after inoculation. Plasma was collected at weekly intervals for viral plaque neutralizing (PN) titer determination. In another group of rats, the rate of lymphocyte entry into regional LN 24 hr after adjuvant inoculation was determined by radioautography of LN removed 3-180 min after IV infusion of 3×10^8 [^{3}H]uridine-labeled TDL.

Unlike CFA and MDP in oil base, MDP in buffered saline (alone or in combination with C-84 vaccine) did not evoke sterile abscess formation at the local injection site. MDP in saline caused diapedesis of mononuclear cells, local hyperemia and accumulation of lymphoid cells in afferent lymphatics as early as day 2 or 3. Peak local mononuclear infiltration occurred on day 14 for MDP and on day 21 for CFA; the amounts of inflammatory cells in the CFA depots were considerably greater at all days. Granulomas and fibrosis were not seen following inoculation of MDP and/or C-84 in saline, while foam cells and epithelioid cell clusters were often seen by days 14 and 21 after MDP in IFA or A65.

Inoculations of CFA or MDP in an oil base produced a regional LN response characterized by rapid node enlargement for the first 48 hr, followed by a more gradual increase in size which peaked on day 21. The early peak was associated with mast cell degranulation in the LN medullary sinuses, vascular congestion, and dilation of intranodal lymphatic spaces, suggesting increased local and regional blood flow and fluid transudation possibly caused by inflammatory mediators from the injection site. Morphological and radiokinetic indicators of the rate of lymphocyte entry into LN during this 48-hr period demonstrated that MDP caused a marked increase in lymphocyte traffic, occurring whether or not the MDP had been introduced in a saline or oil base. The fact that this increased emigration was

not reflected by a significantly increased accumulation of labeled cells in tissue at 24 hr may be explained by the fact that tissue accumulation depends on the existence of a difference between the rate of lymphocytes entering and exiting the node. The present studies suggest that MDP-containing inocula promote acceleration of the net transit of lymphocytes across LN which would be reflected by exposure of increased numbers of immunoreactive cells to antigen. The hypothesis of increased transit is supported by our recent observation in vitro of the ability of MDP to affect chemokinesis of lymphocytes in a migration-under-agarose chemotaxis assay.

Muramyl dipeptide used in these experiments caused significant elevations of the primary and secondary PN antibody response to VEE, but did not exceed that for CFA; potentiation of antibody titers exceeding the titer produced by an ED_{50} dose of C-84 vaccine was not consistently shown for all doses of MDP used.

Poly(ICLC) Study.

Female Lewis rats (200 gm) were injected subcutaneously with inactivated VEE vaccine (C-84), vaccine mixed with CFA or vaccine combined with poly(ICLC). Poly (ICLC) was also given alone or on opposite sides from the antigen. These rats were bled serially for PN titers. LN changes were studied after serial sacrifice of similarly treated littermates. Additional rats were inoculated SC with graded doses of poly(ICLC). After 24 hr, 3 X 10^8 [^{3}H]uridine-labeled lymphocytes were infused IV, regional and contralateral nodes were removed from lymphocyte traffic kinetic studies.

Poly(ICLC) at 1.0 mg/kg clearly produced potentiation of the VEE PN titer, which equalled or exceeded titers of antibody found with VEE-CFA combinations. Inoculation of poly(ICLC) at distant sites from the antigen depot did not increase early antibody titers, but PN titers after week 1 were slightly elevated over those produced by an ED_{50} dose of vaccine alone.

Regional LN doubled in weight by 7 days after inoculation with VEE vaccine, followed by gradual return to normal weight by day 35. In contrast, poly(ICLC) and VEE + poly(ICLC) caused pronounced regional LN enlargement (4-5 X normal weight) between 6 and 48 hr. Planimetry of histological preparations of these nodes indicated that the bulk of this enlargement was due to accumulation of small lymphocytes in the paracortex. LN draining poly(ICLC) injection sites returned to normal weight in 10 days, while nodes draining VEE + poly(ICLC) injection sites continued to increase in size until 15 days; they were still nearly 2 X normal size on day 35.

Morphological and radiokinetic studies of lymphocyte traffic into similarly treated LN suggested that increased lymphocyte entry in the presence of a constant egress rate had resulted in net accumulation of labeled cells in the paracortex. No other signs of inflammatory changes, such as mast cell degranulation or sinus dilation were seen after the first 48 hr. The presence of some radiolabeled lymphocytes in the afferent lymphatics included in the node sections for autoradiography suggested that the tempo of lymphocyte traffic through the injection site might also be enhanced by the poly(ICLC) mass, but specific studies of the local site were not done.

It is suggested that poly(ICLC) may behave as an adjuvant by encouraging increased lymphoid cell traffic, but it is unclear at this time how this would be similar or different from the increased cell traffic produced by particulates or CFA.

CP-20 961 [N,N-Dioctadecyl-N,N-bis (2-hydroxyethyl) propanediamine]. In studies performed in collaboration with CPT Reynolds, we have examined the inflammatory and immunopotentiating effects of CP-20 961, and have attempted to illustrate the role of migratory cell populations in the acquisition and regulation of immunity to viral vaccine.

Leukocyte migration into the injection sites and regional LN of inbred mice was studied by morphological and autoradiographic techniques following SC inoculation of C-84 VEE vaccine combined with or without biodegradable lipid vehicle containing CP-20 961. Adjuvanticity was indicated by the ability of these combinations to elevate PN titers and to enhance protection from lethal virus challenge (Table V).

TABLE V. EFFECT OF ADJUVANT CP-20 961 ON PROTECTION FROM VEE CHALLENGE 14 DAYS AFTER VACCINATION (n=20/group)

VEE Vaccine diluted 1:5 in:	SURVIVORS Total	%
MEM	10	50
Peanut oil lipid emulsion (LE)	9	45
Intralipid (IL)	11	55
LE + 0.01 mg. CP-20 961	12	60
LE + 0.1 mg. CP-20 961	15	75
LE + 1.0 mg. CP-20 961	11	55
LE + 0.01 mg. CP-20 961 + TWEEN	10	50
LE + 0.1 mg. CP-20 961 + TWEEN	12	60
LE + 1.0 mg. CP-20 961 + TWEEN	10	50
IL + 0.01 mg. CP-20 961	18	90
IL + 0.1 mg. CP-20 961	18	90
IL + 1.0 mg. CP-20 961	17	85
IL + 0.01 mg. CP-20 961 + TWEEN	19	95
IL + 0.1 mg. CP-20 961 + TWEEN	18	90
IL + 1.0 mg. CP-20 961 + TWEEN	20	100
Unvaccinated controls	0	0

The results indicated that vaccine combined with CP-20 961 in soybean oil lipid emulsion (Intralipid) induced transient acute inflammation in the injection site which was followed by lymphoid and monocytic infiltrates by day 7. The injection mass was cleared by lipid-laden macrophages; subsequently macrophages containing brown lipid degradation products were located in the subcapsular sinus and near germinal follicles of regional LN. Rapid expansion of the paracortex by recently emigrated small lymphocytes occurred in a dose-dependent fashion 6-48 hr after SC inoculations with vaccine containing graded doses of CP-20 961. Only modest cellular accumulation was seen in nodes draining aqueous vaccine, or vaccine mixed with the lipid vehicle. Despite continued elevation of the rate of lymphocyte entry into these nodes they returned to nearly normal size by day 3 because of massive efflux of lymphocytes via efferent lymphatics. Proliferative activity and continued cellular influx in the paracortex resulted in a secondary peak in LN size by day 14. The

magnitude of this second peak also varied according to the dose of CP-20 961. Thus CP-20 961 appears to behave as an immunological adjuvant by enhancing cellular traffic through antigen depots and by potentiating the expansion of immunoreactive clones.

Presentations:

1. Anderson, A. O. Mechanism of action of MDP: lymphocyte traffic and antiviral immunity. Presented, International Symposium on Potentiation of Immune Response to Vaccines (NIAID, NIH, BOB, and FDA), Washington, DC, 20 Feb 1979.

2. Anderson, A. O., J. A. Reynolds and D. Harrington. Immunomodulating effect of polynucleotides on lymphocyte traffic and antiviral immunity. Presented, International Symposium on Potentiation of Immune Response to Vaccines (NIAID, NIH, BOB, and FDA), Washington, DC, 21 Feb 1979.

3. Anderson, A. O., and J. T. Warren. Lymphocyte chemotaxis under agarose. Presented, Annual Meeting, AAP/FASEB, Dallas, TX, 3 Apr 1979, (Fed. Proc. 38:1429, 1979).

4. Anderson, A. O. Lymphocyte motility, orientation, and chemotaxis. Presented, Joint Immunobiology Meeting among Johns Hopkins Immunology Council, University of Virginia and University of Maryland, Blackwater Falls, WV, 21 Apr 1979.

5. Anderson, A. O., and J. A. Reynolds. Adjuvant effects of the lipid amine CP-20 961. Presented, Newer Aspects of Immunology with Therapeutic Prospects; Symposium, Branford House, Groton, CT, 14 May 1979.

6. Anderson, A. O. Address to Wagner College Alumni following receipt of the Wagner College Alumni Achievement Award, Staten Island, NY, 19 May 1979.

Publications:

1. Anderson, A. O., and J. A. Reynolds. Adjuvant effects of the lipid amine CP-20 961. J. Reticuloendothel. Soc. In press.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0B6420	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	011
b. CONTRIBUTING				
c. //////////	STOG 80-7.2:2			

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

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 79	1.5	292.0
FISCAL YEAR — CURRENT 80	2.0	275.3

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

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22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Arboviruses; (U) Vaccine development; (U) Dengue virus; (U) Chikungunya virus

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

23 (U) Develop and produce inactivated and attenuated arbovirus vaccines which may then be combined or administered sequentially to military forces for prophylaxis in geographically or BW oriented ways.

24 (U) Arboviruses are propagated in primary or certified diploid cell cultures 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) 78 10 - 79 09 - A reproducible rhesus monkey viremia model has been developed for dengue (DEN-1) vaccine thus enabling us to test candidate clones for the most important marker of avirulence, the inability to induce monkey viremia. An excellent vaccine candidate, TP8-P1-2, was obtained by plaque-to-plaque passage and terminal dilution passage in fetal rhesus lung cells (FRhL). This candidate has shown the following markers; temperature sensitivity (TS) in growth-curve assays and direct plaquing; no reversion to large plaques and no TS after 3 passes in cell culture; identity as DEN-1 in breakthrough neutralization tests and ability to produce only TS virus in suckling mouse brain. A monkey viremia test with TP8-P1-2 is in progress. Chikungunya vaccine development studies were begun. A series of 4 plaque-to-plaque passages were made in FRhL cells. Six more plaque-to-plaque passages were made to purify and select for attenuation. Four clones have been selected for further study; all are small plaque formers and are TS.

* Available in contractor's own organization's approval

BODY OF REPORT

Project No. 3M162276A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 011: Development of Arbovirus Vaccines for Diseases of Military Importance

Background:

The need for attenuated dengue (DEN) virus vaccines is self-evident in view of the health problems posed by DEN in many tropical and semitropical areas. As part of a joint effort coordinated and supported by USAMRDC, attenuated vaccines for all 4 DEN serotypes are being developed at WRAIR (DEN-2 and -3), at the University of Hawaii School of Medicine (DEN-4), and at USAMRIID (DEN-1).

Our DEN-1 program is at an earlier stage of development than are the others. Three human isolates have been adapted to acceptable vaccine substrate (DBS-103 cells) and subjected to plaque-to-plaque passage and terminal dilution techniques to obtain and purify avirulent vaccine candidate clones. The resulting clones are being selectively examined for markers of presumed avirulence for man, viz, small plaque size, temperature sensitivity and inability to induce viremia in rhesus monkeys, etc.

Studies were also initiated late this year on the development of an attenuated Chikungunya (CHIK) vaccine. An earlier attempt (1969) to produce a killed product at WRAIR resulted in a moderately successful vaccine, supplies of which have since been depleted. In view of the severity of this disease and its active presence in both Africa and Asia the need for an attenuated vaccine that may be expected to provide excellent protection after a single dose is obvious. Such a vaccine may be used to protect US military personnel and laboratory workers at risk to infection.

Thus far CHIK virus strain 15561 has been subjected to 3 series of massive plaque-to-plaque selection/purification schemes in MRC-5 cell culture, an acceptable vaccine substrate. The results of these efforts are discussed.

Progress:

Part I. Attenuated DEN-1 Vaccine

Rhesus monkey DEN viremia studies. As previously reported (1) the concensus at the 1978 Dengue Vaccine Task Force meeting was that the ultimate test of avirulence for man is the inability of vaccine candidate strains to elicit viremia in rhesus monkeys. Mouse virulence, plaque size, and temperature sensitivity (ts) markers are considered excellent for use as "screening" aids, but are of marginal value in the absence of the monkey "marker." Therefore, this past year emphasis was placed on increasing the sensitivity of the viremia assay procedure.

The first study was designed to reevaluate the modified viremia assay system. Two DEN-1 strains were supplied by WRAIR expressly for this purpose i.e., DEN-1 #13802 and DEN-1 Carec A. Two monkeys received $\sim 10^4$ PFU of Carec A on day 0 and an additional 2 monkeys received $\sim 10^{2.8}$ PFU of #13802, SC. Serum samples from days 1-11 postinoculation were tested by the direct plaquing technique on LLC-MK_2 cultures inoculated with 1:2 dilutions of these sera.

Shown in Table I are the results of these plaque assays. As indicated, the direct plaque technique detected viremias with both strains, although #13802 is obviously more virulent for rhesus monkeys, considering the long term viremias (7 and 8 days) and the higher levels of viremia elicited. Further study of these sera by the outgrowth or "enrichment" technqiue normally used to document extremely low level viremias was held in abeyance to permit a second study to be carried out with available LLC-MK_2 cells.

TABLE I. RHESUS MONKEY VIREMIA STUDIES ON DON-1 STRAINS CAREC A AND #13802

MONKEY NO.	INOCULUM Strain	INOCULUM Titer (PFU)	DAYS OF VIREMIA	RANGE OF VIREMIA LEVEL (PFU/ml)
T-341	Carec A	10^4	10	5
B-6939			4, 6, 8	5
5605	#13802	$10^{2.8}$	1-7	5-305
X791			1-8	5-1,165

The purpose of the second monkey study was 2 fold: (a) to serve as a further evaluation of the viremia assay system and (b) to compare the viremia inducing capability of the 3 original human serum specimens, from which all our DEN-1 "parents" and clones were derived, to the viremia inducing capability of the parents and PL-2 clone themselves. Since we must be able to document a marked decrease in monkey viremia induced by vaccine candidates vs. "parent" virus, this study was of great significance to the DEN-1 program.

Due to holding space limiations only 14 rhesus monkeys could be employed in this pilot study. Two monkeys each were inoculated SC with the viruses listed in Table II. To preserve the little remaining original human serum specimens, these were diluted 1:15 prior to inoculation. All of the pass-8 (DBS-103) "parents" and the vaccine candidate strain (PL-2) were used undiluted in an attempt to ensure that any virulent subpopulation, even if present at a very low level, would have the opportunity to express itself in terms of viremia induction. Assay for viremia was as described above. The 80% plaque reduction serum neutralization (PRN_{80}) test was used to confirm infection of the monkeys.

As illustrated in Table II all monkeys were infected except T-268, which exhibited neither viremia nor DEN-1 neutralizing antibody. Significantly, all 3 original serum specimens (with the one exception noted above) and the 3 "parent" viruses derived produced viremias in the monkeys lasting 3 - 6 days (most often on days 3 - 8), with peak viremias ranging from 45 to 1585 PFU/ml. In contrast the vaccine candidate strain PL-2 from the DEN-1 #1 parent, produced only 25 - 55 "questionable" PFU/ml on days 7 and 8 in only 1 of 2 monkeys. Both monkeys responded with 1:40 PRN_{80} titers. These results compare favorably to those obtained with WRAIR's DEN-2 S-1 vaccine strain. It should be pointed out that use of the outgrowth or enrichment procedure confirmed the presence of very low level viremia in monkeys C-580, a PL-2 recipient, on days 6, 8 and 9; all plaques were $\leq$ 2 mm, indicating the stability of the PL-2 candidate in terms of plaque size marker after one passage in monkeys. When monkey holding space was made available another study of DEN-1 #1 "parent" and the PL-2 vaccine candidate was conducted.

TABLE II. VIREMIA AND ANTIBODY RESPONSES OF RHESUS MONKEYS INOCULATED WITH UNPASSAGED, PASSAGE 7 "PARENTS" AND PL-2 CLONE OF DEN-1 VIRUSES

MONKEY NO.	INOCULUM Strain	INOCULUM (PFU)	VIREMIA Duration[a] (days)	VIREMIA Peak (PFU/ml)	RECIPROCAL PRN$_{80}$ TITER ON DAY 30
T-329	DEN-1 #1	1.9×10^3	5	545	20
T-340	Orig. human serum		4	190	>80
C-64	DEN-1 #1	5.0×10^5	5	125	20
B-7461	P-7 "parent"		5	260	>80
C-580	DEN-1 #1	3.8×10^5	2(?)**	25-55(?)[b]	40
195A	PL-2 clone		0	0	40
T-301	DEN-1 #2	1.3×10^5	6	730	>80
T-347	Orig. human serum		4	70	>80
T-34	DEN-1 #2	7.5×10^5	5	1585	>80
505	P-7 "parent"		3	80	40
5997	DEN-1 #3	7.0×10^3	6	1050	40
T-268	Orig. human serum		0	0	0
T-348	DEN-#3	2.3×10^4	6	45	20
C-68	P-7 "parent"		5	680	>80

[a] As determined by the direct plaque technique on LLC-MK$_2$ cells.

[b] Two to 7 questionable "plaques" on days 7 and 8.

Effect of alternate passage in CEC and DBS-103 cultures on virulence of DEN-1 virus. For a period of about 5 months we investigated the alternate passage of DEN-1 viruses in CEC and DBS-103 cultures in an effort to attenuate these viruses by adapting them to cells from an unnatural host (chickens). A similar approach was used with some success in the Machupo vaccine program (2). The sequence of passages used was: CEC, DBS-103, CEC, DBS-103, CEC, CEC, CEC. The first passage from CEC to DBS-103 was made with undiluted culture fluid from freeze-thawed cultures that had been infected with either DEN-1 #1 P-7 "parent" or the DEN-1 #1 PL-2 vaccine candidate; a control culture passage was also made. All subseauent passages from CEC to DBS-103 were made by adding a trypsinized suspension of the infected CEC cultures to DBS-103 cultures. All passages from DBS-103 to CEC cultures were made with culture fluids from the infected DBS-103 cultures.

During the course of these passages the following observations were made: (a) during the first passage in CEC culture, after only 2 days incubation, CPE was 2+ to 4+; (b) small (0.5 mm) faint plaques appeared on subsequent titration in SW-13 cultures that were uncharacteristic of DEN virus; (c) with passage, slightly higher yields of virus were obtained from CEC than from DBS-103 cultures; and (d) plaquing the virus on LLC-MK_2 cells thus obtained required an additional 3 - 6 days of incubation after staining to permit visualization of plaques, rather than the usual overnight incubation seen with DEN.

From the beginning the plaque-forming agent was presumed to be virus and therefore several possible sources of the virus were suggested including: (a) DEN viruses with altered characteristics (highly unlikely); (b) avian leukosis viruses originating in the CEC culuture; (c) unknown viruses present in the DBS-103-grown DEN-1 virus preparations, heretofore not expressed in standard plaque assay and growth systems; (d) bovine viruses from fetal calf serum used to prepare CEC and DBS-103 cultures; and (e) contamination of media or culture during the initial passage of virus in CEC culture in the Biological Development Suite. In view of the possible importance of these observations to other projects at USAMRIID, Salk and WRAIR, should the source of the presumed contaminant prove to be fetal calf serum, CEC cultures or DBS-103 cultures, the major portion of our work during the fall involved studies designed to determine the nature and source of the presumed contaminant.

To determine whether the "unknown" agent was indeed a modified DEN virus, both direct and indirect FA (DFA, IFA) and PRN_{80} were run with known positive DEN-1 to -4 monkey sera and/or mouse ascitic fluids. The FA tests were conducted by Mr. Bagley using coded specimens prepared as follows: Leighton tube cultures of LLC-MK_2 cells were inoculated with fluids from either the 1st CEC passage of the viruses shown in Table III, or the 2nd passage (i.e., CEC_1, 103_1 CEC_2, 103_2). As controls, additional Leighton tube cultures were inoculated with the standard seeds of parent viruses of DEN-1 (#1, #2 or #3), the PL-2 candidate vaccine strain or the WRAIR DEN-2 attenuated vaccine; additional sham-inoculated cultures were included as negative controls.

As shown in Table III both the IFA and DFA tests documented the presence of DEN-1 virus in all of the standard seed preparations of DEN-1; DEN-2 virus was also demonstrable by IFA in the standard DEN-2 vaccine-inoculated Leighton tube cultures. Significantly, no DEN-1 or DEN-2 virus could be demonstrated by IFA in any specimens that had been passaged in CEC (CEC, pass) or CEC and DBS-103 (103_2 pass) cultures. These results suggested the presence of another agent either alone or in such a concentration as to mask the growth of DEN viruses.

To further test for the presence of DEN virus in the PL-2 and DEN-2 vaccine specimens used for the FA studies, PRN_{80} tests were conducted with DEN-1 #1 and DEN-2 positive monkey sera. The former monkey had been inoculated with the original human serum specimen from which DEN-1 #1 was obtained, while the latter had received a standard laboratory strain of DEN-2 virus. Preinoculation sera served as controls. Testing was limited

to the PL-2 strain and the DEN-2 vaccine due to: (a) our particular interest in PL-2; (b) our need for a control virus that had been prepared outside of the BD suite; and (c) the finite availability of cells for these tests.

TABLE III. RESULTS OF IFA AND DFA TESTS ON CEC- OR DBS-103-GROWN DEN VIRUSES

VIRUS SOURCE	VIRUS	RESULTS[a] IFA Against: DEN-1	DEN-2	DEN-3	DEN-4	DFA Against: DEN-1
CEC, Pass	DEN-1 #1	-	-	-	-	
	#2	-	-	-	-	
	#3	-	-	-	-	
	PL2	-	-	-	-	
	DEN-2 vacc.	a	-/-[b]	-	-	
	Neg. Cont.	-	-/-[b]	-	-	
103_2 Pass	DEN-1 #1	-	-	-	-	
	#2	-	-	-	-	
	#3	-	-	-	-	
	PL2	-	-	-	-	
	DEN-2 vacc.		-/-[b]	-	-	
	Neg. Cont.	-	-	-	-	
Standard seed	DEN-1 #1	3+				3+
	#2	4+				3+
	#3	3+				4+
	PL2	4+				3+
	DEN-2 vacc.		4+			4+
	Neg. cont.	-				-

[a]Blank denotes not tested.

[b]Results of repeat tests.

The results of these tests can be simply stated; neither the PL-2 nor the DEN-2 vaccine viruses could be neutralized by DEN-1 and DEN-2 monkey antisera, respectively after the viruses had been passed one (CEC_1) or more (103_2) times in CEC and DBS-103 cultures. The plaques that were observed, threfore, must be considered to be due to the presence of another "plaquing" agent in the passaged material. A confirmatory PRN_{80} test using DEN-1 immune mouse ascitic fluid against the CEC_1 passage fluid of PL-2 gave identical results.

Since three of the common denominators of the emergence of the "unknown agent" were CEC cutlures, the fetal calf serum used to produce them, and possible contaminants in the BD suite, additional attempts were made to elicit this "unknown agent" with a single passage of standard seed viruses in CEC cultures. Such passage was carried out 3 more times yielding erratic results

with regard to the rapid CPE seen previously in CEC. For example, on the final attempt even 2 of 3 or the DEN-1 isolates previously grown only in primary green monkey kidney (PGMK) culture produced 4+ CPE within 2 days, whereas DEN-1 #2 and #3, the PL-2 vaccine candidate, and the DEN-2 vaccine produced little or no CPE even after incubation for 5 days.

The inclusion of the PGMK-grown viruses with positive results and the general nonreproducibility seen in the system seemingly reduced the common denominators to fetal calf serum or CEC cultures. Ultimately, the discovery of a long-term and apparently widespread Mycoplasma contamination problem in the Tissue Culture Section and its obvious impact on these studies led to a complete halt in the alternate passage investigations. In view of more pressing problems and interesting results in the DEN-1 program, Chief, Virology Division verbally countermanded the earlier decision to go all out on the CEC/103 study. No further work in this area was conducted, nor is anticipated.

Further purification of DEN-1 #1 PL-2 vaccine candidate by terminal dilution passage in DBS-103 cultures. In view of the encouraging monkey viremia data (see Table I) and the general interest in and partial acceptance of the PL-2 vaccine candidate (1, and COL Russell, personal communication), it was quite obvious that this strain was the most promising with regard to its lack of virulence markers, such as induction of high monkey viremia and ability to replicate at high temperatures (i.e., 39-40°C). In addition, the PL-2 strain has been a consistent small-plaque former in a variety of cell systems including LLC-MK_2, BS-C-1, and SW-13 as compared to parent (DEN-1 #1, pass DBS-103).

Since the promised strains of new DEN-1 viruses from the Caribbean had not yet been received it was decided to immediately proceed with further purification of the PL-2 strain. For this we used the terminal dilution passage technique that had worked well for Dr. Halstead in the DEN-4 virus vaccine program.

Starting with the standard PL-2 preparation which titers $\sim 5 \times 10^6$ PFU/ml, a 10^{-5} dilution was prepared; from this a further 1:40 dilution was made to permit inoculation of each of 36 T-75 flasks of DBS-103 cultures with about 1 PFU. Groups of 18 flasks were placed at either 35 or 39.3°C with a fluid maintenance medium (MM) for 7 days. CPE was recorded on day 7 and samples were taken from CPE + flasks for titration, plaque sizing and for use in subsequent passage in DBS-103 culture. As shown in Table IV only 4 of 18 flasks incubated at 35°C showed CPE. As expected only these 4 exhibited plaquable virus when subsequently titrated in LLC-MK_2 cultures. A sample from flask #17 was used for the next terminal dilution passage at 1/2 $\log_{10}$ dilutions from $10^{-2.5}$ to $10^{-6.5}$. By day 7 the $10^{-2.5}$ to $10^{-6.0}$ flasks incubated at 35°C showed 1 to 3+ CPE and yielded virus at titers of 1×10^5 to 7×10^5 PFU/ml; only 1 of 2 flasks at the $10^{-6.5}$ dilution yielded virus at 35°C and was therefore used as the starting material for the next terminal dilution series. It should be noted that virus neither grew at 39.3°C nor produced CPE within the 7-day incubation period employed for growth. When the flasks were fed and incubated for a total period of 11 days at 39.3°C, virus replicated which would normally plaque at 35°C, but not at the higher temperature. (This is referred to as "leakiness;" these progeny could not be plaqued at that temperature). Similar observations have been made both

at WRAIR and the University of Hawaii (personal communication, Dr. Brandt and Dr. Halstead). It should be noted that virus of this type is present in the AIDRB-approved DEN-2 attenuated vaccine and is considered acceptable.

TABLE IV. REPLICATION OF PL-2 STRAIN IN DBS-103 AT 35 AND 39.3°C AT 1ST LOW INPUT PASSAGE

	INCUBATION AT:					
	35°C			39.3°C		
FLASK NO.	CPE	PFU/ml[a]	Plaque size (mm)	CPE	PFU/ml[a]	Plaque size (mm)
1- 3	-	-	-	-	-	-
4	+	1.7×10^4	1.0	-	-	-
5-11	-	-	-	-	-	-
12	±	8.0×10^1	1.0	-	-	-
13-14	-	-	-	-	-	-
15	+	2.0×10^4	1.0	-	-	-
16	-	-	-	-	-	-
17	+[b]	7.0×10^4	1.0	-	-	-
18	+	7.0×10^4	1.0	-	-	-
4 controls	-	-	-	-	-	-

[a]Assayed at 35C in LLC-MK_2.
[b]Used for 2nd passage in DBS-103 cultures.

The terminal passage (TP) series was carried out through TP-8, at each passage taking samples of virus from culutres that had received the highest dilution of virus to be then diluted and used to inoculate a fresh set of cultures. Although work was halted for 3 weeks in February to permit installation of new HEPA filters and was then stopped completely to permit work on CHIK virus in March (see below) some data were accumulated to strongly suggest that use of the TP method had resulted in an excellent candidate vaccine clone.

Data were obtained at each TP stage with regard to plaque size and temperature sensitivity. We will present some representative results comparing the parent virus (DEN-1 #1), PL-2 candidate strain, and TP-5, -6, and -7. Results of direct plaque assays on LLC-MK_2 cultures of various TP samples and plaque sizes when viruses were plaqued at 35 and 39.3°C are shown in Table V.

These results show that TP-5, -6 and -7 viruses are ts in terms of direct plaquing and that they are consistently of the small plaque type (1-2 mm) on LLC-MK_2 cells.

The inclusion of DEN-2 parent and vaccine viruses from WRAIR was fortuitous in that it revealed much lower titers for parent virus than had been observed previously both at 35 and 39.3°C. Discussions of these results with WRAIR personnel brought to light similar problems they were having with these standard assay cells since Nov-Dec 1978. New cultures were then put into use here and at WRAIR, which seemed to increase the accuracy of subsequent ts studies at both Institutes.

TABLE V. DIRECT PLAQUE ASSAY IN LLC-MK_2 AT 35 and 39.3°C OF VIRUS SAMPLES OF TERMINAL PASSAGES OF PL-2

VIRUS	PFU/ml (SIZE,mm) AT: 35°C	39.3°C
DEN-1 #1 (Parent)	4.4×10^5 (1-2) (3-4)	3.4×10^2 (1.0)
DEN-1 #1 PL-2	1.2×10^6 (1-2)	1.8×10^2 (1.0)
TP-5	3.3×10^4 (1.0)	-[a]
TP-6	4.0×10^6 (1.0)	-
TP-7	3.6×10^4 (1.0)	-
DEN-2[b] (Parent)	2.3×10^4 (1-2) (3-4)	1.5×10^2 (1.0)
DEN-2[b] (Vaccine)	5.1×10^3 (1-2)	-

[a] No plaques with undiluted inoculum or 10^{-1}.

[b] WRAIR's DEN-2 parent and human vaccine strains included as controls.

Ultimately the TP-8 virus (designated TP-8-PL-2) was selected for more extensive testing as a vaccine candidate strain. Tests on TP-8-PL-2 for specific "markers" either in progress or completed during FY79 included the following:

(a) Growth curves were conducted by inoculation of LLC-MK_2 cultures with either parent virus (DEN-1 #1 P-7) or TP-8-PL-2 followed by incubation at 35, 37, 38, 39.3 and 40°C. Samples of culture fluids were taken on days 1-7 postinoculation and held at -70°C until titrated in LLC-MK_2 at both 35 and 39.3°C. Results of these assays showed a difference between parent and TP-8-PL-2. When samples from each were assayed at 35°C, TP-8-PL-2, as compared to parent, showed decreased yield overall from flasks incubated at 38°C and yielded no detectable virus in the samples from the 39.3°C assays of these same culture fluids. With the parent, plaquable virus was seen at 39.3°C in all samples except those from flasks orginally incubated at 40°C. In contrast, TP-8-PL-2 produced no virus at any temperature which would subsequently plaque at 39.3°C.

(b) In vitro reversion studies were conducted to determine whether TP-8-PL-2, unlike DEN-2 vaccine virus (Dr. Eckles, WRAIR) and DEN-4 vaccine virus (Dr. Halstead, Hawaii), is stable even at high MOI through a series of 3 serial passes in DBS-103 cultures. We used DBS-103 cultures with liquid medium and incubated at 35°C for 6 days at each of 3 virus inputs (undilute, 10^{-2} and 10^{-4}). Samples were taken from flasks on day 5 to inoculate fresh cultures using the same virus input at each passage. The remaining sample was used to titrate virus at both 35 and 39.3°C to establish plaque size and ts. Table VI summarizes these results. Clearly no in vitro reversion is seen in terms of either marker.

TABLE VI. SERIAL PASSAGE OF TP-8-PL-2 IN DBS-103.

PASSAGE LEVEL	VIRUS INPUT	PFU/ml ON LLC-MK_2 35°C	PFU/ml ON LLC-MK_2 39.3°C	SIZE AT 35°C (mm)
1	Undil	6.8×10^3	0	1-2
	10^{-2}	2.3×10^3	0	1-2
	10^{-4}	1.0×10^3	0	1-2
	Undil	4.8×10^3	0	1-2
	10^{-2}	1.0×10^4	0	1-2
	10^{-4}	9.8×10^3	0	1-2
2	Undil	1.2×10^4	0	1-2
	10^{-2}	3.1×10^4	0	1-2
	10^{-4}	6.3×10^3	0	1-2
DEN-1 parent	Conrol titration	8.3×10^5	7.3×10^3	1-4
TP-8-PL-2	Control titration	3.5×10^6	0	1-2

(c) A serum neutralization breakthrough test was also conducted to demonstrate not only the DEN-1 identity of the TP-8-PL-2 seed virus but also to establish that no other viruses were present in the preparation. In the first of several tests, NIH-certified DEN-1 mouse AF (MAF) gave a PRN_{80} titer of 1:80 against 223 PFU of TP-8-PL-2. All observed plaques were typical of the vaccine candidate strain. Since the 1:10 dilution of MAF neutralized all the virus in a 10^{-3} dilution of TP-8-PL-2, the test will be repeated using higher concentrations (lower dilutions) of virus and undiluted or a 1:10 dilution of MAF. Any plaques that "breakthrough" will be amplified in cell culture and retested with the NIH, DEN-1 MAF to complete the identification.

(d) A test of the relative ability of parent and TP-8-PL-2 to grow in suckling mouse brain (SMB) is in progress. Although neither virus kills mice regularly on 1st passage, it is possible that a difference may be seen in levels of virus produced in SMB by day 9 or 10. At the time of writing the mouse brains have been removed, titrated and are on plaque assay in LLC-MK_2 culture. Since the brain suspensions are being titrated at both 35 and 39.3°C, data on reversion with regard to plaque size and ts will also be obtained.

(e) At the time of writing a monkey viremia study is in progress; 3 monkeys each were inoculated with parent virus or TP-8-PL-2. Since undiluted virus was used, the TP-8-PL-2 monkeys received about 10-fold more virus (10^6 PFU) than did the monkeys that received parent virus. This is a severe test, but weighted towards detection of any viremia-producing virus in the TP-8-PL-2 preparation. During the course of the study 1 monkey in each inoculation group developed whole body rash, which may negate any results obtained by plaque assay of monkey serum specimens taken on days 1-14. Because DEN virus does not cause rash in monkeys, pre- and postinoculation sera are now being tested for measles antibody by Dr. London, NIH. This apparently is a common cause of such rashes in monkeys. Moreover, intercurrent infection with measles virus would most probably result in adverse reactions to the DEN-1 viruses (Dr. London, NIAID, NIH, personal communication).

In vitro studies with ribavirin. Although not an integral part of the DEN-1 vaccine program, studies were initiated at the request of COL Russell, WRAIR, to determine the effect of ribavirin on all 4 DEN serotypes as an antecedent to in vivo studies. It was also possible that differences in sensitivity to ribavirin might have served as another "marker" of virulence/avirulence for DEN strains.

In this test SW-13 cell cultures were used with liquid medium to determine the ability of selected concentrations of drug to restrict growth of various DEN virus strains. Replicate cultures were inoculated with 10^5 to 10^6 PFU of a given virus and then maintained with standard maintenance medium or medium containing 25 to 100 µg of ribavirin/ml. Samples were taken on days 3-7 and plaqued on either BSC-1 or LLC-MK_2 cultures. The data shown in Table VII are for day 4 only, since anomalous, erratic results were obtained after day 4.

TABLE VII. EFFECT OF RIBAVIRIN ON GRWOTH OF DENGUE VIRUSS IN SW-13 CELL CULTURE FOR DAY 4

	LOG_{10} PFU/ml YIELD BY:			
	CONCENTRATION OF RIBAVIRIN IN MEDIUM (µg/ml)			
VIRUS	0	25	50	100
DEN-1:				
Isol. 1[a]	7.5	6.4	4.6	3.0
Isol. 2[a]	6.0	5.3	4.2	3.2
Isol. 3[a]	6.6	5.1	4.7	3.2
P1-2 (Isol. 1)	7.9	7.2	6.1	3.9
Carec-A[a]	6.7	5.2	3.9	0
DEN-2:				
N-C[a]	6.6	5.4	3.9	0
WR Parent (PR-159)[a]	5.2	4.7	4.3	0
WR Vaccine	3.4	3.7	2.3	0
DEN-3:				
H-87[a]	3.6	2.6	2.9	0
WR Parent[a]	3.6	3.8	3.5	2.9
WR Vaccine[b]	3.3	0	0	0
DEN-4:				
Hal-4328 S[a]	7.7	7.4	7.1	6.9

[a]Considered to be virulent strains; others less virulent or avirulent.
[b]Day-6 yield.

No demonstrable difference between strains regarded as "virulent" and those believed to be "attenuated" was found. Although ribavirin was effective in retarding the growth of each of the virus strains tested, it was found to be useless as a marker for determining their "virulence" or "attenuation."

Heparin (polyanion) sensitivity of DEN viruses. Sensitivity to polyanions, such as heparin, has been used to differentiate virulent and avirulent strains of other group B arboviruses such as JE virus (LTC French, personal communication). To determine if this might be of value as a "marker" of attenuation for DEN-1 viruses, studies were initiated with the 3 "parent" DEN-1 virus and the PL-2 vaccine candidate. At the request of COL Russell, WRAIR, other DEN serotypes including vaccine strains were also evaluated.

In this test, virus is diluted to contain 100 to 200 PFU. After filtration through a 0.2-μ Millipore pad (to remove aggregates t at confuse results), the virus preparations were added to equal volumes of hep rin diluted in antibotic and protein-free medium. Three-fold dilutions were employed to yield final heparin concentrations in the test of 1.4 - 3000 μg/ml. The virus-heparin mixture was then directly plaqued on BS-C-1 or LLC-MK_2 cultures. Endpoint titers of the heparin effect were expressed as the lowest concentration of heparin that resulted in a 50% plaque reduction (PR_{50}) compared to controls. As seen in Table VIII the degree of sensitivity for "attenuated" viruses is considerable, whereas the "virulent" viruses may be either resistant or totally sensitive. The higher values presumably indicate "virulence" and the lower values "attenuation." (LTC French, personal communication). These results demonstrate that strains assumed to be "virulent" do not consistently correlate with regard to the sensitivity marker, i.e., resistance to > 1000 g/ml = "virulence," < 100 g/ml = "attenuation," with resistance to 100 - 1000 μg/ml being considered "intermediate." Heparin sensitivity, therefore, apparently cannot be used as a marker for determining virulence of attenuation of DEN viruses.

TABLE VIII. HEPARIN (POLYANION) SENSITIVITY OF DENGUE VIRUSES.

VIRUS	CONCENTRATION (μg) OF HEPARIN PRODUCING A PR_{50} TITER
DEN-1:	
Isol. 1[a]	74
Isol. 2[a]	30
Isol. 3[a]	37
P1-2 (Isol. 1)[b]	25
Carec-A[a]	>3000
DEN-2:	
NG-C[a]	64
WR Parent[a]	>3000
WR Vaccine[b]	1.9
DEN-3:	
H-87[a]	3.8
WR Parent[a]	c
WR Vaccine[b]	c
DEN-4:	
Hal-4328 S[a]	3.0

[a]Said to be virulent.
[b]Believed to be attenuated.
[c]Titer too low for testing.

Part II. Attenuated CHIK Vaccine

In March 1979 studies were begun with strain #15561 CHIK virus that had been used at WRAIR in the late 1960s to prepare a killed vaccine for human use (3). For working seed we used WRAIR's 11th PGMK cell-grown virus after 1 additional passage in certified MRC-5 culture to prepare sufficient virus.

The general plan for selection of a suitable vaccine candidate clone was to make 3 or more plaque-to-plaque passages (PL-PL) in MRC-5 culture with no intermediate outgrowth. Based on experience with other alphaviruses our original criteria for selection of promising clones were: (a) small plaque size; (b) ts on direct plaquing; (c) little or no growth in liquid overlay culture at higher temperatures (e.g., 39-40°C); and (d) inability to induce viremia in rhesus monkeys.

Three series of PL-PL passages have been carried out thus far. It should be noted that the 15561 working seed titers were 2.4×10^8 PFU/ml at 35°C and 2.7×10^5 PFU/ml at 39.3°C on MRC-5 culture. This working seed showed 3-4 mm plaques at 35°C routinely..

In the first series ("A series") the 15561 working seed, at Dr. Jahrling's suggestion, was passed through an hydroxyapatite (HAp) column and virus from column fraction #8 - #15 were plaqued on MRC-5 cultures. This series evolved as follows with all plaquing at 35°C:

1st plaque pick - 6 plaques were selected for the 1st PL-PL passage.

2nd plaque pick - 10 plaques were selected for the 2nd PL-PL passage.

3rd plaque pick - 18 plaques were selected for the 3rd PL-PL passage.

4th plaque pick - 26 plaques were selected for outgrowth in MRC-5 cultures for subsequent sizing and ts determination on MRC-5 culture.

Only 9 of the 26 plaque outgrowths from the 4th plaque pick series were tested for ts by direct plaquing at 35 and 39.3°C. However, all 9 showed no ts marker, and although apparently pure, were all large (3-4 mm). This series was discontinued for obvious reasons.

In the second series ("B series") the 15561 working seed was used without prior passage through the HAp column for PL-PL purification. That procedure may have, in fact, selected for more virulent subpopulations, as has been Dr. Jahrling's experience with other alphaviruses. The "B series" evolved as follows:

1st plaque pick - of 12 plaques picked, 5 were selected for the 1st PL-PL passage.

2nd plaque pick - of 32 plaques picked, 8 were selected for the 2nd PL-PL passage.

3rd plaque pick - of 25 plaques picked, 11 were selected for the 3rd PL-PL passage.

4th plaque pick - 45 plaques were picked and several were selected for outgrowth on MRC-5. Through the course of selected ts and sizing studies, it was again found that all those tested were not ts on direct plaquing at 39.3°C and all were 3-4 mm in diameter. However, in addition to direct plaquing a few selected clones were grown at 35 and 39.3°C in liquid overlaid culture for yield studies at the 2 temperatures. One of the clones, 74B, when grown at 39.3°C produced virus that was small plaque (∿ 1.0 mm) when subsequently plaqued at 35°C.

For the third series ("H series") a total of 4 plaques were initially picked from 74B and amplified to 10^6 - 10^7 PFU/ml in MRC-5 culture. One of these, 74B (PL-2), was chosen for further purification in MRC-5 culture and for further evaluation. Throughout the entire passage series the 74B (PL-2) subpopulations were all 0.5 - 1.0 mm at 35 °C and in limited tests clearly were restricted at 38.5°C.

Studies are continuing with 4 of the selected clones (#177, 178, 181 and 194) to establish ts, mouse virulence, in vitro stability of ts and size markers, and monkey virulence. In all studies, we are using PGMK pass 2 15561 "parent" virus for comparison.

Presentations:

1. Cole, F. E,, Jr. Progress in Dengue I Vaccine Development. Presented at Viral Vaccine Development Committee, USAMRIID, Fort Detrick, MD, Mar 79.

2. Cole, F.E., Jr. Chikungunya Vaccine Studies. Presented at Viral Vaccine Development Committee, WRAIR, Washington, DC, Apr 79.

3. Cole, F. E., Jr. Status of Dengue I Vaccine. Presented at Viral Vaccine Development Committee, USAMRIID, Fort Detrick, MD, May 79.

Publications:

None.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION	3. DATE OF SUMMARY	REPORT CONTROL SYMBOL
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78 10 01	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

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	CURRENT 80	1.0	224.9

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Respiratory diseases; (U) Aerosols; (U) Particle size; (U) Immunoprophylaxis; (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) Investigate basic mechanisms of aerogenic immunization against respiratory infections. Most BW agents are transmissible in aerosols. Since this is the most feasible agent dissemination method for a large-scale covert BW operation, the need for establishing immune defenses in the respiratory tract make this work unit essential in a comprehensive BW defense program.

24 (U) Animals are immunized by respiratory or parenteral inoculation with inactivated or attenuated respiratory pathogens with and without adjuvants. Efficacy is determined by seroconversion, development of CMI responses, and protection against respiratory challenge.

25 (U) 78 10 - 79 09 - Modeling studies for respiratory melioidosis have shown that respiratory doses of 10,000 or more P. pseudomallei cause predictable disease and death in adult guinea pigs, with both the number dying and the mean time to death a function of dose. At lower doses the organism is highly variable in its effects. Guinea pigs vaccinated with killed P. pseudomallei developed significant but short-lived levels of humoral antibody. Lymphocyte transformation assays gave no evidence of increased blastogenesis in immunized guinea pigs. Vaccinated guinea pigs were afforded only minimal protection against aerosol challenge with virulent P. pseudomallei. The only criterion suggestive of vaccine-derived protection was an increase in the mean time to death of vaccinated guinea pigs. Initial studies were completed which demonstrated that aerogenic administration of tetracycline is advantageous in the treatment of lethal P. pseudomallei infections in hamsters.

* Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M16277A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 012: Studies in Immunization of the Respiratory Tract

Background:

Models for respiratory meliodosis in hamsters and mice described in previous reports (1) are satisfactory in terms of producing a rapidly fatal disease after exposure to low aerosol doses of Pseudomonis pseudomallei. Infection by the respiratory route has been shown to cause predictable disease and death in hamsters in 2-3 days. A similar response occurs in mice infected with a mouse-adapted strain. The rapidly fatal course of the disease in these rodents limits their usefulness for studies of immunogenesis or the evaluation of therapeutic agents. Neither species has proven suitable for examining the role of lymphokines in host defense against P. pseudomallei infections.

We therefore have initiated studies to develop a model for this disease in guinea pigs. Guinea pigs are generally less susceptible to P. pseudomallei infection than hamsters, survive for longer periods, and have proven useful for measuring cell-mediated immune (CMI) responses to other diseases (2). We have initiated immunogenesis studies in the guinea pig, and have extended earlier studies concerning the immunoprotective efficacies of both passive and active immunizations against respiratory P. pseudomallei infection in the hamsters.

Also, initial studies were completed which demonstrated that aerogenic administration of tetracycline is potentially advantageous in the treatment of lethal P. pseudomallei infections in hamsters.

Progress:

Development of a respiratory melioidosis model in guinea pigs. Outbred Hartley strain guinea pigs were given graded doses of P. pseudomallei, strain 23343, ranging from $10^{2.5}$-$10^{4.8}$ colony forming units (CFU) administered as either small particle aerosols (SPA) or by IP inoculation. Only 63% of the adult guinea pigs exposed to the highest aerosol dose died. The geometric mean time of death (MTD) was 7.7 days. Fifty percent of those exposed to $10^{4.2}$ CFU died with a MTD of 17.1 days. Lethal responses in guinea pigs that received a dose of $\leq$ 800 organisms were extremely variable. Mortalities from 0-30% of those infected occurred without discernible correlation between dose level and the number dying.

Nevertheless, microbial assays of tissues obtained from guinea pigs infected by the aerosol route with $10^{2.5}$ CFU indicated that the organisms replicated to 10^{6} CFU/lung within 4 days. Thereafter, concentrations diminished to $10^{3.7}$ CFU/lung at 14 days; no viable P. pseudomallei were detected on day 21. Viable P. pseudomallei were detected only sporadically in liver and spleen samples. All challenged guinea pigs had developed fever by 3 days; temperatures remained 1-2° above normal through 13 days. In spite of the unpredictable lethal response

in guinea pigs infected with low doses, the longer survival times suggested that these rodents may be more useful than hamsters for immunogenesis and drug therapy studies. Perhaps the lethal response of juvenile guinea pigs to low doses will be more uniform than those observed in adult animals. Subsequent experiments will examine the lethal response as a function of guinea pig age.

Immune response of hamsters and guinea pigs. Previously reported data demonstrated that hamsters vaccinated with 10^9 formalin-killed P. pseudomallei cells developed significant antibody titers, and 33-38% of the vaccinated hamsters were protected against lethal respiratory challenge. Four inoculations given at weekly intervals enhanced the antibody titers of recipient hamsters 1.6-fold over that of a single vaccination, and 70% survived a challenge that killed all nonvaccinated hamsters. We extended these experiments to determine if significant immunization could be accomplished with vaccine doses lower than 10^9 killed cells. Groups of hamsters were given 10^6, 10^7 or 10^9 killed cells as a divided dose administered by the IN and IP routes. A similar number of hamsters received the same volume of sterile saline. Two weeks after vaccination, the hamsters were tested for antibody using indirect fluorescence (IFA), then challenged with a lethal dose of P. pseudomallei administered as SPA. Data obtained are summarized in Table I. Both the antibody titers and the degree of protection observed following vaccination with 10^9 cells were consistent with earlier experience. Only small amounts of specific antibody could be detected in hamsters inoculated with 10^6 or 10^7 cells, but these lower doses provided some protection as evidence by 25-33% survival of vaccinated hamsters following a virulent challenge that killed all nonvaccinated hamsters.

TABLE I. EFFECT OF VACCINE DOSE ON RESISTANCE OF HAMSTERS (n-12) TO LETHAL AEROSOL CHALLENGE WITH P. PSEUDOMALLEI

VACCINE DOSE (No. killed cells)	GEOMETRIC MEAN IFA TITER AT CHALLENGE (n=4)	MEAN LUNG LESION SCORE (moribund hamsters)	% SURVIVAL
0	0	3+	0
10^6	> 5	3+	25
10^7	5	3+	33
10^9	83	3+	33

Experiments were conducted to determine if passively acquired antibody would protect hamsters against lethal P. pseudomallei infections administered by the respiratory route. Undiluted anti-pseudomallei hamster serum with an IFA titer of 1:405 was administered as a divided inoculum, 0.1 ml IN and 0.4 ml IP to groups of hamsters. Antiserum was administered either 1 hr prior to challenge, or 1 hr prior to and against 16 hr after challenge with 10 LD_{50} of virulent P. pseudomallei.

Serum antibody titers measured 1 hr after the antiserum was administered averaged only 1:5, and all of the challenged hamsters died with 3-4 days. The extremely low antibody titers in recipient hamsters were unexpected. Based on simple dilution of the injected serum within the hamster's circulation we would

have expected titers of at least 1:30. Apparently direct IV administration of antiserum will be required to establish effective levels of passively acquired antibody.

Outbred, Hartley strain guinea pigs were given two, 0.1-ml injections of killed antigen, 7 days apart by the IP route. At selected times after inoculation, samples of whole blood were collected and assayed for lymphocyte transformation (LT), and serum samples were assayed for specific IFA antibodies.

The mean IFA titer (n-4) measured after 7 days was 1:45 and reached 1:90 at 14 days; by 40 days the average IFA titer was only 1:15. At this point the guinea pigs were reinoculated with a 1-ml booster dose (7×10^9 killed cells). This elicited an anamnestic response as evidenced by a rapid increase in mean antibody titer from 1:15 to 1:405 within 2 weeks. The high titer was short-lived, however, and diminished to 1:60 within 50 days after the booster dose.

In a follow-up experiment guinea pigs were vaccinated with 4 doses, each containing 10^{10} cells, given IP at 4-day intervals. Despite repeated vaccinations, IFA titers obtained were lower than expected, and effective immunization was not achieved. The mean IFA titer (n=4) measured 7 days after the final vaccination was 1:405 but decreased to 1:78 by 14 days. By 56 days the mean titer decreased to 1:34. LT assays were performed on the same schedule as were IFA assays, but no evidence of increased blastogenesis in immunized guinea pigs was detected. Although the T-lymphocyte mitogen, phytohemagglutinin, stimulated transformation indices ranging from 7 to 15 for cells from both vaccinated and nonvaccinated guinea pigs, indices obtained using the killed pseudomallei antigen were < 2 at all sampling periods. These findings were not unexpected, since it is known that killed vaccines are generally poor stimulators of CMI responses. Vaccinated guinea pigs were afforded only minimal protection against aerosol challenge with virulent P. pseudomallei; 61% of vaccinated guinea pigs succumbed to a challenge dose that killed 65% of the nonvaccinated guinea pigs. The only criterion suggestive of vaccine-derived protection was survival time. Vaccinated guinea pigs lived longer (average time to death of 12.4 days) than nonvaccinated guinea pigs (5.8 days). Examination of the lung, liver, and spleen from both vaccinated and control guinea pigs that survived challenge revealed no grossly visible lesions even though viable P. pseudomallei were isolated from these tissues at 28 days.

Aerosol therapy of respiratory medioidosis in hamsters. Tetracycline is among the drugs most active against P. pseudomallei in vitro (minimum inhibitory concentration 0.8 µg/ml) (3), but when administered parenterally to mice its therapeutic value is marginal. Since following a respiratory challenge with this organisms the initial site of replication is in the lungs, we are examining the feasibility of enhancing the in vivo activity of tetracycline by depositing aerosols of the drug directly in the respiratory tract. In the initial experiment, 15 hamsters infected by the aerosol route with an LD_{90} of P. pseudomallei were treated twice daily with 10 ml tetracycline/kg of body weight by either the aerosol or IM route. Therapy was started 15 hr after infection and continued for 2 days; 33% of aerosol-treated hamsters survived compared to 13% for IM treatment and 7% for untreated hamsters. These differences were not significant at $P \leq 0.05$, but the data are encouraging. The 15 hr delayed therapy and short duration of treatment constituted a rigid test for the efficacy of tetracycline.

Experiments by Dr. Berendt have indicated that tetracycline administered as an aerosol is rapidly cleared from the lungs and blood of recipient hamsters. Perhaps earlier initiation of therapy, with more frequent doses administered over a longer treatment period, may be required to cure an established infection.

Publications:

1. Scott, G. H., G. T. Burger, and R. A. Kishimoto. 1978. Experimental Coxiella burnetii infection of guinea pigs and mice. Lab. Anim. Sci. 28:673-675.

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1. U. S. Army Medical Research Institute of Infectious Diseases. 1 July 1978. Annual Progress Report FY 1978, pp. 119-125, Fort Detrick, MD.

2. Kishimoto, R. A., and G. T. Burger. 1977. Appearance of cellular and humoral immunity in guinea pigs after infection with Coxiella burnetii administered in small-particle aerosols. Infect. Immun. 6:518-521.

3. Hezebicks, M. M., and C. Nigg. 1958. Chemotherapy of experimental melioidosis in mice. Antibiotics Chemother. 8:543-560.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0E6416	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	013
b. CONTRIBUTING				
c. [redacted]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Enhancement of inactivated viral vaccines 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
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	79	1.0	174.9
FISCAL YEAR — CURRENT	80	1.0	251.4

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Viruses; (U) Immune enhancement; (U) Adjuvant; (U) Vaccine; (U) Mice; (U) Monkeys

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

23 (U) Numerous inactivated vaccines have been developed to control infectious diseases; these vaccines often have marginal antigenic potency and cannot be used to stop a disease outbreak or in a BW situation. Immunological adjuvants plus inactivated vaccines frequently evoke more rapid and prolonged development of protective immunity. This work will develop new methods to enhance immunogenicity of presently available, marginally antigenic, inactivated viral vaccines for military personnel.

24 (U) Laboratory rodents and monkeys are inoculated with potential immunologic adjuvants combined with inactivated virus vaccines. Various immunologic responses and resistance against homologous virus challenge are determined to assess adjuvant efficacy and relative toxicity.

25 (U) 78 10 - 79 09 - Lipid emulsion (LE) adjuvant was shown to be highly effective in potentiating the immunologic response to the following inactivated vaccines: Rift Valley fever (RVF), Venezuelan equine encephalomyelitis (VEE), and Western equine encephalitis (WEE). A government patent is being sought for LE. Compound CP 20,961 was also an effective adjuvant when used with RVF and WEE. Pilot studies using liposomes as carriers of VEE vaccine were not encouraging. A guinea pig model was developed to measure both cell-mediated and humoral responses to vaccines and will be valuable in assessing relationships between various components of the immune response. The humoral immune system was shown to be the protective component of the total response to VEE antigen.

Publication: Infect. Immun. 24:160-166, 1979.

*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

* U.S. GPO: 1974—520-141/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 013: Enhancement of Inactivated Viral Vaccines of Military Importance

Background:

Prophylactic immunization using vaccines is one of the most effective means for control of infectious diseases. Inactivated vaccines, however, frequently produce only low to moderate levels of immunity for relatively brief duration. Adjuvants which stimulate immune responsiveness can potentiate antibody titers and extend the duration of immunity. Such agents can markedly expand the potential use of vaccines which exist in only limited supply and can stimulate a very rapid immunological response when immediate protection is needed. Lysine-stabilized poly(I)·poly(C) [polyICLC)] continues to be one of the most potent adjuvants examined to date; however, it is now being challenged for effectiveness by a lipid emulsion developed under this work unit. Another promising adjuvant is a muramyl dipeptide-like compound developed by Pfizer. Recent advances in liposome research make these artificial lipid membranes interesting potential adjuvants.

Progress:

Lipid emulsion. The new metabolizable lipid emulsion (LE) developed last year was evaluated further with selected viral antigens. We believe this new LE formulation offers several notable advantages over other water-in-oil adjuvants, namely: (A) the components are readily metabolizable or are normal host constituents; (B) it is easily emulsified with aqueous vaccine by gentle agitation; (C) dosages of the emulsion between a ratio of 1:1 to 1:4 vol/vol with vaccine appear to be highly effective; (D) the sequence of combining vaccine with LE does not appear to be critical for adjuvant effects; and (E) the suspension appears to be virtually nonreactogenic in recipient animals.

The encouraging results of initial studies with VEE and RVF led to studies in hamsters to assess the adjuvant activity of LE with inactivated WEE vaccine. The hamster-WEE virus model was chosen for study because the inactivated WEE vaccine currently used to immunize at-risk laboratory personnel is marginally antigenic and unlike mice, hamsters are highly susceptible to lethal peripheral (IP, SC) challenge with either WEE or EEE virus. The adjuvant effect of LE on humoral antibody response and resistance in WEE-vaccinated hamsters to homologous virus challenge are shown in Table I. Clearly, LE given in conjunction with marginal concentrations of WEE antigen significantly ($P < 0.001$) enhanced percentage survival in hamsters when compared to vaccine control animals. Survival rates in treatment groups given vaccine (diluted 1:5 or 1:10) were 100 and 94%, respectively, compared to 10 and 6% for vaccine control groups. Furthermore, detectable levels ($<1:4$) of antibody were present on day 14 postvaccination only in groups given vaccine plus LE. Results of these studies suggest that LE may be a potentially important adjuvant for use with inactivated alphavirus vaccines.

TABLE I. ADJUVANT EFFECTS OF LIPID EMULSION ON HUMORAL ANTIBODY RESPONSE AND SURVIVAL OF HAMSTERS CHALLENGED ON DAY 14 POSTVACCINATION WITH 1200 HIPLD$_{50}$ WEE (B-11) VIRUS

VACCINE[a]	LE (gm/mouse)	GM PRN$_{80}$[b] (range) Day 14 (n = 4)	SURVIVORS/ 16	% SURVIVORS Day 28
1:5	0.3	8 (4-32)	16	100[c]
	Saline	2 (<4)	3	19
1:10	0.3	4 (4-32)	15	94[c]
	Saline	2 (<4)	1	6
Saline	0.3	2 (<4)	0	0
Saline	None	2 (<4)	0	0

[a]WEE vaccine (0.3 ml of either 1:5 or 1:10 dilution) SC on day 0 alone or combined with 0.3 gm (~ 0.3 ml) of LE.
[b]Antibody titers (<1:4) assigned a value of 2 for computing GM titers.
[c]P = < 0.001 compared to vaccine control group.

Additional studies were designed to determine the optimum dosage of LE for use with prototype antigens and the sequence of combining the LE with vaccine to obtain maximum adherence or entrapment of antigen with lipid particles, for peak immunologic responses. In brief, the experimental design entailed combining VEE vaccine, undiluted or diluted 1:4, with graded dosages of LE. Inocula for half of the treatment groups of mice was prepared by first combining vaccine with graded doses of undiluted LE. The inocula were then thoroughly mixed and saline added to dilute the LE to the desired final concentration. Inocula for the other treatment groups was prepared in a different sequence. The LE was first diluted to the desired final concentration in saline and then combined with vaccines. Results of direct challenge studies of vaccinated mice (Table II) suggest that neither dosage of the new adjuvant, nor the manner in which it is combined with vaccine appears to be critical for attaining good in vivo adjuvant activity.

In studies performed in collaboration with CPT Reynolds, AA Division (BS03 00 025), and LTC Peters, Virology Division, (A91C 00 131), LE was shown to be highly effective in enhancing humoral antibody responses in nonhuman primates to the NDBR-103 RVF virus vaccine. Results of these studies were reported by CPT Reynolds. Since LE was highly effective as an adjuvant with RVF vaccine in monkeys, a study was conducted in sheep to assess further the adjuvant activity of LE for RVF vaccine in another species and determine if one injection of adjuvant-vaccine might provide effective immunization, by measuring the kinetics of RVF plaque reduction neutralizing (PRN) antibody production. Results are summarized as follows: (A) LE enhanced PRN antibody responses in sheep to RVF vaccine. (B) Animals given 1.0 ml undiluted vaccine plus LE had significantly ($P < 0.05$) higher PRN titers by day 7, compared to vaccine controls. Antibody titers in the undiluted vaccine-LE group remained significantly elevated over

controls for at least 7 months. (C) Antibody titers in sheep given 1.0 ml of a 1:5 dilution of RVF plus LE were only slightly higher than titers in vaccine controls. (D) Antibody responses in sheep given a very low (25 μg/kg) dose of previously untested lot of poly(ICLC) with undiluted vaccine or with vaccine diluted 1:5 were not different from controls.

TABLE II. EFFECTS OF DOSAGE AND SEQUENCE OF COMBINING LE WITH VEE VACCINE ON RESISTANCE OF MICE CHALLENGED ON DAY 14 POSTVACCINATION WITH 7×10^3 $MIPLD_{50}$ VEE (TRINIDAD) VIRUS

VACCINE (SC) (0.4 ml total vol)	RATIO Vaccine/LE	SURVIVORS/ 16	% SURVIVORS Day 28
[a]VEE (undil) + LE	1:1	12	75
[b]VEE (undil) + LE	1:1	15	94[c]
VEE (undil) + LE	2:1	15	94[c]
VEE (undil) + LE	2:1	16	100[c]
VEE (undil) + LE	4:1	15	94[c]
VEE (undil) + LE	4:1	15	94[c]
VEE (controls)		7	44[c]
[a]VEE (1:4) + LE	1:1	11	69[c]
[b]Vee (1:4) + LE	1:1	15	94[c]
VEE (1:4) + LE	2:1	13	81[c]
VEE (1:4) + LE	2:1	13	81[c]
VEE (1:4) + LE	4:1	12	75[c]
VEE (1:4) + LE	4:1	9	56[c]
VEE (1:4) controls		3	19
LE Controls		1	6
Saline controls		4	25

[a]VEE vaccine (0.2 ml of either undilute or 1:4 dilution) first combined with LE, then saline added to adjust final concentration of LE.
[b]LE first diluted in saline to desired concentration then 0.2 ml combined with an equal volume of VEE vaccine (undiluted or diluted 1:4).
[c]$P = < 0.05$ compared to vaccine control group.

To date, 16 of the vaccinated sheep with the lowest PRN antibody titers along with 8 nonvaccinated controls have been challenged with 5×10^5 or 1×10^6 PFU of Zagazig strain virus. Unchallenged contact controls were also included. Comprehensive statistical analyses of hematologic and serologic data are in progress. Results of direct challenge studies completed to date are summarized as follows: (A) All nonvaccinated sheep challenged with RVF were viremic for ≥ 1 day. (B) Several pregnant ewes with PRN titers $\leq$1:10 aborted following virus challenge. (C) Previous observations on direct transmission of RVF from infected

sheep to contact control animals were confirmed. (D) None of the vaccinated sheep with titers $\geq$ 1:20 at the time of challenge became viremic. (E) Lambs born to vaccinated ewes were shown to have substantial titers of maternal antibody to RVF virus.

Studies are planned to challenge the remaining 8 vaccinated animals approximately 12 mon postvaccination to gain additional data on the efficacy of RVF immunization in sheep.

CP 20,961. The new muramyl dipeptide-like compound synthesized by Pfizer (CP 20,961) was evaluated for potential use with RVF vaccine. The experimental design entailed inoculating groups of 20 mice each with vaccine combined with graded, low doses of adjuvants tested. Poly(ICLC), Freund's complete (FCA) and incomplete (FIA) adjuvant groups were inoculated for comparison. On day 14 postvaccination, 4 mice from each group were bled for RVF neutralizing antibody. The remaining 16 mice per group were challenged SC with 750 PFU of RVF (Zagazig) virus. Table III shows the results of direct challenge studies in mice.

TABLE III. EFFECTS OF VARIOUS ADJUVANTS ON SURVIVAL OF VACCINATED MICE CHALLENGED ON DAY 14 POSTIMMUNIZATION WITH 750 PFU OF RVF (ZAGAZIG) VIRUS

TREATMENT (SC)			DOSE/kg	% SURVIVORS
Vaccine[a]		Adjuvant	(µg/0.1 ml)	Day 35 (n = 16)
Vaccine	+	Poly(ICLC)	20	50
			100	50
			200	13
Saline	+	Poly(ICLC)	200	6
Vaccine	+	CP 20,961	10	25
			100	56
			1,000	19
Saline	+	CP 20,961	1,000	0
Vaccine	+	FCA		6
Saline	+	FCA		6
Vaccine	+	FIA		6
Saline	+	FIA		0
Vaccine Controls				19
Saline Controls				0

[a]RVF vaccine 0.1 ml (1:2 dilution) inoculated SC alone or combined with 0.1 ml of various adjuvants.

Poly(ICLC) given at 20 or 100 µg/kg body weight or CP 20,961 given at 100 µg/kg were quite effective as adjuvants in enhancing protection in mice against homologous virus challenge. It is interesting to note that both FCA and FIA provided no adjuvant activity when inoculated in the conventional 1:1 (vol/vol) ratio with vaccine. Results of these studies suggest that very low doses of poly(ICLC) or CP 20,961 may effectively enhance the immunogenicity of the new production lot of inactivated RVF vaccine.

CP 20,961 was also assessed in hamsters for its ability to potentiate inactivated WEE vaccine. Results of WEE serum neutralizing antibody and protection data for vaccinated hamsters challenged on day 14 with WEE (B-11) virus are shown in Table IV. Low titers of antibody were detected on day 14 in all treatment groups given vaccine with graded doses of CP 20,961. Protection was significantly ($P < 0.001$) enhanced in the high (1.0 mg/kg) adjuvant-vaccine group compared to vaccinated controls.

TABLE IV. ADJUVANT EFFECTS OF CP 20,961 ON ANTIBODY RESPONSE AND SURVIVAL OF VACCINATED HAMSTERS CHALLENGED ON DAY 14 POSTVACCINATION WITH 2300 $HIPLD_{50}$ WEE (B-11) VIRUS.

TREATMENT (SC)	DOSE OF CP 20,961 (mg/kg)	GM PRN_{80}[b] (range) Day 14 (n = 4)	% SURVIVORS Day 35 (n = 16)
Vaccine[a] + CP 20,961 + Intralipid	0.001	3.5 (<5-10)	31
	0.01	6.0 (<5-40)	38
	0.1	3.0 (<5-5)	25
	1.0	4.0 (<5-5)	88[c]
Vaccine + Intralipid		3.5 (<5-5)	31
Vaccine Control		2.5 (<5)	13
CP 20,961 + Intralipid		2.5 (<5)	0
Saline Control		2.5 (<5)	0

[a]WEE (0.3 ml of 1:5 dilution)
[b]Antibody titers (<1:5) assigned a value of 2.5 for computing GM titers.
[c]$P < 0.001$ compared to vaccine control group.

Liposomes. Pilot studies conducted in collaboration with LTC Alving, WRAIR, to assess the adjuvant effects of liposomal entrapment of inactivated VEE vaccine in charged liposomes (positive, negative, and negative plus lipid A) on antibody responses and protection in mice to homologous virus challenge were completed. Results of antibody response and protection data (Table V) in mice inoculated either IV or SC with serial 2-fold dilutions of the vaccine-liposome preparations were not highly encouraging. Antibody responses and percentage survival in treatment groups of mice given vaccine incorporated into positive liposomes were not different from responses in vaccine controls. In contrast, both antibody response and protection in treatment groups given vaccine incorporated into negative liposomes alone, or combined with lipid A were significantly lower than responses in vaccine controls. Similar responses in protection were seen in guinea pigs vaccinated with the positive and negatively charged liposomes (Table VI). Additional studies are required to determine the quantity of VEE antigen incorporated into the charged liposomes. Furthermore, factors such as electrostatic charge, concentration and purity of the antigen, etc., must be optimized to achieve the most effective entrapment of antigen within liposomes.

Botulinal toxoid. In conjunction with MAJ Lewis and COL Metzger, Pathology Division, (A841 00 020), pilot studies to assess the adjuvanticity of 5 compounds with pentavalent botulinal toxoid were completed. Because of problems encountered in obtaining a suitable challenge dose of type B bolutinum toxin, results of guinea pig protection studies were inconclusive. There are no current plans to repeat the adjuvant-screening studies with the botulinal toxoid.

Correlation of cellular and humoral immune responses. Studies were conducted in collaboration with LTC Ascher, Bacteriology Division and Dr. Jahrling, Virology Division, to develop assays for determining the relationship between cellular and humoral immune responses, and the protective efficacy of a prototype inactivated alphavirus vaccine, VEE. In brief, a VEE guinea pig model was developed to measure delayed-type hypersensitivity skin reactions, lymphocyte transformation, humoral antibody responses, and vaccine-induced resistance to homologous virus challenge. Previous studies by LTC Ascher and others have shown that pretreatment of guinea pigs with cyclophosphamide (CY) prior to vaccination, selectively suppress humoral immune responses, which in turn results in potentiation of DTH skin reactions to killed tularemia and Q fever antigens. We have recently shown potentiation of DTH reactivity to VEE virus antigen by CY pretreatment in guinea pigs.

The latter observation led to studies using CY-induced selective immunosuppression as an immunologic probe to determine the major protective component of the total (cellular and humoral) immune response of guinea pigs to an inactivated VEE virus vaccine (C-84).

The effects of CY pretreatment on day -3 on DTH skin reactions, LT, and humoral antibody responses of guinea pigs to inactivated C-84 VEE virus vaccine are shown in Tables VII-IX. Results of these studies indicate that CY pretreatment prior to vaccination did not decrease DTH skin responses to a subimmunogenic dose of killed VEE test antigen (Table VII); LT responses were positive [stimulation index (SI) > 2.0] only in CY-pretreated animals (Table VIII); and VEE virus humoral antibody responses measured by both radioimmunoassay and virus plaque reduction were abolished by CY-pretreatment (Table IX).

TABLE ". EFFECTS OF LIPOSOMAL ENTRAPMENT OF INACTIVATED VEE VIRUS VACCINE ON HUMORAL ANTIBODY RESPONSE AND RESISTANCE IN VACCINATED MICE CHALLENGED WITH 1.9×10^4 $MIPLD_{50}$ VEE (TRINIDAD) VIRUS.

TREATMENT (0.1 ml)		IV ROUTE			SURVIVAL	
		Survival		GM SN Antibody Titer		
Vaccine	Lipsosome	No./16	%	(range)	No./16	%
Undil.	Neg.	3	19	<8	0	0
1:2		3	19	<8	0	0
1:4		0	0	ND[a]	1	6
1:8		1	6	ND	1	6
Undil.	Pos.	12	75	23 (16-64)	3	19
1:2		7	44	11 (8-64)	3	19
1:4		10	63	ND	1	6
1:8		5	31	ND	0	0
Undil.	Neg. + Lipid A	2	13	8	0	0
1:2		3	19	8	0	0
1:4		1	6	ND	1	6
1:8		0	0	ND	3	19
Undil.	None	14	88	45 (16-128)	4	25
1:2		8	50	10 (<8-32)	1	6
None	Neg.	1	11	ND	0	0
	Pos.	0	0	ND	0	0
	Neg. + Lipid A	0	0	ND	0	0
Saline Controls		0	0	ND	0	0

[a]ND = Not done.

TABLE VI. EFFECTS OF LIPOSOMAL ENTRAPMENT OF INACTIVATED VEE VIRUS VACCINE ON RESISTANCE IN VACCINATED GUINEA PIGS CHALLENGED WITH 2 x 10^3 PFU OF VEE (TRINIDAD) VIRUS.

TREATMENT (0.1 ml) SC		SURVIVORS/	
Vaccine	Liposome	TOTAL	% SURVIVAL
Undil.	Neg.	0/7	0
	Pos.	7/7	100
	Neg. + Lipid A	0/7	0
Undil.	None	6/7	86
Saline controls		0/6	0

TABLE VII. EFFECT OF CY PRETREATMENT ON DELAYED-TYPE HYPERSENSITIVITY TO VEE VIRUS VACCINE IN GUINEA PIGS (6/GROUP)

		mm x 10^{-1} INCREASE IN SKIN THICKNESS ± SE (diameter)		
IMMUNIZATION	CY (mg/kg)	4 hr	24 hr	48 hr
None	0	0.67 ± 0.21 ($\overline{0}$)	0.0 ± 0.16 ($\overline{0}$)	0.0 ± 0.42 ($\overline{0}$)
VEE-FIA[a]	0	0.50 ± 0.43 ($\overline{0}$)	4.40 ± 0.61 ($\overline{8}$)	2.17 ± 0.54 ($\overline{5}$)
	250	1.08 ± 0.35 ($\overline{0}$)	4.17 ± 0.61 ($\overline{9}$)	2.17 ± 0.46 ($\overline{6}$)

[a]C-84 vaccine diluted 1:6 with saline and emulsified with an equal part of FIA.

TABLE VIII. EFFECT OF CY PRETREATMENT ON LYMPHOCYTE TRANSFORMATION RESPONSES OF GUINEA PIGS TO VEE VIRUS VACCINE

			CPM/well (SI)		
IMMUNIZATION	CY (mg/kg)	N	None	VEE[a]	PHA[b]
None	0	3	56	59 (1.07)	1675 (42.1)
VEE-FIA[c]	0	6	70	115 (1.78)	949 (15.7)
	250	5	63	198 (3.24)	1499 (24.9)

[a]Purified VEE viral antigen 1 µg/ml.
[b]PHA, 1:1000 final concentration.
[c]C-84 vaccine diluted 1:6 with saline and emulsified.

TABLE IX. EFFECT OF CY PRETREATMENT ON HUMORAL ANTIBODY RESPONSE OF GUINEA PIGS (5/GROUP) TO VEE VIRUS VACCINE

IMMUNIZATION	CY mg/kg	RIA_{50} (range)	PRN_{80} (range)
None	0	<80 (-)	<10 (-)
VEE-FIA[a]	0	246 (160-709)	17 (<10-80)
	250	<80 (-)	<10 (-)

[a]C-84 vaccine diluted 1:1 with saline and emulsified with an equal part FIA.

Results of direct challenge studies in guinea pigs and mice inoculated 3 days where CY treatment with graded doses of VEE vaccine and challenged 14 days later with a lethal dose of VEE (Trinidad) virus are shown in Tables X and XI.

Clearly, CY pretreatment decreases the protective efficacy of VEE vaccine in guinea pigs and mice. We have also shown in direct challenge studies that guinea pigs given VEE immune sera prior to virus challenge were afforded solid resistance to VEE virus challenge.

Results of these studies suggest that the humoral immune system is the protective component of the total immune response to VEE antigen, since a strong correlation was shown between the presence of specific humoral immune response and the protective efficacy of a killed VEE virus vaccine. The guinea pig model should provide an important tool for assessing the relationship between the various components of the immune response and the protective efficacy of candidate vaccine-adjuvant combinations.

TABLE X. EFFECTS OF CY PRETREATMENT ON SURVIVAL OF GUINEA PIGS CHALLENGED ON DAY 14 POSTIMMUNIZATION WITH 8,000 PFU VEE (TRINIDAD) VIRUS

IMMUNIZATION[a]	CY[b] (mg/kg)	GM PRN_{80}[c] Day 10 (n = 4)	SURVIVORS Total	% SURVIVORS Day 28
None	0	<1:4	0/8	0
VEE (1:5)	0	7	7/7	100
VEE (1:15)	0	5	6/7	86
VEE (1:50)	0	5	3/7	43
VEE (1:5)	250	<1:4	2/7	29
VEE (1:15)	250	<1:4	2/7	29
VEE (1:50)	250	<1:4	0/7	0

[a]VEE vaccine (0.4 ml of either 1:5, 1:15, or 1:50 dilution) SC on day 0.
[b]Cytoxan pretreatment IP on day -3.
[c]Antibody titers (<1:4) assigned a value of 2 for computing GM titers.

TABLE XI. EFFECTS OF CY PRETREATMENT ON SURVIVAL OF AKR/J MICE CHALLENGED ON DAY 14 POSTIMMUNIZATION WITH 6×10^4 PFU VEE (TRINIDAT) VIRUS

IMMUNIZATION[a]	CY (mg/kg)[b]	SURVIVORS Total	% SURVIVORS (Day 35)
NONE	0	0/13	0
VEE (1:2)	0	13/13	100
VEE (1:4)	0	8/11	73
VEE (1:8)	0	12/12	100
VEE (1:2)	250	2/9	22
VEE (1:4)	250	1/11	9
VEE (1:8)	250	0/13	0

[a]VEE vaccine (0.1 ml of either 1:2, 1:4- or 1:8 dilution) SC on day 0.
[b]Cytoxan pretreatment IP on day -3.

Publications:

Harrington, D. G., C. L. Crabbs, D. E. Hilmas, J. R. Brown, G. A. Higbee, F. E. Cole, Jr., and H. B. Levy. 1979. Adjuvant effects of low doses of a nuclease-resistant derivative of polyinosinic acid polycytidylic acid on antibody responses of monkeys to inactivated Venezuelan equine encephalomyelitis virus vaccine. Infect. Immun. 24:160-166.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6415	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	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	62776A	3M162776A841	00	016
b. CONTRIBUTING				
c. (////////)	STOG 79-7.2.2			

11. TITLE (Precede with Security Classification Code) (U) Interaction of viruses with peripheral host leukocytes as an index of immunity against infections of BW 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
76 08	79 04	DA	C. In-house

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR — PRECEDING 79	0.9	57.0
FISCAL YEAR — CURRENT 80	0	0

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ASSOCIATE INVESTIGATORS
NAME:
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21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Arboviruses; (U) Leukocytes; (U) Viral diseases; (U) Human status; (U) Phagocytosis

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

23 (U) Examine the interaction of human peripheral leukocytes with viruses of military importance with regard to the growth of the virus and its effect on the host cells. Determine the role of the immune state induced by military vaccines on virus-leukocyte interaction.

24 (U) Examine in detail the virus adsorption and growth characteristics of each type of peripheral blood leukocytes from both vaccinated and nonvaccinated individuals. Measure the effect of these cells on the infectivity of these viruses.

25 (U) 78 10 - 79 04 - Venezuelan equine encephalomyelitis (VEE) vaccine virus (TC-83) grew in donor cells of immune and nonimmune guinea pigs in the presence of human serum albumin. The results differed from those seen using human donor peripheral blood leukocytes. There is no explanation for this difference.

In summary, it appears that not all immune macrophages react identically when exposed to different strains of VEE virus. It is possible that the history of prior VEE virus exposure may determine how an individual's macrophages will react with VEE virus substrains.

The work unit is terminated because the investigator has been transferred to work on vaccines of higher priority.

*Available to contractors upon government approval

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6420	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	017
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) South American hemorrhagic fevers; pathogenesis, therapy and 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
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 79	2.0	234.5
	CURRENT 80	1.5	107.0

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NAME: Barrera-Oro, J. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Argentine hemorrhagic fever; (U) Bolivain hemorrhagic fever; (U) Vaccine development

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

23 (U) Continue attempts to develop a combined vaccine against Argentine hemorrhagic fever (AHF) and Bolivian hemorrhagic fever (BHF). Confirm cross-protection studies between AHF and BHF. Develop an attenuated virus suitable as a candidate vaccine strain. Characterize a primate model for AHF. Vaccines and methods of treatment are essential for these highly militarily important diseases.

24 (U) Compare 2 attenuated strains of Junin (JUN) virus (etiologic agents of AHF) and select one for further development as a vaccine strain. Assess other strains of JUN for potential vaccine development. Challenge a second group of AHF-immune monkeys to assess protection against BHF. Inoculate a variety of monkeys with virulent JUN virus to develop a primate model.

25 (U) 78 10 - 79 09 - A known attenuated strain of JUN virus, XJ-44, has been selected for development as an attenuated candidate vaccine strain. Further attenuation attempts are underway. Twelve isolates have been recovered from human serum and are being assessed for virulence. The capacity of JUN virus to protect against BHF was confirmed in monkeys 7 months postimmunization. Rhesus, cynomolgus and vervet monkeys were inoculated with virulent JUN virus. None provided a reproducible or satisfactory model suitable for pathogenesis studies nor for models capable of distinguishing virulent and avirulent virus strains. This effort continues.

Publication: Am. J. Trop. Med. Hyg. 27:1232-1239, 1978.

*Available in instructions upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 017: South American Hemorrhagic Fever; Pathogenesis, Therapy and Immunization

Background:

Our earlier data indicated that Junin virus infection would protect against both Junin and Machupo virus challenge; on this basis we have undertaken a joint project involving the Argentine Secretariat of Health and the Panamerican Health Organization for the development of an attenuated vaccine against Argentine hemorrhagic fever (AHF) that would offer protection against Bolivian hemorrhagic fever (BHF) also. The report for this year describes our efforts to establish the initial bases and protocols for this undertaking.

Although not reported herein, approximately one-half of our efforts during this year were devoted to the support of Work Units A84100009, Dr. Jahrling and A84100026, MAJ Stephen, dealing with the antiviral drug ribavirin and Lassa fever. Those efforts are reported under the respective Work Units.

Progress:

AHF protects against BHF. In our previous report we presented evidence that an attenuated strain of Junin virus protects against BHF. We repeated this study, except that we challenged 4 monkeys with Machupo virus 7 mon, rather than 3 mon, after Junin virus inoculation. The results were similar to our previous experiment; there was no illness; viremia did not occur; and antibody responses to Machupo virus were brisk. There appeared to be excellent cross-protection against BHF.

These data confirm our earlier experimental results and indicate that live-attenuated vaccine against AHF may protect humans against both AHF and BHF. The immunizing virus used in these experiments was Clone-3, an attenuated strain of Junin virus which was inoculated as an investigational vaccine into 600 persons in Argentina in 1969 and 1970. The use of Clone-3 virus in humans was halted in 1970 because its passage hsitory was not known with certainty and because it was grown in mouse brain and used as a live attenuated vaccine. These latter two problems preclude acceptability as an internationally acceptable vaccine. The current task is to find a virus as attenuated as Clone-3 with an acceptable passage history and to identify a certifiable substrate for its further attenuation and vaccine production.

