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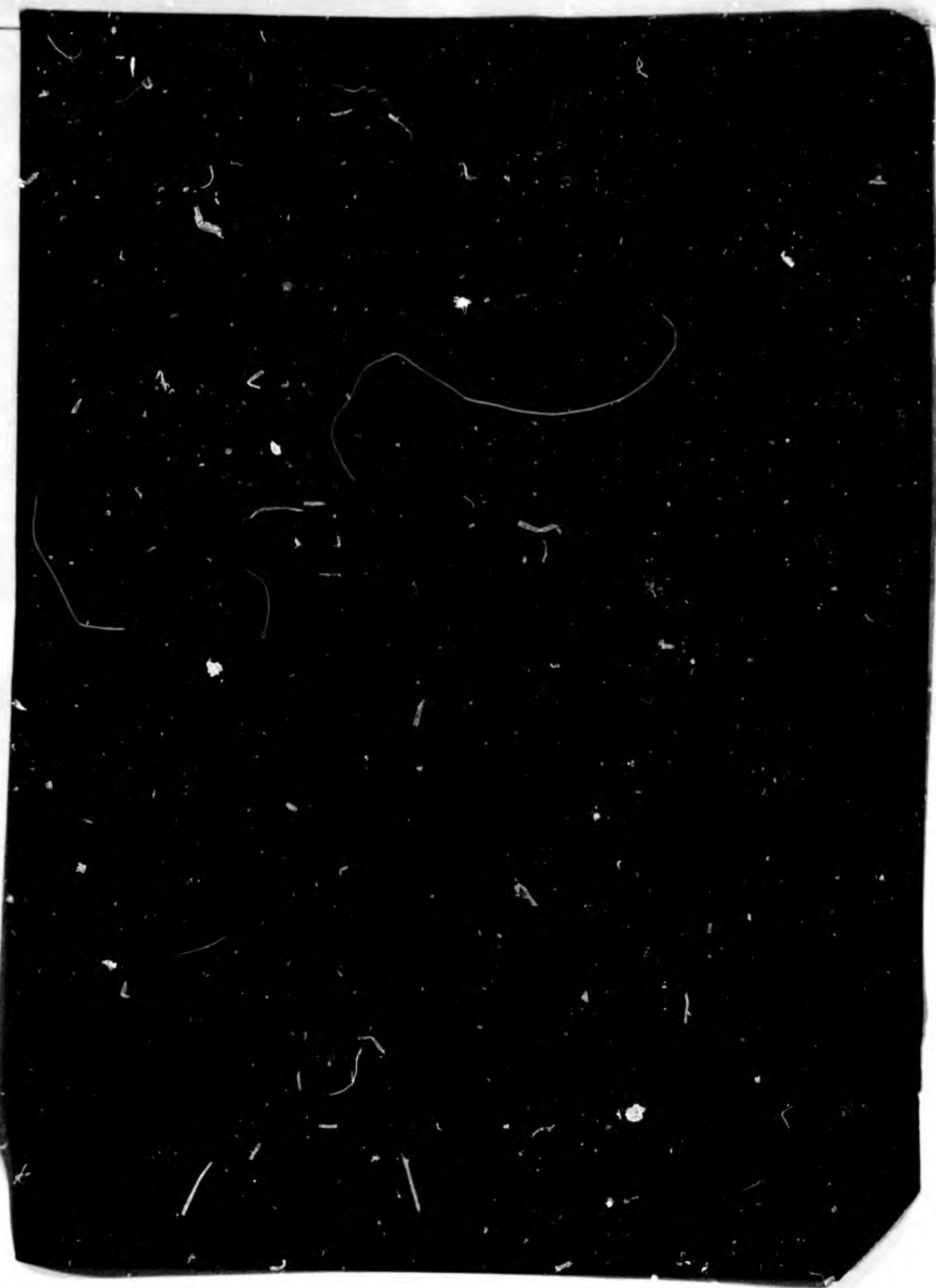
USAMRIID PLANNING SESSION WITH THE AD HOC STUDY GROUP FOR SPECIAL INFECTIOUS DISEASE PROBLEMS (1975), 20-21 NOVEMBER 1975

Army Medical Research Institute of Infectious Diseases
Frederick, Maryland

January 1976



1975 USAMRIID PLANNING SESSION

WITH THE

AD HOC STUDY GROUP

FOR

SPECIAL INFECTIOUS DISEASE PROBLEMS

20-21 November 1975

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other official documents.

U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FORT DETRICK
FREDERICK, MARYLAND 21701

January 1976

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Staphylococcal exfoliative exotoxin	Arenaviruses	Vaccines
Lymphocyte transformation	Q fever	Inflammation
Machupo virus infection	Spotted fevers	Influenza
Klebsiella pneumonia	Antiviral therapy	

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

Progress is reported in selected areas of research in medical defense aspects of biological agents by the U. S. Army Medical Research Institute of Infectious Diseases.

TABLE OF COMMENTS

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Ad Hoc Study Group

Abram S. Benenson, M.D.

Bennett L. Elisberg, M.D.

Ralph D. Feigin, M.D.*

Neal Nathanson, M.D.

A. M. Pappenheimer, Jr., Ph.D.

Jay P. Sanford, M.D.

William D. Sawyer, M.D.

Theodore E. Woodward, M.D.

Armed Forces Epidemiological Board
as Guests

LTC Dunae G. Erickson, MSC

Herschel E. Griffin, M.D.

William S. Jordan, M.D.

Charles H. Rammelkamp, Jr., M.D.

Reuel A. Stallones, M.D.

* Absent

AUTHOR INDEX

INTRODUCTORY COMMENTS

BG Kenneth R. Dirks, MC

My purpose this morning is to tell you about a few things which are happening in the US Army Medical Research and Development Command program and some things outside the Command which impinge on it. If I were to attempt to be comprehensive I would need at least one full day, so I'll limit myself to events which may influence the part of the program with which you are concerned.

