AD

U S ARMY MEDICAL RESEARCH INSTITUTE
OF INFECTIOUS DISEASES

ANNUAL PROGRESS REPORT FY 1981

RCS-MEDDH-288(RI)

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UNITED STATES ARMY MEDICAL RESEARCH INSTITUTE
OF INFECTIOUS DISEASES

Frederick, Maryland 21701

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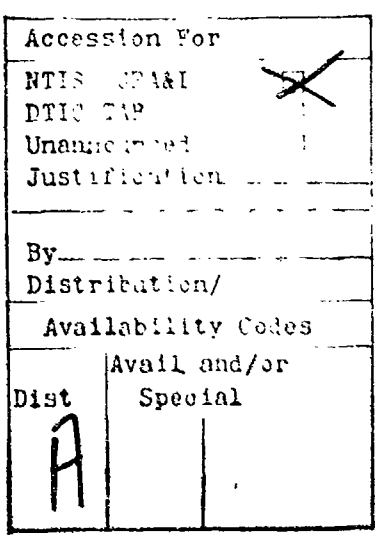
FREDERICK, MARYLAND 21701

ANNUAL PROGRESS REPORT

FISCAL YEAR 1981

RCS-MEDDH-288(R1)



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Projects 3M162770A70
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3A161101A91C1

1 October 1981

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Biological warfare	Rickettsial diseases	Pathology	Host
Viral diseases	Laboratory animals	Defense	
Prophylaxis	Parasite	Metabolism	
Identification	Biochemistry	Malaria	

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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 1981 is presented.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

EDITOR'S NOTE

This FY 1981 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 3M162770A870 and 3M162770A871, and 3M161102BS10 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.

AUTHOR INDEX

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APPENDICES

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

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
Keystone virus
Influenza virus

Rickettsial

C. burnetii

Parasitic

P. falciparum

Toxins

Pseudomonas A
Diptheria
Botulinum A-G
Anthrax toxins
Mycotoxin T-2
Saxitoxin
Tetrodotoxin
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 81:

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 81: FY 1981 was a most productive and eventful year for USAMRIID, with its research programs producing much new data as well as undergoing major realignments and shifts of emphasis in response to USAMRDC guidance, SPEF/SPF guidelines and newly emerging information on Russian BW capabilities and their presumed intent to employ this form of warfare following the reports of the Sverdlovsk accident. A markedly expanded program on anthrax was undertaken to improve the existing vaccine and to develop more information on the pathogenesis of

this disease, particularly the pulmonary form of anthrax. Newspaper accounts of the Russian-supported use of deadly trichothecene toxin (T-2 mycotoxin) in Indochina and possibly Afghanistan have accelerated the implementation of research on medical defenses against small molecular weight toxins within Physical Sciences Division. This new program, which concentrates on T-2 and other mycotoxins, saxitoxin and tetrodotoxin, is being funded at the expense of reduced effort on "Enhancement of Host Defense" studies. Anticipated medical infectious disease needs of Rapid Deployment Forces (RDF) are being supported by a new program concerned with the seroepidemiology of field samples collected from potential trouble spots of the world. Exotic, high-hazard viral diseases endemic to some of these trouble spots constitute a genuine threat to the RDF.

C. Research areas given added emphasis during FY 81: An entirely new program was initiated to study tricothecene fungal toxins, marine toxins and other small molecular weight toxins of microbial origin.

New and expanded emphasis was placed on developing rapid diagnostic methods for the identification of microorganism-derived antigens and/or host antibodies. This is aimed at the diagnosis in the field of both naturally and artificially acquired infectious diseases or toxemias.

A new entomology program, also designed to support RDF, will attempt to define vectors/exotic disease relationships.

Three new live, attenuated viral vaccines are in the final stages of development and include Dengue-1, Chikungunya, and Argentine hemorrhagic fever (Junin virus). The new technology of monoclonal antibodies from lymphocyte hybridomas is being exploited in an effort to develop an entire new generation of more effective, highly specific, and safe vaccines. A major advance has been made in defining the interrelationships among the three toxins of anthrax; that is, protective antigen (PA), lethal factor (LF) and edema factor (EF). The most surprising and interesting information from this program is that edema factor was proved to be an adenylate cyclase. Because EF is an enzyme (like cholera and diptheria toxins) it derives its potency by catalytically altering essential molecules in animal cells.

The botulinum program has continued to progress with the creation of a new neurophysiology laboratory and the hosting of an International Conference at USAMRIID on "Biomedical Aspects of Botulism." Aerosol risk assessment studies have shown that Legionella pneumophila and Lassa fever and Bolivian hemorrhagic fever viruses can each be disseminated as infectious, small-particle aerosols and each is relatively stable. Unfortunately, it was learned that animals previously immunized with killed Rift Valley fever (RVF) vaccine, developed disease when challenged with virulent aerosols of RVF virus. These data suggest that some nonliving vaccines may not be adequate in protecting against virulent aerosols.

Hematological studies on ribavirin, an antiviral drug with effectiveness against some hemorrhagic viruses, have been completed and indicate that high concentrations of the drug arrest maturation of red-cell precursors; however, this effect is reversible when the drug is discontinued.

Finally, the safety loop has been closed to permit the evacuation (air and surface), diagnosis, and treatment under full biological (P-4) isolation conditions of patients suspected of or actually having an infection with such high hazard agents as Lassa, Junin or Ebola virus.

D. Research Areas completed or for which efforts are diminishing in FY 81: Methods were successfully developed for: (a) growing L. pneumophila in artificial media to high concentration while preserving infectivity, (b) isolating a toxin common to Legionella and Legionella-like microorganisms; (c) identifying the organism by serological techniques. This highly successful Legionella program has now been partially phased-down during this fiscal year to permit expansion of the anthrax program.

The long-term highly productive USAMRIID investigations into infection-induced alterations in host metabolism, biochemical and hormonal responses, and performance capacity were also phased-down to permit the initiation of the small molecular weight toxin program within USAMRIID. Studies of endogenous pyrogen and leukocytic endogenous mediator were terminated.

Failure to identify an effective vaccine candidate against P. pseudomallei lead to termination of this effort. Work was also reduced in studies of encephalitic viruses to permit increased eforts in hemorrhagic ones.

E. List of significant accomplishments for FY 81:

1. The NRC/NAS Postdoctoral Research Fellowship Program was fully initiated at USAMRIID.

2. A total of 64 research papers were published.

3. USAMRIID remained among the top three Army laboratories in the ILIR competition for the third consecutive year.

4. A markedly expanded research program on anthrax was initiated to improve the existing vaccine and to develop more information on the pathogenesis of the disease. Toxic exoproteins of anthrax were separated to a high degree of purity. Edema factor was found to be a potent adenylate cyclase. Methods were established for permitting test studies with virulent strains under maximum containment conditions.

5. A new program was begun on small moleuclar weight toxins with initial emphasis on T-2 mycotoxins and selected marine toxins.

6. A new program was introduced to study the sero-epidemiology of field samples collected from potential spots in the world where rapid deployment forces might be assigned. The diagnostic program was expanded to provide rapid agent identification and disease diagnosis capabilities under field conditions. The efficacy of spot slide diagnostic procedures for large assortment of viruses was tested effectively with double blind methodologies.

7. A new entomology program, also designed to support the rapid deployment forces, was initiated to define insect vectors and exotic disease relationships. Studies were initiated to elucidate the vector potential and taxonomy of suspected arthropod vectors of Rift Valley fever virus. Other studies were targeted toward determining the vectors for Ebola and Lassa fever viruses.

8. Approximately 80 potential antiviral drugs were evaluated in a screening program against Rift Valley fever, VEE, Pichinde, sandfly fever, and yellow fever viruses.

9. The production of monoclonal antibodies from lymphocyte hybrodomas was increased for the diagnostic program as well as for the development of new generations of vaccines.

10. A new neurophysiology laboratory was established to study botulinum and other neurotoxins and their therapy. Two experimental drugs were shown to reverse temporarity botulinum Type A neurotoxicity.

11. An international conference was held at USAMRIID on the biomedical aspects of botulinism.

12. The causative virus of Korean hemorrhagic fever was identified and characterized in tissue culture in terms of its electron microscopic appearance and physical characteristics. Attempts to culture this organism and identify it serologically were improved.

13. Methods were developed for growing high concentrations of Legionella-like bacteria in artific[illegible]ia.

14. A toxin was isolated which was common to Legionella and to Legionella-like bacteria.

15. Aerosol-risk assessment studies showed that L. pneumophila, Lassa fever virus and Bolivian hemorrhagic fever virus were each infectious in a small particle aerosol. A component of media in which blue green algae were grown was shown to stablize Legionella organisms while in aerosol.

16. The available formalinalized Rift Valley fever vaccine was found to give inadequate protection to animals challenged with aerosols containing the virulent virus. Improved techniques were established to separate the individual glycoproteins of Rift Valley fever virus in an attempt to produce an improved vaccine and for moving toward gene cloning technologies for this purpose. Studies were initiated to determine the reasons for the genetic differences in susceptibility to Rift Valley fever virus infection among different strains of rats.

17. Various combinations of intravenous nutrients with altered energy and amino acid content were tested for their efficacy for treating infected animals.

18. A jacket and tether system was developed and perfected for permitting long-term infusions of monkeys during studies. This system replaced the use of restraint chairs for comparable studies.

19. A Pichinde virus infection of Strain 13 guinea pigs was established as a useful animal model for studying the hemorrhagic mechanisms of various dangerous hemorrhagic fever viruses.

20. Studies were conducted to support the biological detection and alarm program of the Chemical System Laboratory for testing XM-2 biological sampler.

21. The hematological toxicity of ribavirin, an antiviral drug, was shown to be reversible in monkeys.

22. Operating procedures were perfected and tested and personal training practices were adapted to permit the safe and effective transportation of highly contagious patients, full hospital care, and clinical laboratory testing under P-4 level maximum biological hazard containment conditions.

23. Studies were continued using Pseudomonas exotoxin to determine how similar toxic molecules enter a cell.

24. Additional studies were conducted to define the primary amino acid structure of Staphlococcal enterotoxins.

25. Studies were continued on the Phase I antigen of C. burnetii to separate the protective components from those which cause adverse vaccine reactions.

26. Studies were continued in volunteers to test new antimalarial drugs against resistant species.

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 academia and industry. Individual contractor's research is synopsized in reports which are filed with the Defense Technical Information Center (DTIC). A list of contracts in place during FY 81 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

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0G1536	81 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
0 10 02	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	62770A	3M162770A870	BA	070
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

TITLE (Procede with Security Classification Code)* (U) Risk Assessment and Evaluation of Viral Agents and Their ectors that Pose a Potential BW Threat

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

003500 Clinical medicine; 004900 Defense; 010100 Microbiology; (U) Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 81	5.3	577
	CURRENT 82	6.0	564

19. RESPONSIBLE DOD ORGANIZATION

a. NAME:* USA Medical Research Institute of Infectious Diseases

b. ADDRESS:* Fort Detrick, MD 21701

c. RESPONSIBLE INDIVIDUAL

a. NAME: Barquist, R. F.

b. TELEPHONE: 301 663-2833

d. GENERAL USE

Foreign intelligence considered

20. PERFORMING ORGANIZATION

a. NAME:* Virology Division USAMRIID

b. ADDRESS:* Fort Detrick, MD 21701

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

a. NAME:* Bailey, C. L.

b. TELEPHONE: 301 663-7241

c. SOCIAL SECURITY ACCOUNT NUMBER:

d. ASSOCIATE INVESTIGATORS

a. NAME: Watts, D. M.

b. NAME: POC:DA

21. KEYWORDS (Procede EACH with Security Classification Code) (U) Military medicine: (U) BW defense; (U) Viral diseases; U) Arthropod transmission; (U) Entomology

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

3. (U) Identify specific arthropods and vertebrates associated with the maintenance and ransmission of medically important arboviruses to man and define ecologic and intrinsic actors influencing the ability of arthropods to transmit viruses. Information will llow for development of methods for predicting relative risk of military personnel to rbovirus infections and for designing specific vector control strategies.

4. (U) Conventional and innovative field and laboratory procedures are employed to mplicate specific arthropods and vertebrates as vectors and hosts of arboviruses, espectively. Ecologic and intrinsic factors relating to vector competence are deter- ined experimentally by elucidating the dynamics of virus-vector interactions during he extrinsic incubation period.

5. (U) 80 10 - 81 09 - 50-100% of 8 different Egyptian strains of Culex pipiens mos- quitoes became infected after feeding on about 100,000 PFU of Rift Valley fever (RVF) virus in the blood of donor hamsters. Transmission rates for these mosquitoes ranged from 0-60%, but data are not sufficiently complete to determine differences in geo- graphic origin of mosquitoes, virus, or a combination. Virus transmission was also accomplished by 10 individual mosquitoes which probed for a bloodmeal from hamsters, but were unsuccessful in obtaining that bloodmeal. RVF studies were conducted on the sandfly, Lutzomyia longipalpis, by feeding on hamsters viremic with 1 and 10 million PFU/ml of virus. Relatively low levels of virus were recovered from them on days 1-7. Techniques have been developed to measure salivary output which should enable quantitative transmission studies to be conducted without the need for a monkey model.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U)

Work Unit No. 870 BA 070: Risk Assessment and Evaluation of Viral Agents and Their Vectors That Pose a Potential BW Threat.

Background:

Numerous viral diseases normally transmitted by arthropod vectors are potential threats to the mission of U.S. military forces. Some of these diseases are transmitted to humans in nature only through the bite of an infected arthropod, i.e., dengue (DEN). Others, such as Rift Valley fever (RVF) and Chikungunya (CHIK) can be transmitted not only by an infected arthropod, but also by aerosol. Effective vaccines are not currently available for many of these diseases. Therefore, prevention or control of virus transmission is often achieved only by reducing or avoiding vector populations. It is the scope of this work unit to understand the life cycles of viral diseases which pose a known or potential threat to military personnel. Included are not only vector and reservoir identification, but also ecological and environmental factors which predispose virus transmission. Lack of a clear understanding of all these factors limits our ability to prevent or control more effectively these diseases. With this scope in mind the following program of research was developed. (a) Determining the RVF virus (RVFV) vector potential of several different species of mosquitoes and sandflies from Africa and the United States, and (b) determining the effect of environmental temperature and geographic strain of the vector on transmission of DEN, CHIK and eastern equine encephalitis (EEE) viruses.

Progress:

Rift Valley Fever Virus

Until 1977, RVF was considered to be a disease mainly of domestic animals in sub-Saharan Africa, while in humans it was considered serious, but rarely fatal. RVFV was first reported north of the Sahara Desert in 1977, when it caused an epidemic in the Nile Delta of Egypt, resulting in considerable animal and human mortality. Meegan et al (1), reported results of experimental transmission studies with a single geographic strain of Egyptian Culex pipiens, and implicated this species as a vector of the virus during the 1977-78 epizootics. However, the authors eluded to the possibility that different transmission rates were obtained with differences in vector potential that we are interested in pursuing. Such information should clarify the role of this species in the epidemiology of RVF in Egypt.

Several geographic strains of Egyptian Cx. pipiens have been colonized by us. Results of preliminary transmission experiments are presented in Table I. From 50 to 100% of most strains of this mosquito became infected after feeding on hamsters circulating approximately $10^{5.5}$ PFU of RVFV/ml of blood. Although infection rates were generally high, virus transmission rates varied form 0 to 60%. The data are not yet complete enough to draw definitive conclusions regarding whether these differences reflect differences in geographic strains, the amount of virus ingested, or a combination of both factors. Virus titers for individual mosquitoes that transmitted virus ranged from $10^{4.0}$ to $10^{7.1}$ PFU/ml while average titers for nontransmitters were in the 2-3 $\log_{10}$ PFU/ml range or lower (Table II). Transmission of the virus was also accomplished by 10 different mosquitoes which probed for a bloodmeal from hamsters, but were unsuccessful in obtaining that bloodmeal. Virus titers for these mosquitoes ranged from $10^{4.8}$ to $10^{5.7}$ PFU/ml. It is therefore possible that if this behavior occurs in nature, these mosquitoes could transmit to a larger number of susceptible animals or humans

than those mosquitoes which are successful in bloodfeeding on the first try by transmitting with each attempt to feed. It appears that virus transmission rates were not influenced by the length of the extrinsic incubation after day 7, since there were no differences among days 7, 12 and 15. This observation needs to be extended, however, to test transmission rates on days 20 and 25.

Preliminary data suggest that North American strains of Cx. pipiens and Aedes taeniorhynchus as well as an African strain of Eretmapodites quinquivittatus are susceptible to RVFV infection, but transmission trials were hampered by low virus titers or complete failure of these mosquitoes to refeed on hamsters.

Contrary to the report by Meegan et al. (1), a lag-phase or drop in titer was observed on day 1 or 2 pc infectious bloodmeal for 4 strains of mosquitoes tested. This 2-3 log decrease was followed by titer increases in most species and strains reaching maximum titers by days 6-8.

Even though there is substantial epidemiologic and laboratory evidence to implicate mosquitoes as vectors of RVFV during epizootic/epidemic outbreaks of the disease, there is little available information describing the enzootic/endemic maintenance of the virus. An understanding of the broad range of susceptible vertebrates to the virus and the diversity of environmental regions in which this virus has been recovered may indicate that other biting arthropods are involved in virus transimssion, i.e., Culicoides and sandflies. Supportive evidence for vectors other than mosquitoes is suggested, not only by the recovery of RVF virus from Culicoides in nature, but also the fact that the virus has now been classified as a sandfly fever group virus. With these observations in mind, studies have been initiated to test the susceptibility of sandflies to RVFV and to conduct taxonomic studies on Kenyan species of Culicoides.

Due to the difficulty involved in rearing sandflies and the unavailability of African species, preliminary experiments with these insects were conducted with small numbers of a New World species. In an effort to determine whether RVF virus would replicate in sandflies, a nanoliter pump was used to inoculate a predetermined quantity of virus via a micro-needle into anesthetized sandflies. Results from this preliminary experiment are shown in Table III. Virus was recovered from only 1 of 18 sandflies in this experiment. Although results were essentially negative, it is believed that with additional refinement of inoculation methodologies, this metnod can be developed for routine infection of sandflies with virus.

Since Lutzomyie longipalpis, the sandfly species used in these experiments is relatively short-lived with few surviving the first oviposition cycle, it was determined that initial experiments should be designed to determine if this species could be infected with RVFV by feeding on viremic hamsters prior to conducting actual transmission experiments. Thus, the experimental approach was to assay sandflies at various intervals after feeding on viremic hamsters to determine if they became infected and if so, did the titer of virus in the sandflies increase with time. Table IV shows that RVFV was recovered from sandflies from days 0 through 7 after bloodfeeding on hamsters circulating between 6 and 7 $\log_{10}$/ml of RVF virus. Relatively low titers of virus were recovered from these sandflies from days 1-7 which makes it impossible to infer virus multiplication. It is plausible that the low titer of virus in the sandflies even on day 7 is residual virus in the midgut. However, we have not discounted the fact that these low virus titers may represent significant infections which would allow the sandflies to transmit. Studies are currently underway to establish better virus growth curves in sandflies and to evaluate their potential for RVFV transmission.

TABLE I. TRANSMISSION OF RIFT VALLEY FEVER VIRUS TO HAMSTERS BY DIFFERENT GEOGRAPHIC STRAINS OF EGYPTIAN CULEX PIPIENS MOSQUITOES.

MOSQUITO SPECIES AND STRAINS	VIRUS TITERS OF INFECTIVE BLOODMEAL*	INCUBATION PERIOD (DAYS)**	INFECTION RATE (%)***	TRANSMISSION RATE (%)***
C. pipiens				
Egyptian Strains				
Sharqiya$_{P1}$	8.4-9.4	7,10,12,15	69/92 (75)	17/35 (49)
Sharqiya$_{P3}$	8.9-8.3	7,10	24/26 (92)	1/7 (14)
Egyptian Mix$_{P-11}$	8.6-8.6	7,10,12	95/112 (85)	19/41 (46)
Autogeneous Egyptian Mix$_{P-10}$	9.3-9.9	7	22/30 (73)	1/13 (8)
A-Qalumbiya$_{P-7}$	5.6-6.9	7	32/32 (100)	3/5 (60)
A-Qalumbiya$_{P-8}$	7.2-7.4	7,12	5/10 (50)	0/2 (0)
B-Qalumbiya$_{P-8}$	6.4-7.4	4,5,6,7,8,9	60/59 (85)	2/20 (10)
C-Qalumbiya$_{P-7}$	5.6-6.9	4,5,6,7	2/10 (20)	0/2 (0)
Ismailia$_{P-8}$	5.5-7.1	4,5,6,7,9	15/34 (44)	0/11 (0)

* Virus titer per ml blood in hamsters before and after mosquitoes fed.
** Days after mosquitoes fed on viremic hamsters that infection and transmission observations were made.
***Accumulative infection and transmission rates for observations made for each incubation period.

TABLE II. COMPARISONS OF RIFT VALLEY FEVER VIRUS TITERS IN EGYPTIAN STRAINS OF CULEX PIPIENS MOSQUITOES THAT TRANSMITTED WITH THOSE THAT DID NOT TRANSMIT VIRUS TO HAMSTERS.

MOSQUITO SPECIES AND STRAIN	VIRUS TITERS (PFU_{50}/1.0 ml) Nontransmitters			Transmitters		
	No.	Range	Mean	No.	Range	Mean
$Sharqiya_{F-1}$	18	2.0-3.6	2.8±.6	15	4.5-5.8	5.2±.4
$Sharqiya_{F-2}$	6	1.8-4.3	3.0±.9	1	-	5.1
$Sharqiya_{F-3}$	22	1.9-3.9	2.7±.7	15	4.0-7.1	5.3±.8
Egyptian Mix_{F-11}	12	1.7-3.5	2.5±.6	1	-	4.9
Autogenus Egyptian Mix_{F-10}	2	2.1-4.0	3.1±1.3	3	5.3-6.5	5.8±.6

TABLE III. SUSCEPTIBILITY OF LITZOMYIA LONGIPALPIS TO RIFT VALLEY FEVER VIRUS FOLLOWING INTRATHORACIC INOCULATION OF 1.6×10^3 PFU/ml.

POSTINFECTION PERIOD (Days)	NO. INFECTED/ NO. TESTED		TITER OF VIRUS RECOVERED	
	Live*	Dead**	Live	Dead
1	-	0/5	-	0
4	-	0/2	-	0
5	0/4	1/1	0	4×10^3
7	0/5	0/1	0	0

* All live sandflies were tested individually.
**Dead sandflies were tested in pools containing from 2-7 individuals.

Because RVFV has been isolated from Culicoides in Kenya, and it is anticipated that this laboratory will in the near future initiate studies in Kenya to identify the enzootic cycle of the disease, a definitive systematic manual of the Culicoides of Kenya is being developed. This manual will include a taxonomic key for the identification of the adults of all known species occurring in Kenya. This key will include thorough morphologic description with accurate detailed illustrations with summaries of the known biology and distribution which will provide for accurate identification of potential vector species. Table V provides a tentative list of the known Culicoides of Kenya.

Dengue Virus

Investigations concerning the effects of temperature on the vector efficiency of the mosquito Aedes aegypti for DEN viruses focused primarily on the establishment of assays and development of a technique to be used in lieu of a more expensive primate model for assessing vector efficiency. In brief, the technique involves placing the mouthparts of immobolized virus-infected mosquitoes into the cavity of a 0.8-mm diameter capillary tube. Immersion oil is introduced into the opening of the distal end of

the tube and allowed to flow until contact is made with the mouthparts. Contact with the oil stimulates the mosquitoes to salivate. Each mosquito is allowed to secrete saliva for 3 min; each tube of saliva and oil is then transferred to a vial containing 0.5 ml of medium. The contents of the tubes are mixed with the medium by disintegrating the tube with a glass plunger.

TABLE IV. SUSCEPTIBILITY OF L. LONGIPALPIS TO RIFT VALLEY FEVER VIRUS FOLLOWING BLOOD FEEDING ON 6 VIREMIC HAMSTERS CIRCULATING FROM 3×10^6 - 8×10^7 PFU/ML OF BLOOD.

POSTINFECTION PERIOD (Days)	NO. INFECTED/ NO. TESTED		RANGE OF VIRUS TITERS FROM SANDFLIES	
	Live	Dead	Live	Dead
0	6/8	-	O-EPND*	-
1	-	2/2	-	-
2	1/1	-	1.2×10^2	-
3	2/2	6/6	1×10^2	3×10^1
			2×10^2	2.2×10^4
4	5/9	4/4	$0 - 1 \times 10^3$	$0 - 8 \times 10^3$
5	-	6/6	-	2×10^1
				1×10^3
6	2/3	-	$0 - 1 \times 10^2$	-
7	1/1	1/1	1×10^2	1×10^1

*End point not determined.

After this technique was perfected, saliva was collected from Ae. aegypti infected with DEN-2 virus by intrathoracic inoculation. Virus was not detected in saliva by the plaque assay employing LLC-MK_2 cells, but was recovered from C6/36 cells that were allowed to incubate at 28C for 10 days afte reinoculation with saliva suspensions. Findings suggested tha this technique may be suitable as an indicator of virus transmission.

Transovarial Transmission of Alphavirus

Investigations were initiated to explore the possibility that EEE virus is maintained by inheritance from parent to offspring in Culiseta melanura with the virus surviving in the larvae during the winter season in the Pocomoke Cypress Swamp, MD. A total of 118 third and fourth stage larvae were collected during March and April. The majority of these larvae were retained for approximately 14 days at 26-28C in the laboratory. During the holding period, 21 males and 3 females emerged. The larvae that failed to emerge and the adults were stored at -70C for virus assay. All specimens will be assayed for virus by the mosquito inoculation technique employing Toxorhynchites amblonensis and in C6/36 cells at 27 and 28C, respectively. Due to the low number of larvae collected by the standard dip technique, 10 floating larvae traps were placed in selected breeding sites on 25 March in an attempt to increase the sample size. These traps failed to capture Cs. melanura larvae.

Other studies are in progress to assess the possible role of the follicle epithelium as a barrier to transovarial transmission of alphaviruses by respective vector mosquitoes. An Ae. aegypti-Sindbis virus model was selected with the intentions of extending such studies to other more appropriate vector/virus combinations. Employment of this system depends on results of ongoing experiments designed to obtain indirect evidence of an ovarian barrier by demonstrating

that Sindbis virus is not transmitted transovarially by experimentally infected Ae. aegypti. In this experiment, 120 mosquitoes were inoculated intrathoracicaly with $10^{4.7}$ PFU/ml of Sindbis virus. Approximately one-half of these mosquitoes ingested blood from hamsters 24 hr before inoculation with virus, while the remainder were bloodfed 24 hr after inoculation. Mosquitoes were maintained at 28C and 70-80% relative humidity. During the incubation period, mosquitoes were allowed to feed on hamsters at approximately 2-week intervals until the completion of 3 ovarian cycles. After the 3rd batch of eggs were laid, parent mosquitoes were stored individually at -70°C for virus assay. Eggs from each ovarian cycle were hatched in separate trays and immatures were allowed to develop to adults at 26C. All male mosquitoes were stored at -70C within the first 24 hr of emergence, while females were stored on days 1-3 and on days 11-14 after emergence.

TABLE V. THE CULICOIDES (DIPTERA: CERATOPOGONIDAE) OF KENYA.

Genus Culicoides	
Subgenus Monoculicoides	adersi group
cornutus	adersi
Subgenus Meijerehelea	albovenosus group
distinctipennis	albovenosus
leuocostictus	
pycnostictus	dekeyseri group
	kaimosiensis
Subgenus Beltrammyia	
nivosus	inornatipennis group
	arenarius
Subgenus Trithecoides	ravus
fulvithorax	
	neavei group
Subgenus Avaritia	neavei
grahamii	ovalis
imicola	
kanagai	schultzei group
kibatiensis	rhizophorensis
spinifer	schultzei
tororoensis	enderleini and/or subschultzei
trifasciellus	
	sterocorarius group
Subgenus Diphaomyia	bedfordi
accraensis	gambiae
exspectator	
papillatus	Unplaced species
radiomaculatus	eriodendroni
similis	parvulus
tropicalis	shimoinensis

Sindbis virus was recovered from suspensions prepared from each of 18 parent mosquitoes. Virus titers per suspension ranged from 10^4 to $10^{5.3}$ and the geometric mean titer was $10^{4.7}$ PFU/ml. Attempts to recover virus from suspensions prepared from 101 pools of 1-day-old progeny males consisting of 409 specimens were unsuccessful. Likewise, virus was not detected in suspensions prepared from 18 pools

consisting of 394 1-3-day old progeny females. In addition, of evidence virus was not noted on reassaying these mosquitoes. However, virus was recovered from suspensions prepared from 12 of 15 pools consisting of 139 11-15-day-old progeny females. These virus-positive suspensions and parent mosquito suspensions were confirmed as Sindbis by PRN tests employing virus-specific antiserum. In order to verify further that Sindbis virus was transmitted vertically to offspring, remaining eggs from the parent mosquitoes were hetched and immatures were reared to adults at 26C. Virus was not detected in 45 suspensions prepared from individual males that were 3-7 days old. Females were allowed to lay 2 batches of eggs and then stored at -70C for virus assay. Virus was recovered from suspensions prepared from 31 of 36 individual females. Virus titers per suspension ranged from $10^{2.3}$-$10^{4.5}$ PFU/ml and the geometric mean titer was $10^{3.6}$ PFU/ml. Eggs from the latter females were hatched and immatures were reared to adults. Attempts to recover virus from suspensions prepared from 5 individual males and 5 individual females sacrificed on days 5, 10 and 15 after emergence were unsuccessful. These data suggested that virus was not transmitted to 2nd generation offsprings. An experiment to confirm these findings is in progress.

Table VI provides a summary of the species, strains, origin and estimated monthly production of all species currently reared in our production insectary. All of the mosquito species except the Toxorhynchites are used in virus transmission experiments. The Toxorhynchites are used as a host for virus assays. After considerable initial difficulty in establishing sandfly colonies, techiniques were modified so that it appears tha we will be able to rear 2 different species. Both species are New World phlebotomines, indigenous to the Neotropic and Neoartic regions. Colony production of all species with the exception of Lutzomyia anthrophora are now providing sufficient specimens for both colony maintenance and experimental transmission studies.

Publications.

None.

LITERATURE CITED

1. Meegan, J. M., G. M., Khalil, H. Hoogstraal, and F. K. Adham. 1980. Experimental transmission and field isolation studies implicating Culex pipiens as a vector of Rift Valley fever virus in Egypt. Am. J. Trop. Med. Hyg. 29:1405-1410.

TABLE VI. VECTOR SPECIES AND STRAINS REARED IN THE DEPARTMENT OF ARBOVIRAL ENTOMOLOGY.

MOSQUITO SPECIES	ORIGIN	LABORATORY DESIGNATION	HISTORY	ESTIMATED MONTHLY PRODUCTION
Cx. pipiens	Sharqiya, Egypt	Sharqiya	USAMRIID Colony, 1981	28,827
	Qalumbiya, Egypt	Egyptian mix	USAMRIID Colony, 1981	24,000
	Qalumbiya, Egypt	Autogenous Egyptian mix		
	Qalumbiya, Egypt	A-Qalumbiya	USAMRIID Colony, 1980	8,600
	Qalumbiya, Egypt	B-Qalumbiya	USAMRIID Colony, 1980	7,600
	Qalumbiya, Egypt	C-Qalymbiya	USAMRIID Colony, 1980	9,500
	Cairo, Egypt	Ain Shams	USAMRIID Colony, 1980	9,500
	Ismailia, Egypt	Ismailia	USAMRIID Colony, 1980	10,800
	New Jersey, USA	Mott	WRAIR Colony, 1975	9,500
	Washington, DC	Washington	WRAIR Colony, 1977	13,700
	Washington, DC	Yellow body	WRAIR Colony, 1975	13,500
Ae. taeniorhynchus	Florida, USA	Ae. taeniorhynchus	USAMERDL COLONY, 1975	16,600
Er. quinquevittatus	Dar Es Salam, Tanzania	Eretmapodites	AMBI Colony, 1971	950
Ae. aegypti	Bangkok, Thailand	Aegypti	Field collected eggs, 1981	400
To. amboinensis	Hawaii	Tox	Unknown	200
SANDFLY SPECIES				
Lu. longipalpis	Minas, Brazil			1,500
Lu. anthrophora	Del Rio City, TX			800
			TOTAL	171,000

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA OG3814	81 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
80 10 02	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	62770A	3M162770A870	BB	069
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2	2		

11. TITLE (Precede with Security Classification Code)*

(U) Assessment of Airborne Microbial Agents of Potential BW Threat

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
80 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 81	3.5	401
FISCAL YEAR CURRENT 82	4.0	982

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

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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) Viral diseases; (U) Aerosols; (U) Bacterial diseases; (U) Respiratory immunity; (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) Define aerosol stability characteristics and respiratory infectivity properties of organisms of potential BW importance; elucidate pathogenesis of infections induced by aerosols; evaluate vaccines for protection against aerosol challenge. These studies are used to identify efficacious vaccines which will protect deployed military troops.
24. (U) Determine aerosol survival characteristics of potential BW agents, develop animal models, including LD-50 information as well as clinical, gross and histopathologic changes during infections. Characterize immune defenses within the respiratory tract. Use this information to determine the efficacy of vaccination and therapeutic procedures.
25. (U) 80 10 - 81 09 - Lassa fever virus was relatively stable in aerosol and infected guinea pigs and cynomolgus monkeys. Decay rate of Junin virus in aerosol was rapid; the LD-50 was 0.48 PFU for guinea pigs. Virulence of Lunyo strain of Rift Valley fever (RVF) virus was increased for mice exposed by aerosol, compared to other strains. Inactivated RVF vaccine did not provide complete protection vs. small-particle aerosol challenge; the immune response was effective against IP challenge. Aerosol stabilizing component of algal extract for Legionella pneumophila was lost by dialysis. Sequential infection of influenza virus followed by L. pneumophila caused increased mortality in mice. Immunosuppression of guinea pigs reduced the L. pneumophila aerosol LD-50. Evidence was lacking for cross-infection among guinea pigs. Differences in lethality between Pontiac and Philadelphia-1 strains of L. pneumophila were not related solely to ability to proliferate in lungs. Animal models were verified for Bacillus anthracis, Vollum 1B. 4 B. anthracis subunit protective factor immunogens were as effective as the aluminum-absorbed vaccine in protecting guinea pigs.
Publications: Infect. Immun. 30:497, 1980; 31:1209, 32,:690, 33:848, 1981.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U)

Work Unit No. 870 BB 069: Assessment of Airborne Microbial Agents of Potential BW Threat

Background:

Any microorganism to be employed as an airborne BW agent against US forces must be stable in aerosol and infective via the respiratory tract. Investigations are designed to define the aerosol stability characteristics and respiratory infectivity potential of candidate agents. Additional parameters of the pathogenesis of infections induced by aerosols are elucidated as the need indicates, to include determination of the sequence of events leading to immunity following vaccination or aerosol infection. Vaccines developed for candidate BW agents are evaluated for protection afforded against aerosol challenge. These studies are directed toward identification of efficacious vaccines which will protect deployed troops and at-risk laboratory personnel.

Progress:

Suspensions of Lassa fever virus (LASV) propagated in Vero cells were disseminated at an efficiency of 75%. Aerosol stability of the virus at 24 C was inversely related ro relative humidity with the half-lives ranging from 13-35 min. The LD_{50} in outbred Hartley guinea pigs to respiratory challenge with LASV was $10^{3.3}$ PFU. Susceptibility to infection was greater in inbred strain-13 guinea pigs (LD_{50} approximated $10^{1.0}$ PFU). All cynomolgus monkeys exposed to infectious aerosols died, even at the lowest dose of $10^{2.7}$ PFU. Analysis of selected tissues for virus concentration at various time intervals after exposure suggested that the lungs were the primary site for virus replication. Subsequently, a viremia developed that led to systemic infection. Administration of the antiviral drug ribavirin by either the IM or respiratory routes following aerosol challenge was ineffective in preventing deaths among Strain-13 guinea pigs. The time to death, however, was extended slightly.

Aerosol stability determinations for the Romero strain of Junin virus (JUNV) were performed at 24 C and each of 3 relative humidities (RH) (30, 55 and 80%), using virus stocks that had been propagated in Vero cells. Results of these tests are shown in Table I. An inverse relationship existed between aerosol stability and RH. Between 28 and 56% of virus subjected to dissemination remained airborne and infective after 4 min (39% efficiency), based on plaque-producing properties. The aerosol decay rates were 4.1, 5.0 and 5.8%/min for relative humidities of 30, 55, and 80%, respectively (Table I). These decay rates yielded virus half-life values in aerosol of 16.7 min at 30%, 13.8 min at 55%, and 12.0 min at 80%.

TABLE I. EFFECTS OF RELATIVE HUMIDITY ON PERCENTAGE RECOVERIES, AEROSOL DECAY RATES AND AEROSOL HALF-LIVES ON JUNV

HUMIDITY (%)	AEROSOL RECOVERY (%) BY MIN 4	32	60	DECAY RATE[a] (%/min)	HALF-LIFE[a] (min)
80	32.8	4.0	1.3	5.8	12.0
55	28.1	5.1	1.7	5.0	13.8
30	55.1	13.3	4.6	4.1	16.7

[a]Mean of 4 replicates.

Infectivity of JUNV (Romero) for guinea pigs challenged by aerosols was determined using dynamic aerosol equipment. Serial 10-fold dilutions of virus suspensions were disseminated with a Collison nebulizer (1) into a Henderson-type (2) aerosol transit tube modified by incorporation of an animal exposure box. Groups of 8 guinea pigs, each weighing 295-310 g, were exposed to each dilution. Similarly, groups of guinea pigs were inoculated IP. A summary of the dose-response titrations and the times to death as a function of challenge dose are given in Table II.

TABLE II. MORTALITY AND TIME-TO-DEATH RESPONSES IN GUINEA PIGS AFTER AEROSOL AND IP CHALLENGE WITH JUNV

ROUTE	DOSE (log_{10} PFU)	DEAD/TOTAL (%)		MDTD[a] (range)		LD_{50} (log_{10} PFU)
Aerosol	0.6	5/8	(63)	19.0	(16-23	
	1.6	8/8	(100)	15.7	(14-18)	0.48
	2.6	8/8	(100)	14.6	(14-16)	
	3.6	8/8	(100)	14.1	(13-15)	
IP	0.3	8/8	(100)	16.3	(14-18)	
	1.3	7/8	(88)	14.6	(13-16)	< 0.30
	2.3	8/8	(100)	15.0	(13-20)	
	3.3	8/8	(100)	15.4	(13-18)	

[a]Mean days to death.

As expected, the mean time to death after aerosol exposure was inversely related to the challenge dose of virus, whereas virus concentration exhibited little effect when injected IP. The LD_{50} for aerosol challenge essentially was itenditcal to that exhibited by IP challenge. Although both of the MDTD were slightly greater than previously reported for animals challenged IP (3), there was no statistical difference among the reported results.

Lunyo virus, isolated in 1955 from mosquitoes in Uganda, Africa, is related

antigenically to Rift Valley fever virus (RVFV) and is classified as a strain of RVFV. Available information indicated that this strain readily infects adult mice when inoculated IC while mice inoculated by the IP or SC route exhibited infectivity. Male ICR mice, approximately 7 weeks old, were infected via the respiratory route with graded doses of virus which had been propagated in Vero cells after being passed IC 12 times in suckling mice. The median respiratory LD_{50} was 2.59 $\log_{10}$ PFU which approximated the titers of other RVFV strains tested. The geometric mean time to death (GMTD) ranged from 6.4-12 days. These results indicated a high degree of infectivity following aerosol exposure and were unexpected. In a second study, mice were exposed by the IP or respiratory route with graded viral doses Results show that Lunyo virus was fatal to mice by either route (Table III). The respiratory LD_{50} was 3.1 $\log_{10}$ PFU; however, 100% mortality was not attained at the highest respiratory dose of 4 $\log_{10}$ PFU. Administration via the IP route failed to produce 50% mortality at dose levels up to 6.5 $\log_{10}$ PFU. Mice that survived the initial infection were challenged via the respiratory route on day 21 with 3.5 PFU of RVFV strain ZH-501. The highest rate of survival occurred in mice previously inoculated by the IP route. These data are particularly interesting because all other RVFV strains previously studied were less virulent for mice when administered by the respiratory route.

TABLE III. RESPONSE OF ICR MICE TO INFECTION WITH LUNYO VIRUS BY THE RESPIRATORY AND IP ROUTES OF ADMINISTRATION

INITIAL INFECTION WITH LUNYO VIRUS ON DAY 0		SURVIVORS CHALLENGED BY RESPIRATORY ROUTE, DAY 21 WITH 3.51 $\log_{10}$ PFU, ZH-501 STRAIN	
Dose ($\log_{10}$ PFU)	DEAD/TOTAL	DEAD/TOTAL	% MORTALITY
Respiratory			
0.06	0/20	20/20	100
1.03	0.20	20/20	100
2.08	3/20	15/17	88
3.10	13/20	7/7	100
4.03	11/20	8/9	89
IP			
0.75	0/20	17/20	85
1.75	1/21	10/20	50
2.75	2/21	4/19	21
3.75	5/20	2/15	13
4.74	4/20	4/16	25
5.75	9/20	1/11	9
6.53	2/20	11/18	61
Controls		20/20	100

Preliminary studies showed that Maxx and F-344 rats were susceptible to respiratory induced infection with strain ZH-501. The LD_{50} of Maxx and F-344

rats exposed to graduated aerosol doses of strain ZH-501 was 2.0 and 1.8 $\log_{10}$ PFU, respectively. Also, a small number of Wistar-Furth rats were inoculated IP to determine the LD_{50} dose in preparation for vaccine potency studies. Male rats, approximately 14 weeks old (mean 280 g) were inoculated with graduated 0.4-ml doses of the ZH-501 strain. The rats were highly susceptible as evidenced by an $IPLD_{50}$ of < 2 PFU. In another IP infectivity study, male ICR mice were inoculated with graduated doses of the SA-51 strain, and the median $IPLD_{50}$ was 0.1 $\log_{10}$ PFU.