Searching for substrates. We have attempted to identify acceptable cell culture substrates that will yield with passage a less virulent Junin virus than the parent. We passaged Clone-3 virus 4 to 5 times in primary dog kidney (PDK), human diploid (MRC-5) or in fetal rhesus lung (FRhL) cells. In addition, we passed a virulent human liver isolate (P3551) of Junin virus in PDK cells. The results are shown in Table I. From these data we conclude that PDK cells increased the virulence of P3551, as shown by a shorter time to death. Moreover, the relatively attenuated Clone-3 virus was more lethal for guinea pigs following only 2 PDK cell passages. Thus, PDK cells will not be used for attenuation of Junin virus.

It is difficult to infer anything about MRC-5 or FRhL cells, other than that they did not augment the virulence of Clone-3. Both remain candidate substrates for further passage of a potential vaccine virus.

TABLE I. GUINEA PIG LETHALITY OF JUNIN VIRUSES WITH DIFFERENT PASSAGE HISTORIES

Strain	Passage History		Guinea Pig Lethality Dead/Total (Mean Day Death ± SE)
	Substrate	No. Passages	
P3551	FRhL	1	8/10 (22.9 ± 5.17)
P3551	FRhL	1	
	PDK	5	9/10 (17.6 ± .94)
Clone 2	Original	0	2/10 (21.5)
Clone 3	MRC-5	4	1/10 (33)
Clone 3	FRhL	4	1/10 (36)
Clone 3	PDK	2	4/10 (25 ± 11.4)

Measurements of attenuation. Our comments above described Junin virus, Clone-3. Recently, Dr. Barrera-Oro attempted to determine whether a parallel passage of the same virus strain was equally attenuated and presumably equally acceptable as a vaccine. The advantage of the parallel passage virus, XJ passage 44 (XJ-44), is that it has a well-documented passage history and has never been passed in a continuous cell line. It also has been extensively used in laboratories throughout the world with minimal containment and no history of illness.

Dr. Barrera-Oro compared Clone-3 and XJ-44 by 2 parameters. One was the well-characterized weight loss phenomenon observed by Argentine investigators studying Clone-3 virus. Adult guinea pigs tend to lose or gain weight less rapidly than controls when infected with Clone-3 virus, particularly in high titer. Results showed no significant difference between high virus dose of XJ-44 and Clone-3 with respect to weight gain. Both groups of guinea pigs gained approximately 40% in weight over 29 days postinoculation. Controls gained 56% in weight. Although deaths are less common in adult guinea pigs inoculated with either virus, baby guinea pigs are more susceptible. Clone-3 killed 8 of 29 baby guinea pigs inoculated in a virus titration, whereas XJ-44 killed 6 of 30 similarly inoculated. Thus the XJ-44 is at least as attenuated by these measurements as the virus used experimentally in 600 persons in Argentina 10 years ago.

Strains and virulence studies. We have 18 different specimens from which we are attempting to isolate virus strains for assessment of potential use as vaccines; 9 are from fatal cases and 9 from relatively less severe cases. In addition, we have several different passage levels of the prototype XJ strain. Initially, pools of each strain isolated are being grown in MRC-5 or FRhL cells and titrated. They will then each be inoculated into guinea pigs to assess

virulence. From the most promising strains we will begin to assess their potential as a vaccine candidate. From the XJ strain we are following a separate development scheme. It is known that the strain became attenuated in Dr. Casals laboratory. It was subsequently cloned and then used to immunize 600 persons as described above. Rather than use the Clone-3 virus we plan to assess its parent. To do this we will use the 3 potential substrates mentioned above and passage the XJ virus at high and low multiplicity to derive several different seed pools. These will all be assessed in guinea pigs for virulence.

Presentations.

Eddy, G.A. Cross-protection of monkeys against Bolivian hemorrhagic fever by Junin virus. Presented Annual Meeting, American Society of Tropical Medicine and Hygiene, 6-19 Nobember 1978, Chicago, Il.

Publications.

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6428	79 10 01	DD-DR&E(AR)636

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

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	020
b. CONTRIBUTING				
c. [redacted]	STOG 80-7.2:2			

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 Biolcgy (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 79	3.0	269.0
FISCAL YEAR CURRENT 80	30	305.6

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Botulism, (U) Toxoid; (U) Clostridium botulinum

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. Use the purified toxins to produce toxoids which can then be tested for safety and efficacy as new immunizing agents. This work unit is an essential element in a comprehensive program for medical defense against BW agents because it is aimed at developing and testing new toxoids for use in military forces.

24 (U) Purify neurotoxins of Clostridium botulinum types A-C. Use these to develop a polyvalent toxoid.

25 (U) 78 10 - 79 09 - Colony selection, plasmid curing, phage induction, and manipulation of culture medium were used to identify, enhance and stabilize type A C. botulinum. This strain produces an increased quantity of toxin per ml. The greatest toxin yields were obtained in 4 days in static cultures supplemented with calcium lactate. Maximal toxin production was obtained in only 24 hr in a 50-L fermentor at 35°C, using 5 L/min nitrogen overlay, agitation at 50 rpm and an initial 1% glucose concentration. Chromatographic procedures were found to be useful for purification of large quantities of type A toxin. Over 700 L of Botulism Immune Plasma (Human) collected from volunteer donors. Immune plasma is efficacious in prophylaxis and treatment of botulism in guinea pigs; 50 L of plasma were fractionated to prepare 1 L of immune globulin.

Publications: Rev. Infect. Dis. 1:689-690, 1979;
Toxicon 17 (Suppl.):102, 1979;
Appl. Environ. Microbiol. 38: in press, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M762776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 020: Microbial Toxins and Their Role in the Pathogenesis of Disease

Background:

Bacterial products play a major role in the production of disease. It has been long recognized that the toxin of Corynebacterium diphtheriae produces all the symptoms of the clinical disease diphtheria and that antibody to the toxin will completely prevent the illness, even in the presence of the organism. Other toxins such as the enterotoxins of Staphylococcus aureus and the neurotoxins of Clostridium botulinum are taken into the host preformed. During the past year all efforts have been extended in the area of C. botulinum toxins.

A polyvalent toxoid was prepared in 1958 by Parke, Davis, and Co. under contract to Fort Detrick (referred to here as PD). This PD toxoid contains antigens to types A, B, C, D, and E neurotoxins. At the time of its preparation full knowledge of the neurotoxin was not available and the preparation contains $< 10\%$ of the desired immunogen. Mild side reactions including tenderness, redness, heat, and swelling at the site of the injection are common. The basic course to produce satisfactory antibody levels requires 4 injections over a period of 1 year. In addition, little scientific investigation has been achieved to improve the production and purification of adequate amounts of pure neurotoxins to prepare a new toxoid.

Measurement of antibodies following immunization is accomplished by mouse neutralizalization test. This test has the inherent problems of an animal assay system and in addition requires large numbers of animals.

Treatment of C. botulinum intoxication consists of complete respiratory support and neutralization of circulating toxin. The antiserum currently in use for neutralization of toxin is of equine origin and has a high reaction rate due to sensitivity to horse proteins. (The only U.S. source of equine antiserum notified CDC in July 1978 that they would no longer provide this product.) Although a human antiserum for the treatment of botulism has been proposed for many years, no real effort has been expended to develop such a product.

Progress:

Quantitative consistency of toxin production by all available strains of C. botulinum is notoriously poor and appears to be under extra-chromosomal control. Extensive studies were undertaken in an attempt to identify and stabilize a type A strain that would consistently produce toxin in both static and fermentor conditions of $\geq 1 \times 10^6$ $MIPLD_{50}$/ml of culture.

Colonial morphology and hemagglutinin production were used extensively as markers for the selection of maximal toxin-producing organisms. However, use of this method did not identify a single type A strain that would consistently produce sufficiently large quantities of toxin.

Plasmid curing and phage-induction methods were also employed but failed to either identify a high-producing strain or completely negate strains of toxin production. It has, however, been well-established by similar studies that toxin production by both types C and D strains are under phage control.

Variations in culture medium were then made in an attempt to increase and stabilize toxin production of the available type A strains. These variations included the addition of trypticase soy, proteose peptone, and lactalbumin hydrolysate, without improvement in toxin production. Since in static culture lysis of the organism is thought to play a role in toxin release, divalent cations (Mg and Ca) were added to the medium to promote cell lysis. The addition of Ca resulted in a slight increase in toxin production. Calcium lactate was most effective in inducing toxin release. However, the greatest toxin yields (1-4 x 10^6 LD_{50}/ml of culture supernatant) were obtained by using a concentration of 2 gm of calcium lactate/L of NAK-yeast medium. Decreasing the calcium lactate in steps to 0.5 gm/L resulted in the stepwise lowering of toxin production to the original levels. Although we have been able to formulate a medium which optimizes toxin production, we unfortunately have not advanced our understanding of the qualitative fluctuation in toxin production which continues to occur within a single strain.

Similar nutritional studies with C. botulinum type B suggest that maximum toxin yields in static cultures can be obtained by calcium lactate supplementation with either NAK-yeast medium or lactalbumin hydrolysate medium. Increasing the yeast extract from 0.5 to 1.5% further enhances toxin production.

The time of appearance and the quantity of toxin produced by the Hall strain of C. botulinum type A grown in a 50-L fermentor under various conditions was next examined. Toxin concentrations of 1-2 x 10^6 LD_{50}/ml were attained using this system.

The effect of glucose concentration, using 1.5, 1.0, 0.5 and 0.25% and no glucose, was determined. The medium consisted of 2% casein hydrolysate. 1% yeast extract, and 0.1% thioglycolate, plus glucose as indicated. Growth was dependent on glucose concentration up to 1.0%. Significant lysis of the culture occurred only with 0.5% glucose. The amount of toxin in the culture fluid was identical in cultures supplemented with 1.0 and 1.5% glucose, with the maximum levels occurring in 24 hr. Using 0.5% glucose, maximum levels occurred in 30 hr. In cultures supplemented with 0.25% glucose and those to which no additional glucose was added, considerably less toxin was produced and this was apparently inactivated. This inactivation can be attributed to the rise in pH above 6.8. After 8 hr of growth in medium initially supplemented with 1.0% glucose, an addition of glucose was made to the culture to yield a further 1.0% glucose. This procedure did not increase toxin concentrations beyond those obtained with 1.0% glucose only.

The possibility of increasing toxin yield by controlling the pH of the culture was also investigated. The pH (which after inoculation was 7.1) was uncontrolled until pH 6.0 was reached. This occurred after approximately 8 hr of growth in the presence of 1.0% glucose. The pH was then maintained at 6.0 for the duration of the experiment. pH control had no effect on the growth rate and maximum levels of toxin were not increased.

The effect of temperature was determined. Maximum toxin levels were reached in 48 hr at 30°C, 24 hr at 35°C and 30 hr at 40°C. Considerably less toxin was produced at 45°C. Toxin yields were not increased by substituting CO_2 sparging (1 L/min) for the nitrogen overlay (5 L/min).

To date, optimal conditions for toxin production by the Hall strain of type A are: nitrogen overlay at a rate of 5 L/min, an agitation rate of 50 rpm, a temperature of 35°C and an initial glucose concentration of 1.0% with pH uncontrolled. Under these conditions, the maximum toxin concentration is attained within 24 hr. In contrast, incubation times of 4-5 days are necessary for maximum toxin production by static cultures of this organism.

A purification scheme for type A neurotoxin has been developed further. The toxin is precipitated from 50 L of culture fluid by adjusting the pH to 3.5 with 3 N H_2SO_4. After washing the precipitate with distilled water, the toxin is released from the precipitate by repeated extraction with 0.2 M phosphate buffer, pH 6.0. The extract is dialyzed against 0.05 M citrate buffer, pH 5.5, and is then applied to a 10 cm x 100 cm column containing DEAE-cellulose equilibrated in citrate buffer. The column is eluted with citrate buffer and the toxin emerges at the void volume. This single column chromatographic step allows the removal of nucleic acid from the equivalent of up to 100 L of culture. The toxic peak from the DEAE-cellulose column is then dialyzed against 0.05 M phosphate buffer, pH 7.9. To separate the neurotoxin from the hemagglutinin, this preparation is applied to a column of DEAE-cellulose equilibrated in the phosphate buffer. Upon elution with phosphate buffer, the neurotoxin emerges in the void volume. The neurotoxin is then dialyzed against 0.2 M succinate buffer, pH 5.5. This preparation is free of hemagglutinin activity. However, it does contain small amounts of another protein as demonstrated by SDS-polyacrylamide gel electrophoresis (SDS PAGE).

We have also used DEAE-Sephadex (equilibrated in 0.15 M Tris-HCl, pH 8.0) to separate the neurotoxin from the hemagglutinin and other contaminating proteins. In this procedure, the neurotoxin is adsorbed to the Sephadex and subsequently eluted with Tris buffer containing 0.1 M NaCl. The NaCl causes the resin to shrink; consequently the flow rate of the column is reduced. This factor may preclude the scaling up of this method. Although concentrated samples of neurotoxin prepared by this more tedious method appears in PAGE to be free of contaminating proteins, many batches do however contain traces of hemagglutinins.

Studies have been initiated on the growth and nutritional conditions required for maximum toxin production by type B, using the fermentor system. Also, methods for the purification of type B neurotoxin are currently being developed.

During the past year a collaborative effort was initiated with CDC to determine the quantity and the decay rate of Botulism Antitoxin Trivalent (Equine) in the sera of patients receiving the heterologous antitoxin for prophylactic and/or therapeutic purposes. Data collected from this study are contributing to a critical joint agency evaluation of the quantity of heterologous (equine) and of homologous (human) antitoxin needed for effective prophylaxis and treatment of food-borne botulism. Preliminary data indicate that excessive amounts (> 30,000 U) of equine antitoxin are often administered to Botulinum-intoxicated patients. The effective half-life in man of each component of the trivalent equine antitoxin was shown to be only 5-7 days.

Guinea pigs were utilized to determine the efficacy of Botulism Immune Plasma (Human), IND 1332 (BIP), for the prophylaxis and treatment of type A botulism. The relationship of the time of antitoxin (plasma) administration, appearance of clinical signs of toxemia, detection of serum toxemia and survival were evaluated. Guinea pigs were inoculated IM with 10 IU of plasma 4 hr before or 4, 8, 12, and 34-44 hr after oral administration of 4 guinea pig oral LD_{50} ($GPOLD_{50}$) of buffered

crude type A toxin. Circulating toxin could be detected in the serum of guinea pigs 4 hr after oral ingestion; however, clinical signs of botulism were not evident until 12-24 hr after administration of toxin. When BIP was administered IM 4 hr before or 4 or 8 hr after challenging with oral toxin and clinical signs did not develop, survival was 100%. Plasma administered at 12 hr effected a 67% survival rate and at 34-44 hr, when clinical signs were very prominent, a 45% survival rate. All untreated guinea pigs died within 4 days. However, the time to death for many of those which were treated at 34-44 hr, but eventually died, was extended to as long as 10 days after exposure. These toxemic guinea pigs did not receive any supportive care, as a hospitalized human patient would. The extended survival of toxemic guinea pigs, treated with antitoxin at a time when clinical signs were present, may well be correlated with the survival of intoxicated humans treated with BIP.

A protocol (Med. Div. Protocol FY 79-6, Work Unit A841 00 001) to determine the effective half-life in man of BIP and the relationship between units of BIP administered vs. the maximum resulting circulating titer obtained has been approved and clinical trials have begun.

A pilot lot of 52 L of the BIP collected at Fort Detrick has been fractionated under contract by the Michigan Department of Public Health for the recovery of the IgG portion of this plasma. Approximately 1 L of Botulism Immune Globulin (Human) was recovered and is being held in bulk until all identity, sterility and pyrogenicity testing is completed.

A contract (DAMD 17-79-D0006) was negotiated with Pine Bluff Biological Products, Inc., Pine Bluff, AR, for the collection and delivery of a minimum of an additional 1,000 L of hyperimmune BIP. To date 700 L of BIP have been collected and delivered under this contract.

The anamnestic immune response of 24 of the Pine Bluff BIP (Group #1) Med. Div. Protocol FY 79-3, Work Unit A841 00 001) donors to a booster immunization in Feb 1979 with botulinum toxoid, adsorbed, pentavalent (ABCDE) IND-161 (also designated as the old or PD toxoid) was evaluated by the mouse neutralization test. The geometric mean serum titer for the donor group to type A toxin was 0.82 U/ml at the time of boosting and was 8.98 U/ml for sera collected 28 days later. The group prebooster geometric mean type B titer was 0.06 U/ml while the 28-day postbooster titer was 0.52 U/ml. The type E geometric mean was 0.16 U/ml before and 2.3 U/ml after booster. During the 1960s, the individuals in this Pine Bluff group #1 received toxoid series of 3 immunizations and 4-7 subsequent booster immunizations. The last booster, prior to the February 1979 booster, was given during 1969-1970. The approximate 10-fold increase recorded for both type A and type B neutralizing antibody, and even greater for type E, in response to a single booster, after a 9-year lapse in immunization, is highly significant ($P < 0.001$) and represents an excellent anamnestic response.

In cooperation with the Medical Division an evaluation and comparison of the clinical and immunologic responses of human volunteers to each of 3 lots [1 old (PD) and 2 new (MDPH #1 and MDPH #2)] of pentavalent botulinum toxoid were initiated (Med. Div. Protocol FY 79-4, Work Unit A841 00 001).

Three groups of volunteers comprising a total of 54 individuals were immunized with one of 3 lots of toxoid according to 1 of 3 dosage schedules. Two weeks after completion of the initial toxoid series of 3 injections the new lots of toxoid

(MDPH #1 and #2) had stimulated immune responses (geometric mean of 0.30 and 0.36 type A; 0.08 and 0.10 type B), as measured by titers of serum antibody to type A and B, which were equal to or greater than those elicited by the old toxoid (geometric mean of 0.14 type A and 0.02 type B). Serum titers to types C, D and E botulinal toxins are currently being determined.

Studies were also initiated during this year to develop an enzyme-linked immunosorbent assay (ELISA) system for the immunodetection and measurement of human antibodies to botulinal neurotoxins. Type A neurotoxin was purified, toxoided and adsorbed to multiple well microtiter substrate plates. Sera can easily and consistently be ranked by the "BOT-ELISA" test as to degree of reactivity with type A neurotoxin. The virtues of this test when completely developed will include low cost for supplies, equipment, reagent stability, safety, sensitivity, reproducibility and ease of procedure. Attempts to standardize and apply this test to a diagnostic setting are continuing.

Rabbit antiserum to purified type A neurotoxin was produced and in cooperation with Physical Sciences Division the IgG portion was purified for use in the development of an ELISA for the detection of type A neurotoxin. The sensitivity of this rapid toxin detection test is being manipulated and its application evaluated.

Presentations:

1. Lewis, Jr., G. E., and J. F. Metzger. Botulism Immune Plasma (Human). Presented, Interagency Botulism Research Coordinating Committee Meeting, Beltsville, MD, 2-4 Oct 78.

2. Lewis, Jr., G. E., J. F. Metzger, and R. Wood. Botulism antitoxin: homologous vs. heterologous. Presented, Interagency Botulism Research Coordinating Committee Meeting, Beltsville, MD, 2-4 Oct 78.

3. Siegel, L. S., and J. F. Metzger. Toxin production by Clostridium botulinum type A under various fermentation conditions. Presented, Interagency Botulism Research Coordinating Committee Meeting, Beltsville, MD, 2-4 Oct 78.

4. Siegel, L. S. Botulinum toxin: fermentation studies. Presented, Workshop on Bacterial Toxins, WRAIR, Washington, DC, 28 Mar 79.

5. Metzger, J. F. Botulism program goals and progress. Presented, Workshop on Bacterial Toxins, WRAIR, Washington, DC, 28 Mar 79.

6. Lewis, Jr., G. E., and J. Anderson. Human antiserum for botulism. Presented, Workshop on Bacterial Toxins, WRAIR, Washington, DC, 28 Mar 79.

7. Lewis, Jr., G. E. Clostridium botulinum toxins. Presented, Symposium on Military Veterinary Medicine, WRAIR, Washington, DC, 23-27 Apr 79.

8. Siegel, L. S. Effect of fermentation conditions on toxin production by Clostridium botulinum type A. Presented, Annual Joint Meeting of the Washington and Maryland Branches of the American Society for Microbiology, Frederick, MD, 27 Apr 79,

9. Lewis, Jr., G. E., and J. F. Metzger. Studies on the prophylaxis and treatment of botulism. Presented, 6th International Symposium on Animal, Plant and Microbial Toxins, Uppsala, Sweden, 19-24 Aug 79 (Toxicon 17 (Suppl.):102, 1979).

10. Lewis, Jr., G. E. Production and testing of human botulinal antitoxin. Presented, CDC Production and Use of Human Botulinal Antitoxin Meeting, Atlanta, GA, 26-27 Sep 79.

11. Metzger, J. F. Need for and use of human botulinal antitoxin. Presented, CDC Production and Use of Human Botulinal Antitoxin Meeting, Atlanta, GA, 26-27 Sep 79.

Publications:

1. Metzger, J. F., and G. E. Lewis, Jr. 1979. Human-derived immune globulins for the treatment of botulism. Rev. Infect. Dis. 1:689-690.

2. Siegel, L. S., and J. F. Metzger. 1979. Toxin production by Clostridium botulinum type A under various fermentation conditions. Appl. Environ. Microbiol. 38: in press.

3. Lewis, Jr., G. E., and J. F. Metzger. 1979. Studies on the prophylaxis and treatment of botulism. In: Natural Toxins (B. Eaker and T. Wadstrom, eds.) Pergamon Press, London, in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6425	79 09 04	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 10 01	H. 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	62776A	3M162776A841	00	025
b. CONTRIBUTING				
c. [illegible]	STOG 79-7.2.2			

11. TITLE (Precede with Security Classification Code): (U) Effect of protease inhibitors upon kinin system activation during Salmonella typhimurium infection in monkeys

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
76 10	79 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 79	1.0	79.0
FISCAL YEAR CURRENT 80	0	0

19. RESPONSIBLE DOD ORGANIZATION
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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) BW defense; (U) Kallikrein; (U) Kinins; (U) Bacterial sepsis; (U) Protease inhibitor; (U) Aprotinin; (U) Monkeys

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 effects of a protease inhibitor (aprotinin) upon activation of the kinin system during Salmonella typhimurium infection in monkeys. It is hoped that this new approach to therapy will prove useful in a wide variety of infections with hemorrhagic and/or hypotensive complications which are potential causes of military casualties from either natural or BW disease.

24 (U) Determine pharmacology and appropriate routes of administration of aprotinin to monkeys. Use aprotinin in monkeys infected with S. typhimurium as prophylaxis against kinin activation and disseminated intravascular coagulation.

25 (U) 78 10 - 79 08 - Study of the effect of aprotinin in disseminated intravascular coagulation (DIC) revealed no differences in body temperature, platelet and white cell counts and hematocrit between the drug and saline groups of infected monkeys. Comparison of streptokinase and urokinase activation of plasminogen was conducted in several species of monkeys. Problems were encountered due to inability of the manufacturers to maintain a constant supply of chromagenic substrate and urokinase. In a comparative study with NIH, an attempt was made to determine whether urinary kininogen has a plasma or renal origin. Chromatographic results suggested a renal tubular origin.

Work unit is terminated since the investigator has left the Army.

Publication:

In Kinins II, Part B, p. 341-347, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against BW Agents (U)

Work Unit No. A841 00 025: Effect of Protease Inhibitors upon Kinin System Activation during Salmonella typhimurium Infection in Monkeys

Background:

The infection of rhesus monkesy (Macaca mulatta) with Salmonella typhimurium results in the clinical syndrome of disseminated intravascular coagulation (DIC). DIC is characterized by intravascular activation of factor XII and consequent activation of the pathways for coagulation of fibrinolysis and kinin generation (1).

Theoretically, the use of protease inhibitors would block the sequential activation of the proteolytic enzymes in these pathways. If this effect is proved to be clinically significant, these agents will be a novel approach in the therapy of infectious diseases (2, 3). Protease inhibitors could be adjuvant therapy in the severe bacterial, rickettsial and viral infections associated with DIC (3).

Aprotinin (Trasylol^{R}) is a protease inhibitor obtained from bovine lung; it is a kallikrein, trypsin and plasmin inhibitor. In vitro, it has been shown to inhibit early reactions of blood coagulation and to prolong clotting time. In view of these properties, aprotinin has been proposed as therapy for DIC. However, in vivo studies were contradictory as to the effect of aprotinin in human coagulation; in clinical trials aprotinin was found to be beneficial in uncontrolled studies (4, 5).

Progress:

In a protocol for the study of aprotinin in DIC, 6 groups of 4 monkeys each were studied. The following parameters were assessed (A) general conditions, i.e., temperature, rash, etc.; (B) bacteremia; (C) plasma prekallikrein, fibrinogen, fibrin degradation products, plasminogen, APTT, and prothrombin time; and (D) hematocrit and WBC and platelet counts. We have studied 15 monkeys; significant differences in body temperature, platelet and WBC counts and hematocrit were seen in infected and noninfected comparisons. No differences were found between saline and aprotinin treatment in groups infected with S. typhimurium.

In a study of plasminogen activation, we compared streptokinase (2083 U) and urokinase (776 U) activation in different primate species using plasma from humans and several subhuman primates. Progress was hampered by the inability of the manufacturers to provide a constant supply of the chromogenic substrate and urokinase.

In collaboration with Drs. J. J. Pisano and J. V. Pierce, NIH, we attempted to determine whether urinary kininogen has a plasma (glomerular filtration) or renal (tubular secretion) origin. We injected 125I-labeled kininogen into monkeys and applied their plasma and urine samples to Sephadex columns. When chromatographed, the plasma samples had a sharp early peak of radioactivity that coincided with the blue dextran standard peak. The later (> 5 hr) urine samples had a later peak that coincided with the one for sodium azide (another standard). However, urine obtained

1 hr after injection did not have a sharp peak and the radioactive fractions occurred between the plasma and later urine peaks. The data suggest tubular origin for kininogen found in the urine. A paper entitled, "^{125}I-Kininogen is metabolized prior to urinary excretion when injected in monkeys," was prepared and cleared for publication on 6 Aug 79 and forwarded to Dr. J. J. Pisano, of the National Heart, Lung and Blood Institute, Bethesda, MD, for publication at his discretion.

The work unit was terminated because the investigator left the Army.

Presentation:

de Sa Pereira, M. Kallikrein inhibitor (aprotinin): plasma turnover and effects of coagulation in rhesus monkeys. Presented, International Symposium on Kinins -- KININ 78, Tokyo, Japan, 6-9 Nov 78.

Publication:

de Sa Pereira, M. 1978. Kallikrein inhibitor (aprotinin): plasma turnover and effects of coagulation in rhesus monkeys, p. 341-347. In Kinins II, Part B, 1979.

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1. Wing, D. A., T. Yamada, H. B. Hawley, and G. W. Pettit. 1978. Model for disseminated intravascular coagulation: bacterial sepsis in rhesus monkeys. J. Lab. Clin. Med. 92:239-251.

2. McNichol, G. P., and A. S. Douglas. 1972. Thrombolytic therapy and fibrinolytic inhibitors, pp. 393-428. In Human Blood Coagulation, Haemostasis and Thrombosis (R. P. Biggs, ed.). Blackwell Scientific Press, Oxford.

3. Yamada, T., P. Harber, G. W. Pettit, D. A. Wing, and C. N. Oster. 1978. Activation of the kallikrein-kinin system in Rocky Mountain spotted fever. Ann. Intern. Med. 88:764-768.

4. Amris, C. J., and M. Hilden. 1968. Anticoagulant effects of Trasylol^{R}: in vitro and in vivo studies. Ann. NY Acad. Sci. 146:612-624.

5. Prentice, C. R. M., G. P. McNicol, and A. S. Douglas. 1970. Studies on the anticoagulant action of aprotinin ("Trasylol"). Thromb. Diath. Haemorrh. 24:265-272.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION: DA 0B6423	2. DATE OF SUMMARY: 79 10 01	REPORT CONTROL SYMBOL: DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	026
b. CONTRIBUTING				
c. ~~Schedule~~	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Effectiveness of selected antiviral compounds against diseases of BW 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
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: 79	1.0	345.9
FISCAL YEAR CURRENT: 80	1.0	506.1

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Loizeaux, P. S.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Chemotherapy; (U) Togaviruses; (U) Arenaviruses; (U) Metabolism; (U) Bioavailability; (U) Monkeys

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

23 (U) Evaluate potential antiviral compounds with significant antiviral activity against infectious diseases in animals. Experimental diseases selected for study are important human pathogens of military concern, and the information obtained will be invaluable in control and treatment of viral diseases in military personnel, emphasizing those of possible BW importance.

24 (U) Test candidate compounds against selected viruses in tissue culture, rodents and other subhuman promates.

25 (U) 78 10 - 79 09 - Ribavirin was shown to be an effective antiviral drug for the prevention and treatment of Lassa fever virus infection of rhesus and cynomolgus monkeys. Treatment was effective given late, after the onset of viremia and fever. Similarly, ribavirin was effective in the early treatment of Rift Valley fever virus infection of rhesus monkeys. Initial pharmacokinetic data showed that the distribution of ribavirin followed the major replication sites of arenaviruses, possibly accounting for its effectiveness against these model virus infections. Continuation of the pharmacokinetic experiments will lead to dosage refinements and possible increased efficacy. Ribavirin did not achieve significant levels in the central nervous system and may not, therefore be useful against encephalitis viruses.

Publications: Lancet 1:267-268, 1979;
J. Infect. Dis. 139:267-272, 1979;
Fed Proc. 38:429, 1979;
Abstracts of ASM Meeting - 1979, p. 275.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M1622776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 OC 026: Effectiveness of Selected Antiviral Compounds Against Diseases of BW Importance

Background:

Vaccines are not available for the prevention of most virus infections of military importance. Consequently, a limited program was undertaken to evaluate the potential for use of chemotherapeutic agents as an alternate or adjunct method to prevent or treat salient virus infections.

Infection models have been established in rodent and subhuman primate species to simulate comparable disease entities seen in man. These models have been utilized successfully to demonstrate the great potential of antiviral drugs. The recent approval for use of adenine arabinoside for use against herpes encephalitis and the broadening of approval for use of amantadine against influenza virus in man increases the probability that antiviral drugs will be clinically useful in the treatment of virus-induced diseases of military importance.

Progress:

Preliminary studies were conducted to establish a primate model for Lassa fever virus infection for use in evaluating the antiviral activity of ribavirin. A rhesus monkey model has now been established which appears to reflect the disease seen clinically in man, as characterized by high fever, hemorrhagic signs and death.

Subcutaneous inoculation of 10,000 PFU of Lassa fever virus (LAS) (Josiah strain) induced uniformly detectable viremia by day 5 in all untreated monkeys (Table I). Viremia peaked by days 10-14 and untreated monkeys began to die, with an eventual mortality of 60%. When viremia titers were evaluated in the untreated group of monkeys, deaths occurred when the viremia continued to rise above 10^4 PFU/ml of serum; in contrast, survival occurred when viremia remained below this level. When IM ribavirin treatment (50 mg/kg loading dose, followed by 10 mg/kg 3 x a day) was started on day 5 and continued through day 18, viremia remained low. Therapeutically administered ribavirin prevented hemorrhagic manifestations and death in all treated monkeys. Similarly, monkeys given identical doses of ribavirin on days 0-18 survived. The latter schedule of treatment begun soon after virus inoculation prevented the early appearance of detectable viremia and significantly ($P < 0.001$) suppressed the peak virus titer compared to untreated controls. These data suggest that ribavirin treatment of LAS may be of value for LAS patients. Additionally, this model infection should prove useful in the evaluation of other potential antiviral drugs and various combinations of drugs and specific immune plasma.

TABLE I. VIREMIA TITERS IN LAS-INFECTED RHESUS MONKEYS TREATED WITH RIBAVIRIN

	MEAN TITER ± SE (Log_{10} PFU/ml)				
	Untreated Controls			Ribavirin-treated	
DAY	Total (n = 10)	Lethally Infected (n = 6)	Survivors (n = 4)	Day 5-18 (n = 4)	Day 0-18 (n = 4)
3	0.96 ± 0.18	1.01 ± 0.26	0.87 ± 0.27	1.63 ± 0.41	<[a]
5	2.79 ± 0.16	2.93 ± 0.17	2.57 ± 0.32	3.27 ± 0.10	<
7	3.80 ± 0.16	4.08 ± 0.12	3.37 ± 0.25	3.37 ± 0.15	1.60 ± 0.34
10	4.38 ± 0.36	5.18 ± 0.24	3.17 ± 0.27	3.25 ± 0.29	2.00 ± 0.48
12	4.50 ± 0.56	5.87 ± 0.42	3.12 ± 0.18	3.15 ± 0.41	2.12 ± 0.54
14	3.02 ± 0.36	Dead	3.02 ± 0.36	2.25 ± 0.57	1.37 ± 0.44
17	2.97 ± 0.23		2.97 ± 0.23	1.62 ± 0.51	<
19	2.60 ± 0.40		2.60 ± 0.40	1.45 ± 0.49	1.00 ± 0.40
21	1.62 ± 0.60		2.65 ± 0.32	<	0.95 ± 0.35
24	0.97 ± 0.37		1.35 ± 0.75	<	<
% Survival	60	0	100	100	100

[a]Undetectable at less than 1.7.

Rhesus monkeys challenged IV with RVF virus develop signs of disseminated intravascular coagulation (DIC) including increased PT, APTT, and fibrin degradation product and decreased platelet counts. Viremia was detectable uniformly on day 1 after challenge, reached a peak of 10^5 PFU/ml by day 2 and was undetectable by day 7. Ribavirin was given IM (50 mg/kg loading dose; 10 mg/kg 3 times a day) beginning 2 hours after virus inoculation to 4 additional monkeys. These treated monkeys had significantly ($P < 0.001$) lower viremias when compared to those of sham-treated control monkeys. All infected monkeys had serum neutralizing antibody titers of 1:80 by day 7 even though 2 out of 4 ribavirin-treated monkeys were not detectably viremic.

Continued interest in the potential for use of ribavirin suggested experiments to ascertain the pharmacokinetics of distribution, disappearance and excretion of ribavirin. Ribavirin was injected IV or IM at a dose of 10 mg/kg (0.85 uCi/mg, ^{14}C-ribavirin labeled on the carbonyl position of the triazole ring). The plasma disappearance curve following IV injection was triphasic with an initial half-disappearance time of 24 ± 4, a second of 220 ± 15, and a third of 766 min. For monkeys injected IM, the curve was also triphasic with an initial half disappearance time of 39 ± 1, a second of 252 ± 28, and a third of 1258 min. The urine of IV-injected monkeys contained 46 ± 5% of the injected ^{14}C dose in 8 hr, while 47% was excreted in 24 hr; in monkeys dosed IM, 50 ± 3% was excreted in 8 hr, while 63% was excreted in 24 hr. Monkeys were exsanguinated at 8 hr. Organ levels of ^{14}C-ribavirin were highest in liver, kidney and adrenal; the brain accounted for 0.5% of the residual ^{14}C. The data in monkeys suggests that tissue retention of this antiviral drug was greater than originally indicated by studies with ^{3}H-ribavirin in the rat (studies done by ICN Pharmaceuticals, Inc., Irvine, CA). Final analysis of the distribution of ribavirin following a single dose of 10 mg/kg revealed that the concentration in the blood cells continued to rise beyond the original values reported at 8 hr.

These levels remain constant through 72 hr and then decrease slowly. Analysis of blood samples would suggest that ~2% of the original injected dose of labeled ribavirin is detectable on day 35. Liver biopsies taken at the same time had negligible amounts of detectable ^{14}C. Further studies will be required to determine if the residual ^{14}C counts reflect labeled ribavirin or merely metabolized and redistributed ^{14}C. Additionally, multiple-dose experiments will be required to determine saturation effects and whether accumulation of ribavirin in blood cells can physically contribute to the fall in red and white cell counts.

Similar studies were done in rats to determine plasma disappearance, urine excretion, CO_2 elimination and tissue distribution of ^{14}C-ribavirin over a 72-hour time period. Rats were injected IM with a 10 mg/kg dose (~0.85 μCi/mg). These data will assist interpretation and adjustment of treatment regimens currently used in rats and provide a comparison for rhesus monkeys. It is apparent that rats excrete about 20% more ^{14}C in the urine at 8 hr than do monkeys and that tissue levels at 8 hr are lower than in monkeys. Thus, it appears that the rhesus has increased tissue retention vs. the rat. Cumulative CO_2 excretion plateaus at 2 hr with < 2% of the injected dose excreted by the respiratory route. Studies in rats do not show persistence of ribavirin in blood cells suggesting that rats may not reflect disposition as it might occur in man. The relative affinity of ribavirin for rat, monkey and human red cells will be evaluated *in vitro*.

Previous studies in mice using various togaviruses (VE, YF and JE) have suggested that ribavirin is not an effective antiviral compound against these infections. Additionally, ribavirin does not achieve effective drug levels in the CNS, presumably because of selective exclusion by the blood/brain barrier. In order to evaluate *in vitro* kinetics of ribavirin uptake and excretion, ^{14}C-labeled ribavirin was incorporated into the maintenance media of 3 tissue culture cell lines. Vero cells did not achieve a steady-state level of label until 48 hr, whereas, BW-JM cells achieved nearly maximal levels within 1 hr. Uptake by glial cells was intermediate between Vero and BW-JM cells. Following a washout procedure using maintenance media free of ribavirin, the same trend was apparent in reverse. After 1 hr, only 15% of the counts were retained by the BW-JM cells and only 1% at 24 hr. Glial and Vero cells retained approximately the same amount of label at 1 hr (62% vs. 69%, respectively), but counts were lower in glial cells (27%) than Vero cells (51%) at 6 hr. A corollary to this experiment was a simultaneous yield reduction assay using either 10 or 25 μg/ml of ribavirin incorporated into the maintenance medium and RVF virus. Virus was detectable in Vero and glial cells beginning on day 1 after inoculation and on day 2 in BW-JM cells. Maximum yield occurred on day 3 in both cells and on day 4 in BW-JM cells. Virus yield was somewhat lower in Vero cells treated with 10 μg/ml of ribavirin on days 1 and 2, but yield on days 3 and 4 was similar to control flasks. No virus was significantly ($P < 0.001$) lower in these treated flasks compared to control flasks. By day 4, virus yield in treated flasks again approached control values. No virus was detectable in glial or BW-JM cells treated with ribavirin. These data show that ribavirin is taken up effectively by a cell of CNS origin and that ribavirin effectively inhibits virus replication in these cells. Further, should a method be developed to get ribavirin or an analog to the CNS, the data suggest that CNS infections such as those induced by VEE or RVF viruses might be effectively treated.

Since the interferon inducer poly(ICLC) was shown to be an effective antiviral drug in RVF-infected mice, a corollary experiment was done in vitro to characterize the sensitivity of RVF to interferon using L-929 cells and purified mouse interferon produced by Litton Bionetics, Inc. (∿ 20,000 units/ml). The strains of RVF evaluated were ZZ-501, SA-75, SA-51 and Entebbe using VSV as the control virus. Equivalent amounts of interferon yielded 8% plaque reduction of VSV and SA-75 with the remaining strains requiring about 2.5 times as much interferon for similar plaque reduction. It can be concluded that these strains of RVF were about as sensitive to interferon as a known sensitive virus such as VSV.

As a screening procedure, tissue culture studies were conducted to evaluate the antiviral properties of potential antiviral compounds, primarily analogs or ribavirin. Table II shows the results of plaque reduction assays of these drugs against the different test viruses. CF-100 (Ag salt of sulfadiazine) and Ag metachloridine (WR-235942) in dimethylsulfoxide were ∿ 10 times more toxic to tissue culture cells when compared to ribavirin-related compounds. Compounds designated RKR-01, RKR-02, and RA-13 were more potent against sandfly fever (Sicilian) than ribavirin, but were not active against VEE of YF. Compounds RA-98, RB-122, RB-125, and RA-83 were not as effective as ribavirin against RVF.

Presentations:

1. Stephen, E. L., D. E. Jones and G. A. Eddy. Treatment of Machupo virus infection of monkeys using either ribavirin or its triacetyl derivative. Am. Soc. Trop. Med. Hyg., Chicago, IL 6-19 Nov 78.

2. Ferrara, E. A., R. W. Wannemacher, Jr., and E. L. Stephen. Plasma disappearance, urine excretion and tissue distribution of ^{14}C-ribavirin and its metabolites in rhesus monkeys. Presented, Annual Meeting, FASEB, 6-10 Apr 79. (Fed. Proc. 38:429, 1979.)

3. Stephen, E. L. Antiviral therapy. Presented, Symposium on Military Veterinary Medicine, 23-27 Apr 79, WRAIR, Washington, DC.

4. Jones, D. E., E. L. Stephen, C. J. Peters, and P. S. Loizeaux. Rift Valley fever-infected $C_{57}Gl_6$, mice: evaluation of the antiviral activity of ribavirin. Presented, Annual Meeting, ASM, May 1979. (Abstracts of the Meeting, p. 275.)

5. Stephen, E. L. Ribavirin treatment of Togavirus, Arenavirus, and Bunyavirus infections in subhuman primates. Presented, Scientific Symposium, ICN Pharmaceuticals. Newport Beach, CA, 6-7 Sep 79.

TABLE II. EFFECT OF VARIOUS ANTIVIRAL COMPOUNDS[a] ON VIRUS YIELD AS MEASURED BY PLAQUE ASSAY

VIRUS (Cell type)	CONCENTRATION REQUIRED TO GIVE 80% PLAQUE REDUCTION (μg/ml)								
	Ribavirin	RKR-01	RKR-02	RA-13	CF-100[b]	Ag Meta.[b] in DMSO	RA-98	RD-125	RA-83
RVF (SW-13	15	100[c]	>100[c]	100	5	2.5	>100[c]	>100[b]	
RVF (Vero)									-
YF (Vero)	50	-[d]	-	-	-	>10[e]			
VEE (Vero)	100	-	-	-	10	10	>100[c]	>100[b]	>100[b]
DEN-2 (SW-13)	100	50	100	50	>2.5[c]	>2.5[c]			
SFS (SW-13)	100	25	50	25	>2.5[c]				
PIC (Vero)	100	100	>100[c]	100	> 10[c]	-			

[a]RD-122, no plaque reduction for RVF and VEE in Vero cells.
RB-124, no plaque reduction for RVF and VEE in Vero cells.
[b]Toxic to tissue culture at 5 μg/ml and up.
[c]50-75% plaque reduction, no plaque reduction up to 100 μg/ml.
[e]DMSO control had similar plaque reduction.

Publications:

1. Stephen, E. L., and P. B. Jahrling. 1979. Experimental Lassa fever virus infection successfully treated with ribavirin. Lancet 1:267-268.

2. Stephen, E. L., D. E. Hilmas, H. B. Levy, and R. O. Spertzel. 1979. Protective and Toxic effects of a nuclear resistant derivative of polyriboinosinic-polyribocytidylic acid on Venezuelan equine encephalomyclitis virus in rhesus monkeys. J. Infect. Dis. 139:267-272.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6427	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	029
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Physiological aspects of drug therapy during infection of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 02300 Biochemistry

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76 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 79	0.5	171.5
FISCAL YEAR — CURRENT 80	0.5	270.7

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
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21. GENERAL USE
Foreign intelligence considered

20. PERFORMING ORGANIZATION
NAME: Animal Assessment Division USAMRIID
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ASSOCIATE INVESTIGATORS
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22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Hemoperfusion; (U) Yellow fever; (U) Rocky Mountain spotted fever; (U) Interferon; (U) Monkeys

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

23 (U) Determine and evaluate specific physiologic responses to new drugs of potential military application. Assess mechanisms for potentiating desirable, and inhibiting undesirable, actions of such drugs. Evaluate alterations in physiology induced by infectious agents and their modification through drug therapy.

24 (U) Measure various physiological and biochemical changes in monkeys and other animals during selected viral infections. Evaluate interferon, interferon-inducer and candidate antiviral drugs for ability to prevent or modify adverse virus-induced changes associated with infection.

25 (U) 78 10 - 79 09 - An improved activated charcoal hemoperfusion system was established for use in rhesus and cynomolgus monkeys. Yellow fever-induced changes in tissue concentration of Zn, Fe, Cu, Ca and Mg in monkeys were determined; liver showed the most changes. Decreased cardiac Ca reduced contractile force, which may contribute to YF mortality. Activated charcoal hemoperfusion and hyperalimentation of YF-infected monkeys is ineffective in prolonging survival. Various portion of brain showed decreased concentrations of total, extracellular and intracellular Ca in monkeys with Rocky Mountain spotted fever. Decreased extracellular Ca was also observed in liver, heart, lung, and muscle. Selected physical properties and chemical compositions of poly(ICLC) have been determined; a suspension would be suitable for IV injection except for high viscosity. Increases in rectal and skin temperatures were observed within 1 hr after a single IV injection of fibroblast interferon at a dose of 120,000-140,000 U/kg in monkeys. Initial fever and skin vasodilation lasted approximately 3 hr and decreased below baseline levels for the remainder of the experiment.

Publications: Am. J. Vet. Res. 40:1035-1039, 1979;
Fed. Proc. 38:538, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents(U)

Work Unit No. A841 00 029: Physiological Aspects of Drug Therapy During Infection of Military Importance

Background:

A shift in emphasis of this work unit has been made from studying the physiological aspects of yellow fever (YF) and Rocky Mountain spotted fever (RMSF) to the physiologic aspects of drugs with potential military application. Work was completed on tissue changes of specific minerals and trace metals occurring in YF and RMSF infection and on physiologically directed treatment of YF.

The objectives of the newly-titled work unit are: To determine and evaluate specific physiologic responses to new drugs of potential military application. To assess mechanisms for potentiating desirable and inhibiting undesirable actions of such drugs. To evaluate alterations in physiology induced by infectious agents and their modification through drug therapy.

Interferon. Isaacs and Lindemann (1) were the first to discover interferon during a study of the interference produced by the growth of live virus in fragments of chick chorioallantoic membrane. One activity of interferon is to inhibit the multiplication of many viruses. Since the protection provided by interferon is of limited duration (a few days), possible antiviral mechanisms of interferon include indirect inactivation of virus and inhibition of virus growth.

The possible use of interferon or its inducers [poly(ICLC)] in the prevention and treatment of human diseases continues to be of great interest. Human interferon has been used successfully in the treatment of rabies in cynomolgus monkeys (2). Several clinical implications of using interferon for treatment in patients were reviewed by Marx (3).

De Somer et al. (4) observed that IM and intracutaneous injections of fibroblast interferon in patients caused fever and skin irritation. The development of fever was dose-related and usually reached a peak 6-12 hr after injection. Similar side effects of interferon during high dose clinical therapy were also observed by Tamm and Sehgal (5). These investigators reported that the pyrexial response was transient; whereas, malaise and fatigue continued even during prolonged treatment at lower doses of interferon. Hypotension has also been observed in some patients.

Progress:

Yellow Fever (YF)

Tissue Ca^{++}, Mg^{++}, and tract metal changes in YF-infected monkeys. Tissue concentrations of Ca^{++} and Mg^{++} and trace metals (Zn, Fe, and Cu) of 8 YF-infected rhesus monkeys were compared with concentrations in 5 noninfected rhesus (Table I). The liver showed the most changes including an increase in total Ca^{++} and decreases in Zn, Fe and Cu. Skin showed increase in Mg^{++} and Zn

content. Fe and Zn increased in the medulla oblongata and cerebral cortex respectively. Ca^{++} content in the left ventricular muscle decreased when compared with control values. Decreased cardiac Ca^{++} reduces contractile force, which may contribute to YF mortality.

Treatment of YF. Studies to evaluate the separate and combined techniques of hemoperfusion and hyperalimentation were initiated. This approach was based on the belief that hemoperfusion aids in the removal of toxins resulting from impaired hepatic function, tissue breakdown and possibly viral origin while hyperalimentation would provide required nutrients for physiologic maintenance. Three rhesus monkeys were treated by hemoperfusion 3 days postinoculation. Neither viremia nor survival time was improved. Hyperalimentation also failed to show any beneficial effect on 2 YF-infected monkeys.

Rocky Mountain Spotted Fever (RMSF)

Changes in tissue Ca^{++}, Mg^{++} and trace metals of monkeys with RMSF. Analyses of tissue distribution of Ca^{++} and Mg^{++} and total tract metal (Zn, Fe, Cu) levels in monkeys 6-8 days after inoculation with *Rickettsia rickettsii* (10^2-10^3 PFU) were completed. The greatest changes were observed in various portions of the brain. Total Ca^{++} content including both extra- and intracellular Ca^{++} decreased in the medulla oblongata, thalamus-hypothalamus complex, cerebellum, and cerebral cortex. The medulla oblongata showed an increased concentration of intracellular Mg^{++}, and the thalamus-hypothalamus complex increased total Fe content as compared with normal noninfected tissues. The decrease in the extracellular Ca^{++} concentration was a common finding among several tissues of the infected monkey including the liver, heart, lung and muscle. The decreased Ca^{++} concentration in the brain and other organs may be associated with fever and/or alteration of cell membrane functions.

The Hemoperfusion System

System development. The hemoperfusion system was refined and several technical problems were eliminated. The following procedural changes were developed to provide a mechanically acceptable hemoperfusion system:

(a) Dry saline-soaked charcoal at room temperature for 24-48 hr. (b) Remove charcoal particles < 1.18 mm diameter. (c) Tightly pack redesigned column with charcoal and flush continuously for 1 hr with distilled water to remove any remaining small particles of charcoal. (d) Reduce blood flow rate to 8-10 cc/min. (e) Use extra-corporeal flow probe in the system to continuously monitor flow rate. (f) Improve the air trap by reducing the dead space and removing air more effectively.

System evaluation. Mean blood pressure and biochemical changes in blood and plasma during a 10-hr hemoperfusion were measured in rhesus (n = 5) and cynomolgus monkeys (n = 4). Identical measurements were also made at 24 and 48 hr. All measured parameters were maintained relatively constant in the rhesus monkey except for a transient decrease in leukocytes, slight hemolysis and a gradual decrease in hematocrit. The cynomolgus monkey showed respiratory alkalosis, hemolysis, and a significant decrease in leukocy . However, all changes in the blood returned to baseline levels within 24 .rs after hemoperfusion.

TABLE I. CHANGES IN TISSUE MINERALS AND TRACE METALS IN RHESUS MONKEYS WITH YELLOW FEVER.

TISSUE	GROUP[a]	n	Ca^{++} (mEq/kg FFWT)[e]	Mg^{++} (mEq/kg FFWT)	Zn (mg/kg FFWT)	Cu (mg/kg FFWT)	Fe (mg/kg FFWT)
Liver	C	5	1.74 ± 0.06	7.36 ± 2.51	46.6 ± 1.4	2.08 ± 0.15	57.5 ± 7.1
	I	8	3.01 ± 0.34[b]	5.69 ± 0.59	29.0 ± 3.7[c]	1.05 ± 0.15[d]	38.9 ± 4.0[d]
Heart	C	5	1.81 ± 0.04	7.77 ± 1.11	14.4 ± 2.0	3.54 ± 0.60	34.8 ± 5.2
	I	8	1.51 ± 0.06[c]	8.41 ± 0.52	17.2 ± 0.6	3.87 ± 0.16	39.2 ± 2.0
Skin	C	5	3.23 ± 0.32	1.83 ± 0.33	4.9 ± 0.3	0.95 ± 0.13	25.1 ± 1.8
	I	8	3.32 ± 0.20	4.15 ± 0.51[c]	10.8 ± 0.9[d]	1.00 ± 0.10	20.6 ± 2.8
Cerebral cortex	C	5	2.64 ± 0.62	5.94 ± 1.10	9.9 ± 0.5	4.58 ± 0.43	28.6 ± 3.3
	I	8	1.90 ± 0.07	6.87 ± 0.38	11.4 ± 0.4[b]	4.16 ± 0.18	26.9 ± 2.0
Medulla Oblongata	C	5	1.80 ± 0.05	8.69 ± 0.25	7.2 ± 0.4	5.35 ± 0.66	12.1 ± 1.2
	I	8	1.95 ± 0.12	8.62 ±	.0 ± 0.7	4.33 ± 0.28	18.5 ± 2.1[b]

[a] C = control, I = infected.

[b] $P < 0.05$

[c] $P < 0.01$

[d] $P < 0.001$

[e] FFWT = Fat-free wet tissue.

Interferon

Two lots (No. 2 and 4) of fibroblast interferon were provided by NIH. To determine if the prepared interferon was pyrogenic, 3 chaired, conscious rhesus monkeys each received a single IV injection of interferon via saphenous vein. Rectal temperature and selected skin temperatures, including surfaces on the upper back, lower abdomen, inner thigh, and top of the head, were measured continuously for 24-hr periods before and after interferon injection. The rectal temperature was determined with a rigid thermo-probe and recorded with either a Honeywell recorder or a Yellow Springs tele-thermometer. The output signals from the tele-thermometer were transmitted to a Brush recorder. A linear calibration curve of different temperatures ranging from 23.3 to 41 C had been previously established for this recording system. Skin temperature was measured by precalibrated disk electrodes, welded to the thermocouple wire and recorded on a Honeywell recorder. All disk electrodes were surgically sutured to the surface of skin. Results are summarized in Table II.

Fibroblast Interferon Lot 2. Each of the two monkeys received 121,000 U/kg of interferon. Only one monkey showed an increase in rectal temperature. From a baseline value of 39 C, fever onset occurred within 1 hr after interferon injection and reached a peak response (40.3 C) 2 hrs after injection. Rectal temperature returned gradually to preinjection levels within 3-5 hrs, then decreased below baseline values and fluctuated between 37.2 to 38 C throughout the remainder of the experimental period.

In general, various skin temperatures changed simultaneously with the rectal temperature after interferon administration. Initially, skin showed increases in temperature (vasodilation) which later decreased below baseline values (vasoconstriction). Among all skin temperatures measured the abdominal skin showed the most irregular temperature changes ranging from 32.2 - 35.5 C during a period of approximately 11 hrs. During the 24-hr control period preceding interferon, monkeys received an IV injection of isotonic saline (2 ml) equal to the subsequent interferon volume. No change in either rectal or skin temperature was observed.

Fibroblast Interferon Lot 4. Rectal and various skin temperatures were measured in a single monkey following a single (140,000 U/kg) dose of interferon. Temperatures increased within 30 min after injection and responses were similar to that of the monkey showing fever with lot 2 interferon. Rectal temperature reached a peak of 40.0 C from a 38.5 C baseline value within 1 hr; duration of fever was 3 hrs. Rectal temperature returned to preinjection levels by 3.5 hrs and continued to decrease to lower than baseline values (37-37.5 C) throughout the remainder of the experimental period. From these preliminary observations these 2 lots of interferon do possess component(s) which will induce fever in rhesus monkeys. Although the exact cause of fever is unknown, marked physiological changes may be associated with fever and other undetermined side effects.

TABLE II. THE EFFECT OF HUMAN FIBROBLAST INTERFERON ON RECTAL AND SKIN TEMPERATURE (°C) IN RHESUS MONKEYS.

SITE OF MEASUREMENT	LOT NO.	DOSE X 10^3 U./ MONKEY	TIME POST-IV INJECTION OF INTERFERON (hr) 0	1	2	3	4	10	12	24
Rectum	2	750	39.0	39.9	40.2	39.8	38.7	37.7	37.4	37.9
	4	800	38.5	39.7	39.5	39.0	37.7	37.5	37.2	37.4
Abdomen	2	750	36.5	36.9	37.6	35.0	33.3	34.7	34.7	35.0
	4	800	35.6	35.8	36.9	36.3	35.1	34.8	34.8	34.9
Inner thigh	2	750	33.6	34.1	34.3	33.8	33.2	32.2	32.3	32.2
	4	800	35.1	35.9	36.4	37.0	35.3	35.7	35.7	35.1
Back	2	750	36.2	37.8	38.1	37.3	36.2	35.3	35.1	34.9
Head	4	800	32.6	33.4	33.7	33.0	32.7	32.2	32.3	32.2

Poly(ICLC)

Properties and Composition of Poly(ICLC). Selected physical properties and chemical compositions of poly(ICLC) (lot CC-114C, provided by Dr. H. B. Levy, NIH) have been determined and results are summarized in Table III. It appears that poly (ICLC) in a suspension is suitable for IV injection in animals, except for its high viscosity. The stock suspension solution of poly(ICLC) must be diluted (2-4 X) with isotonis saline before use.

Development of Techniques

Surgical cannulation. Surgical procedures for cannulations of the common carotid artery and internal jugular vein of macaques have been established. With the shortage in rhesus and cynomolgus monkeys, neck and femoral blood vessels must be used for cardiovascular studies and blood sampling. It was demonstrated that both common carotid arteries could be cannulated without threat to life. However, a hypertensive response was always induced with bilateral ligature of common carotid arteries as a result of decreased pressure in both carotid sinuses.

Maintenance of cannula patency. Procedures to maintain patency of cannulas have been established. The technique involves constant infusion of heparinized saline (40 U/ml) to both cannulas separately at a rate of ~ 0.015 ml/min. The main purposes of this approach are to prevent blood clots from forming in the cannula over a long period of time and to avoid cannula flushing 2-3 X daily, which may inject clots into the circulatory system and cause thrombosis and possible death.

Chromatographic separation of total lipids. Significant progress has been made on the separation of tissue or plasma total lipids into various fractions using a silicic acid column. This was achieved by the stepwise elution of the lipid material in the column with different mixtures of solvents, including petroleum ether, chloroform, and methanol. Various lipid fractions have been separated including lysolecithin, a naturally occurring hemolytic substance and cardiostimulant. The development of this technique is essential for studying lipid metabolism and biochemical mechanisms of infection or toxemia-induced hemolysis.

TABLE III. SELECTED PHYSICAL PROPERTIES AND CHEMICAL COMPOSITION OF POLY(ICLC).

PARAMETER	VALUE	PARAMETER	VALUE
Osmolality	320.5 mOsm	Na^+	123.0 mEq/L
Viscosity	25.17 centipoise	K^+	3.55 mEq/L
Surface tension	64 dyne/cm	Cl^-	173 mEq/L
pH	6.583	P	1.1 mg/dl
PO_2	275.40 mm Hg	Ca^{++}	0.06 mEq/L
PCO_2	11.70 mm Hg	Mg^{++}	0.02 mEq/L
HCO_3^-	1.20 mM/L	Cu	14.0 µg/dl
Total CO_2	1.50 mM/L	Fe	40.0 µg/dl
		Zn	40.0 µg/dl

Presentations:

Liu, C. T., and R. P. Sanders. Development of a hemoperfusion system with activated charcoal for rhesus monkeys. Presented Annual Meeting FASEB, Dallas, TX, 1-5 Apr 79 (Fed. Proc. 38:538, 1979).

Publications:

Liu, C. T., R. P. Sanders, C. L. Hadick, and P. Z. Sobocinski. 1979. Effects of intravenous injection of leukocytic endogenous mediator on cardiohepatic functions in rhesus macaques. Am. J. Vet. Res. 40:1035-1039.

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2. Hilfenhaus, J., E. Weinmann, M. Majer, R. Barth, and O. Jaeger. 1977. Administration of human interferon to rabies virus-infected monkeys after exposure. J. Infect. Dis. 135:846-849.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA OE6420	2. DATE OF SUMMARY* 79 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 78 10 01	4. KIND OF SUMMARY D. CHANGE	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING* NA	8a. DISP'N INSTR'N NL	8b. SPECIFIC DATA - CONTRACTOR ACCESS ☒ YES ☐ NO

9. LEVEL OF SUM	10. NO CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
A. WORK UNIT	a. PRIMARY	62776A	3M162776A841	00	030
	b. CONTRIBUTING				
	c. [illegible]	STOG 80-7.2:2			

11. TITLE (Procede with Security Classification Code)*

(U) Physiologically directed treatment of biological toxemias of military 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
73 08	CONT	DA	C. In-house

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a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:* NA

c. TYPE d. AMOUNT:

e. KIND OF AWARD f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	0.5	221.4
FISCAL YEAR CURRENT 80	0.5	293.4

19. RESPONSIBLE DOD ORGANIZATION

NAME:* USA Medical Research Institute of Infectious Diseases

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20. PERFORMING ORGANIZATION

NAME:* Animal Assessment Division USAMRIID

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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) BW defense; (U) Hemoperfusion; (U) Toxemias; (U) Intestinal function; (U) Cyclic AMP

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

23 (U) Study physiological and biochemical responses in animals to selected toxins of military importance. Evaluate pharmacological and/or physiological means and mechanisms for treatment of toxemias.

24 (U) Examine effects of SEB in a hypotonic solution on intestinal transport of water and electrolytes. Determine optimal therapy for food poison-induced diarrhea of staphylococcal origin. Evaluate effects of purified cholera enterotoxin on pathophysiologic changes and survival after IV or aerosol challenge. Treat toxemias by hemoperfusion.

25 (U) 78 10 - 79 09 - A marked species difference was shown in toxicity of cholera toxin given IV to monkeys; in 2 species the toxin was nonlethal, in the other 2 species the toxin was lethal. The heart was the principal organ affected. Cholera toxin was shown to be nonlethal by the aerosol route for 4 species of monkeys. In the Dutch rabbit, IV cholera toxin acts primarily on the CNS in altering water content and electrolyte concentrations. Heart, skeletal muscle, lung, stomach, and kidney are also affected. Tissue changes may be due to alterations of cell membrane functions resulting from increased tissue cyclic AMP levels. Hemoperfusion through an activated charcoal column demonstrated no effect in the treatment of cholera toxemia in Dutch rabbits, but was successful in monkeys when diminished toxicity of SEB was demonstrated when toxin was perfused through rabbit intestine in hypotonic solution (250 mOsm). Hemoperfusion and pretreatment with 6-mercaptopurine or aspirin showed no effect on SEB lethality in Dutch rabbits.

Publications: Am. J. Vet. Res. 39:1692-1694, 1978; 40;2 accepted, 1979; Radiat. Res. 76:402-409, 1978; Toxicon 16:543-550, 1978; Physiologist 22:77, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 030: Physiologically Directed Treatment of Biological Toxemias of Military Importance.

Background:

New Research Title. The original research title (Physiologically Directed Treatment of SEB-Induced Intoxication) limits research to the study of SEB and does not accurately reflect current research requirements. The change allows greater flexibility in examining physiological aspects of certain biological toxins. Main objectives of this research are to study physiological and biochemical responses in animals to selected toxins of military importance and to evaluate pharmacological and/or physiological means and mechanisms for the treatment of toxemias.

Effects of cholera enterotoxin. Effects of cholera enterotoxin on the intact gastrointestinal tract have been well documented (1). Death may ensue from massive rapid intestinal losses of water and electrolytes (2). Increases in adenyl cyclase activity and cyclic AMP concentrations in intestinal mucosal cells are believed to be the causes (3, 4). Whether or not cholera enterotoxin can be transported into the circulation from the intestine still remains controversial (5). Furthermore, no work has been done concerning the effect of administration of cholera enterotoxin aerosols in animals.

Progress:

Staphylococcal Enterotoxin B (SEB)

Rhesus monkeys. Treatment of SEB Toxemia. Lethal toxemia was induced in rhesus monkeys with an IV dose of SEB ranging from 50-100 μg/kg. These intoxicated monkeys were treated 1-5 hr post-SEB by hemoperfusion through an activated charcoal column for 5-8 hr. Results are summarized in Table I. Data show that when treatment was started within 2 hr of challenge, the monkey could be saved. In contrast, if the treatment was initiated later than 3 hr post-SEB, intoxicated monkeys could not survive. It is possible that improvement of the hemoperfusion system by eliminating all major mechanical problems may further enhance the survival rate.

Dutch rabbits. Systemic effects of IM SEB. Dutch rabbits die between 10-24 hr after a single IM injection of SEB (100 μg/kg). It is unknown whether the cause of death is related to pulmonary edema as shown in SEB-intoxicated rhesus monkeys. Experiments were conducted using 8 control and 10 SEB-inoculated rabbits. Water content and electrolyte (Na^+, K^+) distribution in the lung, heart and diaphragm were not significantly different between control and SEB intoxicated rabbits at the time of death. Results indicate that pulmonary edema is not a major factor leading to death.

TABLE I. TREATMENT OF SEB TOXEMIA BY HEMOPERFUSION OF ACTIVATED CHARCOAL IN RHESUS MONKEYS.

DOSE OF SEB (μg/kg)	TIME OF TREATMENT POST-SEB (hr)	DURATION OF HEMOPERFUSION (hr)	SURVIVAL TIME (hr)
100		6	72
50	1	6	Indefinite
		8	10
	2	5	Indefinite
		5	Indefinite
50	3	3	6
	4	8	13
	5	8	10

After a single dose of SEB (50 μg/kg) was injected into each of 6 normal Dutch rabbits, animals responded initially with an increase in rectal temperature and a decrease in O_2 consumption. The fever lasted for 15 hr; hypothermia was observed shortly before death (mean time to death = 18 hrs). The O_2 consumption continued to decrease for 6 hrs after SEB injection and then returned to baseline levels. However, a marked increase in O_2 consumption occurred during the terminal stage. The SEB-induced hypermetabolism may be unique to rabbits, since a persistent hypometabolism was observed in IV SEB-inoculated rhesus monkeys.

Effects of intestinal perfusion of SEB in hypotonic solution. The severity of diarrhea was enhanced when the cannulated intestine of Dutch rabbits was perfused (2.5 ml/min) with a hypertonic solution (350 mOsm) containing SEB (10 μg/ml). Studies were continued to determine the intestinal transport of water and electrolytes during constant intestinal perfusion of the same dose of SEB in a hypotonic solution (250 mOsm) for 6 hr. The intestinal perfusion of SEB in a hypotonic solution (n = 6) did not cause significant functional changes of the intestine when compared to control rabbits (n = 7) receiving no treatment. These findings suggest that SEB-induced changes in the gastrointestinal tract (vomiting and diarrhea) may be diminished by dilution of gastric and intestinal secretions.

Effects of SEB in 6-mercaptopurine (6-MP)-treated rabbits. Previous work indicated that decreased O_2 levels and leukopenia may result in increased resistance to the lethal effects of SEB. To investigate this further, Dutch rabbits (n = 4) were fed with 6-MP (25 μg/kg) for 5 weeks followed by 50 mg/kg for 4.5 weeks. Obvious inhibition of growth was seen and O_2 consumption and leukocyte counts were moderately decreased. At this time, a lethal IV dose of SEB (50 μg/kg) was given to determine survival time and changes in O_2 consumption and rectal temperature during toxemia. It was found that O_2 consumption decreased slightly, with few changes in rectal temperature. Three of the 4 rabbits died between 9 and 31 hrs, indicating that pretreatment with 6-MP had no effect on modifying SEB toxicity.

Effects of acetylsalicylic acid (ASA) in SEB toxemia. To determine if prostaglandins play a major role in the death of SEB-intoxicated rabbits, the prostaglandin inhibitor ASA was tested in rabbits against lethal doses (50 μg/kg, IM) of SEB. Six rabbits each received a 197 mg dose of ASA by

gavage every 6 hrs for 48 hrs or until death. The initial dose was administered a few minutes after SEB intoxication. A control group of 5 rabbits received SEB but no ASA. Mortality within 21 hr was 3/6 and 3/5 for ASA-treated and control groups, respectively. Results indicate that aspirin has little or no protective effect against SEB toxicity and prostaglandins may not be directly associated with lethality during SEB toxemia in Dutch rabbits.

Treatments of SEB toxemia. SEB (25-100 μg/kg) was injected IV into Dutch rabbits. Treatment of SEB toxemia by continuous hemoperfusion for 5-8 hr through activated charcoal was started 0.25-2 hr after SEB inoculation. None of the tested rabbits (n = 6) survived, indicating a possible difference in the handling of SEB by rhesus monkeys and Dutch rabbits. To test the validity of the hemoperfusion system 4 normal Dutch rabbits were subjected to the same treatment as SEB-inoculated rabbits. Normal rabbits did not show ill effects and survived indefinitely (Table II).

TABLE II. SURVIVAL TIME OF IV SEB-INOCULATED DUTCH RABBITS FOLLOWING HEMOPERFUSION TREATMENT THROUGH ACTIVATED CHARCOAL.

SEB DOSE (μg/kg)	n	LENGTH OF HEMOPERFUSION (hr)	SURVIVAL TIME (hr)
None	3	8	Indefinite
None	1	5	Indefinite
100	1	8	27
50	2	8	8-24
50	2	5	6-8
25	1	5	8

Treatment of SEB toxemia with hemoperfusion through a glass bead column. The propensity of leukocytes to adhere to glass offers another mechanism for induction of leukopenia and possible reduction of SEB effects. Hemoperfusion of Dutch rabbits through a column of glass beads produced only a transient decrease in leukocytes, followed by compensatory leukocytosis. SEB-induced death could not be prevented by this approach. In contrast to activated charcoal, the glass beads had no adsorptive capacity for SEB in *in vitro* studies.

Cholera Enterotoxin.

Rats. Rats (Sprague-Dawley) were resistant to cholera enterotoxin. A single IP or IM dose of cholera enterotoxin (100-400 μg/kg, n = 12) did not kill any of the tested rats, although they appeared ill. The mechanism for this toxin resistance is unknown.

Dutch rabbits. Effects on survival time. Dutch rabbits are susceptible to IV cholera enterotoxin inoculation. Results are summarized in Table III. Rabbits with cannulation of the carotid artery and external jugular vein and restraint died earlier than the intact caged rabbit receiving the same dose of enterotoxin. These findings provide evidence that the Dutch rabbit is a suitable model for studying mechanisms of cholera enterotoxin-induced changes.

TABLE III. EFFECTS OF IV CHOLERA ENTEROTOXIN ON SURVIVAL TIME IN DUTCH RABBITS WITH AND WITHOUT SURGERY AND RESTRAINT.

TOXIN DOSE (μg/kg)	SURVIVAL (hr)(No./Total) With	Without
50	48 (0/1)	
100	13 (0/1)	20 - 48 ($\bar{X}$ = 26) (1/7)
200	Not done (0/0)	18 - 40 ($\bar{X}$ = 24) (0/5)

Effects on tissue water and electrolytes. To search for a possible mechanism in causing cholera enterotoxin-induced death, water, electrolytes and total lipids in various tissues of Dutch rabbits were determined following a single IV cholera enterotoxin injection (100 μg/kg). Identical biochemical analyses were also performed in control rabbits for comparative purposes. To summarize, the following effects of cholera enterotoxin were observed (Table IV).

Most changes occurred in the cerebellum, including significant decreases of total and extracellular Na^+ and water. Total tissue K^+ and Cl^- concentrations were also decreased. Total Cl^- concentration and extracellular water content decreased in the cerebral cortex. The thalamus-hypothalamus complex showed decreases in total Na^+ and K^+ levels. The spinal cord revealed a marked decrease of intracellular Na^+ concentration. No significant changes were demonstrated in the pons and medulla oblongata.

Total and intracellular Na^+ content significantly decreased in the skeletal muscle (abdominal region), but increased in the stomach (fundus). The right ventricle showed an increase in total lipids. The left ventricle revealed a decrease in the intracellular Na^+ concentration. The lung demonstrated decreases in total water and Na^+ levels. The liver total water content increased, and the diaphragm showed intracellular dehydration. Both renal cortex and medulla responded with an increase in intracellular water content; and the renal medulla further revealed that total Na^+, as well as intracellular Na^+ and extracellular water content decreased. Skin and jejunum did not show significant changes in the measured parameters.

It appeared that IV-administered cholera enterotoxin acts on the CNS excluding pons and medulla oblongata. Other organs, such as cardiac and skeletal muscle, stomach, lungs and kidneys were also affected. Furthermore, while liver showed an accumulation of water and stomach had a high concentration of Na^+ all other measured tissues demonstrated general losses of water and electrolytes. The possible mechanism in causing tissue water and electrolyte changes may be associated with cell membrane damage, which alters the normal Na^+ transport function.

Tissue cAMP levels following an IV cholera enterotoxin (100 μg/kg) injection in rabbits are presented in Table V. No significant changes were obtained when compared with corresponding tissues of control rabbits. However, limited data revealed that tissue cAMP concentrations increased after a higher dose of cholera enterotoxin (200 μg/kg) was given.

TABLE IV. EFFECT OF CHOLERA ENTEROTOXIN (100 μg/kg, IV) ON TOTAL (T), INTRA-(IN) AND EXTRACELLULAR (E) WATER AND ELECTROLYTE IN NEURAL AND NONNEURAL TISSUES OF DUTCH RABBITS.

TISSUE	GROUP	(n)	$(H_2O)_T$ (g/kg FFWT)[d]	$(H_2O)_{In}$ (g/kg FFWT)	$(H_2O)_E$ (g/kg FFWT)	$(Na)_T$ (mEq/kg FFDT)[e]	$[Na]_{In}$ (mEq/kg H_2O)	$(K)_T$ (mEq/kg FFDT)	TOTAL LIPIDS (g/kg Wt)[f]
Cerebellum	C[a]	(10)	897 ± 9	636 ± 35	261 ± 31	576 ± 50	31.0 ± 5.6	988 ± 144	104.0 ± 5
	I	(10)	861 ± 11[b]	685 ± 27	176 ± 18[b]	401 ± 28[c]	42.2 ± 4.9	608 ± 29[b]	89.6 ± 2.9[b]
Cerebral cortex	C	(9)	856 ± 13	573 ± 38	282 ± 30	488 ± 67	58.2 ± 15.8	724 ± 56	69.0 ± 4.1
	I	(10)	859 ± 6	654 ± 21	205 ± 2.0[b]	373 ± 22	34.1 ± 6.1	713 ± 38	70.9 ± 4.8
Thalamus	C	(10)	894 ± 13	658 ± 31	236 ± 26	644 ± 84	50.0 ± 9.1	1058 ± 139	123.0 ± 7
	I	(10)	861 ± 8	674 ± 17	187 ± 21	380 ± 36[c]	39.7 ± 4.8	699 ± 63[b]	119.0 ± 8
Spinal cord	C	(9)	862 ± 14	662 ± 21	200 ± 24	527 ± 82	66.3 ± 11.1	580 ± 47	198.0 ± 9
	I	(10)	858 ± 11	615 ± 20	243 ± 24	431 ± 37	38.1 ± 6.3[b]	721 ± 61	211.0 ± 6.0
Muscle	C[a]	(10)	761 ± 11	629 ± 16	132 ± 13	274 ± 21	34.6 ± 5.0	377 ± 23	43.8 ± 6.2
	I	(10)	738 ± 10	602 ± 15	136 ± 14	105 ± 7[b]	12.0 ± 2.7[c]	338 ± 29	37.1 ± 2.3
Stomach	C	(8)	816 ± 7.8	483 ± 21	333 ± 23	276 ± 21	14.7 ± 3.0	384 ± 29	36.6 ± 4.8
	I	(9)	832 ± 9.6	489 ± 22	343 ± 21	378 ± 30[b]	31.1 ± 4.9[b]	453 ± 24	36.3 ± 23
Left ventricle	C	(10)	817 ± 4	586 ± 16	231 ± 15	303 ± 26	38.5 ± 6.7	399 ± 33	32.5 ± 2.9
	I	(10)	810 ± 5	545 ± 33	165 ± 34	259 ± 14	19.4 ± 3.7[b]	406 ± 14	32.7 ± 2.0
Lung	C	(9)	847 ± 5	500 ± 45	347 ± 45	560 ± 62	63.6 ± 11.2	489 ± 45	38.6 ± 2.7
	I	(10)	827 ± 7[b]	467 ± 26	360 ± 26	406 ± 24[b]	39.3 ± 7.2	41[illegible] ± 17	38.6 ± 3.8
Liver	C	(8)	765 ± 5	593 ± 16	172 ± 19	155 ± 11	21.1 ± 6.1	333 ± 29	51.7 ± 2.7
	I	(10)	785 ± 6[b]	578 ± 15	207 ± 15	170 ± 11	12.5 ± 3.7	393 ± 45	56.1 ± 4.5
Diaphragm	C	(9)	788 ± 14	646 ± 17	142 ± 14	165 ± 24	20.7 ± 3.7	348 ± 40	34.7 ± 4.1
	I	(10)	754 ± 16	579 ± 21[b]	175 ± 20	139 ± 14	15.0 ± 3.5	311 ± 29	34.2 ± 3.9
Renal cortex	C	(9)	797 ± 7	481 ± 15	316 ± 17	322 ± 24	35.5 ± 7.3	335 ± 23	42.2 ± 2.9
	I	(10)	819 ± 7	540 ± 22[b]	279 ± 21	351 ± 18	52.4 ± 5.4	407 ± 35	42.6 ± 2.2
Renal medulla	C	(9)	850 ± 7	413 ± 42	437 ± 45	564 ± 50	75.7 ± 13.1	381 ± 25	39.7 ± 4.0
	I	(10)	841 ± 4	547 ± 28[b]	294 ± 35[b]	432 ± 18[b]	43.7 ± 5.7[b]	445 ± 57	38.5 ± 4.4

[a] C = control, I = Intoxicated.

[b] $P < 0.05$

[c] $P < 0.001$.

[d] FFWT = Fat-free wet tissue.

[e] FFDT = Fat-free dry tissue.

[f] WT = Wet tissue.

TABLE V. CYCLIC AMP LEVELS IN PLASMA, URINE AND TISSUE OF CONTROL AND CHOLERA-INTOXICATED RABBITS (100 µg/kg).

	pMol/g wet wt.	
SAMPLE	CONTROL (n = 6)	TOXIN (n = 5)
Skin	311.8 ± 65.9	158.9 ± 13.3
Muscle	147.2 ± 39.5	110.0 ± 25.8
Diaphragm	433.0 ± 143.6	409.0 ± 171.2
Heart (Left ventricle)	715.5 ± 256.0	258.3 ± 101.2
Liver	128.0 ± 28.7	169.5 ± 67.1
Lung	430.8 ± 113.3	403.4 ± 91.1
Renal cortex	279.7 ± 64.0	138.3 ± 37.1
Renal medulla	426.8 ± 86.9	269.4 ± 91.2
Stomach	389.8 ± 102.2	271.9 ± 114.2
Jejunum	235.8 ± 32.2	203.1 ± 39.5
Cerebellum	1464.6 ± 498.6	420.2 ± 182.5
Thalamus	766.1 ± 103.7	614.8 ± 163.9
Spinal cord	266.6 ± 19.8	240.4 ± 58.1
Plasma (pMol/ml)	44.5 ± 7.5	40.2 ± 15.3
Urine (pMol/ml)	32,496 ± 26,200	271,872 ± 173,577

Treatment of Cholera Toxin. Two Dutch rabbits were injected IV with lethal doses of cholera enterotoxin (25 µg/kg). Death could not be prevented by using the established technique for hemoperfusion through an activated charcoal column. It is possible tnat the molecular weight of cholera enterotoxin (a protein) is too high or the molecule is not suitable for charcoal adsorption. If the cholera enterotoxin leaves the circulatory system quickly, charcoal would not be able to remove it.

Monkey IV Studies. Four species of monkeys were given IV doses of cholera enterotoxin (50-200 µg/kg). Results on survival time and pathologic changes after toxin inoculation are summarized in Table VI. It was clearly shown that pigtail and cynomolgus monkeys were susceptible; a dose of 50 µg/kg was lethal within 5-6 days. Capuchin and African green monkeys were not susceptible. Only one of 2 African green monkeys at the 200 µg/kg level died and none at the 50 µg/kg level; all capuchins survived at both levels of enterotoxin.

Monkey Aerosol Studies. To examine if cholera entertoxin poses a BW threat, highly purified cholera enterotoxin (20 mg in 10 ml of distilled water) was presented to 4 species of monkeys through aerosolization for 27-30 min. The concentration of cholera enterotoxin in the collecting sampler was determined with a Beckman DB spectrophotometer at 280 m µ . Since the composition of amino acids was not identical among 3 protein standards used, values of optical density were 0.15, 0.24 and 0.51 with the same concentration (250 µg/ml) for human albumin, SEB, and cholera toxin, respectively. Thus, the cholera enterotoxin solution (250 µg/ml) was used as standard and the presented dose of such enterotoxin for each monkey was calculated (Infect. Immun. 9:101, 1974) and is shown in Table VII.

Although the presented dose of cholera enterotoxin reached unusually high levels (85 to 311 µg/kg), none of the monkeys died. However, pigtail monkeys appeared very ill after aerosol exposure. General observations from all monkeys after exposing to aerosols of cholera enterotoxin are summarized in Table VII.

Since this enterotoxin is not easily transported into the general circulation via the lung at such a high presented dose, it may be concluded that cholera enterotoxin is unlikely to be used as a BW agent.

TABLE VI. EFFECTS OF IV CHOLERA ENTEROTOXIN ON SURVIVAL TIME, PATHOLOGIC CHANGES AND GENERAL OBSERVATIONS IN 4 DIFFERENT SPECIES OF MONKEYS.

SPECIES (n)	DOSE (μg/kg)	SURVIVAL TIME (hr)	MAJOR PATHOLOGICAL CHANGES	GENERAL OBSERVATIONS
Pigtail (2)	50 50	142 113	1. Lung: severe hemorrhage 2. Heart and lung: petechial hemorrhage 3. Pericardial sac: fluid	1. Flushed face 2. Decreased activity 3. Anorexia 4. Few stools 5. Epistaxis
Cynomolgus (3)	50 50 50	104 15 92	1. Lung: severe hemorrhage 2. Ascites, severe 3. Heart: petechial hemorrhagic necrosis of papillary muscles (left ventricle) 4. Adrenal: congested 5. Hydrothorax, moderate	1. Anorexia 2. Decreased activity 3. Tremors 4. Few stools
African green (4)	50 50 200 200	Indefinite Indefinite Indefinite 100	1. Lungs: hemorrhages 2. Heart: necrosis of entire left ventricle 3. Peritonitis and ascites	1. Anorexia 2. Few stools 3. Foaming at mouth 4. Tremors
Capuchin (4)	50 50 200 200	Indefinite Indefinite Indefinite Indefinite	No necropsy performed	Appeared normal

Development of Techniques

A technique for the hemoperfusion of Dutch rabbitrs using activated charcoal was developed. Several control rabbits were evaluted for 6 hr showing that the procedures of hemoperfusion did not adversely affect the animal. Thus, the established techniques for hemoperfusion are considered to be safe.

The presently established system for determining cardiohepatic functions had been designed for a rhesus monkey weighing > 3.5 kg. Recently the system has been modified successfully to accommodate a 2-kg rabbit. This newly developed technique may also be applied to small monkeys.

Techniques for determining tissue levels of cAMP and glycogen have been developed. The former was achieved using the principle of competitive adsorption and measuring the radioactivity of ^{3}H-cAMP. The latter was measured in terms of glucose, which is the final breakdown substance after acid digestion. Obtained values for cyclic AMP and glycogen concentrations in various tissues are comparable with published data.

TABLE VII. GENERAL OBSERVATIONS ON 4 SPECIES OF MONKEYS AFTER CHOLERA ENTEROTOXIN AEROSOL EXPOSURES.

SPECIES[a]	PRESENTED DOSE (μg/kg)	OBSERVATIONS 1-4 Days	5-8 Days
Capuchin	117.6, 310.8	No signs	
African green	98.1, 228.3	No signs	
Cynomolgus	88.6, 126.3	Anorexia	Recovered
Pigtail	85.4, 120.8	Anorexia	Recovered
		Glassy eyes	
		Shallow, rapid respiration	
		General depression	

[a]n = 2 for each species. None of these monkeys showed emesis and diarrhea.