First, of course, is money. FY 76 funding levels have proven difficult to live with, to say the least. Funding constraints, due to Congressional reductions this year, have significantly limited not only the expansion of our research Command-wide, but have disallowed even maintenance levels in some areas, particularly in our extramural research program. The US Army Medical Research Institute of Infectious Diseases (USAMRIID) has been fortunate, in that direct reductions have not been levied against any of its research lines. Unfortunately, it must carry its proportionate share of general reductions. We were able to anticipate most of the FY 76 reductions; laboratories have been funded at minimum essential levels consistent with those expectations. Recent trends and attitudes, especially in Congress, have made it resoundingly clear that all of our research must be tailored toward militarily unique requirements. No longer is it sufficient to say research meets a military requirement, or is militarily relevant, --- it must be militarily unique.

This attitude has cost us a significant amount of money in FY 76 ($2.6 million in drug abuse), and promises to plague us in the future if we do not choose and justify our projects carefully.

As you may have heard, Congress has decided that we should not be engaged in research on alcohol and drug abuse, and that line item has been deleted. A survey and investigation team is currently reviewing all DOD medical research to answer these questions: Is it effective? Is it unique? and Does it duplicate other federal effort? This review is looking down to the work unit level.

The Surgeon General's Research, Development, Test and Evaluation (RDTE) program in Figure 1 clearly shows the downward trend in terms of purchasing power. No figures are shown for FY 77. The crystal ball is still too cloudy for that, but it is probably safe not to expect much if any increase in terms of constant dollars. In spite of budgetary constraints we have successfully defended, thus far, at least 4 important construction programs for FY 77 and 78; these are shown in Table I. As you see, one of these is for much needed alterations to the animal holding facility here at USAMRIID. The others are for facilities improvement

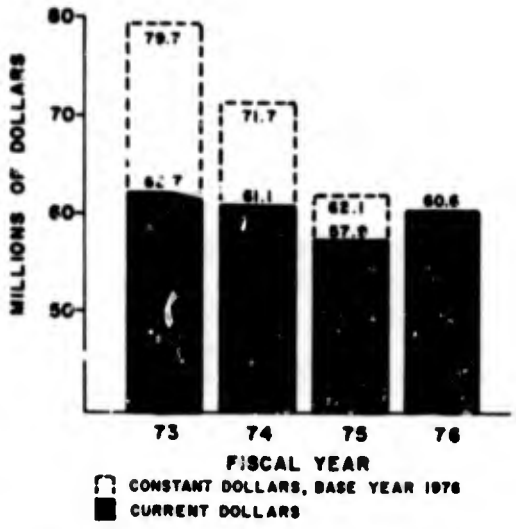

Figure 1. RDTE Program of the Surgeon General, Army, FY 73-76.

TABLE I. FY 77 & 78 MCA CONSTRUCTION PROGRAM, USAMRDC

FISCAL YEAR	UNIT	PROJECT	PROPOSED APPROPRIATION
1977	WRAIR	Improvements to research facilities (bldg 502/503, 512)	$1,108,000
1977	USAMRIID	Alterations to research animal holding facility (bldg 1412)	$957,000
1978	WRAIR	Improvements to research facilities (bldg 40 & 189)	$957,000
*1978	USAIDR	US Army Regional Dental Activity/ US Army Institute of Dental Research, new facility	$12,480,000

*Project to be funded by medical construction dollars and sited at Fort Meade.

at the Walter Reed Army Institute of Research (WRAIR), and for construction of a new facility for the US Army Institute of Dental Research (USAIDR), which will be shared with the Regional Dental Activity. The USAIDR now is housed within the WRAIR building.

Recent trends in manpower authorizations for the Command and for USAMRIID are shown in Table II. The trend is down---and we expect the trend to continue. Table III shows USAMRIID's Authorized vs. Actual strengths. The discrepancies between the two figures for both Medical

TABLE II. MANPOWER STATUS (AUTHORIZED) FOR USAMRDC AND USAMRIID

PERIOD	USAMRDC	USAMRIID
1 Jan 75	3152	550
1 Jul 75	2989	515
1 Nov 75	2932	513
Total reduction	220 spaces (7%)	37 spaces (6.7%)

TABLE III. MANPOWER STATUS OF USAMRIID AS OF 31 OCT 75

	AUTHORIZED	ACTUAL
Medical Corps	26	14
Veterinary Corps	26	23
Medical Service Corps	25	25
Army Nurse Corps	3	1
Warrant Officer	1	1
Enlisted	234	186
(human subjects)	(138)	(3)
(other)	(96)	(183)
Civilians	198	190
(professional series)	(48)	(44)
(other)	(150)	(146)

Corps officers and volunteers are obvious. The MC strength is low due primarily to the programmed reduction of incoming Berry Plan personnel, which of course resulted from the abolition of the draft. Steps have been initiated to retain on-board personnel and to recruit new personnel. This latter includes educating Army Medical Department (AMEDD) recruiting personnel, submission of more information and data to AMEDD recruiting publications, and encouraging R&D professional members to speak to potential candidates or groups of candidates on the professional benefits of military service.

The "Whitecoat" or human research, volunteer program should improve in the near future with the release of guidance by the Deputy Chief of Staff for Personnel (DCSPER) to Recruiting Command for the step-by-step processing of enlistment applications for potential human research volunteers. Until now there had been no instruction in effect for their enlistment. Active efforts by this Command and the cooperation of DCSPER and Recruiting Command resulted in the guidance which is effective 1 December 1975. About 15 candidates are "waiting in the wings" for serious consideration by the Army Recruiting Non-commissioned Officers.

The various reductions in resource authorizations, both people and money, have been imposed upon us in piecemeal fashion. Each reduction proposed must be assessed against ongoing programs. As a result we are continually examining and re-examining programs, tasks and work units. At first glance it would appear to be a simple matter to establish priorities, but basically for each work unit we must answer these questions:

1. Does the Army have the only requirement for this work?
2. Are other agencies supporting work in this area? To what extent? Are we complementing or duplicating others' efforts?
3. Does the Army need it worse, or sooner, than any other agency?
4. Is the Army uniquely capable of supporting or carrying out this research?