Studies were initiated to evaluate efficacy of the RVF vaccine against an aerosol challenge. Male, Wistar-Furth rats, approximately 13 weeks old, were vaccinated with decreasing quantities of the NDBR-103 vaccine prepared in African green monkey kidney cells. Rats were vaccinated on days 0 and 14. Six separate groups of rats were given 4-fold dilutions ranging from 0.4 to 0.0004 ml. Antibody titers on day 21 were directly related to vaccine dose, with a higher does inducing a aproportionately higher titer. Rats were challenged via the respiratory route on day 28 with 4.7 $\log_{10}$ PFU of the ZH-501 strain. Two of 63 vaccinated rats survived, one each in the 0.4 and 0.03 ml vaccine groups.

A second evaluation was performed using a newer vaccine, TSI GSD-200, and the challenge dose was reduced 10-fold. The antibody response was dose-related and comparable to the response elicited by NDBR-103 (Table IV). Against a challenge of 3.87 $\log_{10}$, the ED_{50} was 0.04 ml vaccine/dose (95% confidence interval: 0.00005-0.28). Geometric mean antibody titers in rats that survived the challenge increased 10-fold or greater in those rats given less than 0.4 ml vaccine. Vaccine efficacy against an IP challenge indicated the $IPED_{50}$ was 67 nl (Table V) (95% confidence limits of 31-143 ml).

TABLE IV. RESPONSE OF WISTAR-FURTH RATS TO VACCINATION WITH RVF VACCINE[a] ON DAYS 0 AND 14 AND SUBSEQUENT CHALLENGE WITH 3.87 PRU OF STRAIN ZH-501 VIA THE RESPIRATORY ROUTE ON DAY 28

VACCINATION REGIMEN (ml vaccine)	GM TITER (reciprocal) ON DAY 21 PRN-50	RESPONSE TO RESPIRATORY CHALLENGE DEAD/ TOTAL	GMTD[b]	POSTCHALLENGE PRN-50 titer (reciprocal)
0.4	5,383	2/8	18.0 (14-24)	9,682
0.1	2,198	3/8	10.5 (9-20)	15,946
0.03	1,549	4/6	11.0 (10-11)	14,039
0.01	482	5/8	10.0 (9-11)	14,084
0.0016	164	7/7	10.0 (9-20)	--
0.0004	37	5/8	9.0 (5-15	7,326
Controls	13	7/7	3.0 (3-6)	

[a] TSI GSD-200

[b] Geometric mean time to death

TABLE V. RESPONSE OF WISTAR-FURTH RATS TO VACCINATION WITH RVF VACCINE[a] ON DAYS 0 AND 14 AND SUBSEQUENT CHALLENGE WITH $10^{3.86}$ PFU OF STRAIN ZH-501 VIA IP ROUTE ON DAY-28

VACCINATION REGIMEN (ml vaccine)	PRN-50 (reciprocal), DAY 21	DEAD/ TOTAL	MDTD	POSTCHALLENGE PRN-50 TITER (reciprocal)
0.08	1,950	0/7		2,283
0.016	1,416	0/8		1,550
0.0032	429	0/8		1,488
0.0064	231	0/8		2,538
0.00128	61	3/8	4	7,854
0.0000256	54	6/8	3	6,318
Controls	10	8/8	3	-

[a]TSI GSD-200

These data demonstrate that the protection provided by this vaccine against aerosol challenge is less than that against IP challenge. The significance of the findings relative to disease in man in unknown, although the results show the inactivated vaccine does not provide complete protection against RVFV disseminated in small-partical aerosol.

Extracts of blue-green algae were reported to stabilize Legionella pneumophila in aerosols (4). Study of the nature of the stabilizing component(s) was performed by dialyzing algal extract overnight at 4 C against frequent changes of distilled water. The aerosol stability, at 30% RH, of Legionella suspended in dialyzed extract, nondialyzed extract, mineral broth (used as a growth medium for algae), water, and tryptose saline was determined. The stabilizing effect of algal extract was lost by dialysis, suggesting that at least one component of the stabilizing system had MW of less than 12,000. Mineral broth also showed considerable stabilizing activity after a large initial loss. These observations, taken in conjunction with the previously reported stabilizing activity of 2,2'-dipyridyl, suggest that the survival of L. pneumophila in aerosols is strongly influenced by inorganic ions. Evaluation of 5 algal samples from natural habitats, obtained from Fliermans et al. (4), showed a tendency towards stabilization by the natural products, but the difference was not statistically significant. Apparently the aerosol stabilizing compound was not present in sufficient naturally occurring quantities to produce a marked effect.

Sequential infection of AKR/J mice with intranasally (IN) instilled influenza virus followed 3 days later by IM L. pneumophila caused almost 100% mortality (Table VI) (3). Similar doses of the 2 agents given as single infections were not lethal.

TABLE VI. RESPONSE OF AKR/J MICE TO IN INSTILLATION OF $10^{3.8}$ ED_{50} OF INFLUENZA VIRUS FOLLOWED BY $10^{6.0}$ L. PNEUMOPHILA (5)

TREATMENT	GMTD (days ± log SD)	P^a	DEAD/ TOTAL	P^b
Influenza virus alone on day-3.	8.12 (0.05)	<0.005	6/29	<0.00001
Influenza virus on day 0 followed by L. pneumophila on day 0	6.57 (0.079)		27/29	
L. pneumophila alone on day 0	NA		0/30	<0.00001

[a]Virus control mice vs. sequentially infected mice, Student's t-test

[b]Virus and bacterial control mice vs. sequentially infected mice, Fisher's exact test

Pathogenesis of the sequential infections was studied by serial killing of AKR/J mice at various times after infection. Viable counts of virus and bacteria in the lungs are given in Table VII. In the course of other sequential infections, e.g., influenza followed by Streptococcoccus pneumoniae, the virus suppresses host defenses and permits proliferation of the bacterium. In this study, however, L. pneumophila seems to have inhibited viral clearance. Further work must be done to determine the mechanism of this reaction, but preliminary bacteriologic and histopathologic observations suggest that a toxin may be responsible.

TABLE VII. VIABLE COUNT OF INFLUENZA VIRUS AND L. PNEUMOPHILA IN LUNGS OF SEQUENTIALLY INFECTED AKR/J MICE

DAY AFTER L. PNEUMOPHILA	GM VIRAL CONCENTRATION (log_{10} EID_{50}/lung) (log SD) VIRAL CONTROL	SEQUENTIAL INFECTION	P^a	GM BACTERIAL CONCENTRATION (log_{10} cfu/lung) BACTERIAL CONTROL	SEQUENTIAL INFECTION	P^a
-3	2.2 (0.35)	2.2 (0.35)				
1	6.6	6.6		6.7 (0.09)	6.5 (0.32)	
2	6.6	6.6		5.9 (0.67)	5.6 (0.47)	
3	5.6 (0.13)	5.7 (0.33)		5.1 (0.04)	5.0 (0.22)	
4	4.8 (0.25)	6.1 (0.21)	0.01	4.6 (0.05)	5.8 (0.24)	0.005
7	1.0	3.2	0.001	5.5 (0.64)	3.2 (1.4)	0.05

[a]Probability determined by Student's t-test on geometric means. Three mice per value.

Each of 2 replicate experiments were performed as follows with squirrel monkeys: 4 monkeys were given 10^8 EID_{50} of the influenza virus intratracheally (IT) and exposed to an aerosol dose of 10^8 L. pneumophila 3 days later; 4

monkeys were inoculated with saline IT, and after 3 days, with L. pneumophila (L. pneumophila controls); and 4 monkeys were inoculated with influenza virus IT followed by tryptose saline at 3 days by aerosol (influenza controls). In the second experiment all animals were killed at 6 days for histopathology and determination of viable virus and bacterial concentrations in the lungs. Although sequential infections undoubtedly produced more severe illness than did either agent alone, the viable count data were equivocal. The concentration of virus in the lungs of the influenza controls was approximately the same as that seen in the sequentially infected monkeys (10^3-10^5 EID_{50}/lung). Similarly, bacterial counts were very low in both control and sequentially infected groups ($<10^3$/lung). Although histopathology has not yet been completed, it is possible that the mechanism of action of sequential infection differed from that seen in mice.

The LD_{50} of L. pneumophila was reduced more than 10-fold in guinea pigs treated for 4 days with 40 gm/kg/day of cyclophosphamide and 50 mg/kg/day of hydrocortisone. Cyclophosphamide alone resulted in a 5-fold reduction and corticosteroid alone had no effect (3). Evaluation of the serum microagglutination (MA) titers of immunosuppressed guinea pigs 14 days after receiving graded doses of L. pneumophila showed cyclosphosphamide suppressed the development of humoral antibody when employed alone. Significant suppression of serum MA titers was not seen in animals receiving the combination of drugs (Table VIII).

Guinea pigs challenged with 9×10^4 organisms after 4-day treatment with cyclophosphamide and cortisone evidenced greater concentrations of L. pneumophila in lungs and blood at 72 hr and thereafter than did the controls (Table IX). When guinea pigs were exposed to 5×10^3 L. pneumophila 4 days after immunosuppresive therapy was started and the treatment continued to day 7, all animals died that receoved cyclophosphamide alone or in combination with hydrocortisone. Those given the combined treatment, however, died at a significantly more rapid rate.

TABLE VIII. SERUM MA TITER (14-DAY) IN IMMUNOSUPPRESSED GUINEA PIGS (n = 6)

TREATMENT	RECIPROCAL GM MA TITER BY DOSAGE		
	760	7,600	91,000
Cyclophosphamide	79	256	338
Hydrocrotisone	362*	724	645
Combined	315*	512	no survivors
None (control)	416*	724*	813*

* $P < 0.01$ vs. cyclophosphamide

TABLE IX. EFFECT OF 4-DAY IMMUNOSUPPRESSION ON VIABLE CONCENTRATION OF L. PNEUMOPHILA IN BLOOD AND LUNGS

TIME AFTER INFECTION (hr)	GM CONCENTRATION OF L. PNEUMOPHILA			
	BLOOD (per ml)		LUNGS (total lung)	
	TREATED	CONTROL	TREATED	CONTROL
24	0	0	2.0×10^6	3.3×10^6
48	0	0	4.7×10^7	2.0×10^7
72	1.7×10^2	4.2×10^1	$1.2 \times 10^{9*}$	5.0×10^8
96	4.5×10^3	2.0×10^1	$6.4 \times 10^{9*}$	3.3×10^8

*P < 0.01 vs. control lung

Another factor of interest has been cross-infection by L. pneumophila, i.e., the ability of an infected guinea pig to transmit the infection to a normal cagemate. In 10 experiments, none of the animals housed "downwind" from infected animals became ill or seroconverted, even though bacterial counts in the lungs of infected guinea pigs exceeded 10^9. Air samples were taken with AGI impingers for 30 min daily for 5 days, and the impinger contents injected IP into guinea pigs. None of these animals seroconverted, suggesting that the concentration of organisms shed by the infected guinea pigs was below the limits of infection by the IP route (< 1,000).

The pathogenesis of the Philadelphia-1 and Pontiac strains of L. pneumophila in guinea pigs was contrasted. The former is the strain used in all experiments described to date. The latter is interesting because it was isolated following an epidemic in which no pneumonic signs were described even though the respiratory tract was apparently the portal of entry (6). After aerosol exposure the Philadelphia-1 strain proliferated more rapidly in the lungs for the first 48 hr but by 72 and 96 hr, counts were approximately equal for both strains. In both cases organisms were first detected in the spleen at 48 hr, exceeding 10^6 at 72 hr. Bacteremia was not detected, although hematogenous spread from the lungs must have occurred. Additionally it was shown that neither sex nor age posed significant risk factors for infection of guinea pigs.

A vial of lyophilized Bacillus anthracis, Vollum 1B strain, was obtained. Spore contents of the vial were used to prepare a mass cultures in Thorne's semisynthetic medium (7). Spores, obtained by heat-shocking, were suspended in 1% phenolized gelatin-phosphate and stored at 4 C.

Virulence tests were conducted with the phenolized suspensions of spores to reestablish the validity of laboratory animal models to be used subsequently for candidate anthrax vaccine evaluations. Results of the dose-response studies are shown in Table X. The data obtained for parenteral challenge routes in all 3 species were analogous to the findings reported previously for the Vollum 1B strain. Dose-response values for oral exposure had not been reported previously. The oral LD_{50} in guinea pigs was equivalent to that determined by aerosol challenge, both being approximately 5.0 $\log_{10}$ viable spores. Fischer-344 rats

were quite resistant to lethal infections induced by these spores of this strain of B. anthracis. By contrast, the Fischer-344 rat has been shown to be highly susceptible to the lethal action of anthrax toxin (8). Guinea pigs, on the other hand, are susceptible to lethal infection but resistant to anthrax toxin (8).

TABLE X. ANIMAL DOSE RESPONSE TO B. ANTHRACIS, VOLLUM 1B

SPECIES	CHALLENGE ROUTE	SPORE LD_{50} (log_{10})
Mice (Swiss outbred)	SC	0.7
Guinea pigs (Hartley strain)	IP	2.9
	aerosol	4.9
	oral	4.9
Rats (Fischer-344)	IP	>4.0
	aerosol	>5.7
	oral	>8.0

Four candidate anthrax vaccines were evaluated in guinea pigs against the virulent Vollum 1B strain. Each of the experimental vaccines were comprised principally of the protective factor antigen extracted from the V770 strain of B. anthracis, and each had been prepared by Dr. A. Johnson-Winegar, Pathology Division, USAMRIID. Strain V770 is avirulent, noncapsulated, and nonproteolytic. It was used to develop the Wright human vaccine. This strain produced the protective factor (PF) antigen, but lacks the ability to produce the other 2 antigens (lethal factor and edema factor).

Hartley strain guinea pigs were given 0.5 ml of the experimental vaccines SC for a total of 3 injections at 2-week intervals. The same vaccination regimen was used for 3 control groups of guinea pigs: group 1 received sterile saline, group 2 was injected SC with the Wright human vaccine, and group 3 was injected with the Sterne, attenuated viable spore vaccine (concentration of 1×10^6 spores/0.5 ml). None of the groups showed any adverse reaction to the vaccination procedures. Skin reactions did occur in the guinea pigs that received the Sterne spore vaccine; each of these guinea pigs developed a necrotizing skin ulcer within 1 week of vaccination, yet they appeared alert and healthy with no detectable loss in body weight. The skin lesions were circumscribed with some immediate surrounding edema. The lesions did not appear to penetrate the musculature. By 2 weeks the lesions were essentially healed. With 7 days after the vaccine dose 2 was given in the opposite flank several of the guinea pigs developed a small, minor lesion at the injection site. All induced lesions had healed completely prior to administration of dose 3.

Two weeks after the 3rd vaccine injection each guinea pig was challenged IM with 3.99 log_{10} viable spores. The guinea pigs were examined daily for 10 days, during which time 2 guinea pigs from each group were bled via cardiac puncture at selected intervals. Whole blood was cultured for B. anthracis, and serum was assayed for humoral antibodies using the indirect hemagglutination (IHA) and indirect fluorescent antibody (IFA) techniques. Protective response and

bacteriologic data are shown in Table XI. All of the saline control guinea pigs died within 3 days (MTD = 2.11). Protection afforded guinea pigs against challenge with virulent anthrax by 2 of the experimental vaccine lots (V-10-25 and V-10-13) was analogous to that exhibited by the reference Wright human vaccine. The other 2 experimental vaccines (Fract 12 and V-10-24) failed to protect animals. These latter 2 vaccines, however, contained only 20% of the PF antigen concentration in the previous 2 experimental vaccines. Only 1 of 14 Sterne spore-vaccinated guinea pigs died and this death was delayed to day 10 after challenge. None of the Sterne vaccinated guinea pigs evidenced demonstrable bacteremia. By contrast a consistent bacteremia developed in guinea pigs vaccinated with the Wright vaccine or the 4 experimental vaccines. These data suggest that the immune response from vaccination with viable spores is significantly more protective than the response elicited to any of the subunit immunogens. Serologic assays have not been completed.

TABLE XI. RESPONSE OF VACCINATED GUINEA PIGS CHALLENGED IM WITH VIRULENT B. ANTHRACIS, VOLLUM 1B (3.99 $\log_{10}$ SPORES/0.5 ml)

VACCINE TREATMENT	NO. GUINEA PIGS	BLOOD CULTURE[a]	SURVIVORS/ TOTAL CHALLENGED	% SURVIVAL	MTD
Saline	25	-	0/25	0	2.11
Wright's (PF - soluble)	15	+ (5,7,10)	12/15	80	5.62
Sterne (viable spores)	14	- (2,5,7)	13/14	93	10
PF - soluble (Lot V-10-25)	15	+ (4,7,10)	11/15	73	3.55
PF - alhydrogel (Lot V-10-13)	15	+ (5,7,10)	12/15	80	6.46
PF - soluble (Fract 12)	15	+ (2,5,7)	0/15	0	3.3
PF - alhydrogel (Lot V-10-24)	15	+ (3)	0/15	0	2.7

[a]Numbers in parentheses indicate positive (or negative) blood culture on postchallenge days

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0G3811	81 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8. DISP'N INSTR'N	8A. SPECIFIC DATA-CONTRACTOR ACCESS	9. LEVEL OF SUM
80 10 02	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	62770A	3M162770A870	BC	068
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2:2			

11. TITLE (Precede with Security Classification Code)*

(U) Technology Development for Rapid Detection and Identification of BW 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
80/10	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

e. KIND OF AWARD: NA f. C. I. MT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 81	4.7	317
FISCAL YEAR CURRENT 82	3.0	444

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) Viral diseases; (U) Bacterial diseases; (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) Develop the capability for rapid, early diagnosis of viral and bacterial organisms and biologic toxins of military significance in environmental samples and biologic fluids.

24. (U) Immunologic assays are utilized to include antibody-linked enzymes (ELISA), fluorescence (FA), radioactive materials or chemiluminescence (CL) of white blood cells as a means of specific and rapid identification of organisms. Specific antibodies are produced by traditional means or, if possible, in monoclonal systems.

25. (U) 80 10 - 81 09 - Francisella tularensis and Venezuelan equine encephalomyelitis (both vaccine strains) can be detected at concentrations of 10-100 thousand organisms/ml by ELISA. CL response of polymorphonuclear leukocytes was found valuable in early diagnosis of experimental bacterial (F. tularensis) and viral (Pichinde) infections. The FA test was proven specific and sensitive when antibodies to arboviruses were correctly identified in 12 of 13 unknown sera. Epoxy-coated spot slides containing arbo- and arenavirus-infected cells for use in FA were stable for 1-3 years when stored at -20 or -70 C. Electron microscopy detected paramyxoviral particles in half of infected cell samples examined.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162770A870: Risk Assessment of Military Disease Hazards (U)

Work Unit No. 870 BC 068: Technology Development for Rapid Detection and Identification of BW Agents

Background:

Rapid detection and identification of BW agents or other naturally occurring infectious organisms is essential to prevent or minimize the mortality and morbidity of soldiers in the field. This task has fortunately been made less formidable by recent technological developments such as immunofluorescence microscopy, radioimmunoassay, electron microscopy (EM) and immune electron microscopy, chemiluminescence (CL) and enzyme-linked immunosorbent assay (ELISA). All these assay systems have some advantages and disadvantages. Therefore there is a real need to investigate which method is simple, sensitive and specific for a given field situation.

Progress:

Rapid Diagnosis of Viral Diseases of Military Importance (R. R. Rosato)

Stability study. A study to determine the stability of antigen containing spot slides was started last year and has been continued through days 240 and 365. Although only the 240- and 354-day data are new, all data are presented for completeness (Table I). Rift Valley fever (RVF) and Pichinde (PIC) viruses were stable at both 4 or -20 C for up to 365 days; Tacaribe (TCR) for 123 days at both 4 and -20 C, then stability decreased at both temperatures between 123 and 240 days. West Nile (WN) stability was similar to TCR to 123 days, but then showed a more pronounced decrease at 4 C than at -20 C. The decrease noted at both temperatures on day 61 was not noted on day 123 and was probably related to the conjugate used on day 61. Dengue-2 (DEN-2) was stable to day 123 at 4 C, then decreased at 240 days and 365. On the basis of this study, the decision was made to can spotslides in an inert atmosphere (nitrogen) and store at -20 C. That process is underway.

Inactivation Study. The problem of residual live virus in prepared spotslides has been a continuing source of concern. Methods developed at USAMRIID allow production of inactivated Ebola (EBO), Machupo (MAC) and Lassa (LAS) slides; however, slides produced under contract have not been inactivated and most contain residual live virus. A number of viruses are inactivated during slide fixation by acetone or storage for periods of time, but partial testing at Yale Arbovirus Research Unit (YARU) indicates slides of Japanese encephalitis (JE), eastern (EEE) and western (WEE) equine encephalitis and Chikungunda (CHIK) contain from 3.0 to 5.4 $SMICLD_{50}$/0.02 ml of resuspended virus.

Data obtained from the ^{60}Co inactivation study described in the last annual report indicates that 300,000 rad did not inactivate some alphaviruses when assayed by $SMICLD_{50}$ test.

TABLE I. STABILITY OF FA ANTIGENICITY AFTER STORAGE AT VARIOUS TEMPERATURES

	4 C						-20 C					
	Days						Days					
Virus	0	30	61	123	240	365[c]	0	30	61	123	240	365
TCR	4[a]	3	4	3	1	1	3	3	4	3	1	1
PIC	4	4	4	4	4	4	4	4	4	4	4	4
EEE	3	2	4	3	2	1	3	2	4	4		4
DEN-2	2	1	4[b]	4	2	0	2	1	4[b]	4	3	2
WN	3	4	2	3	1	1	3	3	2	3	2	2
RVF	4	4	4	3	4	3	4	4	4	4	4	4

[a] intensity on a scale of 1 to 4+, average 8 spots
[b] conjugate changed for both temperatures
[c] moisture on all slides when opened

Even so, data is being accumulated that indicates ^{60}Co may be the nondestructive method of choice for the inactivation of most virus spotslides. As new, fresh lots of spotslides are produced we will determine the presence of residual virus, if any, and the effects of ^{60}Co irradiation on viability.

Sensitivity Study. The sensitivity of the IFA test (i.e. detection of antibodies in relation to other standardized methods) is important in assessing its usability as diagnostic procedure. Using standardized reference sera obtained from NIH, homologous IFA titers were determined and compared with published suckling mouse $ICLD_{50}$, ($SMICLD_{50}$) neutralization, complement fixing (CF), and hemagglutinating inhibition (HAI) values. Relative FA sensitivities to each test were calculated by dividing the IFA titer by the specific titer given for each serum by a specific test. Results (Table II) indicate the IFA test is less sensitive in all instances than $SMICLD_{50}$ neutralization which in turn is more sensitive than the CF and HI tests. Equivalence is indicated by a value of 1.0, <1 more sensitive, >1 less sensitive.

This data strongly indicates fluorescent antibody techniques should become the Institute's primary method for routine serodiagnosis for all but vaccine and immunization protocols which are based on neutralization antibody titers. A correlation between FA and NT titers has yet to be demonstrated.

TABLE II. RELATIVE SENSITIVITY OF SELECTED SEROLOGICAL TESTS TO DETECT ARBOVIRUS ANTIBODIES

VIRUS		ASCITIC FLUID	REPORTED VALUES[a]				CALCULATED VALUES IFA/SPECIFIC TEST		
ANTIGEN	STRAIN	STRAIN	NT[b]	CF	HI	IFA[c]	NT	CF	HI
CHIK	Ross	S-27	5.4	32	320	512	0.002	16	1.6
EEE	Alabama	MASS	4.3	64	320	4096	0.205	64	12.8
VEE	TC-80	TC-83	4.1	128	1280	2048	0.153	16	1.6
WEE	McMillian	Fleming	4.1	32	320	4096	0.315	128	12.8
WN	Egypt 101	B-956	3.5	128	160	512	0.160	16	3.2
YF	Asibi	17D	4.8	256	160	1024	0.016	4	6.4
JE	Nakayama	Nakayama	5.3	256	160	512	0.003	2	3.2
DEN-1	Hawaii	Hawaii	3.6	128	160	1024	0.258	8	6.4
DEN-4	H-241	H-241	3.7	256	320	4096	0.819	16	6.4

[a] Cunningham, 1978-80

[b] Suckling Mouse

[c] Experimental Values

Testing of unknowns. Identification of coded, unknown sera supplied by Dr. Casals, YARU, was finished. Initial screening using polyvalent group slides indicated 4 group A (#2, 3, 5, 13), and 7 group B (#1, 4, 7, 8, 9, 10, 11). Two sera (6, 12) were both group A and group B negative. When the latter were tested against monospecific slides for LaCrosse (LAC), lymphocytic choriomeningitis (LCM), PIC, TCR, MAC, Junin (JUN), sandfly fever-Sicilian (SF-S), Oropouche (ORO), RVF, sandfly fever-Naples (SF-N), Hazara (HAZ), Korean hemorrhagic fever (KHF), and LAS: one was positive at 265 for SF-N, and the other was positive at 1024 for ORO. No other reactions were seen with any other antigens tested. All sera were further tested and we concluded: #2 - Venezuelan equine encephalomyelitis (VEE), 3 - EEE, 5 - CHIK, 13 - WEE, 6 - SF-N, 12 - ORO, 1 - DEN-1, 7 - DEN-2, 11 - DEN-4, 8 - JE, 10 - Langat (LGT), 4 - yellow fever (YF), and 9 - a negative, giving a false positive group reaction at low dilution, a low titered group serum or a flavivirus other than those for which tests were run. Dr. Casals verified the correct identification of 12 of the 13 samples, including the specific identification of the 3 dengue serotypes submitted. The single serum not identified was #9 (SF-S) which reacted with the group polyvalent antigen slide and led us astray.

We are pleased that the IFA system developed in conjunction with Dr. Casals for serodiagnosis, is the most viable, broadbased (29 viruses) system available at USAMRIID at this time.

Laboratory Diagnosis of Viral Disease of Military Importance (J. P. McCarthy)

In vitro stimulation of PMN chemiluminescence (CL). Bacterial studies. Polymorphonuclear leukocytes were isolated from pooled whole blood of 4 normal Fischer-Dunning rats. One ml of 10^9 live vaccine strain (LVS) Francisella tularensis, Streptococcus pneumoniae, or Salmonella typhimurium organisms was thoroughly mixed with 1 ml of LVS immune serum (titer >1:256 by microagglutination) and incubated at 37 C (H_2O bath) for 30 min. Following opsonization, 10^7 bacteria were added to 10^5 PMN and the CL was measured for at least 15 min using the Picolite Luminometer. Only LVS bacteria stimulated an elevated PMN CL response when compared to controls. Both S. typhimurium and S. pneumoniae failed to elicit an increased PMN response. Additional studies demonstrated that the PMN CL assay could be used to detect as low as 5×10^6 LVS and 5×10^5 live or heat-killed S. typhimurium.

Viral studies. PMN were isolated from pooled whole blood of 6 normal male Hartley guinea pigs. One ml of 10^{10} VEE virus (TC-83 strain) or 10^9 PIC virus was thoroughly mixed with 1 ml of PIC virus immune serum (titer: >600 by IFA) and incubated at 37 C. Each opsonized virus was spun at 35,000 x g for 1 hr at 25 C, supernatant fluid discarded and the virus resuspended in 1 ml of HBSS. Opsonized PIC virus (5×10^6) and VEE virus (5×10^6 or 5×10^8) were each added to 10^6 PMN and the CL measured for 20 min using a Tricarb Scintillation Counter. As seen in the bacterial studies only the PIC virus stimulated an elevated PMN CL response in vitro. Neither concentration of VEE virus opsonized with the PIC virus immune serum to cause an increase in the PMN CL. Further studies suggest that this assay can be used to detect as low as 10^3-10^4 PIC virus.

These studies suggest that viruses and bacteria must be opsonized with homologous immune serum to stimulate an elevated PMN CL response in vitro. Further, the in vitro PMN CL assay may be a valuable tool for the rapid identification of BW agents.

Effect of exercise on endogenous PMN CL. Studies were performed to measure the effect of exercise on the endogenous PMN CL response of immune and nonimmune rats following challenge with LVS. Male Sprague-Dawley rats were immunized by a single IP injection of 10^4 LVS/100 g body weight and allowed 14 days to develop an antibody titer ($\geq$1:2048 by microagglutination). Rats were then separated into categories of exercised and nonexercised or sedentary with each category containing 4 subgroups: (a) nonimmune noninfected, (b) nonimmune infected, (c) immune noninfected, and (d) immune infected. Each subgroup contained 2 rats. Rats were injected IP with 10^7 LVS/100 g body weight and the PMN CL was measured 24 hr following injection. Control animals were injected IP with an equal volume of tryptose-saline culture medium. Exercised rats were forced to run using a wheel running apparatus for 60 min at 15 m/min just prior to PMN isolation. Significant fever ($P < 0.05$) was measured in nonimmune infected animals 24 hr following injection. The integrated area of the PMN CL response from nonimmune-infected exercised rats was significantly decreased ($P < 0.001$) as compared to the nonimmune infected sedentary animals. The integrated area of the PMN CL response from immune infected exercised rats was also decreased as compared to immune infected sedentary animals.

In vitro effects of Legionella extracts on rats PMN CL. Studies were performed to measure the effect of protein extracts from various strains of Legionella organisms on basal endogenous rat PMN CL in vitro. Briefly, Legionella organisms were sonicated and centrifuged for 30 min at 17,000 rpm. Supernatant fluid was acid-precipated with 2 N HCl to pH 3.5 and centrifuged for 20 min at 17,000 rpm, then placed on a preparative isotachophor column and fractions containing homologous peaks were collected and combined. Extracts were prepared from genetically related Washington, Atlanta and Los Angeles strains of Legionella pneumophila and genetically unrelated Legionella dumoffi, bozemanii and macadeii strains. Although the latter strains are genetically unrelated to Washington, Atlanta and Los Angeles strains, studies by Colonel Hedlund have shown that all the above strains are antigenically related.

Experiments were performed measuring the effect of protein samples from acid precipitation supernatant fluid and preparative isotachophor peaks on basal endogenous rat PMN CL. Chemiluminescence was measured from rat PMN following a 15-min incubation at 37 C with 8.0 µg of protein extract. Controls were treated identically using equal amounts (wt/vol) of bovine serum albumin. Results showed that each Legionella strain tested decreased the basal endogenous PMN CL response compared to BSA control values. PMN suspensions were shown to be greater than 90% viable following incubation with protein extracts using the trypan blue dye exclusion test. These results suggest that although various strains of Legionella have been shown to be genetically unrelated, they are antigenically related and may share a common pathogenicity.

Measurement of PMN CL during bacterial infection using a newly developed whole blood assay. Studies were performed to determine if alterations in endogenous PMN CL of rats during bacterial infection could be detected using whole blood rather than isolated neutrophils. Rats were injected IP with 10^7 live or heat killed LVS/100 g body weight and PMN CL response using whole blood measured 24 hr following injection. Controls received an equal volume of tryptose saline culture medium. Fever was measured in rats injected with live LVS. Heparinized whole blood (80 U/ml) was diluted 1:100 with HBSS and maintained at 5 C. The following were added: 50 µl of opsonized zymosan (4 mg/ml) as a stimulus, 200 µl of luminol (10^{-3} M, pH 7.2) and then 2000 µl of diluted whole blood to a 25-ml glass liquid scintillation vial. Following addition of whole blood, the vial was immediately placed in a Tricarb liquid

scintillation counter and CL was measured for 0.2 min. Following measurement the vial was placed in a water bath at 37 C and PMN CL measured at 1-min intervals for 20 min. Chemiluminescence is reported as cpm/PMN using the mean of 3 white blood counts and whole blood differential (500 cells) to determine the concentration of PMN in the assay vial. In 3 separate experiments, in vitro zymosan stimulation caused a significant increase in the PMN CL response of whole blood isolated from rats injected with live LVS as compared to the PMN CL response from whole blood of rats injected with either heat-killed bacteria or tryptose saline culture medium.

These studies demonstrated for the first time that alteration in PMN CL during bacterial infections can be detected using whole blood rather than PMN isolates. Further studies will be required to completely evaluate the applications of this new technology in rapid diagnosis and prognosis of bacterial infections in the host.

Evaluation of electron microscopy for rapid viral detection (F. Macasaet)

Electron microscopy. The traditional way of recognizing the presence of viruses in cell cultures is to observe for cytopathic effect (CPE) by light microscopy, but such events may take days or even weeks to develop. On the other hand, viral particles can be demonstrated by electron microscope (EM) examination of infected cells as early as several hours following inoculation, thereby providing a potentially rapid means of detection. Although identification by EM is limited to morphological grouping of a virus, this is probably all that is needed to initiate proper therapeutic or preventive measures. A specific treatment modality can follow after complete serological identification of the isolated virus.

The role of EM in rapid diagnosis was evaluated by examining infected cell cultures 24 hr following inoculation with clinical specimens, a time when no visible CPE was detectable. In 8 cultures infected with parainfluenza viruses, 4 were positive for viral particles by EM. However, no virions were seen in 5 entero- or 2 herpes-infected cultures examined in the same manner but at a different time. Preliminary results indicated that direct EM of clinical specimens was rapid but lacked sensitivity. Improved detection of viruses by EM after direct ultracentrifugation of specimens to the grid should next be evaluated for its usefulness in providing early diagnosis.

The enzyme-linked immunospecific assay (ELISA) for antibody determination has also been found useful for antigen detection. One of the drawbacks of this procedure is the need for high-titered specific antibody. However, ELISA is sensitive and rapid; therefore its applicability in the clinical laboratory was explored. Preliminary experiments employed a VEE TC-83 antigen detection system and followed previously published standard procedures.

Evaluation of ELISA for rapid viral detection. Quality of the captured antibody appeared to determine the sensitivity of the test. When untreated rabbit anti-VEE (titer of 1:1280 to 1:2560 by PRN test) or NH_4SO_4-precipated IgG was used as capture antibody, the test could detect VEE TC-83 in concentrations of 10^7-10^8 PFU/ml. However, with affinity chromatography (AC)-purified IgG, the sensitivity increased about 1000-fold. The problems with AC were that large quantities of antiserum and antigen were needed for each purification procedure and the purified IgG were not stable when stored for more than a week at 4 C. These problems should be easy to resolve and it appears that the antigen capture ELISA will be a good technique for rapid viral detection.

Routine clinical virology. Twenty-three viruses from about 400 clinical specimens were isloated and identified in primary monkey kidney and human lung fibroblast cell cultures. Most were enterovirus, herpes or parainfluenza viruses from patients with relatively minor illnesses such as upper respiratory tract infections, sores or gastroenteritis.

Publications:

1. Foulke, R. S., R. R. Rosato, and G. R. French. 1981. Structural polypeptides of Hazara Virus. J. Gen. Microbiol. 53:169-172.

2. Rice, R. M., B. J. Erlick, R. R. Rosato, G. A. Eddy, and S. B. Mohanty. 1980. Biochemical characterization of Rift Valley Fever Virus. Virology 105:256-260.

3. Walder, R., R. R. Rosato, and G. A. Eddy. 1981. Virion polypeptide heterogenicity among virulent and avirulent strains of Eastern Equine Encephalitis (EEE) virus. Arch. Virol. 68:229-238.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0G3810	81 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
80 10 02	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	62770A	3M162770A871	BA	150
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7 2 2			

11. TITLE (Precede with Security Classification Code)*

(U) Prevention of BW Diseases Caused by Microbial Toxins

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

003500 Clinical medicine; 004900 Defense; 016800 Toxicology; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 81	3.8	728
	CURRENT 82	5.0	564

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) Microbial toxins; (U) Vaccines; (U) Prophylaxis; (U) Therapy; (U) Toxoids

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

23. (U) Develop and evaluate biologics and selected compounds for prevention and treatment of disease induced by microbial toxins of military importance. Prepare, characterize and produce toxoids/vaccines suitable for protection against botulism and anthrax. Collect, produce and test immunoglobulins having efficacy in prevention and treatment of microbial toxemias. Develop materials and methods for detection and assay of toxins.
24. (U) Develop new technology for fermentor-system production of small experimental lots of microbial toxins and methodology for isolation, purification, alteration and detection of toxins. Immunogenicity of various antigens will be described and converted to experimental toxoids/vaccines. Improve procedures for immuno- and chemotherapy of toxin-mediated diseases. Action(s) and effects of toxins and their antagonists will be determined and described at the cellular level.
25. (U) 80 10 - 81 09 - Technology was developed for the fermentor-system production of type E botulinal neurotoxin. Kinetics of type A neurotoxoid production were examined. Over 2000 liters of botulism immune plasma, human, were collected and tested. A neuromuscular physiology laboratory was established for study of actions and effects of botulinal toxins and their antagonists.
Publications: Infect. Immun. 30:381, 1980; Appl. Environ. Microbiol. 40:1023, 1479, 42 1018, 1981; Med. Biol. 59:11, 1981; Curr. Microbiol. 6:127, 1981; chapters in: Natural Toxins, Medical Aspects of Botulism, Staphylococci and Staphylococcal Infections.

*Available to contractors upon official approval

BODY OF REPORT

Project No. 3M162770A871: Military Disease, Injury and Health Hazards (U)

Work Unit No. 871 BA 150: Prevention of BW Diseases Caused by Microbial Toxins

Background:

Among the bacterial toxins considered to be potential BW agents, the 7 neurotoxins produced by the bacterium Clostridium botulinum and the lethal toxin complex produced by the bacterium Bacillus anthracis are of the most concern.

Botulinal neurotoxins are the most potent biological toxins known. Their ease of production, stability and lethality qualify them as very high priority toxins for potential use as overt, covert, terrorist and/or BW agents against both military and/or civilian populations. The primary site of action for the botulinal toxins is the cholinergic nerve terminal, where they block the release of the neurotransmitter acetylcholine. Poisoning results in flaccid paralysis; respiratory failure is the usual cause of death. The protection of personnel potentially at risk through exposure to botulinal neurotoxins, the treatment of exposed individuals and the rapid detection of threat toxins are all of considerable concern to both the field commander and the at-risk laboratory worker.

Each of the 7 types of botulinal neurotoxin, designated A-G, is immunologically distinct but pharmacologically similar in action. The amount of botulinal toxin sufficient to be immunogenic in man is thought to far exceed the lethal dose for man. Therefore, botulinal toxoid (toxin which has been chemically modified such that it is no longer toxic but is antigenic) has been traditionally used as an immunogen. A pentavalent (ABCDE) botulinal toxoid was produced by Parke-Davis (PD), under contract to the Army, in the late 1950s. This toxoid, which contains only about 10% neurotoxoid, is in use today. The crude toxoid produces sustained measurable antibody titers in humans only after a series of 4 injections administered over a period of 1 year. Mild side reactions, including tenderness, erythema, induration, and swelling at the site of injection, are common. The PD toxoid, along with the responsibility for its distribution, now belongs to the Centers for Disease Control (CDC), Atlanta, GA. At the current CDC rate of distribution, the Nation's supply (both military and civilian) of PD toxoid will be exhausted by the spring of 1982. A new, less reactogenic yet highly immunogenic, botulinal toxoid prepared from highly purified neurotoxins and capable of inducing protective immunity to all 7 types of toxin is required. Toward this end, optimal fermentor conditions for the production of type A and type B neurotoxin have been developed by USAMRIID scientists (1, 2).

Until recently the only antitoxin available for the treatment of botulism patients was prepared in horses and is provided to the U.S. by a Canadian firm. The equine antitoxin initiates adverse reactions in many recipients and is useful for the neutralization of only 5 (ABCDE) of the 7 known botulinal neurotoxins.

Unfortunately, treatment with specific antitoxin does not reverse existing botulinal toxin-induced paralysis. However, the compounds 4-aminopyridine (4-AP) and 3,4-diaminopyridine (3,4-DAP) are very effective in antagonizing the blockage of transmitter release resulting from botulinal poisoning (3).

The methods now available for detecting and assaying, in a variety of media, nanogram quantities of botulinal toxin are both time consuming and cumbersome procedures and require considerable modernization with new technologies.

Anthrax has long been considered a highly effective potential BW agent. Of particular concern is the aerosol deployment of anthrax spores. The spores are very resistant to inactivation by heat, sunlight and drying. A systemic infection, subsequent to exposure by aerosol, is difficult to diagnose and often fatal. The pathogenesis of anthrax infection is poorly defined; factors to be considered include pulmonary germination of the anthrax spore, systemic multiplication, and persistence of anthrax bacilli, elaboration of the lethal toxin complex, the mechanism of toxin action at the cellular and subcellular levels and the precise mechanisms of action on the host organism as a whole. Earlier work (4) revealed the presence of 3 distinct components of the complex: lethal factor (LF), edema factor (EF), and protective antigen (PA). A biological activity has not been described for PA. However, PA is required for expression of the biological activity of LF and EF (5). The anthrax vaccine licensed for use in man is produced under cultural conditions that support the production and release of substantial quantities of PA by an avirulent strain of B. anthracis (6). The culture supernatant containing PA and numerous other metabolic products is alum-adsorbed, stabilized and then bottled for human use. No attempts have been made to characterize the antigens present in this vaccine or to improve the vaccine by selective purification of PA and/or other components necessary for establishing protective immunity. The only animal vaccine licensed in the U.S. is a live spore vaccine made from the avirulent Sterne strain of B. anthracis. Both vaccines provide protection against laboratory challenge of immunized guinea pigs and against naturally acquired infection (as evidenced by epidemiological observations) of man and animals respectively. The basic mechanisms, and essential antigens necessary for the rapid establishment of optimal active immunity are unknown. A better understanding of the development and establishment of the immune response to anthrax is a necessary prerequisite to an overall approach in the prevention of anthrax by active immunization.