Presentations:

Liu, C. T. and R. P. Sanders. Development of a hemoperfusion system with activated charcoal for rhesus monkeys. Presented Annual FASEB Meeting, Dallas, TX, 6-10 Apr 79.

Publications:

1. Liu, C. T., and M. J. Griffin. 1978. Distribution of tissue water and electrolytes in normal rhesus macaques. Am. J. Vet. Res. 39:1692-1694.

2. Liu, C. T., and D. E. Hilmas. 1978. Effect of X radiation on survival of rabbits with staphylococcal B enterotoxemia. Radiat. Res. 76:402-409.

3. Liu, C. T., R. D. DeLauter, M. J. Griffin, and C. L. Hadick. 1978. Effects of staphylococcal enterotoxin B on functional and biochemical changes of the lung in rhesus monkeys. Toxicon 16:543-550.

4. Liu, C. T., M. J. Griffin, and J. W. Springer. 1979. Alterations of tissue water and electrolytes in rabbits after an intravenous injection of cholera toxin. Physiologist 22:77 (abstract).

5. Liu, C. T., and R. P. Sanders. Modification of lethality induced by staphylococcal enterotoxin B in Dutch rabbits. Am. J. Vet. Res. (Accepted for publication, 5 Sep. 79 MS No. 9233R).

6. Liu, C. T., R. P. Sanders, J. W. Dominik, and S. B. Formal. Effects of intravenous and aerosol administration of crude Shigella toxin to rhesus macaques (Preliminary observations). Am. J. Vet. Res. (Accepted for publication, 5 Sep 79, MS 9323R).

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6. Berendt, R. F. 1974. Simian model for the evaluation of immunity to influenza. Infect. Immuno. 9:101-105.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
	DA 0C6411	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	031
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)*

(U) Mathematical and computer applications in medical BW defense 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 79	1.0	85.0
	CURRENT 80	1.0	116.5

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Oland, D. D.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Diagnosis; (U) Computers; (U) Medicine; (U) Statistics

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 computer programming, biostatistical, mathematical, biomedical engineering, and information management techniques to gather, store, process and interpret biomedical information in a research program for medical defense against BW agents, emphasizing diagnostic, therapeutic and immunoprophylactic studies.

24 (U) 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) 78 10 - 79 09 - The computer support available to investigators at USAMRIID has been significantly upgraded by the purchase of a minicomputer and terminals for data acquisition, storage and analysis.

Research efforts have been concentrated in the areas of biomedical indices of infection, vaccine effectiveness, information management and kinetic modeling of physiological systems, and in experimental design, biostatistical and computational consultation.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 031: Mathematical and Computer Applications in Medical BW Defense Research

Background:

Mission. The research mission of the Computer Science Office (CSO) is to develop and apply computer programming, biostatistical, mathematical, biomedical engineering, and information management techniques to gather, store, process, and interpret biomedical information generated within the Institute. The CSO has responsibilities in three functional areas of support to the Institute: (1) biostatistics and applied mathematics, (2) information management, and (3) research support (implementation of minicomputers for control of experiments, data acquisition and analysis).

ADP equipment. During the past four years, USAMRIID has received its scientific ADP support through the use of a Remote Job Entry (RJE) batch terminal that has been used to communicate with either a Univac 1108 computer (National Bureau of Standards) or the CDC 3500 computer (WRAIR). In Oct 78, the Management Information Systems Directorate (MISD), Fort Detrick, began providing remote communications support for RJE batch terminals and for smaller interactive terminals. The CSO has moved a number of its programs and data bases from WRAIR and NBS to the IBM 360 at MISD, because of their excellent support, close proximity and stability of the ADP service. MISD also has made an interactive file management system (WYLBUR) available. WYLBUR has been used extensively for program development, program maintenance, and execution of data analysis jobs via small terminals by programmers in the CSO and by investigators in 2 research divisions.

In-house computer support has become a reality this year with the installation of PDP 11/34 minicomputer in building 1412 for real-time data acquisition and analysis, and for use by investigators for data analysis via their own terminals. Two terminals are connected to the minicomputer.

The ADP support for USAMRIID is directed toward making the power of the computer more accessible to the end-user, the researcher. One way this is being done is to provide programs and terminals to the researcher so he can process his own data. During the past year, 7 computer terminals have been located in 5 divisions of the Institute to be used by investigators and technicians for data analysis, information retrieval and program development. This not only accomplishes the prime objective of providing better, more cost-effective ADP support to the researchers, but it also frees CSO personnel to spend more time developing and maintaining programs rather than running routine programs. The acquisition of additional terminals for identified functions is planned, continually assuring as much compatibility as possible between all ADP equipment.

Progress:

Early diagnosis of infection. In clinical diagnosis, the assay of a single

some promise of using the approach of profiles of biochemical changes for early detection of infection and/or other stresses.

Normalizing transformations and confidence intervals have been completed for the 37 blood parameters for males and for the 37 parameters for females. Those 43 blood parameters which showed no male-female differences remain to be analyzed.

Immunization system. A computerized system is now operational for recording special immunizations which are scheduled for at-risk personnel at USAMRIID and the results are monitored. The data base consists of immunization data on 319 active and 245 inactive personnel, back to 1972. The programs in this system provide: (1) monthly lists of personnel requiring special immunizations or titers, (2) appropriate forms to record all immunization transactions, (3) statistics and histograms concerning immunization effectiveness, (4) a monthly list of at-risk personnel who are delinquent in receiving immunizations and (5) complete immunization records for each individual in the special immunization program.

The system has been automated to the point where the data base can be queried for summary immunization and titer information. This has proven to be a valuable asset to the USAMRIID Immunization Committee, the Occupational Health Nurse, and the Scientific Advisor in providing information for evaluating the effectiveness of current vaccines and vaccination procedures. The results of one search presented to the Immunization Committee prompted a decision of the Committee to modify the current immunization schedule for EEE and WEE vaccines by eliminating unnecessary immunizations while maintaining high levels of protection.

During the past year, 8 special queries of the data base have been made in response to requests for information:

1. Histograms and statistics on pre-titer and post-titer values of the initial, first 6-month booster and subsequent annual EEE and WEE immunizations.

2. Lists, statistics and histograms, showing titers on all individuals and on individuals whose last VEE titer was $\leq$ 1:80.

3. Lists, statistics and histogram showing titers on all individuals and on individuals whose last yellow fever titer was $\leq$ 1:40.

4. Division listings of all individuals active in the special immunization system before 1974.

5. Statistics on titers of VEE shots given within and after 40 days of EEE and WEE shots.

6. Statistics on individuals who no longer receive EEE and WEE boosters and have had at least 2 titers since the last booster.

7. Alphabetical listing of all personnel active in the special immunization data base and appropriate statistics.

8. Alphabetical listing of all personnel inactive in the special immunization data base and appropriate statistics.

parameter is often used to generate diagnostic information, even though an illness may be associated with many simultaneous changes in the chemical composition of body fluids. Although some changes may individually be biologically insignificant, information on the simultaneous changes in a number of parameters, i.e., biochemical profiles, could be expected to yield information with higher diagnostic relevance.

The ultimate objective of this project is to develop profiles of biochemical values to aid in early detection and diagnosis of infection in man. Current efforts have centered on early detection.

The data base consists of controls and/or patients from 8 hospital and volunteer studies. Each study has values on ∿ 105 biochemical parameters. During the past year, all efforts in this project have been confined to examining control studies, in order to establish reference population data to which ill individuals can be compared. Once this control population is properly evaluated, it will be possible to determine the probability of occurrence of a change in each parameter measured on ill patients. These probabilities will then be combined as a means of increasing the overall probability of detecting biochemical parameter changes in ill patients.

This year all data analysis has been done on a data base of 81 biochemical parameters collected from each participant (130 males and 80 females) in a USAMRIID volunteer control study conducted in August 77. As reported last year, few of these serum chemistry parameters were found to have Gaussian distributions. Age differences were seen in some of the parameters, and 37 of the 81 parameters showed statistically significant differences between male and female populations. The following steps are being used to describe statistically the distribution of each biochemical parameter of the USAMRIID controls:

1. Generate a polynomial equation to fit the data from each parameter.

2. Transform the polynomial curve to a Gaussian distribution.

3. Compute the mean, SD, and confidence intervals on the transformed curve for each parameter.

4. Transform the mean, SD, and confidence intervals back to the original units of the data.

This type of analysis has been used to obtain means and normal ranges (95% confidence intervals) for Zn, Fe, and Cu from the USAMRIID controls. Having defined the control populations, the trace metal responses of ill patients (West Virginia University Medical Center) were each compared to the controls, and the exact probabilities of occurrence of the values of Zn, Fe, and Cu for each of the ill patients were combined for each patient. This procedure combines 3 probabilities which may not be small enough to be significant individually, but which result in a significant probability when multiplied times each other. The power of using a multi-parameter profile can be seen in the case where >80% of the ill patients showed abnormal trace metal (Zn, Fe, Cu) profiles typical of infection, whereas only 25% showed an abnormal response in Zn alone. Then, using a combined profile of trace metal response, this increases the probability of detecting trace metal changes in ill patients.

Analysis of all 81 blood parameters is not complete, but there appears to be

Separate computer programs were written for each of the above queries. This points out a major problem with the current computerized Immunization System, i.e., the data base cannot be queried by a non-ADP user (Medical Division personnel). It is essential that such a capability be incorporated into the system, with a terminal located in Ward 200. A major effort will be initiated next year to implement the Immunization System on a Data Base Management System on the IBM 360, so that a user can query the data base via a terminal by asking English-type questions instead of writing a program for every question.

Consultation. Biostatistical/computer consultations were held with investigators of all divisions of the Institute to develop solutions to experimental design, statistical or computational problems. A large amount of time and resources are spent in meeting these data analysis problems.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6413	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	036
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Spontaneous diseases in laboratory animals used for developing medical anti-BW defense

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
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 79	1.0	39.6
FISCAL YEAR CURRENT 80	1.0	63.5

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

RESPONSIBLE INDIVIDUAL
NAME: Barquist, R. 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: DePaoli, A.
TELEPHONE: 301 663-7211
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Rozmiarek, H.
NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Laboratory animals; (U) Spontaneous diseases

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

23 (U) To evaluate and monitor the health status of laboratory animals on arrival to the Institute and to identir and characterize spontaneous diseases which develop in laboratory animals while in the colony or on research projects. This information is necessary to preclude or minimize the experimental variable of natural diseases, allow selection of adequate animal suppliers, control zoonoses and ultimately the successful completion of the laboratory's BW research defense research mission.

24 (U) Predetermined numbers of animals from the various suppliers will be sacrificed on a monthly basis and monitored histopathologically. In addition, diagnostic techniques to include clinical pathology, histopathology, animal inoculation, etc. will be employed to investigate all natural animal deaths.

25 (U) 78 10 - 79 09 - Quality control monitoring of rodents entering the Institute during this reporting period revealed that most animals supplied to USAMRIID were of acceptable quality, although not disease-free. The principal endemic diseases in incoming animals with greatest potential for disrupting or invalidating experimentation were Sendai virus infection in mice, interstitial pneumonia and encephalitozoonosis in guinea pigs and chronic murine pneumonia in rats. Disease surveillance of Institute colony animals revealed sporadic deaths in the rodent population which were primarily bacterial. Forty-five nonhuman primates were lost, 15 as a result of natural diseases and 30 from complications related to experimental manipulation, primarily vascular catheterization.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 036: Spontaneous Diseases in Laboratory Animals Used for Developing Medical Anti-BW Defense

Background:

The usefulness of laboratory animals in medical research is dependent to a large degree on the reproducibility of experimental procedures. The single most important factor determining data reproducibility in a given laboratory animal species is the variable of concurrent animal diseases. The severity of such diseases varies from clinically silent or chronic enzootic conditions to acute highly lethal states. All, however, may interfere with animal experimentation, compromise experimental data, or worse, preclude planned studies. It is apparent, therefore, that the variables of clinically silent diseases in the experimental animal must be characterized and if possible eliminated. Likewise, disease outbreaks or unexplained deaths of animals on experiment must be investigated and their impact on the ongoing studies evaluated.

Progress:

Monitoring of rodents supplied by vendors to USAMRIID identified a number of subclinical endemic diseases in animals entering the Institute. Most of the conditions were sporadic and of little significance. A number, however, had the potential for disrupting experimental studies in which the affected animals were employed. These included Sendai virus in mice and interstitial pneumonia and encephalitozoonosis in guinea pigs. Evidence of Sendai virus infection in mice was found sporadically in shipments throughout this monitoring period. Typically, 1 or 2 of the animals selected for examination from a given shipment had characteristic lesions of this infection. These lesions usually were mild and consisted of acute necrotizing and/or proliferative bronchiolitis with concomitant patchy interstitial pneumonia. The low attack rate and observed early lesions suggest these animals represent activation of latent Sendai infection by the stress of shipment.

An interstitial pneumonia of unknown origin but morphologically consistent with a viral or Mycoplasma etiology was present in many of the incoming guinea pigs. The full impact of this endemic disease on Institute projects employing guinea pigs cannot be evaluated as these guinea pigs are not necropsied at terminus of some studies. However, there is little doubt that some studies have been badly compromised by the presence of this condition in the guinea pigs under study, i.e., aerosol studies of Legionella pneumophila.

Encephalitozoonosis, a protozoan disease caused by Encephalitozoon caniculi, made its reappearance in the guinea pig population during the last quarter of the year after its apparent absence for a period of a year. This endemic disease characterized by granulomatous encephalitis is a potential problem particularly to virological studies encompassing CNS disease.

Finally, as in previous years early lesions of chronic murine pneumonia (CMP) caused by Mycoplasma pulmonis were present in nearly all rats entering the Institute

and in approximately 5% of mice. This ubiquitous respiratory disease of rats has had little effect on ongoing studies which typically, are short-term experiments. However, because of its progressive nature this condition poses a considerable problem for long-term studies.

Disease surveillance of colony animals during this reporting period revealed sporadic deaths in the rodent population, primarily due to bacterial diseases. Forty-five nonhuman primates were lost, 15 as a result of natural diseases and 30 from complications related to experimental manipulations, primarily vascular catheterization. Causes of death included: bacterial pneumonias, hypovolemic shock, thromboembolism, pulmonary infarction, cerebral infarction, necrotizing aortitis, cerebral edema, septicemia and gentomycin toxic nephrosis.

An outbreak of mucoid enteritis, a sporadic but occasionally devastating disease of rabbits, occurred in one group of rabbits on an immunological study. The cause of mucoid enteritis is not known but enteric bacterial toxins are believed to play a role. The disease is acute, usually lethal, and characterized by watery stools containing copious amounts of mucus.

In summary, monitoring of diseases in laboratory animals revealed that while a number of potentially disruptive health problems were present in some animals entering the Institute, the health status of most animals was acceptable.

Presentations:

1. DePaoli, A. Gastrointestinal neoplasma of nonhuman primates. A review and report of eleven new cases. Presented, Symposium on Gastrointestinal Diseases of Nonhuman Primates, San Francisco, CA, 5 March 1979.

2. DePaoli, A. Pathology of gastrointestinal diseases of nonhuman primates. Presented, Pathology of Laboratory Animals Course, AFIP, Washington, DC, August 1979.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6429	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	040
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Hazards and variable associated with research animals used in medical defense against BW

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 010100 Microbiology; 02600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 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 79	1.0	82.9
	CURRENT 80	1.0	120.7

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

RESPONSIBLE INDIVIDUAL
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20. PERFORMING ORGANIZATION
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
NAME: Rozmiarek, H.
TELEPHONE: 301 663-7221
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Miller, Jr., J. G.
NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Parasites; (U) Laboratory animals; (U) Microbiology; (U) Infectious diseases;

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

23 (U) Monitor clinical health of newly arrived and in-house research animals. Investigate any deviation from normal and evaluate the effect on proposed use in ongoing research at USAMRIID; institute corrective and preventive measures when indicated. This work is essential to assure that the best quality of animal possible is used in all critical infectious disease research of military significance in medical defense against biological attacks.

24 (U) Conduct a quality control program for newly received animals to include evaluation of their viral, bacterial, parasitic, hematologic, metabolic and neurologic status. Histopathologic evaluation will be done where indicated. All primates will be examined upon receipt and semiannually thereafter for symptoms of zoonotic and other diseases, to include testing for tuberculosis. The effects of any deviations from normal on their proposed use in ongoing research will be investigated in detail and evaluated.

25 (U) 78 10 - 79 09 - Significant findings included Salmonellosis in rodents, encephalitozoonosis in rabbits, nematodiasis in rodents, and acute, highly transmissible Klebsiella pneumonia in guinea pigs. All conditions were detected by routine surveillance; animals were diverted from use in research. Chronic respiratory disease continues to be detected in rats, but close surveillance and monitoring of animal suppliers has kept complications to a minimum. No animals with severe CRD lesions were observed. No positive cases of tuberculosis, measles, or herpesvirus B were detected in nonhuman primates. Use of the electron micrscope as an early diagnostic tool shows great potential in virology research. Evaluation of rodent beddings revealed advantages of certain materials for use in closely contained cages and facilities.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 040: Hazards and Variables Associated with Research Animals Used in Medical Defense Against BW

Background:

Even in well-managed, efficient research institutes, the constant hazard of contamination of normal conventional animals with undesired unusual disease entities exists. This is especially a problem for an infectious disease research institute, as the invading condition may be masked by the effects of the conditions under study. This potential for contamination is amplified at USAMRIID where space limitations generally preclude in-house breeding programs, and most conventional animals are purchased from commercial sources. Animals subjected to the stresses of shipping and handling are especially susceptible to a wide variety of bacterial and viral organisims. They are often latent carriers of disease when received. Symptoms may not show up until the animals are stressed in research. An effective system of identifying these latent conditions, characterizing them and eliminating them whenever possible is essential to maintaining the integrity and reproducibility of research.

Progress:

The animal disease surveillance program is continuing as established over the past 2 years and includes routine parasitology, bacteriology, hematology and histopathology. During the year, 668 rodents from 9 different commercial sources were examined. Primates were evaluated before being issued for use in research: 1,430 evaluations were conducted which included parasitology, bacteriology, serology, and electrocardiograms.

Rodents from 3 commercial vendors were infested with pinworms (Syphacia obvelata) when delivered during the year. The vendors were contacted; the infestations were eradicated in 2 of the breeding colonies. In the third, a contract was terminated and animal procurement diverted to another source. Although infestation with the pinworm does not cause fatalities or even clinical signs in healthy rodents, it can cause clinical illness when animals are subjected to experimental stress. Subclinical infestation results in significant changes in metabolic parameters and should not be tolerated in a research animal colony. Infection with salmonellosis was detected in mice from one supplier and the animals were diverted from use in research. Salmonellosis is a frequently reported disease in mice, resulting in a variety of symptoms ranging from mild anorexia to death. Control and treatment of Salmonella-infected mice is impossible, since antibiotic therapy is helpful but does not eradicate the infection. It presents a zoonotic threat, as several serotypes can infect both humans and animals. Future mouse orders were diverted to avoid purchasing from suppliers with potential disease problems.

Chronic respiratory disease (CRD) is found in differing degrees of serverity in virtually all conventional rats; it was found in 21% of USAMRIID rodents surveyed during the year; 26% fell in the mild category and 13% were classified as minimal lesions histopathologically. No severe lesions of CRD were observed.

Persistent respiratory abnormalities in guinea pigs from one commercial vendor resulted in elimination of the entire group and also elimination of other guinea pigs housed in the room with them. An existing contract with this supplier was terminated and orders diverted to other suppliers. Further investigation revealed infection with Klebsiella pneumoniae; lesions of pneumonia were identified on histopathologic examination.

Encephalitozoon cuniculi was detected in one animal this year. Further investigation did not reveal any other infestations. This latent disease not only is notorious for having compromised the results of many animal experiments, but presents a zoonotic hazard as well. It infects a wide range of laboratory animals, including rats, mice and rabbits, and cannot be tolerated in a research animal facility.

No positive cases of tuberculosis, measles or herpesvirus B were detected in primates. A rhesus monkey involved in a human bite exhibited oral lesions and was evaluated for the presence of herpesvirus B. A biopsy of the lesions revealed no inclusion bodies. Specimens taken from the lesion were negative by virus isolation attempts. The monkey did not demonstrate detectable antibody to herpesvirus B on the date of the bite or 7 or 14 days later. It was concluded that the animal was not transmitting herpes B virus at the time it bit the human.

During this year, CPT Heisey began and completed work on a project entitled "The Effects of Isoniazid, and Antituberculosis Drug, on the Guinea Pig (Cavia porcellus)." A thesis on this work was submitted to the graduate school of Pennsylvania State University and fulfilled the research requirement for CPT Heisey to be granted the Master of Science degree in the Department of Comparative Medicine. A scientific paper has been submitted to the Laboratory Animal Science Journal for publication.

A study to evaluate the absorbency and ammonia control potential of 5 different bedding materials in filter-top rodent cages was initiated this year. This comparison was completed in guinea pigs and was presented at the national AALAS meeting by CPT Bryant. A scientific publication is currently in preparation.

Electron microscopy was used to screen primates for viruses before being used in research. As the techniques in use were perfected, CPT Graham expanded this work to include use of the electron microscope as an early diagnostic aid in the study and identification of viruses under investigation in the institute. Close collaboration with Dr. Jahrling of Virology Division resulted in the preparation of two scientific articles for publication.

Presentations:

1. Bryant, J. M. Acariasis causing alopecia in rodents. Presented, 8th Annual Session, NCAB/American Association for Laboratory Animal Science, Hunt Valley, MD, 13-14 Sep 78.

2. Rozmiarek, H. The role of AAALAC in accreditation of biomedical research institutions. Presented, USAF School of Aerospace Medicine, Brooks Air Force Base, TX, 6-11 May 79.

3. Heisey, G. B. MS Thesis. The effects of Isoniazid on antituberculosis drug on the guinea pig (Cavia porcellus). Presented, Pennsylvania State University, May 79.

4. Rozmiarek, H. Waste disposal from a biohazardous containment animal facility. Presented, 30th Annual Session of the American Association for Laboratory Animal Science, Atlanta, Georgia, 16-21 Sep 79.

5. Bryant, J. M., and H. Rozmiarek. Ammonia control potential of five rodent contact beddings. Presented, 30th Annual Session of the American Association for Laboratory Animal Science, Atlanta, GA, 16-21 Sep 79.

Publications:

1. Heisey, G. B., H. C. Hughes, H. Rozmiarek, and C. M. Lang. 1979. The effects of Isoniazid, an antituberculosis drug on the guinea pig. J. Infect. Dis.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6411	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	043
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Respiratory disease mechanisms, pathogenesis and therapy of airborne infections

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
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		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	2.0	137.3
	CURRENT 80	2.0	188.1

19. RESPONSIBLE DOD ORGANIZATION
NAME: USA Medical Research Institute of Infectious Diseases
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20. PERFORMING ORGANIZATION
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PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
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ASSOCIATE INVESTIGATORS
NAME: Dominik, J. W.
NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Prophylaxis; (U) Viral disease; (U) Aerosols; (U) Respiratory infections; (U) Pathogenesis

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

23 (U) Examine respiratory disease mechanisms, including penetration, retention, clearance and replication of pathogens introduced in the host by enemy attack via the respiratory route. The research is essential for determining the pathogenesis and developing promising approaches to prophylaxis and therapy of infections, both respiratory and systemic, acquired by host inhalation, the most likely means of exposure in BW operations.

24 (U) Assess aerosol properties of infectious microorganisms and challenge experimental animals by respiratory route; assess postexposure effects using microbiological, histopathological and immunological methods.

25 (U) 78 10 - 79 09 - Aerobiological research studies have demonstrated that both the Machupo and Lassa fever viruses are relatively stable in aerosol, especially at low relative humidities and both viruses are highly infectious and moderately lethal for guinea pigs and cynomolgus monkeys by the airborne route. Initial information was acquired on the pathogenesis of the airborne infections. To date the data have suggested that the lung represents a primary site for virus replication with virus transport via the circulation to a wide range of organs also capable of supporting virus growth.

Publication: Proc. Soc. Exp. Biol. Med. 161:350-354, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M762776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 043: Respiratory Disease Mechanisms, Pathogenesis, and Therapy of Airborne Infections

Background:

We reported previously on the aerosol stability and respiratory infectivity properties of Japanese B encephalitis (JE) virus (1). The investigations were undertaken to assess the potential threat of JE virus as a biological warfare agent and to determine the possible hazards of airborne infection among research personnel. Limited studies were completed, also, on the pathogenesis of the infections in laboratory animals in order to contrast the characteristics of the airborne infections with infections induced by more conventional routes of challenge.

Continuing efforts were focused on similar studies with selected arenaviruses, specifically the viruses of Bolivian hemorrhagic fever (Machupo, MAC) and Lassa fever (LAS). These investigations were undertaken as the initial efforts of a broadly based program to evaluate the aerosol stability and respiratory infectivity properties of viral agents of potential importance in medical defense.

Progress:

Aerosol stability and respiratory infectivity of Machupo virus. The Carvallo strain (#21677) of MAC virus was employed throughout these investigations. The stock virus was furnished by Virology Division and represented a 1974, 3rd passage harvest raised on BHK-21 cells in roller bottles. Quantitative assays of all virus suspensions, aerosol samples and clinical specimens were performed using established plaque enumeration procedures on Vero cell monolayers. Assays of the stock virus consistently yielded concentration estimates of $10^{6.9}$ PFU/ml.

Studies to investigate the aerosol stability properties of the virus were conducted in one of the Aerobiology Division, environmentally controlled, 6200-L aerosol chambers. Two experiments were completed. In a first study, 4 replicate aerosol trials were conducted at each of 3 relative humidities (RH), 80, 55, and 30%. Aerosol temperature was maintained at 75°F. Undiluted virus (1 ml) was disseminated in each trial using a standardized small particle generator. Aerosol samples were collected at aerosol ages of 4, 32, and 60 min in each trial. Similar conditions were employed in a second experiment with completion of 3 replicate trials at each RH.

Table I presents a summary of the findings and shows that similar results were obtained in both experiments. Approximately 40% of the total virus subjected to dissemination was airborne at 4 min of aerosol age. These results suggested efficient survival of the virus during the processes of dissemination and equilibration with the aerosol environment. The early age aerosol recoveries were not effected by RH. After 4 min, however, aerosol stability was influenced markedly by, and inversely related to, RH. The half-life of the airborne virus at the low RH was ∿ 3-fold that at the high humidity. Aerosol decay rates of 30 and 55% RH were well within the ranges observed with other viral, rickettsial, and bacterial pathogens. The decay rate for 80% RH was relatively high and translated to a

half-life of only 7 min.

TABLE I. EFFECTS OF ENVIRONMENTAL HUMIDITY ON THE AEROSOL RECOVERIES, DECAY RATES AND HALF-LIFE SURVIVAL PROPERTIES OF MAC

AEROSOL AGE (Min)	% RECOVERY					
	Experiment 1			Experiment 2		
	80%	55%	30%	80%	55%	30%
4	40.2	36.2	45.0	46.4	43.3	44.0
32	2.96	8.02	11.6	0.94	4.17	11.9
60	0.17	2.64	9.12	0.18	1.47	7.19
Decay rate (%/min)	9.76	4.68	2.85	9.88	6.05	3.23
Half-life (min)	7.1	14.7	24.2	7.0	11.4	21.4

Table II summarizes the results of studies conducted to quantitate the lethal and infectious dose-responses of Hartley outbred guinea pigs (Buckberg farms) challenged with aerosols of MAC. The aerosol challenges were conducted in a dynamic aerosol apparatus equipped for aerosol exposures and sampling. Aerosols were produced with standard Collison generator operated continuously for 10 min in each exposure (2). Three exposure doses with 16 guinea pigs (average wt, 300 gm)/level were achieved by disseminating 10-fold dilutions (1:4, 1:40, 1:400) of the virus. Aerosol concentrations were determined by assays of all-glass impinger samples collected during exposures. Results of these assays formed the basis for estimating exposure doses. After exposure, the guinea pigs were observed for signs of infection; deaths were recorded through 40 days. Sera for titration of plaque reduction neutralization antibodies (PRN) were collected from all surviving guinea pigs at 40 days.

TABLE II. LETHAL INFECTION RESPIRATORY DOSE RESPONSES OF HARTLEY OUTBRED GUINEA PIGS EXPOSED TO MAC AEROSOLS

EXPOSURE DOSE (Log_{10} PFU)	DEAD/TOTAL	GMTD[a]	SEROCONVERSIONS	
			Pos./total	PRN_{50}
1.0	6/16	23.9	8/10	15
2.3	9/16	25.5	6/7	12
3.2	9.16	23.7	7/7	52

$LD_{50} = 10^{2.23}$; $ID_{50} < 10^{1.0}$

[a]Geometric mean time to death in days

[b]PRN tests performed on 40-day sera of surviving animals

By Reed-Muench methods for median dose response calculation an estimated LD_{50} of $10^{2.23}$ PFU was obtained (3). Death patterns, with a mean time to death of 24-25 days, were relatively orderly with respect to exposure dose. With rare exception the guinea pigs which died exhibited partial paralysis, typical of MAC infection, in the time period of 1-3 days prior to death. Even among the 4-day survivors, several guinea pigs exhibited rough coats, emaciation and partial paralysis at the time of sacrifice. Results of PRN tests suggested that all but 3 of 24 survivors were infected; hence the ID_{50}, while undetermined in this experiment, was < 10 PFU.

Additional sudies were undertaken to determine the primary sites of virus replication in infected guinea pigs and to examine the virus population dynamics in selected tissues as a function of time after a high-dose aerosol challenge. Sixteen guinea pigs were exposed to aerosols disseminated as a 1:4 dilution of virus.

Results of aerosol sampling suggested that guinea pigs inhaled $10^{3.95}$ PFU of virus in 10 min. Two guinea pigs were killed at each of 8 periods extending over 27 days. Specimens of blood, brain, liver, lung and spleen from each guinea pig were assayed for virus concentrations. Table III summarizes the results of these tissue specimen assays.

TABLE III. CONCENTRATIONS OF MAC IN VIRUS IN GUINEA PIG TISSUES AS A FUNCTION OF TIME AFTER AEROSOL CHALLENGE WITH 3.95 $\log_{10}$ PFU OF VIRUS

DAYS POSTCHALLENGE	MEAN LOG_{10} PFU/gm TISSUE (n=2)			
	Brain	Liver	Lung	Spleen
3	Neg	Neg	4.82	Neg
6	Neg	Neg	4.61	Neg
10	Neg	Neg	3.28	3.55
13	3.20	4.35 (1)[a]	4.92	5.91 (1)
17	5.91 (1)	4.13 (1)	3.69 (1)	5.09 (1)

[a] 1 of 2 guinea pigs positive, virus concentration is the level in the positive animal.

Blood samples were consistently negative, suggesting that viremias, if any, were below our assay titer sensitivity (~ 20 PFU/ml). In addition virus was not detectable in any tissue after 17 days following challenge. Lung samples were consistently positive for virus in relative high concentrations through 17 days, and virus was present in spleens by 10 days and in the livers and brains by 13 days. The lack of detectable viremias was unexpected in view of the fact that at least one guinea pig from an earlier study, when sacrificed on day 12, was circulating $10^{3.0}$ PFU/ml. Thus, viremias in guinea pigs following respiratory infection with MAC are transient; the lung is a primary organ supporting virus replication; and virus is transported by way of the blood to infect the spleen, liver and brain.

Nine cynomolgus monkeys (3 groups of 3 each) were challenged with $10^{4.3}$,

$10^{3.3}$, and $10^{2.3}$ PFU MAC as small particle aerosols (SPA). Rectal temperatures were measured twice weekly and blood samples, for viremia and serum neutralizing antibody measures were collected weekly. The mortality and day-to-death results are summarized in Table IV.

TABLE IV. MORTALITIES AND DAYS-TO-DEATH OF CYNOMOLGUS MONKEYS CHALLENGED WITH GRADED DOSES OF MAC AEROSOLS

EXPOSURE DOSE (Log_{10} PFU)	MORTALITIES	
	Dead/total	Day of death
2.3	2/3	14, 17
3.3	2/3	14, 17
4.3	2/3	28, 108

Two of 3 monkeys from both the low and medium doses died in the period between 14 and 17 days after exposure. First signs of illness were observed in these monkeys only with 3 days before death. Clinical signs first included anorexia, occasional diarrhea and fever, followed by hypothermia just prior to death. One monkey exhibited hemorrhagic salivation on the day of death; none showed signs of neurologic disease. Necropsy and histopathologic examinations were conducted by Pathology Division (MAJ Callis) on one medium-dose monkey. Lesions consistent with MAC infections were observed in this monkey with microscopic evidence of necrotizing hepatitis, enteritis and colitis. Two late deaths occurred among the 3 high-dose monkeys and both exhibited signs of primary neurologic disease prior to death including whole-body tremors and paralysis in both legs.

Serum samples collected at weekly intervals for viremia and PRN determinations indicated that all 9 monkeys were viremic at either 7 or 14 days, or both. Virus concentrations were in the range of 10^2 and 10^4 PFU/ml of serum. Virus was not recovered from any sera collected after 14 days. Six of 7 monkeys tested were PRN positive by 14 days; all monkeys surviving thereafter were circulating PRN antibodies. PRN_{50} serum titers exceeded 1:528 in all 4 monkeys still surviving at 97 days.

Aerosol stability and respiratory infectivity of LAS. A freshly prepared harvest of the Josiah strain of LAS, raised on Vero cell monolayers, was furnished by Dr. Jahrling, Virology Division. As with the MAC, quantitative assays of virus suspensions, aerosol samples and clinical specimens were performed using established plaque enumeration procedures on Vero cell monolayers. Again, assays of the stock virus (when taken from frozen storage and thawed) have consistently yielded concentration estimates of $10^{6.9}$ PFU/ml.

An experimental design identical to that employed in the MAC studies was applied in a study to investigate the aerosol stability of the LAS. Four replicate trials in a 6200-L static aerosol chamber were conducted at 75°F and each of 3 RH (80, 55, and 30%). Aerosol samples were collected at aerosol ages of 4, 32, and 60 min in each trial. Table V summarizes the results of these aerosol stability trials.

TABLE V. EFFECTS OF RH ON % RECOVERIES, AEROSOL DECAY RATES AND AEROSOL HALF-LIVES OF LAS

AEROSOL AGE (min)	% RECOVERY 80%	55%	30%
4	83.0	65.0	79.6
32	14.4	11.6	36.0
60	3.9	4.5	16.9
Decay Rate (%/min)	5.45	4.73	2.77
Half-life (min)	12.7	14.6	24.9

This LAS was considerably more stable in aerosol than was MAC. Between 65 and 83% of the virus subjected to dissemination was airborne and infective, based on plaque-producing properties, at 4 min after dissemination. As with other viruses examined, i.e., JE and MAC, aerosol stability was inversely related to RH. On the other hand a decay rate of 5.45 %/min, corresponding to a half-life of 12.7 min at 80% RH, was indicative of markedly better stability with this virus than with MAC.

In an initial study to investigate the respiratory lethal dose reponses of guinea pigs to LAS, 32 Hartley strain guinea pigs (Buckberg Farms), weighing 240 gm each were exposed by aerosol in groups of 8 to graded doses of virus. Exposures were accomplished in a dynamic aerosol apparatus by procedures identical to those used with MAC virus. Extimated exposure doses were $10^{0.7}$, $10^{1.7}$, $10^{2.9}$, and $10^{3.7}$ PFU. By Reed-Meunch computational methods, these results yielded an LD_{50} of $10^{3.12}$ PFU.

Eight heavy (625 gm), strain-13, guinea pigs received from Dr. Jahrling were exposed to the lowest dose in these trials. Three of these animals succumbed, supporting the findings by Virology Division that this inbred guinea pig is highly susceptible to LAS. With both the Hartley and strain-13 guinea pig strains, deaths occurred between 19 and 31 days with a mean time to deat of 25.

The objective in a subsequent study was to examine the virus concentration dynamics in selected tissues of guinea pigs after aerosol challenge. Thirty-two Hartley strain guinea pigs (Buckberg) weighing 192 gm each were challenged with a high dose ($10^{4.1}$ PFU) of virus as SPA. Two guinea pigs were killed at each of 10 periods extending over 33 days; specimens of blood, brain, liver, spleen, lung and upper respiratory tract (lung) from each guinea pig were collected for measures of virus concentrations.

Assays from the first 7 periods extending over 24 days after exposure have been completed; Table VI summarizes these results. Measures of virus concentrations were highly reproducible and generally consistent between animals within a period. High concentrations of virus were measured from lungs throughout the period of measurement with peak concentrations exceeding $10^{7.0}$ PFU/gm. Only the spleen yielded higher concentrations, starting at 6 days and peaking between

$10^{8.0}$-$10^{8.5}$ PFU/gm. The blood, brain, liver and URT containing modestly lower concentrations of virus, though, even in these tissues the virus titers were very significant and persisted through 24 days. These data suggested that the lung represents a primary site for virus replication with virus transport via the circulation to a wide range of organs also capable of supporting virus growth.

TABLE VI. LOG_{10} PFU LAS PER GM OF GUINEA PIG TISSUE AS A FUNCTION OF THE TIME AFTER AEROSOL CHALLENGE WITH $10^{4.1}$ PFU OF VIRUS

DAYS POSTCHALLENGE	MEAN LOG_{10} PFU/gm TISSUE (n=2)					
	Bood	Liver	Spleen	Brain	Lung	URT
3	Neg	2.55	Neg	Neg	3.50	Neg
6	2.37	2.48 (1)[a]	6.03	Neg	6.33	4.85
10	4.30	4.55	8.08	3.17	7.35	6.52
13	4.69	5.30	8.51	3.67	7.41	6.99
18	4.24	4.32	7.77	6.79	7.20	6.71
20	4.31	4.58	7.76	4.45	6.80	6.75
24	3.30 (1)	3.98 (1)	5.79 (1)	4.76 (1)	4.60 (1)	5.19 (1)

[a] 1 of 2 guinea pigs positive, virus concentration is the level in the positive animal.

Three cynomolgus monkeys in apparent good health after recovering from earlier MAC infection, were challenged by aerosol with $10^{4.8}$ PFU of LAS; 2 died with apparent primary LAS fever at 12 days. Sera contained $10^{3.0}$ and $10^{3.4}$ PFU/ml. One monkey survived and showed no signs of illness through 48 days when the monkey was exsanguinated as a source of immune serum. As evidence that this monkey was infected, serum from a 6-day sample contained $10^{2.2}$ PFU of virus/ml.

The research completed in this report period has demonstrated that both MAC and LAS are relatively stable in aerosol, especially at low RH. Both viruses are highly infectious and moderately lethal for guinea pigs and cynomolgus monkeys by the airborne route. Initial information has been acquired on the pathogenesis of these airborne infections. Investigations for the immediate future will focus on completion of the LAS studies currently in progress and continuance of definitive respiratory dose-response studies in cynomolgus monkeys. It is proposed that similar investigations will be undertaken with additional arenaviruses including Junin and Ebola viruses as soon as practicable.

Presentations:

1. Larson, E. W., Hazard potential from laboratory activities: aerosol generation. Presented, National Institute of Environmental Health Sciences, Research Triangle Park, NC, Dec 1978.

2. Larson, E. W., Experimental aerobiology and the transmission of respiratory infections. Presented, Department of Medicinal Chemistry, Medical School, University of Tennessee, Memphis, TN, Feb 1979.

Publications:

1. Wilson, S. Z., V. Knight, R. Moore, and E. W. Larson. 1979. Amantadine small-particle aerosol: generation and delivery to man. Proc. Soc. Exp. Biol. Med. 161:350-354.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6412	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 08 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	62776A	3M162776A841	00	045
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Animal models and animal resources for medical defense studies of diseases of BW importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 002600 Biology; 001700 Animal husbandry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
76 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 79	1.0	107.6
	CURRENT 80	1.0	120.9

19. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Miller, J. G.
NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Primates; (U) Animal models; (U) Animal resources

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

23 (U) Evaluate animals as models for the study of infectious and other disease processes under study at USAMRIID; establish such models when appropriate animal species do not exist. Develop facilities and expertise necessary to raise and produce these animals in-house when not available commercially. Establish expertise to raise rhesus monkeys in-house in the event that present scarce supplies become nonexistent or economically prohibitive. This work is essential to assure an adequate supply of appropriate animal models for the study of infectious diseases of potential BW importance.

24 (U) Establish breeding, maternity, obstetric and pediatric techniques for rhesus monkeys. Establish breeding colonies of other laboratory animals as needs arise for specific entities dealt with by USAMRIID investigators.

25 (U) 78 10 - 79 09 - Production in the Calomys callosus and Sigmodon hispidus colonies stabilized to meet all investigator needs for these species. The Microtus colony was abandoned after a final feasibility study by Virology Division showed the vole to be unsuited to their research needs, and in view of the close proximity and ready availability in this species through FDA investigators at Bethesda. A paper reporting Demodex sp. of mites in C. callosus is in preparation. The 24 female rhesus monkeys in our breeding colony produced 14 offspring during the year, bringing the total number produced to 33. Statistical analysis of data collected to date on growth, maturation and hematologic parameters showed earlier, more rapid growth to higher levels than earlier reports, while complete blood counts correspond very closely with previously reported values.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 045: Animal Models and Animal Resources for Medical Defense Studies of Diseases of BW Importance

Background:

With the many varied and unusual organisms used at USAMRIID, occasional diseases or organisms are encountered for which no animal model or natural reservoir is known. In some instances these facts are known, but no reliable commercial source exists for that particular animal species. If no animal model is known, investigations must be carried out to determine what species would be acceptable. If a model is known but availability is a problem, in-house colonies must be established to meet needs.

Two rodent species not available commercially have been identified as models for diseases under investigation at USAMRIID. Calomys callosus (vesper mouse) is the natural reservoir host for many arenaviruses, including Machupo virus and is the only available known species colonized which allows Korean hemorrhagic fever (KHF) to grow and replicate. Sigmodon hispidus (cotton rat), is a natural reservoir for VEE and Tamiami viruses.

Macaca mulatta has long been the prime nonhuman primate for a variety of research endeavors, and continues as such at USAMRIID. Increasingly stringent export restrictions by the Indian Government over the past several years culminated early in 1978 with a total ban on its export from that country. The large data-base and volumes of experimental results available on the rhesus require that efforts be made to somehow maintain supplies of this research animal. To this end, a program was initiated to develop the expertise necessary to produce and raise the species domestically.

Progress:

Rodent colonies. With improved record-keeping, established breeding pairs, programmed replacements, and systematic outbreeding, production in the C. callosus and S. hispidus colonies attained the levels anticipated to meet all investigator needs. Stabilization of colony size for each of these species was achieved as desired at 50 breeding pairs, with the resultant production of 625 S. hispidus and 594 C. callosus this year. Higher production with the same number of breeders is possible, but declining investigator requests resulted in the decision to allow the number of offspring to decrease for the last two quarters of this fiscal year.

After feasibility studies by Virology Division showed Microtus sp. (voles) not to be suited to their needs, and in view of their continuing reproductive difficulties at USAMRIID, the few remaining vole breeders were sacrificed for complete necropsies. Investigators at FDA laboratories at NIH, have an established colony of these animals and have agreed to supply breeding pairs to USAMRIID for reinstitution of a breeding program if the need arises.

High production levels in the C. callosus colony provided more animals than required and allowed us to utilize these extras in several ways. Complete hemograms were performed on some and a large group was held for signs of clinical acariasis after Demodex sp. of mites were discovered in some colony members. The acaricide study planned did not materialize, but the discovery of this mite in this species of animal has not been reported and a case report for publication is currently in preparation.

Rhesus colony. Reproduction in the M. mulatta colony continued at the anticipated high level for the year, with 14 births, 7 juveniles weaned, and 6 confirmed pregnancies in the 24 females comprising the colony. Revised data collection schedules proved advantageous as collection and analysis were more easily accomplished by the less sporadic input provided. With several of the older juveniles reaching 2 years of age, statistical analysis of the data collected to date was initiated and the results presented at the Animal Resources Division Professional Staff Seminar in May, 1979. These data are currently being incorporated into a research paper being prepared for presentation and publication.

The paper being prepared deals with the study of age related changes in body weight, linear body measurements, skeletal maturation, and hematologic values determined sequentially from the same individuals during their growth period. Our findings from laboratory-born and maternally reared macaques correspond in general with previously reported data from animals under less controlled conditions. Trends are similar, with principal differences seen in slightly earlier maturation, more rapid weight gain to higher levels with corresponding larger linear dimensions in our animals. Hematologic parameters follow very closely those reported previously for infants and juveniles of this species.

The continuing problem of deaths in the colony due to gastric dilatation was manifest in the loss of 4 adult females and 2 infants during this year. No explanation has been proposed or found for the higher prevalence of this condition in the breeding colony than in the general USAMRIID nonhuman primate population, with this being an area bearing future consideration as to predisposition of a somewhat closed colony and possible prophylactic measures.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6422	79 10 01	DD-DR&E(AR)636

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

10. NO 'CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	046
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Role of bacterial exotoxins in disease pathogenesis

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
75 07	CONT	DA	C. In-house

17. CONTRACT GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	1.0	56.0
FISCAL YEAR CURRENT 80	1.0	76.6

19. RESPONSIBLE DOD ORGANIZATION

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NAME:

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Exotoxins; (U) Pseudomonas aeruginosa; (U) Burn infections; (U) Laboratory animals

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

23 (U) Characterize certain potent bacterial exotoxins and identify their role in infection. The exotoxin (PE) used as a model, Pseudomonas aeruginosa, is believed to be responsible for the high frequency and severity of Pseudomonas infections of burn wounds. Both the bacterium and its exotoxin are potential BW agents. Characterization of this exotoxin will improve our ability to prevent, diagnose, and treat bacterial infections or intoxications, and deal with novel uses of these and other pathogenic agents.

24 (U) After characterizing and developing an assay for exotoxins in mice, examine the biological factors involved in toxin synthesis and pathogenesis.

25 (U) 78 10 - 79 09 - PE was prepared in high yields and supplied to investigators studying pathogenesis, somatic cell genetics, and 3-dimensional protein structure. Collaborative studies were begun of the protective role of the PE glutaraldehyde toxoid in pulmonary infections. 125-I-PE binding studies demonstrated that mouse fibroblasts have 100,000 receptors of rather low affinity. Immunocytochemical staining and autoradiography showed that PE receptors are localized in "coated pits," strongly supporting the hypothesis that this toxin enters cells by adsorptive endocytosis. Diphtheria toxin internalized by Vero cells is degraded to amino acids; inhibition by chloroquine indicates that this process occurs in lysosomes. Degradation in cell extracts is blocked by inhibitors of cathepsin B. The diphtheria toxin receptor Vero cells was solubilized from purified membranes and was demonstrated by immunoprecipitation.

Publication: Abstracts, Am. Soc. Microbiol. 1979, p. 17.

*Available to contractors upon originator's approval

☆ U.S. GPO 1974-542-843/8691

BODY OF REPORT

Project No. 3M762776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 046: Role of Bacterial Exotoxins in Disease Pathogenesis

Background:

Bacterial exotoxins are responsible for the pathogenesis of certain infections (diphtheria, cholera, tetanus, anthrax) and may play a role in others. Characterization of certain exotoxins has led to successful immunization with coincident protection from infection.

Pseudomonas aeruginosa infection is a frequent and serious complication in debilitated patients, especially those suffering extensive burn wounds. Of the toxic materials produced by this bacteria, the most potent is the ADP-ribosylating exotoxin A. This exotoxin (PE) is produced by nearly all clinical isolates of P. aeruginosa and is lethal to a variety of animals and cultured cells. Recent evidence suggests that antibody to PE is somewhat protective in human infections (1). We have developed a toxoid which effectively induces neutralizing antibodies in experimental animals. Test of this toxoid in animal models should help determine the role of PE in infection.

As part of a broad ranging study of PE we are examining how it enters cells and becomes enzymatically active. Prior work suggested that diphtheria toxin (DE) and PE enter cells by adsorptive endocytosis and are activated in lysosomes. The resemblance of the uptake of these toxins to that of polypeptide hormones and growth factors allows application of a wide body of knowledge to the study of the mechanism of action of bacterial toxins.

Progress:

We continued to produce and purify PE as required to maintain stocks both for our own use and for distribution to qualified investigators outside USAMRIID. Continued improvements in technique have increased production so that a recent fermentation yielded 300 mg of toxin, the largest batch yet obtained. The method developed here also provides the basis for a purification procedure that recently appeared in Methods in Enzymology (2). The authors, Drs. Iglewski and Sadoff, learned the relevant procedures (fully acknowledged in the review) during a 3-day visit to USAMRIID several years ago.

We received and filled requests from 11 investigators for toxin, toxoid or antitoxins. A graduate student from the laboratory of Dr. C. B. Saelinger, University of Cincinnati, also came to assist in the fermentation and learn the procedure for PE purification. The 140 mg of PE obtained will allow Dr. Saelinger's laboratory to undertake a variety of studies on the role of PE in infection. Many investigators are using PE in combination with DE to select toxin-resistant mammalian cell mutants. Simultaneous resistance to both toxins is a dominant phenotype conferred by mutation in the gene coding for elongation factor 2 (EF-2). Since the mutation is dominant, stable, easily detected and well behaved, it is rapidly becoming popular in studies of somatic cell genetics and mutagenesis. Supplying

Pseudomonas toxin freely to these workers should facilitate progress in this field.

Pseudomonas toxin has also been supplied to Dr. Paul Sigler, an experienced X-ray crystallographer at the University of Chicago. Having been assured that large amounts of toxin could be provided, Dr. Sigler is now actively seeking to produce crystals of this protein. Determination of structure by X-ray diffraction could help explain how PE binds to and kills cells.

Tests of the glutaraldehyde toxoid of PE in burned mice are continuing at NMRI in collaboration with Dr. O. Pavlovskis. Several more preparations of the $Al_2(PO_4)_3$ adsorbed toxoid were prepared for this purpose. It is anticipated that these studies will be completed soon and that a manuscript describing the results will be prepared. New studies of the protective action of PE toxoid were begun by Dr. Klinger, Case-Western Reserve University. Dr. Klinger will use a pulmonary infection model in dogs and rabbits in hopes of better understanding why cystic fibrosis patients are particularly susceptible to P. aeruginosa infections.

Biochemical study of the binding and internalization of PE by sensitive mammalian cells was made feasible by our prior development of a lactoperoxidase iodination method for radiolabeling PE. However this work has been hampered by the high non specific-binding of ^{125}I-PE to cells. In a continuing effort to understand and thereby eliminate this nonspecific binding, we determined the size of the radioactive material bound to L-929 cells at 4°C. In addition to the 66,000 MW peak expected for PE, the SDS gels of solubilized cells showed a peak of MW ~ 25,000. When the labeled PE was bound to Vero cells, which are relatively less sensitive to PE, a higher proportion of the bound radioactivity was found in the low MW peak. Operating on the assumption that the labeled PE contained a trace impurity with high nonspecific affinity for cells, we subjected the PE to several different purifications. Unfortunately none of these led to a consistent elimination of the low MW peak.

Another limitation in binding studies was the apparent difficulty in saturating receptors even at high PE concentrations. These experiments used excessive amounts of PE because experiments with monolayer cells require rather large volumes of culture media per cell. This problem can be circumvented by using highly concentrated suspension L-929 mouse fibroblast cells and harvesting the incubation mixtures by brief centrifugation on sucrose gradients. The L-929 cells have about 100,000 receptors which can be saturated at 10-20 µg PE/ml. While accurate quantitative data (i.e. Scatchard plots) have not yet been obtained, it appears that receptors for PE have a relatively low affinity compared to those for DE (where saturation is achieved at 0.1 µg DE per ml).

A substantial effort has been made to prove our hypothesis that the PE and DE enter cells by adsorptive endocytosis. Microscopic evidence is being sought to show that PE binds to receptors localized in "coated pits" on the cell surface and is then taken into vesicles which later fuse with lysosomes. The largest part of our studies have employed peroxidase methods, since these are highly sensitive and because the effectiveness of a particular protocol can be evaluated by eye or with the light microscope, thereby avoiding the complex sample preparation needed for viewing samples in the electron microscope. Horseradish peroxidase (HRP) was conjugated to the PE and DE and to IgG fractions purified from the respective antitoxins. These conjugates were purified and then tested in solid-phase systems to establish their quality. The HRP-anti-DE conjugate, as an example, was used in an ELISA test on vinyl Microtiter plates and shown to be capable of measuring DE at concentrations below 1 ng/ml.

In the first attempts to demonstrate receptors on cultured cells 2 "direct" (i.e. HRP-toxin) conjugates were bound to sensitive cells in the presence or absence of excess unmodified PE. After washing to remove unbound reagents a chromogenic HRP reagent was added. No specific, competable binding of the conjugates was demonstrated. The failure of this direct method is consistent with our earlier experience with toxin-ferritin conjugates, and suggests that attachment of a large molecule to PE prevents the latter from binding to its receptor.

Subsequent work therefore employed "indirect" or immunochemical "sandwich" methods. These procedures are similar to widely used immunocytochemical techniques except that the antigen being studied is reversibly bound on the cell surface. This imposes a requirement that subsequent steps neither disrupt the PE-receptor interaction nor involve such long incubations that the PE disassociates. One solution to this problem consists of fixation of the cell monolayer, but in preliminary tests it was shown that fixation of Vero cells with either glutaraldehyde or formaldehyde effectively destroyed PE receptors. In subsequent trials, PE was bound to cells at 4°C and the cells were then fixed. After control of several of the variables, successful staining of PE on the surface on L-929 cells was obtained. The cells were treated sequentially at 4°C with PE, 2% glutaraldehyde, rabbit antitoxin, goat anti-rabbit IgG, and the soluble peroxidase-rabbit antiperoxidase complex (PAP). Diaminobenzadine stain was found by light microscopy on cells receiving all the reagents but was absent in controls lacking any layer of the sandwich. When the specimens were examined by transmission electron microscopy the reaction product was found localized over "coated" regions of the membrane. To our knowledge this is the first demonstration that receptors for a bacterial toxin are localized in specific regions of mammalian cell membranes. Since coated membrane is known to invaginate and pinch off to form coated vesicles, this result strongly supports our hypothesis that PE enters cells by adsorptive endocytosis.

Since the biochemical evidence makes it probable that DE also enters cells by the same method, repeated attempts were made to apply the HRP staining procedure to DE bound to Vero cells. Since no rabbit anti-diphtheria toxin was available, the sandwich used was goat anti-toxoid:rabbit anti-goat IgG:peroxidase goat anti-peroxidase. Although a range of reagent concentrations and incubation conditions were tried, no DE-specific staining was obtained. It was suspected that some component of the sandwich was not functioning, so a solid-phase system was used to test the reagents. Using ^{125}I-DE in the sandwich plastic: horse anti-toxin ^{125}I-DE:goat anti-toxin:rabbit anti-goat IgG (RAG):peroxidase-goat antiperoxidase, it was found that the RAG previously used behaved poorly. When a different RAG was substituted, it was possible to get visible color at concentrations of DE < 1 ng. Since this is the amount of DE bound specifically to 2 cm^2 of Vero cell monolayers, this sandwich should be capable of detecting DE on the cell surface. However, in further trials it became evident that the cells were nonspecifically binding IgG in the goat or horse anti-toxins that constitute the first layer of the sandwich. To circumvent this interference we undertook to prepare pure anti-DE antibodies by immunoadsorption. After trying several methods for attaching DE or PE to resins, success was achieved using glutaraldehyde-activated amino-hexyl Sepharose 4B. Essentially all the DE or PE added to this activated resin became covalently attached, and the resulting resins were used to selectively bind and purify the respective horse and goat antibodies. These antibodies will be used in all future immunoperoxidase staining trials.

The HRP method as described above is suitable only for detecting toxin on the cell surface, since cells fixed by standard procedures are impermeable to the IgG reagents. To extend the technique so as to detect toxins inside cells, presumably in vesicles and lysosomes, we began tests of a newly described fixation procedure. Willingham and Yamada (3) have recently shown that cells fixed briefly with 0.25% glutaraldehyde and 1% water-soluble carbodimide and subsequently treated with saponin are permeable to immunoglobulin reagents. We improved the published procedure by removing the Tris (which reacts with glutaraldehyde) so that the fixative solution is stable. When tested on Vero cells saturated with ^{125}I-DE, the improved fixative was found to adequately fix toxin to the cells; no significant loss of ^{125}I was observed. Parallel samples examined by electron microscopy showed excellent preservation of structure. This promising method will therefore be tested with the peroxidase reagents to detect internalized DE.

To corroborate and extend the receptor localization studies employing HRP, we have also performed electron microscopic autoradiography, in collaboration with Dr. John White (Pathology Division). Although beset by initial technical problems, some preliminary experiments have been completed. These confirm that PE on the surface of L-929 cells is principally located in coated pits.

Preliminary results from experiments with ^{125}I-DE seem to indicate that Vero cell-surface receptors are not highly localized to coated pits or other specific membrane regions. In parallel experiments at 37°C where DE was allowed to internalize, silver grains were found predominantly in the nucleus. This latter result was quite unexpected; if confirmed, it would require a reexamination of our hypothesis that most internalized DE is transported to lysosomes.

Studies of the uptake of ^{125}I-DE by Vero cells have been continued in collaboration with Ms. Rebecca Dorland (Work Unit A91C 00 137) and Dr. John Middlebrook, (Work Unit S03 00 006). Previous studies showed that DE was taken into Vero cells at 37°C and progressively released from the cells in a trichloroacetic acid (TCA)-soluble form. Since this process is inhibited by chloroquine, it is presumed that the proteolysis being observed occurs in the lysosome. This evidence is central to the hypothesis that uptake of DE into cells occurs by adsorptive endocytosis, possibly through coated pits. To provide further evidence that lysosomes are involved, the nature of the TCA-soluble material was examined. Cells were saturated with ^{125}I-DE at 4°C, washed to remove unbound DE and then incubated at 37°C. At 1 and 6 hr the supernatant was collected and lyophilized. Samples run on Sephadex G-50 showed that at 1 hr most of the radioactivity in the supernatant was high MW, presumably DE which had dissociated from the receptor. At 6 hr the majority of the radioactivity was low MW. A sample of this material examined by thin layer chromatography was shown to be monoiodotyrosine. Since complete degradation of proteins to amino acids is considered to occur principally in lysosomes, this result is further evidence of lysosomal processing of DE.

Further evidence that lysosomes are involved in DE degradation was sought in several ways. A number of protease inhibitors were tested on intact cells preloaded with ^{125}I-DE. Degradation, measured as release of acid-soluble radioactivity into the culture medium, was substantially blocked by tosyl-lysine chloromethyl ketone (TLCK), an active site-reagent specific for trypsin-like proteases. Though TLCK probably affects other cellular processes, this result is suggestive that a trypsin-type protease such as cathepsin B, is involved.

To further characterize the role of lysosomes in the intracellular processing of toxins, degradation of ^{125}I-DE was examined in Vero cell extracts. The extracts were found to be vigorously proteolytic under acidic conditions, with the optimum pH at 3.4. A number of protease inhibitors blocked the degradation, as shown in the table.

INHIBITOR	CONCENTRATION (μg/ml) REQUIRED TO GIVE 50% INHIBITION OF DE DEGRADATION
Antipain	< 0.1
Leupeptin	< 0.1
Pepstatin	10.0
TLCK	2.0
TPCK	> 100.0
Soybean trypsin inhibitor	50.0
Bovine serum albumin	100.0

The pattern of inhibition found is fully consistent with that expected for cathepsin B. The susceptibility of the degradation to antipain, leupeptin, and TLCK suggests that these inhibitors may be useful in determining whether degradation of DE within cells is a necessary step in cytotoxicity.

Previous work from Pathology Division showed that Vero cells have well-defined cell membrane receptors for DE. Two logical extensions of this work are to demonstrate that purified membranes contain receptors and to solubilize and purify the receptors. To detect membrane-bound receptors we have sought to show that ^{125}I-DE binds to purified membranes. A simple 2-phase polymer system was employed to isolate membranes. Binding assays were processed by rapid centrifugation through a 0.3 ml sucrose gradient, a method that achieves a complete separation of membranes and DE. Specific binding of DE to membranes was observed in a number of experiments but was absent in others. Efforts to associate the absence of binding with particular experimental variables have not yet identified the source of variability.

A recent report claims partial purification of the DE-receptor from guinea pig lymph node cells (4). W sought to confirm and extend these results in Vero cells. Purified membranes solubilized in nonionic detergents yielded samples which effectively blocked ^{125}I-DE binding to Vero cells, indicating the presence of soluble receptors. Methods were developed for lactoperoxidase labeling of cell surfaces with ^{125}I. Following the published report cited above, the labeled cells were lysed and the dialyzed extract containing putative labeled receptor was chromatographed on an agarose-lentil lectin column to obtain a glycoprotein fraction. Immunoprecipitation of this fraction with DE, antitoxin, and protein A-bearing staphylococcal cells yielded specific (i.e., toxin-dependent) precipitated radioactivity as claimed in the published report. Extension of this method may allow the receptor to be assayed routinely, thereby aiding in its purification and characterization.

Presentation:

Leppla, S. H., R. B. Dorland, and J. L. Middlebrook. Uptake of Pseudomonas exotoxin A by cultured mammalian cells. Presented, Annual Meeting American Society of Microbiology, Los Angeles, CA, 4-8 May 1979 (Abstracts of the meeting p. 17).

Publication:

Walker, A. L., C. G. McLeod, Jr., S. H. Leppla, and A. D. Mason, Jr. 1979. Evaluation of Pseudomonas aeruginosa toxin A in experimental rat burn wound sepsis. Infect. Immun. 25:828-830.

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4. Proia, R. L., D. A. Hart, R. K. Holmes, K. V. Holmes, and L. Eidels. 1979. Immunoprecipitation and partial characterization of diphtheria toxin-binding glycoproteins from surface of guinea pig cells. Proc. Natl. Acad. Sci. USA 76:685-689.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OD6421	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 04 18	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	62776A	3M162776A841	00	047
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Physiochemical and biological characterization of components of Coxiella burnetii

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

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b. NUMBER: NA

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18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	1.0	114.1
	CURRENT 80	1.0	143.0

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NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Vaccines; (U) Q fever; (U) Rickettsia

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

23 (U) Demonstrate vaccine potential of components of Coxiella burnetii to protect troops against Q fever from natural contact or from potential employment as a biological warfare agent.

24 (U) Isolate purified components of C. burnetii, determine antigenic, immunogenic, allergenic and physiocochemical properties; investigate effectiveness for aerosol immunization. Examine pathophysiology of disease in appropriate animal models.

25 (U) 78 10 - 79 09 - In guinea pig protection tests to determine the effects of enzymes on the biological properties of the soluble phase I antigen of C. burnetii lysozyme produced a marked enhancement of immunogenicity in 2 experiments (66 and 83% decrease in fever days/animal). This increase in protection apparently was not the result of enzymatic activity but of an adjuvant effect by lysozyme. Preliminary evidence from macrophage migration inhibition tests suggest that lysozyme may function by augmenting the induction of a cellular immune response.

Lysozyme was also found to effect a reduction in reactogenicity (less induration in skin test with sensitized guinea pigs), not only for the phase I antigen, but especially for the particulate phase I Q fever vaccine, NDBR-105.

Publications: Infect. Immun. 22:627-628, 1978;
Abstracts, Annual Meeting ASM - 1979, p. 71.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 047: Physicochemical and Biological characterization of Components of Coxiella burnetii

Background:

The soluble phase I antigen of Coxiella burnetii tested successfully as a vaccine for man in Czechoslovakia (1) and Romania (2), is far less reactogenic than cell-wall or intact preparations of this organism (3). One objective of this work unit is to evaluate the potential of the soluble antigen for use as a vaccine. This may be accomplished, in part, by demonstrating the following in connection with the antigen: enhancement of immunogenicity by adjuvants, capacity to trigger a cellular immune response, and lack of significant reactogenicity.

We have demonstrated that the immunogenicity of the soluble antigen can be enhanced by a modified polyriboinosinic-polyribocytidylic acid (4), that it has the capacity to induce a cellular immune response, and that the antigen is about 100 X less skin-reactive than the Merrell-National Laboratories particulate, phase I vaccine (NDBR-105). The unique phase I antigen of C. burnetii is composed of carbohydrate, protein and lipid. We initiated an investigation on the relationship of these components to the biological properties of antigenicity, immunogenicity, and reactogenicity by treating the antigen with enzymes. This approach had, as a rationale, the idea that an enzymatically modified antigen might possess enhanced immunogenicity or be free of reactogenicity. Also, information obtained with this antigen might be applicable to other rickettsial or bacterial antigens.

Results obtained in an initial experiment using proteinase K, wheat germ lipase and lysozyme were described in the previous Annual Report. in subsequent tests, lysozyme produced a marked enhancement of immunogenicity of the phase I antigen and also an apparent reduction in skin reactogenicity; therefore, most effort has been focused on the effects of lysozyme.

Progress:

Modification of the antigenicity/immunogenicity of the soluble phase I antigen of C. burnetii. In a continuing investigation of the effect of enzymes on the relationship of the carbohydrate, protein, and lipid components of the soluble phase I antigen of C. burnetii to the biological properties of antigenicity, immunogenicity and reactogenicity, a second guinea pig protection experiment was conducted. In this test much more definite data were obtained.

Guinea pigs (10/group) were inoculated SC with 2 doses, 15 days apart, of 4 µg of enzyme-treated or untreated phase I antigen, as the dialyzed and lyophilized trichloroacetic acid (TCA) extract, suspended in Freund's incomplete adjuvant (FIA). Enzymes used were the same as in the first experiment: proteinase-K (Beckman), wheat germ lipase (Worthington), and lysozyme (Sigma), all all at a concentration of 25 µg/ml. Serum samples were taken 14 and 21 days after the

second inoculation. Guinea pigs were challenged IP 46 days after the second dose with 5 x 10^5 egg ID_{50} of phase I C. burnetii. Temperatures were recorded daily for 10 days; guinea pigs with temperatures >40.0°C for ≥ 2 consecutive days were considered unprotected. Serum samples were obtained 14 days post-challenge.

Table I summarizes the antibody responses in guinea pigs 2 and 3 weeks after the second dose of antigen in terms of the percentage of animals positive per group and the geometric mean microagglutinating (MA-I and MA-II) and complement fixing (CF-II) antibody titers of each group. (Phase I CF antibody is not found in response to the phase I antigen or to inactivated particulate C. burnetii vaccines, but only to the live rickettsia).

TABLE I. EFFECT OF ENZYME TREATMENT OF PHASE I ANTIGEN OF C. BURNETII ON ANTIBODY RESPONSE IN GUINEA PIGS

	MA-I				MA-II				CF-II			
	% pos		Titer[a]		% pos		Titer[a]		% pos		Titer[a]	
TREATMENT	2[b]	3[b]	2	3	2	3	2	3	2	3	2	3
None	30	30	1.7	1.5	20	50	1.3	2.3	0	0	0	0
Lysozyme 37C/18 hr	88	100	12.3	9.7	88	100	9.5	9.7	30	71	1.0	4.9
Proteinase-K 53C/24 hr	56	50	2.2	2.2	0	50	0	2.0	0	0	0	0
Lipase 37C/18 hr	50	90	2.6	4.6	60	90	2.3	4.6	10	10	1.2	1.2

[a]Geometric mean MA titer

[b]Weeks after second dose of antigen.

Treatment with proteinase-K did not change the antibody response significantly. Lipase treatment resulted in a 2- to 3-fold increase in MA-I and MA-II titers, and in the percentage of positive guinea pigs, plus a very minimal level of CF-II antibody. The most pronounced difference was effected by lysozyme: there was a 3-fold increase in MA antibody titer; also, 50% of the guinea pigs that received lysozyme-treated antigen possessed CF-II antibody compared to none of those that received the untreated antigen.

Data in Table II illustrate the degree of protection against challenge afforded guinea pigs by treated and untreated phase I antigen. Proteinase K and lipase treatment reduced immunogenicity. Lysozyme treatment produced a remarkable increase in protection.

TABLE II. EFFECT OF ENZYME TREATMENT ON THE IMMUNOGENICITY FOR GUINEA PIGS OF THE PHASE I ANTIGEN OF COXIELLA BURNETII

TREATMENT	ANIMALS WITH FEVER/TOTAL (%)		TOTAL FEVER-DAYS	FEVER-DAYS ANIMAL
Saline controls	9/9	(100)	44	4.9
Untreated antigen	4/8	(50)	16	2.0
Lysozyme 37°C/18 hr	0/9	(0)	3	0.33
Poteinase-K 53°C/24 hr	6/9	(67)	34	3.8
Lipase 37°C/18 hr	8/10	(80)	32	3.2

At 14 days postchallenge the highly protected group of guinea pigs, those that received lysozyme-treated antigen, had overall lower titers of CF and MA antibodies than other groups that received antigen; these lower titers might be related to the less severe illness experienced by animals that received this antigen.

We have recognized that the action of lysozyme to enhance protection might not be enzymatic in nature. For example, lysozyme, a basic protein, is known to form complexes with bacterial polysaccharides as well as with some other macromolecules (4). The phase I antigen in the form of a complex might function more effectively by remaining in the recipient host for a longer period of time. We have shown by following the CF activity of the antigen in filtration and centrifugation experiments that a complex of lysozyme and antigen can be formed. When antigen and lysozyme were mixed in water and subsequently centrifuged at 15,000 rpm for 3 hr, 90% of the CF activity of the antigen was sedimented; however, in a buffer system 94% of the activity remained in the supernatant. Skarnes and Watson (5) reported that inhibition of lysozyme by acidic polymers occurred only in systems devoid of buffer or salt.

An alternative explanation for the potentiation of immunogenicity by lysozyme is that lysozyme acted as a adjuvant; this could have occurred with lysozyme-treated antigen samples since lysozyme had not been entirely removed from these samples.

Adjuvant effect of lysozyme on the phase I antigen of C. burnetii. To investigate the possibility that lysozyme enhancement of immunogenicity with the phase I antigen might result from an adjuvant mechanism rather than enzymatic activity, we conducted a guinea pig protection experiment in which lysozyme was adminsitered to guinea pigs 5 hr before the antigen. In this test guinea pigs were inoculated SC with 2 doses, 14 days apart, of antigen only for group 1 and lysozyme followed by antigen for group 2. Saline and lysozyme control groups were included. For the first dose 3.5 mg (protein) of antigen and 25 mg of lysozyme were administered, and for the second dose, 7.0 mg antigen and 25 mg lysozyme were administered. Freund's adjuvant was not employed in this test. Serum samples were taken 14 days after the second dose with 5×10^5 egg ID_{50} of phase I C. burnetii. Temperatures were

recorded daily for 10 days; guinea pigs with temperatures >40.0°C for ≥ two consecutive days were considered unprotected. Serum samples were obtained at 14 days.

Data in Table III illustrate the greater degree of protection against challenge afforded guinea pigs that received lysozyme prior to antigen. All saline and lysozyme control guinea pigs were unprotected with fever-day/animal ratios of 4.1 and 3.3, respectively. Forty percent of the guinea pigs that received antigen alone were unprotected with a fever-day/animal ratio of 1.2. In the group that received lysozyme prior to antigen 1 of 10 animals was unprotected, with only 4 fever-days in the entire group for a ratio of 0.4. Results of this test suggest that lysozyme acts to increase protection by triggering a defense mechanism of the host.

TABLE III. ADJUVANT EFFECT OF LYSOZYME ON THE IMMUNOGENICITY OF THE PHASE I ANTIGEN OF COXIELLA BURNETII

PREPARATION	NO. FEBRILE/ TOTAL	TOTAL FEVER-DAYS	FEVER-DAYS/ ANIMAL
Saline	6/6	31	5.1
Lysozyme (25 µg)	7/7	23	3.3
Antigen (3.5 µg	4/10	12	1.2
Antigen (2nd dose 7 µg)	1/10	4	0.4
Lysozyme (25 µg) 5 hr after	-	-	-

In earlier tests we found that lysozyme-treated phase I antigen produced higher antibody titers in guinea pigs than did untreated antigen, e.g., MA antibody titers were as much as 6 X higher. In this test the MA-II antibody response was only slightly higher in guinea pigs that received lysozyme prior to antigen but CF-II antibody titers were 10 X higher (Table IV).

TABLE IV. EFFECT OF LYSOZYME ADMINISTRATION TO GUINEA PIGS ON SUBSEQUENT ANTIBODY RESPONSE TO PHASE I ANTIGEN

	MA-I		MA-II		CF-II	
PREPARATION	%+	Titer[a]	%+	Titer	%+	Titer
Antigen only	80	5.7	40	1.9	40	2.6
Antigen 5 hr after lysozyme	100	4.2	90	3.7	100	24.2

[a]Geometric mean titer

Preliminary macrophage migration inhibition tests, conducted through the cooperation of LTC Richard Kishimoto, suggest that lysozyme may enhance protection by inducing or augmenting the induction of a cellular immune response.

Effect of lysozyme on the skin reactogenicity of the phase I antigen of C. burnetii and the particulate phase I Q fever NDBR vaccine. In initial tests to assess the effect of enzymes (proteinase-K, wheat germ lipase, and lysozyme) on the reactogenicity of the phase I antigen of C. burnetii, lysozyme reduced the skin reaction. Because of this, additional tests were conducted with lysozyme.

Sensitized guinea pigs employed in these tests had previously received injections of the phase I antigen followed by challenge with phase I C. burnetii. Lysozyme-treated and control samples of the antigen were compared by ID injection along the shaved flanks of the same guinea pig. Reactions were assessed at 24, 48, and 72 hr postinjection by measuring the diameter and intensity of erythema and the increase in skin thickness using skin calipers (Schmelltäster, A02T, Kröplin). The results obtained using 2.5 µg of lysozyme (egg white, crystalline, Sigma) and 5.0 or 7.0 µg of antigen per injection are shown in Table V.

TABLE V. MODIFICATION BY LYSOZYME OF THE REACTOGENICITY OF THE PHASE I ANTIGEN OF COXIELLA BURNETII

ANTIGEN (Protein) (gm)	GUINEA PIG NO.	LYSOZYME	INDURATION (mm x 10^{-1}) 24 hr	48 hr	72 hr
5.0	1	-	2.5	2.0	0
		+	0	1.0	0
	2	-	7.0	8.5	7.0
		+	6.0	8.0	3.0
	3	-	6.0	2.0	3.5
		+	5.5	3.0	2.0
7.0	1	-	21.5	21.5	15.0
		+	9.0	14.0	9.0
	2	-	11.0	9.5	9.0
		+	9.0	5.5	3.0
	3	-	18.0	20.0	15.5
		+	6.0	9.0	6.5
	4	-	4.0	1.5	0
		+	2.0	0	0

Induration proved to be a better index of difference than erythema. Induration measurements, presented for individual guinea pigs, illustrate the variation in sensitivity among animals. Lysozyme had only a minimal effect with 5.0 µg of antigen, but caused a pronounced reduction in all with 7.0 µg. Lysozyme controls were negative for induration at all hours.

We also investigated the effect of lysozyme on the reactogenicity of the particulate phase I Q fever vaccine NDBR-105 (Merrell-National Laboratory). The results, shown in Table VI, are presented as the average induration readings obtained with 8 guinea pigs over a period of 31 days. Each guinea pig received 0.25 µg of the vaccine that had been treated with lysozyme (2.5 µg/0.1 ml, 37°C for 18 hr) at one injection site and a control of the same amount of vaccine at an adjacent site.

Treated and control samples were heated at 60°C for 1 hr before being injected. Most determinations of skin reactions include readings from 1-4 days post-injection (3). We found much larger areas of induration at later intervals, with maximum readings at 7 days. Although individual guinea pigs varied in their response, as in the tests with the phase I antigen (Table V), the data indicate a definite reduction in reactogenicity, ranging from 40-65% from the 3-day interval on.

TABLE VI. REDUCTION OF THE REACTOGENICITY OF THE Q FEVER VACCINE (NDBR-105) BY LYSOZYME

TREATMENT	INDURATION (mm x 10^{-1}) BY DAYS						
	1	2	3	7	15	24	31
None	3.0	4.8	7.1	16.9	13.4	10.9	10.3
Lysozyme (0.25 µg 37°C/18 hr)	3.0	3.4	3.6	7.1	8.0	6.1	3.5

Presentations:

Wachter, R. F., and G. P. Briggs. Enzymatic modification of the antigenicity and reactogenicity of the soluble phase I antigen of Coxiella burnetii. Presented, Annual Meeting American Society of Microbiology, Los Angeles, CA, 1-6 Apr 79. Abstract, p. 71.

Publications:

Wachter, R. F., G. P. Briggs, and C. E. Pedersen, Jr. 1978. Enhancement of the immunogenicity of phase I antigen of Coxiella burnetii. Infect. Immun. 22:627-628.

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4. Wachter, R. F., G. P. Briggs, and C. E. Pedersen, Jr. 1978. Enhancement of the immunogenicity of phase I antigen of Coxiella burnetii. Infect. Immun. 22:627-628.

5. Skarnes, R. C., and D. W. Watson. 1955. The inhibition of lysozyme by acidic polymers from pathogenic bacteria. J. Bacteriol. 70:110-112.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
	DA 0G6412	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	049
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Pathogenesis of hemorrhagic vascular lesions induced by nonindigenous rickettsiae

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
76 03	79 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 79	1.0	27.8
FISCAL YEAR CURRENT 80	0	55.8

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21. GENERAL USE

Foreign intelligence considered

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NAME: Kenyon, R. POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Nonindigenous rickettsiae; (U) Vascular permeability; (U) Laboratory animals; (U) Immunity

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

23 (U) Investigate the pathogenesis of the vascular lesions induced by dangerous nonindigenous rickettsiae. Knowledge of the pathogenesis of such lesions is essential to the understanding of the pathophysiology and planning of rational therapy for the soldier infected by any one of a number of these potential agents. Such adjunctive therapy, to be utilized along with antibiotics in treating infected soldiers, will reduce hospitalization time and enable the individual to return to a duty status more rapidly.

24 (U) Using an appropriate rodent or primate model system, study morphologic sequence and location of vascular changes and the influence of immune processes, kinins, etc. on them. Use this knowledge to improve therapy.

25 (U) 78 09 - 79 10 - Using a chick embryo model for rickettsial infection, it was found that lesions could be induced by Rickettsia conorii in vessels of the chorio-allantoic membrane as well as in other blood vessels. Organisms were always associated with vessels containing lesions but were also found in vessels without lesions. Ontogenetically, lesions appear before complement and coagulation proteins are fully developed, and before the embryo is fully competent immunologically, this indicating a direct relationship between rickettsiae and the development of vascular lesions. A similar sequence of events was observed in chick embryos infected with Rickettsia rickettsii. This model should enhance our knowledge of the pathogenesis of these rickettsial diseases.

* Available to contractors upon originator's approval

☆ U.S. GPO: 1974-540-881/8681

BODY OF REPORT

Project No. 3M762776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 049: Pathogenesis of Hemorrhagic Vascular Lesions Induced by Non-Indigenous Rickettsiae

Background:

The lesion typical of rickettsial spotted fevers is segmental vasculitis characterized by inflammatory cell infiltration into the affected portion of the vessel, necrosis and endothelial cell proliferation. The pathogenesis of the lesion is not known. Wolback hypothesized that damage is initiated by direct infection of endothelial cells by the rickettsiae with subsequent endothelial necrosis and inflammation (1). In later stages of the disease, it was shown that intravascular coagulation and immune mechanisms play a role in lesion severity and development (2, 3). Whether the vascular damage is caused directly by the rickettsiae or their products or indirectly by host response against the organisms remains to be elucidated.

Progress:

A model of spotted fever rickettsial disease was established in the chick embryo, wherein the pathogenesis of the vascular lesions induced by the rickettsiae could be followed in a temporal fashion by inoculating the rickettsiae into embryos of various ages; various cellular and humoral factors could be eliminated from disease pathogenesis, thus providing a relatively clearcut model for determining the sequence of events. It was previously reported that early events in the lesions occurred probably in the absence of total hemolytic complement and coagulation proteins.

The primary lesion induced by Rickettsia conorii was characterized by inflammatory cell infiltration of a segmental nature. These lesions were first seen in the chorioallantoic membrane of the chick embryo 48 hr after inoculation. This lesion was felt to be caused by one of 2 mechanisms: (A) direct chemotaxis by the rickettsiae or its products, or (B) degeneration of cells containing the rickettsiae with subsequent leukocyte chemotaxis.

Chemotaxis. The technique utilized to measure chemotaxis was the Nelson technique whereby agarose plates are poured, wells are punched in them, and a suspension of leukocytes and chemotactic agent is added to individual wells (4). Various sources of leukocytes were tried: guinea pig peritoneal cells obtained by administering shellfish glycogen, guinea pig peripheral blood leukocytes and human peripheral blood leukocytes. Several techniques for separating leukocytes from the rest of the blood were attempted. The one which was most favorable was to collect human peripheral blood in heparin, allow the blood to settle at room temperature and remove the leukocyte-rich plasma. Leukocytes were then washed with medium 199 and added to the agarose plates. For some reason, lysis of neutrophils occurred from most leukocyte sources. Several attempts to correct this were made, but the cause could not be determined. Leukocytes from one individual consistently remained intact and were utilized throughout the experiments. Rickettsia rickettsii were

grown in chick embryo cell culture for 5 days. Infection was verified by staining a portion of the culture cells with Gimenez stain to demonstrate the organisms. Following growth, the supernatant was removed, centrifuged to remove the rickettsiae and frozen in aliquots for testing. Infected and uninfected chick embryo culture cells were lysed by freeze-thawing and frozen in aliquots. Chemotaxis was not observed with either portion of the infected cell cultures nor with uninfected cultures. Chemotaxis was observed in controls using Escherichia coli culture filtrate, or purified C3 incubated 1.5 hr with human serum. Results indicated that either R. rickettsii and cultural products were not chemotactic or that the concentration of chemotactic products was too low to measure chemotaxis. Further work with these substitutes mixed with human serum as a source of complement were initiated but not completed.

Direct cell injury induced by the rickettsiae. In a group of chick embryo specimens which had been inoculated with high numbers of R. conorii, a myriad of organisms were observed at 5 days in vessels by immunofluorescence. By electron microscopy, rickettsiae were found within the cytoplasm of endothelial cells of numerous vessels, often in high numbers. Several rickettsiae were observed just beneath the cell membrane, producing a bulge on the host cell surface. Others were observed, completely detached from the cell surface in the lumen of this vessel but still enclosed within a host-derived portion of cell membrane. The majority were found free within host cell cytoplasm. Most infected endothelial cells showed no morphologic alteration whatsoever. However, an endothelial cell was rarely observed in which there was dispersion or loss of the electron-dense material normally found in the cytoplasm and reduced density of the cell membrane. Adjacent cells appeared essentially normal except for the presence of organisms within them, as described above. These changes appear to be degenerative. The reason for the paucity of such cells is probably related to the fact that the degenerating cells are exposed to the flow of blood within the vascular lumen which would wash them away from the site of injury. Thus, it appears that Wolbach's hypothesis of endothelial injury followed by inflammation may be the means of initial vascular damage.