Answers to these questions will help us to establish relative priorities, along, of course, with consideration of relative cost, relative likelihood of useful result, and last but not least, scientific merit in today's climate. It is important to recall that we are producers of a product, sometimes materiel, but usually knowledge. Our priorities must depend upon the priorities of our customers, the users of the knowledge or materiel we produce. Oftentimes our customers don't know what they need except in very general terms---so we must tell them what we think they need, and why, and then supply it.

I have recently appointed a committee to consider redistribution and consolidation of military infectious disease research to avoid duplication of effort and conserve resources. This will ultimately require realignment of missions among the three Command laboratories which have infectious disease programs (Table IV). Studies directed toward Defense against Biological Agents will continue to be the responsibility of USAMRIID, but its mission should be broadened to include all work involving highly hazardous organisms, for which its specialized facilities can be best utilized.

TABLE IV. PROPOSED INFECTIOUS DISEASE MISSIONS OF USAMRIID, LAIR AND WRAIR

COMMAND LABORATORY	PROPOSED INFECTIOUS DISEASE MISSION
USAMRIID	Highly hazardous microogranisms Host response to aerosol transmission Vaccine development Studies in volunteer subjects
LAIR	Infectious dermatoses
WRAIR	All other militarily significant infectious disease Microbiologic and parasitologic immunology Epidemiological research and surveillance Entomology OCONUS Laboratory Command and support

It is also likely that all vaccine preparation work will be transferred to USAMRIID. WRAIR will continue to be the lead laboratory for work with less hazardous agents, including militarily important respiratory diseases, tropical protozoal infections, diarrheal disease, some rickettsiae and Togaviruses of military significance. Included in this concept will be discontinuation of work in subjects which are not considered militarily unique, such as typhoid fever, streptococcal infections and venereal disease. The Navy has a much more active program in schistosomiasis, so Army efforts in that area will be drawn to an orderly conclusion by 1977.

As you are also aware, there is considerable interest currently in the question of the use of human volunteers in military research programs. I have recently issued a Command-wide regulation establishing new standards for policies and procedures governing the use of human subjects in Command-sponsored or -conducted research. This regulation is a rather lengthy one, dealing as it does with a complex, and dynamic area of medical law, regulations, and ethics. It extends requirements for informed written consent to broader areas than heretofore; limits use of special classes of humans as volunteer subjects and requires establishment of local human use committees at in-house laboratories for review of protocols. Contractors are required to have on file with DHEW (under part 45, Title 46) or with the Command, written assurances that they will comply with the provisions of the regulation.

Finally, the Chief of Staff has directed The Surgeon General to establish a special study group to review use of human subjects in all Army research efforts including weapons development and testing new equipment. This group, composed of members of each Army element

conducting such research is under the able Chairmanship of Colonel E. Buescher, who was the personal choice of The Surgeon General for this important task. The study group is to recommend policy to the highest levels in the Army in order to insure that the highest ethical standards prevail.

I'm sure that this necessarily perfunctory overview has raised more questions than it has answered, but I believe it serves to give you some idea of the magnitude and scope of problems with which we are currently trying to cope.

DISCUSSION

Dr. Griffin asked how many of the USAMRDC total of 1,500 work units were assigned to USAMRIID. Colonel Metzger replied 83.

General Dirks recalled a meeting on 29 September 1975 with members of the Food and Drug Administration (FDA) in which vaccines unique to the military and which had limited use were discussed. He remembered that Dr. John Jennings and Mr. Fonds had reacted favorably to this concept, and General Dirks asked Dr. Elisberg if he could supply additional information. Dr. Elisberg could not. Dr. Beisel stated under current guidelines each administration of every one of the Institute's experimental vaccines required a signed and witnessed formal consent form, including explanation of adverse reactions plus complex record keeping.

Dr. Woodward noted that VEE vaccine development was not on the agenda and asked Dr. Beisel if the Institute were completely satisfied with the vaccine. Dr. Beisel replied that the live, attenuated vaccine has been an extremely useful one when used appropriately. However the attenuated vaccine has numerous limitations, i.e., it appeared capable of inducing abortion if administered to pregnant women, and because of greater potential reactogenicity, USAMRIID would not recommend the administration of this vaccine to children. Thus, this attenuated vaccine could not be recommended for use in the general population. He reported that the Institute had recently obtained approval to begin initial testing of a new killed, TC-83 vaccine in man. Dr. Woodward stated that he had asked this particular question to illustrate the perfect example of how USAMRIID could develop an important vaccine that would not have been developed elsewhere.

Dr. Jordan wanted to know the relationship between the mission of this Institute and the mechanisms used for surveillance of infectious diseases of potential military importance; also, he was interested in how priorities were established relative to the mission. Colonel Metzger replied that this represented a real problem area because there was a complete lack of usable medical intelligence on BW. The Institute attempts to compensate for the lack of information and also a lack of BW defense precedent by developing a broad program of animal models for study and testing and he felt that if the country had to face an infec-

tious agent not previously encountered, perhaps the Institute would have worked with a related model infection which would have bearing and some significance. Regarding the setting of priorities, Colonel Metzger felt that emphasis should be given to the problem of early and reliable diagnosis of an infectious disease. He said that this was also a problem because new concepts and new approaches were seldom being generated in this area of medicine.

General Dirks made several points regarding Dr. Jordan's two questions. He commented upon the multiple sources of information about infectious disease incidence around the world. The Command utilized the World Health Organization, information supplied to CDC, USAMRDC laboratories scattered throughout the world, particularly along or near the equator, and information supplied by the daily newspaper. General Dirks felt that all of this information gives us some anticipation of new problems. He indicated that in dealing with military contingency planning, we have an obligation to consider virtually any and every disease that might occur anywhere in the world. General Dirks felt that it was a large and complex task to interpret all information, apply it to a manageable mission, and then to execute programs that accomplished the mission. He reported that he and his staff had recently completed an exercise in which all USAMRDC programs were assigned priorities, and that little agreement was achieved between laboratories, since each placed greater importance on their own mission.