Progress:

Two experimental lots of type A botulinum toxin were produced using the standard fermentor conditions developed at USAMRIID (1). Each lot was highly purified using a procedure developed in this laboratory. The first toxin lot yielded 100 mg of neurotoxin, representing 20% recovery from the starting material (culture fluid). The overall purification achieved was approximately 800-fold on the basis of LD_{50}/mg protein. The second lot contained 66 mg of neurotoxin, a 13% recovery and a 500-fold purification. These preparations were used to develop a toxoiding methodology for type A botulinal neurotoxin. Purified type A neurotoxin in 0.2 M succinate buffer, pH 5.5, was filtered through a nitrocellulose membrane (0.2-μm pore size) and mixed with an equal volume of sterile 1.2% formalin in succinate buffer (final formalin concentration 0.6%). The preparation was mixed, placed at 35°C, and sampled with time. The samples were dialyzed in succinate buffer for 24 hr to remove formalin, and injected into mice to determine residual toxicity as LD_{50}/ml.

An evaluation of the efficacy of a fluid neurotoxoid vs. $Al(OH)_3$- adsorbed neurotoxoids was begun. (Only aluminum compounds are used as adjuvants in toxoids licensed in the U.S.) A small quantity of type A neurotoxoid prepared with 0.6% formalin (28-day incubation) was adsorbed to $Al(OH)_3$ (final concentration 4 mg/ml). This adsorbed neurotoxoid was uded to immunize mice (0.5 ml, sc). A 2nd group of mice received 0.5 ml sc of the corresponding unadsorbed neurotoxoid. Control groups of mice were injected with $Al(OH)_3$ (4 mg/ml in succinate buffer) or with buffer only. At 4 weeks postinjection, all mice will be challenged with one of several toxin doses and the level of protection provided by each neurotoxoid will be determined.

One lot of neurotoxoid (0.6% formalin for 28 days) was dialyzed to remove formalin and stored at 4°C. This preparation is being tested weekly for toxicity to determine the stability of the neurotoxoid.

During this year studies were also initiated with C. botulinum type E. Unlike type A neurotoxin, type E neurotoxin is produced as a progenitor molecule which must be activated to attain maximum toxicity. The progenitor toxin can be activated by treatment with trypsin. C. botulinum type E was cultivated in a fermentor system for the first time (7). The culture medium consisted of 2.0% casein hydrolysate, 0.5% yeast extract and 1.0% glucose. Fermentation conditions were: nitrogen overlay (5 L/min), agitation rate of 50 rpm, and a temperature of 30 C. Under these conditions, the maximum amount of toxin (1.5×10^5 mouse IP LD_{50}/ml after trypsin activation) was produced with only 12-24 hr. In contrast, the static cultures traditionally used to produce toxin must be maintained for 96-100 hr to yield comparable amounts of toxin. Increasing the concentration of yeast extract in the fermentor culture medium to 1.5% resulted in an increase in toxin production (2.8×10^5 LD_{50}/ml after activation). Such yields are adequate to begin the development of methods for the purification and toxoiding of type E neurotoxin.

In response to a telephonic request from CDC, 496 vials (4,960 doses) of botulinum toxoid adsorbed pentavalent (ABCDE), Michigan Department of Public Health (MDPH) Lot #A-2, were transferred to CDC. The quantity of toxoid provided them should temporarily replenish the Nation's supply of pentavalent botulinal toxoid. This pentavalent toxoid, and several monovalent toxoids, were produced in the 1970s by MDPH. The toxoids were prepared by a method almost identical to that used by Parke-Davis in the 1950s to prepare the pentavalent toxoid currently used. This MDPH pentavalent toxoid was bottled for USAMRIID in 1978 and during 1979-1981 was evaluated in USAMRIID volunteers (8). The toxoid is now stockpiled at USAMRIID and at CDC. However, it serves only as a "stop gap" measure until the new and improved USAMRIID developed neurotoxoids become available.

The Alaska Investigations Division of the DHEW requested assistance from USAMRIID in developing and executing a program for the immunization of Alaskan natives with a monovalent type E botulinum toxoid. USAMRIID volunteers (22) were immunized with an MDPH-produced monovalent type E botulinal toxoid. No significant adverse reactions occurred. Determination of the immunogenicity of this toxoid has begun.

A program for the collection by plasmapheresis of Botulism Immune Single Donor Plasma (Human) (IND-1332) was completed during December of FY 1981; 2,381 liters of plasma were obtained and screened for antibody activity to botulinal toxins during the course of this program. One thousand sixty-one liters of this plasma

were selected and shipped to Dr. Richard Condie, Director of the Minnesota ALG Program, for the preparation of Botulism Immune Globulin (human).

The Army-owned horse "First Flight" was hyperimmunized, by the use of botulinal toxoids of all common toxin types (ABCDEFG) and subsequently with the homologous botulinal toxins. This horse was transported to, and is now housed at, the University of Minnesota, Minneapolis. Over 200 L of Heptavalent Botulism Immune Plasma (equine) were collected by plasmapheresis from First Flight. A pilot lot of the plasma was processed in Dr. Condie's laboratory, from which 350 ml of immunoglobulin was prepared. The immunoglobulin is of IV quality, has substantial neutralizing activity for all 7 botulinal toxin types and is now available for emergency use.

Mice poisoned with type A toxin develop signs of botulism and die within a much shorter time period than do mice poisoned with an equivalent quantity of type B toxin. Clinical observations also suggest a difference in the effects of types A and B botulinal toxins. For example, the mean duration of hospitalization is reported to be longer for individuals poisoned with type A toxin, 39 days, than for those poisoned with type B toxin, 24 days (9). A study was designed to determine if a difference in the potency of these toxins occurs at the cellular level in a defined animal model. All experiments were performed in vivo on the extensor digitorum longus (EDL) muscle of male Wistar rats (150-200 gm). Crystalline C. botulinum type A (hemagglutinin, MW 500,000 + neurotoxin, MW 150,000) and purified type B toxin (neurotoxin, MW 167,000) were prepared for injection in a phosphate-gelatin buffer at pH 6.2. A single 0.25-ml bolus of either type A or B toxin was injected sc into the anterolateral region of the right hind leg, superficial to the distal portion of the tibialis anterior muscle. Two precisely determined dosages of each toxin were used: 20 and 4 LD_{50} of type A and 234 and 24 LD_{50} of type B. At various times after toxin injection, the rats were anesthetized with α-chloralose (75 mg/kg, IP). The deep peroneal nerve was stimulated with short duration (2 msec) and maximal amplitude (4-7 V) square pulses using a Grass S88 stimulator. Muscles were considered paralyzed when their average single twitch tension was less than 5% of normal.

Both 20 and 4 LD_{50} of type A botulinal toxin produced muscle paralysis within 24 hr of injection. The muscles receiving 20 LD_{50} remained paralyzed through 10 days after injection, but showed some signs of recovery at 14 days. The muscles treated with 4 LD_{50} of type A began to recover at 10 days and were about 30% of normal at 14 days.

The muscles injected with 235 LD_{50} of type B were also paralyzed within one day and remained so through day 3. However, at 6 days, these muscles began to recover and were not significantly different from normal at 14 days. In contrast, the muscles treated with 24 LD_{50} of type B toxin were never paralyzed, but showed a reduction in twitch tension at 6 days.

These results corroborate laboratory findings in mice and clinical observations of man regarding the action of types A and B botulinum toxin; i.e., the duration of effect for type B is less than that of type A. The present findings also demonstrate that a greater amount of type B toxin is required to produce an effect similar to type A. The reason for this difference in action is unclear, but may result from type B dissociating from its binding site more readily and/or degradation occurring at a more rapid rate.

In a preliminary experiment, type A neurotoxin was rendered completely nontoxic for mice in 48 hr by 0.6% formalin, and remained nontoxic for the duration of the experiment, 28 days (Table I). This experiment was repeated, with sampling done at shorter intervals. Again the preparation was nontoxic for mice in 48 hr. However, toxicity was detected at 3 and 5 days, but not at 7 days, nor for the duration of the experiment (28 days). A study is currently in progress comparing the kinetics of detoxification of a single lot of toxin (2.5 X 10^6 LD_{50}/ml) by 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0% formalin.

Table I. TOXOIDING KINETICS OF TYPE A BOTULINAL NEUROTOXIN IN THE PRESENCE OF 0.6% FORMALIN.

PRELIMINARY[a]		REPEAT[b]	
Time (hr)	Toxicity (LD_{50}/ml)	Time (hr)	Toxicity (LD_{50}/ml)
0	6.3 X 10^5	0	5.6 X 10^5
6	6.3 X 10^2	2	1.6 X 10^4
12	6.3 X 10^1	4	3.2 X 10^3
24	2.0 X 10^1	6	1.6 X 10^3
48	Nontoxic	12	3.2 X 10^2
5 days	Nontoxic	24	2.8 X 10^1
7 days	Nontoxic	36	6
14 days	Nontoxic	48	Nontoxic
21 days	Nontoxic	3 days	6
28 days	Nontoxic	5 days	6.3 X 10^1
		7 days	Nontoxic
		14 days	Nontoxic
		21 days	Nontoxic
		28 days	Nontoxic

[a]Initial toxin concentration: 1.4 X 10^6 LD_{50}/ml

[b]Initial toxin concentration: 1.6 X 10^6 LD_{50}/ml

In addition, it was observed that 3,4-DAP was less effective in reversing the paralysis induced by type B toxin than with type A. However, further experiments are needed to determine the nature of the difference between types A and B neurotoxin.

In an effort to establish a nonhuman primate model suitable for evaluating the efficacy of aminopyridine compounds in the treatment of botulism, 6 cynomolgus monkeys were poisoned IV with partially purified type A toxin. Each monkey was maintained separately and fitted with a leather metabolic jacket which allowed complete freedom of movement within the cage and free access to food and water. An indwelling catheter extending from the jugular vein, through a flexible steel cable attached to the jacket, and extending outside the cage, provided a route for the infusion of toxin and 3,4-DAP, as well as a route for the collection of whole blood.

Monkeys were inoculated with 100-371 mouse LD_{50} of neurotoxin/kg body wt. Onset of signs of botulism correlated with the dosage of toxin given, ranging in time of first appearance from 10-31 hr after poisoning. The administration of 3,4-DAP, which has been reported to be less of a convulsant than 4-AP, was delayed from 3-6 hr after appearance of the first signs of botulism, and the interval between the 1st and 2nd treatment extended from 1.75 to 5 hr. 3,4-DAP was administered as a single bolus of 1.1-2.1 mg/kg given before poisoning, and/or as multiple doses after the development of severe signs, or as a continuous IV drip of 0.78-0.95 mg/kg/hr.

The first sign observed in all of the poisoned animals was choking while attempting to swallow bits of dry food. Muscular weakness and, in some monkeys, ptosis were the next signs to appear; respiratory distress followed soon thereafter. Next, the acceptance of food ceased and swallowing of water became difficult. As the illness progressed, a closed-mouth rhythmic respiratory pattern gave way to an open-mouthed, shallow, abdominal, gasping pattern. Oral and nasal discharges, white, forthy, and often stringy, became increasingly evident. This was probably due to an increasing inability to swallow, rather than an increase in oral secretions. Death, attributed to respiratory insufficiency, and in one monkey possibly complicated by drug overdose, soon followed.

All inoculated monkeys responded favorably within 2-10 min after treatment with 3,4-DAP at 1.0-2.6 mg/kg. The clinical response of each monkey varied according to the degree and duration of paralysis existing at the time of treatment. In general, muscle tone returned, strength of grip increased, prostrate animals were again able to stand, ptosis was reduced or negated, and respiratory patterns improved unequivocally. Unfortunately, within 2 hr after the initial 3,4-DAP treatment, signs of paralysis returned. Treatment with a second equivalent dose of 3,4-DAP induced a response considerably less dramatic than that observed after the initial dose. The continuous IV administration of 3,4-DAP did not prevent the return or the progression of the paralytic signs of botulism.

Although the small number of monkeys utilized in this pilot study was insufficient to establish many important factors, such as the IV LD_{50} for untreated cynomolgus monkeys, numerous promising trends were observed; (a) cynomolgous monkeys present a very uniform sequence of events when inoculated IV with type A botulinal toxin, and the sequence parallels that seen in man; (b) treatment with 3,4-DAP provided a definite improvement in muscle strength, mobility, and respiratory pattern of poisoned monkeys; (c) the drug-induced regression of clinical signs

was consistently transient, lasting only about 2 hr; (d) response to subsequent treatment with 3,4-DAP was less than that induced by the initial treatment; (e) at the doses of toxin and drug administered, 3,4-DAP was not convulsive, possibly prolonged survival, but did not prevent death; and (f) the metabolic jacket apparatus, while allowing for freedom of movement, provided a convenient and nonrestrictive means for the administration of drugs, the sampling of body fluids, and most importantly, the uninhibited observation of the natural progression of botulism in this nonhuman primate model.

An enzyme-linked immunosorbent assay (ELISA) was developed for the detection and quantification of C. botulinum type G toxin. Using a "double-sandwich" technique and alkaline phosphatase as the enzyme indicator, type G toxin was detected in quantities equaling that required for 1 mouse LD_{50}. Time required for the procedure was approximately 6.5 hr, but this requirement could be reduced to 5.5 hr or less by using precoated plates stored at -70 C.

Numerous procedures were investigated for use in the production and purification of anthrax protective antigen (PA). For the fermentor production of PA, B. anthracis strain V770-NP1-R was grown in a synthetic medium (6). A standard purification procedure developed at USAMRIID is routinely employed for the isolation of PA from culture supernatant. Bacteria are removed by centrifugation. Next, an ion-exchange resin (DEAE-cellulose) is added to the culture supernatant to bind, along with other proteins, the PA. Adsorption and elution are accomplished in ammonium acetate buffer (pH 8.0); the PA is subsequently eluted from the resin using 1 M NaCl. The protein is recovered in one large peak, consisting of 8-10 components, one of which is PA. Analysis of this partially purified material has shown that it is antigenic and affords a certain level of protection in animals. Rats were immunized with this material and have survived an IV toxin challenge; guinea pigs were immunized with this same partially purified antigen and survived an IM challenge (200 LD_{50}) of the Vollum strain anthrax spores. Another lot of this partially purified PA was adsorbed on Alhydrogel (a commercially available aluminum hydroxide gel suspension); animals were immunized in an identical fashion. These data are summarized in Table II.

Table II. EVALUATION OF ANTHRAX IMMUNOGENS IN RATS AND GUINEA PIGS

TREATMENT	NO. SURVIVORS/NO. CHALLENGED	
	RATS	GUINEA PIGS
Saline	0/8	0/15
Wright's vaccine (licensed human vaccine)	8/8	12/15
Soluble PA (USAMRIID)	8/8	11/15
Alhydrogel (USAMRIID)	4/4	12/15
Anvax Sterne vaccine (licensed animal vaccine)	4/4	13/15

Sera were obtained from animals surviving challenge and antibody titers were measured using the indirect hemagglutination IHA assay, as directed by Buchanan et al. (10). Geometric mean titers for surviving rats in all groups were ≥4096. Final titers in the guinea pig groups described above were: Wright's vaccine >2048, <4096; soluble PA >1024, <2048; and PA-Alhydrogel >512, <1024; Sterne vaccine >2048, <4096.

HA titers are obtained using PA antigen prepared by MDPH under contract to USAMRIID. A new lot of this antigen was prepared in June 1981 and subsequently delivered to USAMRIID. Analysis of this material by standard biochemical techniques showed that the protein concentration was about 140 μ/ml and the protein pattern on an SDS-acrylamide gel indicated the product was >95% pure, and composed primarily of a single band having a MW of about 85,000-90,000. Guinea pigs have been immunized with purified material in order to determine its immunogenicity. Substantial quantities of anthrax antiserum have been harvested from horses, burros, and goats immunized with Sterne strain spores. These antisera are used to detect various components of anthrax toxin present in culture supernatants and in column fractions collected during purification procedures. PA seems to produce the most predominant band of precipitate in an Ouchterlony double diffusion gel when reacted with the horse serum. Several hundred milliliters of horse serum have been supplied to various investigators at USAMRIID and to Dr. Curtis Thorne, a USAMRDC contractor, who is investigating the genetics of anthrax. Levels of PA antigen production have been determined for several strains of anthrax. The V770-NP1-R strain, when produced in totally synthetic medium, produced PA having an Ouchterlony titer of 1:1-1:2. Thorne has reported Ouchterlony titers as high as 1:8 using the Sterne strain in casamino acid medium.

Using the indirect HA assay, the sera of over 100 USAMRIID personnel receiving anthrax immunizations have been evaluated. Numerous individuals are now receiving booster immunizations at one-year intervals. The serum antibody titers existing prior to and following booster immunization are being compared. Statistical evaluations are not completed, since data are still being collected. However, a trend is developing which seems to indicate only a slight decline in titer by one year. As anticipated, a modest increase in titer followed booster immunization. Another group of individuals who had received extensive immunization in earlier years and subsequently were not immunized for periods of 5-10 years, were screened for existing anthrax titers. Essentially all of these individuals were seronegative, indicating a need for booster immunizations.

In addition, a new phase of research which focuses on the role of macrophages in the host's ability to process anthrax spores was initiated very recently. This work is in its earliest stages, and no data are as yet available.

Presentations:

1. Lewis, Jr., G. E. A review of the botulism research program at USAMRIID. Presented, Interagency Botulism Research Coordinating Committee Meeting, New Haven, CT, 8 Oct 1980.

2. Sellin, L. C. Pre- and postsynaptic actions of botulinum toxin at the rat neuromuscular junction. Presented, New Jersey Medical School, Newark, NJ, Dec 1980.

3. Sellin, L. C. Pre- and postsynaptic actions of botulinum toxin at the rat neuromuscular junction. Presented, Cornell University, Ithica, NY, Dec 1980.

4. Lewis, Jr., G. E. Approaches to the prophylaxis, immunotherapy and chemotherapy of botulism. Presented, Int Conf on the Biomedical Aspects of Botulism, Frederick, MD, 16-18 Mar 1981.

5. Siegel, L. S. Fermentation kinetics of botulinum toxin production (types A, B and E). Presented, Int Conf on the Biomedical Aspects of Botulism, Frederick, MD, 16-18 Mar 1981.

6. Sellin, L. C. Postsynaptic effects of botulinum toxin at the neuromuscular junction. Presented, Int Conf on the Biomedical Aspects of Botulism, Frederick, MD, 16-18 Mar 1981.

7. Johnson, A. D., Cross, A. S., and DeCicco, B. T. Isolation of exfoliatin from a non-group II strain of Staphylococcus aureus. Presented, Annu Mtg, ASM, Dallas, TX, 1-5 Mar 1981 (Abstracts, B31, p. 19, 1981).

8. Siegel, L. S., and J. F. Metzger. Effect of iron on growth and toxin production by Clostridium botulinum type A. Presented, Annu Mtg, ASM, Dallas, TX, 1-5 Mar 1981 (Abstracts, B32, p. 20, 1981).

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10. Lewis, Jr., G. E. Approaches to the prophylaxis, immunotherapy and chromotherapy of botulism. Presented, Imperial College of Science and Technology, London, England, 20 Jul 1981.

11. Lewis, Jr., G. E. Botulism. Presented, Center for Applied Microbiolcgy and Research, Porton Down, England, 23 Jul 1981.

12. Lewis, Jr., G. E. and R. M. Wood. Effects of 3,4-diaminopyridine in cynomolgus monkeys poisoned with type A botulinum toxin. Presented, Int. Symp on the Effects of Aminopyridines and Similarly Acting Drugs on Nerves, Muscles and Synapses, Paris, France, 27-29 Jul 1981.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0G3813	81 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
80 10 02	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	62770A	3M162770A871	BB	149
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)*

(U) Prevention of Bacterial and Rickettsial Diseases of Potential 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
80 10	CONT	DA	C. In house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 81	3.5	865
	CURR: 82	9.0	409

19. RESPONSIBLE DOD ORGANIZATION

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TELEPHONE: 301 663-7341
SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS
NAME: Rozmiarek, H.
NAME: Jemski, J. V. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) Military medicine; (U) BW defense; Bacterial diseases; (U) Rickettsial diseases; (U) Vaccines; (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) Develop a base of technology and when possible, specific measures that either prevent or effectively counteract bacterial and rickettsial diseases that fall into 2 categories: have significant BW potential and could be used against this country because of its vulnerability; and are highly virulent and dangerous agents which require special containment laboratories for safe study and are uniquely important to the U.S. military forces.

24. (U) Employ state of the art clinical and diagnostic techniques to ensure and maintain healthy research laboratory animals. Identify and establish special animal colonies when unique requirements are identified. Employ both chemical and immunologic studies to identify major effective antigenic components in potential BW agents, such as Bacillus anthracis, Francisella tularensis and Coxiella burnetii.

25. (U) 80 10 - 81 09 - Conditions were determined for using febrile reactions to establish whether or not C. burnetii vaccine is effective. Antibodies were shown to play a protective role in Fischer-344 rats challenged with a lethal dose of F. tularensis. A peptidoglycan was isolated and purified from B. anthracis; it affords partial protection. It may have a role as a supplemental immunogen. USAMRIID's multifaceted research animal colonies were maintained by intense surveillance and treatment of diseases. The animal model repertoire was expanded by obtaining and breeding Calomys callosus, Sigmodon hispidus and Clethrionomys gappert.

Publications: Infect. Immun 34, In press, 1981.

*Available to contractors upon originator's approval

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

BODY OF REPORT

Project No. 3M162770A871: Military Disease, Injury and Health Hazards (U)

Work Unit No. 871 BB 149: Prevention of Bacterial and Rickettsial Diseases of Potential BW Importance

Background:

This annual report is the summary of 5 individual research unit by 4 principal investigators. Since they are not really bound too tightly by a common thread, the work must be specified for each investigator.

Dr. Johnson determined conditions for maximum reliability of the febrile reactions of guinea pigs infected with Coxiella burnetii and used this information to estimate a PD_{50} for lot 5 of the NDBR-105 phase I Q fever vaccine. He initiated biochemical studies to confirm or replace the febrile reactions in guinea pigs as a measure of response to Q fever infection.

Dr. Jemski showed that Francisella tularensis LVS strain vaccination by small particle aerosol inhalation, intranasal instillation, or by IP, SC or IM injection provided practically total and equal protection against respiratory tularemia. He found that passively administered F. tularensis whole antiserum or the total immunoglobulin fraction protected F-344 rats against lethal tularemia and finally that F-344 rats vaccinated with LVS aerosols or by IM injection with LVS were fully protected against aerosol challenge for at least 2 years.

Dr. Ezzell successfully isolated and purified cell wall material from Sterne strain of Bacillus anthracis primarily composed of peptidoglycan (PG) as determined through chemical analysis. He demonstrated the partial efficacy of PG enriched cell wall fragments as a vaccine in protecting guinea pigs against lethal challenge with spores. He isolated and purified capsule material, poly-D-glutamyl peptide from Vollum 1B strain of B. anthracis. He determined that capsular material in solution does not inhibit PMN chemiluminescence (CL) during phagocytosis of opsonized Sterne cell wall fragments. He also demonstrated the expression of peptidoglycan cell wall antigens on surface of gamma-irradiated Sterne strain cells using fluorescent antibody (FA) techniques.

COL Rozmiarek has as part of his efforts to identify hazards and variables associated with research animals diagnosed and treated pasteurellosis in rabbits, encephalitozoonosis in guinea pigs and rabbits, hexamitiosis (spironucleosis) and infant diarrhea in mice, murine respiratory mycoplasmosis (MRM) and nworms in rats, malaria (Plasmodium inui) in cynomolgus monkeys, and shigellosis and salmonellosis in newly arrived African green monkeys.

Breeding colonies of Calomys callosus and Sigmodon hispidus were maintained in a stable configuration. Several Clethrionomys gapperi were obtained from a trapper in northern Canada; breeding efforts to date have resulted in over 50 births. The strain 13 guinea pig breeding colony has stabilized at approximately 700 breeding females. An outbreak of bordatellosis was effectively halted with use of an autogenous bacterin. Production has stabilized at 250-300 offspring per month for issue. The rhesus breeding colony recorded 6 live births and 6 successfully weaned

infants during the last 12 months. The number of actively breeding rhesus monkeys in the colony was reduced to 2 males and 10 females and the collection of normal growth parameters on developing infants and adolescents continued.

Progress:

Regarding Dr. Johnson's research on rickettsial disease, initial experiments comparing the protective properties of the DP-7 and NDBR-105 Q fever vaccines in guinea pigs indicated that a number of problems needed solving before adequate precision in the comparisons could be made. One problem was the occurrence of brief sporadic increases in the body temperature of guinea pigs before vaccination or challenge. Since temperature after challenge was the criterion used for determining infection, these changes increased errors. It was found that an acclimatization period of at least 2 weeks was needed, during which time the animals were kept in individual cages, handled frequently, and allowed to become adjusted to new surroundings. Optimal temperature of the holding rooms was determined to be 70 F (21 C) because higher temperatures caused occasional temperature spikes and lower temperatures tended to increase the incidence of respiratory infections with associated febrile responses. The source of guinea pigs was important. In this study, 3 sources were tested, West Jersey Biological Co., Buckburg, Inc., and Charles River, Inc. The West Jersey animals were in poor condition on arrival; most had or soon developed, respiratory infections and several died. Buckburg guinea pigs arrived in good condition and remained so throughout the holding period, except for an occasional temperature spike. These animals were used to assay lot 5 of the NDBR-105 vaccine. Charles River guinea pigs were in excellent health on arrival and remained so throughout the holding period. They were used to assay the challenge inoculum.

As indicated, lot 5 of the NDBR-105 Q fever vaccine was assayed for its protective and serologic properties in Buckburg guinea pigs. Four groups of 10 animals were each inoculated SC with various concentrations of vaccine using serial 10-fold dilutions from 20 to 0.02 µg per animal. Four weeks later, they were challenged with 1×10^5 FD_{50} of the Henzerling strain of C. burnetii. Temperatures of each vaccinated, challenged animal along with 9 infected, unvaccinated controls and 7 unchallenged controls were recorded daily for 10 consecutive days. Data comparing the number of fever days (> 40.0 C) in each vaccinated group with the number of fever days in the infected control group were analyzed statistically using probit analysis; PD_{50} was calculated to be 0.012 µg. Each guinea pig in the infected control group evidenced infection, while none in the uninfected control group developed elevated temperatures. The PD_{50} in this study was smaller than that obtained in a similar experiment several years ago by Dr. Ormsbee at the Rocky Mountain Laboratory, USPHS. He used the same lot of vaccine, but a different time schedule and another source of guinea pigs, to obtain a PD_{50} of 0.32 µg. The two values are different statistically; however, they are close enough, considering variation in the procedures, to suggest that estimates of the protective doses of the vaccine lots can be obtained.

Because febrile reactions in guinea pigs are extremely sensitive to environmental and other uncontrollable influences, biochemical parameters were investigated as supplements or alternatives to this indicator of infection. Heggers et al. (1) reported the effect of Q fever infection in the guinea pigs on a number of serum components. Among these, glucose, glutamic-oxalacetic transminase (GOT) and creatinine phosphokinase (CPK) appeared to be most affected by the infection and were chosen for further study. In an initial experiment, serum from 10 normal

guinea pigs were analyzed for the 3 components in collaboration with the Clinical Laboratory. Results indicated that serum glucose levels were within normal limits for guinea pigs and showed a variation of about 10% about the mean. The GOT levels were less consistent with about a 22% variation, and the CPK were least consistent, with a variation of about 36%.

Dr. Jemski reported previously (2) that in 3 experiments, 98-100% of F-344 rats passively vaccinated with F. tularensis immune serum from donor F-344 rats immunized with the live vaccine strain (LVS) of F. tularensis survived an aerosol or IP challenge with virulent F. tularensis, SCHU-S4. Only 10% of control that received normal rat serum survived the challenge. In contrast, published reports by others (3, 4) attributed no protective capacity to passively transferred antiserum. Experiments were conducted, therefore, to define the critical factors responsible for the protection afforded to the recipient F-344 rats in our experiments.

Serum preparations consisted of immune whole serum (Code I), and immune whole serum dialyzed against a 25,000-dalton membrane (Code II) to remove possible soluble T-cell receptors or other soluble lymphokines with a MW of 25,000 or less. The immune serum fraction preparations tested consisted of a reconstituted $(NH_4)_2SO_4$ precipitate containing the total immunoglobulin components (Code III); a purified and resolubilized IgG fraction (Code IV), and a purified resolubilized IgM fraction (Code V). In addition, whole LVS immune serum was treated with 2-mercaptoethanol (2-ME) to eliminate IgM but retain potential soluble protective factors or IgG if present (Code VI). For control purposes, whole normal rat serum (Code VII) and a reconstituted preparation of a nonspecific precipitate (Code VIII) were included.

Rats were passively immunized by IP injection with 0.7 ml of the various serum preparations, then challenged IP 16-18 hr later with 1×10^6 cells of virulent F. tularensis, SCHU-S4. Just before challenge, blood was obtained via orbital sinus bleeding from 2 rats of each group; blood was assayed for the presence of LVS serum agglutinins. All animals were observed twice daily throughout a 20-day holding period. The response data obtained from these rats are shown in Table I.

All control rats given normal rat serum (Code VII) died within 9 days after challenge. All rats passively immunized with the nondialyzed whole immune serum (Code I) or with the dialyzed immune serum (Code II) survived challenge. The total protection afforded with the dialyzed immune serum indicated that a soluble lymphokine, such as a proposed T-cell receptor with a MW of 25,000 or less was not involved in conferring passive protection. Of significance was the 90% survival rate (9/10) obtained with the reconstituted precipitate fraction containing all immunoglobulins (Code III). The protection achieved for the rats receiving only the IgG fraction (Code IV) or only the IgM fraction (Code V) was 60% and 55%, respectively, which compared favorably to the 90% protection observed for the rats which received the total immunoglobulin-containing fraction (Code III). These results suggest that in the rat, at least, the total immunoglobulin portion of tularemia antiserum may play a role in acquired resistance to lethal tularemia, particularly in the absence of any induced cell-mediated immunity. These findings also are compatible with the premise that IgG is subsequently induced by the 3x vaccination regimen over a 4-week period. The IgG acting in combination with the earlier produced IgM (still detectable at low titer at 28 days after vaccination) results in a cumulative protection above that conferred by either IgG or IgM alone. The results obtained with the immune serum treated with 2-ME (Code VI) also seem to be additional evidence for the protective effect of IgM, if one accepts that IgM

TABLE I. EFFECT OF PASSIVELY TRANSFERRED WHOLE TULARENSIS IMMUNE SERUM AND ANTISERUM FRACTIONS ON SURVIVAL OF TULARENSIS CHALLENGED RATS

TRANSFER SERUM		RAT RESPONSE				
Code	Fraction	Agg. titer[a]	Surv./ chall.	Prechall. agg. titer[a]	% Surv.	MTD (days)[b]
I	Whole immune serum	1020	6/6	20	100	-
II	Whole immune serum (dialyzed)	200	15/15	20	100	-
III	Total Ig [$(NH_4)_2$ SO_4 ppt]	400	9/10	40	90	6.0
IV	IgG (Fract. 0.01M)	30	6/10	0	60	4.9
V	IgM (Fract. 0.16M)	60	6/11	0	55	6.3
VI	Immune serum + 2-ME	80	5/6	20	83	6.0
VII	Normal rat serum	0	0/11	0	0	6.4
VIII	Nonspecific ppt. (Fraction 0.1 M)	20	4/12	0	33	5.3

[a]Reciprocal geometric mean titer.

[b]Mean time to death.

indeed was neutralized by the 2-ME treatment. The agglutinin titer obtained for the 2-ME fraction, therefore, would be accounted for by the presence of IgG. The role of IgG, however, may be important in the very early stages of a challenge infection. Proctor et al. (6) have shown that peripheral neutrophils, which are rapidly mobilized in the first 18 hr of tularemia infection, can limit or prevent the early dissemination of F. tularensis organisms by phagocytosis but only in the presence of immune serum. It may well be that it is IgM that acts as the specific opsonin to prevent hematogenous dissemination in the challenged animals. Bacteremia is present in nonvaccinated control rats.

Vaccination with small particle aerosols of F. tularensis, LVS, or by IP, IM, or SC injection effectively protects experimental animals against disease and death when challenged by aerosols or IP inoculation with virulent F. tularensis.

A selected number of rats from the 2 vaccinated and sham-vaccinated control groups were challenged with aerosols of virulent F. tularensis, SCHU-S4, at various time intervals over a 2-year period. The first challenge was 3.5 months after

vaccination and the last, at 26 months. Two days prior to each challenge time the animals were bled from the orbital sinus and the serum assayed for agglutinins. Humoral agglutinin titers and the survival response for the 3 groups of rats during the 26-month study period are shown in Table II.

TABLE II. LONG-TERM IMMUNE RESPONSE OF LVS VACCINATED RATS TO CHALLENGE WITH AEROSOLS OF VIRULENT F. TULARENSIS

POSTVACCINATION CHALLENGE TIME (MONTHS)	VACCINATION METHOD	INHALED CHALLENGE DOSE (LOG_{10})	PRECHALLENGE AGGLUTININ TITER[a]	NO. DEAD/ CHALLENGED
3.5	MCPH	-	0	3/5
	Aero.-LVS	6.1	129	0/10
	IM-LVS	6.1	85	0/10
8	MCPH	-	0	4/5
	Aero.-LVS	5.9	32	0/10
	IM-LVS	5.9	28	0/10
12	MCPH	-	0	5/5
	Aero.-LVS	5.6	46	0/10
	IM-LVS	5.6	26	0/10
18	MCPH	-	0	3/5
	Aero.-LVS	3.7	12	0/8
	IM-LVS	3.7	18	0/5
26	MCPH	-	no data	3/3
	Aero.-LVS	5.2	no data	0/4
	IM-LVS	5.2	no data	0/3

[a]Reciprocal geometric mean serum titers.

Over the 5 challenge periods, over 22% (5/23) of the sham-vaccinated control rats survived in contrast to the 100% survival of the aerosol and IM vaccinated rats. The 2 groups of vaccinated rats were as fully protected against challenge with virulent F. tularensis at 26 months after vaccination as at only 3.5 months. None of the vaccinated rats showed overt signs of illness even though 2-4 log_{10} of F. tularensis organisms were recovered from the lungs at 14 days. As we previously reported, active immunization of F-344 rats with LVS did not prevent colonization of the lungs following aerosol challenge, but illness in these animals was not apparent and only minimal pulmonary histopathology was observed.

A diminution of serum agglutinin titers occurred over the 26-month observation period for both groups of vaccinated rats. A mean titer of 1:15 for these groups was measured at 18 months. The overall data suggested a lack of correlation between titer levels and protection. It is of interest to note, however, that a single LVS vaccination by either the aerosol or IM route provided significant protection to F-344 rats throughout the 29-month span of the experiment.

Dr. Ezzell reported that significant insight has been obtained into the immunogenicity of Bacillus anthracis cell wall antigens. Primarily studied to date has been a cell wall fraction of the Sterne strain of B. anthracis which was purified with sodium dodecyl sulfate (SDS) extractions and trypsin treatment. This peptidoglycan-enriched cell wall fraction was composed of glutamic acid, alanine and diaminopemilic acid in a ratio of 1;1;5;1, respectively, which is in agreement with previous findings (5). In addition N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) were detected (Table III). Excluding the percent total nitrogen, the total weight percentage of the cell wall accounted for equals approximately 75%. Studies are presently underway using the high pressure liquid chromatograph of Dr. Bob Seid at WRAIR to determine the presence of another anthrax polysaccharide composed of equimoles of N-acetylglucosamine and galactose which may account for the remaining 25%. The cell wall fragments were essentially protein free as determined by Lowry and Bio Rad assays, spectrophotometry, and the presence of only trace amounts of amino acids other than those listed in Table III. No nucleic acid was detected through spectrophotometric determinations and the diphenylamine and orcinol tests for DNA and RNA, respectively. The low amount of phosphate indicates the absence of teichoic acid.

TABLE III. PERCENT COMPOSITION OF STERNE STRAIN CELL WALL

COMPONENT	% WEIGHT COMPOSITION	NMOL/MG CELL WALL
Glutamic Acid[a]	4.38	300
Alanine	3.92	448
Diaminopemilic acid	5.76	303
NAM and NAG	60.64	ND[b]
Organic phosphate	0.76	ND
Total nitrogen[c]	5.02	ND

[a]Determined by R. Dinterman, Physical Science Division, USAMRIID.

[b]ND, not determined.

[c]Determined by Karen Bostian, Physical Science Division, USAMRIID.

The material was used to vaccinate female, Hartley guinea pigs through a series of weekly ID foot-pad injections over a 1-month period and then challenged with viable Vollum 1B spores. The results of these trials are shown in Table IV. Challenges in trials A, B, and C (3 vaccinations/week) were performed by Dr. Jemski, Aerobiology Division, whereas trials D and E (1 vaccination/week) were challenged by Dr. Ezzell in Suite B-3, Bacteriology Division. Since the LD_{50} for Hartley guinea pigs is 500 Vollum spores, the challenge dose was dropped to 1500 spores in trials D and E in order to prevent "overriding" the vaccine with too large a

TABLE IV. RESPONSE OF GUINEA PIGS VACCINATED WITH VARIOUS ANTHRAX ANTIGENS TO CHALLENGE WITH VIRULENT B. ANTHRACIS

TRIAL NUMBER	NO. GUINEA PIGS	VACCINE TREATMENT	CHALLENGE DOSE	NO. DEAD/ CHALLENGED	MTD (days)[a]
A	5	Sterne PG[b]	3,020	5/5	3.18
	5	Saline	3,020	5/5	2.00
B	6	Sterne PG	6,025	6/6	4.12
	5	Saline	6,025	5/5	2.55
C	5	Sterne CW[c]	3,160	5/5	2.17
	5	Saline	3,160	5/5	2.00
D	5	Sterne PG	1,500	2/5	4.75
	5	Sterne PG & Vollum capsule	1,500	1/5	6.00
	5	Vollum capsule	1,500	4/5	2.25
	5	Saline	1,500	5/5	2.50
E	5	Sterne cells[d]	1,500	5/5	3.70
	5	Sterne cells & Sterne spores	1,500	4/5	2.75
	5	Sterne cells & Sterne spores & Vollum capsule	1,500	4/5	3.00
	5	Sterne spores	1,500	3/5	4.50
	5	Saline	1,500	5/5	3.20

[a]MTD, mean time to death; surviving guinea pigs excluded from value determination.

[b]PG, peptidoglycan-enriched Sterne cell wall.

[c]CW, cell wall material (peptidoglycan and associated proteins, lipid, etc.), gamma-irradiated.

[d]Gamma-irradiated Sterne vegetative cells.

[e]Gamma-irradiated Sterne spores.

challenge. It is interesting that with larger challenges as in trials A and B some of the animals survived up to 3 days beyond the controls. In trial D, with the exception of 1 guinea pig (1:4 titer), no animals showed a titer against protective antigen prior to challenge and only 2/8 had one after surviving challenge (1:8 and 1:128) as measured by Dr. Anna Johnson-Winegar, Pathology Division, using hemagglutination. The positive control was 1:4096. Therefore, the partial protection afforded by the cell wall vaccine could not be attributed to protective antigen antibodies. As can be seen in Table IV, vaccination with whole vegetative Sterne cells and/or spores gamma-irradiated by CPT Urbanski, Virology Division, were not as effective as the Sterne cell wall fragments. Interestingly, Sterne cell wall fragments from

which proteins and other components had not been extracted (Trial C) did not significantly delay death as in Trials A and B. Therefore, the purified cell wall fragments preparations were more effective than were the less purified cell wall fragments with other associated material or as part of whole cells.

Rabbit antibodies directed against the purified Sterne cell wall fragments were fluorescein-conjugated by Pete Bagley, Virology Division. Using FA techniques, the expression of these and other antigens on the cell surface of virulent and avirulent anthrax strains is being studied. Expression of cell wall antigens on intact Sterne and Vollum 1B vegetative cells has been demonstrated using the above described FA conjugate.

Procedures for isolating purified capsular material, poly D-glutamyl peptide from the Vollum 1B strain are now being performed on a routine basis using anion exchange and gel filtration chromatography. The purity of this material has been repeatedly verified using amino acid analysis and spectrophotometry. Once purified, this material is used in vaccine trials, in macrophage activation studies by LTC Friedlander, Bacteriology Division, and in PMN CL studies, originally performed by CPT McCarthy, Physical Science Division, and now by Dr. Ezzell.

Colonel Rozmiarek noted that the animal disease surveillance program functioned at a normal level this past year, with the quality control monitoring of 390 rats, mice, guinea pigs and hamsters. This total represents 20 strains and stocks from 14 commercial sources. Animals were examined for gross and histologic pathology, bacterial pathogens, parasites, and hematologic parameters. The hematologic data are used to establish normal values for each strain/stock of rodent used at USAMRIID and to determine abnormal values which may indicate latent metabolic or infectious disease processes. Nonhuman primates, rabbits, and poultry were received from vendors during the year, and along with in-house populations were screened for the presence of various disease conditions.

The following diagnostic specimens were examined during the past year from incoming and in-house animals:

Examination	Number
Hematology	1,376
Parasitology	481
Bacteriology	440
Histopathology	390
Serology	135
Serum Biochemistry	41
Urinalysis	15

A summary of the histopathologic examinations of incoming rodents revealed a relatively low incidence of latent changes (Table V).

TABLE V. SUMMARY OF PATHOLOGIC CHANGES IN LABORATORY ANIMALS RECEIVED

SPECIES	NO. EXAMINED	NO. WITH PATHOLOGIC CHANGE			
		Pulmonary	GI	GU	Miscellaneous
Rats	149	38	2	2	1
Mice	71	0	6	0	2
Guinea pigs	35	4	4	0	0
Hamsters	15	1	0	0	0
Total	270	43	12	2	3

Part II of Colonel Rozmiarek's report deals with animal models and resources. He reports the primary function of the S. hispidus and C. callosus colonies are to meet the documented demands of the Institute for these commercially nonavailable animals for investigative needs. We have achieved this goal and are continuing to provide vesper mice and cotton rats to using investigators. No major problems associated with disease, husbandry, or reproduction were encountered during the reporting period.