Previous work with the chick embryo had been performed with R. conorii and Rickettsia sibirica, members of the spotted fever rickettsiae. Comparable work had not been done with R. rickettsii. In order to tie previous in vitro work together with these studies, 11-day chick embryos were inoculated IV with varying doses of R. rickettsii. Embryos given high doses died on days 4 and 5. Numerous organisms were demonstrated within vessels of all sizes in the chorioallantoic membrane. They appeared to be localized to the endothelium and in some cases were in such quantities as to be found throughout the section of vessel lumen. By electron microscopy, organisms were found within endothelial cells, occasionally within macrophages and also within the vessel lumen but surrounded by host cell membrane and cytosol. By immunofluorescence, 48 hr following inoculation, an occasional focus of rickettsiae was observed in the chorionic epithelium and in a rare small vessel adjacent to the capillaries of the chorionic epithelium. At 68 hr, foci of infection of the chorionic epithelium were more numerous and organisms were occasionally located in small and large vessels. By 92 hr and subsequently, organisms were found most frequently in the capillaries of the chorionic epithelium, and randomly in larger vessles of the membrane. Results indicate that capillaries are infected first followed by infection of larger vessels. Reasons for these findings may be that because of slower blood flow in capillaries of the chorionic epithelium, rickettsiae may be more readily able to attach to endothelial cells with subsequent penetration into the cytosol. Work on rickettsiae has been transferred to WRAIR. The work unit is terminated.

Presentation:

Hall, W. C. Respiratory diseases of nonhuman primates. Presented, Pathology of Laboratory Animals Course, Armed Forces Institute of Pathology, Washington, DC, Sep 1979.

Publications:

None.

LITERATURE CITED

1. Wolbach, S. B. 1919. Studies on Rocky Mountain spotted fever. J. Med. Res. 41:1-198.

2. deBrito, T., S. Hoshino-Shimizu, M. O. Pereira, and N. Rigolon. 1973. The pathogenesis of the vascular lesions in experimental rickettsial disease of the guinea pig (Rocky Mountain spotted fever group). A light, immunofluorescent and electron microscopic study. Virchows Arch. (Pathol. Anat.) 358:205-214.

3. Moe, J. B., D. F. Mosher, R. H. Kenyon, J. D. White, J. L. Stookey, L. R. Bagley, and D. P. Fine. 1976. Functional and morphologic changes during experimental Rocky Mountain spotted fever in guinea pigs. Lab. Invest. 25:235-245.

4. Nelson, R. D., P. G. Quie, and R. L. Simmons. 1975. Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. J. Immunol. 115:1650-1656.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6416	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 04 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	62776A	3M162776A841	00	050
b. CONTRIBUTING				
c. ~~CONTRIBUTING~~	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Therapy of disease transmitted by aerosol: Legionnaires' disease

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
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 79	1.0	204.6
FISCAL YEAR — CURRENT 80	1.0	253.9

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NAME:
NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Respiratory diseases; (U) Antibiotic therapy; (U) Legionnaires' disease bacillus

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

23 (U) Devise more efficient methods for treating and monitoring the effects of treatment of respiratory bacterial infections. This information is essential for the expeditious therapy of troops subsequent to BW attack.

24 (U) Determine feasibility of aerosol therapy. Compare efficacy with conventional techniques; determine pharmacokinetics, toxicity and biochemical sequelae to monitor progress of disease.

25 (U) 78 10 - 79 09 - Final experiments using the Klebsiella pneumoniae-squirrel monkey model showed that aerosol administration of kanamycin was significantly more effective prophylactically than the IM route. When treatment was delayed for 24 hr, however, both routes of antibiotic administration were equally effective.

Research was initiated to determine the aerosol infectivity and stability properties of the agent of Legionnaires' disease (Legionella pneumophila). A respiratory disease model was developed in guinea pigs; response criteria were fever, weight loss and seroconversion. The aerosol median lethal dose was significantly lower than that for IP. Preliminary studies with monkeys indicated that a disease model was feasible, experiments are continuing. Aerosol survival studies will be initiated in the near future.

Publications: Infect. Immun. 22:275-281, 1978;
Am. J. Vet. Res. 40:1231-1235, 1979.

*Available to contractors upon originator's approval

* U.S. GPO: 1974-540-943/8691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 050: Therapy of Disease Transmitted by Aerosol

Background:

In the previous annual report (1) the effect of aerosol and IM kanamycin therapy in Klebsiella pneumoniae-infected in squirrel monkeys was compared. No differences in mortality or morbidity were observed. In addition, the prophylactic effects of the routes of treatment were compared in mice challenged with K. pneumoniae over a period ranging from 0.5-72 hr after a single kanamycin treatment of 15 mg/kg. The aerosol was significantly more effective than the IM route. In this report, the prophylactic effect of the 2 routes of administration is compared in squirrel monkeys. In the final experiment with K. pneumoniae, a dose-response study is described.

The major emphasis has been expended upon dose-response studies with both aerosols and IP Legionella pneumophila (the agent of Legionnaires' disease). In addition, the development of a guinea pig model for respiratory L. pneumophila and preliminary studies with nonhuman primates are described.

Progress:

To elucidate the reason for the superiority of aerosol over IM kanamycin prophylaxis against K. penumoniae, the concentration of the antibiotic in the lungs of mice given an ED_{90} dose by either route was determined. The ED_{90} dose was first established by treatment groups of 15 mice each with graded doses of aerosols or IM kanalycin 6 hr after exposure to 10 aerosol LD_{50} of K. pneumonia. The ED_{50}, calculated by the method of Litchfield and Wilcoxon (2), was 20 and 200 mg/kg for the aerosol and IM routes, respectively. These doses were then administered to uninfected mice which were killed in groups of 3 at selected times between 0.5 and 72 hr. The results of this experiment, given in Table I, show that significantly more kanamycin persisted in the lungs of aerosol-treated than IM-treated mice, even though the total dose given to the latter was 10X that of the aerosol.

TABLE I. AMOUNT OF KANAMYCIN IN THE LUNGS OF MICE (n=3) FOLLOWING AEROSOL (20 mg/kg) OR IM (200 mg/kg)

HOURS AFTER ADMINISTRATION	mg KANAMYCIN/LUNG	
	Aerosol	IM
0.5	14.3	11.7
4.0	2.2	0.4
24.0	0.9	trace
48.0	1.2	trace
72.0	0.9	trace

A similar series of experiments was carried out with squirrel monkeys. Twelve monkeys were given a single IM dose of kanamycin of 11.25 mg/kg; 12 additional monkeys were exposed by aerosol to the same dosage and 8 monkeys were given no treatment and were reserved as controls. Six hr after treatment, 8 of each of the 2 antibiotic-treatment groups as well as the 8 controls were challenged intratracheally (IT) with 10 LD_{50} of K. pneumoniae. The remaining 4 monkeys in each therapy group were similarly challenged at 24 hr. The results are given in Table II. Very little protection was afforded the IM-treated monkeys and the aerosol was clearly superior in respect to prophylaxis.

TABLE II. KANAMYCIN PROPHYLAXIS (11.25 mg/kg) AGAINST KLEBSIELLA PNEUMONIA INFECTION OF SQUIRREL MONKEYS

ROUTE OF KANAMYCIN ADMINISTRATION	INFECTED CONTROLS Dead/total	RESPONSE TO CHALLENGE 6 hr Dead/total	P[a]	24 hr Dead/total	P
None	8/8	-		-	
IM	-	7/8	< 0.005	4/4	0.05
Aerosol		0/8		0/4	

[a] χ^2 test (yates correction), aerosol vs. IM response.

To determine lung clearance of kanamycin, 4 monkeys were given 11.25 mg/kg by aerosol and 4 additional were treated IM with the same dose. Two monkeys in each group were killed at 6 hr, and 2 additional at 24 hr; their lungs were removed, homogenized and assayed for kanamycin. The data, shown in Table III, again illustrate that the clearance rate of the antibiotic after IM administration was significantly greater than after aerosol treatment. It is interesting to note, however, that the concentrations at both 6 and 24 hr after IM treatment were greater than the previously reported (3) minimum inhibitory concentration of kanammycin for this strain of K. pneumoniae (0.625 µg/ml). Despite this, IM-kanamycin was almost without effect, even at 6 hr.

TABLE III. CONCENTRATION OF KANAMYCIN (11.25 mg/kg) IN THE LUNGS OF SQUIRREL MONKEYS AFTER AEROSOL OR INTRAMUSCULAR ADMINISTRATION

ROUTE OF ADMINISTRATION	mg OF KANAMYCIN IN LUNGS 6 hr	24 hr	CLEARANCE RATE[a] (%/hr)	p[b] (Clearance rates)
Aerosol	87.0	47.0	5.56	< 0.025
IM	21.5	1.6	14.66	

[a]Calculated from the equation: $k(\%/hr) = \frac{\text{Ln conc (6 hr)} - \text{Ln conc (24 hr)} \times 100}{18}$

[b]Probability based on t-test calculated on means of clearance rates (2 monkeys/point)

The final study carried out with K. penumoniae was an attempt to compare the aerosol and IM ED_{50} doses of kanamycin. Because of limitations imposed by the number of monkeys available at any one time, this study was carried out in several phases. Three monkeys were given kanamycin 2X daily by aerosol for 4 days, beginning 24 hr after IT instillation of 10 LD_{50} of K. pneumonia, 3 additional monkeys were given IM treatment at the same times. One monkey was infected and untreated, and one was reserved as a uninfected control. This experiment was carried out for a total of 3 dose-levels for each route. In one case, an error in determining IM-dosage necessitated an extra experiment. The data are given in Table IV. Unfortunately, 0 and 100% effects cannot be employed for ED_{50} determinations. The data indicated, however, that the IM ED_{50} was < 6.9 mg/kg and the aerosol EL_{50} fell between 6.8 and 3.8 mg/kg. Thus, it can be concluded that, in contrast to the prophylactic situation, aerosols do not offer an advantage over the more conventional therapy route.

TABLE IV. MORTALITY OF K. PNEUMONIAE-INFECTED (10 LD_{50}) SQUIRREL MONKEYS AFTER TREATMENT WITH KANAMYCIN.

ROUTE OF KANAMYCIN AMINISTRATION	DOSAGE[a] (mg/kg/day)	DEAD/TOTAL	MEAN TIME TO DEATH (days)
None (uninfected control)		0/4	
None (infected control)		4/4	2.0
IM Treated	6.9	1/3	7.0
	12.5	2/6	7.5
	22.5	0/3	-
Aerosol Treated	3.8	3/3	3.3
	6.8	1/3	6.0
	9.0	1/3	7.0

[a]Antibiotic was administered for 4 days

Legionnaires' disease. The development of charcoal-yeast extract media (CYE) has greatly expedited investigation of Legionnaires; disease by permitting accurate estimation of viable organism distribution. Table V presents the mortality data observed in 2 dose-response experiments carried out with the L-1 strain of L. pneumophila. In the first, 16 guinea pigs were employed at each of several selected dose-levels of aerosolized L. pneumonph la; in the second, IP inoculated organisms were employed. The data suggest that guinea pigs are at least 10X more sensitive to aerosols of L. pneumophila than to IP inoculation. This tentative conclusions, however, must be verified with replication since we do not have an estimate of between-experiment variations.

TABLE V. MORTALITY OF GUINEA PIGS FOLLOWING AEROSOL OR IP ADMINISTRATION OF L. PNEUMOPHILA

ROUTE	DOSE	% MORTALITY	LD_{50} (95% Confidence Limits)	GEOM. MEAN TIME TO DEATH (days)
Aerosol (n=16)	$3.0x10^3$	0		
	$2.4x10^4$	6	$1.4x10^5$	10.0
	$6.5x10^4$	25	($7.1x10^4$-$2.9x10^5$)	7.5
	$2.3x10^5$	100		3.2
IP (n=6)	$1.9x10^5$	20		5.0
	$1.9x10^6$	33	$3.0x10^6$	5.0
	$1.9x10^7$	83	($6.x10^5$-$1.5x10^7$)	3.6
	$1.9x10^8$	100		3.0

In Table VI are presented the serum microagglutination (MA) titers obtained in several experiments. Of interest here was the observation that all guinea pigs that were exposed to viable organisms developed titers, suggesting that all had been infected. The poor coorelation between dose and serum MA titers in the guinea pigs that received 3,000-65,000 cells, indicated that antibody formation was still going on, or that a plateau had been reached. More infestigations on the development of humoral and cell-mediated immune mechanisms will be carried out.

TABLE VI. SERUM MA TUTERS IN GUINEA PIGS EXPOSED TO AEROSOLS OR INJECTED IP WITH L. PNEUMOPHILA

ROUTE	DOSE (organisms)	DAY OF SERUM COLLECTION	GEOM. MEAN RECIPROCAL MA TITER	COEFFICIENT OF CORRELATION (r)[a]
Aerosol	129	28	34.9	
	516		77.0	0.97
	2,100		194.9	
	2,000 killed		0	
Aerosol	3,000	13	197.4	
	24,000		140.4	-0.47
	65,000		181.0	
IP	1,500	13	34.6	
	15,000		59.7	0.98
	150,000		111.4	

[a] Log dose vs titer

In addition to MA titers, all infected guinea pigs had transient fever and lost weight (not shown). This data, therefore, indicated that the median aerosol infectious dose (ID_{50}) was very low, certainly < 129 organisms, whereas the LD_{50} was very high, ∿ 140,000 (see Table V). The data also suggested that the criteria of fever, wiehgt-loss and seroconversion could be used to establish a model in guinea pigs that could be employed for a variety of studies such as immunoprophylaxis and therapy.

To develop the model, an arbitrarily chosen dose of 7,000 viable bacteria was administered by aerosol to 32 guinea pigs. Sixteen animals were followed daily for fever, wiehgt change, dyspnea, reffled fur and lethargy. Eight were bled at weekly intervals (starting before exposure) to obtain serum for MA determinations, and 8 were bled daily for bacteremia and total leukocyte concentrations. Adn additional 8 guinea pigs served as uninfected controls. Separate guinea pigs were employed for determinations involving blood withdrawals. No consistent patterns of bacteremia or leukocytosis were noted alghough both occurred sporadically. Weight change and rectal temperature data are given in Table VII. Every infected guinea pig showed significant loss of weight and fever; these signs were not seen in the uninfected controls.

TABLE VII. WEIGHT CHANGES AND RECTAL TEMPERATURES OF GUINEA PIGS INFECTED WITH AEROSOLS CONTAINING 7 x 10^3 L. PNEUMOPHILA BY AEROSOL

DAY AFTER INFECTION	% WEIGHT			RECTAL TEMPERATURE (^oF)		
	Infected	Control	P[a]	Infected	Control	P[a]
Baseline	-	-	-	102.3	102.4	
1	+0.4	+1.7	*	102.4	102.2	
2	0.0	3.8	*	103.2	102.0	*
3	-4.2	5.5	***	105.0	102.4	***
4	-10.7	0.8	***	104.4	101.6	***
5	-13.9	3.4	***	103.6	102.2	***
6	-14.2	4.9	***	102.9	102.2	**
7	-12.0	7.8	***	102.0	102.0	
8	-10.5	8.7	***	102.3	102.0	
9	-8.4	7.6	***	101.9	102.0	
10	-9.5	8.5	***	101.8	101.9	
11	-5.5	9.5	***	101.9	102.1	
12	-4.7	9.3	***	102.0	102.5	
13	-4.5	9.3	***	101.9	102.0	

[a] *P $\leq$ 0.05; ** P $\leq$ 0.01; ***P $\leq$ 0.001; t test infected vs. control

Peak MA titers were achieved on day 14 and then declined (Table VIII). Every infected guinea pig also showed this pattern. The apparent decline in titer by day 24 indicates a need for even longer holding in order to answer the question of immunity on rechallenge. This question of immunity to rechallenge will be investigated.

TABLE VIII. SERUM MA ANTIBODY TITERS OF GUINEA PIGS (n=8) EXPOSED TO AEROSOLS OF 7 x 10^3 L. PNEUMOPHILA

DAY AFTER EXPOSURE	GEOMETRIC MEAN RECIPROCAL MA TITER*	LOG GEOMETRIC STANDARD DEVIATION
0 (Baseline)	< 8.0	-
7	71.8	0.12
14	287.4	0.12
21	161.3	0.14
24	128.0	0.30

Preliminary experiments on the pathogenesis of L. pneumophila in guinea pigs were carried out by exposing groups to 4,400 organisms in experiment 1 and 230,000 in a second. At 1, 3, 5 7, 11, and 14 days, 3 guinea pigs were killed for viable organism determinations in blood, spleen and lungs and 3 were killed for histopathological examination (in collaboration with CPT Knutsen, Pathology Division).

No guinea pigs died in the first experiment; in the second only 2 guinea pigs of the 48 exposed survived as long as 7 days. Uninfected control guinea pigs were also sacrificed at each time period. Only sporadic isolation of organisms was achieved from blood and spleen. The viable counts calculated for the lungs are presented in Table IX. The count reached a maximum on day 7 in the case of the 4,400-cell doses and on days 1 and 5 in the high-dose guinea pigs. The drop in count on day 5 in the low-dose occurred in all 3 guinea pigs. Perhaps the most interesting observation was the persistence of the organisms for at least 14 days. A second experiment, lasting considerably longer than 14 days is planned.

TABLE IX. NUMBER OF BACTERIAL IN LUNGS OF GUINEA PIGS AFTER AEROSOL CHALLENGE WITH 2 DOSES OF L. PNEUMOPHILA

DAYS AFTER EXPOSURE	GEOM. MEAN OF L. PNEUMOPHILA LUNG COUNT AT INDICATED CHALLENGE DOSE	
	4,400 Organisms	230,000 Organisms
1	1.4×10^6	2.4×10^8
3	4.8×10^6	ND
5	2.3×10^5	2.8×10^8
7	5.5×10^6	1.9×10^7
11	4.0×10^5	No survivors
14	1.0×10^5	No survivors

Histopathologic examination of guinea pigs of the low-dose group revelaed only an early chemotactic response 24 hr after exposure. On day 3 all animals had a mild to moderate interstitial pneumonia. The picture on day 5 was much the same except that larger areas of the pulmonary parenchyma were involved. On day 7 there was consolidation of major portions of lobes of the lung. The character of the inflammatory cell infiltrate had changed to predominately mononuclear, but there were moderate amounts of fibrino-necrotic exudate within the air spaces. By day 11, there was early resolution in many portions of the lung characteristized by decreased cell numbers in air spaces and the alveolar interstitium. Resolution was more apparent by day 14. In other portions of the lung, however, active foci of suppurative inflammation remained with early abscessation. On days 11 and 14 several extrapulmonary lesions were observed, with suppurative infiltration of the colonic mucosa, subacute disseminated peritonitis, mediastinitis and mild acute splenitis.

Guinea pigs exposed to 2×10^5 organisms had lesions that were morphologically similar to the low-dose group. The inflammatory response, however, was greatly accelerated and intensified. For example, peak inflammation occurred on day 7 in low-dose group and on day 3 in the high-dose.

Regardless of dose, the pulmonary lesions were more characteristic of viral or rickettsial etiology than bacterial infection. Also, the fact that early fibrosis of the interstitium occurred on days 11 and 14 was indicative that the lung parenchyma never completely recovered, but remained somewhat comprised. The pathogenesis phase of this work will also be greatly extended.

The results of preliminary experiments with both squirrel and cynomolgus monkeys were somewhat equivocal. Organisms were isolated for at least 7 days from

the blood and oropharynx of cynomolgus monkeys exposed to aerosol doses of 10^5 organisms, but other signs of illness were minimal. IT instillation of 10^6 L. pneumophila caused transient leukocytosis, tachypnea, reduced food consumption and loss of weight. Squirrel monkeys exposed to aerosols of 3×10^6 organisms showed much the same reactions but also had a transient (days 2-4) fever. Further experiments are planned for both species of nonhuman primate in the hope of establishing a useful model for therapy and immunoprophylaxis

In addition to the future experiments already mentioned, intensive effort will be expended in the near future to determine the survival of L. pneumophila in aerosols and to determine whether animal-to-animal transmission can occur.

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1. Berendt, R. F., G. L. Knutsen, and M. C. Powanda. 1978. Nonhuman primate model for the study of respiratory Klebsiella pneumoniae infection. Infect. Immun. 22:275-281.

2. Berendt, R. F., M. A. Schneider, H. W. Young, and F. R. Frola. 1979. Protection against Klebsiella pneumoniae respiratory tract infection of mice and quirrel monkeys given kanamycin by aerosol and injection. Amer. J. Vet. Res. 40:1231-1235.

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1. U. S. Army Medical Research Institute of Infectious Diseases. 1 October 1978. Annual Progress Report, FY 1977, pp. 363-373. Fort Detrick, MD.

2. Litchfield, Jr., J. T., and F. Wilcoxon. 1949. A simplified method of evaluation dose-effect experiments. J. Pharmacol. Exp. Therap. 96:99-113.

3. Berendt, R. F., M. A. Schneider, H. W. Young, and F. R. Frola. 1979. Protection against respiratory Klebsiella pneumoniæ respiratory infection of mice and squirrel monkeys given kanamycin by aerosol and injection. Amer. J. Vet. Res. 40:1231-1235.

4. Freeley, J. C., G. W. Gorman, and R. J. Gibson, 1978. Primary isolation media and methods, pp. 107-117. In "Legionnaires" the Disease, the Bacterium and Methodology, G. L. Jones and R. A. Hebert (ed.), Center for Disease Control, Atlanta, GA, p. 107-117.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0C6428	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	051
b. CONTRIBUTING				
c. //////////	STOG 80-7.2:2			

11. TITLE: (U) Analysis of subcellular structures in microbial infections of potential BW importance

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: b. EXPIRATION:
b. NUMBER: NA
c. TYPE: d. AMOUNT:
e. NO OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		A. PROFESSIONAL MAN YRS	B. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	1.0	178.0
	CURRENT 80	1.0	218.1

19. RESPONSIBLE DOD ORGANIZATION
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20. GENERAL USE
Foreign intelligence considered

20. PERFORMING ORGANIZATION
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TELEPHONE: 301 663-7211
SOCIAL SECURITY ACCOUNT NUMBER

ASSOCIATE INVESTIGATORS
NAME: Shirey, F.
NAME

POC:DA

23. KEYWORDS: (U) Military medicine; (U) BW defense; (U) Rickettsia; (U) Influenza; (U) Mycoplasma; (U) Ultrastructure; (U) Toxins; (U) Microscopy

24. TECHNICAL OBJECTIVE, 25. APPROACH, 26. PROGRESS (Provide brief technical summary of work done with security classification code.)

23 (U) Study infections and toxemia states at the ultrastructural level by scanning and transmission electron microscopy so as to elucidate mechanisms by which infectious microorganisms enter and leave cells and to identify target organelles damaged by microorganisms and toxins. These studies should provide basic information relative to specific therapy and protection against diseases caused by these agents and could lead to early detection of agents of potential BW importance.

24 (U) Infected animals and cell cultures provide experimental material for examination by scanning and transmission electron microscopy. Conventional techniques and more sophisticated approaches, i.e., immunolabeling, freeze fracture, replication by metal casting, and stereology, are used in these studies.

25 (U) 78 10 - 79 09 - R. rickettsii produced a vascular lesion in hen egg chorioallantoic membrane similar to other spotted fever rickettsiae, except that there is endothelial cell necrosis. The Korean hemorrhagic fever agent is thought to be a Bunyamwera-like virus. This conclusion is based upon negative contrast studies and examination of ultrathin sections of infected A549 cells in culture. Electron microscopic autoradiography showed that Pseudomonas exotoxin A is bound to membrane receptors in coated pits and receptors for diphtheria exotoxin are diffusely located in the plasma membrane. Legionella pneumophila ingested by alveolar and peritoneal macrophages, destroys the phagocyte by prolific multiplication in the cytoplasm. Sandfly fever virus infection does not alter ultrastructure of human skeletal muscle to a significant extent.

DD FORM 1498

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 051: Analysis of Subcellular Structures in Microbial Infections of Potential BW Importance

Background:

Mechanisms and phenomena by which infectious microorganisms and toxins enter cells and damage or alter subcellular structure and function are studied by various methods utilizing electron beam microscopy (EM). We have shown that vesicular stomatitis virus (VSV) was recognized easily by scanning electron microscopy (SEM) on infected cells and that smaller viruses and pleomorphic ones are difficult to identify unless a specific immune marker is used. The respiratory disease caused by Mycoplasma pneumoniae was studied in golden Syrian hamsters infected with a small particle aerosol (SPA). Bronchopneumonia developed within 5 days and mycoplasmal filaments were seen and identified by immunolabeling on ciliated respiratory epithelium. Influenza virus (A/NJ/76), another small pleomorphic respiratory pathogen, was localized on ciliated respiratory epithelium of infected mice using phage T4-labeled immunoglobulins. Coxiella burnetii were demonstrated in the respiratory tract of guinea pigs exposed to aerosols of this rickettsia. These observations demonstrated the usefulness of SEM and immunomarkers for studying infectious diseases. Other rickettsial infections which have been studied include Rickettsia rickettsii and Rickettsia akari in cell cultures and Rickettsia conorii and Rickettsia sibirica in hen egg choriollantoic membrane (CAM). The vascular lesion of rickettsial spotted fever seen in the CAM is markedly similar to that seen in mammalian hosts and the surface changes associated with entrance or release of rickettsiae, either from endothelial cells or cells in culture and regardless of rickettsial species, were practically identical.

Progress:

The pathogenetic study of rickettsial spotted fevers was terminated as a result of program changes. It was possible to obtain CAM from hen eggs which had been infected with R. rickettsii before the cessation of laboratory work. The early temporal sequence of changes and the morphologic expression of vascular disease were identical to those reported earlier for CAM infected with R. conorii or R. sibirica. Within 3 days, rickettsiae were in the cytoplasm of endothelial cells where they multiplied, producing vacuolization and moderate hypertrophy. The presence of rickettsiae within endothelial cells attracted circulating heterophils and mononuclear cells that migrated through the vessel wall and produced a typical segmental lesion in adjacent stroma. In contrast to the changes induced in CAM by R. conorii or R. sibirica, however, some endothelial cells became necrotic late in the disease induced by R. rickettsii.

Korean hemorrhagic fever (KHF) is one of several similar febrile diseases that have wide geographic distribution and are designated as hemorrhagic fever with renal syndrome. The recent work of Dr. Ho Wang Lee in Korea (Grant No. DAMD 17-80-G-9461 and LTC George R. French (Work Unit A841 00 054) at USAMRIID has established methods for propagation and study of KHF. In particular, LTC French's discovery of a cell

culture for propagation of KHF virus has enabled him to develop serological methods for diagnosis and quantitation as well as to determine various physical characteristics of the virus.

Our collaborative work with LTC French was started with the goal of obtaining morphologic evidence for a definitive characterization of KHF virus. Several approaches were used which utilized transmission electron microscopy of either ultrathin sections or negatively stained preparations from infected and normal A549 cells. The negative stains were made from centrifuged pellets of disrupted cells and various purified or enriched preparations derived by gradient centrifugation and/or chemical treatment. The source of material for ultrathin sections included intact, whole cells from cultures or centrifuged pellets of disrupted cells. In addition, some of the material for ultrathin sections was used in immunolabeling experiments with either ferritin or the peroxidase-antiperoxidase (PAP) system as the probe; some negatively stained specimens were made from material suspended in specific immune serum.

In a large number of specimens, the most critical and limiting factor has been the comparatively low concentration of virus. In spite of this, we have been able to determine the morphology and probable classification of KHF virus.

Negatively stained preparations illustrated the close association of KHF virus for host cellular material. A huge amount of cellular debris was present in each sample in which virions were found. This has been most disconcerting because it becomes very difficult to recognize and interpret structures as viral in a sample which is predominantly nonviral. The cellular material either obscures detail or consists of many "virus-like" structures, which are sources of confusion. The signal-to-noise ratio is of an order which makes it extremely difficult to discriminate significant features. In an attempt to suppress the background and enhance the signal, we added antisera with KHF virus antibodies to some samples prior to negative staining. This resulted in the clumping of particles similar in size and shape to those previously assumed to be KHF virus in untreated samples. We have concluded, therefore, that the KHF virus is a fringed spherical particle, 80-100 nm in diameter.

We have also examined ultrathin sections prepared from the sedimented, disrupted cells which were used as the starting material for virus purification and enrichment and which were stained by negative contrast (supra vide). Again, low concentrations of virus relative to the enormous amount of cellular debris were seen. Round particles consistent in size with the negatively stained particles were found in membranous vesicles. Attempts to use immunolabeling were not successful because it appeared that the reagents did not penetrate the densely aggregated material. This was confirmed when we examined cellular debris from cultures infected with Rift Valley fever (RVF) virus in an identical manner. Only the virions at the periphery of each sample were labeled. The chances of finding KHF virus, present in extremely low concentrations, and located in sufficient numbers to be labeled at the periphery seemed slim indeed.

When sections from infected cultures were examined, we were impressed again with the paucity of virus particles and with the healthy appearance of the cells. After arduous examination of numerous specimens from many infected cultures, it was possible to find virus particles in vesicles within the Golgi region and other areas of the cytoplasm. The particles were usually 95-100 nm in diameter and the range in size was from 80-105 nm. These observations led to the conclusion that the KHF agent is most probably a Bunyamwera-like virus. This is consistent with the physicochemical

characteristics which have been determined by LTC French. Additional work is planned to confirm and expand these observations.

We have collaborated in a number of other projects, including the ultrastructural localization of toxins, phagocytic studies of Legionella pneumophila, and an evaluation of skeletal muscle in sandfly fever infection. Autoradiographs for EM were prepared from various cell cultures treated with either ^{125}I-labeled diphtheria (DE) or Pseudomonas (PE) exotoxins. The results showed that under conditions which enhance membrane binding of the toxins, PE was bound to receptors located in coated pits on the plasma membrane and DE was bound to receptors diffusely located on the plasma membrane. The binding of PE in coated pits was confirmed using immunolabeling techniques with either PAP or ferritin as the probe.

After demonstrating that L. pneumophila multiplied within the cytoplasm and destroyed phagocytic alveolar macrophages, we studied by TEM and SEM the interaction between this organism and peritoneal macrophages from normal animals and animals which had survived infection with L. pneumophila. It was shown that like alveolar macrophages, few cells regardless of source, were active phagocytically. Those which did take up bacteria were destroyed by the bacteria that multiplied rapidly in the cytoplasm. The addition of immune serum to mixtures of bacteria and macrophages potentiated the phagocytic activity of both cell types but did not affect the ultimate fate of macrophages that ingested bacteria.

Biopsies of the quadriceps femoris muscle were obtained from volunteers who participated in the sandfly fever project (Medical Division Protocol FY 79-6, Work Unit A841 00 0) to evaluate the effect of infection on skeletal muscle ultrastructure. With one exception, there were no profound changes which could be attributed to the infection. There were variations in the volume of interfibrillar space and amount of lipid and the presence of focal myofibrillar disarray of biopsies taken during the baseline, febrile, and convalescent periods from the control and 6 of 7 infected volunteers. One volunteer who was infected with virus had extensive edema between fibers in biopsies taken during the febrile and convalescent periods. It was felt that this edema was of vascular origin rather than from the muscle mass. Many small vessels had thickened basement membranes and disruptions in the tight junctions between endothelial cells. The muscle fibers contained many focal areas of myositis in which there were varying degrees of fibrillar necrosis and Z-disc streaming. The significance of these findings is not clear at present, but the reactions may be part of an allergic manifestation.

These studies have been possible through the collaboration of the following individuals: MAJ W. C. Hall, R. rickettsii; LTC G. R. French, KHF virus; LTC R. A. Kishimoto, L. pneumophila; Drs. S. H. Leppla and J. L. Middlebrook, PE & DE; Dr. G. Friman, sandfly fever study.

Presentation:

White, J. D. Electron microscopy in the study of infectious diseases. Presented, Dept. of Biology, Western Maryland University, Westminster, MD, May 1979.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6423	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	052
b. CONTRIBUTING				
c. ~~XXXXXXXX~~	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code) (U) Therapeutic manipulation of metabolic-endocrine controls during infections of unique military 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
75 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 79	1.0	86.7
	CURRENT 80	1.0	169.5

19. RESPONSIBLE DOD ORGANIZATION
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SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME: Merrill, G. A.
NAME: POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Infections; (U) Hormone therapy; (U) Metabolic defects; (U) Rats; (U) Receptors; (U) Virus

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 role of insulin and glucagon in the metabolic effects and feedback control during infection and infection-induced stress. Characterize the optimum manipulation of the various hormone axes involved in carbohydrate, fat and protein supply and use as a means of eliminating the undesirable metabolic changes associated with disease in either the physiological manifestations of that disease or a prolonged recovery period. In addition to the relation of this product to BW defense the expertise gained through work in this area is immediately applicable to the soldier infected secondary to either combat or noncombat injuries.

24 (U) Using isolated islets of Langerhans, the effects of bacterial and viral infection on insulin production and release will be measured. The physiological action of insulin in vivo will be assessed by use of glucose tolerance and insulin tolerance testing. In addition, the effect of infectious stress and its associated metabolic changes on binding of insulin to its receptors and thus the physiological action of insulin will be characterized.

25 (U) 78 10 - 79 09 - Continuing studies on the potential diabetogenic effect of the TC-83 VEE vaccine virus in animal models have failed to demonstrate replication or pathophysiological changes. Virus attaches to the cells of the isolated islets of Langerhans, but does not replicate. In vivo glucose tolerance tests and insulin release from isolated islets have all been shown to be normal in animals up to 180 days postinoculation. Insulin receptor studies have shown that physiological increases in serum glycoproteins can inhibit in vitro insulin binding to cultured monocytes. Sandfly fever virus infection in humans was shown to impair glucose tolerance and to inhibit insulin binding due to a marked reduction in receptor concentration on the cell surface. Publication: Clin. Res. 26:773A, 1978.

*Available in controlled form upon originator's approval

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE AND WILL BE USED UNTIL EXHAUSTED. ☆U.S. GPO 1974-546-843 8001

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 052: Endocrine-Metabolic Controlled Responses to Infection of Unique Military Importance

Background:

Previous observations have noted an unexplained relative hyperinsulinism during infection or experimentally induced endotoxemia. During infection, the liver seems unable to produce ketones, which may be a reflection of this hyperinsulinism. A vital area of concern in the clinical management of soldiers who might be BW victims, or who develop wound-related infections, is the supply of energy substrates during periods of high fever-induced metabolic needs and a reduction in the catabolic effects that prolong recovery from illness.

Insulin is the principal hormone involved in energy supply and reversal of catabolism. Previous investigations dealing with insulin response in, and effect on, infectious diseases have been confined to measurements of insulin production, circulating concentrations and target organ effects. With the radioreceptor assay we now have the capability to explore binding of insulin to its cellular receptor (necessary for the action of insulin) and thus can evaluate many facets of insulin pathophysiology during infection-associated events, e.g., changes in various proteins, pH, trace-metal concentration and other hormone systems.

In a living animal, the beta cell, the major component of the islets of Langerhans, is subject to the changing concentrations of stimulants and inhibitors in the circulation as well as neurogenic effects on the endocrine pancreas. With the introduction into the laboratory of the technique for isolating islets of Langerhans, we now have available a tool for directly measuring the effects of specific hormones, chemicals, toxins and/or microorganisms on the beta cell itself without unmeasurable interference from other substances. This gives us the ability to evaluate the effect of microorganisms on the synthesis and release of insulin as well as their effects on circulating insulin and insulin binding.

Progress:

Modification of hormone response. Major work in the past year has included the investigation of LiCl as selective inhibitor of insulin release from the islet. The effects of both chronic and short-term administration of LiCl were evaluated by glucose tolerance and insulin response testing in rats. The chemical was administered orally in all experiments with equivalent volumes of saline administered to controls. In several experiments a high protein liquid diet was given concurrently by means of an intragastric catheter every 4 hr to both control and experimental rats.

Rats treated for 18 days with 60 mg of LiCl/kg/day divided equally into 6 feedings per day demonstrated marked glucose intolerance and impaired immunoreactive insulin (IRI) response. LiCl-induced impaired IRI response involved a delayed peak response as well as a decrease in total IRI release. Both experimental and control rats demonstrated initial weight loss which stabilized after 5 days; then both groups

demonstrated consistent weight gains. This suggested that the results were not due to dietary differences. Rats given LiCl in equivalent doses for only 5 days did not demonstrate significant glucose intolerance. However, severe glucose intolerance and impaired insulin release was observed in rats given a higher dosage (400 mg LiCl/kg/day). These data suggest a gradual accumulation of Li in the beta cell of the pancreatic islets as a possible explanation for the impaired immunoreactive insulin release and glucose intolerance seen in the treated rats.

VEE (TC-83) viral studies. Investigation continued on the effect of the TC-83 vaccine virus strain of VEE on carbohydrate metabolism. Numerous studies failed to demonstrate the presence of vaccine virus in the pancreas of acutely infected hamsters. Pancreatectomies were performed on hamsters 24, 48 and 72 hr after inoculation with 5.0-5.3 $\log_{10}$ PFU of VEE TC-83. Both direct and indirect fluorescent antibody staining was performed on multiple pancreatic sections and the slides examined in cooperation with Dr. Jahrling of the Virology Division. We were unable to demonstrate the presence of virus utilizing either method.

To examine the possibility of viral replication in the islet itself, isolated islets of Langerhans from rats, hamsters and guinea pigs were obtained, put into culture and incubated with both labeled and unlabeled VEE TC-83 virus. In each study there was no indication of viral replication. By using the radiolabeled virus in conjunction with Dr. Jahrling, we were able to determine that the virus did, in fact, adsorb to the cells in the islet.

Studies were also conducted on the IRI release from isolated islets obtained from hamsters which had previously been infected with VEE TC-83. Islets were obtained from hamsters 2, 3, 60 and 90 days postinfection with 5.2 $\log_{10}$ PFU TC-83. These islets were then incubated in Krebs-Ringer bicarbonate solution with 300 mg/dl glucose as the stimulus. There was no significant impairment of insulin release from islets from the infected hamsters as compared to those from controls. Demonstration of infection in the hamsters was obtained both by elevation of temperature and isolation of virus from the spleen.

The in vivo glucose tolerance test given hamsters 4 and 6 mon postinfection with TC-83 virus was not significantly different from control hamsters of the same age.

Receptor studies. Preliminary studies of insulin binding to receptors on isolated hepatic plasma membranes have been completed in conjunction with CPT Little of the Bacteriology Division. Insulin binding was shown to be pH-dependent with an optimum binding at pH 7.4, temperature-dependent with best results at 16°C, which maximized binding and minimized insulin degradation, and time-dependent with no increase in binding after 90 min. Preliminary experiments indicate increased binding on membranes isolated from rats 48 hr after inoculation with S. pneumoniae. To eliminate possible artifactual variations in results using the membrane preparation, large batches of control and infected membranes were prepared and pooled. Small aliquots were frozen for future experiments. The combined plasma membrane pool has been assayed and shown to be extremely pure with no significant contamination by other cellular organelles. Studies are continuing to confirm these original observations.

Specific insulin binding to isolated hepatic nuclei has been demonstrated in conjunction with Mr. E. Hauer of the Physical Sciences Division. All work has been of a preliminary nature in an attempt to characterize optimal binding conditions for the assay. In all of the studies done thus far demonstrating specific binding, nuclei obtained from Streptococcus pneumoniae-infected rats have shown increased receptor

binding. The relation between the increased binding and the increased RNA synthesis previously reported during S. pneumoniae infections remains to be characterized.

Initial in vitro experiments to determine the effect of glycoproteins on insulin receptor binding have been conducted using the culture dye M9 mononuclear cells. Various glycoproteins are known to be elevated in plasma during stress conditions (including infection) and these substances have been shown to inhibit cell division and lymphocyte transformation. Our research has demonstrated that cultured monocytes incubated 18 hr in the presence of 166 mg/dl ovomucoid (a purified glycoprotein from egg white) in the growth media, significantly depressed insulin binding if the cells were not washed free of ovomucoid during the preparation for the assay. It was also observed that the cell diameter of ovomucoid-treated cells was significantly larger than untreated cells. When these treated cells were washed prior to assay, the cell diameter returned to that of the control cells and the inhbiition of binding was abolished. Thus, elevations of glycoprotein during infection may inhibit insulin binding to its receptor by coating the cell surface. This may be an explanation of the insulin resistance seen in infection. It is also important to note that assays of insulin binding, as ordinarily performed, may not accurately reflect in vivo conditions.

Planned studies to evaluate the effect of infection in adrenalectomized animals, which would be unable to produce the increase in glycoproteins, have been hampered by the inability to adrenalectomize a rabbit, the minimum size animal needed for the receptor measurements planned.

Human viral infection study. Human volunteers were studied for changes in insulin receptor binding and for oral glucose tolerance during acute infection with sandfly fever virus (Medical Division Protocol FY 79-6, Work Unit A84100001). Sandfly fever virus produces a short duration, nonlethal, well-characterized clinical illness. Each individual in the study served as his own control for both the receptor and glucose tolerance studies. Individuals were studied prior to inoculation and at the peak febrile period. Our studies demonstrate a significant decrease in glucose tolerance, following an oral glucose challenge of 1.75 gm glucose/kg/ideal body weight. Plasma glucose values were uniformly higher at each point in the 2-hr study during the time of infection as compared to the preinfection controls in spite of the significantly higher insulin values at each measured time point. In each of the subjects, glucose values failed to return to basal levels by 120 min.

Insulin receptor binding in each of the volunteers was markedly decreased. The decrease in labeled insulin binding ranged from 7.5-33%. By Scatchard analysis, plotting bound:free ratio the function of the amount of insulin bound, changes in insulin receptor concentration per peripheral mononuclear cell can be determined. In this study we demonstrated a 25-75% decrease in receptor concentration in those individuals infected with the virus. To examine changes in binding due to alterations of receptor affinity, a DeMeyts affinity plot was prepared on each individual. There were no consistent results demonstrated, with no significant change in receptor affinity in 2 of the 3 infected individuals. However, the one individual having the largest decrease in receptor numbers demonstrated a significant increase in binding affinity at low insulin concentrations. This may be the result of physiological mechanisms of the cell to maintain homeostasis by increasing receptor affinity to compensate for the severe receptor loss.

Antibody receptor assay. Initial isolation of insulin receptors from hepatic plasma membranes of rats for use in the development of an assay to measure receptor number directly by binding of a labeled antireceptor antibody has been performed. It is anticipated that the antibodies to the receptor will be labeled with horseradish peroxidase for detection by chemiluminescent technique or radiolabeled for a radioimmunoassay method, either of which we anticipate will produce a greater sensitivity in determination of receptor numbers than is presently possible by the radioreceptor assay currently used. Purification or receptor preparations by affinity chromatography is presently underway.

Endorphin study. Various preparations of endorphins and enkaphalins have recently been shown to have the ability to alter glucagon and insulin release from pancreatic islets. To investigate the possibility that these substances may be responsible for the hyperinsulinemia associated with an infection, hamsters were given endotoxin or saline. Rat tissues were studied at 3 and 5 hr postinoculation. During collection of blood, Trasylol, a proteinase inhibitor, was added to minimize endorphin degradation. Endorphin levels were determined by radioimmunoassay. There was no significant change in endorphin levels detected in the endotoxemia animals compared to control. These studies are still in the very preliminary stages; the amount of cross-reactivity between hamster endorphin and the antigen used in the assay has not been completely determined.

Endorphin levels were also measured during the human volunteer study with sandfly fever virus. No significant changes in measurable endorphin levels were noted during the illness.

Publication:

Anderson, Jr., J. H. and G. A. Merrill. 1978. Impairment of glucose tolerance by lithium chloride. Clin. Res. 26:773A.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6418	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUMRY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 04 18	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	62776A	3M162776A841	00	053
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Characterization of nonindigenous tick-borne rickettsiae for vaccine development

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 79	1.0	152.4
	CURRENT 80	1.0	140.8

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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) Military medicine; (U) BW defense; (U) Vaccines; (U) Tick-borne rickettsioses; (U) Q fever

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

23 (U) Characterize in vitro and in vivo biological and chemical markers of rickettsial cultures to enhance vaccine efficacy and to facilitate identification of strains and species. Assess low virulent organisms or potential military importance for use as live vaccines for protection of troops exposed to a biological warfare environment.
24 (U) Grow rickettsiae in tissue culture, embryonated eggs or animals to obtain sufficient quantities of organisms for chemical or physical fractionation. Vaccinate animals with various fractions and test for protective capacity by challenge with virulent agents.
25 (U) 78 10 - 79 09 - Work continued on attempts to separate and characterize the protective antigen of Rickettsia rickettsii. The antigen was found to be stable in, but not precipitated by, 0.1 N HCl. It was inactivated by 1.0% merceptoethanol in 1.0% sodium dodecyl sulfate solution as used in gel electrophoresis, and it did not pass through a G-200 Sephadex column. The protective factor was not affected by alpha-amylase or lysozyme digestion, or by 0.01 sodium periodate, but was destroyed by the enzyme Pronase. It was only partially sedimented by 8 hr centrifugation at 35,000 x g. No cross-protection among other members of the spotted fever group of rickettsiae was observed in guinea pigs vaccinated with the trichloroacetic acid precipitated antigen.

Due to transfer of certain aspects of rickettsial diseases to WRAIR, only studies with Coxiella burnetii phase I vaccine will be reported in the future in this work unit.

*Available to contractors upon originator's approval

☆ U.S. GPO: 1974-533-043/3691

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 053: Characterization of Non-indigenous Tickborne Rickettsiae for Vaccine Development

Background:

Current vaccines against rickettsial agents are made from partially purified, killed whole organisms suspended in an isotonic medium. Some vaccines, such as those of spotted fever and typhus also contain substantial amounts of host material as well as soluble rickettsial products. More vigorous purification to remove host material also removes most of the soluble (S) antigen of these organisms which contains much of the protective factor. Little is known of the nature of the S antigen, but available information indicates that it is a protein-carbohydrate complex residing principally in the slime layer surrounding the organism. It can be easily separated from rickettsial bodies by ether extraction of rickettsial suspensions followed by sedimentation, and it can be partially sedimented by very high-speed centrifugation of the supernatant. This material is being used as a starting point in this investigation to isolate and characterize the protective antigen of Rickettsia rickettsii which causes Rocky Mountain spotted fever (RMSF). Methods used for the investigation of the protective antigen were discussed previously (1).

Progress:

Freund's incomplete adjuvant (FIA) was employed in earlier studies to increase the immune response of guinea pigs vaccinated with various fractions derived from the rickettsial preparations (1). To determine whether the adjuvant was necessary, a sample of soluble antigen was dialyzed, concentrated and divided into 2 equal portsions. One part was mixed with FIA and injected into a guinea pig. The second part was used alone for injection into another guinea pig, using a 2-dose schedule with half given on day 0 and the remainder on day 7. After challenge, both guinea pigs were resistant to the infection, which indicated that immunization without an adjuvant could be used if desired.

In a related experiment, a sample of soluble antigen was dried thoroughly by evaporation from a celluiose tube, rehydrated and inoculated into a guinea pig using the same 2-dose schedule. This guinea pig was also protected against virulent challenge, showing that the drying did not inactivate the protective antigen.

One procedure considered for fractionating the soluble rickettsial antigen was polyacrylamide gel electrophoresis (PAGE). In this method, protein complexes are usually dissociated prior to electrophoresis by solutions of sodium dodecyl sulfate (SDS) containing mercaptoethanol (ME), a process that sometimes denatures, irreversibly, certain types of proteins. To determine whether this treatment inactivated the protective antigen, a sample of whole rickettsial suspension was divided in half, and one part treated with SDS + ME according to a method reported by Eisemann and Osterman (2). Both samples were then dialyzed, concentrated and inoculated into guinea pigs. After challenge, the guinea pigs vaccinated with the treated sample succumbed to the infection while the animal receiving the untreated

sample was protected. These results suggested that gel electrophoresis probably will be of little use in separating the protective antigen from other components in the mixture.

In another experiment, an attempt was made to fractionate the S antigen solution using gel exclusion chromatography. A column (0.9 x 20 cm) of G-200 Sephadex was prepared and equilibrated with phosphate buffered saline, pH 7.3. A sample of the S antigen suspended in a small amount of saline was applied to the top of the column. Slightly less than one void-volume of saline was passed through the column and discarded. The solvent flow was then continued until 5 void-volumes (∿ 60 ml) were collected. Second and third samples of this amount were also collected. Each of the 3 samples was then reduced in volume, mixed with FIA and injected SC into guinea pigs. As a positive control, a sample of the original material was also inoculated into a guinea pig. The guinea pigs were rested for 4 weeks and challenged with 1000 EID_{50} of the R strain of R. rickettsii. The control guinea pig was protected from the infection as shown by its lack of febrile response. However, the animals injected with samples from the column all showed the typical febrile response of RMSF infection. The protective antigen was either small enough to penetrate into the gel and was not eluted by 15 void-volumes of saline, or was not dispersed in the saline and simply filtered out.

The so-called S antigen associated with typhus rickettsiae has been reported to be particulate in nature, and capable of being sedimented by high-speed centrifugation (3). To determine whether the soluble antigen of RMSF rickettsiae which contained the protective factor could also be sedimented, a sample of S antigen was centrifuged at 9,000 x g for 30 min to ensure that all rickettsial bodies were removed from the preparation. The supernatant was then centrifuged at 35,000 x g for 4 hr. A small clear gelatinous precipitate formed in the bottom of the tube. It was resuspended in about 1 ml of K-7 buffer, mixed with an equal volume of FIA and inoculated SC into a guinea pig. The supernatant from this centrifugation was concentrated to a small volume, mixed with FIA and injected into another guinea pig. Both guinea pigs were challenged with R. rickettsii one month after vaccination; they were immune. In another similar experiment, using the same g force, but centrifuging for 8 hr, protective material was again found in both the sediment and supernatant. Apparently this antigen is either partially soluble in aqueous solutions or is in the nature of a sol which can be sedimented only with great difficulty.

Since trichloroacetic acid (TCA), a strong acid as well as a protein precipitant, has been useful in precipitating the protective antigen of R. rickettsii, an attempt was made to determine whether acid alone would cause a similar reaction. A solution of soluble antigen was made 0.1 N in HCl and incubated at 4°C for 24 hr. A small amount of precipitate formed which was separated by centrifugation and resuspended in ∿ 5 ml of distilled water. The supernatant and the resuspended sediment were then dialyzed exhaustively against distilled water to remove the acid. Another precipitate formed in the dialyzed supernatant; it was also separated by centrifugation and was resuspended in 2.0 ml of saline. The supernatant was concentration to ∿ 2.0 ml by evaporation through dialysis tubing. Each preparation was then treated and injected into guinea pigs as described earlier. On challenging the guinea pigs with virulent R. rickettsii, it was determined that the

original TCA precipitate contained no protective activity, but the supernatant and the precipitate that formed in it during dialysis were protective. Results of these experiments indicate that the protective antigen is very stable in the presence of strong acids and that other properties of TCA appear responsible for its precipitating effect on the antigen.

Several attempts were made to determine some characteristics of the protective antigen by chemical or enzymatic digestion methods. In the first attempt, a 100-ml sample of soluble antigen was digested for 24 hr at 34°C with 25 mg of a protease (Pronase®). This sample was dialyzed against distilled water, concentrated and injected into a guinea pig as described previously. The guinea pig when challenged with R. rickettsii, became ill with the disease, indicating that the sample had lost its protective activities. A control sample of soluble antigen without the protease digestion protected a guinea pig against the challenge

In another experiment, 25 mg of the enzyme, amylase, which hydrolyzes the -1,4-linked glucose units in polysaccharides, was added to another 100-ml sample of the coluble antigen. The mixture was incubated at 34°C for 4 hr, dialyzed against distilled water, concentrated and injected into a guinea pig. On challenge, the animal was found to be protected aginst the infection, indicating that amulase-sensitive charbohydrate components were not present or were not necessary for protective activity.

A 100-ml sample of a whole rickettsial suspension was made 0.01 M with sodium periodate. The sample was incubated at 4°C for 24 hr, dailyzed against distilled water and concentrated to ∿ 2.0 ml. It was injected into a guinea pig and the animal was challenged 4 weeks later. No loss of protective activity was observed; the animal reacted similarly to an unimmunized control guinea pig. As a control for the periodate, a sample of the soluble phase I antigen of Coxiella burnetii was treated with periodate and injected into a guinea pig. The protective effect of this sample was destroyed, as indicated by the development of an infection when challenged with live phase I C. burnetii. A control guinea pig receiving an equal amount of untreated antigen was protected. Since periodate reacts with and chemically changes carbohydrates, it would appear that a carbohydrate is not associated with the protective factor of R. rickettsii.

Another attempt was made to characterize this protective antigen by enzymatic digestion. A sample of the S antigen precipitated by TCA and a sample of the antigen sedimented from infected tissue culture fluid, were treated with a lysozyme solution (200 mg/ml) for 4 hr at 37°C. These samples, along with untreated samples along with untreated samples of each type were injected SC into guinea pigs. The animals were rested for 28 days, then challenged with 1000 ID_{50} of the R strain of R. rickettsii. All guinea pigs were protected from the infection, suggesting that lysozyme treatment had no apparent effects on the protective antigen.

From the data reported here, the protective antigen of R. rickettsii appears to be largely protein in nature, with little or no active lipid material had no effect on activity, and amylase, lysozyme and periodate treatments were also ineffective. In a previous report (1), 20% sodium sulfite was shown to partially precipitate the antigen, a property suggestive of the presence of carbohydrate. However, at the concentration used, some protein could also be precipitated. Overall, the evidence suggests that the protective antigen is large protein.

Guinea pigs that survive infection with R. rickettsii are usually protected against infection by other members of the spotted fever group. Since the antigen precipitated by TCA protected guinea pigs from challenge by R. rickettsii, an experiment was conducted to determine whether this antigen conferred any protection against other members of the spotted fever group. Fifteen guinea pigs were vaccinated SC with the TCA-precipitated antigen suspended in FIA, using enough antigen to protect the animals against 1000 EID_{50} of R. rickettsii. One month later, groups of 3 guinea pigs each were challenged with 1000 $E D_{50}$ of virulent R. rickettsii, R. akari, R. australis, R. conorii, and R. sibirica, along with an unvaccinated control guinea pig for each rickettsial species. Temperatures were recorded daily for 10 consecutive days. The guinea pigs challenged with R. rickettsii remained afebrile during the test period while the unvaccinated control animal became ill. Those challenged with the other rickettsiae developed febrile responses similar to those of the controls for each organism. Apparently, the TCA-precipitated vaccine did not contain sufficient cross reactive antigens to produce protective immunity against the other organisms.

Due to a reorganization of the Rickettsiology Division and a transfer of activities, project in this work unit involved with rickettsiae other than C. burnetii will be terminated. Remaining under this work unit will be those studies concerned with further testing of the NDBR-105 phase I Q fever vaccine.

Publications: None

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2. Eisemann, C. S., and J. V. Osterman. 1976. Proteins of typhus and spotted fever group rickettsiae. Infect. Immun. 14:155-162.

3. Cohen, S. S., L. A. Chambers, and J. R. Clawson. 1950. Studies on commercial typhus vaccine. 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 0G6422	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	054
b. CONTRIBUTING				
c. 1HHHHH	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code) (U) Characterization and evaluation of selected hemorrhagic fever agents for vaccine development

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

13. START (YR MO)	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY			16. PERFORMANCE METHOD
76 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 79	1.0	220.1
	CURRENT 80	1.0	213.6

19. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME:
NAME:

21. GENERAL USE: Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede each with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Congo-Crimean hemorrhagic fever (CHF-C); (U) Korean hemorrhagic fever; (U) Fluorescent antigen

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

23 (U) Complete characterization of the agent of Korean hemorrhagic fever (KHF) and assessment of the squirrel monkey model for human disease. Assess the potential of ribavirin for chemotherapy. Initiate KHF vaccine development. (U) Study CHF-C virus. 24 (U) KHF virus will be disrupted to isolate and examine viral RNA. Determination of the number of RNA strands and segments will be utilized to complete the classification of the virus. The efficacy of ribavirin as a chemotherapeutic agent will be assessed in the squirrel monkey model of KHF. Vaccine development will be initiated by re-isolation of the virus in vaccine certified cells from known infectious human blood. 25 (U) 78 10 - 79 09 - Six vaccine certifiable cell lines have been tested for growth of CHF-C virus and the related Hazara virus. Hazara virus replicates to good titer in all lines tested; however, significant but low yields of CHF-C virus were obtained in WI-38 cell lines. Examination of the structural proteins of Hazara virus revealed an important difference from the reported characteristics of Bunyaviridae virus proteins. This completes the current phase of CHF-C.

The etiologic agent of KHF has been characterized as a heat-stable, enveloped, cell-associated RNA virus. Electron micrographs have revealed spherical virus particles 95 ± 15 nm in diameter. These and other characteristics are consistent with viruses of the family Bunyaviridae but do not rule out the possibility that KHF is an arena-virus. Extensive serologic comparisons performed in the past year failed to relate KHF virus to any other known virus or group but do link KHF to a similar disease of unknown etiology in Sweden. The squirrel monkey (Saimiri sciureus) has been identified as a potential host for an animal model of the human disease.

DD FORM 1498

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 054: Characterization and Evaluation of Selected Hemorrhagic Fever Agents for Vaccine Development

Background:

Part I. Congo-Crimean hemorrhagic fever (C-CFF).

The basic laboratory parameter of Hazara (HAZ) virus and Congo (CON) virus strain IbAR 10200 were reported in the previous annual report. Plaque assay procedures were developed for both viruses and growth characteristics for HAZ virus in BHK-21 cells and Congo virus in Vero cells were described. Reciprocal plaque-reduction neutralization (PRN) tests, indirect fluorescent antibody (IFA) tests and mouse cross-protection tests all indicated that the 2 viruses were antigenically similar and either virus might be usable as an immunogen for group protection. Utilizing the methodology developed last year, our objectives for this year's work were (a) to determine the replicative characteristics of both viruses in vaccine-certifiable cells, and (b) to examine and characterize the structural proteins of HAZ virus to determine their correspondence to reported values for other known Bunyamwera or Bunya-like viruses.

Part II. Korean hemorrhagic fever (KHF).

In previous annual reports we described the first successful isolation of the etiologic agent of KHF in cell cultures. In this cell line, designated A-549 and described as a type II alveolar cell derived from a human lung carcinoma, KHF establishes a noncytolytic persistent infection readily detectable by direct fluorescent antibody (DFA) or IFA test. The in vitro isolation of KHF made possible for the first time the opportunity to begin characterization of the agent on a biochemical as well as a physical level and to develop new and more rapid assay systems for the detection and serologic diagnosis of virus.

Progress:

Part I: C-CHF

Growth characteristics of C-CHF in vaccine-certifiable cell lines. Preliminary investigation of replication of these viruses in human diploid lung cells and growth characteristics in continuous laboratory cell lines revealed that the best yields were obtained at low input multiplicities and that yield was directly proportional to increasing percentages of fetal calf serum (FCS) in the cell medium. Thus, optimal yields were achieved at input MOI of 0.005 PFU/cell and 10% FCS in the medium. Utilizing these same conditions virus growth curves for HAZ and CON IbAr 10200 viruses were constructed for 6 vaccine-certifiable cell lines; chick embryo fibroblasts (CEF), duck embryo fibroblasts (DEF), fetal rhesus lung (FRhL), dog kidney (DK) and human diploid lung lines, WI-38 and MRC-5. The results, showing peak titer and timing are shown in Table I.

TABLE I. C-CHF VIRUS YIELD IN VACCINE-CERTIFIABLE CELL LINES

CELL LINE	HAZ		CON (IbaR 10200)	
	Peak $\log_{10}$ PFU/ml	Hr	Peak $\log_{10}$ PFU/ml	Hr
CEF	6.3	108	<2.0	-
DEF	5.6	120	<2.0	-
FRhL	5.5	108	<2.0	-
DK	5.4	96	<2.0	-
WI-38	6.8	72	4.4	144
MRC-5	5.8	72	<2.0	-

Maximum yield for HAZ virus occurred on WI-38 at 6.8 $\log_{10}$ PFU/ml at 72 hr. This compares to a yield of $10^{7.7}$ PFU/ml on BHK-21 cells. Yields for CON virus were insignificant on all cell lines tested except the WI-38 which yielded $10^{4.4}$ PFU/ml at 12 days; yields on Vero cells were $10^{5.7}$/ml at 5 days.

Physicochemical characterization of the structural proteins of HAZ. HAZ virus was chosen for a study of the structural proteins because of its relative capability to replicate to high titer, some 10-fold higher than strain 10200 of CON. The 11th mouse brain passage of HAZ was cloned for study by 3 terminal dilution passages in BHK-21 cells. The pooled working stock was prepared from the 3rd terminal dilution virus by inoculation of 5 ml onto roller bottles of BHK-21 cells. The supernatants (200 ml/bottle) were harvested 48 hr postinfection, clarified by centrifugation at 8000 x g for 30 min, divided into 5-ml aliquots and stored at -70°C. Growth curves for this working stock (clone 579), were constructed at various MOI and it was determined that maximal titers ($\leq 10^8$ PFU/ml) were achieved at 24 hr at an input MOI of 0.1 PFU/cell. Virus for study was propagated therefore by infection of BHK-21 monolayers in roller bottles (MOI of 0.1). After absorption and removal of the inoculum, 50 ml of E-199 medium containing 1/40 normal concentration of amino acids, 5% dialyzed FCS with HEPES, antibiotics and the appropriate radioisotope (500 µCi of tritiated amino acids or glucosamine/roller bottle or 200 µCi of ^{14}C-labeled compounds/roller bottle were added. Preparations dually labeled with [^{3}H] uridine or ^{14}C-L-amino acids were propagated in H-BME media with 1/10 normal amino acids rather than the E-199 medium. Labeled control supernatants (not infected)were prepared in the same manner.

Labeled infected and control supernatants were clarified by centrifugation at 380 x g for 10 min followed by centrifugation at 800 x g for 30 min. Concentration of the virus was accomplished by $(NH_4)_2SO_4$ (50% v/v) precipitation at 0°C. The precipitate was recovered by pelleting at 10,000 x g and resuspended in the buffer to 1/100 of the original volume; 5 ml of the virus concentrate was purified on 30 ml continuous 20-50% sucrose gradients formed above a 2.6 ml 60% sucrose cushion and centrifuged for 4 hours at 115,000 x g in the SW 27 rotor. Fractions (1 ml)

were collected from the bottom of the tube using positive displacement by mineral oil pumped in at the top; 50-µl samples of each fraction were counted for radioactivity by liquid scintillation; every other fraction was assayed for virus content by plaque titration. Peak fractions were pooled, diluted 2-fold in TNE, and layered on to a second continuous 20-50% sucrose gradient. Centrifugation, fractionation and assay procedures were repeated and peak fractions were stored at -70°C until needed. Purified virus was prepared for PAGE by the addition of 1% SDS and 2-mercaptoethanol and heating at 100°C for 10 min. Samples were adjusted to yield 10-20,000 CPM/gel or 5 µg of each standard protein for stained gels. Bromophenol blue was added to a final concentration of 0.001%. Laemmli gels were prepared in borosilicate 5 x 12-cm tubes using a 1.2-cm stacking gel above a 9.8 cm-resolving gel. Resolving gels of 6, 8, 10 and 12% (w/v) acrylamide and stacking gels of 3% acrylamide were prepared in the appropriate buffer with an acrylamide: BIS ratio of 30:0.8 (w/v) and 0.1% SDS polymerized with 0.025% (w/v) ammonium persulfate and 0.1% TEMED. Radiolabeled gels were sliced for counting or fixed and stained with 0.2% coomassie brilliant blue in 7% acetic acid. The isopycnic density of nucleocapsid was determined on sucrose gradients after disruption of the virus with 2% NP-40 detergent. Extrinsic labeling of virus with ^{125}I was accomplished by incubation at ambient temperature for 15 min with 500 mg of glucosoxidase in 0.5 ml of purified virus with 250 mg lactoperoxidase and 1.9 mCi of carrier-free ^{125}I. Three equal 50-µl portions of 1% D-glucose were added at 5-min intervals. After incubation, 0.1 ml of 1M KI was added; unreacted materials were removed by successive centrifugation in 20-50% sucrose gradients containing 10 mM KI. Fractions were assayed for DPM on a Beckman 8000 gamma counter; a portion of the peak sample was analyzed by PAGE as previously described.

Analysis of the data resulting from the experimental methods applied to HAZ virus revealed its similarity to other Bunyaviruses as expected, but also revealed some very important and as yet unexplained differences. These comparisons are presented in Table II prepared by SP5 Foulke of this laboratory. The values for all viruses other than HAZ were taken from Obijeski and Murphy (1).

TABLE II. BIOCHEMICAL CHARACTERISTICS OF SELECTED BUNYAVIRUSES[a]

PARAMETER	BUN	ORI	PHL	LAC	CE	HAZ
Proteins (MW) ($x10^3$)						
Large (L)	--	--	--	180	--	--
Glycoproteins						
G1	125	125	125	120	82	84
G2	30	30	75	35	38	45
G3	--	--	--	--	30	30
Nucleocapsid	22	22	26	25	17.5	52
Sucrose buoyant density (g/cm^3)						
virus particle	--	1.18-1.19	--	1.18	1.18	1.16
Nucleocapsid	--	1.31	--	1.21	1.25	1.25-1.26

[a]Value for all viruses except HAZ virus taken from Obijeski and Murphy (1977).
BUN = Bunyamwera, ORI = Oriboca, PHL = Phlebotomus fever, LAC = La Crosse.

TABLE III. PLAQUE NEUTRALIZATION DATA FOR HAZ CLONE 579 AND SELECTED ANTISERA[a]

SERUM	TREATMENT[b]	RECIPROCAL TITER			
		CON 10200		HAZ CLONE 579	
		PRN_{50}	PRN_{80}	PRN_{50}	PRN_{80}
Mouse anti-HAZ #1	None	270	150	1400	450
	Kaolin	190	70	900	300
Mouse anti-HAZ #2[c]	None	60	35	450	190
Normal mouse	None	35	15	<10	<10
	Kaolin	<10	<10	<10	<10
Guinea pig anti-CON[d]	None	940	400	60	20
Normal guinea pig	None	<10	<10	<10	<10
Polyvalent anti-CON serogroup mouse ascitic fluid[e] (2 trials)	None	650	300	50	20

[a]Unless otherwise noted 50% and 80% plaque reduction titers were calculated from 3 trials using probit-log plots.

[b]25% kaolin clay to remove nonspecific inhibitors.

[c]Anti-HAZ (1087) prepared by Dr. J. Casals, YARU.

[d]Anti-CON 10200 prepared by Dr. R. Rosato.

[e]NIH reagent #G221-601-567.

virus is first detectable in these cells about 72 hr as small discrete foci that spread very slowly in size and do not increase significantly in number for a few more days. These foci are easily detected utilizing a 16X low power objective and can be counted in much the same manner as plaques, provided the time and area counted are held relatively constant. Thus, the system was easily modified from the 14-day $TCID_{50}$ titration in which infected monolayers of cells were scored as positive or negative, to a fluorescent foci forming unit (FFU) assay in which infectious centers per unit area were counted at 72 hr. The FFU assay system presently in use is performed by adding 0.1 ml of diluted virus suspension to each of 2 wells of an 8-chambered "lab-tech" slide culture apparatus, 4 dilutions per chamber slide. A 549 cell suspension (0.1ml,1-2 x 10^5 total cells) is then added to the virus. The slides are incubated for 72-78 hr at 36°C, fixed in the usual manner with cold acetone and stained by DFA or IFA. The fluorescent foci in 5 fields are counted, averaged for the 2 wells multiplied by an "area observed" factor and corrected for dilution and volume factors to yield FFU/ml. It has been found most productive to test unknown virus suspension in 4-fold increments which cover a 2.7 log_{10} span on one slide apparatus. Dilutions are selected for actual count where foci average 2-10/field. FFU have been found to be linear with dilutions and the assay system has proved to be as reproducible as plaque assay systems.

The development of a FFU assay system made possible a foci-reduction neutralization test as an adjunct to our present IFA test for KHF antibody. This neutralization test is performed in exactly the same manner as a PRN test. Our experience with this procedure is quite limited, but we do obtain reduction in foci with KHF immune serum; it will be important to determine the correspondence of the antibody it measures to that measured in the conventional IFA test.

Characterization of the etiologic agent of KHF. Existing data relative to characterization accumulated by Dr. Ho Wang Lee,(Korean University Medical School, Seoul, Korea) indicates that the KHF agent is a lipid solvent sensitive virus with a minimum diameter of < 100 nm. We confirmed the lipid solvent sensitivity characteristic with the standard chloroform inactivation test and turned our attention to determination of the type of KHF virus nucleic acid utilizing established yield quantitation procedures in the presence of 10^{-6} 5-bromodeoxyuridine (BUdR) and varying concentrations of actinomycin D. Herpes simplex (hominis) type I and CON virus strain IbAr 10200 were employed as DNA and RNA virus controls, respectively. The yield data of KHF virus in the presence of these DNA virus inhibitors unequivocally established that KHF virus is an RNA virus. In the presence of 10^{-6} M BUdR the yield of CON virus, the RNA control, and the KHF virus was not reduced whereas the yield of herpesvirus was reduced > 99.9% at peak. Similarly, KHF virus replication is not sensitive to actinomycin D at concentrations that clearly inhibit the DNA virus control. The actinomycin D yield data are shown in Table IV. KHF virus was tested at 2 input MOI, ~ 0.001 and 0.1 infectious particle/cell. Only the data for yield at 72 hr are shown in the table because even the 0.01 mg/ml concentration of the drug is toxic to A-549 cells at periods later than this and incorrectly gives the impression of significant inhibition of all virus replication. Similarly, concentrations of the drug > 0.02 mg/ml are not shown because they are clearly cell toxic at 72 hr. Note the apparent stimulation of KHF virus replication at the low MOI and low concentration of the drug. This repeatable phenomenon is unexplained but may be a result of interferon suppression by actinomycin D. CON virus was not tested at low MOI, so there is no basis for comparison with the RNA control virus. On the other hand, herpesvirus replication was clearly inhibited at this low concentration.

TABLE IV. EFFECT OF ACTINOMYCIN D ON VIRUS YIELD 72 HR POSTINFECTION.

	YIELD IN PFU OR $TCID_{50}$/ml			
ACT. D	Herpes simplex,	CON	KHF	
µg/ml	Type I	IbAR 10200	Low MOI	High MOI
0	4×10^6	2×10^4	7×10^2	2×10^5
0.01	7×10^4	3×10^4	5×10^4	2×10^5
0.02	8×10^3	4×10^3	3×10^2	7×10^4

The question of the nature of KHF virus RNA, i.e., single or double stranded, in one piece or multiple segments, has not been approached in any definitive manner. However, a clue to the first question of strandedness has been provided by chlorpromazine long-wave UV inactivation studies with KHF which indicate that KHF RNA is probably single stranded. Dr. Carl Hanson, a contractor from the University of California, has shown that DNA viruses and single-stranded RNA viruses are inactivated by long-wave UV (20 mW/cm^2 at 10 cm distance) in the presence of 10 mg/ml of the drug. The double-stranded RNA viruses such as Colorado tick fever or Bluetongue are not inactivated under these conditions. When KHF virus inactivation was compared to HAZ virus and WEE virus in this test system, KHF clearly was inactivated in a manner similar to the single-stranded RNA viruses.

Thermal inactivation studies with KHF virus have shown it to be a relatively stable virus, much more so than VEE or JE viruses for example, and comparable to Rift Valley fever in this respect. The biological half life of KHF in the presence of 10% serum at pH 7.5 is $\sim$ 2 weeks at 4°C, $\sim$ 10 hr at 37°C, and 10 min at 50°C. Dr. Ho Wang Lee, utilizing infectivity data in Apodemus, has shown half-lives of 48 hr at room temperature and < 1 min at 56°C.

Several trials with KHF virus-infected A-549 cell monolayers have failed to demonstrate hemadsorption with rhesus monkey, guinea pig or goose RBC. Similarly crude alkaline or sucrose-acetone extraction of high titered cell culture infectious fluids has failed to yield a hemagglutinin.

One characteristic of KHF virus that has been found to be very useful is its strong cell association. At the time of peak virus yield in infected A-549 cell cultures 2/3 of the virus yield remains in, or is firmly attached to the cell. Destruction of infected cells by mechanical maceration or freeze-thaw releases this virus from the cell but it remains firmly attached to small fragments of cell membrane or is interiorized in cell vesicles. As a consequence, virus is easily concentrated by low speed ($\sim$ 2000 x g) centrifugation. Attempts to separate the virus from cell components after concentration by this procedure have not been entirely successful. Purification of the virus is necessary for further characterization of viral structural proteins and nucleic acid and is highly desirable for definitive morphologic characterization by electron microscopy. Partial success has been achieved by proteolytic digestion of cell components and filtration through 0.8μ membrane filters. Electron microscopic observation of concentrated unpurified or partially purified virus pellets has revealed spherical particles, occasionally elongated or oval, $\sim$ 100 nm in diameter. This size is quite variable, however, and particles as small as 80 nm or as large as 130 nm in diameter are frequently observed. The presence of peplomers or a prominent fringe on the virus surface is not a usual finding; the fringe that is observed is totally removed by attempts to purify with genetron treatment or proteolytic digestion. An interior structure of partially degraded, presumably KHF, virus has been occasionally seen and gives one the impression of individual capsomers on the virus surface with a tightly coiled helical nucleocapsid of $\sim$ 10-12 nm diameter in the interior of the particle. Spherical particles have also been observed in thin sections of infected cells intracytoplasmically in the region of the Golgi apparatus. No evidence of ribosome-like structures within the virus particle have been observed; nor have we seen clear evidence of virus in initmate association with Golgi structures. Virus has not been seen to bud into vesicles or from cell membranes, but is frequently observed in cell

In summary, the structural proteins of HAZ most closely resemble the reported values for California encephalitis (CE) virus with one important difference: the nucleoprotein of HAZ is 52,000 daltons compared to 17,500 for CE virus and 22,000-26,000 for other Bunyamwera or Bunya-like viruses. This immediately suggested that HAZ virus nucleoprotein was in a dimer form; however, reexamination of the data failed to reveal the presence of monomers of 26,000 MW. Like CE, HAZ appears to have 3 glycosylated proteins exposed at the surface of the virion. The MW are 84,000, 45,000 and 30,000, quite similar to the values reported for CE. The sucrose buoyant density of intact HAZ virus was 1.16 g/cm^3 compared to 1.18 g/cm^3 for other Bunyaviruses; the nucleocapsid banded at 1.26 g/cm^3, similar to CE, but less than the 1.31 g/cm^3 of the other supergroup of Bunya-like viruses. The identity of clone 579 virus was confirmed by PRN tests to be HAZ virus and was compared to CON strain IbAr 10200 (Table III). As reported last year, the 2 viruses are similar antigenically with 4-fold differences in PRN_{50} and PRN_{80} titers, when utilizing antiserum prepared against HAZ virus; larger differences were apparent when utilizing antisera against CON virus. Electron micrographs of clone 579 HAZ virus obtained in cooperation with Dr. John White (Pathology Division) revealed a somewhat heterologous population of principally oval particles 90-130 mm in diameter when observed from gluteraldehyde-fixed direct pellets. Predominantly round particles 100-130 mm in diameter were observed in infected BHK-21 cells. These particles typically had an electron-dense core with an equally dense outer coat separated from the core by a thin translucent zone. An outer fringe was not a prominent feature. Purified virus observed from sucrose, CsCl or Renografin gradients were highly distorted with all particles elongated, 80 x 160 mm, blebbed and fuzzy.

The establishment of plaque assay procedures for the 2 viruses, in vitro serologic procedures in the form of IFA or DFA and PRN tests, growth characteristics in vaccine-certifiable cells, and characterization of the structural proteins of HAZ virus completes all the objectives set forth in the research plan for this group of agents. It is anticipated that any future work on the C-CHF viruses in the coming year under this work will be of a very limited nature.

Part II. KHF

Fluorescent foci assay system and neutralization test. The initial in vitro studies of KHF virus replication in A-549 cells depended upon a $TCID_{50}$ assay system that was performed much in the same manner as systems that utilize CPE as their indicator of infection. The KHF-A-549 cell assay system utilized the presence of IFA detectable antigen as the indicator of infection in place of CPE. This assay system has several inherent disadvantages; the most important being the semiquantitative nature of the test, wherein virus titer is determined by a Reed-Muench statistical estimate of infectious virus present in the titrated sample, and secondly, the length of time, 14 days, required to complete the assay. The replicative characteristics of KHF virus in these cells, however, suggested the possibility that a direct infectious center or fluorescent foci assay system could be developed that would be identical in principal in its quantitative aspects to the PFU assay utilized for other viruses. Viral specific antigen with A-549-adapted

vesicles or vesicle-like structures. We do not have immunoelectron microscopic evidence to confirm the fact that the particles described above are indeed KHF virus, but we have every reason to think that they are. No other virus or virus-like particle has been observed consistently or frequently enough to alter this view.

In summary, data obtained within the last year have shown or strongly indicated that the etiologic agent of KHF is a medium sized, lipid-solvent sensitive, single-stranded RNA virus with other characteristics that are consistent with the Bunya-like or Bunyamwera group viruses.

Antigenic relatedness of KHF virus to other RNA viruses. An extensive effort has been made during the past year to test polyvalent and monovalent antisera of groups and individual RNA viruses by IFA test or KHF spotslides in an attempt to relate KHF virus antigenically to another known RNA virus or group of RNA viruses. To date, antisera of 154 viruses have been tested against KHF-infected A-549 cells. These viruses are representative of groups that comprise a total of 331 togaviruses, bunyaviruses, orbiviruses, arenaviruses, rhabdoviruses or unclassified viruses described in the International Catalog of Arboviruses, including certain other viruses of vertebrates. The number of viruses within groups and the corresponding number of viral antisera examined within each group are shown in Table V.

TABLE V. MAJOR GROUPS OF RNA VIRUSES AND NUMBER OF REPRESENTATIVES OF EACH GROUP EXAMINED BY IFA TEST AGAINST KHF VIRUS SPOT-SLIDES

GROUP	NUMBER		
	Serologic Groups	Known Viruses	Antisera Examined
Togaviruses	2	77	6
Bunyamwera supergroup	13	107	72
Bunya-like viruses	11	70	38
Orbiviruses	11	35	11
Arenaviruses	1	10	5
Rhabdoviruses	3	16	2
Unclassified tick-borne	3	9	7
Unclassified mosquito-borne	1	4	4
Unclassified unidentified	-	-	9
Total	45	331	154

Particular emphasis has been placed on the Bunyamwera supergroup or bunya-like viruses in that the physicochemical and morphologic characteristics of KHF most closely resemble these viruses. A positive result which appears to be specific has been repeatedly demonstrated with a Bunyamwera group polyvalent antisera which titers 1:20 on KHF spotslides. Individual antisera for the 18 recognized members of this group (Table VI) were negative in IFA tests against KHF spotslides. The Bunyamwera group of viruses is one of 13 groups of serologically related viruses within the supergroup. The supergroup viruses often share very tenuous relationships in that many times only one-way cross-reactions are observed, or in other instances, only one member of a group cross-reacts with a second group, thus tying the 2 groups together into the supergroup. Thus, the uniformly negative results obtained with relatively high titered monovalent Bunyamwera group virus antisera may reflect a one-way cross-relationship that will require individual Bunyaviruses to be tested against KHF antiserum in order to demonstrate relatedness.

TABLE VI. BUNYAMWERA GROUP VIRUS ANTISERA TESTED BY IFA AGAINST KHF VIRUS SPOTSLIDES

SUBGROUP	VIRUS	SOURCE	HOMOLOGOUS TITER[a]	KHF IFA
Bunyamwera	Bunyamwera	YARU	1:128	<1:10
	Germiston	CDC-Denver	1:256	<1:5
	Shokwe	CDC-Denver	1:810(NT)	<1:5
	Batai	YARU	1:128	<1:10
	Ilesha	YARU	1:64	<1:10
	Birao	CDC-Denver	1:10240(NT)	<1:5
	Tensaw	CDC-Denver	1:128	<1:5
	Cache Valley	CDC-Denver	1:512	<1:5
	Maguari	CDC-Denver	1:80(NT)	<1:5
	Northway	CDC-Denver	1:1024	<1:5
	Santa Rosa	CDC-D	1:1100(NT)	<1:5
	Lokern	NIH	1:64	<1:5
Wyeomyia	Wyeomyia	NIH	1:128	<1:5
	Taiassui	YARU	1:256	<1:10
	Anhembi	CDC-Denver	1:320(NT)	<1:5
	Sororoca	CDC-Denver	1:244(NT)	<1:5
Main Drain	-	NIH	1:256	<1:10
Kairi	-	YARU	1:256	<1:10

[a]Homologous titers determined by CF test unless designated (NT), determined by PRN test.

Utilization of KHF virus spotslides and the IFA test for serologic diagnosis of nephropathia epidemica in Sweden. Nephropathia epidemica (NE) is a disease sporadically present throughout many portions of the Scandinavian countries; it is clinically similar, although generally less severe, than KHF. Through the cooperation of Dr. Goran Friman, a Swedish physician on sabbatical leave to USAMRIID and Swedish clinicians present in endemic areas of the disease in

Sweden, we have had the opportunity to test a number of sera on NE patients, and areas of Sweden for antibody reactive in the IFA test with KHF virus spotslides. We have tested a total of 115 sera on 70 Swedish persons to date. Of sera from 24 patients with suspected or clinically compatible NE, 19 were positive and 5 were negative or equivocal. Of the 46 non-NE or not ill persons, 45 were negative. Sera from KHF-reactive patients had a peak geometric mean titer of 1:709 with a median titer of 1:160. Three of the 5 clinically suspected persons with nonreactive sera (KHF titer < 1:20) had only one serum available for test. Thus, it would appear that KHF-infected spotslides are a suitable test vehicle for the retrospective serologic diagnosis of NE. Dr. Ho Wang Lee has obtained similar results utilizing frozen sections of lung tissue from KHF-infected Apodemus agrarius corea and sera from patients with KHF-like illness in Sweden, Finland, far eastern Russia and Japan (2).

Presentations:

French, G. R. Recent advances in Korean Hemorrhagic fever research. Presented, Annual Meeting, American Society of Tropical Medicine and Hygiene. 10 Nov 78.

Publications:

None

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6416	79 04 24	DD-DR&E(AR)636

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

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	055
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c. //////////	STOG 79-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Immunologic studies with typhus fever rickettsiae

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
77 06	79 04	DA	C. In-house

17. CONTRACT GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	0.5	33.5
	CURRENT 80	0	0

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS
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21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Vaccines; (U) Laboratory animals; (U) Immunopathogenesis; (U) Typhus fever

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

23 (U) Develop an effective, inactivated whole organism, cell culture grown, non-reactogenic vaccine for immunoprophylaxis of epidemic, louse-borne typhus fever. This disease is a major epidemic problem for military personnel.

24 (U) Develop purified master and working seeds of Rickettsia prowazekii in leukosis virus-free eggs. Simultaneously, growth characteristics will be examined. Testing will be conducted in appropriate laboratory animals.