General Dirks felt that other factors were important in the assignment of priorities; i.e., (1) the ability to perform work within a given technological area; (2) the state of the art; and (3) the availability of personnel. He stated that the driving force is not always perceived as clear-cut, immediate requirements for information. He attempted to balance all of these inputs into an array that might place the Army Medical Department in a position to solve problems facing our troops wherever they might be committed in the world. General Dirks reminded the group that specific contingency plans were classified; however, newspapers constituted a good resource. General Dirks indicated that these problems were constantly reviewed and asked the group for their recommendations since he felt that there were sufficient problems in this area to share with everybody.

Dr. Benenson reported that since 1898 typhoid fever has been considered a disease unique to the military and that 99% of the research had been accomplished by the military. He wanted to know who will work on this disease since it has now been declared to be a nonmilitary problem. General Dirks responded by stating that USAMRDC would have difficulty in persuading anyone on Capitol Hill and in the Executive Branch that typhoid fever is a military problem based on the number of cases since World War II and the following factors: (1) information is available on sanitation and disease prevention; (2) vaccines are available although their efficacy could be discussed; and (3) a method of antibiotic treatment is known. General Dirks felt that with this body of information available, we would have trouble convincing his superiors that typhoid fever required continued research.

Dr. Benenson indicated that the civilian community had solved the smallpox problem and wondered if, because of a declining "herd immunity" it would now become a prime military problem. He expressed his concern that as long as Russia vaccinated its forces, the U. S. must also continue its vaccination program. He considered smallpox as a prime candidate for BW. General Dirks responded to this comment by dividing it into two parts: (1) from the operational aspect, smallpox could be a problem among large numbers of unprotected troops; and (2) from the R&D aspect, monies could not be justified to perform additional research. General Dirks pointed out that since there is sufficient knowledge to eradicate smallpox from the world scene, R&D monies should not be spent; however, monies to purchase stocks of vaccine should be considered.

Dr. Benenson reported that Colonel Metzger's assessment was correct; that is, early diagnosis is needed because it takes at least one week to differentiate smallpox from certain other infections.

Dr. Griffin stated that since venereal disease (VD), by fiat, was no longer considered to be a unique military problem he wondered if now military commanders would not "get off the backs" of preventive medical officers and staff surgeons regarding VD rates. General Dirks replied that we are gaining some understanding from military commanders on this subject. He thought that the decision regarding VD was not by fiat. Based on recent mortality and morbidity reports, VD has become more of a civilian problem. General Dirks noted that these figures could change overnight if troops were committed to combat. He concluded this part of the discussion by stating that it would be very difficult to obtain monies for VD research from a critical Congress when VD rates among military personnel were declining.

CURRENT STATUS OF USAMRIID AND ITS RESEARCH ACTIVITIES

William R. Beisel, M.D.
Scientific Advisor

Again, this year, we will begin the scientific portion of our Planning Session with a general overall review and progress report of the current status of USAMRIID. I will summarize the nature of our mission, the status of our laboratory facilities, the composition of our staff, our current research efforts, and problems. We want your opinions and recommendations on these last two points.

Last year we stated that the professional program at USAMRIID had reached a new level of relative stability and maturation. We assess our overall research productivity in a similar manner again this year; this assessment is supported by the publication record shown in Figure 1.

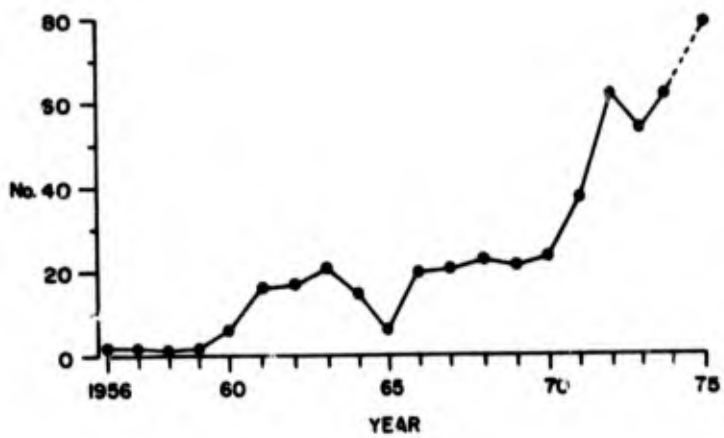

Figure 1. USAMRIID publications by calendar year.

Minus factors seem to have been balanced out fairly well by plus factors. Institute staff members are continuing to produce a variety of solid research achievements and these seem to be having a growing impact upon the field.

Our building continues to provide us with the safe, yet flexible type of laboratory facility needed to conduct complex microbiological research studies in an environment that is relatively free from operational problems. The annex building has now been upgraded to have virtually the same type of functional capabilities. The renovated laboratory space there is operational and has provided additional open- and containment-type laboratories. The latter include a large, specially designed

Class 3 Suite that will permit expanded research efforts with several highly dangerous microorganiums. Engineering plans have been drawn up to allow eventual removal of the huge stainless steel tanks in the annex so that eventually the space still unavailable to us can be converted to modern animal holding areas. If this renovation is included within the next Department of Defense budget, actual work could begin in FY 1977.

Fort Detrick has been a medical post for several years now; we are finding it increasingly easy to obtain full cooperation with respect to the procurement of supplies, equipment, and other services. The administrative relationships between the Institute and the Post have benefited by a progressive tightening up of budget accountability.

Several other areas of support for ongoing research efforts have been improved. These include the establishment of a team of medical maintenance specialists with the capabilities needed for trouble-shooting most of our laboratory equipment. In addition, our biomathematical capabilities have again become stabilized through the acquisition of a computer terminal. This terminal interacts with the large computer facility at Walter Reed as well as with the one at the National Bureau of Standards. Our facilities for maintaining farm animals have been upgraded also, with the acquisition of additional pasture land and improvements in the barn and corral.

Our research mission shown here has remained unchanged during the past several years.

To conduct studies on the pathogenesis, diagnosis, prophylaxis, treatment, and epidemiology of infectious diseases with particular emphasis on problems associated with medical defense against biological warfare, on naturally occurring diseases of peculiar military importance, and on microorganisms the study of which requires special containment facilities.