S. hispidus - approximately 25 breeding pairs were maintained. There were 85 born, 82 weaned, and 55 issued.

C. callosus - approximately 25 breeding pairs were maintained. There were 220 born, 212 weaned, and 18 issued.

The breeding colony of strain 13 guinea pigs (Cavia porcellus) has undergone significant change during this year. The colony is stabilized at 35 breeding racks or 175 harems of 4 females and 1 male per harem. The colony has moved from the inadequate facility in Bldg 1412 to 7 rooms in Suite AR 3 in Bldg 1425. There were 3,770 offspring born in average litter sizes of 3.78. There were 2,023 weaned in average litter sizes of 2.66. These figures compare favorably with figures available from commercial production inbred guinea pig colonies. There were 1,788 animals issued to investigators during the year. Production is steadily increasing and should continue to do so for the coming quarter. To maintain genetic uniformity, replacement breeders for this colony will continue to come from first or second generation stock from inbred colonies maintained by the Division of Research Services, NIH. Animals issued to investigators will usually be from the F3 and occasionally from the F4 generation away from true inbreds. Investigation has shown this to be acceptable for use in the USAMRIID program. In October-December, 1980, an epizootic of pneumonia swept through the colony resulting in sigificant morbidity and some mortality. The causative agent, Bordatella bronchiseptica, is highly transmissible and very difficult to control with therapeutic treatment. Working with Dr. James Ganaway of the Veterinary Resources Branch, NIH, an autogenous bacterin was produced and all animals in the colony, vaccinated. This quickly brought the outbreak under control and no new cases have been detected. A total of 39 adult breeders died during this time. We are now vaccinating all potential breeding

animals with the bacterin before they are introduced into the colony. Moving the colony from one large room to 7 smaller rooms greatly reduces the risk of severe losses by disease outbreak. Cesarean derivation of all animals to be newly introduced into the colony has been considered as an additional disease preventive measure. Because genetically monitored breeding stock are extremely difficult to obtain, it has not been adopted, but will remain under consideration.

The need arose during the period for a regular supply of gappers red backed voles (Clethrionomys gapperi) to support virology studies. Several efforts to breed these animals in captivity at other locations have been unsuccessful and there is no available commercial source. Six pairs have been obtained from a trapper in Canada and efforts have begun to create an environment in which they will reproduce here. The outcome is uncertain as very little is known about the husbandry or diseases of this animal and will be learned with time. Breeding to date appears to be going well, with 80 births and 66 animals weaned thus far. There are now second generation progeny in the colony, and 20 breeding pairs have been established. No animals have been issued from the colony, but preliminary investigations will begin soon to clarify the role of this animal species in the USAMRIID research program.

Primates. The rhesus breeding colony has continued to produce healthy offspring, provide valuable experience in the management and maintenance of a non-human primate breeding colony and allowed for the collection of growth, maturation and hematologic data on colony-born and -reared rhesus monkeys. The colony now has a growing number of sexually mature female offspring and a group of male offspring which are nearing sexual maturity. These offspring will be used at a later date to evaluate the potential of a second generation breeding colony. At present, the colony consists of 10 breeding adult females, 2 breeding adult males, 3 nursing infants, 31 weanlings, 7 female juveniles, 7 adolescent females (menustrating), and 9 male juveniles. During the past 12 months there were 6 live births and 6 successful weanings. One surviving neonate was delivered via cesarean section followed by the unfortunate demise of the mother 2 days after surgery. The cesarean section was indicated by acute profuse vaginal hemorrhage within days of the estimated date of delivery caused by a condition of placenta praevia. A case of psychogenic polydipsia was diagnosed in an infant monkey. Urinalysis and electrolyte disturbances were the basis for the diagnosis which was probably due to an incompatible pairing of weanlings. The monkey recovered following supportive care. The colony-reared females which have begun to cycle are within normal limits on the basis of physiological parameters. To date, we have not observed the abnormal behavior patterns, e.g., spinning, etc., that have consistently been reported in nonhuman primates individually housed at an early age. This may be attributable to our limited gang housing system for newly weaned monkeys which allows for additional socialization after separation from the mother. Observations on these animals will continue as they approach sexual maturity.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0G1537	81 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
80 10 02	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	62770A	3M162770A871	BC	148
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2	2		

11. TITLE (Precede with Security Classification Code)*

(U) Prevention of Viral Diseases of Potential 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
80 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 81	4.0	2585
	CURRENT 82	10	2813

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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) Viral diseases; (U) Vaccines; (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) Study pathogenesis of selected toga-, bunya- and arenaviruses unique in their capacity to cause widespread epidemics of human diseases. Research focuses on identification of the determinants of viral virulence and elucidation of mechanisms of natural and acquired resistance to viral disease. Understanding will allow development of attenuated and/or inactivated vaccines or therapeutic regimens for use in military personnel.

24. (U) Suitable animal models are developed, preferably using discrete genetic differences, to highlight key steps in pathogenesis. Viral replication, antigen expression, and immune responses are monitored during infection to allow inferences about critical determinants which may be tested by experimental manipulation.

25. (U) 80 10 - 81 09 - Efforts centered on lethal hemorrhagic fever viruses. Methods were developed to measure neutralization (N) of Lassa virus in vitro and validated in vivo, using antibody transfer; results explained failure of human serotherapy, allowed detection of unsuspected strain differences, and may provide a method to monitor efficacy of vaccines. Initial studies with Ebola virus demonstrated protection with an inactivated vaccine, in vitro N with serum, in vivo protection with serotherapy and biological differences between Sudan and Zaire strains. Epidemiological studies suggest that a Korean HF-like virus may be present in rats far removed from classical endemic habitats. Several sandfly fever viruses are under study; resistance to fatal infection lies in early nonimmunological events reflected in infected isolated macrophages. Prototype vaccines being examined a[t] Junin, Chikungunya, and Dengue-1.

Publications: Virology 106:250, 1980; Lancet 2:1215, 1980; J. Infect. Dis. 143:291, 1981; J. Clin. Microbiol. 13:791, 1981; Intect. Immun. 32:872, 1981; Contrib. Epidemiol. Stat. 3:21, 42, 124, 1981; chapter in Viral Zoonoses, vol. 1, p. 403, 1981.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M162770A871: Risk Assessment of Military Disease Hazards (U)

Work Unit No. 871 BC 148: Prevention of Viral Diseases of Potential BW Importance

Background:

Many of the viruses under study in this work unit share the combinations of aerosol infectivity and the ability to induce severe human disease. This, of course, enhances their BW potential, but simultaneously makes their study dependent on tedious work under rigid microbiological containment. Moreover, relatively little is known about most of these dangerous agents, necessitating careful model building and pathogenetic studies to plan strategies for prevention or treatment of their diseases. Other viruses under consideration are less virulent for humans and perhaps better understood. This is reflected in the increased weight given to the area of vaccine development with the less virulent viruses. This work unit will be discussed in categories by taxonomic status of the virus under study, since the emphasis and approach is quite variable among the groups.

Progress:

The major thrust has been in the Arenaviridae family. These viruses cause severe and even fatal disease, often associated with the hemorrhagic fever (HF) syndrome. Lassa fever, the most geographically widespread member of the group, has been shown to comprise a heterogeneous group of virus strains with varying antigenic properties and pathogenicity for laboratory animals. Indeed, if some strains of lymphocytic choriomeningitis (LCM) virus were isolated in Africa, they might well be classified as Lassa fever (LAS) virus by present criteria. The understanding of antigenic and virulence relationships will shape future concepts of the nature and control of LAS. Meanwhile, major progress has been made in understanding of passive serum therapy of LAS. An in vitro neutralization (N) test has been developed which can predict success in treatment or prophylaxis of experimental LAS. This test has several unusual properties compared to other common viral N tests: (a) stringent complement requirement, (b) activity lost rapidly with serum dilution, and (c) first appearance of positivity weeks after clinical disease. These findings combined with the very low N titers found in humans (compared to monkeys or guinea pigs) explain why serotherapy for LAS has not been particularly successful. The existence of a validated N test would allow the development and monitoring of a killed vaccine by conventional means; but, to date inactivated LAS virus preparations have failed to elicit either N antibody or protection.

The role of cellular factors in resistance to arenavirus disease has been attacked more directly in guinea pigs infected with Junin (JUN), (causative agent of Argentine HF, AHF). A cellular cytotoxicity assay has been developed for both JUN and LCM and shows specific cytotoxicity of spleen cells from virus-infected animals. This is the first antiviral cytotoxic system and one of the few of any type reported for the guinea pig. Killing is largely due to antibody-dependent cellular cytotoxicity for the following reasons: (a) it is not abrogated by a monoclonal anti-T cell antiserum, (b) aggregated human gamma globulin blocks, and (c) kinetics of activity resemble several other ADCC viral systems. Thus the guinea pig stands in marked contrast to the rat and mouse infected with LCM, but resembles hamster (LCM, vaccinia) and human (vaccinia, measles) systems. This assay should allow us to probe the role of cellular factors in pathogenesis in more detail.

Vaccine development efforts for AHF continue to be promising. The previously reported "XJ-44" mouse-passaged vaccine candidate had been shown to be free of adventitious agents and as attenuated as an experimental mouse brain vaccine (Clone 3) formerly used in humans in Argentina. Further attempts to clone the virus yielded strains (candidate 1) which are even more attenuated when inoculated intracranically (IC) in baby strain 13 guinea pigs and 9- to 14-day-old mice. This highly attenuated virus is, however, immunogenic for guinea pigs. Moreover, candidate 1 continues to be avirulent after in vitro passages. This virus will be used to prepare an experimental vaccine for nonhuman primate studies and eventually for immunization of humans.

Comparison of conventional histopathology of both human and animal arenavirus models reveals 3 common themes: (a) the IC inoculated rodent (e.g., LCM in the mouse) has abundant cellular infiltrates which probably are essential to trigger disease. This model may have little or no relevance to HF research; (b) in human or realistic animal models of HF there are relatively minor cellular infiltrates (suggesting that the role of cellular immunopathology may be less), and, most importantly, (c) there are no lesions which explain the demise of the host. Therefore, we have undertaken a physiological study of a model of this HF type, the Pichinde (PIC)-infected guinea pig. Results to date have indicated important cardiovascular perturbations as early as day 7-10. These results may lead us to understand the early lesion in arenavirus HF and suggest useful therapeutic alternatives.

Considerable interest in the Phlebovirus genus of the Bunyaviridae family has been stimulated by the discovery that Rift Valley fever virus (RVF) is a member of the genus and by the extensive RVF epidemic in Egypt between 1977-79. Ongoing studies have shown that in a rat model a single dominant gene determines resistance to fulminant RVF. This gene has now been shown to be strongly expressed in vitro in isolated peritoneal macrophages. Leading candidates for mechanisms (defective-interfering particles and interferon) are under study. A surprising feature of the rat model was its specificity for Egyptian RVF isolates. Viruses from different regions, although fully virulent in other respects, were not lethal for rat strains, which were exquisitely sensitive to Egyptian isolates. Quantitative studies of the ontogeny of resistance and virological observations on moribund animals, however, indicate that the gene controlling Egyptian virus replication is also operative to some extent on non-Egyptian viruses. Presumably there is another host-virus genetic interaction which limits the lethality of non-Egyptian viruses.

Similar principles to those elucidated in detail for RVF also apply to other Phlebovirus rodent systems: (a) different geographic isolates of what is apparently a single virus have a different spectrum of pathogenicity for a single defined host (Punta Toro, (PT), strains in hamsters); (b) early host-virus interactions determine the outcome of infection before classical immune mechanisms are operative (PT in hamsters); (c) immune mechanisms may be important in eventual recovery or in protection, but have surprisingly little to do with natural resistance (cyclophosphamide treated or nude athymic mice infected with severe Phleboviruses); (d) interferon inducers such as Poly IC are very effective prophylactically (PT-infected hamster).

An antigen-specific ELISA has been developed for RVF. It holds promise for rapid diagnosis, vaccine standardization, and studies of antigen metabolism in infected animals.

The newest putative member of the Bunyaviridae family, Korean hemorrhagic fever (KHF) virus has been the subject of intense interest. Major efforts have been devoted to obtaining and characterizing reagents free of adventitious agents. This has

allowed us to begin virus strain characterization. Methods for preparation of virus pools and antisera, radioimmunoassay, plaque assay, and plaque reduction N (PRN) test are being refined.

A Swedish guest investigator in our laboratory has confirmed that KHF-infected cell cultures can be used to detect antibodies to the related nephropathia epidemica (NE) agent and has begun a country-wide sero-survey of Sweden for infection. He has also identified the NE agent in the lungs of wild-caught voles in endemic areas of Sweden.

The Ebola-Marburg group of viruses continues to present a vexing problem. Effective treatment for these lethal agents has been as elusive as the natural reservoir. Ribavirin has now been thoroughly tested in the guinea pig model and is of no value. In vitro studies with interferon have not been hopeful, but the prophylactic value of this substance has been disassociated from its in vitro antiviral effects in other infections. Furthermore, there are monkey therapy studies in the literature which suggest some helpful effects of interferon. Serum interferon assays in lethally infected monkeys are negative until the late stages, suggesting that early passive interferon treatment could have some effect.

Passive antibody treatment (with human plasma) has not been effective in monkeys or guinea pigs in past experiments. However, hyperimmune guinea pig serum pool has been prepared and used to successfully protect lethally infected guinea pigs. Administration of approximately 6 ml/kg on days 0, 2, 4, 6, and 8, were necessary. The PRN titer of the serum was 1:320. Further work on the quantitative relations between antibody titer and serum volume may shed light on why these attempts succeeded and past efforts failed.

Further work characterizing the basic biology of Sudan and Zaire isolates and their relationships continue, as do attempts to standardize the N test and improve virus assays.

Two vaccines for Togaviridae are in late stages in development. An experimental lot of live attenuated dengue-1 vaccine has been prepared and partially tested. Pending final testing and subhuman primate data, this vaccine is ready for volunteer studies.

A live-attenuated Chikungunya vaccine under development has been shown to possess desirable experimental characteristics: small plaque size, temperature sensitivity, low viremia in nonhuman primates and avirulence for suckling mice. After additional cloning, it should be possible to prepare seeds and a prototype vaccine lot.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0B6410	81 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
80 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	62770A	3M162770A871	BD	147
b. CONTRIBUTING				
c. CONTRIBUTING	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:*
c. TYPE: d. AMOUNT:
e. KIND OF AWARD: NA f. CUM. AMT.

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 81	0.6	476
FISCAL YEAR CURRENT 82	4.0	716

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE

Foreign intelligence considered

ASSOCIATE INVESTIGATORS
NAME:
NAME: POC:DA

22. KEYWORDS (Procede 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. Procede 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 in 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 and medical ethics reviews.

25. (U) 80 10 - 81 09 - Preparations for testing Rift Valley fever vaccine (TSI-GSD-200) and determination of dose-response curves are underway. A protocol comparing the reactogenicity and immunogenicity of the Parke-Davis pentavalent botulinum toxoid and the Michigan Department of Public Health (MDPH) monovalent B toxoid was completed as was immunization of USAMRIID volunteers with MDPH type E monovalent toxoid. The program for the collection of botulism immune single donor plasma (human) IND-1332 was completed. Phase II trials using the antimalarial halofantrin (WR 171,669) continued. The Ward's high containment isolation suites underwent remodeling to upgrade them to P-4 isolation capabilities. They were used after a possible accidental exposure to Ebola virus. USAMRIID's Medical Isolation Team conducted 2 aeromedical evacuation exercises with the Air Force. In-house training exercises of the isolation facilities continued.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3M162770A871: Prevention of Military Disease Hazards (U)

Work Unit No. 871 BD 143: Evaluation of Experimental Vaccines in Man for BW Defense

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 involving human volunteers.

Progress:

Vaccines

Rift Valley Fever Vaccine (RVF). A protocol, (82-1) Addendum 79-5 to determine the reactogenicity and antigenicity of RVF vaccine (TSI-GSD-200), lots 9-20 in healthy military volunteers and to determine a dose-response curve of several of these lots and previously tested lots is being prepared and will be presented to the Professional Staff and Human Use Committees for review.

Botulinum Toxoids. Another important area with which USAMRIID has been concerned has been the production of Botulinum Immune Plasma, Human Origin. One problem has been relatively low concentration of antitoxin generated in human plasma relative to the equine product. This is particularly true in respect to the type B antitoxin response in humans.

It has been suggested in animal studies that the use of a monovalent toxoid would elicit a higher response than the same amount of antigen contained in a multivalent preparation. A modification of this hypothesis was examined in the USAMRIID plasmapheresis program for the collection of botulinum immune plasma of human origin. Under contract to the US Army Research and Development Command, the Michigan Department of Public Health, (MDPH) bottled one lot of the monovalent B toxoid produced at the same time as the B toxoid that was used in the pentavalent MDPH toxoids. A research protocol with fully informed consent was then conducted with volunteers in the USAMRIID botulism immune plasma program. These volunteers, who had been immunized and received 8-12 annual boosters of pentavalent botulinum toxoid prior to 1970, were given a booster immunization with the Parke-Davis pentavalent toxoid in 1979. Twelve months later (1980), these same individuals received another booster of the Parke-Davis pentavalent toxoid and, in the opposite arm, a 0.5 ml dose of the MDPH monovalent B toxoid. The volunteers uniformly reported much less immediate pain with MDPH monovalent B toxoid. Local reactions in the volunteers were no greater in frequency than in the pentavalent series. It is interesting to note that

the 3 individuals who did have moderate reactions had received the monovalent B toxoids. The 1980 B titer response, at 4 weeks following immunization was measured by the standard mouse neutralization test. The increase in B titer compared to that elicited from pentavalent toxoid alone (1979), was not statistically significant. Additional studies must be done in terms of numbers and timing of immunizations.

USAMRIID also obtained a MDPH type E monovalent toxoid produced at the same time as the MDPH monovalent B toxoid. Subsequent to approval for the appropriate protocols, 22 USAMRIID volunteers were immunized with the type E monovalent toxoid under protocol FY 81-2. No significant adverse reactions occurred. Determination of the immunogenicity of this toxoid has begun.

The USAMRIID Program for the collection of Botulism Immune Single Donor Plasma (Human) IND-1332 was completed during December of this year.

Chemotherapeutics

Malaria. Results of Phase II trials (Protocol FY 80-7) of the candidate antimalarial halofantrine, (WR 171,669) conducted to date are summarized in Table 1. Recrudescence of malaria occurred at single doses of 1000 and 1500 mg initially followed 6 hr later by 500 mg. Only 2 volunteers experienced side effects of drug administration consisting of mild nausea and diarrhea.

Department of Epidemiology

A Department of Epidemiology was created within the Division of Medicine during the last quarter of FY 81. Functions of this department may be grouped under three sections: research serology, test and evaluation of rapid diagnostic techniques, and epidemiology of selected infectious diseases. The research serology section is primarily a service facility which provides serological support for the USAMRIID immunization program. This function was formerly provided within the Bacteriology Division and has been laterally transferred to Medical Division. The section conducts titrations on sera from vaccine recipients using the hemagglutination-inhibition (HI), complement fixation (CF) and plaque-reduction neutralization (PRN) tests. A radioimmunoassay (RIA) is also being developed.

Antigens routinely tested include EEE, WEE, VEE and RVF viruses and tularemia. Many other antigens are tested on an as-needed basis. Two populations are serviced by this section, the in-house recipients of USAMRIID vaccines and extramural recipients of selected vaccines. Results provided by the research and serology section determine the efficacy of the USAMRIID immunization program and provide the serological basis for determination of immune status for personnel entering "hot suite" work areas.

Aside from the purely service-oriented tasks of providing immune status reports for vaccine recipients, the research serology section also conducts a number of research protocols directed at determining optimum immunization schedules, elucidating interactions between various antigens used to immunize USAMRIID personnel and other investigations which utilize serological techniques.

TABLE I - TREATMENT OF P. FALCIPARUM WITH WR 171,669

PATIENT NO.	DOSE	TOXICITY	At treatment	Highest	Parasite	Fever	Outcome
1	250 mg Q6H x 12	None	220	222	28	90	Cure
2	250 mg Q6Hx 12	None	1200	1200	70	108	Cure
3	250 mg Q6H x 12	None	1620	1620	71	81	Cure
4	250 mg Q6H x 8	Mild diarrhea	230	230	27	54	Cure
5	250 mg Q6H x 8	None	190	600	40	98	Cure
6	250 mg Q6H x 8	None	150	430	63	104	Cure
7	250 mg Q6H x 4	None	270	280	63	104	Cure
8	250 mg Q6H x 4	None	230	420	69	104	Cure
9	250 mg Q6H x 4	None	60	330	62	98	Cure
10	500 mg Q12H x 2	None	30	350	56	102	Cure
11	500 mg Q12H x 2	None	250	260	44	94	Cure
12	500 mg Q12H x 2	Mild, nausea diarrhea	880	2290	65	124	Cure
13	1000 mg x 1	None	280	1050	82	124	Cure
14	1000 mg x 1	None	100	100	21	63	Recrudescence
15	1000 mg x 1	None	130	130	36	87	Indeterminate[a]
16	1500 mg x 1	None	2740	2740	28	116	Cure
17	1500 mg x 1	None	1150	1150	45	94	Cure
18	1500 mg x 1	None	190	280	12	70	Recrudescence
19	1500 mg x 1	None	1150	1150	42	79	Recrudescence

20	1000 mg followed in 6 hr by 500 mg	None	2310	4200	46	84	Cure
21	1000 mg followed in 6 hr by 500 mg	None	2010	2010	38	84	Cure
22	1000 mg followed in 6 hr by 500 mg	None	1130	1130	70	104	Cure
23	1000 mg followed in 6 hr by 500 mg	None	4420	9490	75	28	Cure
24	1000 mg followed in 6 hr by 500 mg	None	3190	3520	70	104	Cure

[a]Patient treated prophylactically with Mefloquine HCl at time of recrudescence in patient 14. No evidence of recurrence at time of treatment.

The test and evaluation of rapid diagnostic techniques is a newly created section. This section was specifically created in response to the need for rapid diagnosis of infectious diseases and biological warfare agents, especially in support of the Rapid Deployment Forces geographically directed to Southwest Asia. Preliminary work is in progress to determine the utility of the FIAX^{R} fluorescence solid-phase immunoassay system. Agents to be considered first will be those endemic in Southwest Asia.

The Department of Epidemiology is newly created. The objective is to investigate the occurrence and distribution of selected infectious diseases of military importance on a global scale. At present, 2 projects receive the majority of efforts. The first is Korean hemorrhagic fever (KHF). Studies are currently in progress to elucidate the dissemination of KHF among personnel of major medical centers who have been exposed to infected laboratory animal colonies. A second study is to determine if KHF has been introduced into the United States through transport of infected wharf rats via international shipping. Both studies are in their preliminary phases and results are too fragmentary to present.

The second topic of investigation by the epidemiology section is the serologic testing of specimens obtained from US military personnel deployed on exercises overseas. This project is being conducted in collaboration with the Department of Epidemiology, Preventive Medicine Division, WRAIR. Here both pre- and post-deployment sera are drawn from individuals who participate in military exercises overseas. In addition, health survey questionaires are administered to each individual and an attempt is made to correlate clinical illness with serologic evidence of infection acquired while deployed. Specimens assayed to date have been from individuals deployed to Southwest Asia, northern and tropical Africa.

Isolation Unit Clinical Laboratory

The Isolation Unit Clinical Laboratory was utilized when Dr. Eugene Johnson was isolated in the Ward 200 Isolation Suite for observation after a possible accidental exposure to Ebola virus in Zaire. The Isolation Unit Clinical Laboratory performed numerous laboratory procedures in support of the isolation/observation of Dr. Johnson from the time of his admission, 24 Apr 1981, until his discharge. This exercise illustrated numerous problems with the P-4 operational readiness of the Isolation Unit Clinical Laboratory. Under the responsibility of Captain Lyerly, these problems are currently being addressed and appropriate measures, such as training, revision of Standard Operating Procedures, procurement of equipment, and personnel actions are being taken.

The role of the Isolation Unit Clinical Laboratory is currently being expanded to include not only responsibilities for P-4 level laboratory support for patients in the Isolation Suite, but also for support of animal research protocols throughout the Institute. Two protocols are currently being supported at the P-3 level utilizing veterinary specimens. The agents involved are hepatitis A and gamma-radiation-inactivated Lassa Virus. Coordination is currently ongoing to initiate more collaborative projects with appropriate investigators throughout the Institute.

Preliminary developmental work has also been initiated for the deployment of a mobile, high-containment clinical laboratory for field use in support of the Vickers Isolator Team.

Hospitalized Exposures

Ebola Virus. Dr. Eugene Johnson was placed in reverse isolation in the Medical Officer of the Day suite on Ward 200, USAMRIID, for protective observation after a possible accidental exposure to Ebola virus while in Zaire. He remained totally asymptomatic, and received no therapy. He tolerated his hospitalization well.

USAMRIID Medical Isolation Team

The USAMRIID Medical Isolation Team conducted 10 monthly in-house training exercises utilizing Vickers portable and bed isolation units. In addition, 2 aeromedical evacuation exercises were conducted, in conjunction with the Air Force, at Lages Air Field in the Azores, 17-19 Apr 1981, and at McDill AFB, Florida, 20-21 Jun 1981. These training missions utilized the Vickers portable isolation system with a pseudo-patient on C-141 and C-130 aircraft, respectively. The following equipment/procedures were evaluated: (a) loading and securing of isolators in the AMBUS and aircraft; (b) in-field decontamination procedures for isolators and personnel; (c) Air Force oxygen delivery and suction systems in conjunction with the aircraft transport isolator; and (d) in-flight capabilities of the Life-Pak 5 cardiac monitor and battery operated rectal probe thermometer.

The Ward 200 high containment isolation suite underwent remodeling. The following additions/revisions were made to upgrade the suite to P-4 isolation capabilities: (a) Chemturion ventilated isolation spacesuits; (b) a Lysol chemical decontamination shower for use with the isolation spacesuits; (c) 2 air-lock piggy-back steam autoclaves; (d) windows and speaking diaphragms between each patient room and adjoining rooms outside the isolation suite; and (e) pass-through Lysol "dunk-tanks" and UV boxes in each patient room to allow passing items to, or from, patient rooms and adjoining rooms outside the isolation suite.

Publications

None.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0G3815	81 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
80 10 02	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	62770A	3M162770A871	BE	146
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2;2			

11. TITLE (Procede with Security Classification Code)*

(U) Exploratory Antiviral Drug Development

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

003500 Clinical medicine; 004900 Defense; 010100 Microbiology; 012600 Pharmacology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
80 10	CONT	DA	C. In house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 81	4.5	735
FISCAL YEAR CURRENT 82	5.0	716

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

21. GENERAL USE

Foreign intelligence considered

ASSOCIATE INVESTIGATORS

NAME: Huggins, J POC:DA

NAME: Kastello, M.D.

22. KEYWORDS (Procede EACH with Security Classification Code) (U) Military medicine: (U) BW defense; (U) Antiviral drugs; (U) Pharmacology; (U) Viral diseases; (U) Laboratory animals

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

23. (U) Identify effective drugs against viruses that are potential threats to military personnel; obtain data on toxicology, pharmacology and metabolism of antiviral drugs and conduct preclinical and clinical studies to assess safety and efficacy in compliance with FDA regulations; conduct laboratory studies to develop novel antiviral drugs by identifying potential targets for pharmacologic intervention.

24. (U) Assess efficacy of potential antivirals against viruses in tissue cultures and in rodent models for Rift Valley fever (RVF) and VEE. Evaluate toxicity and pharmacology of promising compounds in preclinical protocols conducted in rodents and non-human primates. Describe mechanisms of action and metabolism of new drugs and provide analytical support for drug analysis. Apply approaches of molecular virology and cell biology to define the molecular basis of virus-cell interactions.

25. (U) 80 10 - 81 09 - Screened 79 novel nucleoside drug analogues against 5 viruses in tissue culture; 38 were tested in rodents with RVF; 24 drugs had antiviral activity. Preclinical toxicological studies included evaluation of the effects of ribavirin on hematopoiesis, RBC survival and fragility. The drug decreased both RBC half-life and the rate of maturation in bone marrow; effects were fully reversible. Biochemical characterization of a viral receptor on a macrophage cell line was completed; the receptor was found to have pH optimum for binding of 7.4-7.8, binding constant of 2.0 billion, and 40,000 copies/cell. Binding of virus to the cell is eliminated by proteolysis or plant lectins. The receptor appears to be a glycoprotein.

Publications: Fed Proc. 40:634, 1981; 2 chapters In "Infection," 1981.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3M162770A871: Military Disease, Injury and Health Hazards (U)

Work Unit No. 871 BE 146: Exploratory Antiviral Drug Development

A. Background:

Viral infections are a significant military health problem in the United States and throughout the world. To date, vaccination has provided the only approach for defense against viral illnesses. However, the prophylactic use of vaccines as the only source of defense present serious limitations. Vaccines are virus-specific and not available for protection against most viral infections of military importance. They are ineffective if given after onset of infection and can be easily circumvented through naturally occurring antigenic shifts or deliberately through application of contemporary genetic engineering technology. Chemotherapeutic agents, on the other hand, circumvent many of these limitations; hence, they are critically needed for the prevention and treatment of virus induced diseases (1,2,3).

Use of antiviral drugs in military personnel requires a comprehensive understanding of their pharmacology to ensure selection of the correct drug which combines low toxicity and high efficacy for treating specific viral infections. Civilian pharmaceutical companies, in general, have been unwilling to invest in development of drugs which do not have a large market. Antiviral compounds of military importance have a limited market, have not attracted the interest of the pharmaceutical companies and, therefore, have been included in a group of drugs referred to as "orphan drugs." The purpose of this Work Unit is to develop and evaluate new antiviral drugs for prophylaxis and treatment of viral infections caused by potential BW agents or by strategically important viruses.

The broad spectrum antiviral drug ribavirin (Virazole, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been shown to be effective against Lassa fever (LAS) and Rift Valley fever (RVF) in laboratory animals. One objective of this research has been to determine the safety of this drug for use in high hazard viral infections in man.

Drug screens. Seventy-nine analogs of ribavirin and other antiviral drugs were screened and evaluated in vitro against RVF, VEE, PIC, SFS and YF viruses (Table I). In vivo studies were done in mice against RVF and VEE viruses with approximately one-half of the drugs. Twenty-four of the compounds were found to be inactive against the test viruses. Drug BJ-84143 (RA-71) and BJ-84125 (RV-13) were more effective in vitro than ribavirin. Pyrazofurin was effective against all of the viruses at concentrations ranging from 2.5 to 25 μg/ml.

Expanded in vivo tests (drug treatment b.i.d., for 10 days) were performed with 4 analogs of ribavirin; BJ-4511 (RA-114), BJ-45526 (RA-116), BJ-29893 (RA-98), and BJ-58826 (RV-33), which in earlier screening tests, had been shown to have comparable antiviral activity to ribavirin. Results indicated they were as effective as ribavirin at the 100 mg/kg dose level, but less so at the 25 mg/kg dose level. Of the 4 compounds BJ-45526 was nearest to ribavirin in activity, against RVF virus. An additional study was performed with this drug and ribavirin to compare anti-RVF virus activity with drug treatment doses of 20, 40 and 80 mg/kg b.i.d. over a 10-day period. Both of the drugs extended the time to death at all 3 dose levels. Ribavirin appeared to be most effective at the 40 mg/kg level, whereas, BJ-45826 was somewhat better at the 80 mg/kg level. Hematocrit levels were higher with BJ-45526, than they were with ribavirin at the 80 mg/kg dose level.

TABLE I. IN VITRO EVALUATION OF RIBAVIRIN ANALOGS AND OTHER POTENTIAL ANTIVIRAL DRUGS AGAINST VARIOUS VIRUSES.

COMPOUND	CONTRACT NUMBER	RESPONSE OF VIRUS[a]				
		PIC	RVF	SFS	YF	VEE
BJ58518**	RA-136	-	-	-	-	-
BJ58527**	RV-22	-	-	-	±	
BJ58536**	RV-33	++	+	+	+	-
BJ58554**	RP-67(III)	-	-[c]	+	-	-[b]
BJ58545**	RV-39	-	-	-	-	-
SV3937** CN-48,085-2, Lot P		++[c]	++[c]	++[c]	±[c]	++[c]
SV-6736** CN-56,506, Lot P & Lot Q		-	-	-	-	-
SV-8611** CN-65,624, Lot Q & Lot T		-	+	-	-	-
SV-11,736 CN-72,917, Lot P		+++[c]	++[c]	++	+++[c]	+
Sodium petroselinate*		++[c]	++[c]	-	++	-[c]
BJ63859**	RA-116 R	++	+	++	++	-
BJ63868**	RA-148	-	-	+	+	-
BJ64877**	RA-150	-	-	-	++[c]	-
BJ63886**	RA-73	-	-	-	++[c]	-
BJ63895**	RP-75	-[c]	-	-	-	-
BJ63902	RA-137	-	-	++	+[c]	-
BJ63911	RV-24	++	+	+++	+++	-
BJ63920**	RV-57	+[c]	++[b]	+[c]	+++	±
BJ-63939	RI-91	+	++	++	+[c]	-
BJ-63948**	RI-36	+	+	-	-	-
BJ-63957**	RI-33	-	+	-	-	-
BJ-64966**	RV-66	-[b]	+[b]	-[b]	++[b]	++
BJ-36496**	MPL-XI-230	++	++	-	-	+
BJ-76212**	RI-20	±	-[b]	-	-	-
BJ-76267**	RP-77	-	+	+	±	-
BJ-81553**	RP-91	+	++	-	-	+
BJ-81544**	RV-116	+	++	+++	+++	+
BJ-36450**	DSC-III-237	-	-	-	-	-
BJ-76196**	RA-151	-	-[b]	+[b]	+[b]	-[b]
BJ-76203**	RA-152	-[c]	++[b]	+[c]	+	-
BJ-81535**	RA-159	-	±	-	-	±
BJ-81517**	RA-135	-	-	-	-	-
BJ-2241*	DSC-III-170	++[c]	++[c]	+++[c]	++[c]	++[c]
BJ-44532*	MPL-XI-240	-[c]	-[c]	-[c]	-[c]	-[c]
BJ-57995*	(Dow) Niclosamide	-	-	-	-	-
BJ-76187*	RA-149	+	+	++	+++[c]	-
BJ-76221*	RI-23	+	-	++	++	-
BJ-76230*	RI-61	+[c]	-[b]	-[c]	-[c]	-[c]
BJ-76249	RI-63	+	-	-	-	-
BJ-76258	RI-66	-	-	++	-	-
BJ-76276*	RP-80	+[c]	++[c]	-[c]	++[c]	±[c]

COMPOUND	CONTRACT NUMBER	RESPONSE OF VIRUS[a] PIC	RVF	SFS	YF	VEE
BJ-76301*	RP-86	++	+	-	+	
BJ-76294*	RP-83	+	++	+	+[c]	-
BJ-81526*	RA-155	+	+	+++	++	-
BJ-85060	EDC-1	-	-	-	-	-
Pyrazofurin (Lilly)[d] Lot H26-58G-AD-82		++	+++	++++	++	+++
Clofibrate (Ayerst) NDC-046-0243-81 Control No. INPR		+[b]	+	-	+[b]	++
Nafenopin (Ciba) 63-394		++[b]	+[c]	++[c]	++[c]	++
BJ-86326*	RY-29	+++	+	-	-	-
BJ-86334*	RY-43	++	-	±	-	-
BJ-86281*	RA-138	+++	+	+	+	-
BJ-86290*	RA-180	++	-	-	±	-
BJ-86308*	RA-181	-	-	+[c]	-[c]	-
BJ-86316*	RY-6	-	-	-	+	-
BJ-82701*	NCI/ING	++[c]	++[c]	±[c]	++[c]	-[c]
BJ-84205*	RA-36	-	-	-	-	-
BJ-84198*	RA-33	+	+	+[c]	±	-
BJ-841345*	RA-66	++	++	+	-	-
BJ-84143*	RA-71	+++	+++	+++[c]	++++	+
BJ-84152*	RA-72	-	-	-	-	-
BJ-84161*	RA-174	+[c]	+[c]	-[c]	+[b]	-[b]
BJ-84170*	RA-175	-	-	-	±	-
BJ-84189*	RA-176	-	-	-	+	-
BJ-84116*	RV-126	-	+	-	+	-
BJ-84125*	RV-133	++	+++[c]	+[c]	++++[c]	++[c]
BJ-84090*	RY-2	-	-	-	-	-
BJ-84107*	RY-22	+[c]	-[c]	-[c]	+[c]	++[c]
Lycoroysone* (Glycyrrhetic acid) Lot 22925		++[c]	++[c]	++[c]	++[c]	±[c]
BJ-91139*	RY-59	-	-	-	+++	-
BJ-91102*	RV-176	-	-	-	-	-
BJ-91111*	RV-177	-	-	-	-	-
BJ-91120*	RV-178	-	±	-	+	-
BJ-91040*	RA-187	-	+	-	-	-
BJ-91059*	RA-189	-[c]	++[c]	-[c]	++[c]	-
BJ-91068*	RA-190	-	-	-	-	-
BJ-91095*	RV-171	±	-	+	-	-
BJ-91077*	RA-191	-	-	-	-	-
BJ-91086	RA-192	-	-	-	-	-
RKR	-	-	-	-	++++	-
BJ-92449	RA-194	-	-	-	±	-
BJ-92458	RA-196	-	-	-	-	-
BJ-92467	RA-197	-	-	-	-	-
BJ-92476	RA-198	-	-	-	±	-
BJ-92485	RA-199	-	-	-	-	-

COMPOUND	CONTRACT NUMBER	RESPONSE OF VIRUS[a]				
		PIC	RVF	SFS	YF	VEE
BJ-92494	RA-200	-	-	-	-	-
BJ-92501	RA-201	-	-	-	-	-
BJ-92510	RA-202	-	±	-	-	-
BJ-92529	RY-84	-	-	-	-	-
BJ-92538	RY-92B	-	-	+	-	-
BJ-92547	RV-189	-	-	-	-	-
BJ-92556	RV-190	-	-	-	-	-
Ribavirin**		+++	++	+++	+++	±

* Indicates compounds also tested in vivo (RVFV).
** Indicates compounds also tested in vivo (RVFV and VEE).
a Graded as follows:
± Questionable activity;
\+ 10-30% plaque reduction, high drug concentrations (250-500 µg/ml);
++ 31-60% plaque reduction, middle drug concentrations (100-250 µg/ml);
+++ 61-90% plaque reduction, middle drug concentrations (256-100 µg/ml);
++++ 90% plaque reduction, low drug concentrations (10-25 µg/ml)
b Drug toxic at 500 µg/ml
c Drug toxic at 250 µg/ml
d Graded as follows:
\+ 10-30% plaque reduction (25 µg/ml)
++ 31-60% plaque reduction (10 µg/ml)
+++ 61-80% plaque reduction (5 µg/ml)
++++ 81-99% plaque reduction (2.5 µg/ml)

The in vitro results of combinations of amantadine and rimantadine with ribavirin show an enhanced effect against RVF, VEE and SFS viruses, above that of ribavirin against RVF and VEE viruses. Drug concentrations of 25, 50, 100 and 200 mg/kg did not enhance the effect of ribavirin. Rimantadine and amantadine as single drugs or in combination with ribavirin were toxic at the 200 mg/kg level.

Punto Toro virus, a bunyavirus related to RVF virus, was tested in hamsters to determine the antiviral activity of ribavirin, BJ-29893 and BJ-45520 against this virus. The results of this study showed that 90% of the hamsters survived when treated with ribavirin 25 and 100 mg/kg (-1 through +3) and 50% of the animals treated with 12.5 mg/kg of ribavirin survived. Those animals which did die in all drug-treated groups had an extended time to death of several days over those in the virus control groups. Similar results were obtained wtih animals treated with BJ-45520. BJ-29893 treated hamsters showed 30% survival at 25 mg/kg and 90% survival at 100 mg/kg.

Pre-clinical toxicology. Previous studies have shown that rhesus monkeys and man develop anemia and thrombocytosis during multiple, high-dose treatments with ribavirin. The mechanism by which the anemia occurs is unknown and may involve destruction of peripheral RBC, suppression of hematopoiesis or both. During this period we studied the effect of ribavirin on hematopoiesis. This study was conducted in compliance with The Good Laboratory Practices (Regulation GLP).

Two groups of rhesus monkeys were injected IM with ribavirin for 10 days at a dose of 30 or 100 mg/kg; monkeys served as their own controls and treatment began on day 0. Analysis of hematologic data showed that both groups developed a normochromic, normocytic anemia that was mild in the low-dose group and severe in the high-dose group. RBC count, hematocrit, and hemoglobin decreased significantly ($P < 0.001$) by day 15 and then returned to control levels by day 42 (Table II).

Examination of bone marrow aspirates revealed significant changes in both groups. Monkeys treated with 100 mg/kg shows significant ($P < 0.05$) hyperplasia on day 22. Monkeys treated with 30 mg/kg did not show erythroid hypoplasia but exhibited significant (P 0.05) hyperplasia on day 22. Table III lists percent erythroid precursors. Myeloid precursors were affected by ribavirin treatment. Myeloid erythroid ratios (Table III) increased significantly ($P < 0.05$) on day 10, reflecting the erythroid hypoplasia and returned to control levels by day 22. Differential counts of erythroid precursors revealed the erythroid hypoplasia was due to a significant (P 0.05) decrease in late erythroid forms in particular the polychromatophilic normoblast; early erthroid forms were unchanged or increased. Table III lists the ratio of late to early erythroid precursors. Megakaryocytes were significantly ($P < 0.05$) increased in both groups on day 10.

In addition to quantitative changes, bone marrow examination revealed qualitative changes in both the low- and high-dose groups of animals. There was vacuolization of early red cell precursors, including pronormoblasts and basophilic normoblasts and occasional cells had a megaloblastoid appearance. Other qualitative observations seen on day 10 included an apparent increase in the number of bone marrow histiocytes and phagocytosis of red cells and red cell precursors. Apart from an occasional vacuolated white cell precursor, there were no noteworthy changes in these cells. Occasional megakaryocytes also contained vacuoles.