25 (U) 78 10 - 79 04 - Feasibility of an improved typhus fever vaccine of tissue culture origin and development of a suitable animal model to study candidate vaccines were examined. Highest titers of rickettsiae were attained in CEC and MRC-5 cell cultures (200 and 300 million, respectively). Inactivated vaccines were prepared from rickettsiae grown in these cells and tested in guinea pigs. Such vaccines offered no significant advantage over the present commercial vaccine. Since titers approaching 5 billion can be attained in chick yolk sacs, superior efficacy from cell-culture grown vaccines may be difficult to achieve. This differs from spotted fever rickettsiae where titers from cell cultures exceed those from yolk sac cultures. Numerous small laboratory animals were tested as models for epidemic typhus. Cotton rats and guinea pigs appear susceptible, with signs of illness and some deaths only at high doses. Cynomolgus monkeys seem suitable as a primate model; when infected with as few as 1000 rickettsiae they develop clinical illness, but deaths occur only at high doses. Rickettsiology research on typhus is being transferred to WRAIR. This work unit has achieved its primary objective of comparing a killed tissue culture grown whole organism vaccine against the existing commercial one. Further research on typhus immunoprophylaxis should be focused on subunit vaccine development.

* Available to contractors upon requestor's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 055: Immunologic Studies with Typhus Fever Rickettsiae

Background:

Epidemic typhus fever (caused by Rickettsia prowazekii) has affected mankind since ancient times; it is usually associated with wars and famine. The disease was believed to occur naturally only in man and human body and head lice, but recent evidence suggests that flying squirrels may harbor the organism (1). The disease is debilitating and prolonged with a mortality rate in untreated cases of ∿ 20% (2). Tetracycline therapy is extremely effective, but diagnosis of the disease is often dealyed. Recrudescence may occur many years after the original infection (Brill-Zinsser disease); such an occurrence could conceivably initiate an epidemic (3). Relative infectivity of R. prowazekii in some small laboratory animals has been examined (2), but data are sparse on infectivity for subhuman primates.

There are 2 vaccines available. The first, a commercial product, is a chicken yolk sac-grown formalin-inactivated product. This vaccine reduces mortality to 0%,but contains large amounts of egg proteins, and may not reduce the incidence of disease (4). The second, Madrid E strain, is a live, attenuated vaccine; safety remains an issue so that it is still considered an investigational drug.

Our purpose is to develop and test an efficacious cell-culture-grown inactivated epidemic typhus vaccine. In concert with vaccine development, a suitable small laboratory animal model will be chosen, and the feasibility of a primate model will be examined.

Progress:

Test inactivated vaccines were prepared in MRC and chick embryo cells. Neither vaccine was superior in efficacy to the present commercial vaccine. Further efforts with epidemic typhus vaccine prophylaxis were halted and attention has been focused on development of animal models for the disease.

Twenty-six strains of inbred mice were evaluated as possible models for Breinl strain R. prowazekii. All strains were resistant to an IP challenge of 1.0×10^6 PFU.

A cynomolgus monkey (Macaca fascicularis) model for epidemic typhus infection was defined; 16 cynomolgus monkeys were inoculated IV with varying concentrations of Breinl strain R. prowazekii. Results are summarized in Table I. All infected monkeys became clinically ill and developed a fever by day 3. Total leukocyte counts increased by day 7 and remained elevated through day 14 in the surviving monkeys. The luekocyte response did not change in the 3 monkeys that died. All infected monkeys developed transient neutrophils and lymphopenia.

TABLE I. R. PROWAZEKII INFECTION IN CYNOMOLGUS MONKEYS

CHALLENGE DOSE (PFU)	NO. FEVER/ TOTAL	DAYS (range)		DEATH	
		Incubation	Duration	No.	MTD[a] (days)
0	0/2	0	-	0	-
10^3	4/4	5.0(3-9)	3.4(1-6)	0	-
10^5	4/4	1.5(1-3)	4.7(2-6)	0	-
10^7	6/6	1.2(1-2)	6.3(6-10)	3	7.3(5-11)

[a] MTD = mean time to death (range)

Serum alkaline phosphatase values were elevated from days 6 - 9 in all 3 infected groups. By day 10, values began to return to normal in the surviving monkeys, but continued to rise until death in 3 animals. There was no significant change in BUN and SGPT values in the monkeys infected with 10^3 and 10^5 PFU. Monkeys in the 10^7 PFU group demonstrated elevations in these values from days 4 - 9. Uninfected control monkeys remained clinically normal throughout the study. Hematology and serum chemistry values remained within baseline limits.

Necropsy of the monkeys that died revealed multifocal vasculitis in lung, spleen, kidney, urinary bladder, pancreas and myocardium. Typical "typhus nodules" were present within the cerebellar molecular layer, cerebral cortex and brain stem.

The cynomolgus monkey appears to provide a suitable model for epidemic typhus infection and may be useful in the testing of experimental vaccines. A manuscript has been prepared entitled "Epidemic Typhus Infection in the Cynomolgus Monkey (Macaca fascicularis)," for submission to Infection and Immunity.

No further studies are planned with epidemic typhus. This work unit was terminated because of higher priority tasks and responsibility for rickettsial agent research other than Q fever has been assigned to WRAIR.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6417	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
	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	62776A	3M162776A841	00	056
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Provide with Security Classification Code)

(U) Effects of experimental respiratory infection on pulmonary function

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
77 06	CONT	DA	C. In-house

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b. NUMBER: NA
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e. KIND OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	1.0	114.2
	CURRENT 80	1.0	190.8

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21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Provide EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Immunology; (U) Respiratory diseases; (U) Lung physiology; (U) Tularemia; (U) Melioidosis

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 respiratory infection on mechanical and immunologic functions of the lung. A better understanding of these effects will aid in improvement of prophylaxis and treatment of diseases of military importance. This is especially important in defending against possible airborne BW pathogens.
24 (U) Study the effect of nonspecific stimulation of host defenses prior to experimental respiratory infections.

25 (U) 78 10 - 79 09 - The effects of nonspecific stimulation of the reticuloendothelial system were evaluated in laboratory animals during experimental bacterial infections. Inbred rats and outbred mice were pretreated with glucan, a polysaccharide component of yeast cell wall, before infection with Francisella tularensis or Pseudomonas pneudomallei, respectively. Pretreatment with glucan IV significantly increased survival in rats infected with F. tularensis intraperitoneally or by low dose aerosols; pretreatment with glucan intranasally failed to increase survival in either intraperitoneal or aerosol-infected rats. Intranasal glucan pretreatment significantly increased survival of mice infected by aerosols of P. pseudomallei; IV glucan did not increase survival. These studies demonstrate modification in the course of virulent bacterial infections by nonspecific stimulation of host defenses.

Publications: Fed. Proc. 38:909, 1979;
Am. J. Vet. Res. 40:271-273, 1979.

* Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 056: Effects of Respiratory Infections on Selected Nonrespiratory Functions of the Lung

Background:

Infections in U. S. military and civilian populations resulting from operational use of biological weapons by enemy forces are likely to be induced by inhalation of infectious agent aerosols. Infection of the respiratory tract also represents the initial mode of natural entry of a nonpathogen into the susceptible host.

The lung performs numerous nonrespiratory functions in addition to its primary function of gas exchange. Important among these is protection of the lung from infection by immunological defenses, including both humoral and cellular components. The present investigations were designed to determine the effect of nonspecific stimulation of host defenses prior to experimental respiratory infections.

Progress:

Glucan, a β-1, 3-glucopyranose polysaccharide component of yeast cell wall, is a potent reticuloendothelial (RE) stimulant whose immunobiological activity is mediated in part by an increase in the number and function of macrophages. Glucan, obtained from Dr. N. R. DiLuzio, Department of Physiology, Tulane University, School of Medicine, was used to determine if nonspecific stimulation could enhance host resistance to bacterial infections.

Inbred Fischer 344 rats were divided into experimental groups and treated with glucan suspended in sterile water (1 mg/100 gm) either IV or IN on days 5, 3, and 1 before infection. The IN treated groups were anesthetized with halothane prior to inoculation of the calculated dose of glucan. Rats were challenged with the virulent SCHU S4 strain of Francisella tularensis by either IP inoculation of 10^5 organisms or inhalation of 10^5 or 10^3 organisms in small-particle aerosol (SPA); and the aerosol was generated by a Collison spray device and delivered into a modified Henderson tube. Mortality rates were calculated on day 21 (Table I).

TABLE I. EFFECT OF GLUCAN PRETREATMENT ON SURVIVAL OF RATS INFECTED WITH F. TULARENSIS

PRETREATMENT[a]	INFECTION	SURVIVAL No./Total	SURVIVAL %	P
Glucan (IV)	None	24/24	100	
(IN)	None	16/16	100	
None	IP	0/16	0	< 0.0001
Glucan (IV)	IP	11/16	68	
(IN)	IP	0/8	0	
None	10^5 aerosol	3/8	38	
Glucan (IV)	10^5 aerosol	3/16	19	
(IN)	10^5 aerosol	3/8	38	
None	10^3 aerosol	6/16	38	< 0.001
Glucan (IV)	10^3 aerosol	16/16	100	
(IN)	10^3 aerosol	10/16	63	

[a]Glucan (1 mg/100 gm) given IV via the tail vein or IN on days 5, 3, and 1.

Pseudomonias pseudomallei by inhalation of 30 organisms in SPA. Mortality rates were calculated on day 21 postinfection (Table II). IN glucan pretreatment significantly increased survival of infected mice compared with IN water-treated controls. IV glucan pretreatment failed to increase survival among challenged mice.

TABLE II. EFFECT OF GLUCAN PRETREATMENT ON SURVIVAL OF MICE INFECTED WITH MOUSE-ADAPTED P. PSEUDOMALLEI BY SPA

PRETREATMENT[a]	INFECTION	SURVIVAL No./Total	SURVIVAL %	P
None	+	3/40	18	
Glucan (IV)	-	40/40	100	
	+	11/41	28	
(IN)	-	40/40	100	
	+	34/40	85	
Water (IV)	-	20/20	100	< 0.001
	+	2/20	10	
(IN)	-	20/20	100	
	+	8/20	40	

[a]Pretreatment on days -7, -4, and -1. Glucan (0.5 mg/mouse) was given in 0.1 ml total volume.

Increased resistance to infection with SCHU S4, F. tularensis was seen in rats treated IV with glucan but increased resistance was not seen in systemically treated mice infected with P. pseudomallei. Conversely, increased resistance to infection with P. pneudomallei was seen in mice treated locally (IN), but increased resistance was not seen in rats treated locally and infected with F. tularensis.

Data from our studies suggest that the efficacy of glucan treatment prior to bacterial infections may depend upon its route of administration and the specific host-organism interaction. Experimental tularemia infection of the rat results in systemic dissemination of virulent organisms within 24 hr after IP inoculation and within 48 hr after aerosol challenge (J. F. Jemski, Aerobiology Division, unpublished data). Prior activation of the RE system by IV glucan pretreatment resulted in marked potentiation of resistance to IP tularemia infection, whereas, local deposition of glucan in the pulmonary tract tailed to significantly increase resistance to an overwhelming respiratory or systemic challenge with virulent organisms.

Conversely, respiratory meliodosis, which usually does not become systemically disseminated until the terminal stages of disease, did not respond to IV glucan pretreatment. Local administration of glucan to the respiratory tract, however, significantly enhanced the resistance of aerosol-challenged mice. P. pseudomallei, unlike F. tularensis, is not an obligate intracellular pathogen and readily proliferates in the pulmonary alveoli and bronchioles. The extracellular growth of these organisms was apparently markedly suppressed.

The ability of macrophages to alter the fate of intracellular microorganisms, especially bacteria, has been described by others, although the precise mechanisms are not entirely understood. Most studies demonstrating glucan-induced immunopotentiation have dealth only with infectious agents that are primarily opportunistic such as Staphylococcus or Candida. The present report demonstrates a modification

in the course of selected infectious diseases by glucan pretreatment using highly virulent bacteria.

Presentations:

1. Kastello, M. D., J. V. Jemski, and J. A. Reynolds. Response of glucan-pretreated rats to experimental infection with F. tularensis. Presented, Annual Meeting FASEB, Dallas, TX, 6-10 Apr 79. (Fed. Proc. 38:909, 1979).

2. Kastello, M. D. A comparative approach to regulation of body fluids and electrolytes. Presented, Uniformed Services University of Health Sciences, Bethesda, MD, 10 Apr 79.

Publications:

1. Kastello, M. D., A. D. Emmert, R. F. Denson, and R. A. Kishimoto. 1979. Recovery of alveolar macrophages from rhesus and cynomolgus macaques by lung lavage. Am. J. Vet. Res. 40:271-273.

2. Gonder, J. C., R. A. Kishimoto, M. D. Kastello, C. E. Pedersen, Jr., and E. W. Larson. 1979. Cynomolgus monkey model for experimental Q fever infection.

3. Kishimoto, R. A., M. D. Kastello, J. D. White, E. G. Shirey, V. G. McGann, E. W. Larson, and K. W. Hedlund. In vitro interaction between normal cynomolgus monkey alveolar macrophages and Legionnaires' disease bacteria. Infect. Immun. 25:761-763.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6418	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	057
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Metabolic alterations in fatty acid metabolism during infection of military importance

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
77 07	CONT	DA	C. In-house

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	1.0	117.4
	CURRENT 80	1.0	135.2

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Wannemacher, Jr., R. W.
NAME: Neufeld, H. A. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Inflammatory stress; (U) Tularemia infection; (U) Viral infection; (U) Lipid metabolism; (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) Identify mechanisms responsible for the altered fatty acid metabolism and decreased ketogenesis observed during bacterial or viral infections. Study the muscle-liver-adipose tissue interrelationships during starvation and militarily important infections. Determine if lipids or enzyme replacement are potentially useful in supportive therapies during severe infections. When completed this work will provide the basis for more rational and more effective therapy for the soldier suffering from an infectious disease of natural or BW origin.

24 (U) Using the established rat Streptococcus pneumoniae and Francisella tularensis models, examine mechanistic changes in lipid metabolism by in vivo and in vitro methods. Extend work to study viral encephalitis.

25 (U) 78 10 - 79 09 - Hepatic mitochondria isolated from rats exposed to F. tularensis are able to oxidize long-chain fatty acids (FA) and the carnitine and coenzyme A derivatives of them to ketone bodies at a rate comparable to controls. Hepatic peroxisomes, fractionated from liver of rats given S. pneumoniae oxidize long-chain fatty acyl-CoA at a rate similar to controls, despite the apparent 2-fold decrease in peroxisomal marker enzymes. Determination of the subcellular distribution of CoA and carnitine indicates that approximately 30% of the CoA and 70-90% of the cellular carnitine is cytoplasmic while 10-20% of the CoA and only 4-8% of the carnitine is associated with the mitochondria. The cellular carnitine concentration appears to be shifted toward the peroxisomal fraction during a bacterial infection.

Publications: Fed. Proc. 38:354, 1979;
Clin. Res. 26:627A, 1978; 27:589A, 1979.

*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

* U.S. GPO: 1974-540-447/R491

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 057: Metabolic Alterations in Fatty Acid Metabolism During Infections of Military Importance

Background:

Infectious illness is generally coupled with starvation. Under such conditions the carbohydrate reserves of the body can provide fuel for < 24 hr. Therefore, the role of fat as a reserve fuel becomes very important. In starvation, the oxidation of free fatty acids (FFA) and ketone bodies spares the oxidation of glucose. However, in a bacterial infection coupled with starvation, there is a diminished ketone production(1) and an influx of amino acids to the liver for the production of energy-yielding fuels. This inability to use endogenous fat stores for energy production may be responsible for the protein-wasting state that accompanies infectious illness.

This study is attempting to identify the intrahepatic regulatory site responsible for the diminished ketogenesis of infection. Previous studies have shown that the liver has a decreased capacity for long-chain FA oxidation and ketogenesis during a Streptococcus pneumoniae or Francisella tularensis infection (2). However, isolated hepatic mitochondria isolated from rats infected with S. pneumoniae appear to function normally (2). Coenzyme A (CoA) and carnitine, 2 cofactors important in FA metabolism are thought to be involved with the intrahepatic control of ketogenesis. Hepatic carnitine concentrations increase while CoA decreases during a pneumococcal infection (3). Determining the subcellular distribution of these cofactors will provide insight into the regulation of fatty acid metabolism during bacterial infections.

Progress:

FA oxidation was studied in isolated mitochondria fractionated from the liver of rats exposed to F. tularensis. These studies agree with previously reported studies on S. pneumoniae-infected rats, in that there was no effect of infection on the processes of β-oxidation, the TCA cycle or acetoacetate production. The control site for ketone production thus appears to be either cytosolic or dependent on the cellular integrity.

Intrahepatic control of ketogenesis has been postulated to involve the carnitine-dependent transport of FA across the mitochondrial membrane, as well as the cellular distribution of CoA and its derivatives. Recently malonyl-CoA has been cited as still another regulatory metabolite (4).

The distribution of CoA and carnitine between the mitochondrial, peroxisomal and cytosolic compartments was determined in rat liver under control and infected conditions. Under both conditions a greater percentage of the cofactors was cytosolic. The percentage of the cellular carnitine associated with the mitochondria decreased from 8 to 4% during the infection while a net transfer of carnitine occurred from the cytosol to the peroxisomal fraction. At the present time neither the significance nor the mechanism of this observation is known. The overall ability of

peroxisomes to oxidize palmityl-CoA to acetyl-CoA does not appear to be affected by a pneumococcal infection despite the reported decrease in peroxisomal number (5).

Malonyl-CoA concentration was measured in liver from S. pneumoniae-infected rats and found not to be significantly different than that of fasted control liver. Together with the finding that acyl carnitine concentrations are increased during a bacterial infection, these results suggest that carnitine palmityltransferase I, a regulator of ketogenesis during starvation, cannot be rate limiting for ketogenesis during infection. A definitive measure of this enzyme's activity during infection is in progress.

Presentations:

1. Pace, J. A., F. A. Beall, M. D. Foulke, H. A. Neufeld, and R. W. Wannemacher, Jr. Regulation of fatty acid utilization in isolated perfused livers from Streptococcus pneumoniae infected rats. Presented, American Federation for Clinical Research, Boston, MA, 20-21 Oct 1978 (Clin. Res. 26:627A).

2. Pace, J. G., M. D. Foulke, S. Sokol, F. A. Beall, H. A. Neufeld, R. W. Wannemacher, Jr. Carnitine and coenzyme A distribution in liver of meal-fed, fasted and Streptococcus pneumoniae-infected rats. Presented, Annual Meeting, American Society of Biological Chemists, Dallas, TX, Mar 1979 (Fed. Proc. 38:354, 1979).

3. Pace, J. G. Effects of infection on fatty acid metabolism. Presented, The George Washington University, Washington, DC, Sep 1979.

Publications:

1. Foulke, M. D., J. G. Pace, P. G. Canonico, J. S. Little, and R. W. Wannemacher, Jr. 1979. The effect of Streptococcus pneumoniae infection on rat liver peroxisomal fatty acid oxidation. Clin. Res. 27:589A.

2. Pace, J. G., F. A. Beall, H. A. Neufeld, and R. W. Wannemacher, Jr. Fatty acid metabolism and ketogenesis in rats exposed to Streptococcus pneumoniae. Can. J. Biochem. in preparation.

LITERATURE CITED

1. Neufeld, H. A., J. A. Pace, and F. E. White. 1976. The effect of bacterial infections on ketone concentrations in rat liver and blood and on free fatty acid concentrations in rat blood. Metabolism 25:877-884.

2. Pace, J. A., F. A. Beall, H. A. Neufeld, and R. W. Wannemacher, Jr. 1977. Alterations in carnitine (Car) acylation states in S. pneumoniae infected (INF) rats. Fed. Proc. 36:788.

3. Pace, J. A., M. D. Foulke, S. Sokol, F. A. Beall, H. A. Neufeld, R. W. Wannemacher, Jr. 1979. Carnitine and coenzyme A distribution in liver of meal-fed, fasted and Streptococcus pneumoniae-infected rats. Fed. Proc. 38:354.

4. McGarry, J. D., C. P. Mannaerts, and D. W. Foster. 1977. A possible role for malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. J. Clin. Invest. 60:265-270.

5. Canonico, P. G., J. D. White, and M. C. Powanda. 1975. Peroxisome depletion in rat liver during pneumococcal sepsis. Lab. Invest. 33:147-150.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6425	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	059
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Pathogenesis of anthrax

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
77 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 79	1.0	61.1
	CURRENT 80	1.0	77.2

19. RESPONSIBLE DOD ORGANIZATION

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NAME: Middlebrook, J. L.

NAME: Spero, L. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Anthrax; (U) Toxin; (U) Toxoid; (U) Vaccine; (U) Laboratory animals

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

23 (U) To elucidate the mechanisms of intoxication of anthrax toxin and to define its relationship to the overall course of anthrax infections. Our ultimate goal is the development of effective countermeasures including, but not limited to, the production of a toxin-derived vaccine suitable for human use, which will protect military personnel from anthrax used as a BW agent.

24 (U) Produce toxin in desired quantity. Fractionate and purify anthrax toxin produced in large quantities (either by fermentation or alternate culture conditions) Study the pathophysiology of toxin in laboratory animals. Prepare and evaluate vaccines and/or toxcids.

25 (U) 78 10 - 79 09 - Certain strains of Bacillus anthracis produce one or more of the 3 components described as lethal factor (LF), protective antigen (PA), and edema factor (EF). When injected in combination, LF and PA cause death in rats in 1-2 hr. Similarly EF in combination with PA causes local edema. PA alone is immunogenic, but has no biological activity. Conditions have been established in a fermentor system to produce anthrax toxin in vitro. A slightly modified synthetic medium is used, and unconcentrated culture supernatants contain one or more of the 3 components. Rat and guinea pig bioassays are used to test for LF and EF and a serological system, PA.

Preliminary purification of LF and PA has used ion-exchange resins. Selected materials were removed by increasing molarity of buffers; some fractions were found to contain LF and/or PA.

Publication: Infect. Immun. 24:679-684, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M762776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 059: Pathogenesis of Anthrax

Background:

Although the incidence of anthrax infection in man is low, the devastating effects of the disease are paramount in consideration. Mortality rates for septicemic anthrax exceed 80% in man (1). Coupled with a difficult diagnosis, the seemingly irreversible effect of the anthrax toxin makes it a prime candidate for in-depth study of its mechanism of action.

Bacillus anthracis toxin has been previously described as composed of 3 components: lethal factor, protective antigen, and edema factor (2). The toxin can be produced in vitro using chemically defined media for cultivation of B. anthracis strains (3). Some preliminary work has established that each of the 3 components exhibits a characteristic phenomenon. Lethal factor (LF), when combined with protective antigen (PA), is toxic to several species of animals, including rats, guinea pigs, and monkeys. The biological action of edema factor (EF) is the production of local edema in rabbits or guinea pigs; PA has no biologic activity, but is reported to be highly immunogenic.

Each of the components may play an important role in the pathogenesis of anthrax. They may be produced separately, then combined in some form or they may act synergistically in the course of infection. A significant part of the research, therefore, is to produce, isolate, and characterize each of the components of anthrax toxin. Later studies are planned to include cellular actions, in-depth biochemical characterization of each molecule, and eventually to develop prophylactic and therapeutic measures against anthrax using these materials.

Progress:

Several strains of B. anthracis were tested under a variety of conditions for their ability to produce toxin. The Sterne strain was selected for use since it produced LF in addition to the other components. Strain V770, the strain currently used for vaccine production, was selected for production of PA alone. To date, we have not been able to demonstrate either LF or EF activity in cultures of strain V770.

A series of experiments was conducted in order to determine optimal growth conditions in our 10-L fermentor system. A synthetic medium base was used to facilitate toxin purification studies. After numerous attempts to produce toxin were unsuccessful, a slight modification of the base medium succeeded in providing the necessary nutrients for toxin elaboration, therefore, 0.2-0.5% yeast extract is added routinely to culture medium. All fermentations since that change have contained some amount of crude toxin.

Assay tests for presence of crude toxin include both biological and serological systems. Culture supernatants are injected IV into strain F344 rats; death usually occurs in 1-2 hr, indicating presence of LF and PA. Agar gel diffusion is used to determine the presence of PA. Anti-anthrax serum was obtained from Porton, England; an additional amount of antiserum is being prepared in-house by injecting rabbits with either culture supernatants or partially purified antigens. A supply of standard antigen was prepared for USAMRIID under contract, and this material is used as a reference in the agar gel diffusion plates, and as an antigen for rabbit immunizations.

Once production of crude toxin was standardized, preliminary experiments were conducted in an attempt to purify 1 or more of the components. This avenue of research has 2 major branches: the first involves production of standard antigen only. Cultures of strain V770 were absorbed onto Dowex resin. Several protein-containing peaks were eluted from these columns, but none were antigenic. Further work is in progress to determine whether the antigen is unstable. More favorable conditions for isolation may be required, or a more sensitive assay system may be needed to detect small amounts of the antigen.

The second branch involves purification of LF from Sterne strain cultures. Unconcentrated crude supernatants contain enough LF and PA to kill rats in 1-2 hr. These supernatants have been adsorbed onto a variety of ion-exchange resins, including Dowex and DEAE-cellulose. A number of buffer systems have been used, varying both pH and molarity of the selected buffer. Some recovery of LF and PA has been obtained using 20 mM ammonium acetate buffer at pH 8.0. The toxin components have been eluted using 0.5 M NaCl. Experiments conducted thus far have shown that the active material is only partially purified (as evidenced in polyacrylamide gel electrophoresis), and, additionally, that the material is womewhat unstable. Further steps in purification are being studied.

Other aspects of the work in progress include standardization of an indirect hemagglutination assay for anthrax antibodies and further characterization of the strains of B. anthracis which produce the toxin.

Publications:

Johnson, A. D., L. Spero, J. S. Cades, and B. T. Decicco. 1979. Purification and characterization of different types of exfoliative toxin from Staphylococcus aureus. Infect. Immun. 24:679-684.

LITERATURE CITED

1. Wright, G. G. 1965. The anthrax bacillus, pp. 530-544. In Bacterial and Mycotic Infections of Man, (R. J. Dubos and J. G. Hirsch, eds). J. B. Lippincott, Philadelphia.

2. Lincoln, R. E., and D. C. Fish. 1970. Anthrax toxin, pp. 361-414. In Microbial Toxins, Vol. III, (T. C. Montie, S. Kadis, and S. J. Ajl, (eds). Academic Press, New York.

3. Puziss, M., L. C. Manning, J. W. Lynch, E. Barclay, I. Abelow, and G. G. Wright. 1963. Large-scale production of protective antigen of Bacillus anthracis in anaerobic cultures. Appl. Microbiol. 11:330-334.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6426	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	060
b. CONTRIBUTING				
c. //////////	STOG 80-7.2:2			

11. TITLE (Include with Security Classification Code): (U) Identification of bacterial BW agents using a chemiluminescent immunoreaction procedure

12. SCIENTIFIC AND TECHNOLOGICAL AREAS: 003500 Clinical medicine; 004900 Defense; 012300 Biochemistry; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
77 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 79	1.0	107.3
FISCAL YEAR CURRENT 80	1.0	147.6

19. a. RESPONSIBLE DOD ORGANIZATION
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ASSOCIATE INVESTIGATORS
NAME: Miller, Jr., R. J.
NAME: POC:DA

21. GENERAL USE: Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Bacterial diseases; (U) Chemiluminescence; (U) Rapid detection; (U) Viral diseases

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

23. (U) Develop reproducible and reliable techniques for the rapid detection of small numbers of bacteria or viral particles in biological and environmental samples using a chemiluminescent procedure. Following detection of a biological attack, rapid identification of the causative agent is essential in order to begin the proper and most efficacious therapy.

24 (U) Antigen specific antibodies are bound to appropriate support media by absorption or covalent linkage. The media are incubated successively with antigen and horseradish peroxidase (HRPO)-labeled second antibody. Analyses were made for peroxidase using luminol and hydrogen peroxide. HRPO activity should be proportional to antigen concentration.

25 (U) 78 10 - 79 09 - Studies elsewhere have demonstrated the capability to detect small numbers of microorganisms by luminescent immunochemical procedures. These findings have been confirmed, and further investigations have established workable chemiluminescent immunoreactive assays for Francisella tularensis, LVS, and Venezuelan equine encephalomyelitis. Development of these assays has required identification of compatible support media for absorption or covalent linkage of primary antibodies, production and purification of antigen-specific primary antibodies, preparation and purification of HRPO-labeled second antibodies, and determination of proper assay conditions for greatest sensitivity and reproducibility. Refinements in the assay procedures are continuing as is progress in the development of assays for other microorganisms.

Publication: Fed. Proc. 38:1013, 1979.

* Automatic computer processing information is required.

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 060: Identification of Bacterial BW Agents using a Chemiluminescent Immunoreaction Procedure

Background:

The use of the chemiluminescence of luminol as a system for detection of small amounts of hematin Fe and small numbers of microorganisms has been reported (1-3). Recently, immunoreactive techniques utilizing chemiluminescence (CL) to identify and to quantitate small numbers of microorganisms have also been reported by Halmann et al. (4,5).

Previous work on this unit concerned itself with instrumentation, antibody production and sensitivity in the detection of horseradish peroxidase (HRPO)-labeled antibodies. Investigations were also made on the Aclar plastic strip assay reported by Halmann et al. (5). Other research involved developing assays based on polycarbonate membranes and activated glass beads. In all initial studies negative results were obtained primarily due to high background chemiluminescence and nonreproducibility.

Progress:

Due to various failures in attempts to reproduce literature procedures, a new approach was adopted. Polystyrene tubes were coated with DEAE-cellulose-purified antibodies specific to Francisella tularensis live vaccine strain (LVS). After coating, log dilutions of LVS were incubated for 2 hr. The LVS was then removed, the tubes washed and aliquots of HRPO-labeled second antibody added. Following a 2-hr incubation, the amount of second antibody in solution was determined. The amount remaining in solution should be proportional to the LVS concentration. Satisfactory but sometimes nonreproducible results were obtained. The high degree of nonreproducibility present from batch to batch was associated with the adsorption of antibody onto the tube surface. Therefore, a better support medium was sought.

The assay has evolved until at present it involves the evaluation of 2 systems: adsorbed antibodies on frosted polystyrene beads, and covalently linked antibodies on polyacrylamide beads of less than 10u (Bio Rad Immunobeads).

In the first instance, 6-mm frosted polystyrene beads are coated with Protein A-Sepharose-purified specific antibodies. After washing, the beads are incubated with antigen-containing solutions. The antigen solutions are then removed, the beads washed, and a HRPO-labeled second antibody is added and incubated. After an appropriate time period, the second antibody is removed and the beads are washed. The beads themselves are then analyzed for HRPO using luminol and H_2O_2. In LVS studies, such an assay procedure requires about 5-hr total reaction time, with a detection limit of 10 - 100 bacteria/ml.

The covalently-linked antibody system has been used in the development of a VEE assay. The procedure is the same as described and gives an assay with 10^4 PFU/ml detection limits. The VEE assay procedures compare quite favorably with other systems.

(RIA, tissue culture) presently available for assays but is more rapid. Further refinements in both of these assays are in progress and an assay for Coxiella burnetii is under investigation.

Coordination with the Early Detection Group, Chemical System Laboratory, Edgewood Arsenal, has also been actively pursued and aerosol collecting media used by that group is continuing to be analyzed for compatability with the CL immunoreaction assay.

Presentation:

1. Reichard, D. W. and R. J. Miller, Jr. Chemiluminescent immunoreactive assay for rapid detection of Francisella tularensis. Presented, FASEB, Dallas, TX, 1-10 Apr 1979 (Fed. Proc. 38:1013, 1979).

Publications:

None

LITERATURE CITED

1. Neufeld, H. A., C. J. Conklin, and R. D. Towner. July 1965. The luminescence of luminol as a tool for biodetection. Technical Report 67, United States Army Biological Laboratories, Fort Detrick, Frederick, MD (AD 467 160).

2. Neufeld, H. A., C. S. Conklin, and R. D. Towner. 1965. Chemiluminescence of luminol in the presence of hematin compounds. Anal. Biochem. 12:303-309.

3. Neufeld, H. A., G. E. Hatfield, and A. D. Brumbaugh. 15 August 1971. Investigation of chemiluminescence detection approaches, Project 1W663720D165, Edgewood Arsenal, MD.

4. Halmann, M., B. Velan, and T. Sery. 1977. Rapid identification and quantitation of small numbers of microorganisms by a chemiluminescent immunoreaction. Appl. Environ. Microbiol. 34:473-477.

5. Velan, B., and M. Halmann. 1978. Chemiluminescence immunoassay: a new sensitive method for determination of antigens. Immunochemistry 15:331-333.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6410	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	063
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Rapid diagnosis of viral diseases of military importance

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 0100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 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 79	1.0	226.2
FISCAL YEAR CURRENT 80	1.0	250.2

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Arboviruses; (U) Arenaviruses; (U) Virus diagnosis (FA, RIA, ELISA)

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 standardize immunofluorescent or alternate techniques for isolation, detection and identification of viruses of military importance and high-hazard viruses. Provide diagnostic reagents and protocols to implement the objectives.

24 (U) Produce specific virus antigens and antisera for use as reagents for virus identification and diagnosis; evaluate clinical specimens for diagnosis of virus infections; develop specific SOPs, systemize procedures, and develop protocols for virus isolation and identification.

25 (U) 78 10 - 79 09 - Production or acquisition of working lots of spot slides of 200 each for most viruses of interest is essentially complete. Efforts on spot slides involve maintenance and replacement of existing stocks when necessary. Direct FA conjugates have been produced for 22 viruses and indirect conjugates are available for all viruses. As a prelude to isolation of viruses from clinical specimens, 4 cell lines have been infected with 16 different viruses to assess minimum virus inputs detectable by FA within specified time periods. The specificities of arenavirus reagents by DFA and alphavirus by IFA have been determined. Usefulness of our reagents and procedures in isolating and identifying viruses during infections of humans and animals will be determined as models as clinical specimens become available.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M176776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 063: Rapid Diagnosis of Viral Diseases of Military Importance

Background:

Specific diagnosis of arbo- and arenavirus infections in man is usually made by the isolation and identification of the virus, or by the detection of a significant increase of specific antibodies between acute and convalescent serum samples. The former requires days, the latter, weeks. The most rapid virus diagnosis is made by the detection of specific viral antigen in clinical specimens, such as blood, urine, throat washings, feces and conjunctival scrapings. Biopsy materials are only occasionally available for this purpose. Successful isolation of most arboviruses from patients is the exception, reasons being that the specimen is not obtained soon enough or is not properly handled or transmitted quickly to the laboratory for virus isolation. Viremia for many arbovirus infections in man, if detectable at any stage, generally ceases by the time of, or soon after, onset of symptoms. Mosquito-borne viruses, such as Japanese B. encephalitis (JE) and St. Louis encephalitits (SLE) have very rarely been isolated from the circulating blood of patients, while West Nile (WN), yellow fever (YF), dengue (DEN), VEE and the tick-borne viruses have routinely been isolated from sera collected up to a week following onset of symptoms (1). Because of the transient nature or absence of viremia after disease onset, diagnosis is usually dependent upon a rise in antibodies; it is therefore considered to be a retrospective procedure with regard to treatment.

Recent developments have generated increased interest in fluorescent antibody techniques (FA) for both the detection of viral antibodies and the direct visualization of viral antigen in clinical specimens. The availability of recently developed equipment, the current state of the art, and our desire to develop procedures for diagnosis of viral diseases of military importance, prompted initiation of this work.

Progress:

Antigen spot slides. Spot slides prepared with cells containing specific viral antigens are primary reagents for serodiagnosis of viral infections by FA techniques. We have essentially completed the production or acquisition of working lots of approximately 200 slides for each of the following viruses of interest: alphaviruses: EEE, WEE, VEE, Mayaro (MAY), Chikungunya (CHIK) and O'nyong-nyong (ONN); flaviviruses: JE, SLE, langat (LAN), DEN 1-4, YF, WN; arenaviruses: lymphocytic choriomeningitis (LCM), Junin (JUN), Pichinde (PIC), Tacaribe (TAC), Machupo (MAC) and Lassa fever (LAS); bunyaviruses: La Crosse (LAC), sandfly fever-Naples (SFN) and-Sicilian (SFS), Oropouche (ORO), Rift Valley fever (RVF), hazara (HAZ) and Congo-Crimean hemorrhagic fever (C-CHF); and ungrouped: Korean hemorrhagic fever (KHF). Each lot has been preevaluated, packaged and stored at -70°C; every 3-6 months they are tested for stability and specificity.

Safety testing for residual live virus has been discontinued. Generally, all spot slides contain live virus and are only used in containment facilities commensurate with the recommendations of the Subcommittee on Arbovirus Laboratory Safety (SALS).

Inactivation studies. Initially, it was deemed desirable to produce nonviable antigen-containing spot slides. Preliminary studies were begun both in-house and under contract (YARU, DAMD17-77-C-7035) to find a suitable virus-inactivating substance that allowed retention of antigenicity as determined by FA. All substances tested thus far destroy antigenicity to varying degrees.

Short-wave (2650 nm, UV) irradiation is generally considered virucidal when its effects on the FA reaction are tested. Using PIC-infected Vero cells and uninfected Vero control cells, spot slides were prepared according to our SOP. Slides containing spots of both infected and control cells were irradiated for periods of 5, 10, 20, 40 and 60 min at a distance of 10 cm. Unirradiated slides were used for controls. Results indicated that uninfected Vero control cells were negative at 0 and after 5 min of irradiation; after 10 min while still negative, the cells started to "brown up" at 20 min, cells appeared greenish-brown, and after 40 min, the cells were a lighter greenish-brown that began to approach the green component of positive-infected cells, i.e. a false positive reaction. Infected cells gave a 3-4+-FA reaction at 0 and 5 min, decreased to a 2+ at 10 min, 1+ at 20 min, were $\pm$ at 40 min and appeared negative at 60 min of irradiation indicating the change from a true positive to a false negative. All studies using UV irradiation have been terminated.

Data obtained from contract DAMD17-77-C-7048, Development of Psoralen Photoinactivated Alphavirus and Arenavirus Vaccines (3), indicated that the psoralen, 4-aminomethyl-4, 5, 8-trioxisalen (AMT), and a substance with similar photoreactivity, chlorpromazine (CPZ), might be useful for inactivating virus contained within infected cells.

Studies to define the intracellular virucidal activity of CPZ were most disconcerting. CPZ used at 10 µg/ml is totally virucidal to free PIC virus within 60 sec, when long-wave irradiation is used at a distance of 5 cm. Similar experimental conditions using virus-infected cells are equally virucidal after 240 sec of irradiation. Unfortunately, the unirradiated control sample containing CPZ + virus-infected cells is also negative for live virus. Unlike the psoralen compounds being investigated as virus inactivation agents, CPZ is reported to interact only with virus and cell RNA and DNA, but also with cell membranes, proteins and lipids. Examination of virus-infected cells by IFA shows that those cells exposed to CPZ either in the presence or absence of UV irradiation are only slightly FA-positive, few in number, and in various stages of disruption, whereas identically prepared infected cell controls are FA-positive, present in normal numbers and intact. CPZ appears to be disrupting cellular and/or antigenic integrity at the concentrations used. It is improbable, given the reported mechanism of CPZ interaction with cellular membranes, that an effective minimal virucidal dosage will be found that allows retention of FA antigenic integrity.

Since both short-wave irradiation and long-wave irradiation (in connection with CPZ) were not suitable, we began a series of studies to determine the usability of AMT, a substituted psoralen, for inactivation of viruses contained in cells. Table I gives some results.

TABLE I. EFFECT OF AMT AND LONG-WAVE IRRADIATION ON PIC -INFECTED VERO CELLS AFTER 1 FREEZE-THAW CYCLE

	PFU[a]/ml, by sec (x 10^5)					
Sample	0	30	60	120	240	280
Infected						
AMT + Irr.[b]		44	55	0.63	0.02	Neg
AMT	630					140
Irradiation	230		140		160	130
Controls	140					130

[a]10 µg/ml AMT; long-wave irradiation, 20 µW/cm^2 at 5 cm.

AMT when used in conjunction with the long-wave irradiation appears to be a potent inactivator of PIC virus in Vero cells. Neither AMT nor irradiation alone produced any appreciable effect, as shown by the retention of 10^7 PFU/ml over the 490-sec treatment period. Additionally, FA studies on the treated samples indicate that the treatment does not appreciably diminish the FA reaction nor produce unwanted background or false-positive or -negative reactions, as described in the studies using UV. AMT was investigated further.

We have built a constant-flow inactivation apparatus for AMT inactivation studies. A closed system is needed due to the volume used and for containment of hazardous viruses. An initial experiment using PIC-infected cells indicated that partial inactivation occurs within 12 min (10^7 decreased to 10^4 PFU/ml) but that an additional 84 min of irradiation gave but one additional log decrease in titer. The inactivation curve became asymptotic. Discussions with Dr. Jahrling (USAMRIID) revealed that such a curve also occurs with LAS- or PIC-infected cells. Increasing the concentrations of AMT from 10 to 400 µg/ml, pretreatment of the virus cell mixture with AMT or sequential additions of AMT (AMT is reported not to be degraded or inactivated by irradiation) increases the amount of observed inactivation but does not significantly change the asymptotic nature of the curve. In light of this information, the initial preliminary experiment reported above will be repeated for it does not seem reasonable that changes in volume, geometry or subsequent experimental changes could account for the discrepancy.

Dr. Casals (YARU) reported (2) that treatment of EEE-infected Vero cells with 0.05% β-propriolactone (BPL) for 60 min at 37°C, decreased the titer from $10^{9.7}$ to $10^{4.0}$ $TCID_{50}$/ml. When tested by IFA, spot slides prepared from the BPL-treated cells had fewer cells (≈ 50% less), were distorted and showed some margination of fluorescence; the titer of the serum, however, was the same and equally easy to read, when compared to slides prepared from untreated infected

cells. Similar IFA results were obtained using DEN-2 and LLC-MK_2 infected cells, although DEN-2 was totally inactivated as assayed by IC inoculation of suckling mice. Although most encouraging of the substances tested, BPL is classified as a zero-level carcinogen and therefore stringent requirements surround its use. Currently, we are not treating spot slide or infected cells used to prepare slides with virucidal substances. As commercially available agents are found and methods that do not affect FA techniques are developed, they will be used.

Conjugates. With the exception of DEN-2 and -3, ORO, SFN, HAZ and LAC, direct conjugates, i.e., specific anti-species immunoglobulins, are available for all systems.

Specificity of reagents arenaviruses. We have prepared and standarized all of the necessary reagents for the arenaviruses of interest to USAMRIID programs. Our remaining tasks are to determine the homologous and heterologous specificity of the reagents in the IFA and direct FA (DFA) tests. Initial determinations by FA are given in Table II.

TABLE II. ARENAVIRUS CROSS-REACTIONS BY DFA TESTS

Antigen	Reaction by Antibody					
	MAC	PIC	LCM	LAS	TAC	JUN
MAC	4+	-	-	-	1+	1+
PIC	1+	4+	-	-	2+	-
LCM	-	-	3+	-	-	-
LAS	-	-	-	4+	-	-
TAC	4+	-	-	-	3+	$\pm$
JUN	4+	-	-	-	4+	4+

The results are in general agreement with those reported by Casals (2) with the exceptions of the reported crossing of LCM antibody with LAS and to a lesser degree with PIC antigens, and LAS antibody with LCM and a lesser degree with PIC and JUN antigens. All of these reported crosses were negative due in fact to our objective of producing monospecific sera rather than demonstrating cross-relationships. These will be looked at using Leighton tube slides of virus-infected cells rather than with spot slides. Arenavirus specificity by IFA will eventually be determined using the same reagents.

Alphaviruses. Similar studies have been done using alphavirus reagents and IFA techniques. As indicated in Table III, homologous reactions are strongest. In relation to the homologous reaction, strongest cross-reactions occurred between CHIK (4+ homologous) and ONN (2+ heterologous), MAY (3+ homologous) and CHIK (2+ heterologous) and ONN (3+ homologous) and CHIK 2+ heterologous). The reactions show and confirm antigenic relationships previously reported by neutralization tests. Cross-reactions by DFA will eventually be performed.

TABLE III. ALPHAVIRUS CROSS-REACTIONS BY IFA.

Antigen	Reaction by Antibody					
	EEE	VEE	CHIK	WEE	MAY	ONN
EEE	3+	P[a]	-	P	-	-
VEE	1+	4+	-	-	-	-
CHIK	P	1+	4+	1+	P	2+
WEE	1+	P	-	4+	P	-
MAY	±	1+	2+	P	3+	-
ONN	P	1+	2+	P	1+	3+

[a] P = partial.

Cell sensitivity tests. We have started a series of studies to determine the minimal amount of virus (determined by input $SMICLD_{50}$) required to infect a given type of cell monolayer. These determinations are necessary and directly applicable to the isolation of virus from clinical specimens, since seldom do they contain more than 10^4 PFU of virus. Using a standard procedure, 24-well plates containing various cell monolayers grown on coverslips, virus infections were initiated at specific minimal virus inputs and the time-course of virus and/or virus antigen development followed by fluorescent techniques. The decision to use $SMICLD_{50}$ input rather than PFU input was based on the known variability of cell cultures to a given virus. Thus 10,000 $SMICLD_{50}$ of WEE strain 72V4768 corresponds approximately to 150 for Vero, 9.1 for chick fibroblasts, 2.0 BHK-21 and 0, $LLC\text{-}MK_2$ PFU, respectively.

VEE. Tests with strain 69Z1 indicate that viral antigen was detectable (i.e., slightly + to ±) in Vero and BHK-21 monolayers at inputs of 10 $SMICLD_{50}$/0.1 ml within 24 hr; $LLC\text{-}MK_2$ and chick fibroblasts were negative. At 48 hr, BHK-21 were positive 2+, Vero, 3-4+ and chick fibroblasts, 3-4+, with the same input. $LLC\text{-}MK_2$ cells remained negative until 72 hr, at which time 2-3+ was observed at 10^0-10^1 input. We are unable to detect <10 $SMICLD_{50}$/0.1 ml dose within 48 hr. Infection by inputs of 10 $SMICLD_{50}$ are detectable at 24 hr, using BHK-21 or Vero cells and definitely observed by 48 hr. Generally, 1 PFU is equal to 100 $SMICLD_{50}$.

EEE. NJ 1135 strain of EEE could not be detected at inputs of ≤10 PFU within 24 hr. By 48 hr, $LLC\text{-}MK_2$ and Vero monolayers were positive (2-3+) at inputs of 10^0-10^1, whereas BHK-21 and chick fibroblasts remained negative. The latter cells became positive at 72 h. It is possible to detect 1 $SMICLD_{50}$/0.1 ml of EEE virus within 48 hr, using either $LLC\text{-}MK_2$ or Vero cells.

WEE. Strain 72V4768 of WEE can be detected in Vero cells at an input of 10^1 (3-4+) and 10^0 (1+) within 24 hr. BHK-21, $LLC\text{-}MK_2$ and chick fibroblasts were negative. By 48 hr, antigen intensity in Vero cells increased at a MOI of 10^0 (3+) and involved ≈40% of the cells; the other 3 cell types remained negative, although chick fibroblasts became positive (3+) at a MOI of 10^1 by 72 hr. Vero cell sensitivity extended to inputs of 10^{-1} and 10^{-2} by 72 hr, but

involved few cells (≤ 5%). $SMICLD_{50}$:PFU is 10^3-10^4:1.

ONN. H12628 strain cannot be detected in BHK-21, $LLC\text{-}MK_2$ or MRC-5 cells within 24 hr, in contrast to Vero cells, which are slightly positive at an input of 10^1. Vero cell infections increased to 2-3+ at 10^1 input and was also apparent at an input of 10^0 (2-3+) by 48 hr. MRC-5 cells were slightly positive at an input of 10^1. BHK-21 and $LLC\text{-}MK_2$ cells were negative and remained so at 72 hr. At this same time period, MRC-5 cells are 2+ at inputs of 10^1 and 10^0, whereas Vero sensitivity was indicated by 1-2+ at inputs as low as 10^{-1}, although <1% of the cells were involved. $SMICLD_{50}$:PFU is 10^0-10^1:1.

We have also done preliminary experiments with CHIK, JE, DEN-1, YF, MAY, SLE, LAN, WN, ORO, LAC, RVF and JUN viruses. Although the data are indicative and would be used to isolate and identify clinical unknowns if required, confirmatory experiments should be done. It was our purpose to obtain usable initial information on a number of viruses, rather than finitely determine cell sensitivities on but a few viruses.

Virus stocks. With a few exceptions (7 of 29), all viruses of interest have been prepared in suckling mouse brain and $SMICLD_{50}$ titers obtained. Of the 7 not in our inventory, 3 are P-4 agents; consequently seed stocks are maintained by the respective investigators. The remaining 4, CON, HAZ, LCM and PIC, are not high-priority agents and will be propagated in suckling mice as time permits. Thirteen viruses have also been propagated in cell culture systems, $SMICLD_{50}$ titers determined and PFU titers obtained on BHK-21, $LLC\text{-}MK_2$, Vero and chick fibroblasts or MRC-5 cell lines. Most suckling mouse brain virus preparations have also been plaqued on the various cell lines mentioned. As time permits, we will assemble the above data for publication.

Model systems. The isolation and identification experiment to be described was done during the routine inoculation of a cynomolgus monkey for the purpose of obtaining immune sera. After prebleed (day 0), the monkey was inoculated with 1 ml of RVF virus, strain ZZ-501, pool 900026 Hu/1,FRL/2,BHK/1 as divided doses given SC and IM. Samples of blood and throat washings were obtained daily for 5 days. Throat washings were obtained and processed as previously described (4). Smears were prepared, fixed and tested by DFA. Serum and throat wash supernatants were inoculated onto coverslip-containing 24-well plates, in accordance with the SOP for cell-sensitivity testing. Coverslips were removed at 24, 48 and 72 hr and tested for the presence of virus antigen by direct FA. Serial 10-fold dilutions of both sera and washings were assayed for virus by plaquing on Vero cell cultures. Results are shown in Table IV. When tested under the conditions stated, all throat wash supernatants were uniformly negative both by DFA and PFU assays. Cells obtained from throat washings did not contain demonstrable viral antigen. Sera obtained on days 1 and 2 were viremic reaching maximum titers of 10^4 PFU/ml. Viremia was transient and disappeared by day 3. The presence of virus was demonstrated on the Vero cell amplification system within 24 hr. Although only a small percentage of the cells were infected, the fluorescent staining characteristic of RVF virus made tentative identification possible. This was a preliminary experiment in which data obtained from prior minimal virus input cell sensitivity determinations

TABLE IV. ISOLATION AND IDENTIFICATION OF RVF VIRUS FROM AN EXPERIMENTALLY INFECTED MONKEY

Sample	Day	REACTION[a] (% POSITIVE) 24 hr	48 hr	72 hr	PFU/ml
Throat washing	0-5	-	-	-	-
Serum	0	-	-	-	-
	1	2-3+(<10)	2-3+(2-5)	1+ (10)	$5.5,5.8 \times 10^4$
	2	2+(<1)	2-3+(<10)	1+ (5)	$1.7,8.8 \times 10^4$
	3-5	-	-	-	-
Controls					
Negative		-	-	-	-
Positive					
10^5 $SMICLD_{50}$		± Foci	1+ (10)	1+(≈50)	4.2×10^7
10^3 $SMICLD_{50}$		2+(<)	2+ (25)	1+(≈25)	4.0×10^7

[a]Graded on a scale of 0-4.

allowed the isolation and tentative diagnosis of RVF infection in an animal ostensibly being used for other purposes. Similar experiments will continue to be run in conjunction with studies being done by other investigators at USAMRIID.

Other studies. As reported last year, we found that the use of silicone-treated Vacutainer tubes tended to increase background levels of PIC virus-negative sera when tested by IFA. The use of the new silicone plug Vacutainer tubes by the clinical laboratory prompted a study to determine their effect on RVF sera of known titers. Accordingly, personnel with known antibody titers were bled into tubes devoid of any known chemical additives and into silicone plug tubes. All sera were tested by IFA and PRN tests. The data indicated that the silicone plug tube caused RVF-positive sera (titers 1:8-1:16) to appear negative by the IFA test. No comparable effect was observed in the PRN test. Both tests accurately recorded all positive and negative sera, except as noted. In all cases the IFA titers were much lower (1:8-1:16) than obtained by the PRN_{80} test (>1:320). Direct comparisons were not possible between the titers obtained by IFA and PRN tests, e.g., strong sera giving the highest titers by both tests, moderate sera giving medium titers, etc. Suffice it to say, silicone has been implicated as a source of increased background levels and the suppression of low levels of RVF antibody, thereby producing false negatives in the IFA test.

We have begun adapting enzyme-linked immunosorbent assay (ELISA) tests for antibodies now determined by FA. A small effort was expended acquiring reagents, equipment, etc., familiarization with the test, and determining the working dilution for the goat anti-human IgG peroxidase-labeled conjugate.

Training and service work. Ms. C. Eisermann, Dr. Osterman's group, WRAIR, was trained in the use of the cold cryostat for processing RMSF-containing biopsy materials. Ms. S. Smith, Aerobiology Division, spent 2 weeks being trained in a variety of FA techniques. LTC Lowry and Mr. Mangiafico (Bacteriology Division) received instruction on the use of FA for Legioneila pneumophila. We supplied 192 ampoules of various conjugates (18 agents, and various species) to other investigators at USAMRIID. Support continued to provide technical FA support to 8 investigators involved in 12 different studies.

We began a series of studies in cooperation with other investigators. One such study involved the assay of RVF virus antibodies in human sera by FA using spot slides and slides prepared as monolayers by PRN, RIA and ELISA tests. High, medium and low titers of RVF antibody, true negatives and sera containing possible cross-reacting antibodies were included. When completed, it should be possible to assess the sensitivity and specificity of each test relative to all others.

Although no publications are indicated, it is apparent that sufficient data are being obtained for a number of papers dealing with cross-reactions of arena-, alpha- and flaviviruses; sensitivity of cell systems to the above virus groups; plaquing characteristics of virus groups on various cell lines; and experimental model systems for isolation and identification of virus infections. We hope to publish soon on some of these topics.

Publications:

None.

LITERATURE CITED

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4. Lyerla, H.C., and F. T. Forrester. 1979. Immunofluorescence Methods in Virology, Center for Disease Control, Atlanta, GA. pp 88-8[illegible].

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6416	79 10 01	DD-DR&E(AR)636

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

10. NO CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	62776A	3M162776A841	00	065
b. CONTRIBUTING				
c. //////////	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Mechanism of action of antimicrobial agents

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
78 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 79	0.5	57.5
FISCAL YEAR CURRENT 80	0.5	50.8

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

20. PERFORMING ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Jahrling, P. B.

NAME: Stephen, E. L. POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Ribavirin; (U) Antiviral drugs; (U) Arenaviruses; (U) Togaviruses

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 mechanism of action of ribavirin (1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide). This drug shows considerable promise as an antiviral agent. How it exerts its effect will be useful in treatment of viral diseases of military importance.

24 (U) Test in vitro effect in tissue culture of ribavirin on m-RNA structure of arena- and togaviruses. Various biochemical and radiolabeling techniques will be used.

25 (U) 78 10 - 79 09 - The molecular mechanism of action of the antiviral drug ribavirin was investigated in a Venezuelan equine encephalomyelitis virus (VEE)-infected BHK-21 cell model. The following observations were made: ribavirin inhibited virus growth by more than 90%; tritiated uridine-labeled poly-A-containing RNA (mRNA) isolated and sub-fractionated by SDS polyacrylamide gel electrophoresis showed that the quantity of viral 42S genome was not diminished by ribavirin treatment; tritiated guanosine-labeled mRNA was isolated and treated with ribonuclease. The resulting polynucleotides chromatographed on DEAE-cellulose yielded a radioactive peak which eluted from the column at the same salt concentration as ppp7mG "cap" standard. The magnitude of the peak was reduced or absent from mRNA preparations isolated from virus-infected cells treated with ribavirin; and mRNA from ribavirin-treated infected cells was less efficient in its capacity to direct protein synthesis in an in vitro translation system.

These observations suggest that ribavirin does not inhibit viral transcription, but may interfere with translation of viral messenger by replacing or inhibiting formation of the cap structure on the viral RNA genome.

Publication: Abstract 977, In: Proc. 11th Int. Congr. Chemother. and 19th Intersci. Conf. Antimicrob. Agents Chemother., 1979.

* Available to contractors upon requestor's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 065: Mechanism of Action of Antimicrobial Agents

Background:

Ribavirin (1-β-D-ribofurasyl-1,2,4,triazole-3-carboxamide) is a nucleoside analogue having broad spectrum antiviral activity against both DNA and RNA viruses (1). A variety of specific effects on host cell metabolism have been attributed to ribavirin or its metabolites. Ribavirin monophosphate (RMP) appears to be a competitive inhibitor of inosine monophosphate (IMP) dehydrogenase activity and guanosine monophosphate (GMP) synthesis, while ribavirin triphosphate (RTP) is a selective inhibitor of influenza virus RNA polymerase. Additional effects of ribavirin include inhibition of both thymidine phosphorylation and synthesis of DNA, RNA and proteins (2). Contrasting reports, have been published, which contradict many of the alleged cellular effects of ribavirin (3). As a result, the pharmacological mechanism of action of ribavirin remains obscure. It is not yet clear whether this compound is specifically antiviral in its mechanism of action or whether it inhibits virus replication as a result of its effects on host cellular metabolism.

In an attempt to clarify the mode and specificity of action of ribavirin, we have examined its effects on cellular metabolism and on the replication of VEE virus grown in BHK-21 cells as a model system.

Progress:

VEE virus in the BHK-21 system rapidly replicates with a 3-log increase in virions within 5 hr after infection at a multiplicity of infectivity of about 2 virus particles/cell (Table I). A 4-log increase in virus titer is found after 17 hr. Ribavirin, at concentrations as low as 10 µg/ml inhibits virus replication by more than 99% as compared to nontreated cultures. The inhibition of virus growth is not complete; a 10-fold increase in virus concentration is found 17 hr after infection in the presence of ribavirin. This increase in virus titer, however, is only 1/1000 the virus concentration found in untreated cultures.

The uptake of radiolabeled precursors into trichloracetic acid (TCA)-soluble and -insoluble material was then examined in control and infected cells after 5 hr of incubation with ribavirin at concentrations of 25-800 µg/ml. At the lowest concentration ribavirin inhibits both the TCA-soluble and -insoluble uptake of uridine by control and infected cells. This inhibition is reversed at higher concentrations. Guanosine uptake is enhanced at low doses of ribavirin but declines at higher concentrations of drug to ≈ 80% of the uptake of untreated control and infected cultures. Incorporation of label into TCA-insoluble material is inhibited by ≈ 35% at 100 µg/ml of ribavirin. Guanosine incorporation in infected cells is 25% less than that of control cells. The addition of ribavirin at concentrations of 100-800 µg/ml further reduces the extent of guanosine incorporation by 15-30%.

TABLE I. INHIBITION BY RIBAVIRIN OF VEE VIRUS REPLICATION IN BHK-21 CELL CULTURES[a]

RIBAVIRIN (μg/ml)	LOG_{10} PFU/ml[b] BY HOURS AFTER VIRUS ABSORPTION			
	0	3	5	17
0	4.4	4.4	7.2	8.6
10	4.6	4.5	4.8	5.6
25	4.6	4.3	5.0	5.4
50	4.7	4.5	5.2	5.3
100	4.6	4.4	4.8	5.4

[a]For absorption, 2×10^6 cells were incubated for 1 hr with 4×10^6 PFU of VEE strain TC83 (MOI = 2). Following absorption, inoculum was removed, cells washed once, and medium was replaced with EMEM containing 5% FCS and the stated final concentration of ribavirin.

[b]Virus in cell culture supernatants assayed by counting PFC on Vero cells. Titers are geometric means of 6 determinations (2 from each of 3 experiments).

The complex nature of these results are not readily explainable. They suggest, however, that ribavirin may alter cellular nucleotide pools and cause significant changes in the specific activity of isotopic labeling. Contributing to such effects is the inhibition of inosine uptake and incorporation by low doses of ribavirin. Inosine utilization is inhibited by ≈ 85% at the lowest dose of ribavirin tested. This observation is consistent with the inhibition of IMP dehydrogenase by ribavirin and a subsequent change in the cellular guanosine pool. However, the effect of ribavirin on IMP dehydrogenase activity is not thought to be a principle site of antiviral activity. We are led to this proposal because inosine utilization in virus-infected cells is decreased by > 80% when compared to uninfected cultures. Doses of ribavirin which reduce virion production by 99% have only a minimal additional inhibitory effect on inosine incorporation. It is clear that virus-infected cells, with their DNA-synthesizing capacity nearly shut off, have a reduced requirement for purine synthesis. In a virus-infected cell, therefore, a reduction in GMP synthesis via inhibition of IMP dehydrogenase need not be inhibitory to viral mRNA production.

Inhibition of nucleotide incorporation in ribavirin-treated cells could result from the inhibition of RNA polymerase. Therefore, we determined the rate of radiolabeled uridine triphosphate (UTP) incorporation in BHK-21 cells made permeable to large as well as charged molecules by exposure to lysolecithin (200 μg/ml) at 4°C. Permeable cells incorporate [^{3}H]UTP at a linear rate for at least 15 min. When this approach was used to determine the effects of ribavirin and its phosphorylated derivatives on RNA synthesis, little if any inhibitory effects was observed, suggesting that they did not inhibit RNA polymerase activity in VEE infected BHK-21 cells.

[^{3}H]Uridine labeled mRNA from control and infected cells treated with varying concentrations of ribavirin was then isolated on oligo-dT columns and subfractionated by SDS polyacrylamide gel electrophoresis. The 42s viral RNA genome is readily recognized as a single peak at a relative migration distance of about 0.1 and represents about 10-11% of the total mRNA. Treatment of infected cell cultures with 300 µg/ml of ribavirin did not diminish the relative quantity of viral mRNA. Intermediate concentrations of ribavirin (50 and 100 µg/ml) gave similar results. These observations indicate that ribavirin has no specific inhibitory effects on the synthesis of viral genome. Furthermore, chemical determination of the quantity of mRNA isolated from ribavirin-treated VEE-infected BHK-21 cells did not differ from untreated controls thus supporting the concept that ribavirin does not inhibit viral mRNA synthesis.

Since interference with synthesis of viral genetic information is not a probable mechanism for ribavirin's antiviral activity, we examined the capacity of mRNA from ribavirin-treated cells to direct the synthesis of proteins in an in vitro, rabbit reticulocyte, cell-free translation system. mRNA from infected cells treated with 100 µg/ml of ribavirin was significantly less efficient than mRNA from infected but untreated cells in its ability to direct the incorporation of [^{3}H] leucine into TCA-preciptable material.

Recently, Goswani et al. (4) reported that RTP is a potent inhibitor of the 5' terminal guanilation of vaccinia mRNA. Since the 5' terminal 7-methyl guanine in mRNA appears to be required for efficient translation (5), we studied the effects of ribavirin on the capping of mRNA in our model system. [^{3}H]Guanine-labeled mRNA was isolated on oligo-dT columns and digested with ribonuclease. The resulting mononucleotides were chromatographed on DEAE-cellulose using a linear NaCl gradient. A minor radioactive peak eluting subsequent to the major mononucleotide peak and corresponding to the elution profile of a 7mGppp "cap" standard was found in all chromatographs of mRNA digests from control and VEE-infected cells. The area under the cap peak which was resolved from mRNA preparation isolated from virus-infected cells treated with ribavirin at concentrations of 50-300 µg/ml was reduced nearly 10-fold.

We conclude, therefore, that the pharmacological mechanism of action of ribavirin is not specifically antiviral. We propose that ribavirin does not inhibit viral transcription, but interferes with translation of viral mRNA by replacing or inhibiting formation of the "cap" structure on the viral RNA genome. This mechanism can account for the antiviral potency of ribavirin against both DNA and RNA viruses and explain its ineffectiveness against certain viruses, such as polio, whose mRNA is not capped (6).

Publications:

Canonico, P. G., J. S. Little, P. B. Jahrling, and E. L. Stephen. Molecular aspects of the antiviral activity of ribavirin on Venezuelan Equine Encephalomyelitis Virus (VEE), Abstract No. 977, Proc. of 11th In. Congr. Chemother. and 19th Intersci. Conf. Antimicrol. Agents Chemother.

LITERATURE CITED

1. Sidwell, R. W., R. K. Robins, and I. W. Hillyard. 1979. Ribavirin: an antiviral agent. Pharmacol. Therapeut. 6:123-146.

2. Smith, R. A., R. W. Sidwell, and R. K. Robins. 1980. Antiviral mechanisms of action. Annu. Rev. Pharmacol. In press.

3. Browne, M. J. 1979. Mechanism and specificity of action of ribavirin. Antimicrob. Agents Chemother. 6:747-753.

4. Goswami, B. B., E. Borek, O. K. Sharma, J. Fujitaki and R. A. Smith. 1979. The broad spectrum antiviral agent ribavirin inhibits capping of mRNA. Biochem. Biophys. Res. Commun. 89:830-836.

5. Muthukrishnan, S., B. Moss, J. A. Cooper, E. S. Maxwell. 1978. Influence of 5'-terminal cap structure on the initiation of translation of vaccinia virus mRNA. J. Biol. Chem. 253:1710-1715.

6. Nomoto, A., Y. F. Lee, and E. Wimmer. 1976. The 5' end of poliovirus mRNA is not capped with $m^7G(5')$ ppp (5') Np. Proc. Natl. Acad. Sci. USA 73:375-380.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6418	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
78 12 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	62776A	3M162776A841	00	066
b. CONTRIBUTING				
c. [redacted]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Characteristics of aerosol-induced Rift Valley fever 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
78 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 79	1.0	52.5
FISCAL YEAR CURRENT 80	1.0	53.2

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

NAME:

NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Aerosols; (U) Rift Valley fever; (U) Laboratory animals; (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 potential for aerosol transmission of Rift Valley fever (RVF) isolates. Investigate pathogenesis, therapy and prophylaxis in relation to aerosol challenge. RVF is a disease of military importance in Africa where it is a serious problem for man.

24 (U) Initally, infect mice with dynamic aerosols; follow with other appropriate animal species. Determine effectiveness of the currently available vaccine against Egyptian strains of virus.

25 (U) 78 12 - 79 09 - In a dynamic aerosol system equipped with a Collison device, physical characteristics of the small particle aerosol (SPA) generated from the virus carrier fluid were studied. The SPA consisted of particles 5 microns or less in size with a mass median diameter of 0.964 micrometers.

Aerosol infectivity trials were performed using young adult ICR mice. The logarithmic median lethal dose for 4 strains of RVF virus was ZZ-501, 2.54 PFU; Entebbe, 1.76 PFU; SA-51, 2.59 PFU; and SA-75, 1.86 PFU.

Aerosol stability properties of the ZZ-501 strain in a carrier fluid of EMEM with 10% fetal calf serum was determined at 75 F. Expressed as per cent decay per minute the geometric mean values were 11.9, 6.9, and 2.6 at 80, 55, and 30% relative humidity respectively.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 066: Characteristics of Aerosol-Induced Rift Valley Fever Infections

Background:

Rift Valley fever (RVF) is an acute arthropod-borne disease first described in Kenya in 1931. The causative agent is an arbovirus classified in the family Bunyaviridae. The complete enzootic cycle is unknown; however, a maintenance cycle involving mosquitoes and vertebrates is hypothesized. RVF infects man and several mammalian species, primarily sheep, cattle, and goats.

Historically, RVF has been confined to central and southern Africa and regarded as a disease problem of livestock. Although humans were recognized as being highly susceptible, RVF in humans was described as either a "dengue-like" fever or an "influenza-like" illness noted on occasion in livestock workers. However, in 1977 an outbreak occurred in Egypt which produced widespread, and severe human infections.

Evidence supports a conclusion that RVF virus is an infectious agent which military forces operating in Africa would encounter. Also, there are scattered reports that RVF has spread beyond the African continent into the Middle East.

Potency testing of a currently available inactivated vaccine was performed by IC and IP challenge of mice. Further testing was accomplished using cynomolgus monkeys. Efficacy of the vaccine against an aerosol challenge is undetermined.

Progress:

Four strains of high titered RVF virus stock were prepared and stored separately in EMEM containing 10% FCS and antibiotics. Physkcal characteristics of small-particle aerosols (SPA) generated from the EMEM carrier fluid were then determined. Measurements were made in a dynamic aerosol system in which a Collison spray device was used for generation of the SPA. In 5 separate trials a SPA was generated using stainless steel and plastic spray heads. Results are shown in Table I. Over 98% of the SPA was composed of particles $\leq$ 5 µm in size with their mass median diameter of 0.964 µm.

TABLE I. RECOVERY OF AEROSOLIZED EMEM CARRIER FLUID IN A DYNAMIC AEROSOLIZATION SYSTEM

AEROSOL SAMPLING DEVICE Single-Stage Impactor	% RECOVERY OF SODIUM FLUORESCEIN	
	Stainless Steel Head	Plastic Head
1 μm	54.0	57.9
3 μm	93.9	92.1
5 μm	100.0	98.7
AGI-30	100.0	100.0

Aerosol infectivity trials were performed with the ZZ-501, Entebbe, SA-41, and SA-75 strains of RVF. Five doses of each strain were aerosolized in a dynamic aerosol system. The animal model was young adjult, male, ICE mice, weighing 30-34 gm. Groups of 20 mice were exposed to graded doses ranging from 0.5-4.8 $\log_{10}$ PFU/mouse. The aerosol LD_{50} for the 4 strains, as calculated by the method of Reed and Muench, was ZZ501, 2.54 $\log_{10}$ PFU; SA-51, 2.59 $\log_{10}$ PFU; Entebbe, 1.76 $\log_{10}$ PFU; and SA-75, 1.86 $\log_{10}$ PFU. Complete data are shown in Tables II and III.

TABLE II. RESPONSE OF ICE MICE TO SPA OF ZZ-501, ENTEBBE, SA-51, SA-75 STRAINS OF RVF VIRUS

STRAIN	DOSE (Log_{10} PFU)	RELATIVE HUMIDITY (%)	NO. DEAD/ NO. EXPOSED	MORTALITY (%)	LD_{50} (PFU)
ZZ-501	0.7	58	2/20	10	
	1.7	54	4/23	17	
	2.8	59	10/20	50	2.54
	3.8	55	19/20	95	
	4.8	58	17/17	100	
Entebbe	0.9	51	0/20	0	
	1.9	51	12/20	60	
	2.9	51	19/20	95	1.76
	3.8	51	20/20	100	
	4.8	48	20/20	100	
SA-51	0.5	54	0/20	0	
	1.6	54	2/18	11	
	2.7	54	10/20	50	2.59
	3.8	54	20/20	100	
	4.8	54	20/20	100	
SA-75	0.5	69	0.20	0	
	1.5	69	4/20	20	
	2.2	71	16/20	80	1.86
	3.1	68	20/20	100	
	4.3	62	20/20	100	

TABLE III. RESPONSE OF ICR MICE TO SPA OF RVF VIRUS

DAY	ZZ-501 (n=52)	ENTEBBE (n=71)	SA-51 (n=54)	SA-75 (n=60)
2	0	0	0	0
3	7	53	15	0
4	33	64	37	43
5	42	66	48	49
6	48	67	49	54
7	50	68	51	55
8	50	68	51	56
9	52	70	52	58
10		70	52	58
11		70	52	58
12		70	53	59
13		70	54	59
17		71		59
18				59
19				60

Aerosol stability properties of the ZZ-501 strain were studied in a 6,200 liter static aerosol chamber. In all studies, temperature within the chamber was controlled at 75°F. On 4 separate days, aerosol trials were conducted at RH of 80, 55, and 30%. During each trial 5 ml of high titered RVF stock was disseminated using an FK-8 nozzle. The aerosol stability of the virus varied inversely with RH (Table IV).

TABLE IV. AEROSOL STABILITY PROPERTIES OF ZZ-501 STRAIN OF RVF VIRUS

TRIAL NO.	AEROSOL DECAY RATES (%/min) 80% RH	55% RH	30% RH	TITER OF RVF STOCK (log_{10} PFU/ml)
1	-	11.4	2.8	7.6
2	11.4	6.0	3.0	8.1
3	10.1	5.6	2.1	7.9
4	14.8	5.8	2.8	8.2
Mean % Decay	11.9	6.9	2.6	

Results indicate that the ZZ-501 strain, isolated from Egypt in 1977, is highly infectious for mice and comparable to the infectivity of 3 other strains which have been studied more extensively. The inverse relationship between aerosol stability and RH observed for the ZZ-501 strain is similar to that reported for other viruses, e.g., Japanese B encephalitis, Lassa fever, and Machupo viruses. Studies are being planned to identify a rat strain which is susceptible to RVF exposure by the respiratory route, and then evaluate efficacy of the RVF vaccine against an aerosol exposure.

Publications: None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6419	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. ORG'N HISTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 01 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	62776A	3M162776A841	00	067
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Procede with Security Classification Code): (U) Effect of infection on muscle enzymes in relation to physical training

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
79 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 79	1.5	81.1
FISCAL YEAR CURRENT 80	1.5	74.0

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign intelligence considered

20. PERFORMING ORGANIZATION
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NAME: Ilback, N.-G.
NAME: Crawford, D. J. POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Infectious disease; (U) Exercise; (U) Stress; (U) Physical performance; (U) Laboratory animals

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

23 (U) Determine influence of (a) infectious disease on exercise capability and on biochemical variables related to performance; (b) exercise on the course of infection; and (c) exercise on host-defense mechanisms. This research is essential for ascertaining a soldier's ability to perform physical work, assessing impact of physical conditioning, and developing approaches for successful immunoprophylaxis against potential BW agents. 24 (U) Infect rats with Francisella tularensis, Salmonella typhimurium, and Streptococcus pneumoniae. Measure a variety of oxidative, glycolytic and lysosomal enzymes, substrates and by-products of energy metabolism in red and white thigh muscles, and lymphocyte transformation, macrophage-migration inhibition, antibody titers, and acute-phase proteins in exercise- and disease-stressed rats. Compare voluntary and forced exercised groups with inactive groups and well-conditioned with nonconditioned groups. 25 (U) 79 01 - 79 09 - In the F. tularensis study (LVS) there was a trend for the enzymes (oxidative, glycolytic and lysosomal) to decrease more in those muscle fibers normally having a low enzyme activity. In S. typhimurium, somewhat less pronounced alterations were observed and in S. pneumoniae significant changes were observed only in some of the variables. In the exercise studies exhaustion was monitored using whole blood lactic acid levels. S. pneumoniae and F. tularensis do not alter muscle glycogen metabolism. Beta-glucuronidase and OCT were also elevated as a result of exercise during infection, indicating serious liver involvement. Alpha-2-macrofetoprotein showed pronounced elevation in infected-exercised animals; antibody titers to F. tularensis were lower in this same group when compared to infected controls.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 067: Effect of Infection on Energy Metabolism, Muscle Enzymes and Host Immune Response in Relation to Physical Performance and Training

Background:

Static muscle strength and endurance decrease in febrile infection (1) and a significant impairment persists, although less pronounced, for several weeks afterwards (2). This performance reduction is temporarily correlated to depressions in the activities of glycolytic and oxidative enzymes in muscle tissue and focal derangement of muscle ultrastructure in a number of viral and Mycoplasma pneumoniae infections; similar enzyme changes have been observed in bacterial infection (3). The fundamental cause and nature of these changes in infection have not been clarified.

There are even fewer studies on the influence of exercise and infection (in combination) on the host's immune response. Paralysis in poliomyelitis in humans occurs more frequently and severely if the patient exercises during the disease prior to the paralytic phase. Virus multiplication in the heart occurs more frequently in coxsackie B-3 viral infection in the adult mouse if the animal is exercised during the early period of the infection. Likewise, coxsackie B-3 myocardiopathy in weanling mice shows virus multiplication increased 530 x when infected mice are forced to swim. In a more recent study, exercise during this viral infection was found to postpone the appearance of interferon in the serum and caused a less pronounced antibody response (4).

Preliminary studies were designed to: evaluate existing rodent exercise models in terms of reproducibility, accuracy and degree of exhaustion; assess the influence of Francisella tularensis LVS, 10^7, and Salmonella typhimurium, 10^8, given IP and Streptococcus pneumoniae 10^4, given sc on exercise capacity and biochemical variables related to performance; study the effects of physical conditioning on performance capacities and establish whether the severity and duration of the illness is affected; and investigate influence of exercise on infectious-disease stimulated cellular and humoral immune responses.

Progress:

Evaluation of swimming exercise model. Rats were exercised by having them swim in 50-cm deep, 33°C water in 50-cm diameter metal sheet barrels. Initial studies using this model are nearly complete. Results show a disturbing amount of variability between like groups of animals; this has presented a problem in obtaining accurate and reproducible results. To overcome these problems we are presently evaluating and modifying a wheel-running model. This model should provide a more consistent means of exhausting the rats by a variable-speed motor-driven system.

Influence of infection on exercise capacity and effect of physical conditioning. In an effort to study whether moderately severe exercise is detrimental to muscle cells in early convalescence of an infection and, if so, whether physical training may have a protective effect, the activities of a number of lysosomal enzymes were

measured in red and white skeletal muscle of rats after S. pneumoniae infection (10^4). Thirty-six hours after the exercise, rats were sacrificed and muscle was isolated from the thigh.

The following untrained groups of rats were investigated: nonexercised-noninfected controls, exercised-noninfected, nonexercised-infected, and exercised-infected. In addition, other rats had been trained by daily swimming for 2 weeks prior to the experiment. The following trained groups of animals were investigated: nonexercised-noninfected controls and nonexercised-infected.

Enzyme assays in each of these 6 groups were performed on the day of sacrifice of that particular group of rats except for cathepsin C and D, each of which was measured in thawed cell homogenates one day after the conclusion of the experiment. Results have been expressed in relation to wet weight, protein content, and DNA content of the muscle.

Exercise during the early convalescent phase of infection did not cause any additional activation of the lysosomal system when compared to resting-infected levels. In fact, for cathepsin C a significantly lower enzyme response was recorded in the exercised-infected group compared to the nonexercised-infected group. These results seem to be compatible with the present finding of a "protein sparing" (or rather synthesis-stimulating) effect associated with moderately severe exercise in early convalescence.

The enzyme findings during infection in trained show the same trend as in untrained rats. The somewhat unexpected trend for the activities in trained nonexercised-noninfected controls (compared to trained, nonexercised-infected rats) to be higher when expressed per unit DNA than when expressed per wet weight of protein was not significant. The infection-associated protein loss per cell was the same (on a percentage basis) in trained and untrained rats. The effects of exercise in trained rats during sepsis has not been investigated.

Effect of acute infectious disease on glycolytic, oxidative and lysosomal enzyme systems in muscle tissues. Predominantly red (slow twitch, type I) and white (fast twitch, type II) muscle tissues were sampled separately from the thigh and immediately homogenized and placed on ice for enzyme assays. The rats had been ill for 48 hr with S. pneumoniae (10^4) and S. typhimurium (10^8) and for 72 hr with F. tularensis (10^7) at the time of the investigation. For each infected and control group, n=6. The following variables were measured: cytochrome c oxidase, citrate synthetase, glycerol-3-phosphate dehydrogenase, β-glucuronidase, p-nitrophenylphosphatase, RNAse, protein and DNA.

The results of the tularemia study show a trend for the enzymes to decrease more in those fibers normally holding a low absolute enzyme activity than those holding a high. Thus, cytochrome c oxidase decreased to 53% of the control in white, but to only 70% in red muscle tissue. In S. typhimurium infection, somewhat less pronounced alterations were observed and in S. pneumoniae infection, significant changes were observed only in some of the variables. DNA was constant in all 3 infections.

Cardiac muscles showed the same pattern of changes as skeletal muscle. To determine if a period of physical training prior to infection alters the patterns of response, the F. tularensis LVS model was used. Less pronounced decreases in the activities of oxidative enzymes were observed in heart muscle than in red or white

skeletal muscle of untrained rats, while in trained rats no changes at all seemed to occur.

Influence of exercise on infection. Longitudinal experiments were carried out using F. tularensis LVS (10^7, IP) model to study effects of forced exercise during the course of an ongoing infection in untrained rats. A group of 180 rats was divided into 4 groups: exercising-infected, resting-infected, exercising-sham-inoculated and resting-sham-inoculated. Exhaustive swimming was used. Rats were fed ad libitum. Rats were sacrificed on days 2, 4 and 7 of the infection.

Beta-glucuronidase and OCT were elevated as a result of exercise in the infected rats, possibly indicating more serious liver involvement. Exercise in healthy animals had no significant effect. Further, an acute-phase protein, α_2-macrofetoprotein, showed a pronounced elevation during the combined infection and exercise, suggesting an enhanced rate of de novo transcription. Antibody titers to F. tularensis (LVS) were lower in the infected-exercised group compared to infected controls.

Influence of infection on biochemical variables related to performance. A group of 100 Sprague-Dawley rats were divided into exercising and nonexercising groups; they were further subdivided into infected and noninfected. Infected rats were inoculated with S. pneumoniae (A-5, 10^4, SC). Rats were sacrificed at 24, 48, 72 hr post inoculation. The exercised groups swam for 3 hr prior to sacrifice. Gastrocnemius and heart muscles, liver and plasma were obtained from each rat for analysis. Degree of exhaustion was determined by whole blood lactic acid concentration after exercise. Glycogen determination in white skeletal and heart muscles were used as an additional indicator in characterizing the "intensity" of the exercise and to what extent the rat was exhausted. Blood lactate levels increased during the first 15 min of swim, dropped at 30 min and progressively increased over the 3-hr period of exercise. Skeletal and cardiac muscle glycogen dropped during exercise but was not affected by the infection.

In order to determine the influence of infection and exercise on host energy stores and related hormones, the following plasma variables were measured: glucose, free fatty acids (FFA), ketone bodies, insulin and glucagon.

To meet their increased energy needs, exercising muscles can draw upon intracellular stores of fuels such as triglycerides and glycogen and increase their uptake of glucose or FFA from circulating blood. Simultaneously, appropriate homeostatic adjustments take place, leading to an enhancement of glucose secretion by the liver and to stimulation of FFA production by adipose tissue. In the postabsorptive state, owing to their low concentration, ketone bodies play only a minor role in supplying muscle with energy. On the other hand, when ketonemia rises with fasting, acetoacetate and β-hydroxybutyric acid become major metabolic substrates for exercising muscles.

During exercise alone both plasma glucose and FFA concentrations were decreased below the nonexercised values, while bacterial infection alone decreased plasma glucose and FFA; the combination of infection and exercise elevated only the FFA levels. The plasma glucose remained constant. Plasma β-hydroxybutyric acid and acetoacetate concentrations were decreased during the infection and exercise, but there was no significant difference between non-exercise and exercise-infected levels.

Muscular exercise has been shown to induce an increase in plasma glucagon in animals. It is generally considered that this rise associated with a fall in circulating insulin, contributes to enhance the supply of metabolic substrates for exercising skeletal muscles via mobilization of hepatic glycogen stores and activation of both gluconeogenesis and lipolysis.

Exercise reduces plasma insulin levels and increases glucagon levels. While the exercise-induced reduction of insulin is thought to be mediated via an α-adrenergic inhibition of insulin secretion, the mechanism(s) responsible for increased glucagon secretion has not yet been fully clarified. Infection in conjunction with exercise had no significant effect on insulin levels. The plasma glucagon was too variable in exercising animals to make any concrete observation.

Publications:

None

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1. Aström, E., G. Friman, and L. Pilström. 1977. Human skeletal muscle in bacterial infection: enzyme activities and their relationship to age. Scand. J. Infect. Dis. 9:193-195.