We continue to group our individual research work units into the three major areas shown. No classified research is conducted in the Institute.

USAMRIID Research Approaches

Pathogenesis
- Microscopic, biochemical, physiological, aerobiological, and radiobiological techniques
- Animal models
- Bacterial toxins

Prophylaxis and treatment
- Vaccine development
- Cellular and humoral immunogenic mechanisms
- Viral chemotherapy
- General supportive care

Diagnosis
- Rapid, new immunologic and nonimmunologic techniques

Our individual Divisions are shown in the organization chart (figure 2). Aerobiology, Animal Assessment, Bacteriology, Medical, Pathology, Physical Sciences, Rickettsiology and Virology divisions have research productivity as their primary mission. They may also have some support functions.

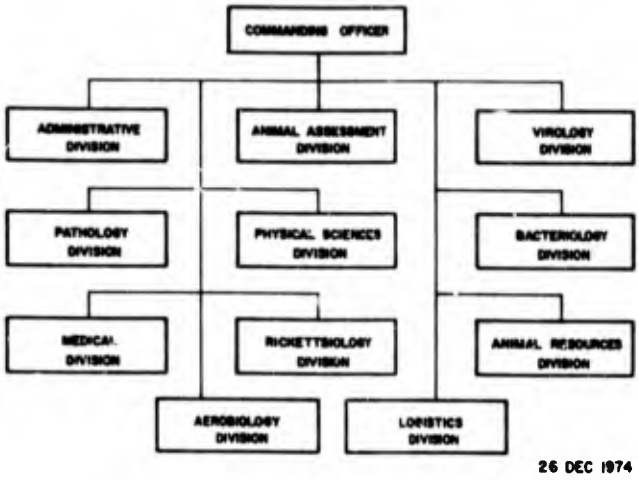

Figure 2. Organization chart of USAMRIID.

Principal research approaches and projects

We continue to employ the philosophy that no boundaries or administrative walls will be created within the Institute that could preclude the full utilization of all our facilities and scientific capabilities in the solution of research problems. Thus, almost all research efforts are based upon the collaborative efforts of scientists and support personnel in more than one division. This philosophy allows a great deal of flexibility for planning and implementing research activities and for gaining maximum utilization from the scientific talents.

Newly arrived professional staff members are assisted in their development of research plans in order to combine their own unique skills and research interests with the military medical requirements as stated in our mission.

Bacteriology Division

The Bacteriology Division is directed by COL Harry G. Dangerfield, MC; to it are assigned the majority of our physician investigators. The ongoing research efforts of the division primarily involve studies in the field of immune responsiveness. Within the broad area, attempts are made to improve methods for detecting cell-mediated immunity and to

investigate the role of various host-defensive systems. These include the interrelated roles of the complement, coagulation, and kinin systems. This latter work is coordinated with investigators in the Pathology and Animal Assessment Divisions. In addition, Major Ascher and Captain Andron (Physical Sciences Division) are attempting to isolate and characterize the molecular species responsible for the transfer factor phenomenon. These two officers played major roles in serving as the local hosts for the Second International Symposium on Transfer Factor which was recently held at the Institute.

In addition to division research aimed at devising methods for measuring host capabilities for mounting a cell-mediated immune response, studies are continuing to determine the requirements for a positive transfer of cell-mediated immune responsiveness from a sensitized donor to a nonsensitized recipient host. This work was initiated by Dr. Eigelsbach, who has now retired, and is being carried on at the present time by Colonel Hunter, Major Harold Hawley and Dr. Virginia McGann. Their current findings suggest that when spleen cells from a sensitized donor are transferred to a nonimmune host, they must receive continuing stimulation by the specific antigen in order to make the transferred cells effective in conferring adoptive immunity. This principle has been demonstrated most clearly using tularemia as a model in inbred groups of mice. The hypothesis that the phenomenon is influenced importantly by lymphokines originating from the transferred cells is being investigated in a search for the underlying mechanism.

Dr. Canonico and several others are continuing their productive work to describe and interrelate the complex pattern of molecular response within key single cells of the host. The chief emphasis of this group is placed on phagocytic cells and on hepatocytes which remain metabolically intact when isolated and grown in tissue culture systems. Major recent emphasis is placed upon the role of the peroxisome in regulating energy metabolism of the infection-stimulated cells. Other aspects of this research in cellular physiology involve the study of cellular enzyme and membrane responses.

Virology Division

The Virology Division is headed by LTC Gerald A. Eddy. It conducts the program for developing, testing and certifying new viral vaccines. Once a candidate vaccine has passed all safety tests and is successful in experimental animal models, all details must be written up for submission to the Army Investigational Drug Review Board (AIDRB). Their permission for human study is given only after coordination and agreement with the Food and Drug Administration, and all new vaccines must meet FDA standards.

Studies in this division are also aimed at defining immunogenic mechanisms involved in virus-host interaction. The pathogenesis of diseases due to virulent and attenuated arbovirus pairs is studied by Captain Jahrling. LTC Edelman the next speaker will describe studies in man with respect to the rapid responsiveness to immunization of an apparently unique subpopulation of T lymphocytes.

Another major area for continuing study involves the work with Machupo virus and other arenaviruses. This topic will be the subject of detailed presentations later this morning, and of some of our questions for the committee.

Rickettsiology Division

This newly established research division has had a productive first year. It is headed by Major Carl E. Pedersen. Most work centers on Rocky Mountain spotted fever (RMSF) and other rickettsial spotted fevers. Some work continues with Q fever and some has been initiated with scrub typhus rickettsiae. Hopefully new rickettsial vaccines will emerge from these studies.

Our new RMSF vaccine has been approved for testing in man and the initial safety study is underway. It is too early to know if this new vaccine will stimulate more immunity than the Lederle vaccine.

We also emphasize the basic studies needed to improve our overall understanding of rickettsiae; this includes attempts to improve methods for detecting both cell-mediated and humoral immunity. Because we wish to become the center of recognized excellence in the study of rickettsial illnesses, studies in this area will be described this afternoon.