To help define the mechanism by which ribavirin induces anemia, RBC survival was studied in groups of treated rhesus monkeys. Blood was taken from each of 9 monkeys and RBC labeled in vitro by incubation with 500 µCi of ^{3}H diisopropylfluorophosate (DFP). After washing, the cells were reinjected IV into appropriate donor monkeys. Monkeys were divided into 3 groups and injected IM with either 15 or 60 mg/kg ribavirin or saline for 10 days. Treatment began on day 0.

A dose-related anemia developed by the end of the treatment period. Hematocrit, RBC count and hemoglobin decreased 12-26% in the low dose group and 53-60% in the high dose group. All values returned to control levels by day 42. A dose-related decrease in RBC survival was observed from day 0 to 28; thereafter, RBC survival was comparable to control values. At 60 mg/kg, ribavirin also inhibited release of RBC from the bone marrow. In summary, ribavirin apparently induces anemia by decreasing RBC survival and by inhibiting release of RBC from the bone marrow. These effects are dose-dependent and are fully reversible when treatment is withdrawn.

Studies were conducted under contract with Hazleton Laboratories, Vienna, VA, to assess acute toxicity of ribavirin in mice, rats and guinea pigs and subacute toxicity in rats and dogs. In the acute toxicity studies, animals were treated with a single dose of ribavirin administered orally or IP and observed for pharmacotoxic signs and death. LD_{50} was calculated and results are shown in Table IV. These studies were conducted in compliance with GLP and the results are comparable to previous work which was similarly monitored.

TABLE II. MEAN HEMATOLOGY VALUES (± SD AND SE) OF MONKEYS GIVEN RIBAVIRIN (N=4 EXCEPT DAYS 42 AND 65, N=3).

GROUP AND DOSE AND LEVEL		BASELINE DAY -7,0	DAY 10	DAY 15	DAY 22	DAY 42	DAY 65
RBC X 10^{-6}							
Group I	Mean	5.35	0.62**	1.96***	2.29**	4.76	5.22
100 mg/kg/day	SD	0.093	0.405	0.509	1.346	0.320	0.092
	SE	0.047	0.302	0.255	0.673	0.185	0.053
30 mg/kg/day	Mean	6.01	4.46**	3.96**	4.80**	5.80	5.83
	SD	0.506	0.759	0.710	0.482	0.303	0.357
	SE	0.253	0.379	0.355	0.241	0.151	0.179
HCT (%)							
Group I	Mean	41.5	21.5***	15.5***	21.8***	41.0	41.7
100 mg/kg/day	SD	1.00	3.42	4.43	12.20	3.00	1.53
	SE	.50	1.71	2.22	6.10	1.73	0.88
Group II	Mean	45.0	34.8***	32.3***	39.3*	46.3	45.5
30 mg/kg/day	SD	2.00	4.42	5.32	4.65	2.22	3.70
	SE	1.00	2.21	2.66	2.32	1.11	1.85
HGB (gm/dl)							
Group I	Mean	13.50	6.78***	5.20***	6.25***	12.40	12.97
100 mg/kg/day	SD	0.383	1.091	1.283	3.733	0.794	0.635
	SE	0.191	0.545	0.642	1.867	0.458	0.367
Group II	Mean	14.80	10.83***	9.85***	12.20**	14.20	14.00
30 mg/kg/day	SD	1.169	1.723	1.526	1.236	0.622	1.046
	SE	0.585	0.862	0.763	0.618	0.311	0.523
RETICULOCYTES/1000 RBC							
Group I	Mean	5.00	0.75	30.25	68.00***	8.67	4.67
100 mg/kg/day	SD	1.15	0.96	34.51	16.87	6.51	2.52
	SE	0.58	0.48	17.25	8.44	3.76	1.45
Group II	Mean	8.75	7.00	34.25*	38.50*	10.00	5.75
	SD	2.75	4.97	18.87	15.52	3.16	1.50
	SE	1.38	2.48	9.44	7.76	1.58	0.78
PLATELETS x 10^{-3}							
Group I	Mean	204.7	479.8	663.6**	727.7**	214.3	265.3
	SD	62.7	154.3	102.9	404.5	41.9	51.8
	SE	31.3	77.1	51.5	202.2	24.2	29.9
Group II	Mean	280.0	341.3	519.7*	332.4	261.3	256.9
30 mg/kg/day	SD	54.6	131.5	260.5	53.4	22.1	93.6
	SE	27.3	65.7	130.3	26.7	11.0	46.8

*P < 0.05
**P < 0.01
***P < 0.001

TABLE III. BONE MARROW MEAN VALUES OF MONKEYS GIVEN RIBAVIRIN (N = 4).

GROUP AND DOSE LEVEL		BASELINE DAY -7,0	DAY 10	DAY 22	DAY 42	DAY 65
% RBC PRECURSORS						
Group I	Mean	33.08	13.83*	52.83*	49.50	30.53
	SD	4.935	8.243	18.304	13.284	6.948
	SE	2.468	4.121	9.152	6.642	3.474
Group II	Mean	43.50	34.20	61.65*	39.08	40.30
30 mg/kg/day	SD	6.894	4.802	7.910	9.590	13.532
	SE	3.447	2.401	3.955	4.795	6.766
MYELOID/ERYTHROID RATIO						
Group I	Mean	1.52	7.43*	0.75	0.92	1.74
100 mg/kg/day	SD	0.31	7.09	0.49	0.58	0.32
	SE	0.16	3.55	0.24	0.29	0.16
Group II	Mean	0.88	1.32	0.48	1.41	1.20
30 mg/kg/day	SD	0.20	0.16	0.19	0.61	0.58
	SE	0.10	0.08	0.09	0.30	0.29
RATIO OF LATE/EARLY ERYTHROID PRECURSORS						
Group I	Mean	6.025	2.775	4.425	10.450*	8.650
100 mg/kg/day	SD	2.141	1.391	2.559	4.106	1.401
	SE	1.070	0.696	1.280	2.053	0.701
GroupII	Mean	9.650	3.757**	11.175	8.300	8.875
30 mg/kg/day	SD	3.387	1.599	3.580	0.416	1.886
	SE	1.693	0.799	1.740	0.208	0.943

*P < 0.05
**P < 0.01

TABLE IV. ACUTE LD_{50} OF RIBAVIRIN IN RATS, MICE AND GUINEA PIGS.

ANIMAL	SEX	MEAN ± RIBAVIRIN LD_{50} (mg/kg) Oral	IP
Rat	Male	4116 ± 749	1758 ± 99
	Female	5827 ± 391	1554 ± 99
	Combined	5006 ± 463	1655 ± 72
Mouse	Male	10,000	1268 ± 120
Guinea Pig	Male	2313 ± 278	823 ± 60

Ribavirin was evaluated for subacute toxicity when administered orally for 28 days at levels of 30,60 and 120 mg/kg to rats (10/sex/group). An additional group served as controls and received only the vehicle in sterile water for injection. No rats became clinically ill or died as a result of ribavirin treatment. In the low dose group, findings were limited to indications of a decrease in the circulating RBC mass. At the mid-and high-dose levels, suppressive effects on body weight and/or food consumption occurred along with decreases in the circulating RBC mass. It should be noted that although the decrease in circulating RBC mass was statistically different from controls, the values remained within normal limits. In addition at the high-dose level, lymphoid depletion was seen in the thymus.

Ribavirin was evaluated for subacute toxicity when adminstered orally for 28 days at levels of 15, 30 and 60 mg/kg to dogs (3/sex/group). An additional group served as controls and received only the vehicle in sterile water for injection. All treated groups exhibited clinical signs of toxicity increasing in severity with dose. Mid- and high-dose groups showed anorexia, emesis, diarrhea and weight loss. The low dose group exhibited only diarrhea. In addition, the high-dose group had dehydration and 5 of 6 dogs died by the end of the treatment period. Circulating RBC cell mass decreased to the low-normal range in the mid-dose group. No alterations were observed in clinical chemistry values. Compound-related histopathological changes were observed at all dose levels, increasing in severity with dose. The most common finding was enteritis in all treated dogs. Lymphoid depletion occurred in the thymus of mid- and high-dose groups, as did hypoplasia of bone marrow. The dog appears to be the species most susceptible to the toxic effects of ribavirin. Ribavirin was also tested in the Salmonella typhimurium/mammalian microsome mutagenicity test (Ames Test) and found to be nonmutagenic.

Pharmacology. Ribavirin has been shown to concentrate in RBC (Table V). The uptake of the drug varies quantitatively between species, monkey RBC being more effective at concentrating ribavirin than human or rat cells. The sequestration of ribavirin is apparently due to phosphorylation of the drug as it enters the cells. The slow rate of release of ribavirin from within RBC may be due to the absence of phosphatases required to dephosphorylate the metabolic intermediates.

To infect a cell, a virus must transfer its genome from the extracellular space to the cytosol. This process requires transport through one or more membrane barriers (4). Mechanism for entry of virus into a cell, termed receptor-mediated endocytosis, involves the binding to specific receptors on the cell surface followed by internalization of the virus-receptor complex via coated pits (5). Investigation of the basic regulatory mechainsms of virus-specific receptors and coated vesicle functions may reveal a primary site susceptible to modification or therapy. Accordingly, studies on the mechanism of binding of VEE (TC-83) to its cell surface receptor and its subsequent internalization, as a first step in understanding the mechanism of entry into a cell were initiated. The first objective was to establish the presence of a receptor and to characterize it biochemically.

A specific receptor for VEE was identified on BW-JM cells, a macrophage-like continuous cell line. The receptor has pH optimum of 7.4-7.8; binding is complete within 1 hr at 0 C, approaches saturation, and the binding of radio-labeled virus can be competed for on a one-to-one basis by unlabeled virus. The equilibrium binding constant is $2.0 \pm 0.7 \times 10^{12}$ (M^{-1}) and the number of receptor binding sites if found to be $4.0 \pm 0.8 \times 10^{5}$ sites per cell. Non-specific binding is estimated at 22 ± 11 % using the program LIGAND, a computerized approach for characterization of Ligand-Binding Systems. The data was best fit to a single class of receptors.

TABLE V. THE INTERNAL CONENTRATION OF RIBAVIRIN AFTER 150 MIN INCUBATION AT 37C AND THE PERCENT CHANGE IN THE INTERNAL CONCENTRATION OF DRUG AFTER WASHING.

	EXTRACELLULAR CONCENTRATION		
	0.04 mM		
	Uptake (μM)	Post Wash (μM)[a]	% Change
Rat	44.95	12.40	72.4
Human	79.32	55.73	29.7
Monkey	141.76	124.45	12.2
	0.4 mM		
Rat	416.75	82.52[b]	80.2
Human	502.14	225.44	55.1
Monkey	939.46	722.14	23.1
	2.0 mM		
Rat	1654.91	417.84[b]	74.8
	4.0 mM		
Human	3811.38	766.95	79.7
Monkey	4717.66	1966.35	58.3

[a] Post Wash values are mean values calculated from the internal concentrations of ribavirin observed during the release period.
[b] Since the internal concentration of ribavirin decreased throughout the release period, the last value observed was used.

Studies to determine the biochemical nature of the cell receptor show binding can be eliminated by gentle proteolysis resulting in loss of only a limited number of surface proteins and will regenerate in 12-18 hrs. Pretreatment of BW-J-M cells with some lectins but not others will block binding. Binding is reduced in a dose-dependent manner by pretreating cells with soy bean agglutinin or wheat germ agglutinin to less than 10 % of the original value.

Studies of virus binding to cells whose virus receptors have been enzymatically digested revealed that virus failed to associate with the cell surface in the absence of the putative virus receptor. Another study revealed that virus disappears from the cell surface in the absence of the putative virus receptor. Another study revealed that virus disappears from the cell surface and is presumably internalized within 30 minutes. Further studies are necessary to trace the pathway of the virus within the cell.

Another aspect of this work unit concerns the basic mechanisms that regulate the coated vesicle pathways within cells. Calmodulin, a ubiquitous intracellular calcium regulatory protein, has been implicated in the function of coated vesicles. Calmodulin has been isolated from cow brain and purified to homogeneity. A bioassay for the calmodulin regulated Ca^{++}-Mg^{++} ATPase of erythrocytes, was established and used to assess the biological activity of the purified calmodulin. Once it was established that the calmodulin was pure and biologically active, the protein was iodinated for use in studies of the interactions of calmodulin with purified coated vesicles. Further studies have been directed towards defining which of the constituent proteins of coated vesicles is the site of this interaction. The coated vesicle proteins can be fractionated into two populations by column chromatography in the presence of 2M urea. One population contains the principle coated vesicle protein, clathrin (m. wt. 180 K daltons) and two proteins with m. wts. of 30-35 K daltons. The second population contains variable amounts of proteins in the molecular weight ranges of 50 and 100 K daltons. It is thought that these proteins participate in the interaction of the coat structure with intracellular membranes which is necessary to form coated pits and coated vesicles. It was found that radiolabeled calmodulin will interact with coated vesicles that have been disrupted using urea. Fractionation of the disrupted coated vesicle-calmodulin mixture on Sepharose CL4B revealed that calmodulin preferentially interacts with the population of coated vesicle proteins that includes the 50 and 100 K dalton peptides. This obsevation is truly significant in that it suggests that calmodulin and calcium regulate the formation of the coated pits which become the vehicles for intracellular transport of various macromolecules and viruses. Further identification of the calmodulin binding site of coated vesicles is being attempted using a derivative of calmodulin which can be photoactivated to form a covalent bond to its binding protein. This "photoaffinity" derivative is being prepared currently and should yield an unambiguous identification of the site of calmodulin interaction with coated vesicles.

The research described here should enable us to better understand (a) the mechanisms by which viruses infect cells and (b) the mechanisms by which coated pits and coated vesicles transport viruses, growth factors and certain toxic macromolecules.

Presentation:

Kastello, M. D., G. L. Wannarka, and P. G. Canonico. Distribution and excretion of ^{3}H ribavirin in rhesus monkeys. Presented, Annu. Mtg., FASEB, 12-17 Apr 1981 (Fed. Proc. 40:634, 1981).

Publications:

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5. Goldstein, J. L., R. G. W. Anderson, and M. S. Brown. 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature 279:679-685.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G1519	81 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
80 10 02	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	3M161102BS10	AN	200
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Characterization of Microbial Toxins of Potential BW Importance

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
80 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 81	4.1	471
FISCAL YEAR CURRENT 82	4.0	385

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) Microbial toxins; (U) Biochemistry; (U) Therapy; (U) Prophylaxis; (U) Toxoids; (U) Lab. animals

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. Toxins under investigation are staphylococcal enterotoxin C, botulinum toxin, Pseudomonas aeruginosa exotoxin A and diphtheria toxin.

24. (U) Find and characterize cell line(s) or tissue(s) susceptible to the toxin of interest; study 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) 80 10 - 81 09 - The amino acid sequence of staphylococcal enterotoxin C was completed. Several analogues of diphtheria toxin were tested for toxicity, ADP-ribosylation capability and NAD hydrolysis activity. Toxin analogues deficient in each of these activities were found. It was found that reticulocytes were sensitive to diphtheria, but differentially so to the nicked and unnicked forms. Several amines were tested for ability to protect from diphtheria toxin and those which did uniformly altered lysosomal pH, suggesting some involvement by this organelle in the toxicity process. Hybridoma cell lines (uncloned) which appear to make antibodies to P. aeruginosa and botulinal toxins were isolated. 2 nerve cell culture lines suitable for study of botulinum toxin's mechanism of action were acquired and adapted to our needs. Publications: J. Biol. Chem. 256:7898, 1981; 2 chapters in Receptor-Mediated Binding and Internalization of Toxins and Hormones; Exp. Cell Res. 137: in press, 1981.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)

Work Unit No. S10 AN 200: Characterization of Microbial Toxins of Potential BW Importance

Background:

Toxins can play an important role in the pathogenesis of many bacterial infections. Cholera, diphtheria and botulism are examples of bacterial diseases in which the bacterial toxin is solely responsible for the clinical synptoms. There are other examples such as Pseudomonas, Escherichia coli and Bacillus anthracis where toxins are apparently involved, but their degree of importance is not firmly established. Clearly, successful treatment and/or prevention of the disease must deal somehow with the toxins that have an important pathogenic role.

With certain bacterial toxins, there is also a concern with their use as BW agents. Botulinum toxin, for example, is one of the most poisonous substances known to man. Moreover, it is a relatively easy task to prepare large quantities of the toxin making its potential use a real threat. Likewise, the staphylococcal enterotoxins are considered as potential BW agents and could be obtained without too much effort or technology.

The principal function of this work unit is to study and characterize bacterial toxins with an ultimate aim of developing protective measures. To meet this goal we are employing a two-pronged approach. First, we are studying several of those toxins proven to be potential BW agents as regards structure, mechanisms of action, etc. Secondly, based on a large amount of evidence that most bacterial toxins have several common features to their mechanisms of action, we have employed a well characterized model (diphtheria toxin) to study certain questions that could not presently be addressed using the specific BW toxins.

Progress:

Staphylococcal Enterotoxins. Three large peptides are produced by limited proteolytic digestion of staphylococcal enterotoxin C (SEC) with trypsin. They have approximate molecular weights of 4,000, 6,500, and 19,000. The complete sequence of 4,000 was described in the previous report. Since then, the primary structure of the 6,500 fragment has also been determined. This was accomplished by cleaving it with cyanogen bromide, isolating the carboxy-terminal peptide, and further cleaving that peptide with chymotrypsin. Purification and sequencing of these subfragments completed the structural analysis of this peptide.

Given the large size of the 19,000 fragment, further degradation was necessary before extensive sequencing could proceed. To this end, it was reacted with cyanogen bromide; the digest was subsequently chromatographed on columns of Sephadex G-50. Four peptides were isolated whose homogeneity was assessed by peptide mapping on HPLC and amino acid analysis. Peptides of sufficient purity were placed in the automatic sequencer, and the PTH derivatives from this instrument were identified with HPLC and thin-layer chromatography.

The first of the cyanogen bromide fragments to be sequenced was carboxy-terminal in the 19,000 and is therefore carboxy-terminal in SEC. Two sequencer runs yielded the following: Y.N.D.N.K.T.V.D.S.K.S.V.K.I.E.V.H.L.T.T.K.N.G.

The second fragment was penultimate to the carboxy-terminal region. It contains 14 residues placed by means of automatic sequencing and compositional data. The 15th residue shown below (methionine) was placed there by virtue of homology with staphylococcal enterotoxin B (SEB), and the fact that free hemoserine was obtained in the original isolation of these peptides. The sequence is: P.A.P.G.D.K.F.D.Q.S.K.Y.L.M.M.

The amino terminal cyanogen bromide peptide from 19,000 contained 8 residues, and included the second half-cystine of the single disulfide bond in SEC. The sequency of this peptide is: V.T.G.G.K.T.C.M.

The last cyanogen bromide fragment to be analyzed is by far the largest one, containing about 90 residues. The following partial structure was obtained: Y.G.G.I.T.K.H.E.G.N.H.F.D.N.G.N.L.Q.N.V.L.I.R.V.Y.E.N.K.R.N.S.I.S.L.E.V.Q.T.N.K.K.S.V.T.A.Q.

Amino acid analysis of this peptide indicated that there were about 40 residues remaining to be sequenced. In order to gain access to this area, whole SEC was maleylated and digested with trypsin. This was done because inspection of the known sequence predicted that restriction of tryptic cleavage to the 3 arginine residues by maleylation would provide peptides containing the unstructured areas in readily purified forms. However, the digests were much more complex than expected, indicating that in addition to the arginyl bonds, several other loci were also affected. Nonetheless, one peptide containing an unsequenced segment was purified, spanning the area between the second and third arginine residues: S.V.T.A.Q.E.L.D.I.K.A.R.

To reduce the number of peptides produced, the 19,000 fragment (instead of whole SEC) was maleylated and treated with trypsin, followed by chromatography on Sephadex G-50. This approach produced a peptide that began with the next residue after the third arginine and ended at the carboxy-terminal residue of SEC. That is, it contained all of that part of SEC remaining to be sequenced, plus a previously structured area. It was placed in the automatic sequencer, and the following results were obtained: N.L.L.I.N.K.K.N.L.Y.E.F.N.S.S.P.Y.E.T.G.Y.I.K.F.I.E.N.

Production of useful data from this peptide ceased quite abruptly with the last asparagine (N) above, instead of gradually in the usual way. This suggests that the residue following the asparagine is unreactive toward the sequencer reagents, leaving a gap of 7 or 8 residues to be determined in the structure of 19,000.

To investigate this further, the largest peptide from a cyanogen bromide digest of the 19,000 was cleaved with trypsin; a peptide beginning with the last phenylalanine (F) above was purified from this by chromatography on Sephadex G-50 and BioGel P-2. When this was placed in the sequencer, the production of useful data once again ceased at the same asparagine residue, i.e., at cycle number 4 in this case.

Eventually the problem yielded to chemical cleavage of reduced, carboxamidomethylated SEC with hydroxylamine. That is, the blockage was the result of an asparaginyl glycine linkage that cyclized upon removal of the penultimate residue, in this case another asparagine. Chromatographic purification of the desired peptide

allowed the final sequence in this area to be determined, completing the sequence of the 19,000 peptide: N.G.N.S.F.W.Y.D.M.M.P.A.P.G.....etc.

As previously described, limited proteolysis of SEC by trypsin produces the 3 major peptides listed. However, sequencer runs on unfractionated digests indicated that 2 small peptides were overlooked by Spero and coworkers in the original isolation procedure. These were purified chromatographically and sequenced.

The first is a dipeptide: L.K. Alignment of peptides in SEC with homologous areas in SEB produces a gap of 2 residues between the 6,500 and 4,000 fragments of SEC. The dipeptide fills this gap on the basis of homology with SEB, the enzymatic specificity of trypsin, and the fact that in one case a small percentage of the 4,000 fragment was found to have this dipeptide still attached.

The second small peptide contains 5 residues: D.N.V.G.K. Alignment of homologous peptides in SEB and SEC leaves a gap of 8 residues between the 4,000 and 19,000 segments of SEC. By elimination, the pentapeptide should fit here, but it is 3 residues shorter and shows little homology with the corresponding area of SEB. This question must be answered more definitively before the sequence of SEC can be considered complete.

Since this area is spanned by the single disulfide bridge of SEC, specific excission could be accomplished by reaction of intact toxin with reagents that cleave to cysteine residues. One such reagent is cyanide, but its effectiveness is unpredictable. Another is 2-nitro-5-thiocyanatobenzoic acid, which we have synthesized. Preliminary attempts are underway to optimize the efficiencies of the reactions. To close the gap, the products will be purified by gel chromatography and further analyzed by enzymatic digestion and peptide mapping.

Pseudomonas aeruginosa Exotoxin A. Pseudomonas exotoxin A (PE) prepared at USAMRIID continues to be a valuable resource for investigators studying this protein. During FY 81 toxin and related reagents were supplied to 9 research groups outside USAMRIID, including several DOD laboratories (USUHS, NMRI, USAISR, WRAIR). Several of these investigators have attempted to prepare PE in their own laboratories, but none have successfully made toxin in the large amounts and at the purities achieved here. Extensive advice has been provided to a small biological supply firm, List Biological Laboratories, Campbell, CA, which is attempting to prepare toxin to offer for sale. After some initial difficulties, List has succeeded in making small amounts of toxin.

One of the principal problems in the current PE preparation protocol was overcome during FY 81. Chromatography on hydroxylapatite columns effectively removes contaminants including lipopolysaccharides, but this chromatography support is notorious for poor flow rates. It has now been found that the small particles present in commercial hydroxylapatite can be removed by repeated decantation after pretreatment with alkali. The resulting material gives high and stable flow rates.

The toxoid developed several years ago continues to be studied in other laboratories as a possible vaccine. In a collaborative study performed at NMRI, the glutaraldehyde toxoid was equally effective at inducing antitoxin antibodies in mice, but was less effective than a formaldehyde toxoid in protecting burned mice against infection. Unfortunately, different adjuvants and immunization protocols were used, so that a valid judgment on the relative efficacies of the 2 toxoids could not be made.

A definitive test of the role of exotoxin in virulence could be obtained in passive immunizations with a monoclonal neutralizing antitoxin. All attempts to access the role of an antigen through active immunization are subject to the possibility that any positive protection could reflect immunity to trace contaminants in the immunogen. This problem is significant in Pseudomonas infection trials since lipopolysaccharide is highly immunogenic and the resulting antibodies are protective.

An effort is currently underway to obtain hybridomas producing monoclonal antibodies to PE. Initial efforts involved the immunization of Balb/c mice with the glutaraldehyde toxoid (10 µg/mouse). The animals were boostered 6 weeks later and the spleens removed 3 days following the booster. Isolated spleen cells were fused with either an IgG-secreting or a nonimmunoglobulin-secreting established plasmacytoma line using polyethylene glycol and viable hybrids selected using hypoxanthine-aminopterin-thymidine (HAT) medium. Fusion frequency was approximately 30%, resulting in the growth of 98 hybrid colonies. An ELISA (enzyme-linked immunosorbent assay) was developed for rapid detection of PE-specific antibody in the hybridoma culture supernatants, based on the appearance of a yellow color following incubation of substrate with goat anti-mouse IgG linked to alkaline phosphatase. A number (37) of antibody-producing colonies were detected in this manner and were passaged and grown for cloning.

Diphtheria Toxins. Structure-function studies of diphtheria toxin (DE) performed in FY 81 centered on extending the analysis of the 10 mutationally altered toxins originally selected by Laird and Groman. These serologically cross-reacting materials (CRM) constitute a unique and valuable set of reagents for characterizing DE action. The detailed analysis of the CRM proteins, designated tox 101-110, is shown in Table I. Tox 102, 103, and 107 were found to have normal ADP-ribosylation activity, indicating that their 40-fold or greater decrease in toxicity must result from a defect in the receptor-binding B subunit. This was confirmed in binding studies when it was shown that tox 102, 103, and 107 proteins were about 100-fold less effective than native toxin at blocking binding of ^{125}I-toxin to Vero cells (Table I, fifth column).

TABLE I. PROPERTIES OF DIPHTHERIA TOXIN CRM

PROTEIN	PROTEIN SYNTHESIS $TCIC_{50}$ (ng/ml)	ENZYME ACTIVITY OF A FRAGMENT: Ribosylation EC_{50}[a] (µg/ml)	ENZYME ACTIVITY OF A FRAGMENT: NAD Hydrolysis (pmol)	B FRAGMENT EC_{50}* (µg/ml)	DEFECTIVE SITE
DE	1	0.2	45	0.1	
101	>10,000	>100	41	0.1	EF-2
102	100	0.2	107	10	R
103	40	0.2	79	10	R
104	>10,000	>100	1	0.1	Ad
105	>100	>100	27	--	EF-2
106	>100	>100	0	--	Ad
107	>100	0.2	75	10	R
108	>100	>100	24	--	EF-2
109	>100	30	113	--	EF-2?
110	>100	3	36	--	EF-2?
197	>10,000	>100	4	0.1	Ad

[a]Concentration required for ADP-ribosylation of 50% of EF-2, in standard assay.
*For block of 1251-toxin binding.

Several of the other CRM were, like the previously characterized CRM197, completely unable to catalyze ADP-ribosylation. To characterize these proteins further, tests for hydrolysis of nicotinamide adenine dinucleotide (NAD) were performed. NAD is normally the ADP-ribose donor during ribosylation; in the absence of elongation factor 2 (EF-2), the NAD bound to the toxin catalytic site may be slowly attacked by H_2O. NAD hydrolysis assays therefore measure the integrity of the NAD binding site. However, the rate of NAD hydrolysis is also affected by the conformation of the whole molecule, since the B subunit is adjacent to the NAD binding site and partially blocks it. The data clearly show that 2 of the proteins, tox 104 and 106, are unable to hydrolyze NAD, and are therefore functionally equivalent to CRM197. At least 3 of the other proteins are able to hydrolyze NAD; these CRM (tox 101, 105, and 108) must therefore be defective in either binding of the other substrate, EF-2, or in catalyzing the transfer reaction. These alternatives could be distinguished only through detailed kinetic studies using purified EF-2. In Table II these CRM are listed as defective in EF-2.

The two remaining proteins not yet discussed, tox 109 and 110, may be the most interesting. These proteins have measurable but decreased ADP-ribosylation activity and apparently normal NAD hydrolytic ability. Like 101, 105, and 108, they would appear to have decreased EF-2 binding. However, it is not clear that a 10-fold lower ADP-ribosylation activity can account for 100-fold or greater loss in toxicity of tox 110. Tox 109 and 110 have not been assayed for B fragment activity, so the possibility that these proteins are double mutants cannot be excluded. The intermediate behavior of tox 109 and 110 emphasizes a cautionary note regarding the analysis summarized in Table I. While the *in vitro* assays measure single functions of these proteins, the *in vivo* activities (toxicities) can be expected to reflect some complex summation of these. Thus, an amino acid change detected *in vitro* as a loss in NAD hydrolysis may alter the conformation of the protein so that it is more susceptible to degradation or so that it interacts differently with the subcellular systems of the target cell which are involved in internalization and activation of the toxin protein.

Research with the DE receptor in FY 81 was concerned with 2 main topics. The first involved completion of work with the metabolic inhibitors. Although we had successfully defined many aspects of the problem, an important point remained, e.g., is the metabolic inhibitor-induced loss of toxin-cell binding due to a loss of receptors or a change in the binding affinity? To distinguish between these 2 possibilities, it was necessary to treat cells with the inhibitors and run a Scatchard analysis of the toxin-cell binding. When this was done (for all 4 active compounds), the results pointed to a loss of binding sites as the proper explanation.

The other main effort with the receptor was concerned with its toxicity for rabbit reticulocytes. We found that DE could inhibit protein synthesis in these cells and sought to determine whether or not this is a receptor-mediated process. The choice of these cells is important because they apparently lack lysosomes and it is believed diphtheria toxin (along with several other toxins, hormones, and viruses) may require lysosomal processing to be active. Dose-responses for DE-induced inhibition of reticulocyte protein synthesis indicated a concentration of $\approx$ 100 µg/ml is required for a complete block. This is quite high relative to most other cell systems and raises a serious question of specificity. Our approach to this problem was to test those drugs or chemicals which have been shown to protect from diphtheria toxin in known receptor-mediated toxicity systems and determine if the agents would protect reticulocytes. We tested metabolic inhibitors and found that those agents which protect in the other systems also protect reticulocytes (Table II).

TABLE II. EFFECT OF METABOLIC INHIBITORS ON DIPHTHERIA TOXIN-INDUCED PROTEIN SYNTHESIS INHIBITION[a]

DRUG	CONCENTRATION (mM)	PROTEIN SYNTHESIS (cpm) Drug alone	Drug and toxin
None		2200	400 (18%)[b]
Fluoride	0.5	2150	530 (24%)
	1.0	1970	780 (40%)
	3.0	1400	1300 (93%)
Azide	0.5	1870	670 (38%)
	1.0	2040	1030 (51%)
Cyanide	1.0	1940	550 (28%)
	5.0	1500	430 (29%)
Dinitrophenol	0.05	2000	1000 (50%)
	0.1	1380	1380 (100%)
	0.5	1600	1680 (105%)
Deoxyglucose	30	1980	330 (13%)
	100	1710	350 (20%)
Salicylate	3.0	2650	670 (25%)
	10	2120	1230 (58%)

[a]Cells were incubated with the indicated agent for 1 hr at 37 C. Toxin was added (100 µg/ml) and incubation continued for 2.5 hr. Toxin and drug were washed out and the cells were incubated 1 hr further at 37 C. Protein synthesis was then measured with a 1 hr pulse of [^{3}H]leucine.

[b]% of drug control.

Certain amines known to protect from diphtheria toxin (NH_4Cl, methylamine, and chloroquine) were tested with reticulocytes and found to protect against DE, but only minimally. This is an anticipated result, since the amines are lysosomotropic agents and the reticulocytes have few or no lysosomes. Nucleotides which block DE-receptor binding were tested and found to work with the correct specificity (Table III).

Most interesting in the reticulocyte work was the observation that unnicked DE was not toxic, but if it was nicked with trypsin, the toxin was active. This is the first documented report of a nicked-unnicked DE differential sensitivity and can be explained as due to (a) a cellular lack of the "nicking enzyme" or (b) an improper packaging or location of the enzyme. The latter explanation is particularly attractive considering that the reticulocytes have lost their lysosomes.

Studies of the DE-receptor (or any toxin receptor) system would be greatly facilitated by the availability of an antibody to the receptor. There are several recent reports in the literature where monoclonal antibodies to receptors were obtained using crude receptor preparations and the mouse hybridoma system. We undertook a similar approach using the Vero cell-DE receptor system. A crude preparation of Vero cell membranes was used to immunize mice. After 8-10 weeks, the mice were boostered; 3 days later their spleens were removed and a fusion experiment was carried out with mouse myeloma cells. The resultant cells were seeded in

microtiter plates and the cells were tested for ^{125}I-DE-Vero cell blocking. Four positive colonies were obtained in the second attempt, but these cells stopped producing blocking activity during the cloning operations. We believe the results are highly encouraging and want to repeat the work. However, before doing so, we are making attempts to determine why the hybridoma cells stopped producing the putative anti-receptor antibody and how such recurrences could be prevented in the future.

During this past year, studies on the cellular internalization of DE were continued, with primary emphasis on the mechanism of action of the protective amine compounds. It was shown that NH_4Cl, a potent blocker of cytotoxicity, has no apparent effect on the internalization or degradation of DE, as assayed by previously established biochemical techniques. Autoradiographic studies appeared to confirm these results: after a 2-hr incubation of cells with ^{125}I-labeled diphtheria toxin at 37 C, more than 90% of the silver grains were associated with the cytoplasm either in the presence or absence of protective concentrations of NH_4Cl. However, more complex experiments designed to determine the effect of specific antitoxin addition on the NH_4Cl-mediated protection indicated that, in the presence of NH_4Cl, biologically active toxin molecules were maintained in a position accessible to antibody, presumably on the cell surface. In these studies, Vero cells were preincubated with DE for 18 hr at 0 C, rinsed to remove unbound toxin, and warmed to 37 C in the presence or absence of NH_4Cl. Antitoxin was added either simultaneously with the NH_4Cl (0 time) or 60 min after NH_4Cl addition. After 2 hr at 37 C, the cells were rinsed, incubated a further 2 hr, and assayed for protein synthesis. Results showed that the cells were completely protected from concentrations of DE up to 100 ng/ml when NH_4Cl and antitoxin were added simultaneously at 0 time. In the absence of antibody, NH_4Cl was only partially protective. When antitoxin was added at 60 min, however, the protective effect was increased ≈ 100-fold. Antitoxin alone added at 60 min had no detectable protective effect. These results suggest the existence of dual internalization or dual intracellular processing mechanisms for diphtheria toxin, with the NH_4Cl-sensitive (biologically relevant) patheway encompassing only a small fraction of those molecules internalized and degraded by the major pathway quantitated by our biochemical measurements. In light of more recent evidence, however, it seems more likely that surface-bound toxin is internalized by a single mechanism, with NH_4Cl acting at the level of an intracellular vesicular pool in rapid equilibrium with the cell membrane. This will be discussed below.

As well as NH_4Cl, a large number of alkylated amines and ethylenediamine derivatives were found to protect cells effectively from the action of DE. The parent compound, ethylenediamine itself, had no effect. Though none of the protective amines had measurable effect on the internalization of DE, all blocked toxin degradation, as determined by cellular excretion of radiolabeled trichloroacetic acid-soluble fragments. Subsequent studies using specific antitoxin, however, showed that, as was the case for NH_4Cl, biologically active toxin molecules appeared to be maintained at the cell surface in the presence of the tested alkylamines.

A series of experiments recently published by Draper and Simon (1) showed that in the presence of NH_4Cl, DE was maintained in an antibody-accessible position at 37 C, but not at 4 C. We confirmed these results in our system, using both NH_4Cl and a wide range of alkylamines (Table IV). One reasonable interpretation of these data is that in the presence of NH_4Cl, toxin becomes sequestered in intracellular vesicles; thus, at 37 C, either antitoxin is endocytosed and neutralizes intravesicular DE, or the toxin-containing vesicles are recycled to the plasma membrane, where the toxin-antitoxin neutralizing interaction occurs (Table IV, Column 2).

At 4 C, presumably neither endocytosis nor exocytosis takes place; therefore, either antitoxin cannot enter the cell or intracellular toxin-containing vesicles cannot return to the cell surface, and protection does not take place (Table IV, Column 3).

TABLE IV. PROTECTION OF VERO CELLS FROM DE BY AMINES: EFFECT OF ANTITOXIN ADDITION*

CHEMICAL	% CONTROL PROTEIN SYNTHESIS		
	No antitoxin addition	+ antitoxin, 37 C	+ antitoxin 4 C
Control	0	3	4
NH_4Cl	2	85	17
Methylamine	3	60	30
Ethylamine	1	87	29
Propylamine	1	82	24
Butylamine	1	87	11
Triethylamine	1	63	27
Tributylamine	4	89	21
Ethylenediamine (ED)	0	7	5
Tetramethyl ED	7	75	48
Tetraethyl ED	10	71	40
N,N-dimethyl ED	27	57	39
N,N'-dimethyl ED	30	63	54
Chloroquine	2	60	30

*Cells were preincubated with the indicated amines (10 mM) for 15 min at 37 C. Diphtheria toxin (3 ng/ml) then was added and incubation continued at 37 C for 60 min. At this point, antitoxin (1:500) was added to the control cells (Column 2) or 4 C (Column 3). No antitoxin was added to the control cells (Column 1). The cells then were rinsed, fresh complete medium was added, and incubation continued for 24 hr at 37 C. Protein synthesis then was assayed as described in Materials and Methods.

A further series of experiments may provide support for the intracellular vesicle recycling hypothesis. Inositol hexaphosphate (IHP) is a highly charged compound and probably does not readily cross the plasma membrane barrier. It effectively protects cultured cells from DE, presumably by interfering with toxin-receptor binding; furthermore, when added at 4 C to cells prebound with radiolabeled DE, IHP enhances the detachment of bound toxin molecules from the cell surface. When IHP was substituted for antitoxin in temperature-shift experiments analogous to those depicted in Table IV, similar results were obtained: the amine-treated cells were protected at 37 C, but not at 4 C. These results may indicate that toxin internalized in vesicles is returned to the cell surface in the presence of amines, where it can be neutralized.

DE internalization and processing studies were further pursued this year using a modification of the spectrophotometric fluorescence probe technique of Ohkuma and Poole (2) to investigate the effects of the protective amines on intralysosomal pH. Since the protective alkylamines and ethylenediamine derivatives all markedly blocked the degradation of radiolabeled toxin, it seemed reasonable to hypothesize that these compounds act at the level of the lysosome. Vero cells therefore were allowed to endocytose fluorescein-labeled dextran, which accumulates in lysosomes. The

cells were then rinsed thoroughly, trypsinized, and fluorescence spectra of the final cell suspensions measured in the presence or absence of various amines. The amine-mediated increase in relative fluorescence intensity presumably represents an amine-mediated increase in lysosomal pH. In these experiments, intralysosomal pH was determined using the ratio of fluorescence measured with excitation at 495 nm to that with excitation at 450 nm (Ex 495/450) compared to a standard curve generated from the excitation spectra of stock solutions of fluorescein-labeled dextran adjusted to various pH in the range 2.0-9.0. The data in Table V demonstrate that the protective amines increase intralysosomal pH by approximately 2 pH units. The nonprotective compound, ethylenediamine, did not elicit a pH increase. Since the lysosomal enzymes have pH optima in the range of 4-5, it is possible that the amine-mediated elevation of lysosomal pH leads to the inhibition of some enzyme(s) essential for the generation of active toxin.

TABLE V. EFFECT OF AMINES ON INTRALYSOSOMAL pH IN VERO CELLS*

CHEMICAL	INTRALYSOSOMAL pH ± SE
Control	4.6 ± 0.3
NH_4Cl, 10 mM	6.7 ± 0.8
Methylamine, 10 mM	6.5 ± 0.7
Ethylamine, 10 mM	6.5 ± 0.9
Propylamine, 10 mM	6.2 ± 0.4
Butylamine, 10 mM	6.1 ± 0.3
Triethylamine, 10 mM	5.9 ± 0.3
Tributylamine, 10 mM	5.7 ± 0
Ethylenediamine, 10 mM	4.4 ± 1.0
Tetramethyl ED, 10 mM	6.3 ± 0.5
Chloroquine, 0.1 mM	6.3 ± 0

*Cells were incubated 18 hr at 37 C in complete E199 medium containing FITC-dextran (1 mg/ml). The cells then were rinsed 4 times with HBSS, trypsinized, and resuspended in complete E199 medium. These cells then were rinsed 2X in unsupplemented HBSS by centrifugation at 1000 rpm for 5 min and finally resuspended in warm HBSS supplemented with 25 mM Hepes buffer and 2% FCS. Fluorescence spectra of 2-ml aliquots of the final cell suspensions in the presence or absence of the indicated amines were measured in the range of 400-500 nm using a Perkin-Elmer MPF-4 fluorescence spectrophotometer. The ratio of fluorescence measured with excitation at 495 nm to that with excitation at 450 nm (Ex 495/Ex 450) was determined for each sample. These figures were used to estimate intralysosomal pH based on comparisons to a standard curve generated from the fluorescence spectra of stock solutions of FITC-dextran adjusted to various pH in the range 2.0-9.0. Results are listed as the average of 3 separate experiments.

An alternative explanation for the protective effect of intralysosomal pH elevation derives from the work of Draper and Simon (2), who demonstrated that diphtheria toxin rapidly penetrates the plasma membrane in response to acid extracellular pH. Furthermore, acidification of the extracellular medium bypasses the protective effect of the lysosomotropic amines. The ability of lowered extracellular pH to bypass the amine-mediated protective effect was tested in our system: results using a large number of alkylamines and ethylenediamine derivatives showed that the protective effect was abrogated by exposure of cells to medium titrated to pH 4.0-4.5. Similar results were obtained with NH_4Cl and another lysosomotropic drug, chloroquine.