2. Alluisi, E. A., B. B. Morgan, Jr., L. S. Caldwell, and W. R. Beisel. 1980. Effects of sandfly fever on isometric muscular strength, endurance, and recovery. J. Motor Behavior.

3. Friman, G. 1978. Effect of acute infectious disease on human isometric muscle endurance. Ups. J. Med. Sci. 83:105-108.

4. Reyes, M. P. and A. M. Lerner. 1976. Interferon and neutralizing antibody in sera of exercised mice with coxsackie B-3 virus myocarditis. Proc. Soc. Exp. Biol. Med. 151:333-338.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6420	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. DISP'N INSTR'N	8B. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
79 02 09	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	62776A	3M162776A841	00	068
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code): (U) Ebola virus infection: characterization of virologic, immunologic, and host-parasite relationships

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
79 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 K units)
FISCAL YEAR	PRECEDING 79	1.0	59.7
	CURRENT 80	1.0	66.6

19. RESPONSIBLE DOD ORGANIZATION
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NAME: Cole, Jr., F. E. POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Vaccines; (U) Laboratory animals; (U) Ebola virus

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

23 (U) Characterize Ebola virus in terms of cell culture, animal infectivity, biochemistry and pathogenesis. This highly infectious virus produces high mortality in man, and could be a major problem for the U.S. military if required to operate in Africa.

24 (U) Beginning with Vero cells, attempt to grow Ebola virus in cell culture. Develop plaque assay so that neutralization tests can be used. Infect guinea pigs and measure a variety of parameters, including dose-response, time to death, and pathogenesis.

25 (U) 79 02 - 79 09 - Guinea pigs of either Hartley or Strain 13 were defined as suitable models for Ebola hemorrhagic fever (EHF) infection. Cross-infectivity studies in guinea pigs revealed similarities between the Zaire and Sudan strains of EHF virus; however, cross-protection was not conferred by immune serum transfer. The interferon-inducing compound, poly(ICLC), given prophylactically partially protected against Zaire and completely against Sudan strains of EHF virus in guinea pigs, while ribavirin, in a similar schedule afforded little protection. EHF virus was cultured in vitro and a plaque assay was developed.

* Available in contractor upon originator's approval

BODY OF REPORT

Project No. 3M162776A841: Medical Defense Against Biological Agents (U)

Work Unit No. A841 00 068: Characterization of Virologic, Immunologic, and Host Parasite Relationships

Background:

In August 1976, outbreaks of an acute hemorrhagic illness termed Ebola hemorrhagic fever (EHF) commenced in Southern Sudan and Northern Zaire (1, 2). This illness was characterized by prostration, fever, headache, myalgia, arthralgia, abdominal pain and high mortality (88% in Zaire, 53% in Sudan). To date, there is no vaccine available for preventing EHF, with isolation and barrier nursing techniques being the principal measures taken during outbreaks. Therapy of EHF is likewise an undeveloped subject. Treatment with immune plasma appeared to be beneficial, while interferon did little to alleviate viremia in a British patient who suffered a laboratory infection with EHF virus (3). Because of the lack of either prophylactic or proven therapeutic measures against EHF, studies were undertaken at USAMRIID in order to develop a vaccine and evaluate various therapeutic measures.

Progress:

Animal Studies. The initial studies were designed to define animal models of EHF virus infection and to assess infectivity of the Zaire and Sudan strains of EHF virus. The Boniface (Sudan) strain of EHF received from the Medical Research Establishment (MRE) Porton Down, England, had undergone one guinea pig (plasma) passage and 2 Vero-cell passages at MRE. Following one passage in fetal rhesus lung cell (M-103) this working pool was designated Boniface $G_1V_2M_1$ and inoculated into Hartley and Strain 13 guinea pigs. Results of those studies are shown in Table I. Strain 13 guinea pigs were slightly more susceptible in terms of infectivity and mortality than Hartley strains. However, both strains were highly susceptible and provided good models of EHF infection.

The next animal experiment was designed to determine relationships between the Zaire and Sudan strains. Results of this experiment are shown in Table II. There was evidence of cross-protection between them in actively immunized guinea pigs. However, Sudan-immune serum provided very little protection against challenge with the homologous or heterologous strain of EHF. In summary, although serum therapy needs further evaluation, this study indicated that factors other than humoral immunity were essential in host defense against EHF virus. Despite the evident biological differences between Zaire and Sudan strains of EHF virus, there was sufficient antigenic similarity that most actively immunized guinea pigs were cross-protected.

TABLE I. EHF (BONIFACE STRAIN) INFECTIVITY STUDIES IN GUINEA PIGS.

GUINEA PIG STRAIN-DOSE	NO.	NO. WITH ≥104.5°F	MEAN DAY TO FEVER	NO. DEATHS	MEAN DAY TO DEATH
Strain 13					
10^{-2}	2	2	5.5	1	10.0
10^{-3}	3	3	6.0	2	15.0
10^{-4}	3	3	12.3	0	-
10^{05}	3	3	6.6	3	16.3
10^{-6}	3	1	7.0	1	8.0
10^{-7}	3	3	14.6	0	-
Control	2	1	21.0	0	-
Hartley					
10^{-2}	4	4	5.2	1	18.0
10^{-3}	4	4	3.7	1[a]	15.0
10^{-4}	4	2	7.0	1	13.0
10^{-5}	3	3	7.0	0	-
10^{-6}	3	1	4.0	0	-
10^{-7}	3	1	12.0	0	-
Control	5	0	-	0	-

[a] 2 of 4 sacrificed for virus passage.

The last major animal experiment dealt with attempts to treat EHF virus-infected guinea pigs with antiviral compounds. Because very little information was available, the compounds selected were limited to 2, the antiviral drug ribavirin, and poly(ICLC), an interferon-inducer possessing adjuvant properties. The design and results of this experiment are shown in Table III. although ribavirin appeared to cause a substantial delay in the onset of fever, there was no reduction in its duration. Ribavirin treatment prolonged the time to death with both strains of EHF virus while not significantly reducing illness or mortality. In the ribavirin-treated group, all Zaire strain-infected guinea pigs and one Sudan strain-infected guinea pig died. Conversely, poly(ICLC) only prolonged slightly the incubation period and did not alter duration of fever; it reduced mortality from both strains of EHF virus. While these studies were not conclusive, they indicated promise of a beneficial effect from treating EHF with poly(ICLC).

In vitro work conducted during the year included evaluation of cell lines as substrates of EHF virus, development of a plaque test, and production of slides for detection of EHF antigen or antibodies by fluorescent examination. EHF virus replicated to moderate titers (10^5 to 10^6 PFU/ml) in both Vero and M-103 cells. Through 4 sequential passages, the titers of virus in the M-103 cell line increased ~ 10-fold. Vero cells were the only line in which plaques were successfully produced by EHF virus. Spot slides bearing antigens of Zaire or Sudan strain were produced and made available for use in indirect fluorescent antibody diagnostic tests.

TABLE II. CROSS-PROTECTION IN GUINEA PIGS ACTIVELY AND PASSIVELY IMMUNIZED AGAINST EHF VIRUS

IMMUNE STATUS	SERUM TREATMENT (IP)	CHALLENGE (IP)	NO.	NO. W/ F	ONSET (Days)	DURATION F (Days)	NO. DEATHS	MEAN DAY TO DEATH
Sudan-Immune[a] (active)	None	Zaire (718)	5	1	5.0	3.0	1	9
Nonimmune	Sudan-Immune		6	6	5.0	3.0	4	10.8
Nonimmune	None		6	6	4.3	3.5	4	9.2
Sudan-Immune[a] (active)	None	Sudan (Boniface)	4	0	-	-	0	-
Nonimmune	Sudan-Immune[b]	Sudan (Boniface)	6	5	5.6	5.2	0	-
Nonimmune	None	Sudan (Boniface)	6	6	4.7	6.2	0	-

[a]Developed fever and recovered from previous inoculation of Boniface strain.
[b]Serum derived from guinea pig which developed fever and recovered after inoculation of Boniface strain.

TABLE III. RIBAVIRIN AND POLY(ICLC) THERAPY OF EHF-INFECTED GUINEA PIGS. THERAPY WAS STARTED ON DAY PRIOR TO INOCULATIC

VIRUS GROUPS	N	INOCULATION (IP)	TREATMENT (SC)	NO. W/ FEVER	ONSET (Days)	DURATION (Days)	NO. DEATHS	MEAN DAY TO DEATH
Zaire control	7	E-718 V_4	None	7	4.6	3.1	6	9.5
Sudan control	7	E-B-$G_1V_2M_1$	None	7	3.1	9.0	3	13.7
Z-ribavirin	7	E-718-V_4	20 mg ribavirin/kg b.i.d.	7	7.1	3.1	7	11.1
S-ribavirin	7	E-B-$G_1V_2M_1$		7	7.6	8.1	1	21.0
Z-poly(ICLC)[a]	7	E-718-V_4	2 mg poly(ICLC) b.i.d.	7	5.9	3.4	2	10.5
S-poly(ICLC)[a]	7	E-B-$G_1V_2M_1$		4	4.8	6.8	0	-
Controls								
Room	8	none	none	0	-	-	0	-
Ribavirin	4	none	20 mg ribavirin/kg b.i.d.	2	12.0	1.0	0	-
Poly(ICLC)	4	none	2 mg poly(ICLC)/kg b.i.d.	4	2.5	1.0	0	-

[a]Does not include the early 1-day fever associated with poly(ICLC) therapy.
[b]Low-grade fever, 104.7°F.

Publications:

None.

LITERATURE CITED

1. Report of a WHO/International Study Team. 1978. Ebola haemorrhagic fever in Sudan, 1976. Bull. WHO 56:247-270.

2. Report of an International Commission. 1978. Ebola haemorrhagic fever in Zaire, 1976. Bull. WHO 56:271-293.

3. Bowen, E. T. W., G. Lloyd, G. Platt, L. B. McArdell, P. A. Webb, and D. I. H. Simpson. 1978. Virological studies on a case of Ebola virus infection in man and in monkeys, pp. In Ebola Virus Haemorrhagic Fever (S. R. Pattyn, ed.) Elsiever North Holland Biomedical Press, Amsterdam.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6418	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	001
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code): (U) Effects of suppressor and helper T cell activities on the efficacy of 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
76 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 79	1.0	74.5
FISCAL YEAR CURRENT 80	1.0	77.4

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NAME: POC:DA

21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Immunization; (U) Immune response regulation during infection; (U) Tularemia

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 role of immunoregulatory functions (especially suppressor and helper T cells) during infection or vaccination. Identify these responses and apply them to optimizing immunity to BW agents.

24 (U) Develop techniques to quantitatively and qualitatively assay immunological regulatory functions in a model infectious system postvaccination. Examine and compare their effects in normal and vaccinated animals in an effort to identify major mechanisms influencing immunologic capacity of in vivo immunoregulatory phenomena to maximize immunizing effects of vaccines.

25 (U) 78 10 - 79 09 - The immunomodulatory effects of Francisella tularensis (LVS) on the ability to respond to other unrelated antigens (represented in these studies by the dependent antigen sheep RBC) are both varied and intense. Immune response mechanism studies appearing in the literature suggest that these effects can be produced by variations in antigenic dose. Bacterial titer of the spleen, liver and blood of LVS-infected mice showed maximal bacteremia during the initial stages of the suppressed period of heterologous immune induction. This observation correlates with the literature findings that high antigenic mass causes suppression. Experiments in which streptomycin treatment decreased bacterial titer show decreased suppression. A further set of experiments comparing IV and SC inoculation route effects on bacterial titer and suppression showed that suppression did not appear in IV inoculated mice while bacteria appeared sooner (day 1 vs. day 2) and proliferated to higher levels. These results suggest that a splenic population of cells, if activated by low doses sufficiently early, negates subsequent suppressive effects of later high bacterial titer in the spleen.

Publication: Fed. Proc. 38:1355, 1979.

*Available to contractors upon requestor's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 001: Effects of Suppressor and Helper T Cell Activities on the Efficacy of Immunization

Background:

Aside from the invasive properties of pathogenic intracellular bacteria, a less well characterized effect may be the ability of the bacteria to alter normal immunoregulatory processes. These processes depend on the amount of antigen being presented to critical immune subpopulations. The number of bacteria in an inoculum is relatively easily determined, but it is much more difficult to determine the dose "seen" by particular lymphoid tissues. The importance of these dose effects has been shown in studies on non-proliferating antigen. The effects of dose variation on antibody titer have long been known (1).

Recent research has shown that these effects are operative also at the level of cellular subpopulations (2). In fact it is clear that cellular communication via lymphokines and contact interactions at the cell surface are sensitive to dose phenomena.

In light of these observations, the major focal points for this year have been the study of the kinetics of bacterial proliferation and the correlation of bacterial mass with the ability of the immune system to respond to unrelated antigens during infection by an intracellular pathogenic bacterium.

Progress:

Bacterial recovery studies were performed in order to determine the extent and kinetics of bacterial proliferation in vivo. Both subcutaneous (SC) and intravenous (IV) routes were used in various experiments at an average dose of 1.5×10^3 bacteria/mouse. Organisms given by either inoculation route were recovered in high numbers in both spleen and liver, and in some blood samples. Their presence correlated well with the presence of high bacterial titers from spleen and liver, and illness evidenced in the appearance and behavior of the mice. Bacterial titer peaked on day 5 after inoculation, at titers ranging from 10^3-10^5/spleen among individual mice inoculated by either route. Bacterial recovery continued through day 9 in all animals. Approximately 1 of 6 mice continued to yield viable bacteria as late as day 22.

There were two differences in bacterial recovery data from the two routes. First, bacteria appeared in the spleen and liver on day 1 in IV inoculated mice while in SC inoculated mice bacteria appeared on day 2 or 3. The second difference was higher bacterial titer on day 5 in IV inoculated mice than in SC inoculated mice.

Pathologic differences between mice were also noted. Abdominal muscle and tissue involvement was observed in inguinal SC inoculation, but in IV inoculation. Statistically significant weight loss in SC inoculated mice was shown. Mice weighing 29 gm lost 2 to 3 gm by day 7 after SC inoculation, but not after IV inoculation. It is unknown whether this visceral involvement has a direct effect on the immune

response capability. However, the weight loss coincided with suppression of immune induction during infection.

Differences between IV and SC inoculated animals' ability to mount an immune response to unrelated antigens were also noted. The profile consisting of phase 1: augmentation; phase 2: suppression, and phase 3: augmentation; reported in SC inoculated mice does not appear in IV inoculated mice. Instead, a major augmentation occurs and is sustained throughout these time periods. The deletion of the suppressive phase may be due to one of the following causes: the effect of the visceral involvement in SC infection may affect immune induction by paralyzing a localized immune cell population or the time at which bacteria reach the spleen may be critical in order to mount a sufficient response to systemic infection. The more rapid buildup of bacteria within the spleen of SC inoculated mice may also affect the type and quality of the subsequent immune response.

The LVS strain of Francisella tularensis is susceptible to streptomycin treatment. Two types of streptomycin treatment have been used to limit bacterial proliferation in vivo, while studying immune induction ability. First, a treatment regimen immediately post-LVS inoculation was used. Second, treatment regimens were performed at various times after SC LVS inoculation to coincide with each of the 3 phases of altered immune response induction. Briefly, the results showed that the suppression phase could be deleted if early treatment was continued past day 10. Other phases were moderated. Treatment initiated at later times after LVS SC inoculation had little if any effect on the immune induction ability. Thus, while small numbers of bacteria were recovered from some treated mice, antigenic mass was diminished and suppression of immune induction was reversed.

Immune inductive capabilities during infection were compared in 2 inbred mouse strains. C3H/HeJ mice, when subjected to an identical SC inoculation protocol as AKR/J, showed higher levels of augmentation and greater suppression than the AKR/J group. Zinkernagel (3) has shown that severity of infections caused by Listeria are highly dependent on histocompatibility factors necessary for communication between macrophages and T regulatory cells. Other similarities between Listeria and F. tularensis infections have been noted. If the infections are similar in this regard, the mechanisms being elucidated in this study may give insights into genetic effects mediating virulence of infection.

Other work this year has established that, unlike numerous other Gram-negative bacteria, intact F. tularensis does not have a mitogenic property. Therefore, nonspecific stimulation of B cells need not be a concern in measuring numbers of specific antibody-forming cells in these studies.

A killed LVS preparation known as Foshay-type antigen has been used to test the effects of a constant number of bacteria on the immune induction potential. High doses produced early suppression of immune induction ability, while lower doses tended to augment this ability slightly. This suggests a similarity to immune induction data after SC inoculation of LVS.

Mixing experiments were also initiated this year, in which measured numbers of T cells or macrophages from infected mice were mixed in Mishell-Dutton culture with normal lymphoid cell populations. Sheep RBC were added, and the ability of the cultured cells to produce a primary immune response was measured. A change in degree of immune response to SRBC can be attributed to the particular cell type added. Results are inconclusive at this time.

Presentation:

Howell, H. M., and D. W. Seburn. Effects of streptomycin treatment on suppression of heterologous humoral immune response during tularemia. Presented, Annual Meeting, American Association of Immunologists, Atlanta, GA, 1-10 Apr 1979 (Fed. Proc. 38:1355, 1979).

Publications:

None.

LITERATURE CITED

1. Eisen, H. N. 1974. Immunology: An Introduction to Molecular and Cellular Principles of the Immune Responses. Harper and Rowe, Hagerstown.

2. Pierce, C. W., and J. A. Kapp. 1976. Regulation of immune responses by suppressor T cells, pp. 91-143. In Contemporary Topics in Immunobiology (William O. Wiegle, ed.); Vol. 5, Plenum Press, New York.

3. Zinkernagel, R. M. 1974. Restriction by the H-2 gene complex of the transfer of cell-mediated immunity to Listeria monocytogenes. Nature (Lond.) 251:230-233.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION: DA 0B6429	2. DATE OF SUMMARY: 79 10 01	REPORT CONTROL SYMBOL: DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	006
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Enzymatic and chemical alteration of microbial proteins for toxoid production

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	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 79	1.0	76.0
FISCAL YEAR CURRENT 80	1.0	73.9

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Amino acids; (U) Enzymes; (U) Toxoids; (U) Vaccines; (U) Staphylococcus; (U) Laboratory animals

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

23 (U) Prepare more effective immunogens against militarily important diseases in which proteins of bacterial and viral origin have significant biological effects. The immediate goal is the identification of the elements of the protein structures containing antigenic determinants and toxic sites. The enterotoxins produced by Staphylococcus aureus are now being studied. These toxins are potential agents for biological attack and are responsible for many outbreaks of food poisoning.

24 (U) Enzymatic modification of enterotoxins is the major modification mechanism. Enterotoxin C-1 (SEC-1) undergoes a rapid, limited digestion by trypsin. Studies are concerned with the chemical and physical characterization of the fragments and determination of their role in the serological, emetic, and mitogenic activity of the whole molecule.

25 (U) 78 10 - 79 09 - A comparison was made of the structure of staphylococcal enterotoxins A, B, and C-1 by means of their circular dichroic (CD) spectra. The CD of SEB and SEC-1 are very similar but differ from that of SEA. SEA retained most of the same bands with respect to both location and sign but with significant difference in intensity. It seems probable that all 3 enterotoxins fold in basically the same manner. As analyzed by CD models, the structures contain little alpha-helix. Investigation of the changes in the CD spectra brought about by tyrosyl ionization and chemical modification has identified some of the CD bands.

Publications: J. Biol. Chem. 253:8787-8791, 1978;
J. Immunol. 122:1285-1289, 1979;
Biochim. Biophys. Acta, in press, 1979.

* Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 006: Enzymatic and Chemical Alteration of Microbial Proteins for Toxoid Production

Background:

The staphylococcal enterotoxins are simple proteins elaborated by certain strains of Staphylococcus aureus which cause emesis and diarrhea in a limited number of mammalian species. All appear to consist of a single polypeptide chain with one disulfide bridge and to have a MW of ~ 28,000. Although enterotoxins A, B, and C do not cross-react in classical measurement of immunodiffusion or quantitative precipitin reaction, SEB and SEC_1 (1 of 2 isoelectrically different variants of SEC) do possess common antigenic determinants. One determinant is located in the first 57 amino acids and one in the last 150 residues of the polypeptide backbone. Enzymatic breakdown and physicochemical properties are being studied to define the secondary structure and to locate more precisely the chemical structure and location of the antigenic determinants.

Progress:

A manuscript describing the first phase of our circular dichroism (CD) studies on the enterotoxins has been accepted for publication. Although the CD of SEB (1) and SEC_1 (2) have been published separately, ours is the first systematic comparison of the staphylococcal enterotoxins in a manner where small differences could be detected with relative certainty. Moreover, we were able to attain much higher resolution data for the near ultraviolet CD of SEB than were Muñoz et al. (1) and to extend the far UV measurements 20-25 nm.

It is quite clear that these 3 antigenic variants of the staphylococcal enterotoxins are distinguishable from each other by their CD spectra. SEB differs from SEC_1 in the magnitude of positive bands seen at 235 nm and at 197-198 nm; SEA differs from both in many respects, with the complete absence of a positive band at 235 nm being the most striking divergence.

The similarities among these proteins are, however, equally impressive. For most of the aromatic region and in the major extremum in the far UV, the spectra for SEB and SEC_1 are virtually superimposable. Further, while the CD of SEA in the peptide region is indicative of substantial differences from SEB and SEC_1 in secondary folding, the retention of the location and sign of all the bands from 245-290 nm is surely suggestive of a similar environment of the aromatic residues in the tertiary structure. This resemblance is greater than that seen, for example, between hen egg white lysozyme and human leukemic lysozyme, which possess a 60% sequence homology and an identical secondary structure (3). Crystals suitable for X-ray diffraction studies have not yet been obtained for any of the staphylococcal enterotoxins, so that their 3-dimensional structures are not known. Nevertheless, it seems probable that they all fold in basically the same manner. They are elaborated by the same species of microorganism and they are all single polypeptide chain molecules of approximately the same number of amino acid residues containing one disulfide loop. Furthermore, they evoke the same biological response and have specific

activities of the same order of magnitude. Minimally, one would anticipate an identity of folding of that part of the molecule required to generate the biologically active combining site.

The close likeness between SEB and SEC_1 and the difference of both from SEA evidenced by CD spectra have been demonstrated also in serologic and biophysical studies. SEB and SEC_1 bound very well with each other's antibody (4) and 2 common antigenic determinants were found on tryptic peptides (5). However, neither SEB nor SEC_1 reacted with antibody to SEA, nor did SEA combine with antibody to SEB or SEC_1. Enterotoxins B and C_1 were much more stable to denaturant-induced unfolding (6). The rate constants for unfolding of SEB and SEC_1 were within a factor of 5, while both differed from the rate constant for SEA by a factor of ~ 50.

We were unable to achieve a satisfactory estimate of the enterotoxins' structure from a summation of reference spectra, but this is not unexpected in view of their low helical content. In this circumstance the inadequacy of the models for unordered conformation (7) becomes of increasing import. The contribution of β-turns (8) was neglected and it is noteworthy that a predictive method of secondary structure placed over 1/3 of the amino acid residues in SEB in turns. Both experimental observations (3) and calculated values (9) on model compounds indicate that the aromatic amino acids can make a significant contribution to the CD of proteins in the far UV. The elipticities are positive from 220-240 nm. When estimated by the procedures suggested by Sears and Beychok (3), these contributions to CD correspond to 69, 46 and 40% of the experimentally exhibited values of toxins A, B and C_1, respectively. This correction tends to increase the amount of helix estimated from the spectra and bring the CD results into better accord with the prediction from the amino acid sequence.

We previously attempted to define the CD bands of SEB and SEC_1 in the near-UV. Ionizing reversibly titrating tyrosyl residues resulted in the appearance of a strong new positive band at 248 nm and a weak negative band with an extremum at 295-298 nm. Nitration of these exposed tyrosyl residues with tetranitromethane gave derivatives with a positive CD band which titrated from pH 5-8. This band had a maximum ellipticity at 238 nm so that it was not possible to determine whether it arose from a shift in the intrinsic 235-nm band or from the same absorbing elements which were responsible for the alkali-generated band at 248 nm in the native enterotoxins. These investigations were continued with enterotoxin A. SEA could not be titrated to as high a pH as the other enterotoxins because a conformational change occurred above pH 10.3; fortunately a very strong change in ellipticity took place at this pH with the appearance of the same positive band at 248-249 nm seen with SEB and SEC_1. (The molar ellipticity was 600 compared to 450 for SEC_1 and 800 for SEB.) Since SEA does not present a positive band at 238 nm, this is strong evidence that the 248-nm band is not associated with those structural elements giving rise to the 238-nm band in SEB and SEC_1.

The weak negative band at 295-298 nm resulting from the alkalinization of SEB and SEC_1 did not appear with SEA; a weak negative band, however, did arise at 302 nm. This change does not correspond to either a tyrosyl or a tyrosylate absorption band and is probably not due to an exposed tyrosyl residue. SEA's small positive band at 292 nm was unaffected.

Reaction of SEA with tetranitromethane gave a product with 4-5 tyrosyl residues nitrated. Again, a strong positive band appeared on titrating from pH 5-8, but it was located at 247 nm a somewhat longer wavelength than the presumable identical

band in the other 2 nitrated enterotoxins. As with SEB and SEC_1 changes in the CD spectra in the 260-290-nm region were seen. Since the intensity of the bands in this region was not changed significantly by high pH with any of the unmodified enterotoxins, it is likely that these CD bands arise largely from buried tyrosyl residues. Therefore, although nitration is thought to alter only exposed tyrosyl residues, the environment of at least some of the other aromatic residues is undoubtedly modified. Continuing studies are concerned with modification of tryptophanyl residues and resolution of the near-UV spectra into Gaussian curves to identify the CD bands and calculate their rotational strength.

We have attempted to localize further the antigenic determinants of SEB; the approach selected was to block the lysyl residues so that only the 5 arginyl residues would be susceptible to tryptic digestion. A derivative obtained by reaction with citraconic anhydride was unstable and one obtained with methyl acetimidate was insoluble. A derivative of SEB was prepared by reaction with maleic anhydride in which all of the free amino groups were apparently blocked. Reaction with trinitrobenzene sulfonic acid gave no color. However, when digested with trypsin, SDS polyacrylamide gels in 8 M urea disclosed that by 80 min the hydrolysis had proceeded well beyond the stage anticipated for cleavage of only arginyl peptide bonds. It was possible to demonstrate that the derivative was stable under the conditions employed so it seems likely that some of the ε-NH_2 groups of the lysine residues were not acylated. The derivative was also prepared in the presence of 6 M guanidine HCl, but no differences were observed when it was treated with the enzyme. Steric hindrance is the probable explanation; it may be significant that SEB contains several amino acid segments in its sequence with contiguous lysine residues.

It seems clear that complete digestion of maleylated SEB will not be a fruitful approach. Partial hydrolysis, however, may enable us to obtain suitable polypeptide fragments. After 10 min, a polypeptide with an apparent MW between 15,000 and 20,000 was present in respectable yield. Another option that will be pursued is digestion by enzymes reported to be specific for arginyl bonds. Two such enzymes have been described, one from Clostridium histolyticum and one from the submaxillary gland of mice; both are available commercially.

Presentations:

None.

Publications:

1. Spero, L., and B. A. Morlock. 1978. Biological activities of the peptides of staphylococcal enterotoxin C formed by limited tryptic hydrolysis. J. Biol. Chem. 253:8787-8791.

2. Spero, L., and B. A. Morlock. 1979. Cross-reactions between tryptic polypeptides of staphylococcal enterotoxins B and C. J. Immunol. 122:1285-1289.

3. Middlebrook, J. L., L. Spero, and P. Argos. 1979. The secondary structure of staphylococcal enterotoxins A, B and C. Biochim. Biophys. Acta, in press.

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9. Woody, R. W. 1978. Aromatic side-chain contributions to the far ultraviolet circular dichroism of peptides and proteins. Biopolymers 17:1451-1467.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0A6414	79 10 01	DD-DR&E(AR)636

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

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	007
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

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 79	1.0	364.0
	CURRENT 80	1.0	288.6

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21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Military medicine; (U) BW defense; (U) Amino acids; (U) Protein synthesis; (U) RNA synthesis; (U) Volunteers; (U) Gluconeogenesis

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

23 (U) Changes in the concentrations and pattern of amino acids in blood and tissues during various infectious diseases will be correlated with alterations in RNA, protein and carbohydrate metabolism. The data obtained will be utilized to develop nutrient therapy to prevent the body wasting of convalescence for these illnesses. In addition, alterations in the concentration or ratio of some blood amino acids may be a useful biochemical tool in the early detection of infectious diseases that pose a potential BW threat to this country. Studies are done in rodents, monkeys, and man.

24 (U) Free amino acid concentrations are determined by ion-exchange chromatography in plasma and tissue of experimental subjects and animals infected with bacterial or viral organisms. Radioactive, nonmetabolizable and metabolizable metabolites will be utilized to study amino acid flux RNA and protein synthesis, glucogenesis, glucose turnover and oxidation, and rates of total body catabolism.

25 (U) 78 10 - 79 09 - Marked loss of body protein in the infected host can be prevented by intravenous or oral infusion of amino acid and/or carbohydrates and/or lipid. The septic host can utilize infused lipid even in the presence of high plasma insulin concentrations. Most of the infection-related increase in urinary nitrogen comes from the utilization of amino acids from degradation of proteins of skeletal muscle and connective tissue for energy requirements and synthesis of glucose. Increase in the infusion of branched-chain amino acids reduces protein breakdown in the infected host.

Publications: In Proc., Western Hemisphere Nutr. Cong. V, pp. 202-209, 1978; J. Parent. Enteral Nutr. 2:507-518, 1978, 3:22, 1979; Fed. Proc. 38:429, 354, 764, 1979; Clin. Res. 27:225A, 557A, 1979; Am. J. Clin. Nutr. 32:936, xx, 1979.

*Available to contractors upon originator's approval

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

* U.S. GPO 1973—540-947/8691

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 007: Therapeutic Reversal of Abnormal Host Amino Acid, Protein and RNA Metabolism during Infectious Disease of Unique Military Importance

Background:

Marked losses of body protein are characteristic of infectious disease (1). This loss of body nitrogen is associated with an increased breakdown of skeletal muscle and connective tissue to supply amino acid for a source of energy, substrates for glucose synthesis and anabolic processes associated with host defense and maintenance of hemostasis during periods of reduced food intake (2, 3). Models have been developed in rats and monkeys to evaluate the interaction between substrate supply and wasting of body protein (3). Even a mild, 2-3 day infectious illness can result in severe loss of body protein; it will take 2-3 weeks to return to original work capacity (1). Thus, a therapeutic procedure which could prevent the wasting of body protein and reduce the time of recovery would be of value to a troop commander whose personnel were exposed to BW attack or had to enter an epidemic area.

Progress:

Utilization of lipid calories during sepsis in the parenteral nutrition model in rhesus monkey: Utilizing the parenteral nutrition monkey model, it has been reported previously (3) that during pneumococcal sepsis in monkeys infused with the amino acids (AA) alone there was lost 2.6 $\pm$ 0.5 gm of nitrogen/kg/day or 12.8 $\pm$ 2.7% of their total body proteins over the 6-day experimental period. In contrast, the addition of dextrose or lipid calories (85 cal/kg/day) to the amino acid infusion prevented this loss of body protein during pneumococcal sepsis. Thus, it was concluded that lipid calories with AA could be effectively utilized as an energy substrate during pneumococcal sepsis in the rhesus monkey. These observations did not support the conclusions of Long et al. (4) that lipid calories were not utilized as a source of energy during sepsis in burned patients. Since these investigators infused a combined dextrose and lipid mixture as a source of calories, the possibility was raised that dextrose would increase peripheral insulin concentrations which in turn might inhibit effective utilization of the lipid calories. To test this possibility, monkeys were infused with a mixture of AA + dextrose which supplied only 1/3 of the optimum amount of calories (32 cal/kg/day) during pneumococcal sepsis. With this nutritional regimen, septic monkeys lost 6.8 $\pm$ 1.0% of their body protein over the 6-day experimental period, and plasma insulin was increased 5-10-fold when compared to monkeys receiving AA infusion alone. The addition of 55 cal/kg/day of lipid emulsion to this AA-dextrose solution prevented the wasting of body proteins during pneumococcal sepsis, even though plasma insulin were markedly elevated.

Since approximately 13% of the calories in lipid emulsion were from glycerol, an equivalent amount of this glucose precursor was added to the AA-dextrose mixture to supply approximately 9 cal/kg/day. When infused with this mixture, septic monkeys lost 6.5 $\pm$ 0.5% of their body protein over the 6-day experimental period. This rate of loss of body protein was not significantly different from that observed when

the septic monkeys were infused with the amino acids and 1/3 calorie requirements as dextrose alone. Thus, it can be concluded that the addition of glycerol calories equivalent to that found in the lipid emulsion did not prevent the wasting of body protein during pneumococcal sepsis. Therefore, these observations would indicate that during pneumococcal sepsis the rhesus monkey was able to utilize the lipid calories as a source of energy even in the presence of excess amounts of plasma insulin. Future studies will be geared to determine whether the monkey can utilize lipid calories during a Gram-negative sepsis.

When noninfected monkeys were infused with AA solution alone, they lost approximately 2.3 $\pm$ 0.7% of their body protein over the 6-day experimental period. The addition of 32 cal/kg/day of dextrose prevented this loss of body protein in the noninfected monkey. This suggests that the chaired rhesus monkey would require approximately 0.55 gm of amino acid N and 50 cal (from AA and dextrose)/kg/day to meet its maintenance requirements. In contrast, the septic monkey requires equivalent amounts of amino acid N and almost 100 cal/kg/day to meet its maintenance requirements. This suggests that pneumococcal sepsis imposes an almost 2-fold increase in energy requirements as compared to its noninfected control.

Protein degradation in skeletal muscle and connective tissue during pneumococcal sepsis in rhesus monkey. It has been hypothesized that during calorie-deprivation the host breaks down proteins of skeletal muscle and connective tissue at an increased rate in order to meet this caloric and AA requirement (2). An important unresolved question is whether the nutritional support therapy will spare skeletal muscle and connective tissue protein. In an attempt to answer this question, urinary 3-methylhistidine (3-MeH) and hydroxyproline were utilized to monitor rates of degradation of skeletal muscle and connective tissue during pneumococcal sepsis in the rhesus monkey.

When monkeys exposed to virulent pneumococci were infused with AA (0.55 gm N/kg/day) alone, they went into marked negative balance, which was associated with a significant increase in urinary excretion of both 3-MeH and hydroxyproline. In contrast, control monkeys given the heat-killed pneumococci were maintained in a slight negative balance; there was a small but significant decrease in urinary 3-MeH and no change in hydroxyproline. The addition of 32 cal/kg/day of dextrose reduced the magnitude of the negative balance and elevation in urinary 3-MeH and hydroxyproline in the septic monkey. With this amount of nutritional support the control monkey remained in N equilibrium and no significant changes were observed in rate of urinary excretion of 3-MeH and hydroxyproline. When dextrose calories were increased to 85 cal/kg/day, the septic monkey went into only a slight negative balance and had a small but significant increase in excretions of 3-MeH, with no change in hydroxyproline. The noninfected control monkey was in positive balance and had no change in rate of excretion from urinary 3-MeH or hydroxyproline. The addition of 85 cal/kg/day of lipid emulsion also reduced N loss and had a slight but significant elevation in excretion of urinary 3-MeH in the septic monkey. With this nutritional support, the control monkey remained in positive balance and had no significant alterations in rate of excretion of urinary 3-MeH and hydroxyproline.

The comparative contribution of skeletal muscle and connective tissue protein to the total N loss was estimated during clinical illness in the septic monkey. The nitrogen loss over the 6-day period is the difference between that observed in the infected and that in the control monkey. Even though monkeys receiving 85 kg/day of dextrose + AA were in positive balance over the 6-day experimental period,

their gain in N was less than that observed in the controls; therefore, the excess was considered to be a N loss due to sepsis. If it is assumed that the value for day 1 represents basal rates of excretion of urinary 3-MeH or hydroxyproline, it is possible to calculate the increase in excretion rates of these 2 urinary amino acids as a result of sepsis. In most species skeletal muscle protein contains approximately 4 μmol 3-MeH/gm and contains 16% nitrogen. From these assumptions, it can be calculated that skeletal muscle contributes 80, 82, 43, 71% of the total N which is lost during sepsis when the monkeys are infused with amino acids alone, with AA + 32 cal/kg/day of dextrose, 85 cal/kg/day of dextrose, or 85 cal/kg/day of lipid. From the literature values in man, it can be calculated that every 10 μmol of urinary hydroxyproline represents a degradation of approximately 1 mg of connective tissue protein, which is 16% N. Utilizing these calculations, it can be shown that approximately 10% of N which was lost during sepsis in the monkey infused with the AA or AA with a similar amount of dextrose came from connective tissue. Even though increased caloric infusion reduced nitrogen wasting in the septic host, the major losses that did occur came from the breakdown of skeletal muscle. Thus, increasing the caloric intake while keeping the amino acid infusion constant reduced the loss of total body, skeletal muscle and connective tissue protein during sepsis. However, during illness as a result of pneumococcal sepsis the caloric requirements appeared to exceed 100 cal/day, thus explaining the slight loss of body and skeletal muscle protein in the high dextrose- and lipid-infused group. It can also be hypothesized that the major increases in protein catabolism which occur during sepsis are to meet the elevated caloric requirements of the host.

Protein sparing effects of IV administered branched-chain amino acids (BCAA) during pneumococcal sepsis in cynomolgus monkeys. It has been postulated that during calorie-protein deprivation, the severely stressed host breaks down body protein at an increased rate and utilizes amino acids to meet some of its caloric requirements. The major sources of endogenous AA during sepsis and trauma appear to be skeletal muscle and connective tissue. Further, under these conditions the branched-chain amino acids (leucine, isoleucine and valine) appear to be the major energy substrate for tissues, such as skeletal muscle. Thus, it has been postulated that increasing the endogenous supply of BCAA could spare body protein during sepsis and trauma.

To test this concept, a previously described parenteral nutritional model in the monkey (3) was utilized to evaluate the protein-sparing effects of BCAA during pneumococcal sepsis. After chair-adaptation of cynomolgus monkeys, catheters were implanted in the jugular and femoral veins and carotid arteries. On the morning after surgery monkeys were infused via the jugular vein with solutions that contained either 24 or 48% branched-chain amino acids. The 24% BCAA mixture was a standard FreAmine II formulation, which was infused at the rate of 0.54 gm N/kg/day and supplied approximately 13 cal/kg/day. For the 48% mixture, the concentration of isoleucine, leucine and valine were all increased with the resultant decrease in the other essential and nonessential AA in the FreAmine mixture. The solution was infused at the rate of 0.52 gm N/kg/day. Both solutions supplied equivalent amounts of electrolytes, trace elements and vitamins. Daily blood samples and complete urine and fecal collections were made throughout the study. On day 1 after the start of the nutritional support, the monkeys received an IV injection via the femoral vein of 3×10^8 live or heat-killed Streptococcus pneumoniae. The monkeys given the live organisms rapidly became febrile and all were septicemic by day 2. At this time the monkeys were treated with antibiotics for the next 4 days.

The control monkeys, which were injected with heat-killed pneumococci, did not develop fever, clinical illness, or septicemia. In both 24% and 48% BCAA groups of control monkeys, the urinary N excretion slightly exceeded intake, which resulted in a negative balance. The 48% group tended to excrete slightly less urinary N but the difference was not significant. Both groups of monkeys excreted approximately 1.5-2 mmol/kg/day of β-hydroxybutyrate, indicating that they had developed ketonemia as a result of the reduced caloric intake.

In the monkeys that developed pneumococcal septicemia, a prompt febrile response was observed in both 24 and 48% groups, which was quickly reduced with antibiotic treatment. As reported previously, pneumococcal sepsis in the monkeys infused with a 24% BCAA resulted in a marked increase in urinary N excretion, which remained elevated throughout the study. Urinary N excretion was also elevated in septic monkeys infused with 48% BCAA but the magnitude of the increase was significantly less than that observed in the monkeys in the 24% group. In both dietary groups ketonemia was observed prior to exposure to pneumococci, but there was a rapid decrease in β-hydroxybutyrate secretion with the onset of sepsis, which is in agreement with previously observed inhibition of starvation ketosis during infectious disease (3). The infected monkeys which were infused with 24% BCAA mixture lost approximately 10% of their body protein over the 6-day experimental period, while those infused with the 48% BCAA lost only 4.8% of their body protein. This is a significant reduction in N loss in the monkeys infused with the higher BCAA mixture. In control monkeys, the 24% group lost 2.7% of their body protein while those given the 48% mixture lost 1.4% of their body protein, which does not represent a significant difference. These data indicate that increasing the BCAA content during pneumococcal sepsis in calorie-deprived monkeys represented a true sparing of body protein. As reported by others in man and rats, increasing the BCAA content of the amino acid mixture did not have a marked protein-sparing effect during calorie deprivation in the nonstressed host.

To determine whether the branched-chain amino acids were sparing skeletal muscle and connective tissue protein, urinary 3-MeH and hydroxyproline were utilized to monitor rates of degradation of skeletal muscle and connective tissue. A negative N balance was observed in control monkeys infused with either 24 or 48% BCAA. The excretion of urinary 3-MeH and hydroxyproline was essentially constant throughout the study and no significant differences were observed between the control monkeys infused with 24 or 48% BCAA solution. In contrast, the infected monkeys in the 24% group were in a more severe negative balance than those infused with the 48% BCAA solution. In both dietary groups, urinary 3-MeH was significantly increased with onset of sepsis. But the magnitude of response was significantly less in those monkeys infused with the 48% as compared to 24% BCAA mixture. Urinary hydroxyproline was also significantly increased with the onset of sepsis but no significant differences were observed between the 2 dietary groups.

If it is assumed that the day-1 preexposure values represent basal rates of excretion of urinary nitrogen, 3-MeH or hydroxyproline, it is possible to calculate the increase in excretion rates of these urinary metabolites as a result of sepsis. As observed earlier, protein loss during pneumococcal sepsis in the monkeys infused with 24% BCAA mixture was significantly greater than that observed in the 48% mixture.

In most species the skeletal muscle contains approximately 4 μmol of 3-MeH/gm of protein containing 16% N. From these assumptions, it can be calculated that skeletal muscle in the septic monkey infused with 24% BCAA solution lost significantly more protein than muscle of the monkeys infused with the 48% mixture. However, in

both dietary solutions skeletal muscle contributed approximately 80% of the total protein which was lost as a result of sepsis.

From the literature values in man, it can be calculated that every 150 μmol of urinary hydroxyprotein represent degradation of approximately 1 gm of connective tissue, which is 16% nitrogen. Utilizing these calculations it can be shown that similar amounts of connective tissue protein were broken down in the septic monkeys infused with either 24 or 48% BCAA mixtures. However, the connective tissue contributed approximately 12% of the total protein lost by monkeys infused with the 48% mixture as compared to 6.6% in those infused with 24% BCAA mixtures.

These data suggest that increasing the BCAA spared skeletal muscle protein but not connective tissue protein in the septic monkeys. However, a marked hypoglycemia developed in the septic monkeys infused with the 48% BCAA mixture. This effect was not observed in the septic monkeys infused with the 24% mixture or in the control monkeys infused with the 48% BCAA mixture. A similar transient increase in plasma insulin on day 1 of the infection and marked increase in plasma glucagon was observed in both dietary groups. This suggested that the hypoglycemia observed in the septic monkeys infused with the 48% BCAA mixture was not related to changes in the glucoregulatory hormones but might be due in part to a reduced supply of gluconeogenic substrates in these monkeys. A slight decrease in plasma glucose has also been reported during therapeutic fast in patients given a higher BCAA solution.

Glucose homeostasis during IV alimentation in control and septic monkeys. Sepsis is usually associated with a marked wasting of body protein. It has been hypothesized that during calorie deprivation the septic host breaks down proteins at an increased rate in order to meet its caloric and AA requirements. Recent studies in man and experimental animals have indicated that IV nutritional support spares protein during sepsis and/or trauma. In an attempt to study further the relationship between the rate of breakdown of body proteins and caloric requirements, a parenteral nutritional model (3) was developed for use during pneumococcal sepsis in the rhesus monkey. A constant infusion of radiolabeled glucose and alanine were utilized to measure the kinetics of these 2 metabolites during sepsis and varying nutrient support. The present studies also investigated the effects of these 2 variables on the circulating concentrations of glucoregulatory hormones, insulin and glucagon.

On day 2 after inoculation of S. pneumoniae and on the last day of the studies, the monkeys received a constant infusion of [^{3}H]glucose and [^{14}C]alanine. Periodic blood samples were obtained over the next 3 hr and were analyzed for specific activity of [^{3}H]- and [^{14}C]glucose, as well as [^{14}C]alanine. These data were utilized to calculate glucose and alanine kinetics during pneumococcal sepsis in the monkey.

When infused with the AA alone, glucose production and utilization were significantly elevated during both illness and early recovery stages of pneumococcal sepsis in monkey as compared to the nonfebrile control. When the monkeys were infused with the AA + dextrose, glucose utilization was similar in both the septic and control monkeys and was approximately equal to that seen during illness in septic monkeys infused with AA alone. Glucose production, however, was almost completely abolished from the infused dextrose except for a slight amount of exogenous production during illness in the septic monkey. Thus, this intake of dextrose appears to meet the caloric requirements in the monkey except for the acute illness phase of the infection. Glucose production and utilization was significantly reduced in the monkeys infused with AA + lipid compared to AA alone. However, both parameters were

elevated above those of heat-killed controls during the illness phase of the infection. Thus, both septic and nonseptic monkeys were able to utilize the infused lipid as a source of calories.

When the septic monkey was infused with amino acids alone, alanine production and utilization were significantly increased when compared to heat-killed controls. Also, there was an increased rate of conversion of the labeled alanine to glucose during the illness phase of pneumococcal sepsis. These data suggest that the increased glucose production in the calorie-deprived septic monkey is the result of an elevated supply of gluconeogenic substrates and a capacity of the liver to synthesize glucose.

The rates of alanine production and utilization were significantly decreased when the monkeys were infused with AA + dextrose and production was approximately equal to infused AA content. However, there was a slight but significant increase in these parameters during the illness phase of pneumococcal sepsis. Despite the reduction in alanine production, the gluconeogenic capacity of the liver was significantly elevated in the septic monkey.

The rate of alanine production and utilization in monkeys infused with AA + lipids was greater than that observed in the monkeys receiving dextrose calories. Again, these parameters increased during the illness phase of pneumococcal sepsis, as did the gluconeogenic capacity of the liver. Thus, the nutrient infusion appears to influence glucose and alanine kinetics in both the septic and nonseptic monkeys, but did not appear to influence the infection-related elevation in gluconeogenic capacity of the liver. Therefore, these studies included the effects of these 2 variables on circulating concentration of glucoregulatory hormones, insulin and glucagon.

In the monkeys infused with AA alone, there was a slight, but not significant, decrease in glucose concentration in the septic monkey, while insulin was significantly elevated on day 1 of the infection. In contrast, glucagon was progressively elevated throughout the study in the septic monkey as compared to the heat-killed control. In monkeys infused with AA + dextrose, glucose concentration was slightly elevated in the septic monkey as compared to the control. Both septic and nonseptic monkey showed 10-fold elevations in plasma insulin, with no significant differences between the groups. As noted in the septic monkeys receiving the AA alone, plasma glucagon was significantly elevated throughout the study. In the monkeys infused with the AA + lipid, plasma insulin was slightly elevated in both the septic and control monkeys, while plasma glucagon, as seen previously, was elevated in the septic monkey. The elevated plasma glucagon could explain high gluconeogenic capacity of the liver which was observed during pneumococcal sepsis. It could also explain why the dextrose infusion did not suppress the gluconeogenic capacity of the liver in the septic monkey.

Enteral nutritional model in the monkey. In clinical medicine, it is general practice to utilize an enteral nutrition, if the patient has a functional gastrointestinal tract. This procedure has the advantage of obviating catheterization of a central venous vein, thus reducing the chance of catheter sepsis. Therefore, it was of interest to see whether a similar model could be developed in the monkey to evaluate the supply of substrates via the oral route.

Cynomolgus monkeys, weighing 3-5 gm, were adapted to restraint in metabolic chairs for 3 days. The chairs were designed to allow quantitative collection of fecal samples. During the 3-day adaptation the monkeys were fed ad libitum and had free access

to water. Only those monkeys seen to be eating normally, maintaining their body weight, having normal white blood count and clinical chemistry, and showing no tendencies for excessive hyperactivity were accepted into the study. After adaptation to restraint, an indwelling catheter was placed in the right carotid artery and femoral vein. A mercury tip, 7.3 French, nasogastric tube was inserted into the lower stomach and utilized for the infusion of nutrient solution. On the day after surgery the monkeys were infused with a solution of crystalline AA, complex carbohydrates, and lipid at the rate of 125 ml/kg/day.

Nitrogen balance was maintained in 4 monkeys with pneumococcal sepsis and 2 controls receiving heat-killed organisms. Control monkeys exhibited slight diarrhea but no additional complications. Three out of 4 of the infected monkeys developed reduced rates of gastric emptying, apparent decreased intestinal mobility, and some regurgitation of the infused solution. One monkey developed a very severe regurgitation and died as a result of inhalation of the vomitus. The difficulties with emptying of the stomach and intestinal mobility disappeared when fever lysed in the other 3 monkeys. The hyperosmotic (645 mOsm) solution may have accounted for its reduced acceptability in septic monkeys. Because of the higher osmotic content of the solutions containing crystalline AA and complex carbohydrates, studies were iniated with commercially available sodium caseinate solutions containing both carbohydrate and lipid calories. Initially studies were done with Isocal but difficulties were encountered because of the high lactose content of this preparation. Because of these difficulties, a second solution, Osmolite, which is only 300 mOsm and contains no lactose was evaluated in control and septic cynomolgus monkeys. This solution was well accepted by the monkeys, but difficulty developed with the coagulation of the protein when it was allowed to remain at room temperature for more than 12 hr. To overcome this difficulty, monkeys were fed the total daily caloric and protein requirements over a 12-hr period. By this technique it was found that if the monkeys were adapted during the 3-day chairing period to the solution, both septic and control monkeys could utilize the solution. Currently, the adaptation procedure is being developed and it is hoped that this technique can be utilized to evaluate the use of oral nutrient support to spare body protein during sepsis.

Effect of substrate support during yellow fever (YF) in rhesus monkeys. One of the manifestations of a number of the viral diseases which are of interest at USAMRIID is the marked hepatocellular damage, associated with these infections. In order to develop some insight into the possible therapeutic management of this type of viral disease, studies were initiated to determine the effects of nutrient support during lethal YF in the rhesus monkey. This viral disease causes severe hepatocellular damage during the terminal stages of the infection.

Two rhesus monkeys were adapted to restraint in metabolic chairs for 4 days. The chairs were designed to allow quantitative collection of urine and fecal samples. During this period the monkeys were fed ad libitum and had free access to water. After adaptation to restraint in the metabolic chairs, indwelling catheters were placed in the right jugular and femoral veins. After surgery, the monkeys were returned to the metabolic chairs and the catheters were connected to 0.5% saline which contained 4.5 U/ml of heparin and were infused at a flow rate of 0.5 ml/hr. On day 1 after surgery, the monkeys were infused with a solution that supplied 0.55 gm of amino acid N and 85 cal/kg/day in dextrose via the jugular vein catheter.

On day 1 after starting the infusion of the nutrient solution, the monkeys were injected SC with approximately 10 PFU of the monkey-adapted Asibi strain of YF virus.

Blood, urine and fecal samples were collected for the next 5 days. Both monkeys became viremic on day 2 and febrile by day 3. Marked decreases in circulating granulocytes and platelets were observed by day 4 and the monkeys died 110-120 hr postinoculation.

Blood glucose concentrations increased to 300-600 mg/dl in samples obtained late on day 4 and early on day 5. Serum Fe was initially depressed but markedly increased during the latter stages of the infection. Total blood AA were depressed through day 4 but were elevated 2-3-fold during the next 24 hr. These increases were most marked in the nonessential AA and were even seen in those amino acids which were not present in the infusion medium. The BCAA were markedly decreased through day 4 and then rose to preinfusion concentrations. Arginine tended to decrease in the terminal samples while ornithine was elevated. This suggested that arginase was being released either as a result of granulocyte destruction or hepatic damage.

It had been postulated previously that during YF the hepatic damage shut down gluconeogenesis, which led to hypoglycemia and death due to energy deprivation of cells, such as the brain. The current studies did not support this conclusion but rather suggested that the virus or some other toxic product was blocking the cells' utilization of energy substrates with a resultant buildup in the blood as a result of the exogenous supply of substrates.

Since the monkey-adapted Asibi strain of YF is 100% lethal at < 1 PFU in rhesus monkeys, it is difficult to assess the value of anti-viral and/or nutrient support with this model. Therefore, attempts will be made to see if another strain of YF or a different species of nonhuman primate could produce a model which had a lower degree of lethality.

Substrate support therapy during YF virus infection in bonnet monkeys. Studies were initiated in bonnet monkeys which were either adapted to chair restraint and infused with amino acid-dextrose solution or maintained in normal monkey cages and fed monkey chow.

One monkey was adapted to chair restraint and had indwelling catheters placed in the right jugular and femoral veins. One day after surgery, the monkey was infused with a solution that supplied 0.55 gm of amino acid N and 85 cal/kg/day of dextrose via the jugular vein catheter. The second monkey was maintained in a cage and allowed access ad libitum to water and monkey chow. One day after initiation of IV alimentation in the chaired monkey, both monkeys were inoculated with 20 PFU of the monkey adapted Asibi strain of yellow fever virus.

By day 4 after exposure to the virus the caged monkey had a viremia of 7.5×10^9/ml, a subnormal body temperature and died 8 hr later. In contrast, on day 4 postexposure the monkey maintained by IV alimentation had a viremia of 2×10^7/ml, and was still alive at 24 hr. By day 5 the viremia had increased to 1×10^9/ml, body temperature was subnormal, and the monkey died 12 hr later. Thus, the preliminary studies suggest that IV alimentation with AA and calories delayed onset of viremia and death as compared to the monkey maintained in the cage. Additional studies are planned to confirm these observations. Studies are also planned to initiate antiviral therapy in combination with IV nutrient support when the monkey first demonstrates a rise in body temperature in the presence of viremia, approximately day 3 postexposure.

Plasma glucose was initially elevated 100 hr after exposure to the virus and reached values of almost 1500 mg/dl during the terminal stages in the monkey infused with the AA + glucose solution. Plasma AA were also elevated during the terminal stages in the monkey receiving hyperalimentation solution and the one maintained in the cage. As reported previously, the infusion of high dextrose solution results in a 10-fold increase in plasma insulin, but 1 day after exposure to the YF virus plasma insulin rose to > 1000 μU/ml and remained elevated for the next day, dropping down and reaching values of almost 600 U/ml at the terminal stages of the infection. This marked increase in plasma insulin was observed before the onset of fever or viremia in the monkey receiving IV alimentation. These data support the conclusion that during terminal stages of YF the monkey is unable to utilize substrates from endogenous or exogenous sources. It was of interest that total plasma AA in the chaired monkey were elevated to almost the same concentrations as those observed in the one receiving IV support. This suggested that there was a marked breakdown of body protein and release into circulation of AA in the caged monkey ill with yellow fever.

The marked elevations in plasma insulin were observed very early after exposure to the YF virus and suggested a direct effect on the beta cells of the pancreas during this infection. Future studies will be geared to confirm this observation and to determine if plasma insulin concentrations are also elevated in the caged monkey exposed to the YF.

African green monkey - model for yellow fever infection. Because of the poor supply of rhesus and bonnet monkeys, an alternative species of monkey was sought to continue the studies. The African green monkey is currently in abundant supply and is of suitable size to be evaluated as a possible candidate to study metabolism during YF. The first monkey tested weighed 7.0 kg and was inoculated with 150 PFU of the monkey-adapted Asibi strain of YF virus. This monkey was maintained in a cage with food and water supplied ad libitum. The monkey developed a fever by day 4 which subsided and was followed by a marked elevation in body temperature by day 8-10. The monkey developed viremia on day 2, reached a maximum of 2×10^5 PFU/ml on day 4 and was negative by day 7. A slight leukopenia developed on day 3 and was present through day 9 of the study. Despite a temperature of almost 105°F, the monkey survived the infection and started to develop neutralizing antibody titers by day 10, which reached very high values by day 28.

The second African green monkey received an SC inoculation of 6 PFU of the same strain of YF virus. The monkey had a mild increase in body temperature on day 5 and a subsequent severe elevation in body temperature to 105.7°F on day 10. This monkey developed viremia on day 3 with a maximum of 1.2×10^5 PFU on day 4. Marked leukopenia developed on day 5 and was present through day 13. Despite a very high body temperature, the monkey survived, and antibody titers are currently being determined.

Preliminary data suggests that the African green monkey is susceptible to the YF virus infection and develops an acute nonlethal illness. There is some suggestion of a possible dose response to this virus in this species. Thus, the course of infection following exposure in the African green monkey is closer to that seen in man, which means that it may be a very useful model for studying the metabolic response and effectiveness of nutrient support and antiviral therapy.

Presentations:

1. Wannemacher, Jr., R. W. Alterations in protein metabolism during injury and sepsis. Presented, Texas Nutrition Conference, Texas A&M, College Station, TX, 5-6 Oct 1978 (Proc., pp. 164-165, 1978).

2. Wannemacher, Jr., R. W. Key role of individual amino acids in host response to infection. Presented, Post-graduate Symposium on Impact of Infection on Nutritional Status of the Host, Poughkeepsie, NY, 21 Oct 1978.

3. Wannemacher, Jr., R. W., C. L. Hadick, Jr., and W. R. Beisel. Nutrition and infection interrelationships in monkeys. Presented, Workshop on Primates in Nutritional Research. Regional Primate Research Center, Beaverton, OR, 23-24 Oct 1978.

4. Wannemacher, Jr., R. W., M. V. Kaminski, R. E. Dinterman, and T. R. McCabe. Use of lipid calories during pneumococcal sepsis in the rhesus monkey. Presented, 3rd Clinical Congress of American Society of Enteral and Parenteral Nutrition, Boston, MA, 30 Jan-3 Feb 1979 (J. Parenteral Enteral Nutr. 3:22, 1979).

5. Wannemacher, Jr., R. W., R. E. Dinterman, and T. R. McCabe, Jr. Protein degradation in skeletal muscle (SM) and connective tissue (CT) during pneumococcal sepsis in the rhesus monkey. Presented, Annual Meeting FASEB, Dallas, TX, 1-10 Apr 1979 (Fed. Proc. 38:764, 1979).

6. Wannemacher, Jr., R. W., R. E. Dinterman, B. R. Bistrian, and G. L. Blackburn. Protein-sparing effects of intravenously administered branched-chain amino acids (BCAA) during pneumococcal sepsis in the cynomolgus monkey. Presented, American Society of Clinical Nutrition, Washington, DC, 4-5 May 1979 (Am. J. Clin. Nutr. 32:936; Clin. Res. 27:557a, 1979).

7. Wannemacher, Jr., R. W., R. E. Dinterman, and T. R. McCabe, Jr. Glucose homeostasis during intravenous alimentation in control and septic monkeys (M). Presented, 3rd Joint Meeting, American Institute of Nutrition, American Society of Clinical Nutrition, and The Nutrition Society, Guelph, Ontario, 5-7 Jul 1979 (Am. J. Clin. Nutr. 32:xx, 1979).

8. Wannemacher, Jr., R. W., R. E. Dinterman, K. D. Burman, and L. Wartofsky. Thyroid hormone: a regulator of rates of protein degradation in skeletal muscle. Presented, XIth International Congress of Biochemists, Toronto, 8-13 Jul 1979. (Abstracts, p. 622, 1979).

Publications:

1. Wannemacher, Jr., R. W. 1978. Basic changes in protein metabolism during stress, pp. 202-209. In Western Hemisphere Nutrition Congress V (P. L. White and L. Selvey, eds). American Medical Association, Chicago.

2. Wannemacher, Jr., R. W., M. V. Kaminski, Jr., H. A. Neufeld, R. E. Dinterman, K. A. Bostian, and C. L. Hadick. 1978. Protein-sparing therapy during pneumococcal sepsis in rhesus monkeys. J. Parent. Enteral Nutr. 2:507-518.

3. Bistrian, B. R., D. T. George, G. L. Blackgurn, and R. Wannemacher. 1979. Metabolic response to yellow fever immunization (YFI) during a modified fast + 100g glucose. Clin. Res. 27:225A.

4. Ferrara, E. A., R. W. Wannemacher, Jr., and E. L. Stephen. 1979. Plasma disappearance, urinary excretion and tissue distribution of ^{14}C ribavirin and its metabolites in rhesus monkeys. Fed. Proc. 38:429.

5. Burman, K. D., L. Wartofsky, R. E. Dinterman, P. Kesler, and R. W. Wannemacher, Jr. 1979. The effect of T3 and reverse T3 administration on muscle protein catabolism during fasting as measured by 3-methylhistidine excretion. Metabolism 28:805-813.

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1. Beisel, W. R., 1977. Magnitude of host nutritional responses to infection. Am. J. Clin. Nutr. 30:1236-1247.

2. Wannemacher, Jr., R. W. 1977. Key role of various individual amino acids in host response to infection. Am. J. Clin. Nutr. 30:1269-1280.

3. U.S. Army Medical Research Institute of Infectious Diseases. 1 Oct 78. Annual Progress Report, FY 1978, pp. 355-365. USAMRIID, Fort Detrick, Frederick, MD.

4. Long, III, J. M., D. W. Wilmore, A. D. Mason, Jr., and B. A. Pruitt, Jr. 1975. Comparison of carbohydrate and fat as caloric sources. Surg. Forum 26:108-110.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6412	79 10 01	DD-DR&E(AR)636

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

NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
PRIMARY	61102A	3M161102BS03	00	008
CONTRIBUTING				
~~CONTRIBUTING~~	STOG 80-7.2.2			

(U) Therapeutic correction of energy metabolism alterations during infection of unique importance in military medicine

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

CONTRACT/GRANT

DATES/EFFECTIVE: EXPIRATION:

NUMBER: NA

TYPE: AMOUNT:

KIND OF AWARD: CUM. AMT.

	18. RESOURCES ESTIMATE	A. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 79	1.0	178.7
	CURRENT 80	1.0	157.5

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GENERAL USE

Foreign intelligence considered

20 PERFORMING ORGANIZATION

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NAME: Beall, F. A.

NAME: POC:DA

KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Inflammation; (U) Infection; (U) Metabolism; (U) Ketone bodies; (U) Lipids; (U) Trauma; (U) Stress

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

(U) Maintain by appropriate therapy the body stores which are utilized is a source energy during infectious diseases of unique importance in military medicine and ological warfare. During periods of decreased food intake, the body utilizes its own t and protein to supply energy to various cells. Decreased ability of the host to ilize its fat stores during infectious disease could explain the marked protein wast-g associated with illness. Understanding of these metabolic changes can lead to fective nutrient therapy, thus reducing the protein-wasting and promoting rapid covery. A variety of infections are studied.

(U) Microanalytical methods are applied to the study of various metabolites and eir alterations caused by infection.

(U) 78 10 - 79 09 - Studies have continued on the mechanism of the inhibition of tone body production which occurs during inflammatory stress. This aspect of inflam-tion apparently has an endocrinological basis. Insulin was implicated by 3 important servations: (A) infection in diabetic rats does not result in the inhibition of star-tion ketosis; (B) in all stresses tested, ketone depression is accompanied by ele-ted plasma insulin. The elevation of plasma insulin occurs before the depression of asma ketones; and (C) inflammation in the hypophysectomized rat does not result in evated plasma insulin or a depression in plasma ketones. Although inflammation uses a rise in glucagon, the fact that glucagon in the diabetic-infected rat does t cause an inhibition of ketone bodies obviates its involvement. An inflammatory ress seems to have an effect on the pituitary which stimulates the release of insulin ring the anorexic state causing decreased concentration of plasma ketone bodies.

blications: In Western Hemisphere Nutrition Congress V, pp. 210-219, 1978; Fed. Proc. 38:409, 1979; Inflammation 3:289-294, 1979.

Available to contractors upon originator's approval

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

☆ U.S. GPO: 1974—540-643/8691

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents

Work Unit No: BS03 00 008: Therapeutic Correction of Energy Metabolism Alterations During Infection of Unique Importance in Military Medicine

Background:

Previous reports from this laboratory and others in recent years have shown that one of the prime metabolic alterations accompanying infection is protein wastage. Accompanying this negative nitrogen balance are 2 important and related metabolic alterations. With every lethal inflammatory stress in rats, there is an increase in the rate of gluconeogenesis which persists until the animal reaches the moribund state, at which time the rate decreases; in addition, there is inhibition of the ketosis which normally occurs during the fasting state. The major emphasis of this study has been to assess the importance of the inhibition of ketone production, to attempt to learn as much as possible about the mechanism of this response, and to assess the role of this inhibition in treatment of inflammatory states. In addition, it now appears that this phenomenon may be under endocrine control. A major effort is now being extended in the area of understanding the complex endocrine interrelationship with respect to lipid metabolism.

Progress:

In normal mammals, fasting or starvation causes a rapid and dramatic increase in the plasma concentration of acetoacetate and β-hydroxybutyric acid, the ketone bodies. This high concentration of ketone bodies serves to conserve N and also provides a fuel for such vital tissues at heart, skeletal muscle and brain. The presence of any inflammatory stress so far tested in rats causes marked decrease in the concentration of ketone bodies during the anorexia which accompanies inflammatory stress. Among the stresses which have been studied are the following: (A) bacterial infections as typified by Streptococcus pneumoniae, Francisella tularensis, and Salmonella typhimurium; (B) viral infection as typified by VEE infection; (C) induction of a sterile turpentine abscess; and (D) administration of endotoxin.

It is significant to note that accompanying the decrease in the concentration of ketone bodies during inflammatory stress, there is also a decrease in the concentration of free fatty acids (FFA) and plasma albumin. The decrease in the concentration of FFA appears to be closely related to the decrease in plasma albumin, while the decrease in the concentration of plasma ketone bodies seems to be independent of free fatty acid concentration.

Much of the emphasis has been placed on endocrine involvement. It has now been firmly established that in every inflammatory stress investigated there are accompanying decreases in plasma ketone body concentration and elevations of plasma insulin and glucagon.

A study of the following parameters, plasma zinc, rectal temperature, plasma ketone body concentration and plasma insulin, was made with respect to when deviation from the normal occurred after the imposition of an inflammatory stress. Rat

were subjected to two bacterial infections (S. pneumoniae and F. tularensis), administration of endotoxin, and the induction of a sterile turpentine abscess. Measurements were made on blood samples obtained every 3 hr postinoculation. In all cases an elevation in plasma insulin was apparent 3-6 hr prior to the depression of ketone bodies.

Despite the fact that administration of insulin to ketotic rats causes a rapid depression in the concentration of plasma ketone bodies, no direct action of insulin can yet be described. The liver perfusion of insulin caused no effect on the ability of the liver to produce ketone bodies from long chain fatty acids.

Accompanying the rise in plasma insulin during the inflammatory state is a dramatic rise in plasma glucagon. The glucagon may not be involved in the inflammation-induced inhibition of ketone body production. This is so because it can be demonstrated that administration of glucagon causes a transient increase in the concentration of plasma ketone bodies, transient because glucagon increases the secretion of insulin which immediately seems to cause a depression in ketone body concentration.

The relationship between the hypophysis and the inflammation-induced inhibition of ketone bodies has been investigated. There now seems to be little doubt that there is a definite pituitary involvement. When hypophysectomized animals are infected, there is no depression in the concentration of ketone bodies or FFA, nor is there a rise in plasma insulin. In one experimental infection, F. tularensis caused depression of ketone bodies in vagotomized rats. This experiment together with the fact that no effect of inflammation can be detected before 6 hr suggest that the process is an endocrine one rather than a neural phenomenon.

At the present time experiments are underway to study the effect of various pituitary hormones, in order to attempt to establish a direct endocrine role of the hypophysis on the phenomena under study.

Presentations:

1. Neufeld, H. A. The effect of inflammatory stress on ketone bodies and glucoregulatory hormones. Presented, Biology Dept., Mt. St. Mary's College, Emmitsburg, MD, Mar 1979.

2. Neufeld, H. A., J. A. Pace, M. V. Kaminski, D. T. George, and W. R. Beisel. The endocrine basis for the depression of ketone bodies and free fatty acids (FFA) in the inflamed state. Presented, Annual Meeting, American Society of Biological Chemists, Dallas, TX, Mar 1979 (Fed. Proc. 38:409, 1979).

Publications:

1. Kaminski, Jr., M. V., and H. A. Neufeld. 1978. Similarities and differences between septic and non-septic stress, pp. 210-219. In Western Hemisphere Nutrition Congress V, (P. L. White and N. Selvey, eds). American Medical Association, Chicago.

2. Kaminski, M. V., H. A. Neufeld, and J. G. Pace. 1979. Effect of inflammation and noninflammatory stress on plasma ketone bodies and free fatty acids and on glucagon and insulin in peripheral and portal blood. Inflammation 3:289-294.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OE6422	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	013
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Changes in leukocyte function during the course of viral and bacterial infections

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 79	1.0	258.5
	CURRENT 80	1.0	289.7

19. RESPONSIBLE DOD ORGANIZATION

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NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Leukocytes; (U) Polymorphonuclear leukocytes; (U) Laboratory animals; (U) Bacteria; (U) Viruses

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

23 (U) Determine extent of metabolic and functional alterations in host circulating white blood cells (granulocytes) induced by exposure to potential BW agents and evaluate usefulness of these changes for rapid detection, diagnosis, and prognosis of infectious illnesses of interest in BW defense.

24 (U) Using appropriate experimental animal models for bacterial, viral, and rickettsial infections determine leukocyte functions as measured by chemiluminescence, phagocytosis and chemotaxis.

25 (U) 78 10 - 79 09 - Studies have provided the first experimental evidence that enhanced luminol-amplified chemiluminescence (CL) occurs in peripheral polymorphonuclear leukocytes (PMN) during the course of bacterial infections and endotoxemia in rats. This enhanced PMN CL appears to be attributable to (A) stimulation of cellular oxidative metabolism, (B) production of hydrogen peroxide, and (C) the generation of highly reactive excited oxygen species which include hydroxyl and superoxide radicals and singlet oxygen. The magnitude of the PMN CL response is related to the severity of the infection, in that it increases during the illness phase and decreases with recovery. Studies have been initiated to determine whether viral infection induces similar changes in PMN CL.

Publications: Can. J. Microbiol. 24:834-838, 1978; Proc. Soc. Exp. Biol. Med. 160: 175-179, 1979; Circ. Shock 6:190, 1979; Am. J. Vet. Res. 40:1035-1039, 1979; Toxicol. Appl. Pharmacol. 50: in press, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 013: Changes in Leukocyte Function During the Course of Viral and Bacterial Infections

Background:

Polymorphonuclear leukocyte (PMN) phagocytosis and subsequent microbicidal activity provide the host with a primary defense against many invading organisms. The PMN response upon exposure to phagocytizable and nonphagocytizable stimuli is characterized by the "respiratory burst" which includes activation of hexose monophosphate shunt (HMS) activity and generation of microbicidal agents which include hydrogen peroxide, superoxide anion (O_2^-), hydroxylradical and singlet oxygen ($^1O_2$). Recent in vitro studies reviewed by Babior (1) have demonstrated that stimulated PMN generate light, i.e. chemiluminescence (CL). Chemiluminescence has been attributed to the relaxation of various excited oxygen species as well as other excited compounds produced during the respiratory burst.

Studies were initiated to determine whether PMN CL occurs in vivo during infectious illness and to assess the value of PMN CL as an early detection, diagnostic, and prognostic tool for infectious diseases of interest to military medicine. Experiments were performed with circulating peripheral PMN obtained from rats during various stages of bacterial infection and endotoxemia. These studies resulted in the first observations of enhanced CL from PMN stimulated in vivo as a result of infection or toxemia.

Progress:

PMN CL during Francisella tularensis (LVS) infection. Rats were injected IP with 10^7 LVS organisms/100 g body weight and controls with equal volumes of culture medium. The course of infection was monitored by rectal temperature and plasma Zn depression. At various time intervals following the injections (1, 6, 12, 24 and 48 hr), peripheral PMN were isolated and CL measured. CL measurements were performed in the presence of luminol as previously described (2).

Enhanced endogenous PMN CL, compared to control values, was measured in the infected population 6 hr after injection of microorganisms. Elevated temperatures and plasma Zn depression were evident 12 hr postinjection. Mortality (34%) occurred between 24 and 48 hr. The CL response from infected cells increased significantly during the initial 48 hr of infection. The time-course of the PMN CL response suggests a possible relationship between the magnitude of the PMN CL response and the severity of the infection.

These results prompted subsequent studies using a lower dose of LVS to determine the relationship of PMN CL to the course of the infection. Rats were injected IP with 10^5 LVS/100 g body weight and studied at 24-hr intervals for 9 days. Controls were injected with an equivalent volume of culture medium. Significant fever was present during the 24-72 hr-period of infection. Plasma Zn depression occurred 24 hr following infection and continued throughout the experiment with a trend toward normal concentrations by day 8. A 33% mortality occurred on days 2-4 inclusive. On days 1-4 the PMN CL response in cells of infected rats increased dramatically.

Improvement in clinical signs and recovery were paralleled by reduced PMN CL measured on days 5-9. Thus, the course of the infection was directly reflected in the PMN CL.

Additional studies were performed to determine the effect of immune status on the PMN CL response during an LVS infection. Immune (microagglutination antibody levels > 1:80) and nonimmune rats were injected with 10^7 LVS/100 g body weight; and PMN CL was measured 24, 48 and 72 hr following injection. Fever was not detected in immune rats during the study; a slight plasma Zn depression occurred only at 24 hr. A diminished PMN CL response was measured at 24 hr in the immune compared to that of the nonimmune group. By 48 hr, enhanced CL was not detectable in immune rats. These data suggest that the rapid clearance of bacteria in immune rats may be responsible for the diminished PMN CL response.

Results from these experiments support our earlier contention that there is a correlation between the magnitude of the PMN CL response and the severity of the infection in the host. Further, results suggest that PMN CL may have prognostic as well as diagnostic value during the course of bacterial infections.

Effects of Salmonella typhimurium infection and endotoxemia on PMN CL. PMN CL studies were expanded to other bacterial infections to determine if this phenomenon was a general response during sepsis. Rats injected IP with 10^7 S. typhimurium/100 g body weight were studied 24 hr following injection; PMN CL was measured. Controls were injected with either 10^7 heat-killed organisms/100 g of body weight or an equal volume of medium broth. Equivalent enhanced PMN CL was measured in rats injected with live and heat-killed organisms when compared to controls.

Since the enhanced PMN CL from rats injected with heat-killed organisms could be attributable to the endotoxin content of these preparations, experiments were performed to clarify the CL response during endotoxemia in the rat. Initially, the relationship between PMN CL and the dose of administered endotoxin was studied. Rats were injected IP with doses of Escherichia coli endotoxin ranging from 0.01-250 µg/100 g body weight and sacrificed 24 hr following toxin administration. Only the highest dose used (250 µg) resulted in mortality (20%) at 24 hr. The PMN CL at 24 hr was found to be dose-dependent over the range, 0.01-100 µg, with an apparent maximum response elicited with doses of 100 and 250 µg.

Studies were extended to determine the relationship between the time course of endotoxemia and the PMN CL response. Rats were injected IP with 250 µg/100 g body weight E. coli endotoxin; the PMN CL measured 5, 24, 48 and 72 hr following injection. Control rats received an equivalent volume of sterile water. A significant increase in PMN CL occurred within 5 hr which continued increasing through 48 hr. By 72 hr, PMN CL response was nearly dissipated. Since available evidence (3) indicates that endotoxin is cleared rapidly following IP injection, these results may suggest that humoral factor(s) are involved in the activation of the PMN CL response, or that endotoxin induces extensive residual cellular effects during endotoxemia in the rat. Although further work is required, these observations support the contention that enhanced PMN CL following the administration of heat-killed S. typhimurium organisms is attributable, at least in part, to the presence of endotoxin in the inoculum. In addition, the data accumulated during the aforementioned studies suggest that the PMN CL is a generalized response to bacterial infection and toxemia in the rat.

Effect of storage (5°C) on PMN CL response. Rats were injected IP with either

10^7 LVS organisms/100 g body weight or an equivalent volume of support medium. PMN were isolated 24 hr later and stored for various periods at 5°C. CL of stored PMN was measured at 0, 1, 5 and 8 hr following isolation. Results indicated that no significant difference existed between any of the PMN CL measurements for cells from infected rats. From these results it can be concluded that CL measurements can be made at least up to 8 hr following the isolation of PMN provided cells are isolated and stored at 5°C. Additional studies will be required to determine the maximum possible cell storage time.

PMN Cl during Pichinde virus infection. Studies have been initiated to determine whether viral infection induces alterations in host PMN CL similar to those described for bacterial infections. Peripheral PMN from guinea pigs infected with Pichinde virus are currently being evaluated.

Presentations:

1. Sobocinski, P. Z., W. J. Canterbury, and E. C. Hauer. Hepatic accumulation of metallothionein and its possible role in induction of tolerance to endotoxin-induced alterations in zinc metabolism. Presented, Annual Meeting FASEB, Dallas, TX, 1-10 Apr 1979. (Fed. Proc. 38:5769, 1979.).

2. McCarthy, J. P., and P. Z. Sobocinski. Effect of bacterial infection on endogenous granulocyte chemiluminescence (CL). Presented, Annual Meeting FASEB, Dallas, TX, 1-10 Apr 1979. (Fed. Proc. 38:1023, 1979.).

3. Sobocinski, P. Z. Role of hepatic metallothionein in zinc homeostasis in pathologic states. AFRRI Biochemistry Colloquim, Bethesda, MD, Mar 1979.

4. Beisel, W. R., and P. Z. Sobocinski. Endogenous mediators of fever-related metabolic and hormonal responses. International Symposium on Fever, Dallas, TX, 1-10 Apr 1979.

Publications:

1. Walker, R. I., S. L. Snyder, P. Z. Sobocinski, Kenneth F. McCarthy, and June E. Egan. 1978. Possible association of granulocyte mobilization to the peritoneal cavity with $ZnCl_2$-induced protection against endotoxin. Can. J. Microbiol. 24:834-838.

2. Sobocinski, P. Z., W. J. Canterbury, Jr., E. C. Hauer, and F. A. Beall. 1979. Induction of hypozincemia and hepatic metallothionein synthesis in hypersensitivity reactions. Proc. Soc. Exp. Biol. Med. 160:175-179.

3. Walker, R. I., J. E. French, D. A. Walden, T. J. MacVittie, G. A. Parker, P. Z. Sobocinski, and F. R. Appelbaum. 1979. Protection of dogs from lethal consequences of endotoxemia with plasma transfusions. Circ. Shock 6:190.

4. Liu, C. T., R. P. Sanders, C. L. Hadick, and P. Z. Sobocinski. 1979. Effects of intravenous injection of leukocytic endogenous mediator on cardiohepatic functions in rhesus macaques. Am. J. Vet. Res. 40:1035-1039.

5. Sobocinski, P. Z., G. L. Knutsen, W. J. Canterbury, Jr., and E. C. Hauer, 1979. Altered zinc homeostasis and hepatic accumulation of metallothionein in

indomethacin-induced enteropathy. Toxicol. Appl. Pharmacol. 50:557-564.

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1. Babior, B. M. 1978. Oxygen-dependent microbial killing by phagocytes. N. Engl. J. Med. 298:659-668, 721-725.

2. McCarthy, J. P., and P. Z. Zobocinski. 1979. Effect of bacterial infection on endogenous granulocyte chemiluminescence (CL). Fed. Proc. 38:1023.

3. Nolan, J. P. 1979. The contribution of gut-derived endotoxins to liver injury. Yale J. Biol. Med. 52:127-133.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6411	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	015
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code): (U) Effects of infection/intoxication upon structure and function of cellular membranes

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
76 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 79	1.0	130.4
FISCAL YEAR CURRENT 80	1.0	138.6

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Lipogenesis; (U) Plasma membranes; (U) Enzyme regulation; (U) Macrophages; (U) Cytochemistry

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 effects of infection upon the structure and function of liver cell membranes. The identification of infection-induced abnormalities will be useful in developing improved prophylactic and therapeutic measures aimed at the control of membrane-directed hormonal or metabolic activity for diseases of military importance.
24 (U) A variety of techniques, e.g., cell fractionation, biochemical and cytochemical analyses, and centrifugation analysis, are used to study effects of infection upon structure and function of cellular membranes. Bacteria and rickettsia are studied.
25 (U) 78 10 - 79 09 - The effect of Streptococcus pneumoniae infection on the synthesis of plasma membranes isolated from liver homogenates was determined. During infection a significant increase in the synthesis of hepatic plasma membranes occurs, which may be required to accomodate for the increased liver secretory activity observed.

Hepatic nuclei were isolated and characterized from control and S. pneumoniae-infected rats. An in vitro assay system for incorporation of rabiolabeled precursor into RNA by isolated nuclei was developed. During infection a significant increase in the incorporation of precursor into RNA by isolated nuclei was observed.

Studies were initiated to determine the intracellular fate of Coxiella burnetii in guina pig peritoneal macrophages. The effect of UV treatment on the inactivation of C. burnetii in suspension or growing in macrophages, activity of macrophage organelle enzymes, and equilibrium of macrophage organelle enzymes in linear sucrose gradients was determined.

Studies on the binding of radiolabeled insulin to plasma membranes purified from control or S. pneumoniae-infected animals were initiated.

Publication: Infect. Immun. 22:585-596, 1978.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 015: Effects of Infection/Intoxication Upon Structure and Function of Cellular Membranes

Background:

Maintaining the integrity of cell membranes is essential for normal cell function. Alterations in membrane structure may contribute significantly to cellular malfunction and ultimately enhance host susceptibility to infectious diseases. An understanding of the effect(s) of infection on both the structure and function of cell membranes will prove helpful in obtaining a more thorough understanding of the mechanism(s) involved in the pathogenesis of infectious diseases.

Progress:

Studies on the synthesis of rat liver plasma membranes during pneumococcal sepsis. In order to study the synthesis of hepatic plasma membranes, groups of 6 rats were inoculated with 3×10^5 heat-killed (control) or virulent (infected) Streptococcus pneumoniae; 40 hr later they were injected via the dorsal penile vein with 100 µCi of [^{3}H] leucine. At various time intervals thereafter, plasma membranes were isolated from both groups of rats (1), and radioactively incorporated into plasma membrane protein was determined.

During infection there was a significant increase in both the specific activity (CPM/mg plasma membrane protein) and total activity (CPM/mg total liver plasma membrane protein) of incorporated isotope. These results suggested increased synthesis of hepatic plasma membranes during infection. However, it was of importance to determine if any of the radioactivity associated with the preparations was due to radioactively labeled plasma proteins which could be adsorbed to the plasma membranes or in small vesicles co-isolating with the plasma membranes.