Pathology Division

LTC James Stookey heads this division and conducts a preceptor training program for 5 veterinary pathologists. These men provide critical pathology support for all animal studies conducted throughout the Institute, and maintain continuing surveillance of any incidental lesion or disease among animals arriving in the Institute. Over 3,000 studies are completed each year. The preceptor training program has produced more board-certified veterinary pathologists since its inception than any other military training program in this specialty.

The major research efforts of the division involve the production, purification and characterization of bacterial enterotoxins. Colonel Metzger participates importantly as a primary investigator in this group and the scope of its activities has been expanded during the past year. Several of the more recent findings will be described at tomorrow's session.

In 3 other areas of research, coagulation mechanism changes are studied during various types of infectious illness; attempts are being made to differentiate bacteria by physical means; and Major Arthur Anderson is studying ultrastructural changes in lymphoid tissues that result as a consequence of stimulation by various types of antigen.

The final aspect of the Pathology Division research includes the electron microscopic studies being conducted by Dr. John White and Colonel Benjamin Veltri.

Colonel Veltri's work emphasizes stereoscopic and freeze-etch techniques using transmission microscopy. A large portion of Dr. White's recent work has involved scanning electron microscopy. A slide was shown of a microcolony of Mycoplasma pneumoniae in the ciliated tracheal epithelium of a hamster infected via the intranasal route 3 days earlier by Dr. Jemski, Aerobiology Division. The technique for preserving the bronchial cilia in an intact manner was worked out by Major Hetsko, Bacteriology Division, who bled the animal and then perfused the lung in situ with glutaraldehyde injected into the right ventricle. Such an adequate fixation is needed to maintain the cilia in an intact state for scanning studies.

Physical Sciences Division

Physical Sciences Division is led by LTC Philip Z. Sobocinski. The division continues to study the metabolic biochemical and endocrine nature of host responses during infectious illnesses. Multidisciplined approaches are needed in order to characterize the interrelated molecular mechanisms used by cells for the simultaneous adaptations of all key metabolic pathways. An important new finding during the past year is the observation by Dr. Harold Neufeld and his colleagues that infectious stress seems to have a unique propensity for inhibiting the production of ketone bodies by the liver. Included among other studies of host response during infection are measurements of cyclic AMP, serotonin, the catecholamines, other neuroregulatory factors, and a variety of hormones.

On several occasions in recent years we have described to you a series of studies suggesting that phagocytizing white blood cells liberate endogenous mediators that regulate many metabolic changes of the host, in addition to the endogenous pyrogen that stimulates the hypothalamus to initiate a febrile response. The current status of attempts in the Physical Sciences Division to characterize and purify the unidentified substances will be described this morning by Dr. Carol Mapes who has been with the Institute for approximately one year.

Aerobiology Division

The Aerobiology Division is headed by Major Jerry S. Walker and continues its studies of naturally acquired or experimentally produced respiratory illnesses. Major emphasis has been placed upon the development of suitable animal models, the comparison of localized respiratory tract immunity versus systemic immunity, and most recently, the use of aerosols for therapy.

Among the most important findings of this group is the recent identification of the squirrel monkey as being highly susceptible to aerosolized influenza virus. This primate suffers a form of clinical illness that closely resembles acute influenza in man. This model is being exploited to determine the effects of antiviral chemotherapy for either prophylaxis or treatment. Therapy studies will be described this afternoon.

In other aspects of division work, Dr. Joseph Jemski and Dr. George Scott are each studying different aspects of the local immunity produced within the lung when stimulated by antigens delivered via an airborne route. Mr. Edgar Larson is continuing his manipulation of mathematical models of respiratory virus infections.

Animal Assessment Division

This division is led by LTC Duane Hilmas and engages in a wide range of studies. Work is continuing on the development and testing of several different animal models of infectious disease which are used in turn for the evaluation of immunological or therapeutic effectiveness. As described last year by Major William E. Houston, attempts continue to improve effective immunity produced by relatively weak antigens.

Dr. C. T. Liu and others continue investigations of physiological abnormalities that develop as a consequence of infection. Recent emphasis has been placed on pulmonary functional abnormalities that occur during airborne infections.

LTC Hilmas, himself, conducts the continuing Institute program for studying the interrelationships between radiation exposure of the host and susceptibility to various infectious diseases. This work utilizes either our million-volt x-ray source for acute radiation, or a cobalt source for chronic low-dose radiation.

One of the most active areas of Animal Assessment Division research, antiviral chemotherapy, is being conducted by Major Edward L. Stephen. This topic will be presented later today.

Medical Division

Despite the virtual cessation of our Whitecoat program due to the termination of the draft, the Medical Division continues to have the responsibility for continuing the year-by-year evaluation of the experimental vaccines we employ in the protection of our "at risk" laboratory personnel. Major Michael Ascher is head of this Division. In addition he conducts or coordinates whatever volunteer studies we do.

With the recent publication of the new Research and Development Command Regulation 70-25, Use of Human Subjects in Research, a defined procedure has been established for the initiation of any future volunteer studies by laboratories such as this. As shown on the flow chart, a protocol must first be initiated by a research investigator in coordination with the Chief of the Medical Division. After this protocol has been reviewed by an in-house group consisting of our Division Chiefs, it is reviewed by the USAMRIID Human Use Committee. This committee has now been formally constituted; it meets all requirements set forth in the regulation, including the presence of a lawyer, a minister, and non-Department of Defense members. Protocols that receive unanimous approval by this Committee would then be forwarded to the R&D Command Headquarters.

FLOW CHART FOR VOLUNTEER RESEARCH PROTOCOLS

(Based on USAMRDC Reg. 70-25, Use of Human Subjects in Research, Oct 1975)

Research investigator---Chief, Medical Division

↓

Coordinate protocol development

↓

Review by "Chief's" Committee

↓

Review by USAMRIID Human Use Committee

↓

Submission to HQ, USAMRDC

↓

REVIEW COORDINATED BY SGO HUMAN USE REVIEW STAFF

a. FDA ⇌ AIDRB

b. SGO Human Use Committee

c. SGO Clinical Investigation Committee

Protocols will be staffed by the Human Use Review Staff of the Surgeon General's Office and evaluated by a combined group, including The Surgeon General's Human Use Committee, The Surgeon General's Clinical Investigation Committee, and the Army Investigational Drug Review Board. Only after approval from all of these groups has been obtained will the study be conducted. These recently defined steps will relieve this Ad Hoc committee of the direct need to evaluate each specific research protocol. We will, however, still need your advice and guidance about the overall directions and goals of our volunteer program.