In toto, these results seem to support a model in which surface receptor-bound DE becomes sequestered in endocytic vesicles and then exposed to acid pH, possibly following vesicle-lysosome fusion. At acid pH, DE (or toxin fragment) penetrates the vesicle membrane and enters the cytoplasm, where it catalyzes the ADP-ribosylation of EF-2, leading to cytotoxicity. In the presence of amines, lysosomal pH is elevated so that the escape of toxin to the cytoplasm is blocked. These vesicles (secondary lysosomes ?) may recycle to the cell surface.

The role of lysosome in toxin-induced cytotoxicity, however, is still unclear. We have recently shown that rabbit reticulocytes, which lack defined lysosomes, are sensitive to nicked DE but are unaffected by unnicked molecules. Similar experiments were conducted in this laboratory using 4 lysosomal mutant cell lines obtained from the Human Genetic Mutant Cell Repository. These cell lines were obtained from patients suffering from I-cell disease, a disorder in which the lysosomes, due to a packaging defect, are deficient in 15-30 different lysosomal enzymes. Although these cells grow poorly in culture, the effects of nicked and unnicked DE were compared in each line in both protein synthesis and 3-hr cytotoxicity assays. No differences were detected.

Botulinum Toxin. A major new effort was undertaken this year involving studies on the mechanism of action of botulinum toxin. Delineation of other bacterial toxins' mechanisms of action depended heavily on the availability of a cell culture system. Until recently, there were no such candidates for use with botulinum toxin. The development of 2 new cell lines has changed that picture. One, called NG108-15, is a hybrid clone from a neuroblastoma and a glioma fusion. The other, termed PC-12, is a pheochromocytoma line developed in Lloyd Green's laboratory. Both of these cell lines synthesize acetycholine and release the neurotransmitter in response to pharmacologic or electrical stimuli known to evoke release in vivo. There is therefore a good chance that one or the other will serve as a suitable cell culture system for botulinum toxin studies.

A technical difficulty in working with these cells is their poor adhesion to the plastic or glass surfaces on which they are grown. For the types of studies we intend to carry out, it would be advantageous, if not necessary, to have the cells firmly attached to the tissue culture surface. We therefore spent some time working out the treatments which would render the cells adherent during the course of several washes. We ultimately found that a 0.5 hr treatment of the tissue culture vessel with 40,000-70,000 M.W. poly-l-lysine was very effective.

We next moved on to setting up the acetylcholine assay which is required to measure the amount of neurotransmitter release from the cells. Some modifications of the published methodology are needed for the assay to be satisfactory for our needs. We determined the optimal conditions required to "load" the cells with the acetylcholine precurser [^{3}H]choline. The cellular concentration of choline reached a steady state by 60 min with the NG108-15 cells while 90 min were required with the PC-12 cells. We are presently engaged in optimizing conditions to measure acetylcholine release.

A large effort was also devoted to obtaining monoclonal antibodies to botulinum toxin. Experiments were performed in collaboration with Dr. Lynn Siegel using a formaldehyde toxoid of botulinum type A; to date, 14 antibody-producing hybridoma colonies have been isolated, grown, and frozen for future study. The botulinum studies have been complicated by the difficulty of developing an effective ELISA for rapid screening of specific mouse antibody, but parameters for a usable assay have been established based on the alkaline phosphatase reaction. Future efforts will

involve cloning the antibody-positive parent colonies, assaying the clones for antibody production, and testing the resultant antibody for biological effectiveness in mouse neutralization tests. Preliminary mouse neutralization assays using supernatant samples from the parent cell cultures were equivocal, though in some cases appeared to prolong time to death relative to the controls.

Presentations:

1. Dorland, R. B. Isolation and characterization of diphtheria toxin-resistant mutant cells. Presented, USAMRIID-WRAIR Toxin Review, Frederick, MD, Dec 1980.

2. Leppla, S. H. Structure-function studies of studies of diphtheria toxin and mutationally-altered variants. Presented, USAMRIID-WRAIR Toxin Review, Frederick, MD, Dec 1980.

3. Middlebrook, J. L. Possible sites of protection in the mechanism of action of bacterial toxins. Presented, USAMRIID-WRAIR Toxin Review, Frederick, MD, Dec 1980.

4. Middlebrook, J. L. Diphtheria toxin receptors: binding internalization and regulation. Presented, Harvard University School of Public Health, Boston, Apr 1981.

5. Dorland, R. B. Internalization and intracellular processing of diphtheria toxin: effects of lysosomotropic amines. Presented, Gordon Conference on Molecular Pharmacology, Plymouth, NH, June 1981.

6. Middlebrook, J. L. Cell surface interactions of diphtheria toxin. Presented, Gordon Conference on Molecular Pharmacology, Plymouth, NH, Jun 1981.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
DA 0G1522	81 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
80 10 02	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	3M161102BS10	AO	199
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7,2:2			

11. TITLE (Precede with Security Classification Code)*

(U) Bacterial and Rickettsial Diseases of Potential 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
80 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:*

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 81	3.2	907
FISCAL YEAR CURRENT 82	8.0	928

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Lowry, B. S.

NAME: Ristroph, J. D. 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) Q fever; (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) Study well-recognized and newly discovered pathogens with acknowledged BW potential, requiring special containment. Assess and characterize virulence factors, growth requirements and pathogenesis in animal models and resultant therapeutic implications. These broad-based fundamental studies should provide an essential data-base which can be applied to many groups of organisms considered highly dangerous and with BW potential. 24 (U) Current approaches are broad in scope, and include these examples: development and application of DNA homology to Legionella and related organisms; application of isotachophoretic technology to toxin studies; isolation and characterization of bacterial plasmids; identification of selected pathogens by nucleic acid and hybridization technology; purification and characterization of antigenic components of Coxiella burnetii and characterization of respiratory tract responses to selected antigens and immunogens. 25. (U) 80 10 - 81 09 - A/J mice were used as an animal model for Q fever. A complement-fixing fraction of a TCA-extract of phase I C. burnetii antigen protects guinea pigs from febrile reactions postchallenge. An antiserum-containing medium for simultaneous detection and isolation of Legionella species was developed. Avirulent forms of L. pneumophila are more susceptible to enzymatic breakdown than virulent ones. Early histopathology was examined in guinea pigs after aerosol challenge. Identification of a common Legionella toxin was demonstrated in the 5 species of Legionella. A microhemagglutination assay for detection of Legionella antigen in urine was developed. Specialized techniques were developed to demonstrate plasmids in Legionella species and Bacillus anthracis.

Publications: Acta Virol 25:241, 1981; J Clin. Microbiol. 13:115, 843, 1981; Ann. Int. Med. 94:413, 1981; Pharmacol. Exp. Ther. 15:123, 1981; Infect. Immun. 31:1270, 1981; J. Infect. Dis. 143:562, 1981.

*Available to contractors upon originator's approval.

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)

Work Unit No. S10 A0 199: Bacterial and Rickettsial Diseases of Potential BW Importance

Background:

This work unit consists of 5 research units which will be cited individually. Dr. Scott's studies of immunization of the respiratory tract showed that vaccines composed of nonliving Pseudomonas pseudomallei immunogens stimulate synthesis of specific antibody in guinea pigs; however, immunized animals remain susceptible to lethal respiratory infection while reflecting varying degrees of partial resistance to parenteral challenge (1). Sublethal infection of guinea pigs stimulated higher levels of humor antibodies and a marked peripheral lymphoproliferative response, as contrasted with vaccinated animals. In an extension of these studies he has attempted to potentiate the humoral and cellular responses to chemically inactivated P. pseudomallei immunogens through the use of adjuvants, and has examined the protective efficacy of a pseudomallei vaccine that was inactivated by gamma-irradiation. This work has terminated. Studies were initiated to evaluate an array of genetically standardized mice as potential models for Q fever. A laboratory animal exhibiting increased sensitivity to infection with C. burnetii is requisite to effective vaccine development and evaluation.

The objective of Dr. Wachter's research unit is to evaluate the potential of the soluble phase I antigen of Coxiella burnetii as a vaccine. The antigen, which has been tested as a vaccine in volunteers in Czechoslovakia and Romania, is much less reactogenic than either cell-wall or whole organisms preparations of this organism. The antigen (a) is stable to lyophilization, storage at 4°C and autoclaving; (b) will induce a cellular immune response; and (c) is 100 times less skin-reactogenic than the Merrell-National Laboratories particulate phase I Q fever vaccine. To obtain characterization data that will be required for the antigen to be approved as a subunit vaccine, sufficient quantities of purified antigen are needed. In the initial approach to purification, we found that gel filtration of trichloroacetic acid (TCA) extract preparations of the antigen through Sephadex produced 2 major components, only one of which was antigenic and immunogenic. Two fractions have been separated from this active component. This report summarizes progress in the purification of the antigen and in defining its immunogenic properties.

Dr. Hedlund's research unit deals with efforts on Legionnaires' disease which began in January 1978. Since that time many facets of the diseases have been explored, but with a new commitment to the anthrax program the amount of time and space afforded to the Legionnaires' disease program has been modified. Present efforts are directed towards detection and direct isolation of Legionella species from clinical specimens or the environment (Mr. Janssen). LTC Lowry is performing sequential histopathologic studies using a new technique she has introduced which will follow the events following Legionella aerosolization into guinea pigs. MAJ Ristroph is unfolding basic pieces of information dealing with the maintenance of virulence and has developed some fundamental insights into why certain Legionella strains are virulent and others avirulent.

"Identification of pathogens using nucleic acid hybridization" (J. W. Ezzell) deals with DNA homology studies. The extent of hybridization of DNA single strands from 2 organisms to form double-stranded DNA has been utilized over past decades as a measure of DNA homology and thus relatedness. Although primarily utilized as a taxonomic tool, nucleic acid hybridization can be used to identify an unknown organism by determining the homology of its DNA with organisms of known identity. DNA homology determinations are not affected by minor genotypic alterations which can cause major alterations in an organism's phenotype. Therefore, this method can be invaluable in identification of organisms whose phenotype has been altered through mutation and/or genetic engineering so as to make classical identification methods less effective. Over the past 2 years this investigator has established these techniques at USAMRIID to identify new Legionella species isolates and potential BW agents. Having been established, this research unit is being maintained on a stand-by status until required.

Dr. Knudson and CPT Mikesell have been studying plasmids in diseases of potential military importance. Multiple drug-resistant plasmids complicate the treatment of infectious diseases by rendering their hosts resistant to specific antimicrobial agents. This drug resistance can be conjugally transferred in vivo to other bacteria even across species lines. Virulence plasmids specify properties that contribute directly to the pathogenicity of the bacteria. Extrachromosomal genetic determinants code for botulinum, diphtheria, streptococcus erythrogenic toxins, and the enteropathogenic Escherichia coli enterotoxins and pilus-like colonization antigens. Molecular genetic manipulations using plasmids as vectors will allow the design and construction of new species of pathogens against which are present methods of identification and treatment will be ineffective. This research is designed to establish a technological base for the genetic analysis of pathogens and to help evaluate the BW threat from pathogens which contain naturally occurring plasmids or recombinant DNA molecules. Methods have been developed for rapid detection, isolation and characterization of extrachromosomal DNA and have been applied to the genetic analysis of several pathogens of military importance.

Under a new addendum to the research plan (gene cloning and the molecular genetics of pathogens), a proposal to clone the protective antigen gene from Bacillus anthracis has been submitted and approved by the Institutional Biosafety Committee. This will expand the present research program from one of molecular genetic analysis to one of molecular genetic engineering.

Progress:

"P. pseudomallei and C. burnetii animal model" (G. H. Scott). All work to date suggests that nonliving P. pseudomallei antigens have little or no values as vaccines. These observations together with the variability encountered in the virulence of this organism, have led to an administrative recommendation that research on respiratory meliodosis be terminated. Dr. Scott's second effort involves the development of a new animal model system for Q fever (C. burnetii); this looks promising, based on preliminary data.

"Characterization of C. burnetii components" (R. F. Wachter). It was reported earlier that gel filtration on columns of Sephadex G-200 or Sepharose-4B separated TCA extract preparations of the phase I antigen into 2 major components, only one of which was antigenic and immunogenic. Using Sepharose-2B, good separation was obtained of the active peak from the void volume and from the second (inactive)

peak. Further, the original active peak was separated into 2 fractions: an optically visible peak with a low or negative CF titer and an optically obscure fraction exhibiting a high CF titer. Immunogenicity of the 2 fractions was evaluated in guinea pigs. Differences in guinea pig protection afforded by the CF^+ and CF^- fractions is indicated in Table I.

TABLE I. IMMUNOGENICITY OF TWO COMPONENTS SEPARATED FROM EXTRACT PREPARATIONS OF THE PHASE I ANTIGEN OF COXIELLA BURNETII

EXTRACT BATCH	FRACTION	TOTAL FEVER DAYS[a]	FEVER DAYS/ ANIMAL	POSTCHALLENGE TITER (MA-I)[b]
VI[c]	CF^+	4	0.6	295
	CF^-	18	2.3	25
	Saline	13	1.9	1.4
VII[d]	CF^+	11	1.3	42
	CF^-	25	3.1	27
	Saline	41	5.1	3.1

[a]Body temperature > 40.0°C is considered fever.

[b]Geometric mean of the reciprocal of the microagglutination titer to phase I C. burnetii antigen.

[c]Guinea pigs vaccinated, with 2 weeks between doses, and challenged 6 weeks later.

[d]Guinea pigs vaccinated with 1 week between doses and challenged 2 weeks later.

In a previous report we showed that high speed centrifugation (105,000 x g, 4 hr) of TCA extract of the phase I antigen of C. burnetii yielded sediments that contained about 1/3 of the protein but most of the antigenicity and immunogenicity of the extract. A similar study was performed using the sediment and supernatant fractions obtained by centrifugation at 175,000 x g, 5 hr. Both sediment preparations exhibited similar degrees of protection, although the latter supernatant was less immunogenic than the former. Possibly the increased centrifugal force affected the decrement in immunogenicity.

"Legionella pneumophila" (K. W. Hedlund). An antiserum-containing agar medium has been developed which has permitted the simultaneous detection and direct isolation of all known Legionella species and serotypes of L. pneumophila. It was possible to detect and isolate L. pneumophila in specimens from the lung, liver, and spleen of an aerosol-infected guinea pig sacrificed early in the diseased condition, and from seeded air conditioner cooling tower water containing as few as 25 CFU/ml. This method is a modification of one reported by Albizo and Surgalla (3), for Pasteurella pestis, and employs a modification of the filtered

yeast extract medium of Ristroph and co-workers. Antiserum was obtained from a rabbit hyperimmunized with a formalinized vaccine containing all known Legionella species and serotypes of L. pneumophila. Agar gel precipitin titers were 1:32 for L. pneumophila serotypes 1, 2, 3, 4 and 6, and 1:8 for serotype 5, L. micdadei, L. bozemanii, L. dumoffii, and L. gormanii. Ten ml of filtered yeast extract agar was added to 0.5 ml of sterile antiserum per standard petri dish. The plates were streaked for colony isolation with all available cultures of Legionella species, as well as samples of air conditioning cooling tower water seeded with L. pneumophila, pond water, and tissues from a guinea pig infected with L. pneumophila. After 2-4 day incubation at 35°C well-isolated, fully developed colonies of Legionella species exclusively had a precipitin ring around them which was easily visualized when the plates were viewed under oblique transmitted light against a dark background. Selection of Legionella colonies among contaminants was easy. The precipitin ring could be intensified by exposing the culture surface of the plates to chloroform vapors for 10 min and reincubation at 35°C or room temperature for up to 24 hr; however, this procedure killed the organisms so that subculture of the colonies was impossible. False precipitin rings have not been observed around any colonies of air, water or tissue contaminants, nor around the colonies of Pseudomonas, Proteus and coliform species tested. It is probable that the 1:20 dilution of antiserum with the medium prevents potential cross-reacting antibodies from forming visible precipitin rings with common antigens in contaminant bacteria. The antiserum-agar technique may eliminate the current need to passage contaminated clinical and environmental specimens in guinea pigs and embryonated egg yolk sacs before isolation of pure cultures on laboratory media is possible, and should be especially useful in laboratories not equipped to utilize fluorescent-antibody techniques and animal passage for detecting and isolating Legionella species. Practical application of this method in surveying for potential environmental sources of legionellosis, and in isolating Legionella species directly from clinical material as it becomes available is planned. A manuscript tentatively entitled "Antiserum-agar plate technique for simultaneous detection and direct isolation of Legionella species in clinical and environmental specimens" is in preparation.

The mechanism of virulence of L. pneumophila is at the present time unknown, and is probably a combination of factors like those found in many other microorganisms. One factor that is responsible for virulence in this organism is its ability to survive and replicate within host cells. Therefore, investigations into the nature of the cell walls of this organism were initiated. The effect of hydrolytic enzymes on the cell walls was chosen as an initial experiment (Table II).

Trypsin was the most effective enzyme for both types of cell walls; however, chymotrypsin was more active against cell walls obtained from virulent organisms. Papain showed little effect on either strain, while lysozyme was moderately effective. The attenuated strain's increased sensitivity to these enzymes indicated a change in their cell walls. Both trypsin and chymotrypsin were specific in their action on peptide bonds involving the carboxyl groups of certain amino acids. Because of this specific action, we believe that the virulent strain contains more aromatic amino acids in the cell wall than the attenuated strain. This possible requirement for aromatic amino acids by the virulent strain may explain why virulence is lost on media that are low in these amino acids. This relationship between the aromatic amino acid and virulence is under investigation.

TABLE II. EFFECT OF HYDROLYTIC ENZYMES ON CELL WALLS

ENZYME	RELATIVE DROP IN OPTICAL DENSITY[a]	
	V	A
Papain	0.000	0.025
Pronase	0.004	0.081
Lysozyme	0.073	0.043
Trypsin	0.177	0.239
Chymotrypsin	0.191	0.031

[a]Difference in OD_{578} measured by subtracting the final OD at 50 min from the initial OD. Temperature of experimentation was 37°C.

Pathology of acute legionellosis pneumonia has been well publicized, but the early pathophysiology is virtually unknown. Infection is considered to be via the aerosolized route, but few laboratories are equipped to utilize the technique of aerosolization.

In a collaborative study with the Aerobiology Division lethal concentrations of L. pneumophila were aerosolized into guinea pigs. Results indicated that the bacteria were multiplying in and transported by macrophages. L. pneumophila were identified early in significant numbers in the larynx and upper 1/3 of the trachea. There was a direct relation between time and progressively increasing numbers of the bacteria (usually in macrophages) appearing more peripherally in the respiratory tract. These findings agree with the work of Horowitz and Silverstein (4) which showed a direct relationship between the numbers of macrophages cultured and the numbers of L. pneumophila recovered with time from the infected cultures.

In addition to intra-alveolar pneumonia, interstitial pneumonia was present. Within the alveolar septae specific antigenic material was identified by direct fluorescent antibody technique, and attributed to the vascular dissemination of L. pneumophila by macrophages. The interstitial pneumonia occurred throughout the lung while the bronchopneumonia distribution usually reported as the early disease, was apparently due to bacterial transmission solely through airways.

In 1979 we initially demonstrated that L. pneumophila were lethal for AKR/J mice. Later, we showed that cell-free sonicates of the same organism were also lethal when injected IP into AKR/J mice. Acid partition of this crude toxin preparation followed by gel filtration and preparative isotachophoresis of the resultant supernatant material yielded a 3,400 MW toxin. At the time when these preliminary experiments on toxin purification were being done, the other new genetically distinct Legionella agents were being recognized. It now was a simple matter to extend these findings and techniques to the new members of the Legionella family. Representatives of all of the known Legionella species were kindly provided by both Dr. Pasculle of the University of Pittsburgh School of Medicine and the Centers for Disease Control, Atlanta. These organisms were subjected to the identical

cultural, harvesting and toxin separation procedures that were used on L. pneumophila and reported this year. We drew attention to the fact that cell-free acid supernatants of sonicated L. pneumophila and the genetically distinct L. micdadei (Pittsburgh Pneumonia Agent) were lethal for AKR/J mice. We reported that both contained 3,400 MW proteins which were antigenically identical. These extended studies demonstrated that L. pneumophila serotype 1 shares a common toxic, low MW antigen with all other identified generally distinct Legionella species.

Attempts were made to find in vitro correlates to the AKR/J animal lethality studies that might shed some light on pathogenic mechanisms. New Zealand BW-J-M mouse macrophages were grown for 48 hr in culture flasks containing Eagle's minimal essential medium/nonessential amino acids medium with 10% fetal calf serum, 1% penicillin-streptomycin and 1% sodium pyruvate; incubation was at 37 C. Cells were removed from the growth surface by gently rolling glass beads across them. The suspension had a cell count of 6.4×10^5 cells/ml with a viability of 95%. The control sample contained 1.0 ml of cell suspension and 0.5 ml of preparative isotachophoresis elution buffer (H_3PO_4 Tris, pH 7.04). The treated sample contained 1.0 ml of an identical cell suspension and 0.5 ml of the isolated isotachophoretic peak derived from L. pneumophila organisms. The samples were incubated in tightly capped 5-ml Falcon plastic tubes at 37 C. Viabilities were determined by the standard trypan blue exclusion method. The results show that within 4 hr the number of viable toxin-treated macrophages dropped to less than one-half of the control levels.

Another demonstration of Legionella's toxic impact on cells normally involved in antimicrobial defense is provided by chemiluminescence (CL) studies. Basically PMN emit CL after phagocytosis of certain opsonized particles, like bacteria. Light emission can be detected and quantitiated in a liquid scintillation counter and appears to result from the ground state of electronically excited carbonyl groups, thought to be generated during singlet oxygen-mediated oxidation of the phagocytized substrate; one of the earliest studies by Stevens and Young (5) demonstrated a correlation between resistance of certain strains of Escherichia coli to opsonization and decreased in vitro killing, oxygen consumption, visual phagocytosis and CL responses of human granulocytes. Grebner et al. (6) demonstrated parallel relationships between phagocytosis and CL under a variety of conditions designed to alter opsonization of bacteria. This led the authors to conclude that the biochemical processes controlling phagocytosis and CL may be closely related or interdependent. In addition to phagocytosis, Allen et al. (7) also established the relationship of CL measurements to the microbicidal activity of PMN. As noted by Trush et al. (8) the CL response of phagocytic cells is dependent on cell metabolism and the measurement of CL represents a potentially useful index to assess the effects of pharmacologically toxic agents on phagocytic cells. To study the effects of Legionella toxin on CL, preparative isotachophore peaks were obtained. Similar bimodal peaks could be obtained from the other Legionella species. CL techniques previously described by McCarthy et al. (9), but specifically adapted to use human PMN as well as rat PMN were used to test the toxicity of the toxins. The results show that the second peak consistently inhibits the CL activity of the PMN.

The next question that arose was how readily can these "in vitro" similarities of antigenicity, MW, toxic functions be translated into in vivo models? Could animals immunized with L. pneumophila be protected against a lethal challenge from genetically distinct L. micdadei? The following set of experiments were performed

using the Washington strain serotype I, L. pneumophila and L. micdadei obtained from Dr. Pasculle. DNA homology studies were performed to document their genetic distinctness (10). AKR/J mice inoculated with sublethal aliquots of acid supernatant material from both species of Legionella obtained by methods published this year, were boosted 28 days later. After 10 days they were challenged with either a lethal dose of viable L. pneumophila, L. micdadei or a lethal dose of acid supernatant material from the respective organisms. Nonimmunized mice were included for appropriate lethal challenge controls. Animals immunized with L. pneumophila acid supernatant and challenged with a lethal inoculum of viable L. pneumophila or L. micdadei organisms are protected, as are animals given a lethal inoculation of L. pneumophila or L. micdadei acid supernatant. This cross-protection by a previously demonstrated single shared antigen was also confirmed when animals were immunized with L. micdadei acid supernatant and challenged with a lethal concentration of either viable L. micdadei or L. pneumophila organisms or their acid supernatants. Studies using L. pneumophila preparative tachophore peaks which contain the single shared, common antigen were used to protect animals with the same effectiveness as the acid supernatant preparations, although in this low MW form they are probably less efficient as an antigen and harder to obtain.

"Detection and characterization of plasmids" (G. H. Knudson, P. Mikesell). Seventeen strains from the 6 serogroups of L. pneumophila (including OLDA, which was originally isolated in 1947) and screened for the presence of plasmids. Standard methods used for isolating plasmid DNA from E. coli were not effective when applied to strains of L. pneumophila. Extrachromosomal DNA was isolated from 3 strains of L. pneumophila, Atlanta-1 and -2, and OLDA, by using an optimized lysing and plasmid purification procedures. CPT Mikesell has examined species of Legionella other than pneumophila (Table III).

TABLE III. PLASMIDS AND MOLECULAR MASS OF THREE LEGIONELLA SPECIES

SPECIES	STRAIN	PLASMID	MOLECULAR MASS (Mdal)
L. pneumophila	Atlanta-1	pKM69	30
	Atlanta-2	pKM70	30
	OLDA	pLP3	59.5
L. bozemanii	WIGA	pLB1	54
		pLB2	47.5
L. dumoffii	TEX-KL	pLK1	58
		pLK2	46.5

Indigenous cryptic plasmids, such as those found in strains of L. pneumophila, may acquire transposons which code for drug resistance. The presence of an R-plasmid coding for resistance to erythromycin would severely restrict the present treatment of Legionnaire's disease and Pontiac Fever.

Studies on the genetics of Bacillus anthracis. Initial attempts to detect the presence of plasmids in B. anthracis by conventional techniques were not successful, Two avirulent strains (Sterne and 770) of B. anthracis were screened for plasmid DNA using the composite technique developed for the Legionella bacillus. Plasmid elements were successfully purified with this procedure in both isolates; however, the method did not prove to be consistently reproducible. The method of Lovett (11) with appropriate modifications has proven to be more reproducible for plasmid purification in both avirulent strains of B. anthracis.

In addition, plasmid DNA has also been isolated from 2 virulent strains of B. anthracis, Vollum 1B and a bovine isolate designated AMES. We have noted, like C. B. Thorne (personal communication), that CsCl banded plasmid DNA could not be resolved by agarose gel electrophoresis. This problem has been resolved by growing cultures in a chemically defined medium and altering selected electrophoretic parameters. Work is also in progress to apply these methods to 2 additional bovine isolates (Texas, Colorado) and a human Colorado isolate. Preliminary data using electron microscopy and gel electrophoresis indicate these isolates have MW of $20\text{-}35 \times 10^6$.

Gene cloning. The scope of the present research program has been expanded with an addendum entitled "Gene Cloning and the Molecular Genetics of Pathogens". This research will utilize new developments in recombinant DNA technology to increase our understanding of pathogens of military importance and to produce physiologically important proteins.

A memorandum of understanding and agreement for the cloning of the gene for protective antigen from B. anthracis was submitted and approved by the Institutional Biosafety Committee.

Protective antigen (PA) is the immunizing antigenic component of the B. anthracis toxin. PA is the substance in the present crude anthrax vaccine that is primarily responsible for the development of immunity. The objective is to clone to expression the gene for PA by using recombinant DNA techniques in order to develop a more effective anthrax vaccine which will produce a long-lasting, high level of immunity.

Presentations:

1. Knudson, G. B. Principles of gene manipulation: an introduction to genetic engineering. Presented, Western Maryland College, Westminster, MD, 30 October 1980.

2. Hedlund, K. W. Legionella toxin with in vivo lethality. Presented, 3rd Int. Conf. Bacterial Vaccines, N.I.H., Bethesda, MD, November 1980.

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4. Lowry, S., R. F. Berendt, and K. W. Hedlund. Early tissue invasions by Legionella pneumophila following aerosolization into guinea pigs. Presented, United States - Canadian Division Int. Acad. Pathol., Chicago, IL, March 1981; Lab. Invest. 44:39A, 1981; Ann. Clin. Lab. Sci. 11:452, 1981.

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6. Lowry, B. S. Identification of Legionella pneumophila in tissue by combined strains. Presented, Clinical Laboratory of Washington Medical Center, Washington, D.C., June 1981.

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10. Hedlund, K. W. and R. G. Larson. 1981. Legionella toxin with in vivo lethality. The 3rd International Symposium on Bacterial Vaccines, N.I.H. (In press).

11. Hedlund, K. W. 1981. Legionella toxin. In Encyclopedia of Pharmacology and Therapeutics. Pergamon Press, New York (In press).

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3. Albizo, J. M., and M. J. Surgalla. 1968. Use of the antiserum-agar plate technique for specific identification and isolation of Pasteurella pestis. Appl. Microbiology 16:1114-1119.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G1526	81 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
80 10 02	D. Change	U	U	NA	NL	X [X] YES [ ] NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61102A	3M161102BS10	AP	198
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7.2.2			

11. TITLE (Precede with Security Classification Code)

(U) Biology of Viral Agents of Potential 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
80 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 81	4.8	543
FISCAL YEAR CURRENT 82	4.0	857

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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) Viral diseases; (U) Prophylaxis; (U) Laboratory animals: (U) Chemotherapy (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) Elucidate replicative mechanisms and antigenic composition of selected toga- and bunyaviruses that are unique in their capacity to cause widespread epidemics of military significance. Physical and biochemical characterization of viral constituents allow the identification of immunogens of potential prophylactic value. Clearly defined virus-specific replication mechanisms will be investigated as targets for antiviral chemotherapy and clues to pathogenicity, to provide the scientific base for vaccine and drug development to protect military personnel.

24. (U) Characterize the structural protein antigens of selected pathogens using biochemical and biophysical separation methods, as well as define specific antigens and function using specific antibodies. Characterize nucleic acids and deduce the replication strategy of representative alpha-, flavi- and phlebovirus pathogens in an attempt to evaluate various methods for disease control.

25. (U) 80 10 - 81 09 - The envelope glycoproteins of Rift Valley fever virus (RVFV) were separated by isoelectric focusing and clearly demonstrated them to be separate and unique protein species for future antigenic analysis. We separated, purified and characterized the large, medium and small RNA of RVFV by oligonucleotide mapping. The epidemiology of recent dengue-1 virus outbreaks by this same mapping of different geographic isolates was investigated. 2 small potential messenger RNA species in Japanese encephalitis virus-infected cells were identified and demonstrated virus specificity by hybridization to complementary DNA.

Publication: Chapter in The Replication of Negative Strand Viruses, 1981.

* Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS10: Risk Assessment of Military Disease Hazards (U)

Work Unit No. S10 AP 198: Biology of Viral Agents of Potential BW Importance (U)

Background:

This laboratory has had a long-term research interest in the molecular basis for the antigenic relationships that exist among alphaviruses. While it was initially hoped that studies with Sindbis (SIN) virus (a molecularly well characterized prototype of the alphaviruses) would help explain the antigenic complexity of togaviruses, it is now clearly recognized that the antigenic expression of alphaviruses is completely different from that of flaviviruses. Our research with SIN and related alphaviruses has been most helpful in establishing the techniques and methodologies required for the antigenic analysis of togaviruses and has allowed development of some rather basic concepts and hypotheses concerning the antigenic interrelationships of arboviruses in general. The ultimate goal of this research still remains to isolate and characterize the smallest possible antigenic determinant capable of serving as an immunogen of potential prophylactic value.

Early studies clearly showed that purified SIN virions were high titered antigens, yet possessed all of the alphavirus cross-reactivity associated with classical infected cell homogenate antigens, e.g., acetone-extracted infected suckling mouse brain. The development of sensitive radioimmune assays (RIA) for SIN virus, either by precipitation (RIP) of radiolabeled virion components or detection of unlabeled antigen and antibody using a radiolabeled secondary antibody or protein-A, subsequently allowed analysis of virion components following degradation of virion structure and concomittant loss of biological activity. Isolated nucleocapsids were shown to cross-react broadly with a wide variety of alphavirus antisera and was initially touted as the alphavirus group-reactive antigen responsible for antigenic definition of the genus. Both virus-specific antigens and those responsible for closely related "complex" were identified in solubilized envelope glycoprotein preparations. The purification and separation of the two envelope glycoproteins by isoelectric focusing allowed precise definition of the antigenic specificities of these SIN virion antigens. The isolated E1 glycoprotein was capable of hemagglutination (HA) and complement fixation (CF) and induced antibodies in experimental animals that appeared to cross-react with closely related alphaviruses within the complex. In contrast, the E2 glycoprotein appeared virus-type specific and induced neutralizing antibody, although it was poorly immunogenic in its isolated form. We initially felt all virus-type specific determinants were contained on the isolated E2 glycoprotein component of SIN virus.

A rather lengthy series of experiments attempting to isolate SIN specific antigen on a cleaved fragment of the E2 glycoprotein was uniformly unsuccessful. Obviously, either the antigenic determinant in question encompassed a major portion of the glycoprotein molecule or the procedures employed were too harsh to allow conservation of the appropriate antigenic configuration. These negative results prompted a re-evaluation of our approach to the problem.

The detailed characterization of the flavivirus genome and the biochemistry of flavivirus replication have been insufficiently described, even though many viruses of this group are responsible for severe diseases of epidemic proportions. The primary objective of this research is to characterize flavivirus RNA (of dengue viruses in particular) for the purposes of: 1) applying the techniques of viral RNA analyses in order to study the epidemiology of dengue (DEN) virus outbreaks, allowing more precise identification of different geographic isolates and monitoring genomic changes associated with time and natural spread; 2) using oligonucleotide

fingerprint mapping of the genome to detect markers and monitor stable genetic changes in the generation of live attenuated vaccine viruses, to characterize viruses isolated from vaccine volunteers and to address the question of reversion. Do attenuated viruses revert genetically to the original genotype of wild-type virus, or do further changes occur in the genome of attenuated viruses which may result in wild-type phenotypic expression?; and, 3) describing RNA isolated from DEN-infected cells in order to asses their role in virus replication.

Phleboviruses of the family Bunyaviridae contain numerous human and animal pathogens most notable of which is Rift Valley Fever (RVF) virus. The antigenic analysis of this virus, closely related virus members of the phlebovirus genus and isolated and characterized components of these virions remains the major objectives of this research. These studies assume even greater importance as supporting research for investigations directed toward producing RVF virus immunogens using recombinant DNA technology (gene cloning). It is incumbent upon these investigations to describe the basic molecular characteristics of the components of the virion, isolate, concentrate and purify the nucleic acid segment(s) containing the gene(s) for important immunogenic protein(s) as well as describe, isolate and characterize the antigens necessary for inducing those protective antibodies responsible for protection in the immunized host. The requirements described necessitate that this research be multi-faceted and aimed at a basic molecular characterization of the virus, its chemical constituents and antigenic components.

A. Alphaviruses. Monoclonal antibodies to SIN virus have been prepared using classical lymphocyte hybridoma procedures. All lymphocytes originated from Balb/c mice immunized with intact infectious SIN virus. The number of separate hybridomas producing antibody to this virus is currently in the hundreds; however, less than 50 have been characterized. Monoclonal antibodies have been isolated that are specific for nucleocapsid, E1 glycoprotein or E2 glycoprotein, but antibodies equally reactive with more than a single structural virion component have not been detected. All antibodies examined to date react in radioimmune assays (RIA) and indirect fluorescent antibody (IFA) assays and certain of these are also reactive by CF, HI and/or neutralization. Isotype analysis is incomplete, consequently we cannot evaluate the role of isotype in any given antibody's ability to react in a given serological test.

Monoclonal antibodies to SIN nucleocapsid failed to react with intact virions and were incapable of inhibiting HA or neutralizing infectivity. In contrast to our previous assumptions that nucleocapsid constituted the group-reactive antigen common to all alhpaviruses, 3 anti-nucleocapsid monoclonal antibodies appeared virus specific, i.e., failed to react with isolated nucleocapsids from WEE or EEE viruses. These data clearly suggest further analysis of nucleocapsid virus-specific determinants and their relationship to virus specific determinants expressed on the surface of intact virions.

Monoclonal antibodies to the E1 glycoprotein were the most frequent of the specificities observed. Most (but not all) inhibited SIN virus HA and again most (but not all) cross-reacted with WEE virus antigen. None of the 43 anti-E1 antibodies neutralized SIN virus and although it could be argued that low-level neutralizing activity might not be detected in hybridoma supernatants, more than 20 of these hybridomas have raised high-titered ascitic fluids in mice and are still neutralization-negative. None of these antibodies reacted in CF tests. It should be emphasized that in addition to the expected cross-reactions with the SIN-WEE complex, monoclonal antibodies with anti-E1 specificity were found that reacted only with SIN virus (not WEE or EEE) and one preparation reacted equally with all 3 viruses.

All monoclonal antibodies were screened for neutralization of SIN virus and only 7 preparations were positive. All were specific for the E2 glycoprotein. Of a total of 29 anti-E2 hybridomas examined, all produced virus-specific antibody with the possible exception of 2 that exhibited an extremely low level RIA cross-reaction with WEE virus. These data support our basic concept that a SIN virus type-specific determinant resides on the E2 glycoprotein and it is this virion constituent that is responsible for the induction of neutralizing antibodies.

We have recently extended our characterization of these monoclonal antibodies with the collaborative assistance of Drs. G. Cole and A. Schmaljohn, University of Maryland, Baltimore. These investigators examined the ability of individual SIN monoclonal antibodies to protect adult mice against a lethal, intracerebral (IC) inoculation of neuroadapted SIN virus. The AR339 strain of this virus used in the prparation of the lymphocyte hybridomas will not kill adult mice by the IC route. A single 0.1-ml dose of certain monoclonal antibody-containing ascitic fluids clearly protected mice from 1000 LD_{50} of the neurovirulent SIN virus administered 24 hrs later. Mice given a lethal dose of neurovirulent SIN virus could be protected by subsequent administration of monoclonal antibody-containing ascitic fluids given at 24 and 72 hrs postinjection.

The protective capacity of monoclonal antibodies in the passive protection test did not correlate directly with the demonstration of neutralizing activity (in vitro). Although anti-E2 antibodies with high titered neutralizing activity protected mice, many anti-E1 monoclonal antibody preparations with no evidence of neutralizing activity in vitro clearly protected mice against the lethal challenge of neuroadapted Sindbis virus. An explanation of this phenomenon is not available, but the mechanism of protection by

B. Flaviviruses. Virion RNA of the 4 DEN prototype serotypes were shown to be distinct from one another by oligonucleotide fingerprint analyses. On an average, only 7% of the large oligonucleotides were shared among the 4 viruses. Similar analyses were performed on different geographic isolates of DEN-1 to determine if their oligonucleotides were also unique. Virion RNA fingerprints of each of 10 DEN-1 strains were clearly unique, prototype Hawaiian (1944), Western Pacific (2 isolates 1974, Nauru Island), Bangkok (1975), African (1968 and 1978 Nigerian isolates), Sri Lanka (1968), Jamaican (1977 and 1981 isolates), and Carec (1977, large-plaque isolate from the Bahamas). Fingerprints of DEN-1 isolates from the same geographic area exhibited very little difference. The two Caribbean strains were virtually identical (85-90% homologous spots). For purposes of this discussion: % homologous spots = the number of coincident oligonucleotides which upon co-electrophoresis or superimposition of individual fingerprints, formed a single "spot." The 3 more widely separated Pacific/S.E. Asian strains were similar (40-50% homologous) although considerably less so than the Caribbean strain. The Pacific/S.E. Asian strains exhibited very little relationship (15%) to both the Caribbean strains and to the Sri Lanka strain. Surprisingly the last strain exhibited a striking resemblance (70-80%) to African isolates.

The fingerprint technique was applied to epidemiologic questions concerning the origin of the 1977 Caribbean epidemic, originally thought to have been imported from Africa or Asia. Fingerprint comparisons of the 1977 Jamaican strain with both the 1978 isolate from Nigeria and the 1968 isolate from Sri Lanka clearly demonstrated that these viruses were not identical and that the 1977 Jamaican epidemic was probably not caused by either of these two particular African and Asian viruses. However, the 53% homology displayed between the Jamaican and Nigerian or Sri Lanka DEN-1 strains is striking suggesting that the Jamaican epidemic may well have resulted from a virus introduced from the African/Asian region. Unfortunately, no DEN-1 isolates are available to answer this question definitively.

In the course of this study,we inadvertantly discovered a very close similarity between viruses isolated from 2 geographically separated areas, Africa and Sri Lanka. DEN-1 has been endemic in Africa over several decades but has been observed in Sri Lanka only since 1968. Since we have observed in all other cases that a given DEN-1 fingerprint appears to be characteristic of strains from a particular geographic area, we propose that the DEN-1 virus isolated from Sri Lanka was imported to that country from Africa or a neighboring Asian country.

The DEN virus genome appears reasonably stable, at least over a period of 10 years, since the 1978 African strain of DEN-1 retained 82% of the large oligonucleotides compared with a 1968 isolate from the same locality. Interestingly, DEN virus isolated from 2 different patients during the same epidemic (Nauru Island, Western Pacific) exhibited minor but stable changes in their oligonucleotide fingerprints. Separate geographic isolates of DEN-2 strains also exhibited unique fingerprint patterns. Although preliminary, these results suggest that this technique might be a useful tool for flavivirus epidemiologists.

A requirement for precise identification of vaccine markers and a basic molecular interest in the correlation of a precise genotype with an avirulent phenotype prompted the application of the RNA fingerprinting technique to the selection of DEN-2 vaccine candidate viruses. Comparison of a virulent parent virus (PR-PGMK6) and a derived vaccine virus candidate (PR-159-S1) demonstrated that although the 2 fingerprints were very similar, each contained unique oligonucleotides which represented minor changes in their genomes. The parent virus had been passaged 6 times in primary African green monkey kidney (PGMK) cells while the vaccine virus had been passaged an additional 13 times PGMK (P19a) and subsequently 4 times in fetal rhesus lung (FRhL) cells. Although fingerprint changes were evident between the 6th and 19th passage in PGMK, it should be noted that plaque purification and selection for a temperature sensitive (ts) phenotype was actively pursued throughout these passages. Additional changes were evident following 4 passages in FRhL cells during which the only selective pressure applied was the adaptation to grow in these cells. Interestingly, the FRhL-passaged vaccine virus possessed no unique spots unto itself - all were derived either from the P19a virus or from the parent virus.

To address the question of reversion, a large-plaque (LP) revertant (non-ts) of the small-plaque (SP) vaccine virus was isolated and fingerprinted. The exciting result showed it to be identical to the vaccine virus except for a single large oligonucleotide located in the same position as an oligonucleotide characteristic of the wild type (wt) parent virus but lost during the derivation of the attenuated vaccine candidate.