To answer this question Golgi, plasma membranes, and plasma from control and infected rats were labeled to maximum specific activity with [^{3}H]leucine (2). After an appropriate incorporation period, the Golgi, plasma membranes and plasma were isolated. Each fraction was treated with anti-rat plasma in a system containing detergent to solubilize all proteins. For both control and infected Golgi preparations, 50% of the radioactivity was precipitated with anti-rat plasma antibody. This resulted in the suggestion that the labeled plasma proteins contained in the Golgi were antigenic. In plasma from both control and infected animals 80% of the radioactivity could be precipitated with antibody. No radioactivity was precipitated from plasma membranes isolated from control or infected rats. In addition, no counts were precipitated from any preparation with anti-turkey serum or control rat serum. These results suggest that the radioactivity of the isolated plasma membranes is due to label incorporated into plasma membrane proteins only and not due to contamination by plasma proteins.

Since the increased incorporation of [^{3}H]leucine into isolated liver plasma membranes could not be explained by altered leucine pool size or contamination by labeled plasma proteins, it is concluded that there is increased synthesis of hepatic plasma membranes during S. pneumoniae infection in rats.

The reason for the increased synthesis of the plasma membrane during infection has not been determined. There is, however, an increase in synthesis, transport, packaging, and secretion of plasma proteins during infection (2). This phenomenon may require increased synthesis of the intracellular organelles involved in the process. Since Golgi secretory vesicles fuse with plasma membranes (3) and release their contents into the circulation, it may be necessary to synthesize more plasma membrane to accommodate for this process during periods of enhanced secretory activity.

Studies on the binding of ^{125}I-insulin to plasma membranes purified from control and S. pneumoniae-infected rat liver homogenates. Plasma membranes were isolated from control and infected rat liver homogenates (1) and tested for ^{125}I-insulin binding. Both control and infected plasma membranes bound labeled insulin, which could be displaced with an excess of unlabeled insulin.

Experiments were conducted to show that the binding of labeled insulin to isolated plasma membranes was dependent on the plasma membrane protein concentration. Binding was linear from 0.1 to 0.8 mg/ml of plasma membranes for both control and infected preparations. The time for maximal insulin binding to control and infected plasma membrane preparations was also determined. For both control and infected membranes, maximal amounts of labeled insulin were bound by 60 min. Routine binding assays are now performed for 90 min. The pH optimum of insulin binding to plasma membranes isolated from both control and infected rat liver homogenates was determined. Once all the appropriate control experiments are conducted, it will be possible to determine the number of affinity of insulin binding sites on plasma membranes isolated from control and infected rat liver homogenates. It may then be possible to correlate altered insulin binding with metabolic alterations which occur during infection.

Effect of infection on the action of insulin in isolated rat liver nuclei. During this past year significant progress has been made on studies designed to determine if insulin, binding to specific nuclear membrane receptors, stimulates RNA synthesis in nuclei isolated from control and infected rats. Such stimulation has been found in vivo, but the investigations have not addressed the mechanism of this action. Since nuclei have been reported to have membrane-bound receptors specific for insulin, it was hypothesized that the nuclear binding sites are functional receptors that transmit/transfer messages to the nuclear interior. The approach taken consisted of several phases.

1. Isolate, purify, and partially characterize nuclei from control and S. pneumoniae-infected rats.

2. Determine if nucleic isolated from control and infected rats have specific insulin-binding sites.

3. Characterize an in vitro system for studying the incorporation of RNA precursor into RNA of the isolated nuclei.

TABLE I. BIOCHEMICAL ASSESSMENT OF CONTAMINATION OF ISOLATED NUCLEI BY OTHER ORGANELLES (46-HR FASTED/INFECTED)

	SPECIFIC ACTIVITY					
	5'Nucleotidase[a]		Alkaline phosphatase[b]		Glutamate dehydrogenase[c]	
Cell Fraction	C	I[g]	C	I	C	I
Nuclei	0.332 ±0.0027	0.239 ±0.0108	0.219 ±0.0241	1.036 ±0.151	Not detectable	
Plasma Membrane	30.3 ± 1.48	24.8 ±1.68	23.9 +2.28	51.6 ±10.0		
Mitochrondria					0.032 ±0.003	0.031 ±0.002
Microsomes						
Lysosomes						
Ratio: Nuclei/ fraction %	1.10	0.95	0.92	2.01		

[a] µmol Pi released/20 min/mg protein.

[b] nmol of p-nitrophenol formed/min/mg protein.

[c] µmol of NAD oxidized/per min.

[d] µmol of NAD reduced/min/mg protein.

[e] µmol of cytochrome c reduced/per min/mg protein.

4. Determine the effect of insulin on the incorporation of precursor into RNA of nuclei isolated from control and infected rats.

To date considerable progress has been made on the first 3 phases. Nuclei from control and infected rats have been isolated and their purity determined by biochemical and morphological methods. Table I shows a biochemical assessment of contamination of the isolated nucleic by hepatic cellular organelles. The specific activities of marker enzymes were determined in organelles where these enzymes occur as markers and also in the purified nuclei. The ratio of the specific activity of the enzyme in the nuclei to the specific activity of the enzyme in the organelle where it occurs as a marker was determined. These values are used to access purity of the nuclei preparations. Alkaline phosphatase and 5'-nucleotidase were used as plasma membrane markers, NADH cytochrome c reductase and choline phosphotransferase as endoplasmic

OF ± S.E.M. (N = 6)							
Malate dehydrogenase[d]		NADH cytochrome c reductase[e]		Choline phosphotransferase[f]		Acid phosphatase[a]	
C	I	C	I	C	I	C	I
0.0378 ±0.0015	0.0626 ±0.0025	30.3 ±1.6	38.8 ±2.9	291.2 ±15.8	240.2 ±16.8	0.0513 +0.0053	0.0644 ±0.0108
2.2 ±0.138	1.97 ±0.047						
		341 ±38	564 ±38	2385 ±55.2	2083.7 ±15.0		
						11.1	ND[h]
1.72	3.18	8.9	6.9	12.0	11.5	0.46	

[f] DPM/mg protein.

[g] C = control; I = infected.

[h] ND = not determined.

reticulum markers, and acid phosphatase as a lysosomal marker. As seen in Table I, contamination of nuclei by plasma membranes, mitochondria, lysosomes, and endoplasmic reticulum were all < 12%.

For both control and infected nuclei, 4-5% of total cellular RNA and 60-80% of total cellular DNA was associated with the purified nuclei. These results indicate a respectable recovery.

To further characterize the isolated nuclei, cholesterol and phospholipid were assayed. No significant difference in these two constituents in control or infected nuclei was observed.

Table II shows the percent of the total homogenate activity of the marker enzymes recovered with the isolated nuclei. For all marker enzymes, < 2% of the total homogenate activity was recovered with the isolated nuclei. The results shown

TABLE II. TOTAL HOMOGENATE ACTIVITY OF SELECTED ENZYMES RECOVERED WITH NUCLEI FROM CONTROL AND INFECTED RATS

ENZYME	% ACTIVITY OF RATS FASTED FOR 46 HR[a]	
	Control	Infected
5'Nucleotidase[b]	0.507	0.564
Alkaline phosphatase	0.624	0.391
Malate dehydrogenase	0.077	0.120
NADH cytochrome c reductase	1.42	0.717
Choline phosphotransferase	0.657	0.499
Acid phosphatase	0.115	0.226

[a] $\% \text{ Activity} = \frac{\text{Specific activity of enzyme in nuclei x nucleic yield}}{\text{Specific activity of enzyme in homogenate x homogenate yield}} \times 100$

[b] See Table I for units of activity.

in Tables I and II demonstrate that the nuclei isolated from control and infected rats were very pure. These nuclei were also prepared for electron microscopy. Morphological results confirmed the biochemical results in that very little contamination was observed.

Preliminary results on the binding of labeled insulin to isolated nucleic indicate that binding is linear with respect to time and protein concentration. Scatchard analysis indicates either 2 sets of binding sites (high and low affinity) or negative cooperativity. Initial results suggest that rats from infected nuclei bind twice as much labeled insulin as controls.

Studies have also been initiated to characterize an *in vitro* system for the incorporation of [^{14}C]-UTP into RNA by isolated nuclei. To date several modifications of a previously published method have been necessary. Unlabeled UTP has been omitted and the concentration of labeled UTP has been doubled. It has been determined that nondialyzed cytosol is as effective in the assay system as dialyzed. Four times the yeast RNA concentration improved the linearity of the reaction indicating that endogenous ribonuclease was hydrolyzing newly synthesized RNA. The reaction has also been optimized with respect to temperature and pH. Experiments using isolated nuclei, crude nuclei, or liver homogenates all showed the same consistent 50% increase in [^{14}C]-UTP incorporation into RNA by infected preparations. Phase-contrast photomicrographs showed intact nuclei after 60 min incubation in the assay system. Experiments are now in progress to determine if insulin effects can be shown by using this newly developed assay.

Studies on the intracellular fate of phase I and II Coxiella burnetii in guinea pig peritoneal macrophages. These studies were initiated this year. Since certain steps in this study could not be performed in a Blickman hood, it was necessary as a safety consideration to develop a method for inactivating the rickettsiae without inactivating the macrophage marker enzymes since these enzymes would subsequently be monitored in linear sucrose gradients. Since inactivation by chemical fixation, heat, and a number of other conventional methods was known to affect enzyme activity, UV irradiation was tried. It was necessary to show that this treatment had no significant effect on the activity of macrophage organelle marker enzymes and then show that it inactivated both phase I and II C. burnetii. Irradiation for 5-10 min with a GE 15 W bulb at a distance of 10 cm was reported to be sufficient for complete inactivation of rickettsiae growing in monolayers of chick fibroblast cells. Therefore, macrophages adhered to plastic petri dishes were overlaid with HBSS and exposed to UV light (6 $\mu W/cm^2$ x 100) for 10 min. The activity of macrophage organelle marker enzymes was then measured. This treatment almost completely inactivated all marker enzymes measured. Macrophages were then exposed in the same manner for times ranging from 15 sec up to 3 min. It was found that 15 sec was the maximum length of time that the macrophages could be exposed under these conditions without significantly affecting their organelle marker enzymes. It was then necessary to determine if this same treatment would inactivate phase I and II C. burnetii in suspension and growing in peritoneal macrophages. It was important to show that suspensions of phase I and II C. burnetii could be inactivated since it would be necessary later to determine if C. burnetii contained significant levels of enzymes employed as markers for macrophage organelles. Phase I or II C. burnetii (3 ml, 10^8/ml) were dispensed into petri dishes such that the suspensions were approximately 1 mm in depth. These suspensions were exposed for 15 sec to UV light. In addition, guinea pig peritoneal macrophages were prepared and inoculated under optimal conditions with 10^8 phase I or II C. burnetii. After 2 hr, nonattached and nonphagocytized C. burnetii were removed with 2 rinses of HBSS. HBSS was added and these preparations were then exposed as described to UV light. The UV-treated C. burnetii in suspensions and growing in macrophages were then serially inoculated IP into white Swiss mice. It has been shown that one viable C. burnetii will cause mice to seroconvert by 21 days. After 21 days none of these mice seroconverted. These results showed that this UV treatment inactivated both preparations of phase I and II C. burnetii.

It was next determined if this UV treatment affected the fractionation of macrophages harvested from normal or phase I-immune guinea pigs. Guinea pig peritoneal macrophages from these animals were harvested 4 days after the IP injection of 1.5% (w/v) sodium caseinate (15-20 ml/animal). The peritoneal exudate cells were harvested and processed by standard procedures. Approximately 5 x 10^6 macrophages were dispersed into petri dishes and incubated at 37°C for 1 hr in a humid atmosphere of air containing 5% CO_2. Nonadherent cells were removed by washing in HBSS. Adherent cells were scraped off the petri dishes in HBSS with a rubber tipped scraper and sedimented. The sedimented macrophages were homogenized with a syringe by 4 passages through a 12-inch, 25-gauge cannula (4). The nuclei and unbroken cells were separated from the cytoplasmic extract by differential centrifugation. The pellet was then rehomogenized. This process was repeated 4 times and the extracts of each homogenization were combined.

The combined cytoplasmic extracts were fractionated on linear sucrose gradients. Fractions were collected and assayed for marker enzymes of the plasma membrane, endoplasmic reticulum, lysosomes, and mitochondria. Distribution patterns of

enzymes and proteins after fractionation of the cytoplasmic extracts by density equilibrium in linear sucrose gradients were presented in the form of histograms (5). Results showed that the intracellular organelles of the macrophage had no significant effect on this separation.

The second part of this study was also begun. Suspensions of radiolabeled I and II C. burnetii were UV-inactivated, layered on linear sucrose gradients, and centrifuged to equilibrium. The gradients were fractionated and assayed for radioactivity and macrophage organelle marker enzymes. The equilibrium density of phase I and II in alkaline sucrose was 1.24 and 1.23 respectively. When the gradients were assayed for malate dehydrogenase, 5'-nucleotidase, alkaline α-glucosidase, α-D-galactosidase, alkaline phosphatase and N-acetyl-β-glucosaminidase, no detectable quantities of these macrophage marker enzymes were found in either the phase I or II gradients. The absence in phase I and II C. burnetii of detectable quantities of enzymes chosen as markers for macrophage organelles greatly simplifies and facilitates fractionation studies. It eliminates the requirement for correcting macrophage enzyme activities for rickettsial enzyme background.

Once it became possible to UV-inactivate C. burnetii by UV light in suspension or in cultures of guinea pig peritoneal macrophages and to radiolabel the phases, studies were initiated to determine the intracellular distribution and fate of phase I and II C. burnetii in guinea pig peritoneal macrophages.

Macrophages were harvested from phase I immune guinea pigs and infected with ^{3}H-labeled phase I or II C. burnetii in the presence of immune serum. These macrophages were subsequently homogenized and fractionated on linear sucrose gradients. Fractions were collected from the gradients and assayed for radioactivity and macrophage organelle marker enzymes. It was shown that the radioactivity of both phases peaked in the same fraction as the lysosomal enzymes of the macrophage. These results suggested that under these conditions both phases I and II were in macrophage lysosomes. To support this hypothesis phase I immune guinea pigs were injected IP with Triton WR 1339 and Na Caseinate 4 days prior to macrophage harvest. The macrophages phagocytize this detergent and as a result the density of their lysosomes is decreased. When these macrophages containing lighter lysosomes were infected with ^{3}H-labeled phase I or II C. burnetii, homogenized and fractionated as described above, the lysosomal enzyme markers shifted in the gradient to a lighter density as did the labeled phase I and II C. burnetii. These data show that both phases of C. burnetii are associated with the lysosomes of phase I immune macrophages. Additional experiments are in progress with macrophages harvested from nonimmune guinea pigs infected with phase I or II C. burnetii in the presence or absence of immune serum.

Publication:

Little, J. S. 1978. Synthesis, transport, and secretion of plasma proteins by livers of control and Streptococcus pneumoniae-infected rats. Infect. Immun. 22:585-596.

LITERATURE CITED

1. Little, J. S. 1977. Isolation and partial characterization of plasma membranes from the livers of control and Streptococcus pneumoniae-infected rats. Infect. Immun. 16:628-636.

2. Little, J. S. 1978. Synthesis, transport, and secretion of plasma proteins by livers of control and Streptococcus pneumoniae-infected rats. Infect. Immun. 22:585-596.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OF6424	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	019
b. CONTRIBUTING				
c. [illegible]	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Mechanism of action of bacterial exotoxins

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
75 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 79	1.0	188.8
FISCAL YEAR CURRENT 80	1.0	166.8

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

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NAME:

21. GENERAL USE

Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede each with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Food poisoning; (U) Infected burns; (U) Pseudomonas; (U) Staphylococcus; (U) Prophylaxis; (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) Elucidate the mechanisms of action of militarily significant bacterial exotoxins in order to develop prophylactic or therapeutic measures. Two toxins are under investigation; Pseudomonas aeruginosa exotoxin A (PE), which has been implicated as a (the?) causative agent in the mortality of recuperating burn casualties who develop Pseudomonas infections; staphylococcal enterotoxin B (SEB) which has a demonstrated BW potential. Diphtheria toxin is also studied as a model.

24 (U) Find and characterize cell line(s) or tissue(s) susceptible to the toxin of interest; study the toxin-induced biophysical and biochemical events which lead to the cell/tissue response; test drugs to find those which may protect the cell/tissue from the toxin; and test positive findings in laboratory animals to determine human therapeutic potential.

25 (U) 78 10 - 79 09 - Effects of lectins on diphtheria toxin (DE) receptor binding were studied. It was shown that binding is not blocked as suggested by others; but internalization was prevented by protective lectins. Cellular control of DE receptor levels was studied. Receptor is long-lived and little or no synthesis of new receptor is required to maintain steady-state cell surface levels. Receptor seems to be continually taken inside the cell and reutilized. Reinsertation into the cell membrane is protein synthesis-dependent. Concanavalin A blocks the internalization of the unbound DE receptor and the cell responds by synthesizing additional DE receptor. Regain of receptor levels after their removal by trypsin was DNA- and protein synthesis-dependent. A Pseudomonas exotoxin receptor system was also developed and partially characterized.

Publications: J. Biol. Chem. 253:7325-7330, 1978; 254: in press, 1979; Can. J. Microbiol. 25:285-290, 1979; Exp. Cell. Res. 121:95-101, 1979, In Natural Toxins, in press.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 019: Mechanism of Action of Bacterial Exotoxins

Background:

Bacterial toxins mediate most of the harmful effects of many bacterial infections. In some instances, morbidity results from preformed toxins, e.g., botulinum or staphylococcal food poisoning, while in others, from toxin elaborated as the organism grows in the host, i.e., diphtheria or cholera. In either case, successful treatment of, or protection from, the disease must deal with the toxin. In theory, this could be accomplished either prophylactically (immunization) or therapeutically (specific drug or antidote). While the former approach has been successful in at least one case (diphtheria), there are enormous problems (logistical and medical) with immunizing an entire at-risk population. Moreover, it is not clear that all toxins will be amenable to toxoiding or that immunization will always confer adequate protection. It, therefore, seems prudent to make some efforts towards the development of therapeutic measures. To this end, it is our purpose to study the mechanisms of action of bacterial exotoxins and to test available drugs (or identify or develop new ones) for their therapeutic potential.

At this project's inception, the available evidence indicated that each bacterial toxin was quite unique in its mode of action. Studies published since that time have proven that notion wrong. It now appears that most toxins are bipartite, one portion mediating the binding specificity and the other carrying out an enzymatic function. Moreover, it is of considerable interest that, of the 4 toxins whose molecular mechanisms of action are known, all have similar enzymatic (ADP-ribosylating) activity. Generally speaking, microbial toxin action can be divided into 3 stages: toxin-receptor binding, internalization of the toxin or toxin-receptor complex, and expression of enzymatic activity. In theory, one could develop drugs which intervene at any or all of these stages. During the past year, most of our efforts have been directed toward a first stage. One might possibly obtain toxin analogues which would compete with authentic toxin for receptor occupancy. One might synthesize drugs which block binding for some other reason. Or one might induce the cell to "shed" or remove its toxin receptor, thus conferring protection. Whatever approach is taken, it becomes critical to know how the cell controls its toxin receptor numbers. To our knowledge, no such information exists for any toxin. We therefore undertook such a study using our DE Vero cell model system. The other main laboratory effort this year was the study of a Pseudomonas exotoxin A (PE) receptor system.

Progress:

We began with a study of lectin effects on toxin-receptor binding and internalization of DE based on a report by Draper et al. (1) that lectins block the "binding" of DE. This, in turn, suggested that the receptor for DE may be a sugar such as α-methyl mannoside. Our experiments have proven that notion false and instead point to lectin inhibition of toxin internalization as the proper explanation.

Our first step was to reproduce the initial observations of Draper et al.(1) with our cell lines; namely, that lectins can protect cells from toxin-induced inhibition of protein synthesis. Concanavalin A (Con A) does protect Vero cells from DE although not nearly as well as NH_4Cl or ATP, 2 other agents we previously studied.

Lectin protection cannot be explained as a block of toxin-receptor binding. The kinetics of toxin-receptor binding at 4°C were not detectably altered by the addition of Con A. Since no appreciable toxin internalization takes place at this temperature, yet disassociation of toxin and receptor are clearly demonstrable, it is clear that Con A does not directly compete for DE receptor binding. The kinetics of toxin-cell association at 37°C reflect toxin-receptor binding, internalization and degradation. Paradoxically, Con A addition led to an increase in toxin-cell association at this temperature. Obviously 1 of these 3 phases of toxin action has been markedly perturbed by Con A and, since the 4°C-kinetics rule out direct competition, Con A effects must be at the phases of internalization or degradation.

The key clue to Con A's mechanism of protection was provided by the development of an assay which differentiated between cell-surface bound and internalized DE. Instead of washing the cells and dissolving them in base for scintillation counting (our usual assay), we washed away the unbound toxin and treated cells with a combination of pronase and inositol hexaphosphate (PIHP) to "strip-off" toxin (toxin-receptor?) which was on the outside of the cells. For a positive control, we incubated cells with toxin at 4°C and carried out the same assay. The majority (~90%) of specific cell-associated toxin at 4°C could be released by this treatment indicating that it was located at the cell surface; as expected, Con A had no effect on this pattern. At 37°C, a considerable fraction of the total cell-associated radioactivity could not be released by PIHP treatment early; by 4 hr ~70% was internalized. The addition of Con A brought about a marked change in the 37°C pattern, converting the kinetics to appear much like those at 4°C, e.g., toxin was retained at the cell surface. Thus, we concluded that Con A protects cells by blocking toxin internalization. Several other lectins can block internalization as is shown by the data in Table I.

TABLE I. EFFECT OF LECTINS ON DIPHTHERIA TOXIN-CELL BINDING[a]

LECTIN ADDED (100 µg/ml)	SPECIFIC CELL ASSOCIATED RADIOACTIVITY (CPM ± SE, n = 3)
None	760 ± 140
Con A	3400 ± 300
Con A + α-methyl mannoside (50 mM)	1290 ± 410
α-methyl mannoside (50 mM)	380 ± 50
Wheat germ agglutinin	2520 ± 520
Corse lectin	780 ± 140
Lentil lectin	2710 ± 360
Soybean lectin	1560 ± 380
Ricin I	510 ± 70
Ricin II	740 ± 20

[a]Vero cells in phosphate buffered saline, 10% fetal calf serum were incubated with ^{125}I-toxin (0.03 µg/ml) or ^{125}I-toxin + unlabeled toxin (3 µg/ml) in the presence of the indicated agent for 5 hr at 37°C.

Wheat germ agglutinin and lentil lectin exert this effect, but appear to be less potent than Con A. Soybean lectin has a modest effect while gorse, ricin I and II lectins have no detectable influence on binding. The reversal of Con A's effects by α-methyl mannoside (Table I) provides proof that binding of Con A to the cell is responsible for its effects on toxin-cell association.

Several laboratories have reported that Con can inhibit the mobility of cell surface membrane receptors as well as block patching and capping of lymphocytes. Two interesting features of Con A inhibition of receptor mobility have been described. The first is an inability of the monovalent Con A derivative, succinyl Con A, to exert these effects. We prepared succinyl Con A and tested it in our normal binding assay system. When binding is measured at 5 hr (37°C) the Con A effect is maximum. We obtained dose-response curves for Con A and succinyl Con A at this time and temperature and found them equipotent for their effects on toxin-cell association. This was a rather unexpected and virtually unprecedented finding, so we tested our succinyl Con A preparation to assure its authenticity; favorable results were obtained. Thus, we can conclude that the Con inhibition of DE internalization does not require an extensive cross linking of α-methyl mannoside residues on the cell surface.

The second commonly found feature of Con A inhibition of receptor mobility is its dependence on intact microtubules. Thus, the introduction microtubule depolymerizing agents such as colchicine usually block a response to Con A. Table II shows, however, that colchicine did not work in our system.

TABLE II. EFFECT OF COLCHICINE ON CON A-INDUCED INCREASE OF TOXIN-CELL BINDING[a]

SAMPLE	TIME (hr)	SPECIFIC CELL-ASSOCIATED COUNTS (CPM I SE, n=3)
Control	1.5	4700 ± 140
Control	5.0	2410 ± 100
Con A (100 μg/ml)	5.0	8240 ± 700
Con A (100 μg/ml) +colchicine (10^{-4}°M)	5.0	8150 ± 470

[a]Agents were added to Vero cells followed by ^{125}I-toxin (0.03 μg/ml) or ^{125}I-toxin + unlabeled toxin (3 μg/ml).

By itself, colchicine has no effect on diphtheria toxin cytotoxicity or binding. When colchicine was added with Con A, the lectin remained fully potent. Thus, intact microtubules are not required for the expression of Con A effects on diphtheria toxin-cell association.

To begin our work on cell control of DE receptor levels, we investigated the effect of cell density on the number of receptors per cell. In some hormone systems, such as with epidermal growth factor, the number of cell membrane receptors decrease markedly as the cell density increases. In others, such as with insulin, the inverse was observed. In the DE receptor system, neither a net loss nor gain was observed as the cells grew and became dense.

Cells were plated at different densities and the number of receptors/cell measured at various time intervals afterwards. The observed number of receptors was plotted vs. the cell density and it was obvious that there was no correlation. While not a terribly interesting result, it is nice to know that we need not worry about any artifacts creeping into our experiments due to a cell density-dependent regulation of receptor numbers.

A second area of interest was the effect of glucocorticoids on receptor levels. Again in some hormone systems, steroids (particularly glucocorticoids) can alter the number of receptors a cell maintains on its surface. Glucocorticoids do not have any measurable effect on diphtheria toxin receptor numbers (Table III); 2 other representative steroids, testosterone and estradiol, were also examined with similar negative results.

TABLE III. EFFECTS OF GLUCOCORTICOIDS ON THE CELL MEMBRANE DIPHTHERIA TOXIN RECEPTOR LEVEL[a]

STEROID	TOXIN BOUND	
	4°C	37°C
Control	15580 ± 640	8170 ± 160
Triamcinolone Acetonide (100 µg/ml)	14890 ± 480	7100 ± 360
Testosterone (1 mg/ml)	13030 ± 310	7720 ± 610
Estradiol (1 mg/ml)	14330 ± 1340	6820 ± 390

[a]Cells were incubated with steroids for 24 hr and the receptor level measured by binding for 12 hr at 4°C or 1-½ hr at 37°C.

Although the above 2 conditions were without effect on DE receptor numbers, it became apparent that the cell does have some fine-tune controls. Inhibition of protein synthesis by cycloheximide led to a reduction of 60-70% in cell-surface DE receptors. Neither inhibition of RNA synthesis by actinomycin D (Act-D) nor inhibition of DNA synthesis by mitomycin C produced changes in the steady-state receptor numbers. The effect of cycloheximide is apparently due to inhibition of protein synthesis, not a undefined/non specific drug effect, since puromycin, another protein synthesis-inhibitory drug, also induces a loss of receptors.

To eliminate the possibility that cycloheximide (or puromycin) was simply toxic to the cells, we demonstrated that the loss of receptors was reversible when the drug was washed out. Cells were preincubated with cycloheximide for 2 hr, then washed and the number of cell surface receptor measured as a function of time. The

original receptor number was regained in 1-2 hr. Significantly, the regain could be blocked by readdition of cycloheximide but not by Act D. Thus, the steady-state number of cell surface DE receptor is dependent on protein synthesis. That fact that Act D has no effect on the system leads us to conclude that the protein synthesis-dependent step is at the level of translation, not transcription.

In another approach to studying cell controls of toxin receptor numbers, we first treated cells with trypsin to remove surface receptor and then followed the course of receptor regeneration. Treatment with trypsin reduced the surface receptors to very low numbers. If the cells were maintained at 37°C, receptor availability for binding was regenerated as a function of time. Cells maintained at 4°C did not regenerate receptors. The reappearance of receptor was, at 37°C, blocked by both actinomycin D and cycloheximide but not by mitomycin C. This receptor regeneration requires messenger RNA and protein synthesis, but not DNA synthesis. The straightforward interpretation of this data is that after removal of toxin receptors with trypsin, the cell must synthesize new receptor de novo in order to restore its original numbers. Of necessity this leads to 2 other conclusions: Under normal circumstances, there must be a negligible concentration of receptor-coding messenger RNA in the cell, since regeneration is actinomycin D-sensitive. There must not be a large intracellular pool of presynthesized receptors which could be utilized for regeneration since synthesis of mRNA is clearly required after trypsinization.

We have already shown that Con A blocks the internalization of DE bound to its receptor. The effects of Con A on the unbound receptor were also investigated. After Con A was added to cells, no change in toxin receptor numbers were observed for 1 hr. There then occurred an increase in the receptor number until a new steady-state count was reached (4-5 hr). This new number was variable, ranging between 160-300% of controls depending on the experiment.The continuing presence of Con A on the cell is required to maintain the higher steady state receptor numbers, as was shown by the effect of α-methyl mannoside, a sugar known to bring about the disassociation of Con A from its cell-membrane binding sides.

Of considerable interest was the effect of drugs on the Con A induced receptor increase. If Act D or cycloheximide were added simultaneously with Con A, the lectin-induced increase in receptor numbers was abolished. However, if Act D was added 1 hr after Con A, a point at which the receptor number was still at control values, the increase could not be blocked. Nor did the addition of actinomycin D have any effects on the new higher, steady-state receptor number when added at 4 hr. On the other hand, cycloheximide added 1 hr after Con A still blocked the increase, while added at 4 hr it had no effect on, or slightly reduced, the higher steady-state counts. These effects of drugs on the Con A elicited increase of receptors are consistent with the notion that Con A induces the de novo synthesis of new receptors.

To further define the time frame of Act D sensitivity, we added it at much shorter time intervals after Con A challenge. For about 20 min after Con A addition the drug blocked the increase in toxin receptors. From 20-40 min after lectin addition, it became progressively less effective, until, from 40 min on, the drug no longer worked. We interpret this experiment to indicate that no receptor coding mRNA is synthesized for about 20 min after Con A addition. Then from 20-40 after Con A mRNA is synthesized and enough accumulates by 40 min to supply cellular requirements. From 40 min on, mRNA is probably still synthesized but since the required pool is already available, Act D is no longer effective.

The increase in toxin receptor numbers induced by Con A and the decrease caused by protein synthesis inhibitors may reflect changes in receptor number, affinity or

both. To determine which of these possibilities obtain, we incubated cells with either cycloheximide or Con A and then measured toxin binding as a function of concentration, i.e., a binding isotherm. The data obtained were then plotted by the Scatchard method. It was obvious that the slope of the data points was similar in each study while their relative positions and X intercepts were shifted either to the left (cycloheximide) or right (Con A). This finding is consistent with the interpretation that toxin-receptor binding affinities are not significantly altered by cycloheximide or Con A treatment, but that there is a change in the number of binding sites.

In another series of experiments, we treated the ability of ATP to block toxin-receptor binding after treatment of the cells with Con A or cycloheximide. This was one because we have previously shown that certain nucleotides (ATP among them) competitively block toxin receptor binding; nucleotides thus can serve as a probe of the toxin-receptor interaction. We found that binding of toxin to receptor on cycloheximide- or Con A-treated cells was blocked by ATP in a pattern not detectably different from untreated cells. We, therefore, conclude that by this one measure, the DE receptors present after Con A or cycloheximide treatment are functionally similar to controls.

We propose a model to explain all the drug effects described here. Toxin re-receptors are continually taken inside the cell or into the membrane into a position where they can no longer bind toxin. Reinsertation of these same receptors regularly takes place and is dependent on protein synthesis at the level of translation. The reinsertation process must be highly efficient and conservative since newly synthesized receptors are not required to maintain the normal steady-state as shown by the absence of an effect of Act D. On the other hand, if we remove the receptors by trypsinization, a condition where synthesis of new receptors should be required, Act D works as predicted.

Since Con A clearly blocks the internalization of toxin bound to its receptor, we believe it reasonable to propose that Con A also prevents the internalization of the unbound receptor. Apparently preventing receptor internalization acts as a signal to the cell to synthesize new receptors. The evidence for this is that the Con A effect is blocked by Act D, but only if the drug is added simultaneously or within 20-30 min following the lectin.

We have previously shown that many drugs and chemicals protect cells from DE. It is possible that several of them may somehow reduce the number of DE receptors on the cell membrane. In a preliminary experiment we tested several of these drugs and found many did lower the number of available receptors. Table IV shows that NaF, procaine, lidocaine, arsenite and ruthenium red all function this way; NH_4Cl has no effect. The effects of these drugs will all be examined in detail.

TABLE IV. EFFECTS OF DRUGS ON CELL MEMBRANE DIPHTHERIA TOXIN RECEPTOR LEVELS[a]

DRUG	% OF CONTROL RECEPTOR
NaF (5mM)	1
NH_4Cl (0.5 mg/ml)	108
Procaine (10 μM)	26
Lidocaine (10 μM)	51
Arsenite (100 μM)	26
Ruthenium red (1 mM)	26

[a]Cells were incubated with drugs for 3 hr at 37°C. Cells were washed and receptor measured by binding with toxin at 4°C.

The other toxin under study is Pseudomonas aeruginosa exotoxin A (PE). Work with the PE receptor system has not advanced as we would have liked, but some progress has been made. As with the DE system, only those cell lines which are sensitive to PE will bind it specifically (Table V). Although we originally found that mouse liver lines bind PE specifically, it is clear (and predictable) that the L-929 and 3T3 bind PE as well or better. Thus, we have gone over to the use of the established mouse fibroblast lines in our work. The specificity of PE binding is indicated by the fact that the relatively PE-insensitive lines, Vero and MK-2, do not exhibit significant specific binding.

TABLE V. BINDING OF ^{125}I-PSEUDOMONAS EXOTOXIN TO VARIOUS CELL LINES[a]

CELL LINE	COUNTS WELL	AVERAGE COUNT	SPECIFIC COUNTS	NO. CELLS (X 10^5)	SPECIFIC COUNTS/ 10^5 CELLS
L-929	8890 9073	8982	2244	1.0	2244
	6092 7383	6738			
CCl-6	8868 6833	7851	0	4.0	0
	7773 9756	8765			
HeLa	17729 14195	15962	1048	2.2	476
	13988 15839	14914			
3T3	20488 20066	20277	10243	1.6	6401
	10095 9973	10034			
MK-2	13889 12101	12995	0	1.4	0
	12684 14972	13828			

[a]Cells were incubated overnight at 4°C with ^{125}I-Pseudomonas toxin (2 µg/ml) (first 2 numbers, second column) or ^{125}I-toxin plus 100 µg/ml unlabeled toxin (second 2 numbers, second column). Monolayers were rinsed well and dissolved in base for scintillation counting. Three other wells were trypsinized to obtain cell counts.

The big problem we now face is the high level of nonspecific binding encountered with PE-binding studies. All 3 lines exhibiting specific PE binding show 40-60% nonspecific counts at 4°C, a temperature where all biophysical measurements (association constant, disassociation constant, number of receptors/cells, etc.) must be made. The data at 37°C are easier to work with, nonspecific binding being only 20-30%, but more difficult to interpret since the total cell-associated counts represent a sum of toxin bound to cell surface receptors, toxin internalized and toxin degraded. We are concentrating our efforts towards the lowering of the 4°C nonspecific binding, but thus far have not had much success.

Presentations:

1. Middlebrook, J. L., R. B. Dorland, S. H. Leppla, and J. D. White. Receptor mediated binding and internalization of Pseudomonas exotoxin A and diphtheria toxin by mammalian cells. Presented, 6th International Symposium on Animal, Plant and Microbial Toxins, Uppsala, Sweden, 19-24 Aug 79 (Toxicon 17 (Suppl.): 125, 1979).

Publications:

1. Middlebrook, J. L. and R. B. Dorland. 1979. Protection of mammalian cells from diphtheria toxin by exogenous nucleotides. Can. J. Microbiol. 25:285-290.

2. Middlebrook, J. L. and R. B. Dorland and S. H. Leppla. 1979. Effects of lectins on the interaction of diphtheria toxin with mammalian cells. Exp. Cell Res. 121:95-101.

3. Dorland, R. B., J. L. Middlebrook, and S. H. Leppla. 1979. Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells. J. Biol. Chem. 254:in press.

4. Middlebrook, J. L., R. B. Dorland, S. H. Leppla, and J. D. White. 1979. Receptor mediated binding and internalization of Pseudomonas exotoxin A and diphtheria toxin by mammalian cells. In Natural Toxins (D. Eaker and T. Wadstrom, eds). Pergamon Press, London, in press.

LITERATURE CITED

1. Draper, R. K., D. Chin, and M. I. Simon. 1978. Diphtheria toxin has the properties of a lectin. Proc. Natl. Acad. Sci. U.S.A. 75:261-265.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6414	79 10 01	DD-DR&E(AR)636

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

10. NO CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	021
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2-2			

11. TITLE (Precede with Security Classification Code): (U) Regulation and involvement of acute-phase proteins in infections of BW 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
77 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 79	1.0	71.4
FISCAL YEAR CURRENT 80	1.0	75.7

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21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Laboratory animals; (U) Protein synthesis; (U) RNA synthesis; (U) Early detection

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 hepatic regulation of acute-phase proteins during various types of infectious diseases. Determination of the effect of these proteins on the host's defense mechanism will enable evaluation of their importance during early stages of infection. Additional studies based on these findings would deal with approaches toward controlling these processes to the optimum benefit of the host in dealing with infectious diseases of BW importance.

24 (U) Quantitative and qualitative measurement of hepatic RNA in both in vivo and in vitro systems using control and infected animals will be made. Purification and development of specific antibodies to certain acute-phase proteins will be utilized to determine the type of products made by RNA from infected hepatic cells. The involvement of these proteins in humoral or cell-mediated immune response will also be studied. Various drugs will be tested for their effect on RNA and acute-phase protein production.

25 (U) 78 10 - 79 09 - The appearance of acute-phase proteins in the serum of rats subjected to inflammatory agents has led to the study of hepatic RNA regulation, which results in their production, and the subsequent isolation of several of these proteins in order to study their regulation and function. RNA studies have shown that most, but not all, of the acute-phase proteins are increased as a result of increases in RNA transcription; endotoxin stimulates an early increase in free ribosomal RNA; bacterial infections stimulate and increase in bound RNA; and maintenance levels of hydrocortisone are necessary to observe these responses. Isolation and development of antibodies to alpha-1- and alpha-2-macroglobulin, albumin, haptoglobin, and alpha-1-acid glycoprotein are currently underway for use in future studies on their regulation and involvement in the acute-phase process.

Publication: Am. J. Physiol., 1979, in press.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 65 AND 1423-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 021: Regulation and Involvement of Acute-Phase Proteins in Infections of BW Importance

Background:

Numerous metabolic changes take place during the early stages of an infectious disease. Recent findings indicate that one of the more important responses to infection is an increase in the production of serum acute-phase proteins (1) each of which presumably plays a role in the host's defense. Studies on hepatic RNA production and distribution in response to infection have shown that there is an increase in the synthesis of RNA, which is then directed to the bound ribosomes (2), presumably for the increased production of these acute-phase proteins. Further studies on the transcription of specific mRNA and their protein products are needed to verify these observations and provide information on the regulatory mechanisms involved in this process.

Several of these acute-phase proteins have been implicated in such host responses as antiproteolytic activity, wound healing, protection of hemoglobin, transport of metal ions and regulation of the immune response. However, much is to be learned about the specific function of each of the acute-phase proteins and how this relates to the overall response of the host to inflammatory substances.

Progress:

An initial step in the isolation of most serum proteins is chromatography on a gel filtration column, which separates out the various proteins in groups according to their MW. Large batches of both normal and turpentine-inflamed rat serum were, therefore, processed through a large G-150 Sephadex column. The resulting 4 fractions were pooled, concentrated and stored frozen for future use in the isolation of specific acute-phase proteins. The following rat serum proteins discussed below are currently under study; α_1- and α_2-macroglobulin (MG), haptoglobin, albumin, and α_1-acid glycoprotein.

An additional quantity of α_1- and α_2-MG was isolated from the first fraction of the G-150 column by zonal centrifugation and stepwise elution on a DEAE-cellulose ion-exchange column. Improvement in yield from previous batches was seen due to the use of a larger zonal rotor. The resulting purified proteins were lyophilized for storage until needed.

The 3-stage technique of Lombart et al. (3) for the isolation of rat haptoglobin through DEAE-, TEAE- and CM-cellulose ion exchange columns was used. However, the resulting haptoglobin peaks, when run on thin-layer electrofocusing gels, showed impurities at each stage of the isolation, with little improvement in purity after each step. Therefore, a new technique was devised in which inflamed rat serum was chromatographed through a DEAE-cellulose column by gradient elution. The resulting 11 peaks were each tested for haptoglobin content by binding a portion to hemoglobin and comparing the 2 on thin-layer electrofocusing plates. Although there was a

significant amount of haptoglobin in 5 of the peaks, only 2, which demonstrated no other contaminating proteins at their isoelectric points, were used for further purification. These were subjected to preparative flat-bed electrofocusing, and the purified haptoglobin isolated from the appropriate resulting bands in the gels. Although the yield is low using this procedure, the haptoglobin is free of any contamination. This procedure has been successfully used for the isolation of haptoglobin starting with the #3 fraction from the G-150 Sephadex column (MW range = 70,000 to 85,000 K).

Earlier attempts at the isolation of albumin using preparative flat-bed electrofocusing resulted in an impure product, since it has been shown that there are several other proteins of similar size and isoelectric point that will not separate out on such gels. Therefore, attempts were made to isolate albumin on affi-gel blue, as used by others to remove albumin from serum. However, even with different elution techniques, contaminants still remain in the albumin fraction. Therefore, additional steps will be required to eliminate these contaminants.

A protein having a low isoelectric point, which is seen in very limited quantities in normal rat serum, but is very evident in inflamed serum, was observed eluting from the first peak of the affi-gel blue columns during the attempted isolation of albumin. Since the protein has an apparently strong negative charge, it was further purified on DEAE-cellulose. The resulting material appeared as a single band on electrofocusing plates. Due to its low isoelectric point and MW, it appears that this protein is α_1-acid glycoprotein. However, further tests will be necessary for its positive identification.

Attempts have been made to produce antibodies to each of these rat serum proteins in goats. However, the only proteins that resulted in more than a minimal antibody titer were the α_1- and α_2-MG. These antibodies were purified through a 2-stage procedure of $(NH_4)_2SO_4$ precipitation followed by ion exchange chromatography. Due to a small contamination of α_2-MG in the α_1-MG preparation, this resulted in some contamination of the goat anti-α_2-MG in the anti-α_1-MG sample. This will be removed by immunoprecipitation with α_2-MG. An automated analysis procedure for the detection of rat serum α_2-MG using this purified antibody is now available in the automated chemistry laboratory, as will be the α_1-MG when purified.

Attempts at direct purification of the goat antibodies to α_1- and α_2-MG on protein A affinity columns have been unsuccessful, due either to a lack of IgG antibodies or poor recognition of goat IgG by protein A.

Because of easier accessibility and some of the above mentioned problems with goats, we have begun producing our antibodies in rabbits. This will also facilitate antibody purification since protein A binds rabbit IgG much better than goat. Several of the new adjuvants being tested in Animal Assessment Division are being used to try to improve antibody titer.

The use of goat anti-α_2-MG bound to a support medium for the isolation of α_2-MG from serum was initially unsuccessful. This was probably due to a loss of α_2-MG from the large MW fraction of the G-150 column when stored in the freezer. This was discovered when the thawed fraction showed no response in the automated analysis of α_2-MG while the same refrigerated sample retained its activity. A fresh sample will be utilized to determine whether affinity columns of this type can be used for the isolation of α_2-MG and other proteins for which a purified antibody is available.

The use of a commercially available kit for studies of *in vitro* protein translation has been tested with commercially available mRNA and free and bound mRNA isolated in our lab. Although the other mRNA responded well, the previously observed lack of bound mRNA activity was also noted. Therefore, total extracted RNA from bound ribosomes will be tried in this system in an attempt to determine why this *in vitro* system will not translate bound mRNA.

A new technique is currently being developed for the visual analysis of proteins in a sample. The technique called "Protein mapping" employs 2-dimensional electrophoresis for the separation of proteins. They are separated in the first direction on the basis of charge using electrofocusing, and the second direction on the basis of size using an SDS-gel. This procedure should prove valuable for the comparison of actual protein content between infected and control samples.

Presentation:

Thompson, W. L. The effects of *Streptococcus pneumoniae* infection on hepatic messenger RNA production. Presented 11th Miami Winter Symposium, University of Miami, Miami. FL, Jan 1979.

Publication:

Thompson, W. L., and R. W. Wannemacher, Jr. The effects of infection and endotoxin on rat hepatic RNA production and distribution. Am. J. Physiol., in press.

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2. Thompson, W. L., and R. W. Wannemacher, Jr. 1973. Effects of infection with *Diplococcus pneumoniae* on synthesis of ribonucleic acids in rat liver. Biochem. J. 134:79-87.

3. Lombart, C., J. Moretti, and M. F. Jayle. 1964. Préparation et propriétés physiques des haptoglobines de lapin et de rat. Biochim. Biophys. Acta 97: 262-269.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6420	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	022
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Amino acid sequence analysis of pathological agents

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
77 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 79	1.0	63.3
FISCAL YEAR CURRENT 80	1.0	72.9

19. RESPONSIBLE DOD ORGANIZATION

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Pseudomonas; (U) Staphylococcal enterotoxin C; (U) Amino acids

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 amino acid sequence (primary structure) of specific proteins which are the mediators of diseases of potential BW importance. Knowledge of the covalent chemical structure of the proteins will aid in the understanding of the biochemical basis of their toxic effects. This understanding will improve our ability to treat diseases in military personnel in which these toxins contribute to the pathophysiology of the infection.

24 (U) Initially, proteins or fragments of proteins will be subjected to analysis on the Beckman 890C sequencer, with subsequent identification of the individual amino acid derivatives by gas chromatography and other standard methods. It will be necessary to establish a peptide fractionation system in order to separate peptides resulting from the fragmentation of the polypeptide chain. This is necessary in order to determine the complete amino acid sequence of such high molecular weight proteins as bacterial toxins.

25 (U) 78 10 - 79 09 - Final determination of the amino-terminal primary structures of the Staphylococcus aureus exfoliative toxins DI and TA was accomplished with the use of the Beckman sequencer. The first 26 residues of DI and the first 18 of TA were identified. Progress has continued on the amino acid sequence of S. aureus enterotoxin C-1. The identity of the 45 residues in the amino-terminal of the entire protein was determined, while 21 residues from an internal peptide (possibly its entire structure) were established.

Publication: Infect. Immun. 24:679-684, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 022: Amino Acid Sequence Analysis of Pathological Agents

Background:

The rationale for the investigation of protein amino acid (AA) sequences depends upon the fact that proteins are rich in biological information. For example, the reconstruction of genetic events and the description of evolutionary history and mechanisms is possible through the use of AA sequences of similar proteins from different species (1). The elucidation of structure-function relationships in proteins is also under investigation by the use of AA sequences (2).

Since such sequences are such a multifaceted source of fundamental biological information, the last several years have seen an explosive increase in the number of proteins subjected to such determinations. Considerable work and ingenuity have been put into the improvement of the techniques involved. Even so, the principles employed are still the same as originally developed in late 1940s and early 1950s (3). This process involves the fragmentation of the protein chain into peptides, the purification of these peptides, and then the determination of the AA sequence of each peptide. By using semiautomatic Edman equipment, such as the Beckman 890C sequencer, the work involved proceeds much more rapidly than if accomplished by manual methods alone.

Of the purified bacterial toxic proteins which are readily available and of potential BW importance, the enterotoxin C_1 of Staphylococcus aureus (SEC_1), is presently being worked on. The interest in SEC_1 is due to its role in Staphylococcal diarrhea (4). Other purified protein toxins are available, such as the exotoxin of Pseudomonas aeruginosa and the S. aureus exfoliative toxins (5). Further investigation of the AA sequences of these proteins will depend upon the availability of the proteins and considerations of the time involved.

Progress:

The past year has seen a few basic changes in the operation of the laboratory in its basic mission to determine the primary structures of proteins. During the last quarter of the year it was decided to discontinue the use of the gas chromatograph. The data received from it was supplemental to the thin layer chromatography (TLC) and acid hydrolysis of each phenylthiohydantoin (PTH)-amino acid (HI hydrolysis). In addition, the chromatographic data were usually of poor quality. The eventual use of high performance liquid chromatography (HPLC) will more than offset the discontinuance of the technique.

Use of the gas chromatograph was also discontinued due to a reorganization of the Clinical Laboratory. As a result, all equipment had to be fitted into one room instead of two. This consolidation required maximum use of a limited amount of space. Taking into consideration the above factors, it was decided the time was right to discontinue its use. The establishment of the capability for protein separation and purification has also been delayed. Lack of space and the delay in the relocation of the laboratory to a permanent site are the contributing factors.

Although sequence analysis was concentrated on the SEC_1, 2 other proteins were studied. The number of "sequenced" residues varied for each protein. Each resulting PTH amino acid was identified by TLC and HI hydrolysis. During the first half of the year gas chromatography was also used to aid the identification process.

The sequence analysis of the S. aureus exfoliative toxins DI and TA was completed. Final determination of the amino-terminal primary structures was identified to the extent necessary. The following AA sequence for DI has been deduced: H_2N-Lys(1) Glu Tyr Ala Ala(5) Glu Glu Ile Arg Lys(10) Leu Lys Glu Lys Phe(15) Glu Val Pro Pro Thr(20) Asp Lys Glu Leu Tyr(25) Thr(26) . . . - COOH. For TA, the following primary structure was determined: H_2N-Glu(1) Val Thr Ala Glu(5) Glu Ile Lys Lys His(10) Glu Glu Lys Trp Asp Lys(15) Tyr Tyr Gly Val(20) ? Asx Phe(23) . . . - COOH. The question mark (?) represents a residue for which no intelligent identification could be assigned.

The SEC_1 entire protein was subjected to AA sequence analysis. In addition, the amino-terminal fragment (RCAM-6500) and an internal peptide (RCAM-4000) were studied.

Results from numerous sequence analyses of the RCAM-4000 peptide have yielded the following results: H_2N - Asn(1) Tyr Asp Lys Val Lys(5) Thr Glu Leu Leu Asn(10) Glu Gly Leu Ala(15) Lys Lys Tyr Lys Asx (Lys)(20,21) . . . - COOH. The code name in parenthesis represents a tentative conclusion, while the others are more definitive. Although the analysis of this peptide indicates the presence of additional AA, no residues past the 21st have been found. It was assumed that the remaining segment of the peptide was being washed out of the sequencer cup prematurely. In order to remedy this possibility, a polyquaternary amine, Polybrene, was used in an attempt to hold the entire peptide in the cup. Although literature reference indicated that Polybrene would work, repeated efforts with RCAM-4000 have not produced appreciable results. Further experimentation will be conducted on this peptide.

Progress on the amino-terminal segment of SEC_1 has yielded the following primary structure: H_2N - Glu(1) Ser Gln Pro Asp(5) Pro Thr Pro Asp Glu(10) Leu His Lys Ser Ser(15) Lys Phe Thr Gly Leu(20) Met Glu Asn Met Lys(25) Val Leu Tyr Asx Asx(30) Asx (Tyr) (Val) (Ser) (Ala)(35) ? (Asx) (Val) ? ?(40) ? (Asx) (Glx) (Phe) (Phe)(45) . . . - COOH. The majority of the new results has come about thru the novel application of a familiar technique, that of CNBr cleavage at the carboxy-side of the methionine residues in peptides. By carrying out the procedure while the material is in the Beckman sequencer cup, loss of material is prevented. The 2 methionines are in locations which yield 2 large and 1 small peptide when RCAM-6500 is cleaved. Results of using this technique have enabled the extension of the known AA sequence of the amino terminal of SEC_1. This has occurred from use of the CNBr both before any "sequencing" had taken place on the peptide and after 18-23 cycles had been run. An additional reason for the later procedure was to aid in pinpointing the location of the methionine residues. Polybrene was used each time to aid in the retention of the resulting peptides. Work continues on this peptide and the rest of SEC_1.

Presentations:

None.

Publications:

1. Johnson, A. D., L. Spero, J. S. Cades, and B. T. Decicco. 1979. Purification and Characterization of Different Types of Exfoliative Toxin from Staphylococcus aureus. Infect. Immun. 24:679-684.

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1. Nolan, C., and E. Margoliash. 1968. Comparative aspects of primary structures of proteins. Ann. Rev. Biochem. 37:727-790.

2. Niall, H. D., H. Keutmann, R. Sauer, M. Hogan, B. Dawson, G. R. Aurbach, and J. Potts, Jr. 1970. The amino acid sequence of bovine parathyroid hormone I. Hoppe Seylers Z. Physiol. Chem. 351:1586-1588.

3. Brown, H., F. Sanger, and R. Kitai. 1955. The structure of pig and sheep insulins. Biochem. J. 60:556-565.

4. U.S. Army Medical Research Institute of Infectious Diseases. 1978. Annual Progress Report, FY 1978, pp. 347-353, Fort Detrick, Frederick, MD.

5. U.S. Army Medical Research Institute of Infectious Diseases. 1978. Annual Progress Report, FY 1978, pp. 229-233, Fort Detrick, Frederick, MD.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6427	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	023
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Biochemical events at the cellular level: possible early indicators of 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
77 11	79 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 79	0.5	72.8
	CURRENT 80	0	0

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NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Bacterial diseases; (U) Rapid diagnosis; (U) Blood platelets

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

23 (U) Identify metabolic and functional changes in circulating platelets that might be useful in early detection of infections of importance in BW defense. Since platelets are among the first of the host's cells to interact with invading organisms, this study will monitor alterations in platelet parameters to determine if any are of value for detection of infection well in advance of clinical symptoms, such as fever.

24 (U) Isolate platelets from infected and control animals during the early stages of illness and measure biochemical parameters which are probably early indicators of infection.

25 (U) 78 10 - 79 09 - Changes in rat platelet function and composition in response to bacterial infection have been thoroughly investigated for their potential for early detection. Francisella tularensis (LVS) infection causes a statistically significant decrease in serum free arachidonic and linoleic acids, and platelet-free linoleic acid relative to other long-chain fatty acids. These changes begin before the onset of fever but remain small, differing only a little. Metabolites involved in the platelet-release reaction such as ATP and serotonin show dramatic 10- to 50-fold concentration decreases in platelets of infected rats, but only late in the infection. No statistically significant differences in aggregation were observed between platelets from infected or control rats unless endotoxin was also involved. Based on the parameters investigated to date, the platelet is not useful as a means of early detection of infection, and this work unit is therefore terminated.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 023: Biochemical Events at the Cellular Level: Possible Early Indicators of Infection

Background:

The biochemical events investigated as early indicators of infection in this project have focused on those associated with platelets. This is because platelets are known to be among the first host cells of an infected individual to respond to infection. They respond to an invading organism by a release of constituents affecting aggregation, and by aggregation itself, which can be either reversible or irreversible. Platelet aggregation results in the production of dissociable complexes with bacteria or other foreign particles, thus enhancing the clearance of particles to the reticuloendothelial system.

As a result of interactions with bacteria, individual platelets in circulation may be altered by going through the release reaction, or even a reversible aggregation. This causes changes in platelet composition and function which can be measured. Another way interactions with bacteria could result in measurable differences in platelets is alteration of the circulating platelet population. Irreversible aggregates, which deplete the circulating population of the more dense and reactive platelets, would result in measurable differences in platelet-rich plasma collected for analysis. Changes in platelet function and structure during infection were monitored by measuring aggregation ability and levels of various constituents, respectively.

Progress:

Platelet function. Platelet functions may change in response to subtle interactions that do not alter platelet composition detectably. Platelet function was monitored by following aggregation in response to collagen and other aggregating agents. Both in vivo and in vitro studies were conducted. In order to differentiate between the rate of infection itself and that of endotoxins superimposed on infection, the in vivo studies were done with the live vaccine strain (LVS) of Francisella tularensis. This organism does not produce endotoxin, and causes an illness in rats, in which onset can be controlled to allow adequate time for early detection studies. A 10^6 cell/100 gm dose of F. tularensis was used; this is a concentration which results in fever 24 hr after IP injection. For comparison, the effects of the endotoxin-producing organisms, Escherichia coli and Salmonella typhimurium, on rat platelets were also examined.

Results indicate that platelets collected from fasting rats infected with LVS F. tularensis do not differ significantly in their ability to aggregate from those of fasting control rats receiving heat-killed F. tularensis or saline. To observe significant changes, fairly large aggregation differences would have to be seen, because variability between platelets from different rats produces standard deviations of 10-20% aggregation.

The effect of bacteria upon platelets was rechecked by *in vitro* experiments in which several bacteria were used as aggregating agents. It was found that *F. tularensis* did not cause aggregation of platelets, even at 5×10^9 bacteria/ml. The other bacteria tested (*S. typhimurium*, *E. coli*, and *S. aureus*) produced complete platelet aggregation at 5×10^7 bacteria/ml. These latter bacteria all contain endotoxin.

It is known that endotoxin causes platelet aggregation. Data from experiments on this project indicate that platelets from rats injected with endotoxin lose their ability to aggregate in response to collagen. This loss of platelet function is less pronounced with other aggregating agents, as shown in the table below, which shows data for platelets taken from rats 5 hr after injection with *E. coli* endotoxin. There are 3 rats per group, and platelet concentrations were adjusted to the same level for all experiments.

AGGREGATING AGENT	MEAN ± SE PLATELET CONCENTRATION		Significance
	1 mg/ml endotoxin	saline	P
Collagen	3 ± 4	59 ± 1	$p < 0.001$
Thrombin	35 ± 11	66 ± 2	< 0.05
ADP	49 ± 5	60 ± 1	

Possible explanations of these data are that platelets which respond well to collagen are removed from circulation by endotoxin, or exposure to endotoxin makes platelets less reactive to collagen.

Sialyl transferase. Platelet interactions with collagen and other substrates have been shown to involve the transfer of sialyl residues, a reaction catalyzed by the membrane-associated enzyme, platelet sialyl transferase. During the transfer reaction, collagen is bound to the platelet. If more than one platelet is sialating the same collagen molecule, aggregation can result.

Because platelet sialyl transferase is involved so closely with platelet aggregation, studies were undertaken to see if changes in the activities of this enzyme could be used for early detection of bacterial infection. A 10^7 *F. tularensis*/100 gm IP injection resulted in a transient increase of enzyme activity of 25% ($P < 0.1$) coincident with the onset of fever. The enzyme activity was measured by determining the rate of incorporation of radioactive sialic acid into a suitable substrate, fetuin. There appeared to be no value to monitoring sialyl transferase as a means of early detection, since the change observed did not precede the onset of fever.

Platelet release. Upon stimulation by aggregating agents, platelets release a number of constituents into its environment. These constituents, which are referred to as "release factors", include known aggregating agents and therefore amplify the initial stimulus. Several of these have been examined for use as early detection indicators.

Arachidonic acid is both an aggregating agent and a release factor. Platelets contain relatively large amounts of this fatty acid (FA), and can rapidly replace depleted arachidonic acid by biosynthesis from linoleic acid, hydrolysis of esterified arachidonic acid components of its membranes, or from the serum.

Because of its importance in aggregation, a study of arachidonic acid and other FA was undertaken. Free and esterified FA components of platelets and serum were isolated from F. tularensis-infected and control rats and processed for gas chromatographic analysis. The results for serum FFA indicate that in the infected rats arachidonic acid and linoleic acid are decreased relative to the other long-chain FA present. For instance, at 24 hr postinfection, arachidonic acid is 14% of the total long-chain FA measured in infected rat serum compared to 20% in serum from control rats injected with saline or heat-killed F. tularensis ($P < 0.005$). For linoleic acid, the essential FA precursor of arachidonic acid, there is a parallel decrease with the infected rats' serum FA being composed of 15% linoleic acid and control rats', 20% ($P \leq 0.005$). These changes begin at 12 hr, while fever started at 24 hr.

Platelet FFA show a similar change for linoleic acid, although to a lesser extent. The experiments with esterified FA showed no statistically significant changes with infection.

There is a possibility that serum arachidonic acid can be useful for early detection; however, the changes observed in serum or platelet FA were only a few percent, and did not occur early enough in infection to offset the disadvantage of sample preparation time.

Platelet ATP was assayed by the luciferin assay procedure, using platelets from control rats and from rats infected with LVS F. tularensis. A 30-50-fold decrease in platelet ATP levels was found for infected rats. Unfortunately, dramatic as this change was, it occurred relatively late, at 40 hr.

Platelet serotonin was assayed by monitoring the fluorescence of lysed platelets from infected and control rats. Again, there was a dramatic 10-fold drop in levels of serotonin in platelets from infected rats at 40 hr. This decrease was not significant at 24 hr, when the fever was beginning. Malondialdehyde, a metabolite of arachidonic acid, was monitored in serum and platelets by a colorimetric method. It was found that there was a decrease in platelet malondialdehyde at 40 hr which correlated nicely with an increase in the serum level at the same time. No difference was seen in infected and control rats any earlier.

Platelets are not useful as a method for early detection. The work unit is terminated.

Publications:

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6429	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	024
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Diagnosis and pathology of Legionnaires' 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
78 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 79	2.5	440.4
	CURRENT 80	2.5	403.2

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21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Laboratory animals; (U) Special containment facilities; (U) Legionnaires' disease

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

23 (U) Develop rapid serologic method for diagnosis of Legionnaires' disease organism (LDO), determine optimal conditions for growth and survival of LDO and define factors involved in pathophysiology of the disease. If successful, study aerosol infectivity in appropriate laboratory animals. This study will allow the assessment of the military importance of this organism in either natural infections or biological warfare and may well proceed to vaccine development.

24 (U) Starting with information, procedures and sera from CDC, develop a serum band, assess strain differences, and test a variety of growth media. If successful, use subhuman primates for virulence studies.

25 (U) 78 10 - 79 08 - In the past year we introduced a new liquid medium in which small inocula Legionella pneumophila would grow rapidly and in high concentration without loss of antigenicity and virulence. The histopathologic finding of L. pneumophila infections in the AKR/J mouse model were introduced in the First International Symposium on Legionnaires' Disease (November 1978). The enzyme-linked immunosorbent assay (ELISA) has been successfully used with this organism; the test had better sensitivity than micro- and indirect hemagglutination tests. Serotypes still cannot be differentiated. Additional cooperative studies have been undertaken investigating Legionella-macrophage interactions and the dynamics of aerosolization.

Publications: Ann. Intern. Med. 90:676-679, 1979;
Infect. Immun. 25:761-763, 1979;
J. Clin. Microbiol., in press, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 024: Diagnosis and Pathology of Legionnaires' Disease

Background:

Legionella pneumophila was first recognized as the etiological agent of Legionnaires' disease by McDade et al. (1), following the explosive outbreak of pneumonia among American Legion members attending a convention in Philadelphia in the summer of 1976. As new infectious diseases occur, often only identified by epidemic signals, increasingly sophisticated laboratory techniques must be utilized to identify new microbial agents. Organisms that are biochemically inactive defy our current biochemical taxonomic methods and severely stress all methods to handle them with traditional techniques.

Legionella pneumophila is a gram-negative, highly fastidious pleomorphic bacterium. On the enriched laboratory media presently used, growth requires a large inoculum; single colonies do not reach countable size for 3-4 days. Primary isolation growth is slow or may not occur at all. Experiments in bacterial physiology, virulence and immunology require large numbers of organisms in well-defined stages of growth. Currently, slow growth on solid media prevents a definition of these stages and yields low numbers of organisms. Several investigators have recently described broth media for the growth of L. pneumophila. These media all have extended generation times and require large inocula, which make them difficult to use.

Progress:

Liquid medium. A liquid medium has been formulated in which small inocula grow rapidly and give high concentrations of bacteria without loss of antigenicity and virulence. The lag, logarithmic, stationary and death phases are easily determined and reproducible. The formula per liter of medium is 10 g yeast extract (Difco), 0.4 gm L-cysteine-HCl·H_2O (Sigma), 0.25 gm ferric pyrophosphate (CDC) and 1000 ml distilled water. The ingredients are all added to the distilled water, and then membrane-filter (0.45 µm) sterilized. The pH is adjusted to 6.9 after sterilization.

Strain L-3 was inoculated into 500 ml of yeast extract broth (YEB) in a 1-L Erlenmeyer flask and incubated in a controlled environment incubator shaker at 37°C at approximately 100 oscillations/min without CO_2. Under these conditions the following results were obtained. The strain has a lag phase of approximately 8 hr. Logarithmic growth proceeded from the 8th hour to the 40th hour, attaining a maximum cell population of 2×10^9 CFU/ml. The average generation time was 99 min. The stationary phase began at 40 hr and proceeded through 62 hr. The cell population at 62 hr was 1×10^9 CFU/ml. The pH showed little variation from the initial 6.9. The final pH at 62 hr was 7.04. Fluorescent microscopy has been performed on cells grown in the yeast extract broth. These cells at 40 hr gave 3+ fluorescence. Charcoal yeast extract agar grown cells at 72 hr also gave 3+ staining. Our studies show that YEB autoclaved in the absence of Norite A will not support the growth of Legionella. By filter-sterilizing the medium, the need for autoclaving was eliminated

and the charcoal could be removed. Since the primary function of charcoal is that of an absorbant, it was assumed that an inhibitory compound(s) was produced and removed by the charcoal. Further investigation is in progress to determine the nature of this inhibition. To date 14 strains of L. pneumophila have been grown in YEB. Their growth rates have not been calculated, but they are approximately the same as the L-3 strain. Ferric nitrate has been used in place of ferric pyrophosphate with similar results, but was discontinued because it produced a cloudy medium. Yeast extract broth provides a means of producing large numbers of L. pneumophila in a short period of time. It also provides a system for investigation of antigens and possible toxins. The additions of agar or agarose to YEB has provided a clear medium for immunological studies. It is hoped that further investigation with filtration media will produce a less complex medium, so that further psychological investigations can be completed.

Antigenic analysis. An enzyme-linked immunosorbent assay (ELISA) for detecting antibodies to L. pneumophila has been developed. It involves using a simply prepared antigen from autoclaved organisms.

Antigen is prepared by growing L. pneumophila on CYE agar plates for 72 hr at 37°C. Growth is harvested under a microbiological safety hood by adding 3 ml of sterile normal saline to each agar plate and removing the growth by scraping with a bent glass rod. A suspension of 1 x 10^{10} CFU/ml in normal saline is then autoclaved for 15 min at 121°C. The suspension is centrifuged; the supernatant fluid is decanted and designated as stock soluble antigen.

The test procedure used is that of Farshy et al. (3). We have found this procedure utilizing heat-extracted antigen, to be very serogroup specific. When comparisons are made with bacterial microagglutination and indirect hemagglutination tests utilizing the same antigen preparation, better specificity is seen with the ELISA.

Bacterial antigens have been prepared for all 4 serogroups and a sensitive bacterial agglutination test has been developed. Rabbit antisera produced against all 4 serogroups are now available; these antigens and antisera provide a powerful diagnostic tool. Extraction of the organism by autoclaving has shown that serotype-specific antigens reside on the outside of the organism. Biochemical analysis of the whole bacterium by polyacrylamide gels shows a definite similarity of all our strains. The gel patterns repeatedly give 31 peaks when scanned spectrophotometrically. The protein peaks are identical for all 4 serogroups and provide a way to speciate the Legionella. The test at this time cannot differentiate one serotype from another.

Collaboration with Aerobiology Division has produced an aerosol model in guinea pigs and a publication has been submitted.

Determination of survival time. Determination of survival time and growth characteristics of the L. pneumophila under a variety of laboratory conditions has continued. Isolates obtained from Dr. J. F. McDade at CDC in October 1977 which were stored as received at -70°C continue to survive, as do most of the subcultures prepared at that time on Mueller-Hinton agar supplemented with 1% hemoglobin and 2% Isovitalex (MHA) and suspended in 50% rabbit serum in tryptose saline before storage at -70°C. Some subcultures of the Pontiac isolate stored at -70°C at that time are no longer viable; all subcultures of this isolate on MHA slants covered

with glycerol and stored at -10°C were nonviable after 16 mon. As noted in the 1978 Annual Report, cultures of L. pneumophila on MHA stored at 4°C or in the 35°C 5% CO_2 incubator did not survive longer than 1 mon; however, suspensions of the L-2 isolate held at room temperature survived for 109 days in tryptose saline, 162 days in gel saline or phosphate buffered saline and 420 days in pond water. Suspensions of the Pontiac isolate survived for 109 days under the same conditions. When exposed to UV light in the working area of a Blickman hood, a few organisms of the L-2, and Washington Pontiac strains were able to survive 60-sec exposure, but none survived 65 sec.

Solid media currently recommended by CDC for the cultivation of this organism and filtered yeast extract agar (FYE), were compared for effectiveness in recovering viable Washington isolate in tryptose saline suspensions, and in stimulating rapid growth of the organisms (Table I). The effect of storage on the effectiveness of the media was also evaluated. All of the media were contained in petri dishes placed in sealed plastic bags stored at 4°C. The media prepared with Difco yeast extract and sterilized by filtration (FYE) or with addition of acid-washed, activated charcoal (Norite A) and sterilized by autoclaving (CYE) were supplemented with cysteine and ferric pyrophosphate.

TABLE I. COMPARISON OF MEDIA EFFECTIVENESS IN RECOVERY OF VIABLE LEGIONELLA IN TRYPTOSE SALINE SUSPENSIONS

DAYS STORAGE	VIABLE ORGANISMS/ml X 10^5 RECOVERED ON MEDIA[a]				
	MHA	CYE	FYE	FGA (BBL)	FGA (Difco)
3	12,000	12,000	10,900	4.7	430
7	6,100	7,300	11,000	2.3	320
14	12,000	9,200	10,700	8.0	250
21	12,000	6,200	10,400	1.4	130
35	620	5,300	4,400	1.0	58
49	690	3,700		0.2	0

Direct microscopic count of suspensions = 7.0 - 10.0 x 10^8/ml.

[a] Inoculated with 10^2 - 10^9 Washington isolate in 10-fold increments.

MHA, CYE and FYE consistently permit the development of colonies from all organisms detected in suspensions by direct microscopic count using the Petroff-Hausser method. However, CYE and FYE are the only media tested to date which consistently permit growth of organisms inoculated directly from embryonated egg yolk sac culture, and which do not result in attenuation of virulence. In addition, countable colonies appear on CYE and FYE 36-48 hr after inoculation as compared to 72-96 hr required using other media. Recovery of viable Legionella using Feeley-Gorman (FGA) modified MHA (substitution of cysteine and ferric pyrophosphate for

Isovitalex and hemoglobin) is about 1/10th as effective when FGA is formulated with Difco products, and 1/1000th as effective when BBL products are used.

No marked change in the effectiveness of any media occurred after 3 weeks of storage. After 5 weeks of storage the effectiveness of CYE and FYE remained unchanged, but a definite decrease in the effectiveness of MHA and FGA was apparent. It should be noted that FYE and FGA are the only solid media available to date which are transparent, and only FGA permits detection of the water-soluble brown pigment produced by this microbe.

Attempts to produce selective media have been unsuccessful to date, because Legionella are sensitive to concentrations of antibiotics and chemical inhibitors which prevent growth of usual contaminants in clinical and environment material, e.g. sodium azide, copper sulfate, bile salts, sodium desoxycholate, Vancomycin, Colistin, and Nystatin. In spite of the organisms' apparent low resistance to chemical and physical agents (1), suspensions have survived for at least 2 weeks in local tap water and water from a number of local air-conditioning system cooling towers and public fountains. The organisms were killed between 1 and 5 days after suspension in USAMRIID cooling tower water which had been sample 1 hr after routine addition of ENTEC 340 (a quaternary ammonium biocide), but survived in water sampled immediately before the biweekly addition of the biocide.

Further study of the growth characteristics of L. pneumophila in the liquid phase of a biphasic supplemented MH medium has revealed that the correlation between population density, change in pH, and release of melanin pigment is the same whether the initial inoculum is 2 organisms/ml or 1×10^6/ml of medium. The doubling time during logarithmic growth is 6 hr, and when the population reaches 5×10^6/ml the pH begins to decrease. When the population density reaches 5×10^9/ml no further increase occurs, and during a stationary phase of 3 days the pH continues to decrease until it reaches pH 6.2, at which point the population begins to decrease, melanin pigment appears, and the pH begins to rise. The logarithmic death phase continues until no viable organisms can be detected and the pH reaches 7.2.

Attempts to develop media and methods for more rapid growth, selection and identification of Legionella from clinical specimens and natural reservoirs are continuing.

Presentations:

1. Hedlund, K. W., V. McGann, S. Little, and R. Allen. Humoral protection against the Legionnaires' disease bacterium in a new animal model. Presented, International Symposium on Legionnaires' Disease, 13-15 Nov 78, Atlanta, GA.

2. Janssen, W. A., and R. G. Larson. Studies on the survival and growth of Legionnaires' disease bacteria in vitro. Presented, Annual Meeting, ASM, 4-11 May 79, Los Angeles, CA (Abstr. Ann. Meeting-1979, p. 50).

Publications:

1. Hedlund, K. W., V. G. McGann, D. S. Copeland, S. F. Little, and R. G. Allen. 1979. Immunologic protection against the Legionnaires' disease bacterium in the AKR/J mouse. Ann. Intern. Med. 90:676-679.

2. Kishimoto, R. A., M. D. Kastello, J. D. White, F. G. Shirey, V. G. McGann, E. W. Larson, and K. W. Hedlund. 1979. In vitro interaction between normal cynolmolgus monkey alveolar macrophages and Legionnaires disease bacgeria. Infect. Immun. 25:761-763.

LITERATURE CITED

1. McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, W. R. Dowdle, and the Laboratory Investigation Team. 1977. Legionnaires' disease. Isolation of a bacterium and demonstration of its role in other respiratory disease. N. Engl. J. Med. 297:1197-1203.

2. Wang, W. L. L., M. J. Blaser, J. Cravens, and M. A. Johnson. 1979. Growth, survival, and resistance of the Legionnaires' disease bacterium. Ann. Intern. Med. 90:614-618.

3. Farshy, C. E., G. C. Klein, and J. C. Feeley. 1978. Detection of antibodies to Legionnaires' disease organism by microagglutination and micro-enzyme-linked immunosorbent assay tests. J. Clin. Microbiol. 7:327-331.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6411	79 07 27	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	025
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code): (U) Leukocyte adherence inhibition and macrophage migration inhibition to microbial antigens

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
78 04	79 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 79	0.9	109.3
	CURRENT 80	0	0

19. RESPONSIBLE DOD ORGANIZATION
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NAME:
NAME:

21. GENERAL USE: Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Leukocytes; (U) Macrophages; (U) Cell-mediated immunity; (U) Lymphocyte transformation

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

23 (U) Examine the leukocyte adherence inhibition (LIA) test as a means of detecting specific cell-mediated immunity (CMI) to microbial antigens of military medical significance in man and experimental animals and correlate with the more conventional macrophage migration factor (MIF) and lymphocyte transformation tests. Utilize LIA and MIF tests to study CMI against infectious microorganisms.

24 (U) Examine LIA response of experimental animals with known immune status to tularemia and/or purified protein derivative, i.e., tuberculosis. Perform simultaneous LAI and MIF assays on animals to validate LAI results. The second phase will be to expand LAI and MIF studies to humans in order to examine the temporal course of CMI induction.

25 (U) 78 10 - 79 07 - When immune cells are preincubated with specific antigen adherence stimulation occurs, while cell suspensions plus antigen that are not preincubated show a trend toward adherence inhibition. Marked variation in adherence occurred in animals immunized and tested in exactly the same manner. No definite pattern in the adherence of cells could be established. The overall results of the assay are unpredictable and unreliable. Lymphocyte transformation (LT) assays were also nonspecific and unreliable, primarily a result of extreme variations in harvesting the cell suspensions due to faulty equipment. Because of the unreliability of the LAI test and the difficulties encountered with LT, LAI assays could not be correlated with either the MIF or LT tests. The LAI test is not useful for detection of cell-mediated immunity. Adjuvant properties of a metabolizable lipid emulsion and a muramyl dipeptide-like compound designated CP 20,961 were demonstrated with Rift Valley fever and Venezuelan equine encephalomyelitis vaccines.

The principal investigator has left the Army. The work unit is terminated.

*Available to contractors upon originator's approval

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6413	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	026
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Cell surface expression of viral antigens during the infectious process

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense;

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 07	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER: NA

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR	PRECEDING 79	1.0	35.6
	CURRENT 80	1.0	30.9

19. RESPONSIBLE DOD ORGANIZATION

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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) Military medicine; (U) BW defense; (U) Vaccines; (U) Immunology; (U) Rift Valley fever; (U) Laboratory animals; (U) Viruses

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 cell surface expression of viral antigen in Rift Valley fever (RVF) virus and arenavirus infections. Determine the nature of the humoral immune response to these infections, and available vaccines or vaccine candidates, in terms of quantity and specificity of the antibody elicited. These techniques will be applied to vaccine development for viruses of military importance.

24 (U) Develop solid phase radioimmuno assay (SPRIA) procedures for the quantitation of antiviral antibody and antigen. Develop immunoprecipitation techniques for the determination of antibody specificity and detection of cell surface antigens.

25 (U) 78 10 - 79 09 - SPRIA were developed for both anti-RVF antibody and RVF virus antigen. The antibody SPRIA yields titers equal and sometimes greater than the plaque reduction neutralization test in a shorter time and for less cost. Preliminary studies on the cell surface expression of RVF virus antigens have been completed. The cell surface labeling system has been established, the analytical disc gel electrophoresis system used, has been standardized and the initial screening of candidate antisera has been completed.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 026: Cell Surface Expression of Viral Antigens During the Infectious Process

Background:

The presence of virus-specific surface antigens on infected cells has been demonstrated for a large number of viruses of interest to USAMRIID. These include, VEE, EEE, Dengue (DEN), JE, Pichinde (PIC) and lymphocytic choriomeningitis (LCM). These virus-specific antigenic changes in host membranes have been detected by a number of different procedures: immunofluorescence, immunoradiolabeling, immunoelectron microscopy and assays employing cytotoxic antibody or lymphoid cells. However, the antisera used in a large number of these studies were obtained from convalescent hosts and it was not possible to determine whether the antigenic changes detected were the result of viral proteins expressed on the cell surface, altered or unmasked host proteins, or a combination of both (1-3).

The major objective of this project is to study the role of viral antigens expressed on the surface of infected cells during the evolution of viral infections of military importance, i.e., Rift Valley fever (RVF) virus and the arenaviruses. This will be accomplished using a technique of immunoprecipitation (4) to obtain qualitative measure of the antigens recognized and a system of a solid-phase radioimmunoassay to quantitate the responses.

Progress:

Solid-phase radioimmunoassays (SPRIA) have been developed for both RVF virus antibody and antigen. Both assays use viral antigen attached to the surface of 96-well microtiter plates and ^{125}I-labeled protein A as the detection reagent. These assays are currently being evaluated for their reproducibility and statistical correlation with other serological techniques such as plaque reduction neutralization (PRN) or fluorescent antibody (FA) assays (Table 1). Early experimental data indicate that it may be possible to differentiate between antibody produced against a killed virus vaccine and that produced against a live virus infection using a modification of these SPRIAs. Using the RVF antigen RIA it was possible to demonstrate a detectable antigenic difference between the inactivated vaccines NDBR-103 and GSD-200 and live RVF virus. Both vaccines were unable to inhibit binding of a rabbit serum produced against live virus in this test and produced nonparallel curves when a human serum which was the result of immunization and laboratory breakthrough was used.

Preliminary studies of cell surface antigens have been completed. Cell surfaces have been successfully labeled with ^{125}I using lactoperoxidase and glucose oxidase with 75% of the incorporated label appearing in the cell surface proteins and little or no cell death resulting from the procedure. A number of suspension cell cultures have been examined for use; however, it now appears that using standard cell lines grown on microcarriers will be the system of choice. Initial attempts to establish the parameters of the immuno-

precipitation procedure yielded unusual results. A human serum which contained antibodies against both inactivated vaccine and live virus was used; it precipitated only one detectable protein band which migrated in the region of the core nucleoprotein. The process used for virus purification has been modified, as it appears the antigen preparations were artificially enriched with pure protein as a result of the purification process used.

TABLE 1. COMPARISON OF RIA AND PRN TITERS FOR HUMAN AND MONKEY SERA

Serum No.	Reciprocal Titer	
	PRN	RIA
Human		
1	160	160
2	110	<10
3	160	160
4	320	160
5	<10	<10
6	>312	462
7	160	89
8	80	89
9	10	<10
10	320	160
11	>320	>1280
12	10	<10
13	10	40
14	10	<10
15	>320	160
Monkey		
B6628	1280	2560
6490	1280	3380
6486	1280	2081
027	<10	<10
B6973	640	2560
T309	320	3380

Publications:

None.

LITERATURE CITED

1. Burns, W.H. 1975. Viral antigens, pp. 43-56. In Viral Immunology and Immunopathology (A.L. Notkins, ed.). Academic Press, New York.

2. Burns, W.H., and A.C. Allison. 1975. Virus infections and the immune responses they elicit, pp. 479-574. In The Antigens, Vol. III (M. Sela, ed.). Academic Press, New York.

3. Burns, W.H., and A.C. Allison. 1977. Surface antigens of virus infected cells, pp. 213-247. In Virus Infection and the Cell Surface (G. Poste and G.L. Nicolson, eds). North-Holland, Amsterdam.

4. Pink, J.R.L., and A. Ziegler. 1979. Radiolabeling and immunoprecipitation, pp. 169-179. In Immunological Methods (I. Lefkovits and B. Pernis, eds). Academic Press, New York.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY	1. AGENCY ACCESSION: DA 0J6415	2. DATE OF SUMMARY: 79 10 01	REPORT CONTROL SYMBOL: DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	027
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code): (U) Production and use of endogenous pyrogen antibodies in early detection of infections 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
78 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 79	0.5	57.4
	CURRENT 80	1.0	47.6

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Immunology; (U) Endogenous pyrogen; (U) Radioimmunoassay

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

23 (U) Produce and purify antibodies to human endogenous pyrogen (EP). Using these antibodies to develop an effective radioimmunoassay for EP in serum, as a means of early detection of illness in military personnel exposed to BW agents or other sources of infection.

24 (U) Stimulate monocytes obtained from the blood of volunteers or tissue culture lines to produce EP. Inject this EP into goats or rabbits to produce antibodies, and purify these antibodies by immunoabsorption and column chromatography. Develop a radioimmunoassay for EP in human serum using the purified antibodies.

25 (U) 78 10 - 79 09 - A quantitative study of intracerebroventricular injection of EP into rats as an assay procedure has shown that it has distinct advantages over not only the conventional rabbit assay but also over a recently introduced mouse IV method. It combines the high sensitivity of the mouse assay with the good temperature range and linear log-dose response of the rabbit assay. In preparation for RIA or ELISA methods for measuring EP, antibody production and purification are well under way. Goat antibodies specific for rabbit pyrogen have been produced and partially purified. Rabbit antibodies against human EP are also being produced but are apparently not at detectable levels yet. Even without final purification, these antibodies have been useful for analytic immunoelectrophoresis. Purification of both human and rabbit EP, and antibodies toward them have been aided by the CM- and the DEAE-derivatives of Cibacron-blue affinity columns, respectively.