Our first studies under this new regulation will involve the initial testing of new experimental vaccines to evaluate their safety and efficacy. Since protocols of this type will require only limited numbers of volunteers, we anticipate that they could be obtained from the military and civilian staff of the Institute.

Animal Models Developed at USAMRIID

Because of the recent cutback in volunteer studies we have emphasized the development of suitable animal models of disease. Shown in Table I are a number of the newly developed virus models. These have

TABLE I. RECENTLY DEVELOPED USAMRIID ANIMAL MODELS

ORGANISM	SPECIES	UNIQUE FEATURES
Viruses		
Machupo	Green monkey	Death with prominent hemorrhagic diathesis and disseminated intravascular coagulation
	Guinea pig	Death with hemorrhagic diathesis
JBE	Rhesus or cynomolgus monkey	Lethal infection via intranasal route
VEE	Hamster	Hyperacute death due to bacterial sepsis and endotoxemia
Influenza	Squirrel monkey	Acute respiratory illness. Increased susceptibility to secondary infections
Tacaribe	Adult rat	Lethal infection
Rickettsia		
C. burnetii	DBA/2 mouse	Lethal infection by aerosol or inoculation
Bacteria		
S. typhimurium	Cynomolgus monkey	Lethal infection with hemorrhagic diathesis
K. pneumoniae	Rat	Chronic, severe penumonia

excellent promise for allowing us to study the immune process, therapy, or the pathogenesis of important diseases in a manner not possible previously. I have already mentioned several of these models and you will hear about others during the course of the program today. As an example

the lethal model for Japanese B encephalitis in a primate will allow us to evaluate vaccines and B-group vaccine combinations in a manner not previously possible. Lethal infection with Coxiella burnetii in mice greatly simplifies the testing for protective efficacy of various purified antigens derived from this organism. In addition to these newly developed models, we have standardized a large number of models that are not original with us.

Our professional staff has declined somewhat in total numbers during the past year. This is due to a combination of civilian retirements and the departure of several military physicians who have not been replaced. As we anticipated, the end of the Doctor Draft Law has limited our physician input during the past year chiefly to those individuals who still had Berry Plan obligations. We do not anticipate an improvement in this situation in the immediate future; this will continue to be one of our major problems, inasmuch as the accomplishment of every aspect of our mission is ultimately dependent upon our primary research investigators.

Budgetary cutbacks constitute another problem and will limit acquisition of new equipment and the amount of professional travel to scientific meetings. Figure 3 depicts the schematic distribution of funding for our

Figure 3. Distribution of In-house R&D costs.

in-house research. The outer ring represents the 50% of our total budget consumed by overhead costs; almost 60% of this goes to the Post. Changes in budget this year with respect to in-house research funding is represented by a trend toward more equal distribution of research monies among individual research divisions. The shaded area in each division represents the amount of division funding spent on support type activities. For example, the shaded area in Virology Division is accounted for by the operational costs of the tissue culture suite and its staff, who provide all tissue cultures needed throughout the Institute. We continue to manage the

Institute budget within the Headquarters and Administrative Offices so that none of our Division Chiefs or investigators must be faced with a continuing burden of budgetary problems. This approach allows our Chiefs and primary investigators a type of freedom from funding worries that is rare in most academic research institutes.

In summary, we have maintained our scientific output throughout the past year and are continuing to produce exciting new data in a large variety of research areas related to infectious diseases.

Our volunteer study program will pick up again during the next year with studies directed toward the initial testing of new experimental vaccines for safety and efficacy.

We continue to see a shortage of research physicians and a variety of budgetary constraints as our major problems of an administrative nature.

SUBPOPULATION OF ACTIVELY ROSETTING T-LYMPHOCYTES AS AN INDEX OF CELL-MEDIATED IMMUNITY IN MAN

Robert Edelman, LTC, MC

CPT Felsburg and I have been interested in exploring the human immune response to arbovirus vaccines. Toward that end, we have examined some aspects of cell-mediated immunity (CMI) in man.

The immune system is divided operationally into 2 major components: the humoral immune system of B lymphocytes and the cell-mediated immune system of T lymphocytes. Recently techniques have been developed for the identification and enumeration of human B and T lymphocytes by detection of surface markers. These markers, which are unique to each cell type, have facilitated the study of these subpopulations. Peripheral B lymphocytes bear surface immunoglobulin, receptors for complement, antigen-antibody complexes and aggregated IgG. Human T lymphocytes may be enumerated by immunofluorescence with antithymocyte sera and by their ability to bind sheep red blood cells (SRBC) in a rosette formation. Wybran and Fudenberg[1] have recently quantitated a subpopulation of T cells with high affinity receptors for SRBC. These lymphocytes spontaneously bind to SRBC to form rosettes after a 5-min incubation time. This contrasts to the 60-min incubation time required to detect the total T cell population (T-RFC). These rapidly binding lymphocytes are called active rosette forming cells (A-RFC) and normally comprise about 30% of peripheral blood lymphocytes. The total T cell population normally comprises about 70% of peripheral lymphocytes. The percentage of A-RFC in the peripheral blood is thought by Wybran and his co-workers to reflect the cellular immune competence of patients with cancer and other diseases. By contrast, the percentage of total T-cell rosettes in many of their patients were reported not to reflect immunological competence.

The purpose of our study was to characterize the A-RFC subpopulation of T cells in healthy adults. To do this, we measured changes in circulating lymphocyte subpopulations following induction of delayed cutaneous hypersensitivity (DCH) with microbial antigens. Skin tests were performed, because we had discovered by serendipity that the number and percentage of A-RFC in the peripheral blood of healthy persons rose during positive skin test reactions.