Similarly, when a LP revertant of P19a material (4 passages prior to the vaccine virus) was isolated and fingerprinted, the result showed it to be similar to the vaccine virus but with one very interesting exception. The single large oligonucleotide of the vaccine virus which had shifted towards the right (to the wt parent position) in the vaccine revertant virus, had now shifted to the left in the P19a revertant virus. It is tempting to speculate that this oligonucleotide was in some way associated with the virulent or avirulent properties of these viruses. This shifting oligonucleotide is currently undergoing RNA sequence analysis to deduce the molecular changes involved in its evolution, in the hope that these sequences can be mapped to the virus genome and the protein for which it codes.

Virus isolated from a viremic human vaccine volunteer was similary analyzed. Fingerprints of this isolate showed that although it was missing 2 oligonucleotide spots present in the vaccine virus, there were no spots characteristic of the parent wild-type

virus. However, in the fingerprint of the virus isolated from the viremia, the particular shifting oligonucleotide mentioned previously appeared in the same position as that of the P19a revertant virus.

Similar studies were performed on certain DEN-1 Western Pacific virus passages generated during the production of a candidate vaccine virus. As with the DEN-2 isolates, we were able to demonstrate stable oligonucleotide pattern differences among parent and spontaneous ts SP vaccine virus candidates. However, a SP, ts mutant obtained from high level mutagenesis failed to exhibit any oligonucleotide changes from the parent virus. Therefore, it appears that the oligonucleotide mapping technique could become a very useful tool for detecting certain "marker" oligonucleotides, i.e., those of attenuated or revertant viruses, but its application is somewhat restricted as illustrated by our failure to detect differences in the mutagenized virus.

Density gradient purification of virions released from DEN-infected A. albopictus C6/36 or Vero cells readily resolved 3 optically defined bands. In addition to a purified virion band, a less dense band resembling the slow sedimenting hemagglutinin (SHA) band could be clearly resolved from a band located at the interface of the sample and the gradient. Different species of RNA were detected in these bands upon extraction and subsequent polyacrylamide gel electrophoresis. An RNA of approximately 1-2S was found in the interface band, both 8-10S RNA and 42S vRNA were associated with the SHA band, and, as expected, relatively pure 42S vRNA was obtained from purified virions. Neither of the small RNAs (1-2S and 8-10S) co-migrated with cellular tRNA peaks suggesting that these were not cellular tRNA. Fingerprints of these small RNAs invariably displayed a very obvious heavy trail near the origin in the cytidine/adenine-rich region of the autoradiograph in comparison to a very light trail in this region from fingerprints of comparable extracellular material isolated from mock-infected cells. This trail was found not to be DNA, dsRNA, poly(A) or poly (U) segments. Treatment with pancreatic ribonuclease (RNase) clearly solubilized this RNA, suggesting a poly(C) composition, perhaps similar to that found in picornavirus RNA. Fingerprints of the 1-2S and 8-10S RNA showed them to contain oligonucleotide patterns which differed both from each other and from the 1-2S interface RNA released from mock-infected cells. These small RNA appeared to be virus-specific as indicated by the observations that either the 1-2S or 8-10S RNA derived from the same virus strain (parent and vaccine) displayed almost identical patterns, while small RNA isolated from DEN-1 or DEN-2 prototype viruses or from different strains of the same serotype virus displayed unique patterns. We have not been able to correlate the oligonucleotide patterns of the small RNA with the virion 42S RNA simply because the small oligonucleotides of the viral RNA are not sufficiently separated in this region. We hope to firmly establish the origin of the small RNA (viral or cellular) and to determine the role they play, if any, in DEN virus replication (i.e., primer, leader, cleavage products, etc.).

Studies with Japanese encephalitis virus (JE) have been concerned with investigating the replication strategy of flaviviruses. Current data suggest that flavivirus virions possess a single species of messenger sense RNA (40S) capable of coding for both structural and non-structural virus proteins. Other than a double-stranded replicative intermediate version of the 40S RNA which sediments at 22S, no other intracellular virus-specific RNA have been reported. We routinely observe two additional RNA species in actinomycin-D-treated JE virus infected cells which are not found in uninfected control cells.

Presentation:

Dalrymple, J., G. Cole, P. Jahrling, E. Johnson and S. Harrison. Antigenic determinants of the Alphavirus- Sindbis. Presented, 5th Int. Congr. Virol., Strasbourg, France, 2-7 Aug 1981 (Abstract, w42/02, p. 380, 1981).

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G1529	81 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
80 10 02	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	3M161102BS10	AQ	197
b. CONTRIBUTING				
c. CONTRIBUTING	STOG 80-7,2;2			

11. TITLE (Precede with Security Classification Code)

(U) Enhancement of Host Defense against Agents of Potential 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
80 10	CONT	DA	C. In-house

17. CONTRACT/GRANT

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 81	5.5	1103
	CURRENT 82	7.5	1281

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) Metabolism; (U) Infectious diseases; (U) Toxemias; (U) Rapid detection; (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) Develop prophylactic measures and/or rational means of treatment for individuals exposed to nonprotein toxins or infectious organisms. To achieve these goals, basic studies are conducted on the pathophysiology of these toxins and infectious organisms. Such studies are needed against diseases of importance to the military.

24 (U) Develop animal models to study pathologic, biochemical, immunologic and physiologic alterations produced by toxins or infectious organisms. Use of toxin-haptene complexes to develop antibodies for rapid detection and evaluate the use of antitoxins or immunization to protect against intoxication.

25 (U) 80 10 - 81 09 A number of high-hazard diseases of BW potential do not have agent-specific vaccines or therapeutic treatment to provide effective protection to individuals exposed to these agents. To prove medical defense against these infectious diseases, studies have been involved in elucidating the pathophysiology and use of general supporting therapy in the treatment of these diseases. By use of nutrient and hormonal therapy, it has been possible to reverse the weight loss and protein wasting associated with infectious disease. In addition, therapy has been optimized to stimulate host defense mechanisms and reduce the hepatic complication associated with many of these infections. An animal model has been developed to study hemorrhagic alterations that are associated with many of these infections and to develop possible therapeutic approaches. More recently studies have been initiated on the detection, pathophysiology, prophylaxis and treatment of intoxications with small nonprotein toxins of BW importance, including the mycotoxins.

Publications: Biochem. J. 190:663, 1980; JPEN 4:277, 1980; Am. J. Clin. Nutr. 34:229, 238, 1981; Antimicrob. Agents Chemother. 19:1042, 1981. Fed. Proc. 40:338, 525, 499, 862, 901, 919, 1981; Proc. Soc. Exp. Biol. Med. 166:6, 1981; Endocrinology 108(Suppl.): 328, 1981; two book chapters.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3M161102BS10: Military Disease, Injury and Health Hazards (U)

Work Unit No. S10 AQ 197: Enhancement of host defense against agents of potential BW importance

Background:

A number of the high-hazard diseases of BW potential do not have agent-specific vaccines or therapeutic treatment to provide effective protection to individuals exposed to these infectious agents. To provide some medical defense against these infectious diseases, USAMRIID has been involved in elucidating the pathophysiology and use of general supporting therapy in the treatment of these highly hazardous diseases. One of the universal effects of infectious disease is a marked catabolic response which is characterized by anorexia, increased energy expenditure, weight loss, and protein wasting. Therefore, it was rationalized that nutrient supportive therapy could reverse this catabolic response and enhance host defense mechanisms against infectious disease.

From threat analysis study by U.S. Army Medical Intelligence Agency, it has been determined that certain of the small nonprotein toxins are potential BW agents. To help in the development of medical defense against these toxins, it was necessary to develop a program for safe handling and decontamination; rapid detection and identification; determination of molecular mechanism of action; elucidate the pathogenesis and physiological aberrations; development of methods of prevention, diagnosis and therapy; and evaluate potential aerosol threat from these small nonprotein toxins of BW importance. The following actions have been undertaken by the Department of Pathophysiology and Therapeutics of the Physical Sciences Division to develop this program:

The work unit title will be changed to "Pathophysiology of toxemias/diseases due to marine or fungal toxins or microbes of potential BW importance."

A plan for a research task on study of nonprotein toxins of potential biological warfare importance has been developed and submitted for review.

A dozen or more small nonprotein biological toxins could be of potential BW importance. Because of limitations in personnel and resources, priorities had to be developed which were based on a number of factors including threat assessment; availability of toxin-antitoxin; of toxin antitoxin, and assay; contract program success; dangers to research personnel; and uniqueness of the study. Based on these criteria, it was decided to carry out initial studies on T-2 mycotoxin and marine toxins - saxitoxin and tetrodotoxin. The expertise and techniques developed with these toxins will be of extreme value in future studies on other small nonprotein toxins of BW importance.

Progress:

"Therapeutic reversal and abnormal host amino acid, protein, and RNA metabolism during infectious disease of unique military importance." (R. W. Wannemacher, Jr.)

In a recent workshop on "Nutritional supports of the patient: Research direction for the 80's" at the National Institutes of Health, Drs. Wilmore and Kinney (1) stressed the need for nutrient support in patients with life-threatening infection and who had lost 10% of their body weight. These experts also emphasized the importance of development of support therapy that would optimally stimulate host defense against infectious disease. It was also recommended that research on nutritional support in infections requires investigation in both animals and man. An important factor in developing a rational approach to nutritional supportive therapy in infected individual is a knowledge of the effect of various nutrients on protein dynamics in various cells and tissues of the body.

Previous progress reports (2) had suggested that infusion of amino acids and high dextrose (85% of the calories) solutions during infectious disease in monkeys which caused mild hepatic damage resulted in an increased incidence of glucose intolerance, insulin resistance, thrombosis and death. These complications were observed in 2 normally nonlethal infections, namely the Asibi strain of yellow fever (YF) in the African green monkey and the LVS strain of Francisella tularensis in the cynomolgus monkey. Previous studies demonstrated that infusion of 8% dextrose plus a 48% branched-chain amino acid (BCAA) solution was highly effective in preventing protein wasting during gram-positive or -negative infections in the cynomolgus monkey. Therefore, this combination of low dextrose and high BCAA mixture was studied in both YF and F. tularensis infections in the monkey. With this nutrient support regimen, protein wasting was prevented in both infectious diseases. Further, the monkeys did not develop glucose intolerance, insulin resistance or thrombosis. Lethality was similar to that observed in monkeys infused with electrolyte solution and fed orally. These results support the conclusion that high dextrose infusion during infections which cause mild hepatic damage should be done with extreme care, if at all.

It has been demonstrated that the infected rat has a reduced capacity to convert long-chain fatty acids (LCFA) into ketones (3). This results in a slightly reduced efficiency in utilization of long-chain triglycerides as energy substrate by the septic host. In addition, there is a reduced clearance of these triglycerides in the septic host and a tendency to develop marked hypoglycemia. Thus, we have been interested in trying to find a caloric substrate which could be converted to ketones and thus spare requirements for glucose in the septic host. In attempts to look at other caloric substrates we have evaluated IV infusion of butanediol, which does not have to enter the pathway of beta-oxidation to produce ketones. When infused as a 3 or 6% solution along with the 48% branched-chain mixture, this compound had a hypnotic effect on the monkey, which proved to be lethal. While butanediol can be fed at a concentration of 20% in all diets, it cannot be utilized in IV infusions. Monoglyceride of acetylaceto acid is another energy substrate in which the acetoacetic acid is oxidized independently of the carnitine

transport into the cell mitochondria and its monoglyceride is a water-soluble compound. When substituted isocalorically for dextrose and infused along with 48% BCAA, monoacetoacetin was well tolerated by by the septic monkey and as effective in preventing protein wasting during pneumococcal sepsis as isocaloric amounts of long-chain triglycerides or dextrose. Since monoacetin is water-soluble and has a high caloric density, it may prove to be a good alternative energy substitute in the place of dextrose. The FA of medium-chain triglycerides can enter the mitochondria via a carnitine-independent mechanism. When triglycerides which contain 32-80% medium-chain FA were infused along with 48% BCAA in control and septic monkeys, it proved to be a highly toxic solution and was not very ketogenic. Thus, these data suggest that medium-chain triglycerides are not an effective energy substrate when given by IV infusion.

In order to study the effects of infection in nutrient support therapy on the protein dynamics of various cells of the host, a model was developed which entailed the constant infusion of [14C]leucine for 6 hr. By this time period, the specific activity of the leucine and the protein-free filtrate of the plasma had reached a plateau, indicative of constant precursor pool size. Infusion of an 8% dextrose solution significantly reduced leucine turnover and release from the breakdown of total body protein as compared to the infusion of 4.25% of amino acids. The presence of infection increased the rate of leucine turnover and breakdown of total body protein. As with nitrogen balance, these results told very little as to what is happening in the various tissue compartments in the body. IV infusion of dextrose significantly decreased the fractional rate of synthesis and amount of mixed plasma proteins synthesize compared to the amino acid group. The presence of pneumococcal sepsis increased the synthesis of mixed mixed proteins as compared to the noninfected controls. When the monkeys were infused with amino acids, the rate of synthesis of plasma albumin was significantly increased compared to dextrose alone or with amino acid infusion. This resulted in a significantly greater amount of plasma albumin in the monkeys infused with amino acids compared to those receiving dextrose. While the presence of pneumococcal sepsis did not alter the rate of albumin synthesis, the plasma albumin concentration was decreased in all experimental groups. The fractional rate of synthesis in mixed lymphocyte protein was significantly lower in monkeys infused with dextrose compared to amino acids. Pneumococcal sepsis did not significantly alter this rate of synthesis, but lymphocyte counts were reduced in the blood of all infected groups. The stimulation index for lymphocytes by varying mitogens was significantly greater in monkeys receiving the IV amino acid compared to the infusion with dextrose. In all groups, pneumococcal sepsis markedly reduced the stimulation produced by the various antigens. While dextrose reduces the rate of turnover of total body protein, amino acid infusion stimulates total plasma albumin, and lymphocyte synthesis as well as lymphocyte function, suggesting that amino acid infusions are more important stimuli of host defense mechanisms than dextrose. Despite similar rates of albumin synthesis in both control and infected monkeys, plasma albumin concentrations were significantly reduced in a septic group. These results suggest that the major effect of dietary constituents is on the synthesis of plasma albumin, while infectious disease or trauma appear to have stimulated breakdown of this plasma protein.

In attempting to understand the mechanism by which infectious disease is able to alter protein dynamics in various cells of the body, body, a crude extract of a hormone-like substance which is released from stimulated PMN, so-called "LEM," was infused for 24 hr in the rat. During the last 6 hr of the infusion the rats received a constant amount of [14C]tyrosine. Whole- body turnover of tyrosine was increased in the rats infused with LEM, but not in those given the heat-inactivated product. Breakdown of skeletal muscle protein was slightly increased in LEM-treated rats, while synthesis of liver and plasma proteins was elevated. This is suggested evidence that LEM may play a regulatory role in stimulating the altered protein metabolism in the infected host.

A new research plan is being developed which would change the title of this Research Unit to "Development and characterization of guinea pig and monkey models to study toxicity, prophylaxis, and treatment of intoxications with small nonprotein toxins of potential BW importance."

Before initiating any studies on small nonprotein toxins, it is necessary to develop safe handling and decontamination procedures. A careful review of the literature and queries to some of the leading experts in the area of mycotoxin and marine toxin research did not provide the necessary procedures. Thus, it was necessary to establish our own criteria for safe handling and decontamination for each of the small nonprotein toxins studied at USAMRIID. A mouse bioassay has been developed to measure procedures for decontamination of T-2 toxin, tetrodotoxin, and saxitoxin. A skin test bioassay in young rats and guinea pigs has been developed to measure decontamination of the skin irritability of T-2 toxin. A 30-min contact with 0.01% sodium hypochlorite solution inactivates at least 99.9% of tetrodotoxin. A 30-min contact with 2.6% sodium hypochlorite solution or 16 hr with 0.5% sodium hypochlorite solution decontaminates T-2 toxin. Based on these observations, an SOP was developed for safe handling, decontamination, and disposal of waste from the small nonprotein toxins.

"Therapeutic correction of energy metabolism alterations during infections of unique importance in military medicine." (H. A. Neufeld)

Over the past 6 years, it has been established that most infectious diseases in the rat result in a curtailment of normal fasting ketonemia (4, 5). Since ketones can replace glucose as an energy substrate for cells such as those in the brain and muscle, the inhibition of starvation ketonemia coupled with anorexia means that the infected host must utilize glucose as its major energy substrate. To meet its elevated energy requirements, the infected host must break down body protein and utilize amino acids for the synthesis of glucose. Thus, the inhibition of development of fasting ketonemia in the infected host contributes to the protein-wasting associated with this disease. There appears to be a possible endocrine imbalance which is responsible for the inability of the infected host to develop starvation ketosis (6). Therefore, a program has been initiated to evaluate the use of hormonal therapy to stimulate the ketogenic response in the septic host. Hormones from the thyroid, adrenals, and gonads did not alter ketosis in the infected rat. Currently, some of the pituitary hormones are being evaluated as potential regulators of the ketogenic response in the fasted-infected rat. When pharmacological doses of adrenal

corticotrophic hormone (ACTH) were administered to fed, fasted and fasted-infected rats, it stimulated a significant increase in plasma ketone bodies and free fatty acids. These observations suggest that some of the pituitary hormones may play a role in regulating rates of ketosis in the rat.

A major shift has been made in the research efforts of this research unit. Future studies will be involved in the development and characterization of rat and guinea pig models to study toxicity, prophylaxis and treatment of intoxication with the small nonprotein toxins of potential BW importance. A new research plan is being formulated and work has been initiated to study toxicity of T-2 toxin in Fischer-Dunning rats. LD50 values have been determined for T-2 toxin when administered by the IP, IM, or SC route. The LD50 was approximately 0.5 mg/kg body wt when the toxin was given by the IM or SC route, but was approximately 2 mg/kg body wt when given IP. Depending on the administered dose, all rats died between 10 and 20 hr after inocula- 10 and 20 hr after inoculation. Clinical signs included diarrhea, lethargy, and bloody stools. In those rats that survived the higher doses, anorexia was observed for from 4-7 days after inoculation Histopathologic examination of those rats which died revealed acute and severe necrosis of both the large and small intestine, extensive necrosis of lymph tissue, spleen, and Peyer's patches, marked inflammation of the parotid salivary glands, inflammation of the seminal vesicles, and severe congestion of spleen and adrenals. These observations stress the marked radiomimetic effects of T-2 toxin. This is the first systematic study of the measure of toxicity of T-2 toxin by various routes of administration. The differences observed between the IM and SC routes compared to IP injection stress the need for understanding rates of clearance detoxification of administered T-2 toxin.

"Regulation and involvement of acute-phase proteins in infections of biological importance." (W. L. Thompson)

During most infectious disease, there is an increase in the concentration of certain serum proteins, termed "acute-phase proteins," which is presumably related to a stimulation of synthesis of these proteins by the liver (7, 8). Although several of the acute-phase proteins have been implicated in such processes as antiproteolytic activity, wound handling, protection of hemoglobin, transport of metal ions, and regulation of immune response (9), very little is known about the specific involvement of each of these proteins or how they fit in the overall host response to infection. In order to conduct these studies it is necessary to isolate various acute-phase proteins from the serum of rats. Sufficient quantities of alpha-1- and alpha-2-macroglobulins, haptoglobin, and alpha-1-glycoprotein have been isolated, purified, and characterized. Alpha-1-glycoprotein has not been previously identified in the serum of the rat. Within 48 hr of exposure to Streptococcus pneumoniae infection, serum concentration of this protein is increased 10-fold. Thus, this is a new acute-phase protein that has been purified and characterized. Antibodies have been developed in the rabbit against all of the above cited acute-phase proteins as well as albumin, a non-acute-phase protein. An isolated rat spleen

cell procedure has been utilized to evaluate the effect of the acute-phase proteins on mitogen and antigen stimulation of lymphocyte transformation. Heat-treated (56 C for 30 min) rat serum is required for the lymphocyte stimulation. Heat-treated serum from inflamed treated serum from inflamed or fed rats was inhibitory when compared to serum from fasted controls. In general, alpha-1-globulin enhanced antigen and mitogen stimulation of the spleen cell lymphocytes. These results support the concept that some of the acute-phase proteins can play a role in regulation of immune response of the host.

This research unit is being phased out and a new one is being developed to study cytotoxicity, detection, and mechanism of action of small nonprotein toxins of BW importance. These in vitro systems will also be utilized to evaluate potential procedure for protection and treatment of intoxication with the small nonprotein toxins.

"Production and use of endogenous pyrogen (EP) antibodies in early detection of infections of military importance." (W. Critz)

EP is a low MW protein released from phagocytic cells which is in part responsible for the fever observed in most bacterial and viral infections. It has not been possible to separate the EP components from the other so-called "LEM" properties of the crude extract released by phagocytic cells. A sensitive intracerebroventricular assay has been developed for the detection of EP. Varying procedures have been employed to try and purify EP in order to develop specific antibodies and an immunoassay. Because of the low concentration of a highly biologically active material in the crude extract, attempts at purification and characterization of a single protein of EP have not been successful here or in other laboratories working on this problem. While detection of EP could possibly identify the presence of an infectious disease and could play a role in understanding the pathogenesis of fever, the difficulty of purification and development of a sensitive immunoassay has severely reduced its use as a research tool in infectious disease. Since CPT Critz has been transferred from the institute this research unit is terminated.

"Effects of infection on muscle enzymes in relation to physical training." (H. A. Neufeld and D. J. Crawford)

While there is a great deal known about the physical capacities of healthy men, very little is known about their capacities when ill. Present studies were designed to determine the effects of acute and continuing exercise on the metabolic and immunological response to lethal bacterial and viral diseases of BW potential. Swimming and wheel exercise in the rat were utilized to answer the following questions: (a) Does infection alter performance capacity and what are the effects of training on these infection-induced changes? (b) Since infectious illness is often associated with myalgia, negative nitrogen balance, breakdown of muscle protein and generalized malaise, how does alteration in energy metabolism relate to energy infection-induced changes? (c) What effect does acute exercise and preconditioning prior

to, during, and after inoculation with a lethal dose of bacteria have on dose-related mortality and during what stages of an illness is exercise beneficial or detrimental? and (d) Does acute exercise or conditioning alter host immune responses and does training reduce or increase an individual's susceptibility to disease?

Bacterial infection during endurance training in rats reduced the running performance and altered metabolic response to both stresses. Submaximal exercise during acute bacterial infections increased disease-related mortality, while preinoculation exercise reduced susceptibility to lethal challenge. Acute exercise was immunosuppressive as measured by reduced spleen weights, impaired mitogenic-antigenic dependent lymphocyte stimulation, impaired endogenous PMN chemiluminescence and reduced microagglutination antibody titers in immune rats. Prior conditioning did alter the rats' response to acute running exercise, but did not change infection-related responses or alter susceptibility to lethal challenge. From these observations it was concluded that physical fitness established by endurance training does not enhance immunological resistance against infection and, at times, may render conditioned individuals more susceptible.

While these studies do raise some important questions as to the effect of exercise on the immune response of the host, it has been decided to temporarily terminate this research unit, since CPT Crawford has left the Army. If another military exercise physiologist should join the staff, the research unit may be restructured to involve studies on effect of exercise in animals intoxicated with small nonprotein toxins.

"Growth hormone and infection." (D. L. Bunner)

Both growth hormone (GH) and insulin are major anabolic hormones, which undergo considerable changes in effectiveness and production during infection. It appears that both GH and insulin are elevated in most serious infections; in spite of this, there is a considerable loss of body nitrogen. The actual triggering mechanism for nitrogen losses is not well understood nor have clear cut causes for elevation of GH and insulin been determined. A number of mechanisms have been proposed including fever, elevated adrenal steroids, stress and direct cellular injury. Other, perhaps more physiological, mechanisms have been proposed as well, including the release of EP or an increase in the physiologic CNS responsiveness perhaps due to alterations in the level of metabolic fuels or in the cells. In order to arrive at a reasonable hypothesis to help correct the more basic problems of nitrogen loss, considerable information regarding this mechanism and its pathophysiological relationship to GH and insulin need to be determined. The major thrust of this research unit is to try and collect sufficient information to actually arrive at a hypothesis then, involve direct patient therapy. Certainly at this point in time there is reason to believe that either GH or insulin therapy or both could be of practical benefit in treatment of infectious disease and some very limited studies have been done in this regard. Clinical studies have been reported documenting a favorable effect of insulin in nitrogen loss in burned loss in burned patients. It remains to be shown, however, that a more positive nitrogen balance can be translated to an improved immune response, shortened recovery time or lower mortality.

During the illness phase of sandfly fever virus infection in 2 males and 3 females, marked elevations of serum GH were observed throughout the 24-hr period, with no increase in nocturnal release The actual concentrations of serum GH at night were nearly identical to the basal study. Glucose was elevated with no significant changes in insulin values during the 24-hr study. Glucose tolerance was clearly abnormal, with associated hypoinsulinemia. In another study, one subject was partially immersed in a hot tub at 41 C for 2 hr. Body temperature rose as expected and a prompt increase was observed in serum GH concentration. As soon as body temperature returned to normal, GH concentrations also fell. Although clearly more subjects are needed, it is suggested that the altered body temperature itself may at least play a very important role in regulating GH release.

To run similar 24-hr studies in cynomolgus monkeys, a model had to be developed which would allow for frequent repeated blood sampling. Arterial catheters were originally utilized, but they tended to result to result in repeated embolic phenomena. Venous catheters have not reliable for repeated sampling over time. Recently, it has been possible to successfully catheterize the right atrium by way of the jugular vein and consequently eliminate the problem of sample reliability. Approximately 32 monkeys have been studied using this method, and 31 of them were sampled successfully. There was no mortality and no significant catheter infection. Using this model in cynomolgus monkeys it was possible to demonstrate a periodic release of GH by day and night with no significant increase at sleep onset. EEG monitoring was not done. This response in the monkey is quite different from that observed in man. After endotoxin challenge in cynomolgus monkeys, serum GH concentrations are elevated and hyperglycemia develops, with a suggestion of a mild increase in insulin concentration. F. tularensis was also studied, but less successfully in the sense that the monkeys did not seem to be predictably and reliably ill in sufficient degree to cause any substantial alteration in either plasma GH, glucose or insulin.

A chronically catheterized rat model has been developed which demonstrated normal pulsatile release of GH which occurred approximately every 2 hr. Following endotoxin treatment, the basal GH concentrations dropped to near 0 and the pulsations stopped abruptly. The rat has also been utilized to demonstrate the in vitro role of insulin in inducing RNA synthesis in the liver.

"Metabolic alterations in fatty acid metabolism during infection military importance." (J. G. Pace)

Livers from infected rats have a decreased ketogenic capacity associated with an accumulation of carnitine and a decrease in CoA fatty acyl esters (3). During infection more LCFA are directed away from oxidative pathways and ketogenesis towards esterification. The infection-related decrease in ketogenesis is due to changes in the activities of enzymes involved in hepatic LCFA metabolism. Previous studies have shown that activation of LCFA is unchanged during infection while alpha-glycerol phosphate acyltransferase activity increases and carnitine palmityltransferase activity decreases (10). These findings suggest an increased triglyceride formation and

possible increased activity of enzymes regulating FA synthesis. Since FA-activating enzymes, citrate cleavage enzyme, citrate enzymes, FA synthetase, citrate cleavage enzyme, citrate synthetase are key enzymes involved with metabolism of FA, their activities were measured in livers from rats infected with S. pneumoniae. Citrate cleavage enzyme and citrate synthetase were only slightly elevated during infection compared to fasted controls. However, in other experiments performed earlier (10), the products of citrate cleavage enzyme reaction (oxalocetate and acetyl-CoA) increased, suggesting an elevated enzyme activity. Malonyl-CoA concentration did not increase in the fasted-infected rats compared to fasted controls (3). This may be due to an increase in both the acetyl-CoA carboxylase activity and fatty acid synthetase activity. Changes in the enzymes involved in fatty acid metabolism may result in reduced production of ketone bodies in the fasted rat during pneumococcal infection. A shuttling of acetyl-CoA into cytosol might explain the reduced rate of ketogenesis. This necessary energy-yielding fuel, in the form of FA,is directed away from oxidative pathways and ketogenesis towards pathways designed for the synthesis and storage of fat This may be considered a FA futile cycle in the liver of the infected rat.

The research on FA metabolism has essentially been completed and a new research plan is being developed to investigate the absorption, distribution, elimination, and detoxification of the small nonprotein toxins in appropriate in vivo and in vitro models. Studies will be initiated to elucidate the biochemical mechanisms of toxicity of these toxins. By manipulating the systems to alter the ates of toxin metabolism and toxic response, it is hoped to evaluate potential therapeutic agents against such intoxications. In an effort to understand the biochemical nature of toxic response, preliminary studies have been initiated on the effects of T-2 mycotoxin on hepatic mitochondrial respiration. It was found that 2.2 mM T-2 toxin inhibited state 3 respiration by 40% when succinate or pyruvate plus malate was used as a substrate in this mitochondrial system. These findings suggest that T-2 toxin may affect several mitochondrial functions, and that its toxic effects are due in part to its inhibition of cell respiration. Analytical methods for determination of individual mycotoxins by TLC and GLC are being developed. To date, a TLC procedure has been developed for separation of T-2 toxins and several of its metabolites.

"Changes in leukocyte function during the course of viral and bacterial studies. (J. P. McCarthy)

Circulating PMN have an increased endogenous chemiluminescence (CL) activity during various bacterial infections in rat and guinea pig. CL appears to be a relatively effective procedure for measuring functional activity of circulating PMN. They appear to have an inhibition of activity during exercise or in the presence of toxin from or in the presence of toxin from Legionella pneumophila and an increased activity in the presence of LEM or E. coli endotoxin. Recently it has been possible to demonstrate for the first time that alterations in PMN CL during bacterial infections can be detected using whole blood rather than PMN isolates. This technique may have potential value in the rapid diagnosis and prognosis of bacterial infections in the host. This research effort is terminated due to the departure of Dr. McCarthy.

"Evaluation of hemostatic derangement in infectious disease of military importance." (T. M. Cosgriff)

There is very little information concerning the mechanisms which lead to hemorrhage in the hemorrhagic viral fevers, which are of potential BW importance. In order to study the mechanisms of actions that lead the hemorrhagic state in these viral infections, a model of Pichinde viral infection in strain 13 guinea pigs has been developed for use in these studies. In addition, techniques have been developed for the determination of tissue factors elaborated by mononuclear cells which could stimulate hemostatic abnormalities associated with infectious disease.

In collaboration with the Department of Antiviral Therapy, Virology Division, studies were completed on the hematopoietic effects of the antiviral drug, ribavirin, in rhesus monkeys. Previous studies in experimental animals and human volunteers has demonstrated a dose-dependent anemia with ribavirin treatment. Therefore, studies were initiated to characterize the anemia that develops in monkeys treated with ribavirin. In these experiments, ribavirin produced dose-related hematologic toxicity characterized by normochromic, normocytic, hypoproliferative anemia. Ribavirin treatment in the monkey is also associated with thrombostasis, which is also dose-dependent and appears to be due at least in part, to megakaryocyte hyperplasia. Ribavirin did not have an effect on WBC numbers nor did it significantly alter platelet function. RBC survival studies indicate that the anemia produced by ribavirin treatment is due, in part, to increased RBC destruction, as well as decreased production. This increased destruction is probably extracellular. The hematologic changes which occur with short-term administration of ribavirin in monkey appear to be reversible but reduced marrow cellularity was still observed 65 days after stopping ribavirin treatment. This suggests a possible long-term toxicity associated with the ribavirin-induced anemia.

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3. Bunner, D. L., and G. A. McNamee, Jr. Growth hormone response to intravenous E. coli endotoxin and Streptococcus pneumoniae sepsis. Presented, Annu. Mtg. FASEB, Atlanta, GA, 12-17 Apr 1981 (Fed. Proc. 40:862, 1981).

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G3874	81 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. DIST'N INSTR'N	8b. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
81 05 29	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	131
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Role of Macrophage Proliferation and Activation in the Control of Viral Infections

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 002600 Biology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
81 05	CONT	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 81	0.4	64
	CURRENT 82	1.0	103

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) BW defense; (U) Military medicine; (U) Viral diseases; (U) Macrophages; (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) Study interaction of viruses and macrophages in order to be able to counteract viral diseases for which no therapy or immunization is possible or augment existing available therapeutic measures. The viral diseases of concern are those of high-hazard encountered by a BW attack or in a research laboratory.

24. (U) Develop experimental models of infection in experimental animals. Macrophages will be obtained from mice or guinea pigs of different ages, infected with model viruses to examine proliferative ability and examined for cytotoxicity. If successful, efforts will be made to modulate proliferation or enhance cytotoxicity.

25. (U) 81 05 - 81 09 - The proliferation of macrophages, which is necessary for the host to develop cellular immunity, is inhibited by infection in vitro with the arenavirus Pichinde. This may represent a novel mechanism whereby a virus can subvert host resistance. It helps to explain the immunosuppression observed in human arenavirus infections, occurring with no discernible morphologic damage to the cell. Inhibition is dependent upon the dose and duration of exposure to virus and is blocked by a specific antiserum to Pichinde. The inhibition is not due to soluble mediators, such as interferon or prostaglandins. Analysis of the growth of Pichinde in macrophages revealed that normal macrophages replicate virus. Neither inflammatory macrophages nor those stimulated to proliferate by growth factors showed an increased capability of inhibiting viral replication. These results suggest that macrophages are a target for arenavirus infection; such infection causes profound inhibition of macrophage proliferation which would be expected to suppress the immune response to the infection; and interventions should be directed toward enhancing the macrophage's ability to control the virus and enhance host resistance.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A166101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C 00 131: Role of Macrophage Proliferation and Activation in the Control of Viral Infections

Background:

The critical role of the macrophage in natural and acquired resistance to viruses is well recognized (1). This important role is supported by observations that the genetic and age-dependent resistance or sensitivity of animals, as well as the relative virulence of viral strains such as the arenavirus, lymphocytic choriomeningitis (LCM) (2), correlates with the ability of macrophages to replicate viruses in vitro. Furthermore, agents which stimulate or impair macrophage function can enhance or decrease resistance to experimental virus infections [e.g., Semliki Forest virus (3, 4)]. In addition, some agents such as the arenaviruses, display a tropism for the host's macrophages (5, 6).

The macrophage as part of the reticuloendothelial system is often the first cell encountered by a virus. As such it is the prime effector cell in the nonspecific phase of host resistance. During the development of cell-mediated immunity (CMI) to intracellular pathogens, the macrophage, under the influence of the T lymphocyte, is stimulated to proliferate and to become activated (i.e., possess enhanced microbicidal activity) as shown in the classic experiments of Mackaness. Thus, during the course of a virus infection, there are more macrophages available to resist the virus. Whether the individual metabolically activated macrophage is better able to restrict the infection has not been determined. It is clear though that the macrophage is one of the important effector cells in CMI to viruses. Understanding of the interaction between virus and macrophage, the virulence mechanisms of the virus which impair resistance, and the determinants for control of infection by the macrophage will eventually enable us to modulate macrophage function using natural or synthetic products to enhance resistance to infections in man.

Progress:

Inhibition of Macrophage Proliferation by Pichinde. In our study of the interaction of viruses with macrophages we have concentrated our efforts on the arenavirus Pichinde (PIC) and our original observation that infection with this virus in vitro blocks macrophage proliferation. This may be of particular pathogenic significance since other Pichinde-like arenaviruses have a predilection for the reticuloendothelial system.

Mouse peritoneal macrophages elicited with starch were cultivated in vitro and infected with PIC virus. At various times, L-cell conditioned medium containing macrophage growth factor (MGF) was added along with [^{3}H] thymidine and DNA synthesis measured. Prior infection with PIC caused a dramatic inhibition of the DNA synthesis which occurs in macrophages exposed to MGF. The effect was dependent upon the dose of virus and the duration of infection before exposure to MGF. In various experiments, PIC at an MOI of 1-5 caused a 50-95% inhibition of DNA

synthesis. Controls consisting of mock infected medium and heat-killed PIC showed no inhibitory effect demonstrating that viable PIC is necessary for inhibition to occur. In the next series of experiments we demonstrated that pre-incubation of PIC with immune, but not normal monkey serum, completely blocked the inhibition of DNA synthesis, showing the specificity of the inhibitory effect (Table I). Inhibition of macrophage DNA synthesis was observed with 4 different viral pools.

TABLE I. INHIBITION OF MACROPHAGE DNA SYNTHESIS BY PICHINDE VIRUS[a]

MGF	% DNA SYNTHESIS
Alone	100
PIC (MOI 0.01)	40
PIC (MOI 5)	15
mock medium	100
heated PIC (MOI 5)	100
PIC + immune monkey serum	100
PIC + normal monkey serum	30

[a]A compilation of data from several experiments. DNA synthesis induced by MGF alone in the absence of infection varied from 8,000 - 15,000 [^{3}H] thymidine cpm incorporated/cover slip in various experiments. This has been set at 100% in the different experiments for comparative purposes.

We then showed that the inhibition was not due to simple toxicity of PIC for the macrophage. Cell survival of unstimulated macrophages as measured by adherence and viability was unaffected by infection with PIC 6 days earlier (controls: 54 $\pm$ 2 cells/microscopic field; > 95% viable vs. PIC: 53 $\pm$ 1 cells/microscopic field; > 95% viable). In addition, there was no cytopathic effect of the virus observable by phase microscopy. This is similar to what is reported for LCM in mouse macrophages (7). It should be noted, however, that the increases in cell size, number and spreading induced in unstimulated macrophages by MGF is abrogated by infection with PIC. To eliminate the possibility that the observed inhibition was due to changes in transport or pool size of nucleic acid precursors, we measured DNA using a spectrofluorometric assay. By this method it was also shown that PIC blocked macrophage DNA synthesis (Table II) confirming the results with [^{3}H] thymidine incorporation.

There is evidence that prostaglandins and perhaps interferon secreted by macrophages may function as a negative feedback control mechanism to inhibit the proliferation of macrophages (8). For this reason we determined whether the inhibition by PIC was due to release of inhibitory substances by the macrophages. Macrophages were cultivated in the presence of indomethacin to inhibit their synthesis of prostaglandins. They were then infected with PIC followed by

TABLE II. INHIBITION OF MACROPHAGE DNA SYNTHESIS BY PIC INFECTION

CULTURE CONDITION	PIC INFECTION	μg DNA/dish ± SE (n = 3)
Control medium	-	1.88 ± 0.03
MGF	-	7.50 ± 0.23*
MGF	+	2.22 ± 0.02*

*$P < 0.001$.

stimulation with MGF. Indomethacin at concentrations of 10^{-7} to 10^{-4} M did not eliminate the inhibition produced by PIC infection. The indomethacin by itself had no affect on DNA synthesis (Table III, data are only shown for 10^{-4} M indomethacin). Thus prostaglandins do not appear to be involved in the inhibition produced by PIC infection.

TABLE III. EFFECT OF INDOMETHACIN ON PIC INHIBITION OF MACROPHAGE DNA SYNTHESIS

MGF	DNA SYNTHESIS ([^{3}H] thymidine cpm ± SE/cover slip)
Alone	18,256 ± 919
PIC	167 ± 27
Indomethacin (10^{-4}M) + PIC	199 ± 10
Indomethacin (10^{-4}M)	18,050 ± 1,579

To determine whether interferon or other soluble factors produced by macrophages after infection with PIC was responsible for the inhibition we performed the following experiment. Macrophages were incubated with PIC for periods from 2-6 days. The medium was then removed and freed of virus by ultracentrifugation and cobalt irradiation. This material was then applied to cultures of fresh macrophages to see if it would inhibit MGF-induced DNA synthesis. No significant inhibition was produced by these virus-free supernatants at concentrations up to 50%. These results suggest that soluble mediators possibly including interferon produced by PIC infection are not responsible for the inhibition of DNA synthesis in macrophages. We plan to test these supernatants to see if any interferon is in fact induced by PIC infection and to determine whether interferon will inhibit DNA synthesis in this system. In addition, we will determine whether the activated macrophage produces more interferon in response to virus infection as has been reported to occur with interferon inducers (9). Preliminary experiments with other viruses suggest that the arenaviruses are more potent macrophage inhibitors than toga-viruses. These relationships will be explored further.

In all the experiments described above, macrophages were infected with PIC before being stimulated by MGF. We have also demonstrated that DNA synthesis in macrophages previously stimulated by MGF is inhibited by a subsequent infection with PIC. These experiments and those on growth of PIC outlined below suggest that proliferating macrophages are not necessarily activated in order to have enhanced microbicidal activity. Lymphocyte-derived lymphokines may be necessary for this further differentiation and studies to address this are in progress.

Growth of PIC in Macrophages. These experiments were performed to determine a) if PIC grows in macrophages in vitro and b) if the activated and proliferating MGF stimulated macrophage differs in its ability to support viral growth (e.g., has a restricted ability to replicate virus). Peritoneal macrophages were cultivated in the presence or absence of MGF for one or 4 days. They were then infected with PIC (MOI ≈ 2) and viral growth determined over the next 3 days. Table IV shows that macrophages do support the replication of PIC. However virus grew to the same titer in both MGF-stimulated and nonactivated macrophages. Similar results were obtained with resident and starch-elicited macrophages treated with MGF for periods of one or 4 days prior to infection. Analysis by surface immunofluorescence using a fluorescein-conjugated monkey anti-PIC serum confirmed that macrophages were infected. Furthermore treatment with MGF for 2 days after PIC infection did not prevent the spread of virus to uninfected macrophages as determined by fluorescence. With an MOI ≈ 0.025, < 1% of cells were infected 2 days later. When these cultures were then grown in MGF for 4 days more, 100% of the macrophages expressed PIC antigen by FA. Thus, the proliferating macrophage does not have an enhanced ability to restrict the replication of PIC. Similar studies with other viruses are in progress.