Publication: Fed. Proc. 38:1054, 1979.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 027: Production and Use of Endogenous Pyrogen Antibodies in Early Detection of Infections of Military Importance

Background:

The purpose of this project is development of a sensitive assay for endogenous pyrogen (EP), and use of this assay for early detection of infectious illness. Endogenous pyrogen is a 15,000-dalton protein produced in response to infection as a defense mechanism by phagocytic cells of the host animal. It acts upon the hypothalmic region of the brain, causing fever. The reason that EP is a good candidate for early detection studies is that since its action results in fever, it stands to reason that its appearance in serum precedes the occurrence of fever. The research of this project is directed towards determining whether this appearance of EP in human serum precedes fever sufficiently to be useful in early detection of infection.

The first step in this direction is to prepare methods and materials for assaying EP. Assay methods for endogenous pyrogen fall into 2 general classes: those based upon measuring its biological activity and those relying on immunological methods.

Progress:

Assay methods. An intracerebroventricular (ICV) injection method has been found to be a good way to detect endogenous pyrogen as described last year. Efforts have focused on accurately quantitating the temperature response to varying doses of EP administered ICV, especially in comparison with other assay procedures, IV injection into rabbits (1) and mice (2). The ICV-rat method was found to compare very favorably with both of these alternative assays: the rabbit IV and rat ICV methods both have linear log-dose responses to EP over a range of $\geq$ 0-2°C. However, the ICV method is more sensitive, requiring 1/50 the amount of EP that the rabbit-IV procedure does for a 1°C temperature rise. The rat-ICV assay also has advantages over the mouse-IV method, although both procedures have similar sensitivity; mice injected with high EP doses show false negative results. This causes a concave-downward log-dose response curve and restricts the range of EP concentrations over which the mouse assay procedure is operational. No such problem occurs with the rat-ICV model.

Another advantage to the rat-ICV method is that serum from different species can be injected without causing difficulties during the assay. This is in contrast to the conventional rabbit-IV method where injected human serum has traditionally been found to cause fever whether EP was present or not. In the rat-ICV model, no cross-species problems arose when goat or bovine serum was injected.

The ICV method involves implanting an indwelling catheter in the rat's right cerebral ventrical. Following a 10-day period for recovery from the surgery, the rats can be used several times in the next month. A series of 4 weekly injections of rabbit EP gave comparable results each time. The cannulated rats are kept in

individual cages at normal laboratory room temperatures during the experiments: they do not occupy nearly the space that a rabbit colony would, and no special 35°C incubators are required as in the mouse assay.

Immunological assay methods. Immunological assay methods have potentially much greater sensitivity than biological assay procedures. Highly purified antibodies and antigen (in this case, EP) preparations are required, particularly for radioimmunoassay (RIA) and enzyme-linked immunoabsorbent assay (ELISA) methods. Therefore, a great deal of research effort has gone into producing antibodies to EP and purifying these antibodies and EP itself.

Even without purification, once antibodies are produced, they can be of immediate use through immunoelectrophoresis. This is because proteins other than the antibody-antigen complexes formed are removed before visualizing the results. For instance, fused rocket immunoelectrophoresis has been particularly useful on this project for analyzing fractions of crude EP eluted from chromatographic columns and preparative electrophoresis gels. Also, 2-dimensional immunoelectrophoresis has been helpful for determining the purity of various preparations, and counter-immunoelectrophoresis for detecting the presence of antibodies in serum samples.

Production and purification of antibodies. There are two sources of EP for this project: rabbit and human. The rabbit pyrogen is partially purified by butanol/methanol extraction and injected into a goat for antibody production. After 9 months antibodies towards rabbit EP had built up to detectable levels. The method of analysis was a decrease in fever of rats injected ICV with rabbit EP incubated overnight with the antiserum compared to rats injected with EP incubated with saline.

$(NH_4)_2SO_4$ precipitation produced good initial purification of goat antibodies, separating out the IgG fraction. DEAE-Cibacron blue chromatography was also tried, obtaining even better purification, although this method is more difficult to scale-up. The next step is to combine these 2 methods so that the initial purification provided by the $(NH_4)_2SO_4$ fractionation will allow more sample to be applied to the column each time. An immunoabsorption column has also been prepared to separate out the antibodies specific for the partially purified pyrogen that had been injected.

The source of human EP is the hystiocytic cell line, U-937, which we culture now at USAMRIID from a starter sample obtained from Dr. Peter Ralph at Sloan Kettering Institute. This cell line is mildly phagocytic, grows in suspension, and produces human EP spontaneously. For antibody production, the pyrogen is gel filtered through Sephadex G-50, and the 15,000-dalton fraction (which contains the pyrogen activity) is concentrated and injected into rabbits.

The first injection is ID in Freund's complete adjuvant. Subsequent injections are IP in Freund's incomplete adjuvant. So far antibodies to human EP have not risen to detectable levels, although several precipitating bands are visible in Ouchterlony immunodiffusion plates.

Further purification of human and rabbit EP will take advantage of affinity chromatography. The use of immunoabsorption columns is anticipated as the antibodies specific for EP are separated out. In the meanwhile, Cibacron blue and CM-Cibacron blue affinity columns have been found in efforts done on this project to bind endogenous pyrogen. Salt-gradient elution produces a fraction which contains pyrogenic activity and only 1 or 2 antigenic proteins, as determined by fused rocket and 2-dimensional immunoelectrophoresis. This method shows great promise of speeding up and simplifying purification of human and rabbit EP.

It is interesting also that EP binds to the Cibacron blue affinity material, since it is specific for proteins with a "nucleotide fold," such as kinases. This may say something about the structure of EP, or it may mean that EP binds to albumin, which is known to stick to Cibacron blue.

Presentation:

Critz, W. J. Intracerebroventricular injection of rats: a sensitive assay method for endogenous pyrogen. Presented, Annual Meeting, FASEB, 1-10 Apr 1979 (Fed. Proc. 38:1054, 1979).

Publications:

None

LITERATURE CITED

1. Root, R. K., J. J. Nordlund, and S. M. Wolff. 1970. Factors affecting the quantitative production and assay of human leukocytic pyrogen. J. Lab. Clin. Med. 75:679-693.

2. Bodel, F., and H. Miller. 1975. A new sensitive method for detecting human endogenous (leukocyte) pyrogen. Inflammation 1:103-110.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6417	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
78 11 14	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	028
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Cellular internalization of bacterial exotoxins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 016800 Toxicology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
78 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 79	1.0	36.7
	CURRENT 80	1.0	44.8

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

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NAME: Middlebrook, J. L. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Exotoxins; (U) Therapy; (U) Pseudomonas aeruginosa

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 means by which bacterial exotoxins are internalized and processed by mammalian cells, with the goal of fully characterizing mechanisms of intoxication and proposing effective countermeasures.

24 (U) Conjugate selected toxins with various substances conducive to microscopic localization so as to study interactions with resistant and sensitive cell lines. New cell lines may be developed for toxin-resistance in order to study the interactions better. Diphtheria and Pseudomonas exotoxins are studied.

25 (U) 78 11 - 79 09 - Biochemical techniques were developed for assaying the internalization and degradation of diphtheria toxin (DE) by sensitive monkey kidney cells. Using these techniques, the kinetics of DE internalization and degradation were studied; the half-time of internalization was about 25 min. The biological relevance of the internalization/degradation process was established by the use of compounds such as Concanavalin A and toxin-specific antibody, which blocked both internalization (and/or degradation) and cytotoxicity at comparable concentrations. The effects of a large number of lysosomotropic agents and protease inhibitors on the internalization/degradation process were examined. Results indicated an involvement of lysosomes in cellular intoxication.

Chemical mutagenic procedures were used to select toxin-resistant variants of cell lines sensitive to either DE (Vero) or Pseudomonas (L-929) toxin (PE). Mutants were analyzed by several criteria: DE-resistant Vero cells were all found to have both altered toxin-resistant elongation factor-2 (EF-2) and a 75% reduction in toxin-specific cell surface receptors. The PE-resistant L-929 cells were also all found to be EF-2 mutants and in all cases appeared to be heterozygous for this trait.

Publication: J. Biol. Chem. 254: in press, 1979.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 028: Cellular Internalization of Bacterial Exotoxins

Background:

Many bacterial toxins exert their cytotoxic effects through initial interactions with specific cell-surface receptors, followed by internalization and translocation to intracellular sites of action. The intracellular mechanism of toxin action has been extensively characterized; however, the mechanism by which cells internalize and process these molecules prior to the expression of the cytotoxic effect is still unclear. There are few precedents for means by which macromolecules traverse biological membranes. One hypothesis suggests cytoplasmic entry through a "pore" in the membrane bilayers. Recent results from a large number of laboratories have shown instead that many biologically active macromolecules, including serum lipoproteins, polypeptide hormones, and growth factors, enter cells by a process known as "adsorptive endocytosis." This process is initiated by the binding of ligand to specific cell-surface receptors, which are either prelocalized in "coated" regions of the plasma membrane or are rapidly translocated to coated regions following ligand-receptor binding. Coated regions subsequently invaginate and pinch off to form cytoplasmic coated vesicles. The vesicles eventually fuse with lysosomes, in which ligand or ligand-receptor complexes are degraded in some manner by lysosomal enzymes. The kinetics of this process, detailed in several systems, are consistent with the previously described uptake of radiolabeled diphtheria toxin by cultured monkey kidney cells (1). Much of our current research is based on this concept.

A number of biochemical, pharmacological, and genetic approaches were taken to study cellular uptake of diphtheria toxin (DE) and Pseudomonas exotoxin A (PE). The insights obtained should contribute to the elucidation of the intoxication process and may lead to a definitive understanding of how other types of macromolecules, such as hormones and viruses, enter cells.

Progress:

Initial research efforts centered around developing biochemical techniques for assaying the internalization and degradation of DE by mammalian cells. Development of the internalization assay involved comparisons of a large number of proteolytic enzymes and other chemical agents presumptively able to rapidly release ^{125}I-labeled DE from the cell surface. Agents tested included trypsin, pronase, collagenase, EDTA, heparin, dextran sulfate, inositol hexaphosphate and ATP. Optimal conditions for release of surface-bound toxin were found to be incubation for 1 hr at 4°C with pronase (0.25 mg/ml) + inositol hexaphosphate (10 mg/ml) (PIHP). This treatment elicited release of 85-90% of the surface-bound toxin.

Using the PIHP technique, the kinetics of DE toxin internalization by Vero cells were studied at 4°C and 37°C. At 4°C, PIHP-releasable radioactivity increased with time in the presence of ^{125}I-labeled DE and at all time points represented approximately 85% of the total cell-associated radioactivity, indicating that at this tem-

perature essentially all of the DE remained on the cell surface. At 37°C, the increase in PIHP-releasable radioactivity was accompanied by an increase in PIHP-resistant radioactivity. Both reached maximum values around 1-2 hr after toxin addition and decreased thereafter; after 1-2 hr approximately 50% of the total cell-associated radioactivity was resistant to PIHP. Thus, the biphastic kinetic curve exhibited by Vero cells at 37°C can be resolved into 2 components using the PIHP technique: total cell-associated radioactivity is the sum of PIHP-releasable (surface-bound) and PIHP-resistant (internalized) DE. By a modification of this technique, we were able to determine the $t_{\frac{1}{2}}$ of internalization of DE by Vero cells to be approximately 25 min.

Concomitantly a trichloroacetic acid (TCA) technique was developed for assaying degradation of DE by cultured cells. The appearance of TCA-soluble (degraded) radioactivity in the cultured medium corresponded to the disappearance of intact DE from the cell surface. Degradation was essentially complete after 3-4 hr at 37°C. The nature of TCA-soluble material was investigated using thin-layer chromatography and found to be [^{125}I]monoiodotyrosine.

The biological relevance of the internalization/degradation process was established through the identification of certain compounds which blocked both internalization (and/or degradation) and cytotoxicity at comparable concentrations. Specific antibody was found to retard internalization 35-45% and degradation 60-70%; concanavalin A (Con A), a plant lectin previously shown to block DE internalization (2), inhibited degradation approximately 40% after a 180-min incubation at 37°C.

Such results strongly indicate that the cellular internalization of DE is a prerequisite for degradation. Also, since both specific antibody and Con A effectively block the cytotoxic effects of DE at the concentrations used in these experiments, the data support the hypothesis that DE intoxication of mammalian cells is a multistep process involving sequential receptor binding, internalization and intracellular processing and degradation.

The effects of a large number of lysosomotropic agents and protease inhibitors on the internalization/degradation process were also examined. Lysosomotropic agents studied included chloroquine, quinacrine, NH_4Cl, methylamine, neutral red and Triton WR 1339; protease inhibitors included soybean trypsin inhibitor, TLCK, TPCK, PMSF, leupeptin, antipain, pepstatin and benzamidine. Chloroquine had no effect on toxin internalization as determined by PIHP assay, but markedly blocked the degradation and/or excretion of internalized molecules; Quinacrine, a chloroquine analog, acted similarly. NH_4Cl, although protecting cells from the cytotoxic action of DE, had no measurable effect on either internalization or degradation. The other lysosomotropic agents studied were ineffective. Of the protease inhibitors, only TLCK had any measurable effect; degradation was partially blocked after 12 hr pre-incubation of cells in 0.1 mg/ml TLCK. The results obtained with chloroquine and quinacrine strongly suggest that the lysosomes are the site of intracellular DE degradation. The NH_4Cl data, however, are somewhat puzzling; results from a number of systems have indicated that it blocks protein degradation, presumably by increasing the intralysosomal pH, although this effect was not observed in our DE experiments. It has also been suggested that NH_4Cl somehow functions to maintain receptor-bound toxin at the cells surface, in a position accessible to neutralization by specific antibody (3); our results seem to contraindicate this hypothesis, although the possibility of a minor subclass of DE receptors not detectable by our assay cannot be ruled out. A more recent explanation for the protective effect of NH_4Cl derives from the work of Maxfield et al. (4) who found that it prevents the clustering of

epidermal growth factor and α-macroglobulin molecules on fibroblast cell surfaces which occurs prior to internalization. It may be that such a clustering step is also essential for DE internalization and such a possibility is under investigation.

Along with biochemical and pharmacological methods, genetic techniques have been employed in our study of cellular intoxication with the aim of obtaining toxin-resistant mutant cells blocked at various steps in the process. Such a range of mutant cell types was used to great advantage by Goldstein and Brown (5) in their study of the LDL uptake mechanism.

Optimum conditions were established for mutagenizing Vero or L-929 cells with ethyl methanesulfonate (EMS) (16-18 hr at 300 µg/ml). Initially, 140 toxin-resistant Vero colonies were isolated following selection in DE concentrations ranging from 20-10,000-fold the parental tissue culture $TCLD_{50}$ (0.01 ng/ml). Of these, 35 were successfully grown and characterized by a number of criteria: sensitivity to DE, PE, or abrin as determined by 48-hr cytotoxicity assay, sensitivity to DE and PE as determined by inhibition of protein synthesis assay, ability to bind, internalize, and degrade ^{125}I-labeled DE, and sensitivity of isolated mutant elongation factor-2 (EF-2) to DE and PE as determined by in vitro ADP-ribosylation assay. All the mutant cells were extremely resistant to DE (to concentrations 4-logs greater than the parental $TCLD_{50}$) and to PE (to concentrations 1000-fold greater than the parental $TCLD_{50}$). Parental and mutant cells had essentially identical sensitivities to abrin, however, with $TCLD_{50}$ of approximately 3 µg/ml. All of the mutant clones bound ^{125}I-DE at both 4 and 37°C. At 37°C, however, there were marked differences in the binding characteristics of parent and mutant cells. The uptake of radiolabeled DE by parental Vero cells at 37°C followed a biphasic pattern, increasing to a peak around 1-2 hr and subsequently decreasing to a steady-state level. In their mutants, radiolabel uptake did not follow this biphastic pattern, but continued to increase to final plateau values 4-5 fold greater than the parental maximum. The kinetics of toxin internalization and degradation by mutant cells were assayed using the PIHP and TCA techniques. Parent and mutant cells internalized and degraded DE at the same rate under the conditions of the experiment. The $t_{\frac{1}{2}}$ of internalization of DE by both cell types was approximately 25 min. In both studies, about 70% of the total cell-associated radioactivity had been excreted in the form of TCA-soluble fragments after 3 hr at 37°C.

Since the mutant cell lines bound, internalized, and degraded DE, it seemed likely that their high toxin resistance was due to an altered form of cytoplasmic EF-2. EF-2 was isolated from populations of parent and mutant cells and tested for ability to be ADP-ribosylated by DE or PE in an in vitro assay. Results showed that EF-2 isolated from the mutant cell lines was not ADP-ribosylated, strongly indicating that toxin resistance resulted from an EF-2 mutation.

Saturation studies with parent and mutant cells showed that the mutants have differences other than altered EF-2 and suggested that the mutation may be pleiotropic. The effect of labeled DE concentration on degree of binding was compared in the parent and 2 representative mutant cell lines and results plotted by the Scatchard method.

Both parent and mutant cells possess a single class of binding sites with an affinity constant (k) of approximately 9.3 x 10^8 L/mol.

The total number of binding sites per cell in the parent cell population was about 120,000; the mutant cell populations, however, had only about 25,000 binding sites per cell, a 5-fold decrease from the parental value. It is not clear why EF-2 and surface receptor number are simultaneously altered in the mutant cells; perhaps the EF-2 and receptor molecules share a common biosynthetic step (i.e., a glycosylation) which is the actual site of the mutation.

Application of techniques developed for assay of DE internalization and degradation to PE system has been complicated by lack of an effective 4°C binding assay. Preliminary results indicate, that like DE, PE is also rapidly internalized, degraded, and excreted in the form of TCA-soluble fragments by sensitive (L-929 or 3T3) cells at physiological temperature. The effects of a number of drugs and chemicals on PE uptake were investigated. These included NaF, NH_4Cl, Con A, chloroquine, leupeptin, antipain, TLCK, gangliosides, procaine, lidocaine, and chlorpromazine. Uptake was blocked 80-90% by Con A and lidocaine, and 50% by procaine; the other compounds had no detectable effect. Similarly the effects of chloroquine, NH_4Cl, and leupeptin on degradation were studied: all blocked the process by about 50%, although only NH_4Cl appreciably protected cells from PE-mediated cytotoxicity. The biological relevance of the measured internalization and degradation processes is the subject of further investigation.

Thirty-six PE-resistant L-929 sublines were isolated following mutagenesis with ethyl methanesulfonate (EMS) and selection in concentrations of PE ranging from 20 to 10,000-fold the parental $TCLD_{50}$ (0.1 µg/ml). The mutant lines were examined using 48-hr cytotoxicity, inhibition of protein synthesis, ^{125}I-PE binding and *in vitro* ADP-ribosylation assays. In these mutant cell lines, PE resistance could be attributed to an altered form of EF-2 which could not be ribosylated by PE. In all studies, the cells appeared to be heterozygous for the mutant EF-2 trait; that is, only 50% of the total cellular EF-2 was resistant to PE.

Presentations:

1. Dorland, R. B. Internalization and degradation of diphtheria and *Pseudomonas* toxins. Presented, Workshop on Bacterial Toxins, WRAIR, Washington, DC, 28 Mar 1979.

2. Dorland, R. B. Internalization and degradation of diphtheria toxin by monkey kidney cells. Presented, University of California, San Diego, CA. May 1979.

Publications:

1. Dorland, R. B., J. L. Middlebrook, and S. H. Leppla. 1979. Receptor-mediated internalization and degradation of diphtheria toxin by monkey kidney cells. J. Biol. Chem. 254:in press.

LITERATURE CITED

1. Middlebrook, J. L., R. B. Dorland, and S. H. Leppla. 1978. Association of diphtheria toxin with Vero cells: Demonstration of a receptor. J. Biol. Chem. 253:7325-7330.

2. Middlebrook, J. L., R. B. Dorland, and S. H. Leppla. 1979. Effects of lectins on the interaction of diphtheria toxin with mammalian cells. Exp. Cell Res. 121:95-101.

3. Kim, K., and N. B. Groman. 1965. Mode of inhibition of diphtheria toxin by ammonium chloride. J. Bacteriol. 90:1557-1562.

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5. Goldstein, J. L., and M. S. Brown. 1977. The low-density lipoprotein pathway and its relation to atherosclerosis. Ann. Rev. Biochem. 46:897-930.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6422	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8A. ORG'N INSTR'N	8B. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 05 16	D. CHANGE	U	U	NA	NL	[X] YES [ ] NO	A. WORK UNIT

10. NO 'CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS03	00	029
b. CONTRIBUTING				
c. COORDINATING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code): (U) Detection and characterization of plasmids in pathogens 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
79 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 79	0.4	37.6
	CURRENT 80	1.0	129.5

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Bacterial genetics; (U) Escherichia coli; (U) Legionnaires' disease

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

23 (U) Establish a technological base for genetic analysis of pathogenic bacteria, with emphasis on determinations of the presence and nature of plasmids. Awareness of the threat of recombinant organisms as BW offensive agents is of considerable military significance.

24 (U) Techniques of curing, agarose gel electrophoresis, density gradient centrifugation and electron microscopy will be used to establish a rapid procedure for detection and characterization of plasmids. The first organism used as a model will be E. coli with a known plasmid.

25 (U) 79 05 - 79 09 - Systems are being developed for the rapid detection, isolation and characterization of naturally occurring plasmids and genetically engineered recombinant DNA plasmids which are biologically active in pathogens.

Techniques were developed for the rapid detection of plasmids by the use of agarose gel electrophoresis and ethidium bromide staining. Methods have been established for purifying plasmids by cesium chloride-ethidium bromide ultracentrifugation of cleared lysates. Several methods for the elimination of plasmids from the host bacterial cells have been established with cell lines known to harbor plasmids with drug resistance markers.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 029: Detection and Characterization of Plasmids in Pathogens of Military Importance

Background:

Multiple drug-resistant plasmids complicate the treatment of infectious diseases by rendering the bacterial host resistant to specific antimicrobial agents. This drug resistance can be conjugally transferred in vivo to other bacteria, even across species lines (1). Plasmids have been shown to carry genes which code for toxins such as botulinum, diphtheria, Streptococcus erythrogenic, and the Escherichia coli enterotoxin (2). Recombinant DNA technology could be used to splice foreign genes into a plasmid vector which could then be clones in a bacterial host (3). In this way plasmids could be used to construct genetically engineered bacterial pathogens which theoretically could produce venom or toxin proteins, are drug resistant, have altered surface proteins, or provoke new autoimmune diseases (4). This project is designed to establish the defensive capability of rapidly detecting, isolating and characterizing naturally occurring and genetically engineered plasmids in bacterial pathogens of military importance. This research will also lead to a better understanding of the genetics of novel pathogens.

Progress:

This work unit began with the development of methods for the purification and visualization of plasmids in model species of bacteria which contain plasmids of known MW. These general methods are presently being applied to screen strains of Legionella pneumophila for the possible presence of plasmids and will ultimately then be applied to other pathogens of military importance. Plasmids detected in these studies will then be subjected to molecular genetic analysis.

Chloramphenicol, which indirectly inhibits chromosomal replication, while allowing Col El plasmids to continue to replicate, was used to increase the plasmid copy number. Cleared bacterial lysates were prepared by treating the organisms with lysozyme, to generate spheroplasts, followed by a buffered nonionic detergent such as Brij-58, Triton-X, or Sarkosyl. This treatment renders the spheroplasts permeable to macromolecules in the size range of plasmids but impermeable to the large chromosomal DNA. Approximately 95% of chromosomal DNA is then pelleted with the cell debris leaving the plasmids in the supernatant (5).

Covalently closed circular DNA (cccDNA) plasmids were separated from the remaining chromosomal DNA of the cells by preparative cesium chloride-ethidium bromide (CsCl-EtBr) isopycnic centrifugation. The cccDNA binds fewer molecules of the intercolating dye (EtBr) than linear chromosomal DNA and can thus be separated, based upon the differences in density. The plasmid band was visible in the ultracentrifuge tube under UV light and was extracted for further analysis.

Crude sodium dodecyl sulfate lysates, cleared lysates, and lysates purified by CsCl-EtBr ultracentrifugation were applied to agarose gel electrophoresis followed by EtBr staining in order to visualize the plasmid bands. Plasmids of known MW were

co-electrophoresed with newly isolated plasmids in order to estimate their MW. Electron microscopic methods are presently being developed which will be used to determine the precise MW of these plasmids.

In order to develop protocols for plasmid isolation by gel electrophoresis which could be applied to a wide variety of bacterial species, numerous methodological variables had to be tested. These variables included: method of bacterial cell lysis, type and pH of the running buffer, the type and concentration of agarose, horizontal vs. vertical electrophoresis, voltage and time of the run, and conditions for staining and destaining the gels.

Methods were established for the elimination of drug resistance plasmids. Subinhibitory concentrations of EtBr were used to "cure" Serratia marcescens LST1000 of its 45 megadalton plasmid which codes for resistance to ampicillin, kanamycin, streptomycin and tetracycline.

Publications:

None.

LITERATURE CITED

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2. Maas, W. K. 1977. Genetics of toxin production by bacteria, pp. 1-13. In Perspectives in Toxinology (A. W. Bernheimer, ed.). John Wiley and Sons, New York.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OG0179	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 07 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	61102A	3M161102BS03	00	030
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Identification of pathogens of military importance using nucleic acid hybridization

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
79 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

FISCAL YEAR	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
PRECEDING 79	0.25	11.6
CURRENT 80	1.0	37.9

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

ADDRESS: Fort Detrick, MD 21701

RESPONSIBLE INDIVIDUAL

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20. PERFORMING ORGANIZATION

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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) BW defense; (U) Bacteria; (U) DNA homology; (U) Identification; (U) Legionnaires' disease bacteria

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

23 (U) Establish the technique for determining base sequence homology of DNA of bacterial pathogens. Create a reference band of individual DNA varieties from representative bacterial genera. Develop the capacity to isolate, grow and identify rare or unusual pathogens, which may have been altered antigenically or physiologically. Initially, determine homology of Legionella pneumophila and its 4 serotypes. This effort will contribute to medical defense against BW.

24 (U) Measure reassociation between DNA homology of test and reference strains of bacteria, using radiolabeled material, heated hydroxyapatite columns or heated centrifuge. Initally, determine the guanosine (G) + cytosine (C) percent in the DNA of an unknown and compare to know G+C values. On this basis, select known reference bacterial DNA most similar to the unknown. Label unknown (or known) bacterial DNA with P-35, initiate reassociation between known and unknown DNA and perform calculations.

25 (U) 79 07 - 79 09 - Techniques for determining the base sequence homology of DNA from bacterial pathogens for identification purposes are being established successfully at USAMRIID. Creation of a reference bank of DNA from a variety of known bacteria has been initiated, with DNA from 47 genera already acquired. Application of these techniques to L. pneumophila has shown the 4 serotypes of this organism to be related at the species level. These techniques have also been successfully applied to assessing the relatedness of other bacterial genera.

* Available to contractors upon originator's approval

DD FORM 1 MAY 68 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A NO. 1 AND 1498-1 1 MAR 68 FOR ARMY USE ARE OBSOLETE

* U.S. GPO: 1971-510-911/9691

BODY OF REPORT

Project No. 3M161102BS03: Medical Defense Against Biological Agents (U)

Work Unit No. BS03 00 030: Identification of Pathogens of Military Importance using Nucleic Acid Hybridization Techniques

Background:

Classical identification of bacteria relies primarily on analysis of biochemical reactions produced by the metabolic "machinery" of the organism in question, morphology, staining characteristics and specific antigenic determinants on the cell surface. Clinical symptoms of disease or the environment from which it was isolated may also be utilized. These parameters are of course either directly or indirectly related to phenotypic expressions of the genes carried on the DNA of the organism. Unfortunately identification based purely on phenotypic traits is vulnerable to error in that small changes in the base sequence of the genes (i.e., spontaneous or deliberately induced mutations) can significantly alter one or more of these traits. Difficulty may also result from there being too few or insufficient biochemical markers upon which to base identification. A method of identification which is both insensitive to such mutations and is not dependent on multiple biochemical markers is that of DNA-DNA homology determinations. Like classical identification techniques, where identification is based on comparison of phenotypic traits of the unknown organism with those of known organisms, the base sequence of the DNA is likewise compared through the use of DNA hybridization techniques. Identification is made on the premise that the greater the percent reassociation due to base pair-matching between single stranded DNA from 2 organisms when mixed under appropriate conditions, the more closely the two organisms are related.

Progress:

Establishment of DNA hybridization techniques for the determination of DNA homology and its use in bacterial identification at USAMRIID was originally intended to be used to identify or verify the identity of newly isolated strains or serotypes of Legionella pneumophila. Ultimately, however, this technique will be expanded to identify rare or unusual pathogens and potential BW agents which may be altered physiologically or antigenically.

Using the DNA isolation procedure of Brenner et al. (1), we have isolated and purified DNA from 47 strains of Gram-positive and Gram-negative bacteria including the 4 serotypes of L. pneumophila. An additional 36 species of Gram-negative bacteria have been obtained from Mr. Fanning at WRAIR. Radioactive labeling of DNA with a high specific activity (SA) for use in DNA-DNA hybridization studies has been accomplished using Salmonella typhimurium and Escherichia coli. This was done in vivo using either ^{32}P or [^{3}H] thymidine. However, in vivo labeling of L. pneumophila has met with limited success in that isolation of DNA with a high SA from this organism has yet to be realized. The feasibility of using in vitro labeling is now been explored.

Although initial problems existed in obtaining an optimal system for hybridization of SS-DNAs used in DNA homology determinations these problems have been essentially eliminated. Minor problems still exist in there being too high a level of nonspecific binding with certain preparations of DNA. Nevertheless, using this system and ^{32}P labeled DNA from the Knoxville strain of L. pneumophila we have determined that the representative strains of the 4 serotypes of this organism are closely related at the species level. These findings are in excellent agreement with the findings of Brenner et al. at CDC (2).

Procurement of equipment and establishment of procedures for making DNA homology determinations have been for the most part successful. However, certain pieces of equipment necessary to fully develop the potential of this technique have not yet been acquired. Acquisition of a heated centrifuge in order to use the "batch" method (1) is well underway. Also the acquisition of a Gilford 250 spectrophotometer with the necessary accessories for determing the % guanosine + cytosine of DNA preparations is anticipated. The necessity of being able to determine this percentage of DNA from an unknown bacterium is that since this value is known for most genera of bacteria, it can be used to eliminate a vast majority of organisms which are totally unrelated to the organism in question. This will effectively narrow the range of organisms against whose DNA, the DNA from an unknown bacterium, must be checked for homology in order to determine its relatedness, i.e., its identity.

Publications:

None.

LITERATURE CITED

1. Brenner, D. J., G. R. Fanning, A. V. Rake, and K. E. Johnson. 1969. Batch procedure for thermal elution of DNA from hydroxyapatite. Anal. Biochem. 28:447-459.

2. Brenner, D. J., A. G. Steigerwalt, and J. E. McDade. 1979. Classification of Legionnaires' disease bacterium: Legionella pneumophila, genus novum, species nova, of the family Legionellaceae, familia nova. Ann. Intern. Med. 90:656-658.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6414	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	131
b. CONTRIBUTING				
c. [illegible]	STOG 30-7.2:2			

11. TITLE (Precede with Security Classification Code)

(U) Rift Valley fever virus infection: genetic and cellular aspects

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
78 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 79	2.7	122.0
FISCAL YEAR CURRENT 80	4.0	166.9

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

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NAME: Anderson, G. W.

NAME: Erlick, B. J. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Immunology; (U) Rift Valley fever virus; (U) Laboratory animals; (U) Bunyaviruses

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

23 (U) Define antigenic and other laboratory characteristics of recent Rift Valley fever (RVF) virus isolates to ascertain if previously developed vaccine will be efficacious and to try to explain the emergence of severe forms of the disease as part of the clinical spectrum. Knowledge of this virus will help in medical defense to better understand Bunyaviruses, most of which could be considered of importance as potential BW offensive weapons against U. S. troops.

24 (U) Study pathogenesis of RVF in several hosts, including genetically defined members of the same species; contrast differing virus isolates and clones; attempt to explain phenomena as far as possible at the molecular level.

25 (U) 78 10 - 79 09 - A single gene has been shown to result in a 100,000-fold difference in the susceptibility to fulminant disease in rats, opening the path for important mechanistic studies. A primate model for hemorrhagic fever was identified; disseminated intravascular coagulation was implicated as an important pathogenetic mechanism with attendant therapeutic implications. Minimum protective neutralizing antibody concentrations were defined and the ability of vaccine to induce these antibody titers in humans was shown to differ between vaccine lots. Induction of interferon or administration of the antiviral drug ribavirin was shown to be prophylactic and therapeutic in the mouse model of RVF. Molecular studies of the virus revealed that protein and RNA structure were remarkably conserved over time and space but that these techniques held promise in differentiating virus strains indistinguishable by conventional means.

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, NOV 68

BODY OF REPORT

Project No. 3A161101A91C: Medical Defense Against Biological Agents (U)

Work Unit No. A91C 00 131: Rift Valley Fever Virus Infection: Genetic and Cellular Aspects

Background:

Rapidly changing concepts of virulence and epidemiology of Rift Valley Fever (RVFV) have led us to examine the contribution of host and viral genetics to the outcome of the infection, the effectiveness of the available inactivated vaccine in realistic scenarios for its use, alternate modalities of control of RVF, and molecular correlates of virulence and evolution of RVF.

Progress:

We have previously established that inbred rat strains differ markedly in their response to infection with the Zagazig 501 strain of RVF. Formal genetic studies with F1 and back-cross animals have now identified the inheritance of resistance to fulminant infection as being due to a single Mendelian dominant gene not linked to the major histocompatibility complex. The same pattern of heredity has persisted through 3 back-cross generations onto the susceptible background. This provides a strong motivation to seek a single in vitro marker and, eventually, a single gene product to explain the differences seen and to attempt extension to nonhuman primates and patients. Encouraging results continue to accumulate from family studies on gerbils and mice as well.

Experimental infection of rhesus monkeys has yielded titillating but frustrating results. Hemorrhagic fever has never been reported in nonhuman primates infected with classical RVF strains. Three of 18 rhesus monkeys inoculated with the Zagazig 501 strain (isolated from a fatal case of hemorrhagic fever in Egypt) have developed coagulation abnormalities and 2 of these had frank clinical signs of hemorrhagic fever. The frequency of clotting abnormalities has not been enhanced by passage of virus from affected monkeys, and the prevalence of hemorrhagic fever remains too low for systematic studies. Nevertheless, we have been able to document the early occurrence of abnormal clotting parameters suggestive of disseminated intravascular coagulation (DIC) and demonstrate that 1 of the 2 moribund monkeys had fibrin thrombi in the glomeruli. If DIC is an important pathogenetic mechanism in the monkey model (which most closely resembles human disease) it could have important implications for therapy; immune plasma could potentially result in abrupt endothelial cell lysis or increased immune complex formation. Heparin administration could be considered as primary or adjuvant therapy.

Experimental therapy of RVF using the mouse model of fulminant liver necrosis has received additional study. Cooperative studies with MAJ Stephen (AA Div) had established that ribavirin was active against RVF in mice. Since then ribavirin, poly(ICLC), and antibody have been shown to be effective prophylactically and therapeutically in murine RVF. Successful treatment could begin as late as day 3, a time when controls had already begun to die. Encephalitis occurred in some survivors with all 3 therapeutic modalities, particularly when treatment was delayed or

suboptimal. Histopathologic study of tissues from dying animals confirmed that liver disease was the cause of deaths occurring before day 7 and extensive encephalitis afterwards. To emphasize the lack of effect of all 3 drugs on central nervous system infection, we inoculated mice intracranially with a small dose of RVF (25 PFU) and initiated therapy immediately. There was a 2-day prolongation of survival in treated animals, but this was found at autopsy to result from protection of the liver from destruction by virus escaping into the circulation; all treated animals succumbed to necrotizing encephalitis. These studies suggest a variety of in vitro and in vivo experiments to follow up the findings with RVF, but more importantly show for the first time the applicability of these modalities to Bunyaviridae infections and provide principles which may be exploited with other important viruses in the family, particularly some where no model or no active research program exists.

The previously developed inactivated human RVF vaccine (NDBR-103) has proved to be safe and immunogenic in U.S. Government laboratory personnel, but we have little experience under controlled field conditions. In cooperation with Dr. J. Meegan, we vaccinated laboratory workers at NAMRU-3, Cairo, and obtained serum samples 6 weeks and 1 year later. All 108 vaccinees sampled at 6 weeks had detectable antibodies and > 99% had PRN titers of > 1:40. In contrast to previous reports, Dr. Meegan detected a significant HI response which was roughly proportion-on selectal to the PRN test, but about 4-fold lower. He also performed CF and IFA tests on sera with virtually no response (although sera from natural infections reached titers of 1:64-1:512 in both tests). This observation may provide us with a method to distinguish postvaccination from postinfection antibodies. Forty-three sera were obtained at 1 year; there was an ≈ 8-fold decrease in median titer, and 21% fell to < 1:40, although all still had detectable neutralizing antibody.

Although we now have larger stocks of an improved inactivated vaccine (TSI-GSD-200), there are several problems which remain. The quantities available are unlikely to be sufficient in many scenarios for their use. Therefore, we have begun a joint study with the Israeli Defense Force to carefully define the human alternative immunization schedules which might be more economical or yield earlier immunity. When different lots of the vaccine are subjected to the standard mouse potency test, the test is found to be inexact and large differences are found. CPT Meadors (Med Div) has selected a critical dose of vaccine to allow sensitive screening of these lots in humans; inter-lot differences are beginning to emerge. He is also developing a more precise animal test to replace the older potency test. A parallel line biological assay based on the hamster PRN test has shown promise in this respect.

Even knowing the human dose-response in greater detail, we still need to ascertain the minimal protective titer. In experimental animals, this varies from a barely detectable PRN of 1:10 to 1:80. Since we require a minimum titer of 1:40 to enter the laboratory (and have had no laboratory illnesses), we suppose that the human minimal protective titer is < 1:40. CPT Donaldson in our division has developed a plasmapheresis procedure for rhesus monkeys, stockpiled a quantity of standardized postvaccination serum, and is determining the effects of passively acquired antibody in protecting against a large (10^6 PFU) SC challenge of RVF. A titer of only 1:30 prevents viremia and subsequent active antibody production. A titer of 1:20 completely suppresses viremia, but the monkeys become infected, as evidenced by their serum antibody rises. These primate studies will be pursued to determine the minimal protective titer and also to exclude the occurrence of enhancement of viral replication (suggested by in vitro monocyte culture studies) or induction of encephalitis (a common phenomenon in mouse and hamster vaccine or serum titrations) at borderline antibody levels.

Another facet of our program, to understand the pathogenic potential, has led to the infection of a variety of cell substrates in vitro. Low multiplicities of RVF (100 PFU/10^5-10^6 cells) initiate infection in all cell lines or strains studied, except those of lymphoblastoid origin, which do not become productively infected even at high virus doses. All these cultures once infected replicate RVF to high titer (≈ 5 x 10^7 PFU/ml) and suffer complete destruction, except mosquito and iguana heart cells which replicate virus without cytopathic effect (CPE). Primary macrophage cultures are also of interest since they support RVF growth only at high multiplicity and then only to low titers without CPE. The input Zagazig 501 $FRhL_2$ virus was of mixed plaque size (0.5-4 mm) with large plaques (LP) present in higher titer, however, predominant progeny from several cultures was of small plaque size (SP, 0.5-1 mm). Inoculation of mice with SP from several cultures suggested that some were of decreased virulence, particularly one A549 isolate.

In light of the above findings as well as the published experience suggesting virulence heterogeneity of RVF, we decided to clone Zagazig 501 $FRhL_1$ virus and examine the properties of the progeny. A system for plaquing in FRhL cells was developed and the appropriate conditions for terminal dilution in several cell substrates explored. Both plaque picks and terminal dilution yielded SP virus in apparently pure form. LP virus was almost always accompanied by SP; it is not clear if this represents contamination by higher titered SP with poor plaquing efficiency or reversion after valid cloning. Four multiply cloned SP stocks are ready for virulence and molecular analysis; additional cloning is being applied to develop LP clones or ascertain the basis for our inability to obtain them. These clones should contribute significantly to our understanding of the modulation of virulence in RVF populations by the properties of the genetically distinct members of the population. They should shed light on the determinants of hemorrhagic fever, encephalitis, or attenuation.

RVF has been a serologically unique virus for more than 40 years, when it was first reported. Dr. R. Shope at YARU communicated to us that he had serendipitously discovered that RVF was related to the Phlebotomus fever (PHL) group of viruses by HI tests. By sharing reagents we were able to rapidly confirm this by PRN and IFA tests. This serological affinity may give us further clues as to RVF's natural behavior. The cross-reacting neutralizing antibodies in hyperimmune laboratory reagents also raise the possibility of natural cross-protection between these viruses. Perhaps a less dangerous member could even serve as a naturally occurring attenuated vaccine. Preliminary serological studies with postinfection sera have yielded lower levels of cross-reactivity. In the single experiment completed to date, prior infection with PHL viruses prolonged the lives of mice challenged with a low dose of RVF but failed to increase the fraction ultimately surviving.

The most exciting news for this year comes from CPT Erlick's molecular program. The ultimate goal of this program is to apply sensitive and powerful biochemical techniques to understand the genetics, evolution, epidemiology, and virulence of RVF strains. CPT Erlick is now on the border of completing several complex molecular analyses to compare 4 different RVF strains. These strains were chosen to provide a board spectrum in geographic spread, time, and pathogenicity; they are all indistinguishable by serological tests. So far, a detailed comparison of their protein and RNA composition by PAGE has found them to be identical. Tryptic finger prints (done in collaboration with Frederick Cancer Research Center) have been completed on the nucleocapsid protein and indicate that its primary structure is highly conserved in these viruses which differ 25 years in their time of isolation and thousands of miles in their geographic origin. RNA fingerprints from 2 strains are available and show several differences which may ultimately allow us to distinguish strains and study their patterns of spread and evolution.

CPT Erlick has succeeded in developing a technique which permits analysis of virion proteins by PAGE in one dimension followed by isoelectric focusing in a second dimension. This methodology has not yet been applied to the strain comparison but promises to be sensitive and rapid. It has already raised other questions about virion structure.

Presentations:

1. Peters, C. J., and G. J. Urbanski. Pathogenesis of Rift Valley fever infection. Presented, Annual meeting, American Society of Tropical Medicine and Hygiene, Chicago, IL, 6-19 Nov 78.

2. Peters, C. J. Recent advances in immunology, pathogenesis and treatment of hemorrhagic fevers. Presented, Center for the Study of Argentine Hemorrhagic Fever, Pergamino, Argentina, 11 Apr 79.

3. Peters, C. J. Rift Valley fever. Presented by P. S. Loizeaux, Symposium on Military Veterinary Medicine, WRAIR, Washington, 23-27 Apr 79.

4. Peters, C. J. Recent advances in understanding of Rift Valley fever: epidemiology and control. Presented, Working Committee on Rift Valley fever, Jerusalem, Israel, 9 Aug 79.

5. Peters, C. J. Pathogenesis and treatment of Rift Valley fever. Presented, Medical Research and Development Command, Israeli Defense Forces, Tel Aviv, Israel, 16 Aug 79.

Publication:

Peters, C. J., and J. Meegan. 1980. Rift Valley fever. In CRC Handbook Series in Zoonoses, Sect. B: Viral Zoonoses, vol. 1 (G. Beran, ed.). CRC Press, Boca Raton, FL, in press.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6421	79 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY	6. WORK SECURITY	7. REGRADING	8a. DISP'N INSTR'N	8b. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
79 04 04	D. CHANGE	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	137
b. CONTRIBUTING				
c. [illegible]	STOG 70-7,2,2			

11. TITLE (Precede with Security Classification Code)

(U) Laboratory diagnosis of viral 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
79 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 79	0.85	2.9
	CURRENT 80	1.0	27.3

19. RESPONSIBLE DOD ORGANIZATION

NAME: USA Medical Research Institute of Infectious Diseases

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20. PERFORMING ORGANIZATION

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

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SOCIAL SECURITY ACCOUNT NUMBER:

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NAME: Woodruff, N. H., Jr.

NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Military medicine; (U) BW defense; (U) Virus diseases (U) Serology; (U) Rapid diagnosis

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 capability for rapid diagnosis of viral diseases for a clinical laboratory. There is a need to have methodology for early and accurate identification of virus infections for use by the military, especially for those of BW importance.

24 (U) Apply the enzyme-linked immunosorbent assay (ELISA) to a variety of viral disease specimens, comparing them to other routine, but slower methods.

25 (U) 79 04 - 79 09 - A functioning diagnostic laboratory has been set up using 2 to 3 readily available cell cultures; hundreds of viruses affecting man may be isolated and identified. Routine procedures for viral serology have been developed.

An ELISA for antibody detection has been found to work with Venezuelan equine encephalomyelitis virus antigen. Determination of the parameters of the test is in progress. The commercially prepared antigens for cytomegalovirus, varicella, and rubella are suitable for ELISA.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A166101A91C: Medical Defense Against Biological Agents (1)

Work Unit No. A91C 00 137: Laboratory Diagnosis of Viral Diseases of Military Importance

Background:

Acute viral diseases especially those affecting the respiratory tract, are very common among military and civilian personnel (1). Although the associated mortality is very low it is essential to identify the virus(es) involved before proper control and therapeutic measures can be implemented [e.g., amantadine for influenza (2)]. Since the conventional cell culture system may take a few days to weeks to isolate and identify some viruses, there is a need to develop alternate method(s) for rapid diagnosis.

The laboratory approach to viral diagnosis may be done by any, or a combination of the following procedures: (a) viral isolation and identification, (b) viral serology, (c) demonstration of viral particles (antigen) in clinical specimens and/or associated morphologic changes in tissue (histopathology).

Virus isolation has become a relatively simple procedure since Robbins, et al., (3) described the growth of poliovirus in cell culture in 1951. Many cell cultures of human and animal origin that will support the growth of hundred of different viruses are now available. The presence of viruses in cell cultures may be recognized by the presence of frank cytopathogenic effect, by syncytial or giant cell formation, or by performing hemadsorption test with guinea pig erythrocytes (4). Some viruses which do not readily replicate in cell cultures may be isolated in chick embryo (e.g., influenza) or suckling mice (e.g., coxsackie and arboviruses (5).

Viral aerology is essential in epidemiological investigations and in establishing that an infection existed, especially if the virus was not isolated. In a few instances, it is important to determine the immune status of individuals (e.g., rubella and vaccine recipients). Recently, an enzyme-linked immunosorbent assay (ELISA) has been described for the detection of antibodies (6). This method will be explored for its potential in rapid diagnosis.

Histopathology can provide rapid diagnosis in infections due to cytomegalovirus, herpes simplex, and smallpox (7). However, many viral infections do not produce characteristic morphologic changes; in these instances, immunofluorescence test (if reagents are available) or electron microscopy may be utilized. Initial inoculation of specimens into cell cultures before electron microscopic examination may enhance the chance of detecting viral particles.

Progress:

Major emphasis during the first quarter of this project was placed on purchasing reagents and equipment, establishing routine procedures for the collection and processing of clinical specimens, and the training of a part-time technician.

An immunofluorescent (IF) test for antibodies to Epstein-Barr virus (EBV) (the cause of infectious mononucleosis) has been introduced and is now being used by our clinicians. This test is more specific than those which depend on the presence of heterophil antibodies. Many children and even young adults do not develop heterophil antibodies but virtually all will have detectable antibodies to EBV following recovery from the disease.

Cynomolgus monkey kidney and human embryonic lung fibroblast cell cultures are being used for virus isolation. So far no viruses have been detected from clinical specimens submitted to the laboratory during the past several weeks.

Initial efforts in developing ELISA for detecting antibodies to VEE appear promising. Ten serum samples with known titers to VEE as determined by plaque reduction neutralization test (PRNT) were tested; comparable titers were obtained with the ELISA procedure. In 3 samples, the ELISA titers were higher, indicating that perhaps this test is more sensitive than the PRNT.

An indirect hemagglutination (IHA) test to detect VEE antigen in varying concentrations was performed using sheep erythrocytes fixed in glutaraldehyde and coated with monkey IgG anti-VEE as indicator. This method was used by Gaidamovich et al. (8) to demonstrate Colorado tick fever virus in Vero cell culture fluid. Nonspecific agglutination on occasions interfered with the interpretation of the test. If this can be eliminated, IHA has a real value in rapid diagnosis.

The commercially available complement fixation antigens for cytomegalo- and varicella viruses were found suitable for use in the ELISA test. Rubelisa (Microbiological Associates) was tested and found to be a good screening test for antibodies to rubella.

Publications:

None.

LITERATURE CITED

1. Evans, A. A. 1975. Introduction. Symposium: acute respiratory infections in military personnel. Yale J. Biol. Med 48:167-169.

2. Wingfield, W. L., D. Pollack, and R. R. Grunert. 1974. Therapeutic efficacy of amantadine HCl and rimantadine HCl in naturally occurring influenza A2 respiratory illness in man. N. Engl. J. Med. 281:579-584.

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4. Smith, T. F. 1974. Description of viral cytopathic effects and identification of viruses, pp. 254-277. In Laboratory Procedures in Clinical Microbiology, (J. A. Washington, II, ed.) Little, Brown and Co., Boston.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G6428	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES.	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	141
b. CONTRIBUTING				
c. [illegible]	STOG 70-7,2,2			

11. TITLE (Precede with Security Classification Code): (U) Rapid detection of immune complexes in infectious diseases of unique 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
76 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 79	0.5	23.4
FISCAL YEAR CURRENT 80	0.5	21.8

19. RESPONSIBLE DOD ORGANIZATION
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21. GENERAL USE
Foreign intelligence considered

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Antibody (U) Immune complexes; (U) Isotachophoresis; (U) Immunoglobulins; (U) Early detection

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

23 (U) Detect rapidly the presence of infectious agents or their component parts as well as antibodies directed against them in sera. This would significantly aid in the early diagnosis of infectious agents of military medical interest and BW importance.

24 (U) Immune complexes formed by the addition of well-characterized infectious bacterial or viral antigena and the antibodies directed against them can be readily defined by their migration in an electrical field by isotachophoresis of ion species of the same sign. The selective addition or removal of one specific reagent will elucidate the presence or absence of the other.

25 (U) 78 10 - 79 09 - We have demonstrated that soluble immune complexes could be demonstrated by analytical isotachophoresis. In addition, we have shown that the four subclasses of human IgG can be separated and identified by the same technique. Our most recent efforts have been in conjunction with Drs. Wistar, Ganguly and Veltri in elucidating the functional nature of each subclass.

Publications: J. Chromatogr. 162:76-80, 1979.
J. Immunol. Meth. 25:43-48, 1979.

*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 75, FOR ARMY USE ARE OBSOLETE.

BODY OF REPORT

Project No. 3A061101A91C: In-house Laboratory Independent Research (U)

Work Unit No. A91C 00 141: Rapid Detection of Immune Complexes in Infectious Diseases of Unique Military Importance

Background:

The basic principle of isotachophoresis has been described (1, 2). In isotachophoresis the sample (a mixture of anionic and cationic species) is introduced between a leading electrolyte and a terminating electrolyte. In the analysis of anionic species, the leading buffer is chosen so that its effective mobility is higher than all other anionic species, whereas the terminating anionic species is chosen with a mobility lower than those of all other anionic species. When an electric current is passed through such a system in the first stage, all ionic species migrate with a velocity determined by the pH, ionic strength, mobility and the potential gradient. After this stage in which the anionic species of the sample are separated according to differences in effective mobilities, a "steady state" is reached in which all zones migrate with a velocity equal to that of the leading anionic species. Each zone will contain only one anionic species.

In the past, isotachophoresis has been used to separate inorganic ions, strong and weak acids and their salts. More recently in early 1970 the method was applied to the study of complex protein mixtures.

We have studied for the first time the nature of well-characterized antibody-antigen interactions. In addition, because IgG antibody subclass characterization gives clues to the biological and functional "usefulness and appropriateness" of an antibody response, we have studied human IgG subclasses as well.

Progress:

Our present work is directed toward the elucidation of the functional role of specific patterns of IgG subclass response to a variety of antigens. We are aided in this approach by the interest of Drs. Wistar (Naval Medical Research Institute), Ganguly (N.I.H.) and Veltri (West Virginia University Medical Center). Having previously demonstrated that individual subclasses of human IgG could be separated and identified by analytical isotachophoresis (2), we are now directing our attention to the patterns of IgG subclass responses. This is of importance since IgG subclasses constitute 75% of the total serum immunoglobulins and 80% of antibodies to bacteria, viruses and toxins. An expanding body of data suggests that quite a few IgG antibodies are restricted to 1 or 2 subclasses. What determines the IgG subclass response to any antigen is mostly unknown. One would like to establish the full biological ramifications of each subclass in sequence or by itself and tailor antigens to get a specific desirable subclass-immune response which has favorable protective properties.

Since all 4 classes of IgG can be separated and identified by analytical isotachophoresis, this technique may prove of value in establishing the subclass patterns of response in health and disease.

Publications:

1. Hedlund, K. W., and D. E. Nichelson. 1979. Demonstration of soluble immune complexes by analytical isotachophoresis. J. Chromatogr. 162:76-80.

2. Hedlund, K. W., R. Wistar, and D. E. Nichelson. 1979. The identification of the subclasses of human IgG by analytical isotachophoresis. J. Immunol. Meth. 25:43-48.

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2. Everaerts, F. M., M. Geurts, F. E. P. Mikkers, and Th. P. E. M. Verheggen. 1976. Analytical isotachophoresis. J. Chromatogr. 119:129-155.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0J6412	79 05 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
78 10 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	3A161101A91C	00	142
b. CONTRIBUTING				
c. [illegible]	STOG 79-7.2.2			

11. TITLE (Precede with Security Classification Code): (U) Development of radioimmune assay procedures for quantitation of viral antibodies and antigens

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
78 02	79 04	DA	C. In-house

17. CONTRACT/GRANT
a. DATES/EFFECTIVE: EXPIRATION:
b. NUMBER: NA
c. TYPE: d. AMOUNT:
e. NO OF AWARD: f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 79	0.3	2.1
FISCAL YEAR CURRENT 80	0	0

19. RESPONSIBLE DOD ORGANIZATION
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c. GENERAL USE
Foreign intelligence considered

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ASSOCIATE INVESTIGATORS
NAME:
NAME:

POC:DA

22. KEYWORDS (Precede EACH with Security Classification Code): (U) Military medicine; (U) BW defense; (U) Staphylococcus aureus protein A; (U) Radioimmunoassay; (U) Vaccines; (U) Antibodies; (U) Antigens

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 radioimmune assay for quantitation of viral antigens and antibodies. Such a procedure would be of inestimable value for measuring antibody responses in immunized personnel, so as to know how much protection there is against a disease of military importance.

24 (U) Prepare concentrated radiolabeled virus antigens. Add S. aureus protein A (SaCI) to precipitate labeled material. Test supernatants for residual (unbound) antigen and construct a titration curve. Optimum conditions for each disease virus will be determined. Methods will be compared to conventional serologic assays.

25 (U) 78 10 - 79 04 - Staphylococcal protein A was used as a solid phase immunoabsorbent for radioimmunoassay (PA-RIA). PA-RIA readily differentiates among Venezuelan, western and eastern equine encephalitis antibodies. It has been adapted successfully to Chikungunya, Rift Valley fever, Pichinde and Lassa fever virus antibodies. PA-RIA is generally applicable to routine testing of sera of man and animals. It is an assay which is better than conventional serological systems for all the viruses tested. It is the only one for Chikungunya.

The work unit is completed successfully. Further work will be conducted under work units for specific viruses.

* Available to contractors only with sponsor's approval

BODY OF REPORT

Project No. A161101A91C: Medical Defense Against Biological Agents (U)

Work Unit No.A91C 00 142: Development of Radioimmune Assay Procedures for Quantitation of Viral Antibodies and Antigens

Background:

Protein A is a molecule present in high concentration on the surface of certain Staphylococcus aureus strains (1). It rapidly binds most mammalian IgG molecules (2) through an interaction with the Fc region. Formalin-fixed staphylococci retain protein A on their surfaces (3), and may be used as solid phase absorbents in radioimmunoassay (RIA) procedures to separate virus-antibody complexes from unbound radiolabeled virus by low-speed centrifugation. Protein A-bearing Staphylococcus (SaCI) thus substitutes for the secondary antibody (anti-IgG) in an RIA, and offers several advantages. Since protein A combines with most mammalian IgG molecules, it is not necessary to prepare individual precipitating anti-IgG antibodies for each species tested. The reaction between IgG and SaCI is very rapid (4); 1- to 10-min incubation times are sufficient, compared to the 12-18 hr required for the secondary antibody technique. The SaCI reagent is also easily and economically prepared (4). The first objective of this project is the development of RIA using SaCI to measure antibodies against alphaviruses in human and other mammalian sera. This objective was met (5).

Progress:

The feasibility of utilizing staphylococcal protein A as a solid phase immunoadsorbent for radioimmunoassays (PA-RIA) was completed. Procedures were developed for quantitating both viral antigenic mass, and specific antiviral antibodies, using alphaviruses of military importance as models. In particular, antibody assays were developed for VEE, WEE, and EEE viruses, utilizing ^{3}H-labeled, purified viruses as antigens. The PA-RIA clearly differentiated among antibodies to these closely related viruses; it further differentiated among virus subunits within alphavirus complexes. Thus the PA-RIA is more specific than conventional neutralizing antibody tests; subsequent tests demonstrated that the PA-RIA is more precise, sensitive, and rapid than conventional serology. It is anticipated that the PA-RIA will now replace neutralization tests for routine assay for alphavirus antibodies.

The PA-RIA was further adapted to measure antibodies to Chikungunya (CHIK) virus. The only other serologic tool for this virus is the cumbersome and imprecise mouse potency assay. The availability of a reliable assay for CHIK antibodies has permitted the testing of sera from human recipients of CHIK vaccine, and will be invaluable for testing the efficacies of new lots of experimental CHIK vaccines in laboratory rodents, primates, and ultimately man.

In the course of collaborative studies with investigators at Cornell and at Fort Collins, we determined that the PA-RIA was useful for measuring antibodies in sera from almost all species of mammals. Unfortunately, avian sera did not react in this test. However, the PA-RIA appears to have broad application to

the rapid and precise testing of the large numbers of sera from diverse animal species often collected in the course of epidemiological surveys.

The application of the PA-RIA to other groups of viruses was also investigated. The feasibility of the assay for measuring antibodies to Rift Valley Fever virus (RVF) was demonstrated. Presently, we can determine relative antibody titers, but have not yet standardized the assay (as we did for the alphaviruses). However, antibody titers are inversely proportional to the antigenic concentrations used, and the limiting factor in the sensitivity of this assay is the specific radioactivity of the antigen employed. The technology exists for preparing "hotter" radiolabeled antigens should investigators specifically interested in RVF require a more sensitive assay.

We also used protein A to precipitate infectious virus/antibody complexes, and have explored the possibility of utilizing this technique to measure antibodies to arenaviruses, specifically Pichinde and Lassa viruses. Presently no neutralizing antibody assay exists for these viruses; we are optimistic that the protein A approach will permit the quantitation of protective antibody responses in animals inoculated with experimental, candidate Lassa fever virus vaccines.

The feasibility of utilizing protein A in a competitive binding procedure to measure inactivated alphavirus antigens was also explored. Antigen concentrations of $\geq 10^8$ PFU were reliably quantitated; the test would therefore be useful for predicting relative efficacies of alphavirus vaccine lots. Effective vs. ineffective vaccines to WEE were differentiated in this way, and relative concentrations of antigen in 2 lots of CHIK vaccine have likewise been measured. As for the antibody assays, sensitivity of these competitive binding assays depends on specific radioactivity of the labeled antigens employed. Our experience suggests that viruses intrinsically labeled with ^3H are only marginally useful for these purposes. Attempts to label viruses extrinsically with ^{125}I have been less successful, due primarily to the reversible nature of the binding between ^{125}I and lipid-containing developed viruses.

To circumvent this problem, we have recently begun to employ ^{125}I-labeled protein A in a solid phase RIA (SPRIA). In this test, antigen (which need not be purified since cell culture supernatant is sufficient) is absorbed to wells of a disposable plastic microtiter plate. Antibody (dilutions) are reacted for 1 hr, washed, and ^{125}I-protein A is added. Following a 1-hr reaction time and washing, the individual wells are cut out of the plate, and counted in a gamma counter. The ^{125}I bound is a function of antibody bound, which in turn depends on antigen concentration. Preliminary data suggest that less than 10^6 PFU of VEE may be detected by this procedure (i.e, a 100 X increase in sensitivity over the PA-RIA). However, questions of precision and specificity remain to be determined.

These investigations have demonstrated the general applicability of the PA-RIA to routine testing of sera from humans and animals, both experimentally and naturally infected. The PA-RIA is the only serologic tool available for CHIK, and the protein A-neutralizaton test is a promising approach to measuring protective immunity against arenaviruses.

Further studies to develop PA-RIA or SPRIA procedures for other viruses or antibodies should be conducted primarily by the investigators specifically concerned with those viruses. Such studies would be appropriately conducted under the work units covering those viruses not studied here. The procedures developed under this work unit are demonstrably superior to conventional serologic procedures for the viruses studied, and should now be easily adapted to other viruses and antibodies of mission interest.

LITERATURE CITED

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2. Kronvall, G., and R. C. Williams, Jr. 1969. Differences in anti-protein A activity among IgG subgroups. J. Immunol. 103:828-833.

3. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a straphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. 115:1617-1624.

4. Cullen, S. E., and B. D. Schwartz. 1976. An improved method for isolation of H-2 and Ia alloantigens with immunoprecipitation induced by protein A-bearing staphylococci. J. Immunol. 117:136-142.

5. Jahrling, P. B., R. A. Hesse, and J. F. Metzger. 1978. Radioimmunoassay for quantitation of antibodies to alphaviruses with straphylococcal protein A. J. Clin. Microbiol. 8:54-60.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6423	79 10 01	DD-DR&E(AR)636

3. DATE LAST SUMMARY	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
79 01 24	K. COMPLETION	U	U	NA	NL	☒ YES ☐ NO	A. WORK UNIT

10. CODES	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	143
b. CONTRIBUTING				
c. [illegible]	STOG 79-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Defects in cellular immunity after VEE vaccination and repair with 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
77 10	78 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 79	0.3	15.5
FISCAL YEAR CURRENT 80	0	0

19. RESPONSIBLE DOD ORGANIZATION

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GENERAL USE
Foreign intelligence considered

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NAME: POC:DA

22. KEYWORDS (Include EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Vaccines; (U) Encephalitis equine (VEE); Transfer factor; (U) Enzyme immunoassay

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

23 (U) Compare immunization with attenuated VEE (TC-83) with inactivated TC-83 (C-84) vaccines in regard to cellular immune responses of guinea pigs challenged with strain 68U201 VEE virus. Assess effect of dialyzable leukocyte extracts containing transfer factor on these immune responses. The study is needed before any large-scale trials are carried out in man on the C-84 vaccine. The goal is a more effective vaccine with greater safety for protection of military personnel against VEE used as a BW agent.
24 (U) Inoculate guinea pigs with graded doses of C-84 vaccine along with appropriate controls. Determine cellular and humoral immunity before and after challenge. Several weeks later inoculate with C-84 and repeat testing. Inoculate vaccinated guinea pigs with subfractions of human transfer factor.
25 (U) 78 10 - 78 12 - The practical use of enzyme-linked immunosorbent assay (ELISA) as a diagnostic tool was demonstrated in tularemia, Venezuelan equine encephalomyelitis, and Legionnaires' disease. The assay was sensitive, specific and gave faster results than conventional tests. The initial objectives of the study have been achieved. ELISA will continue to be studied in greater detail by others for its potential use in diagnostic purposes. The primary investigator has left the Institute.

Publication: Clin. Immunol. Immunopathol. 12:72-81, 1979.

26. AVAILABILITY (Limitations/restrictions/special markings)

DD FORM 1498 PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA OH6424	79 10 01	DD-DR&E(AR)636

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

10. NO. CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	144
b. CONTRIBUTING				
c. C[illegible]	STOG 70-7,232			

11. TITLE (Precede with Security Classification Code)

(U) Mechanisms and determinants of microbial pathogenicity

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
77 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 79	0.5	61.8
	CURRENT 80	0.5	70.4

19. RESPONSIBLE DOD ORGANIZATION

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NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; (U) Microbial pathogenicity; (U) Macrophages; (U) Pathogenic determinants; (U) Francisella tularensis

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

23 (U) Characterize pathogenic mechanisms and determinants of virulent microorganisms of military importance. Employ these data to establish biochemical criteria for selection, development and evaluation of vaccine candidates and for design, synthesis and testing of antimicrobial drugs.

24 (U) In vitro culture procedures are employed to evaluate the interaction and fate of pathogenic microorganisms with tissue cells. Fundamental information is obtained from tissue fractionation, enzyme analyses and cellular physiology studies.

25 (U) 78 10 - 79 09 - Studies were conducted to determine the mechanism which permits the virulent strain of F. tularensis (SCHU S4) to survive and grow within macrophages. Rat peritoneal macrophages (PM) infected with SCHU S4 or the avirulent strain, LVS, were used as the experimental model. The following observations were made: (A) PM in in-vitro culture kill LVS but not SCHU S4; (B) subcellular fractionation of homogenates of infected PM show that phagocytosis of both strains results in phagolysosome formation; (C) both strains are resistant to killing by the oxygen-independent and -dependent microbicidal systems of the PM; and (D) SCHU S4, but not LVS, synthesize proteins optimally at the intralysosomal pH. It is concluded that none of the known microbicidal mechanisms of phagocytes are effective in killing F. tularensis. The virulence of SCHU S4 compared to LVS appears to reside in the difference with which these 2 strains can effectively perform anabolic functions within the acid environment of the PM lysosome.

Publication: In: Liposomes in Biology and Medicine, pp. 289-328, 1979.

*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, MAR 68, FOR ARMY USE, ARE OBSOLETE.

BODY OF REPORT

Project No. 3A061101A91C: In-House Laboratory Independent Research (U)

Work Unit No. A91C 00 144: Mechanisms and Determinants of Microbial Pathogenicity

Background:

A great variety of pathogenic organisms have the ability to survive and grow in macrophages (1). Such a facultative intracellular parasite is Francisella tularensis. Its virulent strain (SCHU S4) infects and grows within macrophages. In contrast, the attenuated vaccine strain is readily destroyed by RE cells. The availability and differing pathogenic characteristics of these 2 strains offer a unique opportunity to understand the mechanisms which permit virulent microorganisms to survive and grow intracellularly.

This work unit, however, is not limited to the study of F. tularensis but contributes fundamental information as to the cellular biology and immunology of other pathogenic microorganisms of military medical importance. This information is required to minimize the impact of infectious diseases on military and support personnel by identifying mechanisms and determinants of microbial pathogenesis and assure development of optimal prophylaxis and therapy.

Characterization of the biochemistry, structure and function of phagocytes and their organelles in normal and diseased animals is an essential element in clarifying mechanisms of microorganism-induced pathophysiology (2). Such studies may disclose how microorganisms, by altering normal cellular physiology, can mitigate host defense mechanisms, promote cellular dysfunction and enhance host susceptibility to infectious diseases.

Studies on microbial killing and digestion in phagocytes are in their infancy, but it is important to conceive and characterize the ways in which microorganisms can avoid being killed and digested, not only for their basic biological interest, but because of their application to development of vaccines, understanding of persistent and latent infections, design of new therapeutic agents and our ability to deal with any new microbes that periodicially arise and threaten us (3).

Progress:

Sodium caseinate-induced, rat peritoneal macrophages (PM) ingest opsonized LVS and SCHU S4 strains of F. tularensis. These PM kill the avirulent strain, LVS, but not the virulent S4 strain. Subcellular fractionation experiments of PM were therefore performed to follow the intracellular fate of both strains of bacteria following phagocytosis of ^{14}C-labeled microorganisms. PM homogenates were fractionated on sucrose gradients according to the methods of Canonico et al. (4) and the equilibrium density profiles (EDP) of radiolabeled bacteria and of marker enzymes for plasma membrane, mitochondria, endoplasmic reticulum and lysosomes were determined.

It was first shown that the EDP of the 2 bacterial strains differed slightly but significantly. The EDP for both strains after feeding to PM cultures were shifted to lighter densities coincident with the distribution profiles of lysosomal enzymes. These observations suggested that following ingestion by PM the bacteria

become localized within phagocytic vesicles. However, since the EDP of bacteria and lysosomal enzymes overlap it was not possible to determine if the bacteria is in phagosomes or in phagolysosomes. Therefore, experiments were performed with PM pretreated with the non-degradable detergent Triton WR-1339 which accumulates within lysosomes and markedly decreases their density. When Triton-treated PM were fed LVS or SCHU S4 the ^{14}C-bacterial label equilibrated in a lighter portion of the gradient. These data suggest that in vitro phagocytosis of both strains by PM result in phagolysosome formation. To confirm this conclusion PM were fed Dextran 500 which like Triton WR-1339 accumulates within lysosomes but unlike the detergent increases the density of these organelles. Feeding of bacteria to dextran-treated PM resulted in an increase in the EDP of both strains of F. tularensis coincident with a significant increase in lysosome density.

The microbicidal capacity of Triton- and Dextran-treated macrophages were then determined in order to assess the presence of artifacts or alteration in the way treated macrophages handle F. tularensis. Table I shows that opsonized virulent SCHU S4 organisms infect Dextran and Triton-treated macrophages and increase in number at a rate comparable to that seen when normal macrophages were used.

These data, therefore, support the conclusion that virulence of SCHU S4 is not expressed by an inhibition of phagolysosome formation. Rather, it appears that its virulence may be related to the greater resistance of this strain to lysosomal digestion.

Following localization of F. tularensis within rat macrophages our efforts moved to evaluating the reasons by which the virulent strain of F. tularensis resists killing within the phagolysosomal environment of rat macrophages. This required establishment of an in vitro system which replicates the intralysosomal environment so that the effects of pH and lysosomal enzymes on biochemical processes and survival of F. tularensis could be evaluated. This approach required the isolation of lysosomes from rat macrophages and identification of in vitro assay conditions which optimized the viability of bacteria.

TABLE I. PHAGOCYTOSIS AND FATE OF OPSONIZED SCHU S4 INTERACTING WITH RAT PERITONEAL MACROPHAGES IN IN VITRO CULTURE

MACROPHAGE PRE-TREATMENT	NO. BACTERIA/MACROPHAGE	
	0 hr	18 hr
Normal	16.3	82.7
Normal	10.9	63.7
Triton WR-1339	41.9	87.7
Dextran 500	8.2	21.1

Macrophage lysosomes were successfully isolated and purified about 80-fold by equilibrium density centrifugation of cellular homogenates of Triton WR-1339-treated macrophage. A synthetic media which would support the growth of F. tularensis was defined, tested and used to evaluate the capacity of the bacteria to synthesize protein, DNA and RNA within a lysosomal environment. Initially, the pH requirement for the growth of both strains was determined. It was found that the pH optima for SCHU S4 was between 4.5 and 5.5, while protein synthesis by LVS proceeded maximally at pH 6.5. This unexpected result suggests that SCHU S4 may survive within the intralysosomal environment because it can effectively synthesize protein at the intralysosomal pH, which is known to be about 4.5 to 5.0. In contrast, the intralysosomal pH does not optimize the protein synthesizing capacity of LVS. Such a mechanism is consistent with in vitro observations that SCHU S4, but not LVS, organisms can survive when phagocytized by rat peritoneal macrophages.

Similar in vitro experiments performed in the presence of lysosomal extracts demonstrated that the viability of both strains incubated with lysosomal hydrolases was not altered. When protein synthesis was measured in the presence of lysosomal proteins the relative capacity of the organism to synthesize protein was not changed. Digestion of microorganisms by lysosomal enzymes and interference of protein synthesis due to inhibition of amino acid transport by proteins of the lysosome are the 2 major oxygen-independent antimicrobial systems of the macrophage. On the basis of these data neither system appeared to account for the observed difference in the intracellular fate of LVS and SCHU S4 within macrophages.

It was then necessary to develop a technique to simulate the oxygen-dependent antimicrobial system of the phagocyte. An in vitro system was developed for the generation of superoxide by reacting xanthine oxidase in the presence of hypoxanthine in an appropriate buffer. Viable Escherichia coli or Staphylococcus aureus exposed at 25°C to the superoxide generating system were killed within 180 min. On the contrary, the viability of LVS of SCHU S4 bacteria was not changed following exposure to the same superoxide system. We concluded that superoxide and other activated oxygen intermediates are ineffective microbicidal mechanisms against F. tularensis.

It appears, therefore, that none of the known antimicrobial mechanisms of phagocytes are effective in the intracellular killing of F. tularensis. The survival or death of this bacteria within macrophages simply depends on the ability to assimilate nutrients within the intralysosomal environment. The virulence of SCHU S4 compared to LVS appears to reside in the difference with which these two strains can synthesize protein within the acid environment of the lysosome.

Publication:

Canonico, P. G., A. T. McManus, and M. C. Powanda. 1979. Biochemistry and function of the neutrophil leukocytes in the infected and traumatized host, pp. 284-328. In Lysosomes in Biology and Pathology, Vol. 6 (J. T. Dingle and P. Jacques, eds.). Elsevier North Holland Co., New York.

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APPENDIX A
VOLUNTEER STUDIES
(See Work Unit A841 00 001)

PROTOCOL TITLE AND NO. (No. Volunteers[a])	COMMENTS AND RESULTS
Initial Clinical Evaluation of Rocky Mountain Spotted Fever Vaccine, Formalin-Inactivated Sheila Smith Strain, Chick Embryo Cell Origin, Lot. 1, for Safety and Immunogenicity Protocol 76-1 (6)	Addendum 1: Planned.
Acceptability Study of Venezuelan Equine Encephalomyelitis Vaccine, Inactivated, Dried, MNLBR 109, C-84-1 (IND 914) Protocol 77-1 (7 MRVS)	Addendum 2: Three immunizations produced no greater titer than two.
Evaluation of WR 171,669 in the Treatment of Multi-Drug Resistant P. falciparum Malaria Protocol 77T-1 (3 MRVS + 1 + 8 MRVS)	In follow-up, no recurrence of malaria occurred. All 8 added volunteers became patent.
Evaluation of the Human Response to the Administration of Rift Valley Fever Vaccine, Formalin-Inactivated, Tissue Culture Origin, NDBR 103, Lots 1-6 (IND-365) Protocol 78-1 (13 MRVS + 7)	Serologic responses for lots 1-5 were comparable to lot 6.
Evaluation of Human Response to the Administration of Dengue Virus Vaccine (Type 2) Live, Attenuated (IND 1257) Protocol 78-2 (6)	Careful review was conducted of the results. Another protocol resulted (See 79-1).
Transfer of Cell-Mediated Immunity to Microbial Antigens with Dialysable Leukocyte Extracts (Transfer Factor) Protocol 78-3 (18 MRVS + 1)	There was no significant transfer of CMI.
Proposal for the Clinical Evaluation of a Two Dose Schedule of Inactivated Rocky Mountain Spotted Fever Vaccine, Undiluted (IND 862) Protocol 78-4 (10 MRVS)	Minor local reactions (2 of 13) occurred. Serologic conversion was satisfactory. Addendum 1: Effect of a 3rd booster is planned.

Immunization with Live Attenuated Dengue Virus Vaccines Protocol 79-1 (21 MRVS)	8 of 19 were viremic and seroconverted. 1 of 8 had a DEN-like illness and 3 had mild symptoms. In a feeding experiment, 2 of 72 mosquitoes which fed on one viremic volunteer were positive for DEN-2 virus.
Evaluation of WR 171,669 in the Treatment of Plasmodium vivax (Chesson strain) Malaria Protocol 79-2 (6 MRVS)	2 of 6 became patent. The other 4 were treated with chloroquine.
Evaluation of Immunologic Response to Booster Immunization of Botulinum Toxoid, Adsorbed, Pentavalent (ABCDE) (IND 161) Protocol 79-3 (54)	Booster elicited good serologic response. 4 of 54 had moderate or severe local reactions. Plasmapheresis was carried out on 28 of the group. (See also Work Unit 841 020)
Evaluation of the Human Response to Administration of Botulinum Toxoid, Adsorbed, Pentavalent (ABCDE) MDPH (IND 161) Protocol 79-4 (54)	Types A and B titers were equal to or greater than those of the Parke-Davis product. Titers to types C-E are being determined. (See also Work Unit 841 020)
Evaluation of the Human Response to the Administration of Rift Valley Fever Vaccine, Inactivated, Dried TSI-GSD-200 Protocol 79-5 (7 MRVS)	All 7 had no adverse effect. Lots 1-3 and 5 produced adequate serologic responses. Lot 4 results are incomplete.
An Evaluation of Physical Performance Capabilities during Sandfly Fever Infection Protocol 79-6 (MRVS)	Endocrine and metabolic responses were measured. This mild febrile illness was associated with loss of insulin receptors (See also Work Unit 841 052).
Determination in Humans of the Effective Half-Live of Botulism Immune Plasma (Human) IND 1332 Administered Intravenously Protocol 79-7 (1 MRVS, 8 planned)	Performance and biochemical and histologic examinations of muscle biopsies are being evaluated. Circulating neutralization titers were determined in one volunteer for types A and B, the latter being at a lower level than A the former. Types C-E titers are being determined.

[a]Volunteers are professional staff members, unless indicated to be MRVS (Medical Research Volunteer Subject).

APPENDIX B
PUBLICATIONS OF U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES FISCAL YEAR 1979

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APPENDIX C

CONTRACTS, GRANTS, MIPRs AND PURCHASE ORDERS IN EFFECT

FISCAL YEAR 1979

NO.	TITLE, INVESTIGATOR, INSTITUTION
DAMD17-78-C-8035	Mass Spectrophotometric Rapid Diagnosis of Infectious Diseases. M. Anbar, State University of New York, Buffalo
DAMD17-74-C-4095	Adjuvant Effects on Immune Responses to Biological Agents. N. D. Anderson, Johns Hopkins University
DAMD17-78-C-8017	Genetic and Molecular Studies of the Phlebotomus Fever Group of Viruses. D. H. Bishop, University of Alabama
DAMD17-79-C-9108	Analysis of Flavivirus Replication. M. Brinton, Wistar Institute
DADA17-77-C-1035	Rapid Diagnosis of Arbovirus and Arenavirus Infection by Immunofluorescence. J. Casals, Yale University
DAMD17-78-C-8042	Togavirus - Specific Cellular Immune Effector Mechanisms. G. A. Cole, School of Hygiene and Public Health, Johns Hopkins University
DAMD-17-79-C-9024	Lassa Fever Immune Plasma. J. D. Frame, Columbia University
DAMD17-77-C-7043	Development of Psoralen Photoinactivated Alphavirus and Arenavirus Vaccines. C. V. Hanson, California Department of Health
DADA17-73-C-3090	Studies on the Antigenic Composition of Coxiella burnetii. D. J. Hinrichs, Washington State University
DAMD17-79-C-9032	Regulation of Staphylococcal Enterotoxin Biosynthesis. J. J. Iandolo, Kansas State University
DAMD17-79-G-9455	Korean Hemorrhagic Fever. H. W. Lee, Korea University Medical College, Seoul
DAMD17-77-C-7034	Resident Research Associateship Program (Postdoctoral and Senior Postdoctoral) with the Walter Reed Army Institute of Research. H. W. Lucien, National Academy of Sciences
DAMD17-79-C-9046	The Synthesis and Study of New Ribavirin Derivatives and Related Nucleoside Azole Carboxamides as Agents Active against RNA Viruses. R. K. Robins, Brigham Young University
DAMD17-72-C-2140	Studies on Arthropod-Borne Viruses. W. F. Scherer, Cornell University
DAMD17-77-C-7023	Role of Cellular Components of Mosquito Cells in Viral Replication and Transmission. R. H. Schloemer, Indiana University School of Medicine

DAMD17-78-C-8018	Development of Special Biological Products. A. Shelokov, Salk Institute
DADA17-72-C-2170	World Reference Center for Arboviruses. R. E. Shope, Yale University
DA49-193-MD-2694	Biochemical Changes in Tissues during Infectious Illness - Bioenergetics of Infection and Exercise. R. L. Squibb, Rutgers - State University
DAMD17-79-C-9053	Serological Screening Test for any Botulinum Toxin Type. H. Sugiyama, University of Wisconsin Madison
DAMD17-79-D-0006	Preparation of Hyperimmune Botulinum Toxin. S. Ware, Pine Bluff Biological Products
DADA17-78-G-9451	Investigation and Management of Ebola Virus Infection in Non-Human Primates. A. J. Zuckerman, London School of Hygiene and Tropical Medicine, England

GLOSSARY

ADCC	Antibody dependent cell mediated cytotoxicity
ADP	automatic data processing
AHF	Argentine hemorrhagic fever
BHF	Bolivian hemorrhagic fever
BUN	blood urea nitrogen
CBC	complete blood count
CEC	cnick embryo cell (culture)
CF	complement fixation
CHO	Chinese hamster ovary
CL	chemiluminescence
CPE	cytopathic effect
CPK	creatinine phosphokinase
DEN	Dengue virus
EBO	Ebola
ED_{50}	median effective dose(s)
EEE	Eastern equine encephalitis(virus)
EF	edema factor
EM	election microscope
EP	endogenous pyrogen
FA	fatty acid(s)
GH	growth hormone
GOT	glutamic-oxalacetic transiminase
HA	hemagglutinins, hemagglutination
HAI	hemagglutinating inhibition
HAZ	hazara
HI	hemagglutination inhibition

ID	intradermal (ly)
ID_{50}	median infectious dose (s)
$IPLD_{50}$	infectious intraperitoneal lethal dose (s)
IM	intramuscular (ly)
IN	intranasal
IP	intraperitoneal (ly)
IV	intravenous (ly)
JE	Japanese encephalitis
JUNV	Junin virus
KHF	Korean hemorrhagic fever
LAC	LaCrosse virus
LCFA	long chain fatty acids
LAS	Lassa fever
LD	median lethal dose(s)
LCM	lymphocytic choriomeningitis
MA	microagglutination, microagglutinin
MAC	Machupo virus
MMD	mass median diameter
$MLPLD_{50}$	median infectious intraperitoneal lethal dose(s)
mRNA	messenger RNA
MTD	mean time to death
NIH	National Institutes of Health
ORO	oropouche
PA	protective antigen
PEC	peritoneal exudate cells
PFU	plague forming unit (s)
PGMK	African green monkey kidney

PIC	Pichinde virus
PMN	polymorphonuclear luekocytes
PR_{50} or PR_{80}	50% or 80% plaque reduction
RBC	red blood cells
RES	reticuloendothelial system
RIA	radioimmunoassay
RMSF	Rocky Mountain spotted fever
rRNA	ribosomal RNA
RVF	Rift Valley fever
SC	subcutaneously
SEA	staphylococcal enterotoxin A
SEB	staphylococcal enterotoxin B
SEC	staphylococcal enterotoxin C
SF	Semiki forest virus
SF-N	Sandfly fever - Naples
SF-S	Sandfly fever - Sudian
SGPT	serum glutamic pyruvic
SIN	Sindbis virus
SP	small plaque
UV	ultraviolet
VEE	Venezuelan equine encephalomyelitis (virus)
WBC	white blood count
WEE	Western equine encephalities (virus)
WRAIR	Walter Reed Army Institute of Research
YF	Yellow fever

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