TABLE IV. GROWTH OF PIC IN CONTROL AND MGF-STIMULATED MACROPHAGES

DAYS AFTER INFECTION	LOG_{10} PFU/0.2 ml	
	C	MGF
0	2.0	2.0
1	3.2	3.3
2	4.6	4.9
3	4.8	5.2

Other attempts to assess the growth of PIC in macrophages including an infectious center assay with Vero cells and plaquing virus directly in macrophage monolayers were not successful.

Adherence and Cytotoxicity of Macrophages for Viral Infected Target Cells. Our initial studies have concentrated on developing assay systems for both PIC and Semliki Forest (SF) viruses. Vero and L cell monolayers infected with PIC were overlaid with macrophages at a time when discrete foci of infected cells were observed by FA. In pilot studies individual macrophages did not appear to adhere preferentially to infected cells when analyzed by FA and phase microscopy.

Further studies will quantitate binding with macrophage monolayers grown in the presence or absence of activators and overlaid with labeled infected target cells.

Studies with PIC have used Vero and L cells; but we are attempting to develop other syngeneic target cells, since L cells themselves appear to activate macrophages. To date a mouse mastocytoma and 3 lymphoma lines are resistant to infection with Pichinde. The P815 mastocytoma line can be used as a target for SF and a ^{51}Cr cytotoxicity assay is currently being evaluated to determine the effect of macrophage differentiation and activation on cytotoxicity of infected cells.

Cultivation of Suckling Mouse Macrophages. We have succeeded in cultivating suckling mouse macrophages in sufficient quantities so that we can now compare their ability to replicate PIC and SF with that of adult cells by the use of immunofluorescent techniques. We will also be able to determine if suckling mouse macrophages respond to MGF as do adult cells and whether this proliferation can be inhibited by viral infection.

Presentation:

Friedlander, A., and P. Jahrling. Inhibition of macrophage DNA synthesis by viral infection. Presented, 81st Annu. Mtg, American Society for Microbiology, Dallas, TX, 1-6 Mar 1981 (Abstract T81, p. 251, 1981).

Publications:

None.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G3875	81 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
81 06 05	D. Change	U	U	NA	NL	XX YES ☐ NO	A. WORK UNIT

10. NO./CODES:	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	61101A	3A161101A91C	00	132
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Role of T-Cells in Pathogenesis of Argentine Hemorrhagic fever

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
81 06	83 06	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE		a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR	PRECEDING 81	0.4	51
	CURRENT 82	1.0	173

19. RESPONSIBLE DOD ORGANIZATION

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ASSOCIATE INVESTIGATORS

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NAME: POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code)

(U) BW defense; (U) Military medicine; (U) Argentine hemorrhagic fever; (U) Arenaviruses; (U) Immunology; (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 pathogenesis of Argentine hemorrhagic fever (AHF) in animal models. Application of these principles to testing the effect of immune serum, antiviral drugs and experimental vaccines on the course of disease will be conducted. Such information will be valuable for this and other arenaviruses of BW importance.

24. (U) After determining the effector mechanism for cytotoxicity in a tissue culture system, attempt to correlate such a mechanism in a guinea pig model with protection or disease induction. Efforts then will be made to manipulate the mechanism(s) for the benefit of an infected individual.

25. (U) 81 06 - 81 09 - A cytotoxicity assay for the causative organism of AHF (Junin virus)-infected cells by spleen cells from convalescent guinea pigs has been developed and is believed to be due to antibody-dependent cell-mediated cytotoxicity (ADCC). A possible model for virulent Junin infection has been shown in cyclophosphamide-treated guinea pigs infected with attenuated virus. Results have shown that virulent Junin virus replicates well in vitro in mouse macrophages, while attenuated virus replicates poorly or not at all.

* Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A166101A91C: In-House Laboratory Independent Research (U)

Work Unit No. 91C 00 132: Role of T-cells in Pathogenesis of Argentine Hemorrhagic Fever

Background:

Argentine hemorrhagic fever (AHF), an arenavirus infection due to Junin (JUN) virus, is an acute severe disease with a mortality of about 15%. AHF is maintained in nature in rodent reservoirs, and is probably spread by aerosol routes. Immune plasma treatment reduces mortality, but has been implicated with relapse presenting with neurological manifestations (1). This is similar to observations with immunoglobulin-treated Machupo-infected monkeys.

There are numerous analogies between JUN and lymphocytic choriomeningitis (LCM) viruses. Both are lymphotrophic, immunosuppressive, and cause hemopoietic dysfunction (2-4). Although little work along these lines has been done with Junin, studies have shown that much of the LCM pathogenesis in mice is due to cytotoxic T-cells (5). Due to the analogies of LCM and Junin viruses, the fact that neutralizing antibody cannot be measured until late in disease, and since the only accepted treatment leads to a significant rate of late neurological symptoms, the role of T-cells in pathogenesis of AHF warranted investigation.

Progress:

We examined the ability of peritoneal exudate cells (PEC) from inbred strain 13 guinea pigs to support the replication of virulent (strain 3551) or attenuated (strain XJ-44) JUN viruses (Table 1). With the same Multiplicity of Infection (MOI), growth curves are very similar; virus was first detected on day 3 reaching a peak on days 6-9, after which cultures deteriorated. The yield of virus was nearly the same with both strains of virus. Preliminary experiments yielded similar results using PEC from outbred Hartley guinea pigs. The use of autologous serum vs. fetal calf serum on replication of the 2 strains of virus showed no effect.

TABLE I. JUN VIRUS REPLICATION IN PERITONEAL EXUDATE CELLS FROM INBRED STRAIN 13 GUINEA PIGS.

	VIRUS YIELD (PFU/ml) BY DAYS						
VIRUS STRAIN	3	4	5	6	7	8	9
XJ-44	$8x10^3$	$1.5x10^5$	$5x10^5$	$2x10^5$	$1x10^5$	$2x10^5$	$1x10^5$
3551	$4x10^3$	$3.5x10^4$	$3x10^5$	$5x10^5$	ND	$2x10^6$	$2.5x10^6$

ND = Not done.

We next examined the ability of PEC from inbred mice to support replication of the 2 virus strains. In contrast to virus replication data for guinea pig PEC, the avirulent strain of virus showed very little replication in mouse PEC while the virulent strain replicated to reasonably high titers (Table II). Three inbred strains of mice were used as sources of PEC, and the same pattern of virus replication emerged with each. We next attempted growth of strains

of AHF with differing virulence for guinea pigs in mouse PEC (Table III). The trend of greater replication of more virulent strains was observed. Although this technique is probably not sensitive enough to determine subtle virulence differences necessary in selection between candidate vaccine strains, it may be of value in screening field-isolated strains and as a tool in the laboratory for studying virulence and interactions with macrophages.

TABLE II. JUN VIRUS REPLICATION IN PERITONEAL EXUDATE CELLS FROM C3H/HeJ MICE.

VIRUS STRAIN	VIRUS YIELD (PFU/ml) BY DAYS					
	2	3	4	5	6	8
XJ-44	25	25	25	$1x10^2$	$5x10^2$	$5x10^3$
3551	25	$5x10^3$	$5x10^4$	ND	$3x10^5$	$7.5x10^5$

ND = Not done.

TABLE III. REPLICATION OF STRAINS OF JUN VIRUS IN PEC FROM A/HeJ MICE.

VIRUS STRAIN	VIRULENCE IN GUINEA PIGS[a]	PFU/ml BY DAYS				
		5	6	7	8	12
Coronel	±	$9.0x10^2$	ND	$3.5x10^3$	$9.5x10^3$	$1.3x10^4$
Coronel (1 passage in chick cells)	±	$6.0x10^3$	$1.6x10^3$	$5.5x10^4$	$2.3x10^4$	$1.3x10^4$
3551	+	$5.5x10^4$	$1.1x10^5$	$2.3x10^5$	ND	$1.5x10^5$
Romero	+	$2.0x10^3$	$6.0x10^3$	$2.0x10^4$	$6.5x10^4$	$6.0x10^4$
Suarez	+	$4.0x10^3$	$4.5x10^4$	$1.1x10^5$	ND	$2.0x10^5$
Clone 3	-	$2.0x10^2$	$1.0x10^2$	$1.1x10^3$	$5.0x10^3$	$6.0x10^3$
Clone 3 (passage in fetal rhesus lung cells)	-	$3.3x10^2$	$2.0x10^2$	$1.5x10^3$	$6.0x10^3$	$2.3x10^4$

+ = kills 95-100% of 300-400-g guinea pigs; - = kills less than 5%;
± = kills 30-70%.
ND = Not done.

We have determined that treatment of guinea pigs with cyclophosphamide prior to infection with attenuated XJ-44 strain JUN causes a lethal infection, for the most part clinically indistinguishable from that of guinea pigs infected with virulent strains. Table IV shows the results of organ virus determinations of cyclophosphamide-treated XJ-44-infected guinea pigs. Without drug, our experience has rarely shown either deaths in XJ-44-infected guinea pigs or virus isolations from organ samples. The virus isolation pattern from our treated-infected guinea pigs shows virus first appearing in spleen and bone marrow, and, if survival is sufficiently long, finally appearing in the brain. Many of those surviving beyond day 17 develop signs of encephalitis with paralysis; virus can be isolated from these brains. Frozen sections stained with fluorescent antibody show strong fluorescence in marrow and spleen by day 12, correlating with virus titers in the brain at later time points. Antibody development may be delayed, but is not prevented by cyclophosphamide treatment. Illness and death can be prevented by treating with immune serum or by transfer of spleen cells from immune convalescent syngeneic animals. In contrast to that found with some other arenavirus infections (6), passage of virus in guinea pigs does not enhance virulence for that model. Histopathology of cyclophosphamide-treated XJ-44-infected guinea pigs has shown lesions similar to those of guinea pigs infected with virulent JUN strains, namely, enterocolitis and erythroid-myeloid depletion. We have not observed encephalitis in virulent strain-infected guinea pigs; we have observed it in XJ-44 only or cyclophosphamide-XJ-44-infected guinea pigs, but only as a late event (17-24 days, whereas those infected with virulent strains generally die at 11-15 days). We feel that cyclophosphamide-treated XJ-44-infected guinea pigs offer potential as a model for virulent JUN-infected guinea pigs.

TABLE IV. ORGAN VIRUS DETERMINATIONS ON CYCLOPHOSPHAMIDE-TREATED XJ-44 JUN-INFECTED GUINEA PIGS.

	PFU/g TISSUE OR ml BY DAYS					
ORGAN	7	12	14	17	18	24
Spleen	$5x10^4$	0	$5.5x10^5$	$6x10^5$	$2x10^5$	$4.5x10^3$
Heart	0	0	0	0	0	0
Kidney	0	0	0	0	0	0
Liver	0	0	0	0	$5x10^4$	0
Marrow[a]	ND	0	$3.5x10^4$	$1.5x10^5$	$1.3x10^5$	$1x10^3$
Brain	0	0	0	$2.0x10^6$	$4x10^5$	$3x10^5$
Serum	0	0	0	0	0	0

[a]Less than 1 g of tissue.
ND = Not done.

Bearing in mind the parallels between LCM and JUN viruses, studies were initiated to determine if, as with the LCM mouse model, cytotoxic T-cells play a role in pathogenesis of the AHF guinea pig model. Since for a cytotoxic T-cell assay, target and effector cells must be major histocompatibility matched, a strain 13 guinea pig kidney cell line was developed and found to express virus-specific cell surface antigens when infected with either JUN or LCM viruses. Table V shows the results of ^{51}Cr release from JUN-infected (XJ-44) guinea pig kidney cells (target cells) induced by spleen cells (effector cells) from infected (XJ-44) guinea pigs at various points after infection. Table VI shows results of similar experiments with LCM virus (since cytotoxic T-cells with LCM have been described in mice, our rationale was that guinea pigs infected with LCM might be a model for guinea pig T-cell cytotoxicity). It is seen that specific ^{51}Cr release can be obtained with either system using specific effector spleen cells. In both systems, release could be due to cytotoxic T-cells or to antibody dependent cell-mediated cytotoxicity (ADCC) (antibody produced in vitro by splenic B cells). Treatment of effector spleen cells from JUN-infected guinea pigs with monoclonal anti-guinea pig T-cell serum (courtesy of E. Shevak, N.I.H.) prior to use had no effect on the system (data not presented). Aggregated human IgG reportedly inhibits ADCC by blocking the surface Fc receptors on the effector cells preventing sufficient target cell-effector cell interaction for cytolysis (7). Experiments were performed using aggregated human IgG with both JUN and LCM systems. Table VII shows that the presence of aggregated human IgG block ^{51}Cr release in both systems. These results suggest that, at least in part, the observed splenic cytotoxicity is due to ADCC.

TABLE V. ^{51}CR RELEASE FROM JUN-INFECTED GUINEA PIG KIDNEY CELLS BY SPLEEN CELLS FROM INFECTED GUINEA PIGS.

EFFECTOR: TARGET RATIO	% ^{51}Cr RELEASE BY DAYS						
	0	3	6	9	13	16	30
100	3	2	15	30	71	45	48
50	2	1	5	17	74	40	49
25	2	1	3	13	51	21	15
12	1	4	0	12	55	12	10
6	2	0	1		21	6	2

TABLE VI. ^{51}CR RELEASE FROM LCM VIRUS[a] INFECTED GUINEA PIG KIDNEY CELLS BY SPLEEN CELLS FROM LCM INFECTED GUINEA PIGS.

SPLEEN CELL SOURCE	% ^{51}CR RELEASE BY EFFECTOR: TARGET CELL RATIO				
	100:1	50:1	25:1	12:1	6:1
Armstrong					
10 days	45	21	17	16	2
16 days	55	51	45	29	12
We Strain					
10 days	58	21	24	19	7
Normal Uninfected	17	8	3	0	3

[a]Armstrong Strain

TABLE VII. EFFECT OF AGGREGATED HUMAN IgG ON ^{51}Cr RELEASE FROM LCM AND JUN INFECTED TARGET CELLS BY EFFECTOR SPLEEN CELLS.

CONCENTRATION mg IgG/ml	% RELEASE AT EFFECTOR: TARGET RATIO			
	100:1		50:1	
	Blocked	Unblocked	Blocked	Unblocked
JUNIN				
8	2	55	2	51
4	2	42	32	41
LCM				
8	2	48	15	31
4	18	53	19	42

Publications

None.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G3877	81 10 01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUM'RY SCTY	6. WORK SECURITY	7. REGRADING	8. DISP'N INSTR'N	8A. SPECIFIC DATA - CONTRACTOR ACCESS	9. LEVEL OF SUM
80 12 12	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	133
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Role of Anthrax Toxin Components in Virulence of B. anthracis

12. SCIENTIFIC AND TECHNOLOGICAL AREAS

003500 Clinical medicine; 004900 Defense; 010100 Microbiology; 002300 Biochemistry

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
81 06	85 09	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
FISCAL YEAR PRECEDING 81	0.2	36
FISCAL YEAR CURRENT 82	2.0	108

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) BW defense; (U) Military medicine; (U) Anthrax; (U) Prophylaxis; (U) Therapy; (U) Immunology; (U) Laboratory animals

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

23. (U) Purify and characterize the 3 protein components of anthrax toxin, determining their physiologic and enzymatic actions, so as to allow rational design of measures to prevent and treat anthrax infections. This disease is a BW threat to U.S. military forces.

24. (U) Develop methods to produce and purify useful amounts of anthrax toxin; develop sensitive immunochemical, electrophoretic and enzymatic assays to measure the toxin components; identify cultured cells sensitive to the toxin; determine the physiologic and biochemical changes caused by the toxin and the enzymatic activities of the toxin proteins; and study the genetic mechanisms which control toxin synthesis.

25. (U) 81 06 - 81 09 - All 3 protein components of anthrax toxin were produced and purified to states approaching homogeneity. These proteins have molecular weights of approximately 80,000. The combination of protective antigen (PA) and edema factor (EF) was found to cause rapid, profound and reversible increases of intracellular levels of cyclic AMP, an end result identical to that caused by cholera toxin. EF was found to be an adenylate cyclase enzyme which is active only within animal cells. PA appears to bind to cell surface receptors and enable EF to penetrate into cells.

*Available to contractors upon authorized approval

BODY OF REPORT

Project Number 3A166101A91C: In-House Laboratory Independent Research.

Work Unit Number 91C 00 133: Role of anthrax toxin components in virulence of B. anthracis.

Background:

The factors which make Bacillus anthracis virulent for man and animals are poorly understood. Rational design of preventive and therapeutic measures to control anthrax infections is therefore difficult. Previous work identified 3 proteins, collectively called "anthrax toxin," which contribute to pathogenesis. Of these 3 proteins, the protective antigen (PA) has been purified and used as a vaccine, but the lethal factor (LF) and edema factor (EF) have not been available in amounts adequate for characterization of their chemical structures or determination of their cellular modes of action.

This newly initiated work unit has as its objective (a) purification of the protein components of the toxin, (b) development of immunochemical and physical assays to detect and quantitate the proteins, (c) discovery of animal models and tissue culture systems sensitive to the toxin, (d) identification of the fundamental subcellular processes affected by the toxin, and (e) discovery of the individual enzymatic reaction or critical cellular component altered by the toxin.

Progress:

This work unit, initiated in June 1981, has as its objectives the purification and characterization of the 3 proteins (EF, PA, LF) collectively known as anthrax toxin and discovery of their mechanisms of action. B. anthracis was extensively studied at Ft. Detrick prior to 1968, and most of our current knowledge of anthrax was obtained at that time. The virulence of B. anthracis has been attributed to 2 factors: a polyglutamic acid capsule which promotes invasiveness, and the anthrax toxin complex recognized by its lethal action in rats and other animals. The toxin, initially found in the sera of guinea pigs suffering severe infections, was subsequently produced in broth cultures and resolved into 3 components (1), which have no known toxic effects when administered individually. However, PA mixed with LF is lethal to rats with a minimum time to death of 60 min. PA mixed with EF causes edema in the skin of guinea pigs and rabbits. Studies on the mechanisms of action of the toxin components have been severely limited due to the small amounts and uncertain purities of the EF and LF components. Wright and his colleagues developed methods for the large scale production of PA (2), which are being successfully employed by Dr. Johnson-Winegar (Pathology Division).

The initial work on this project therefore concentrated on purification of the protein components of the toxin. The avirulent, non-capsulated Sterne strain was grown in 10- and 20-L batches in a small fermenter, using the casamino acids media of Haines et al. (3). Following, in part, a published report of Ward and Wilkie (4), the culture supernatants were diluted and adsorbed to DEAE-cellulose. Protein was eluted batchwise, precipitated with $(NH_4)_2SO_4$, dialyzed and chromatographed on

DEAE Sepharose CL-6B. Gel diffusion of fractions against sera raised in a horse and a burro, using PA prepared by Ms. Johnson as a standard, enabled identification of a region containing PA. Injection into rats of fractions containing PA along with other fractions from the column showed that LF activity eluted after PA. SDS-slab gel electrophoresis of column fractions showed several protein bands which eluted coincidently with LF activity. The LF-containing fractions were combined and chromatographed on hydroxylapatite, with LF activity again identified by injection into rats in combination with PA. The LF preparation obtained in this way consists of one major polypeptide of MW 80,000 and a number of other minor components. The 80,000 MW band, which is likely to be the LF protein, constitutes at least 90% of the preparation. The LF preparation reacts strongly in gel diffusion with a horse serum raised by injection of Sterne strain spores. PA preparations, in contrast, react much more strongly with a burro anti-spore serum. This differential reactivity was used to advantage in subsequent chromatographic purifications of PA and LF, replacing the rat lethality test for all except confirmatory analyses.

The PA-rich fractions from the DEAE Sepharose CL-6B column were also further purified on hydroxylapatite. The PA protein was obtained at a purity of about 95%, and was also found to have a MW of about 80,000. The growth of the Sterne strain to produce PA and LF has been repeated several times with slight variations in fermentation conditions. One modification which seems to promote reproducible toxin production is the use of automatic pH control. All previously used media contained high concentrations of sodium bicarbonate to buffer the culture, since it had been shown that shifts of cultures to low pH caused large losses of toxin, probably due to proteolysis. While yields of PA and LF have varied, typical amounts obtained from 20-L cultures of the Sterne strain have been 5 and 2 mg, respectively. Larger amounts of PA have been obtained in separate fermentations of strain V770 in the medium of Puziss (2), which was developed to maximize PA production.

Due to time limitations, little work has been done to study the mechanism of action of the lethal toxin (PA and LF). Titration of the purified components in rats confirmed the view that both components must be combined to cause death. Thus, neither 300 µg of PA or 150 µg LF alone killed rats, while 100 µg PA injected with 30 µg LF caused death in 190 min. In contrast to published reports that B. anthracis culture supernatants are not toxic to cell cultures (5), preliminary experiments have shown that the combination of PA and LF kills mouse epidermal cells and cultured macrophages. Chinese hamster ovary (CHO) cells also appeared sensitive, but effects varied with different PA and LF preparations. Therefore, it appears that cell culture systems can be used to study the action of the lethal toxin. This aspect of the work will be resumed when characterization of EF (described below) is completed.

Concurrent with completion of the LF purification, work began on purification of EF. All prior work on EF depended on bioassay in rabbit or guinea pig skin. An important advance was achieved when it was recognized that the edematous response to EF was similar to that caused by cholera toxin and the Escherichia coli heat-labile enterotoxin. These 2 toxins cause large increases in vascular permeability and are often assayed in rabbit skin. Using a rabbit permeability factor (PF) assay, it was found that certain fractions from DEAE columns expressed PF activity when combined with PA. The results were somewhat variable, but this was anticipated since it had been known from early studies that LF and EF act competitively. Thus, fractions containing LF and EF may not give a positive PF

response. Extending the analogy with cholera toxin further, studies were begun with a CHO cell assay in which elevated cytoplasmic levels of 3', 5'-cyclic adenosine monophosphate (cAMP) cause a profound morphological change. Protein fractions eluting off of DEAE columns after those containing PA caused shape changes identical to those caused by cholera toxin. A systematic study of assay conditions showed that the shape change was most evident in EMEM/NEAA medium containing 5% fetal calf serum. Though initially performed by adding dilute CHO cell suspensions to the toxin samples to be tested, the test also can be done by adding toxin samples to confluent CHO cell monolayers in microtiter plates. This convenient and semi-quantitative assay was used to assay EF during its purification on DEAE and hydroxylapatite columns. The preparation obtained contains a major polypeptide of about 80,000 MW as 80% of the total protein. From a 20-L culture, about 2 mg of this purified EF was obtained in the most recent preparation.

Study of the mechanism of action of EF was based on extending the analogy with cholera toxin. A radioimmunoassay kit for measurement of cAMP was purchased, and the procedure scaled down to decrease its cost. A variety of cultured cells in addition to CHO were treated with graded concentrations of PA and EF, typically for 2 hrs, and cAMP was measured in acid extracts. Parallel cultures were treated with cholera toxin. With two exceptions (LLC-MK_2, Vero), all cells responded to the mixture of PA and EF with large increases in cAMP levels (Table I).

TABLE 1. cAMP RESPONSES IN SEVERAL CELL LINES

cAMP Response (pmol/mg cell protein)

Cell Line	Untreated	Cholera Toxin 100 ng/ml	PA 1 µg/ml + EF 0.1 µg/ml
LLC-MK_2	20	20	31
Vero	25	>330	40
FRL-103	35	300	270
CHO	30	660	770
E69 (mouse epidermal)	<20	65	300
E15 B7 (mouse epidermal)	<50	375	125
Rat embryo	<5	50	60
Duck embryo	<15	60	160
G.P. 13	25	210	360
L929	15	70	95
MRC-5	35	220	710

The cAMP levels, typically 5- to 100-fold control levels, did not parallel those in the cholera-treated cultures. This suggests that the cellular receptors for PA and EF are not related to the ganglioside G_{m1} receptor of cholera toxin. The response was dependent on the presence of both PA and EF, with maximum cAMP levels requiring $\geq$1 µg/ml PA and about the same concentration of EF. However, the data in Table I were obtained using the first EF preparation (EF#1), which is much less active than a second subsequent preparation (EF#2). While cAMP levels could not be increased above those shown by further increasing the EF#1 concentrations, EF#2 at similar concentrations caused 1,000-fold cAMP increases in CHO cells. This difference makes it clear that there are important structural features of the EF protein which remain to be identified.

The ability of PA and EF to elevate cAMP levels reinforced the view that these proteins might act, like cholera toxin, by permanently activating the target cell's adenylate cyclase. However, several types of evidence were obtained to show that the analogy was imperfect. Kinetic studies showed that cAMP elevations in CHO cells were rapid, peaking within 60 min., whereas cholera toxin exhibited a well characterized lag. More striking was the rapid reversibility of the PA and EF response; in cells washed free of toxin, cAMP levels fell to control values within 2 hrs.

Experiments were then designed based on the hypothesis that EF interacted in a reversible manner with the adenylate cyclase in CHO membranes. Large increases in cyclase activity were found in membranes mixed with EF. The stimulation, a 100-fold increase in activity, required the presence of EF and membranes, but was not dependent on PA. However, certain "controls" in these experiments showed that under some circumstances the membranes were dispensable. Pursuit of this result made it clear that EF is itself an adenylate cyclase. The reason this was not evident in the first adenylate cyclate assays is that EF is an inactive proenzyme which must be activated by interaction with materials from eukaryotic cells. The exact nature of the activation process is now being studied.

The important finding that EF is an adenylate cyclase provides a coherent model for its action on cells. PA apparently binds to cell surface receptors and allows entry of EF into the cytoplasm. Interaction with eukaryotic cell materials not present in B. anthracis activates the enzyme. The subsequent large increases in cAMP levels, which can far exceed those caused by any other known agent, will profoundly alter cellular processes. The role of EF in pathogenesis was never clarified from studies in whole animals, but the knowledge that EF raises cAMP levels now allows reasonable predictions to be made of which physiological processes are altered.

In the course of purifying the components of anthrax toxin a brief study was made of proteases in B. anthracis, since it was recognized that these might degrade the proteins during purification. Assay of DEAE column fractions showed that a single peak of proteolytic activity eluted after PA. Since the protease most thoroughly studied in the closely related Bacillus cereus is a metalloenzyme, the effect of chelators was tested. EDTA completely inhibited the B. anthracis activity. Therefore EDTA is now routinely added to culture supernatants after centrifugation to remove the bacteria. This appears to have improved the PA preparations in particular, since a PA sample made before use of EDTA became standard shows extensive nicking.

To facilitate further characterization of anthrax toxin, specific antisera were raised in rabbits to PA and LF. These sera appear to be specific since they do not react with the heterologous component. The first attempt to raise a rabbit anti-EF serum was unsuccessful. A substantial effort has been directed to the selection of mouse hybridomas producing monoclonal antibodies to the toxin components. Following an intial attempt which appeared successful, technical problems have occurred. These seem now to have been controlled so that progress should continue in this effort. A sensitive ELISA test has been developed to detect mouse antibodies to each of the 3 components; the test has been used to show that the immunized spleen cell donors are in fact immune.

Preliminary experiments have been conducted to develop an additional animal model for study of anthrax pathogenesis. Extending a report of Russian workers, it has been found that 2 inbred mouse lines, A/J and DBA/2J, are susceptible to infection by large doses of Sterne strain spores, with LD_{50} of about 10^5. This animal model may provide an economical test system for vaccines based on toxin components. A trial is currently underway to determine if immunization with PA or LF protects these mice.

Publications:

None.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY

1. AGENCY ACCESSION	2. DATE OF SUMMARY	REPORT CONTROL SYMBOL
DA 0G3854	81 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
80 12 12	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	143
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)

(U) Antigenic Analysis of Phlebotomus Fever Group Virus Components

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
81 01	82 12	DA	C. In-house

17. CONTRACT/GRANT

a. DATES/EFFECTIVE: EXPIRATION:

b. NUMBER:

c. TYPE: d. AMOUNT:

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

18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In Thousands)
FISCAL YEAR PRECEDING 81	4.6	378
FISCAL YEAR CURRENT 82	3.0	345

19. RESPONSIBLE DOD ORGANIZATION

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SOCIAL SECURITY ACCOUNT NUMBER:

ASSOCIATE INVESTIGATORS

NAME: Peters, C. J.

NAME: Kondig, J. POC:DA

21. GENERAL USE

Foreign intelligence considered

22. KEYWORDS (Precede EACH with Security Classification Code) (U) BW defense; (U) Military medicine; (U) Phlebotomus fevers group viruses; (U) Rift Valley fever; (U) Immunology; (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) Identify major antigenic determinants responsible for inducing neutralizing and/or protective antibody to selected phlebotomus fever virus pathogens. Antigenic analysis of Rift Valley fever virus will provide specific reagents and new methodologies necessary for comparing geographic isolates, evaluating assortant viruses and estimating the value of experimental vaccines. These studies test a new approach to virus disease control in military personnel.

24. (U) Investigate new techniques of lymphocyte hybridoma production of monoclonal antibodies to detect and characterize virus antigens of importance in protective immunity. Use monoclonal antibodies for rapid identification of specific viruses, immunogenic proteins and evaluation of the immune response.

25. (U) 81 01 - 81 09 - RNA species of Rift Valley fever virus (RVFV) have been isolated purified and analyzed by oligonucleotide fingerprinting. RVFV structural proteins have been isolated and identified with clear preparative separation of the envelope glycoproteins. Preliminary lymphocyte hybridoma experiments yielded numerous monoclonal antibodies, most of which are directed at nucleocapsid antigens.

*Available to contractors upon originator's approval

BODY OF REPORT

Project No. 3A16610A91C: In-House Laboratory Independent Research

Work Unit No. 91C 00 143: Antigenic Analysis of Phlebotomus Fever Group Virus Components

Background:

The Phlebovirus genus of the family Bunyaviridae contains numerous human and animal pathogens most notable of which is Rift Valley fever (RVF) virus. The antigenic analysis of this virus, closely related virus members of the Phlebovirus genus and isolated and characterized components of these virions remains the major objectives of this research. These studies assume even greater importance as supporting research for investigations directed toward producing RVF virus immunogens using recombinant DNA technology (gene cloning). It is incumbent upon these investigations to describe the basic molecular characteristics of the components of the virion, isolate, concentrate and purify the nucleic acid segment(s) containing the gene(s) for important immunogenic proteins, as well as describe, isolate and characterize the antigens necessary for inducing those antibodies responsible for protection in the immunized host. The requirements described necessitate that this research be multifaceted and aimed at a basic molecular characterization of the virus, its chemical constituents and antigenic components.

Progress:

RVF virus (Zagazig Hospital 501) was propagated in Vero cells, concentrated by ammonium sulfate precipitation and purified by rate zonal centrifugation on 15-60% (w/w) sucrose gradients. These procedures were experimentally determined to be superior to a variety of methods tested. Purified virions approaching $2 \times 10_{10}$ PFU/ml were disrupted with 2% Triton X-100, releasing ribonucleoprotein (RNP) complexes which were analyzed on sucrose gradients. As shown previously with other Bunyaviridae, three RNP were clearly distinguished. By comparison to the nucleoscapsids of LaCrosse (LAC) virus (115S, 90S, 65S), the sedimentation coefficients of the 3 RVF virus nucleocapsids were determined to be 115S, 90S, and 74S. Subsequent RNA analysis of these complexes on 2.4% polyacrylamide gels revealed that each contained only a single species of virion RNA, the large (L), medium (M), or small (S) RNA, respectively. Approximately 85-90% of the RNA within the RNP complexes was sensitive to digestion with pancreatic RNAse, suggesting that the protein is not tightly associated with the RNA. The apparent MW of the L, M, and S RNA species were determined to be 2.8×10^6, 1.8×10^6, and 0.74×10^6, respectively. These MW weights are essentially identical to those of Punta Toro virus, a serologically related Phlebovirus. The three RNA species were also found to be unique by RNA fingerprint analysis. There did not appear to be any overlap of the large oligonucleotides among the 3 RNA species. The large oligonucleotides present in one RNA species could not be detected in the fingerprints of either of the other 2 species. Few similarities, if any, existed between the fingerprint patterns of RVF virus and those previously reported for other Phleboviruses. The 5' terminus of all 3 RNA species has recently been determined by sequence analysis to be ppAp——. This observation is consistent with data from other Bunyaviruses with known 5' termini. Virus structural proteins were isolated and identified by electrophoresis in 12% polyacrylamide gels. The apparent MW of these proteins were found to be 65,000 (G1), 56,000 (G2), and 25,000 (NC). The 2 envelope glycoproteins were readily separated by nonionic detergent disruption and column isoelectric focusing in sucrose gradients. The G1 glycoprotein focused at pH 4.8 which was clearly separate from the isoelectric point of the G2 glycoprotein at pH 9.6. Data recently obtained clearly show that the

G1 and G2 glycoproteins are in fact 2 different and unique proteins. This has been accomplished by partial digestion of isoelectrically focused proteins with staphyococcus V8 protease and subsequent separation of protein fragments by polyacrylamide gel electrophoresis (Cleveland maps). The pattern of fragments obtained from the digestion of G1 and G2 were clearly unique and no overlapping peptides could be detected between these 2 proteins. These data collectively provide a basis for comparison of RVF with other Phleboviruses and establish parameters for separation and identification of purified virion components.

Approximately 100 μg of purified M-segment RVF virus RNA has been prepared for gene cloning experiments. The effort involved in the preparation of these reagents has been considerable, especially since procedures for virion concentration, purification and RNA purification were only recently optimized.

Safety testing of all virion components removed from the containment laboratories included injection of suckling mice and weanling hamsters as well as direct plaque assay and assay in cells under liquid overlay. All safety testing procedures required numerous experiments to define the maximum allowable concentrations of the various detergents frequently residual in purified RNA and protein preparations. To date, no infectivity has been detected in any of the numerous preparations examined. Cloning experiments by Molecular Genetics Inc., Minnetonka, MN, under a contract from USAMRDC are currently in progress.

The preparation of monoclonal antibodies to RVF virus using lymphocyte hybridomas has been slower than anticipated. Many experiments employing a nonproducer (immunoglobulin) plasmacytoma cell line have yielded a reasonable number of hybridomas producing RVF virus antibody; however, we have been unsuccessful in the continued cultivation and freezer storage of these cell lines. A small battery of RVF virus antibody-producing hybridomas exist in a frozen repository and mouse ascitic fluids have been prepared to the majority of these, however, they were all prepared from an immunoglobulin-secreting plasmacytoma line. The vast majority of these antibody-positive hybridoma clones react with nucleocapsid antigens and are therefore of limited usefulness in the studies described previously. Approximately 6 hybridoma cultures have been preserved that produce antibody to RVF virus envelope glycoproteins. Preliminary immunoprecipitation experiments suggest that all of these antibodies are directed at determinants on the G1 glycoprotein. In all immunoprecipitation experiments, some G2 glycoprotein was precipitated; these techniques clearly require further refinement. All monoclonal antibody-containing ascitic fluids prepared thus far are negative by neutralization and hemagglutination inhibition testing. Apparently the antigenic determinants important in these tests are not yet detected by our small battery of reagents; further hybridoma experiments are required.

Presentations:

None.

Publication:

Dalrymple, J. M., C. J. Peters, J. F. Smith and M. K. Gentry, 1981. Antigenic components of Punta Toro virus, pp. 167-172. In The Replication of Negative Strand Viruses (D. H. L. Bishop and R. W. Compans, eds.), Elsevier/North Holland, New York.

APPENDIX A
VOLUNTEER STUDIES

PROTOCOL TITLE AND NO. (No Volunteers)	COMMENTS AND RESULTS
Growth Hormone Release and Associated Metabolic Changes Induced in Man by Sandfly Fever M-23-B Protocol 81-1 (6 MRVS)	One subject did not develop Sandfly Fever. No complications. Results showed no significant change in prolaction but distinctive change in growth hormone--elevated and increased.
Evaluation of the Human Response to the Administration of Botulinum Toxoid, Adsorbed Monovalent (E), MDPH LOT 3 #7007. M-25 Protocol 81-2 (19 MRVS, 4 USAMRIID Staff)	Eight native vaccinees had mild local reactions. All in group which received 0.5 ml of toxoid on day 0, 14, 84. Lot #7007 of monovalent (E) toxoid proved to be safe and efficac ious product for the induction of substantial levels of neutralizing activity to type E botulinal toxoid.
Evaluation of WR 171, 669 in the Treatment of Multi-Drug Resistant Plasmodium Falciparum Malaria. M-26 Protocol 81-3 (12 MRVS)	Twelve volunteers infected with Smith isolate of P. falciparum mairia; 10 were cured with WR 171, 669 was effective against multi-drug resistant P. falciparum malaria; in a single day treatment and was well tolerated.
Growth Hormone Release and Metabolic Changes Induced in Man by Mild Brief Artificial Hyperthermia. M-23-A Protocol 81-4 (3 MRVS)	Results suggest that there is a clear-cut release in growth hormone during hyperthermia but no significant change in prolactin.

APPENDIX B

PUBLICATIONS OF U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FISCAL YEAR 1981

1. Anderson, A. O., and N. D. Anderson. 1981. Functions in structure and physiology of lymphatic tissues, pp. 29-76. In Cellular Immunity and Inflammation (J. J. Oppenheim, D. L. Rosenstreich, and M. Potter, eds). Elsevier/North Holland, New York.

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15. Bistrian, B. R., D. T. George, G. L. Blackburn, and R. W. Wannemacher. 1981. The metabolic response to yellow fever immunization: protein-sparing modified fast. Am. J. Clin. Nutr. 34:229-237.

16. Bistrian, B. R., D. T. George, G. L. Blackburn, and R. W. Wannemacher. 1981. Effect of diet on the metabolic response to infection: protein-sparing modified fast plus 100 grams glucose and yellow fever immunization. Am. J. Clin. Nutr. 34:238-244.

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APPENDIX C

CONTRACTS, GRANTS, MIPRs AND PURCHASE ORDERS IN EFFECT

FISCAL YEAR 1981

NO.	TITLE, INVESTIGATOR, INSTITUTION
DAMD17-78-C-8035	Mass Spectrophotometric Rapid Diagnosis of Infectious Diseases. M. Anbar, State University of New York Buffalo
DAMD-17-80-C-1054	Mechanisms of Protective Immunogenicity of Microbial Significance. M. S. Ascher, University of California College of Medicine
DAMD17-74-C-4128	Genetics of the Encephalitis Vector, Culex tarsalis, for Possible Application in Integrated Control. M. Asman, University of California Berkeley
DAMD17-78-C-8017	Genetic and Molecular Studies of the Phlebotomus Fever Group of Viruses. D. L. Bishop, University of Alabama
DAMD17-79-C-9108	Analysis of Flavivirus Replication. M. Brinton, Wistar Institute
DAMD17-78-C-8042	Togavirus - Specific Cellular Immune Effector Mechanisms. G. A. Cole, School of Hygiene and Public Health, Johns Hopkins University
DAMD17-81-C-1120	Preparation of Purifed Botulinal Antitoxin. R. M. Condie, University of Minnesota
DAMD17-80-C-0100	Study of Toxic and Antigenic Structures of Botulinum Neurotoxins. B. R. DasGupta, University of Wisconsin Madison
DAMD17-81-C-1028	Rapid Methods for the Laboratory Identification of Pathogenic Microorganisms. R. J. Doyle, University of Louisville
DAMD17-79-C-9024	Lassa Fever Immune Plasma. J. D. Frame, Columbia University
DAMD17-79-C-9494	Isolation of the Etiologic Agent of Scandinavian Epidemic (Endemic) Nephropathy from Human Patients (and from Wild Rodents) as Presumptive Strain in a Vaccine against Korean Hemorrhagic Fever. G. Friman, Uppsala University Hospital, Uppsala, Sweden
DAMD17-77-C-7043	Development of Psoralen Photoinactivated Alphavirus and Arenavirus Vaccines. C. V. Hanson, California Department of Health
DAMD17-77-C-7018	Vector Competence of Mosquitoes for Arboviruses. J. L. Hardy, University of California Berkeley

MIPR-2025	Preparation and Characterization of Mouse and Human Monoclonal Antibodies to Botulinum Toxins. K. W. Hunter, Uniformed Services University of the Health Sciences
DAMD17-80-C-0091	In Vitro Selection of an Attenuated Variant of Sindbis Virus: Investigation of the Molecular Basis for Attenuation. R. E. Johnston, North Carolina State University
DAMD17-79-G-9468	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
PO-2083	Production of Monoclonal Antibody to Hemorrhagic Fever Viruses. J. McCormick, Centers for Disease Control
DAMD17-81-C-1014	The Preparation of Partially Purified Anthrax Protective Antigen from One Satisfactory 100-Liter Culture of B. anthracis, for Use in Serologic Testing. J. R. Mitchell, Michigan Department of Public Health
DAMD17-81-C-1026	Genetic Characterization of Insect Vectors of Disease. J. R. Jeffrey, Yale University
DAMD17-81-C-1189	Detection, Isolation and Characterization of an Agent from Febrile Patients in Malaysia Serologically Reactive with Rickettsia sennetsu. M. Ristic, University of Illinois Urbana-Champaign
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
DAMD17-80-C-0176	Transovarial Transmission of JE Virus by Mosquitoes. L. Rosen, University of Hawaii
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
DAMD17-78-C-8056	In Vitro Studies of Sandfly Viruses and Their Potential Significance for Vaccine Development. J. F. Smith, University of Maryland
DAMD17-78-C-8023	Regulation of Salivary Output by Mosquitoes. A. Spielman, Harvard School of Public Health

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

DAMD17-81-C-0178 Studies of the Transovarial Transmission of Phlebotomus Fever Viruses in Sandflies. R. M. Tesh, Yale University

DAMD17-80-C-0099 Genetic and Physiological Control of Protective Antigen by Bacillus anthracis. C. B. Thorne, University of Massachusetts

DAMD17-81-C-1156 Rapid Diagnosis of Arbovirus and Arenavirus Infections by Immunofluorescence. G. Tignor, Yale University

DAMD17-79-G-9508 Propagation and Characterization of the Etiologic Agent of Nephropathia Epidemica. C.-H. Von Bonsdorff, University of Helsinki, Finland

DAMD17-79-D-0006 Preparation of Hyperimmune Botulinum Toxin. S. Ware, Pine Bluff Biological Products

DAMD17-80-G-9472 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	chick 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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