Volunteers in our studies were healthy adults from the staff of USAMRIID. Informed consent was obtained. Some individuals were tested with PPD. They were selected from persons enrolled in the USAMRIID tuberculosis control program. Other subjects were tested with the Foshay tularemia skin test antigen. They were selected on the basis of whether or not they had previously been vaccinated against tularemia with the live, attenuated tularemia vaccine. The skin test antigens were applied intradermally on the volar surface of the forearm and read at 24, 48 and 72 hr. The skin tests were considered positive if the diameter of induration was $\geq$ 5 mm.

Individuals were bled for 3 consecutive days before the skin test in order to establish a baseline value for each of the lymphocyte subpopulations. Blood was then collected repeatedly during the first week after skin testing. Total leukocyte and differential counts were done. The percentages of B and T cells in each volunteer were measured as follows: fasting venous blood was collected in heparin. The peripheral blood lymphocytes were isolated on Ficoll-Hypaque gradients. For each test, a washed lymphocyte suspension was preincubated with fetal calf serum. Washed sheep erythrocytes were added and the cell reaction mixture was centrifuged at low speed for 5 min to permit brief contact of lymphocytes with sheep erythrocytes. The cell button was gently resuspended with a Pasteur pipette and the rosettes were counted in a hemocytometer chamber. A lymphocyte was classified as an active rosette if $\geq$ 3 sheep cells adhered to its surface. At least 200 lymphocytes were counted per replicate sample, and each sample was run in triplicate.

The total T cell population was measured using a similar procedure except that the lymphocytes and sheep erythrocytes were kept in close contact for 60 min instead of 5 min before they were resuspended and counted. Peripheral B cells were measured by membrane immunofluorescence for surface immunoglobulin. We used fluoresceinated rabbit antisera specific for the heavy chain of each major human immunoglobulin class.

We first examined a group of 60 normal men, and determined their normal baseline values for the 3 subpopulations of peripheral lymphocytes.

The mean percentages + 1 SD for each subpopulation are shown in Figure 1, active E-rosettes = 28.1 $\pm$ 3.3, total E-rosettes = 66.7 $\pm$ 5.5, and Ig-bearing B cells = 29.3 $\pm$ 4.0. The daily variability in circulating

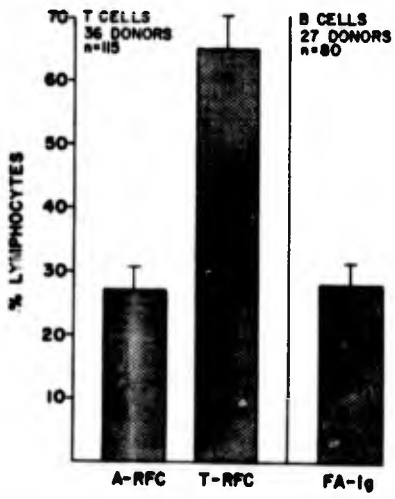

Figure 1. Normal values for human lymphocyte populations.

lymphocyte subpopulations in 10 individuals are shown in Figure 2.

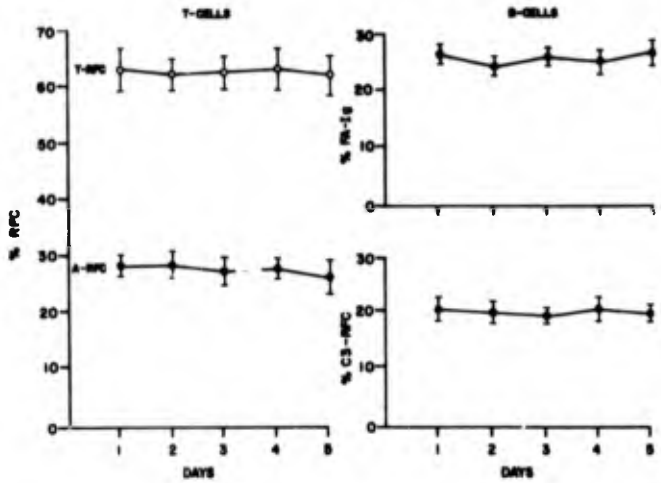

Figure 2. Daily variability of circulating lymphocytes in 10 humans.

We examined the active rosettes after intermediate-strength PPD skin tests in 7 persons. Results are shown in Figure 3.

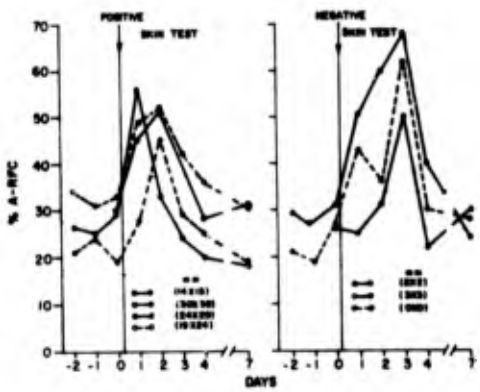

Figure 3. A-RFC response following intermediate strength PPD skin test.

The shaded area represents 2 SD around the baseline mean of the group. Any individual value > 2 SD from the baseline mean was considered significant. Four individuals demonstrated positive DCH reactions, and 3 had negative cutaneous responses. Both groups, however, exhibited a significant rise in A-RFC after skin testing. The A-RFC in the skin-test positive group peaked between day 1 and day 2, whereas the A-RFC in the skin test negative group peaked a day later. The 3 persons skin-test negative to the 5 tuberculin units in intermediate-strength PPD were

later challenged with 250 units tuberculin or, second-strength PPD. All 3 developed positive skin test reactions to the 250-unit dose. This suggests that the earlier test dose of 5 tuberculin units was not sufficiently potent to elicit delayed type hypersensitivity.

Since this study failed to produce any skin test negative controls, we initiated a second one in an attempt to identify truly PPD-negative individuals. Nine persons with negative skin tests to intermediate-strength PPD were challenged with second-strength PPD and their lymphocyte kinetics were examined (Figure 4). Five of the 9 developed positive

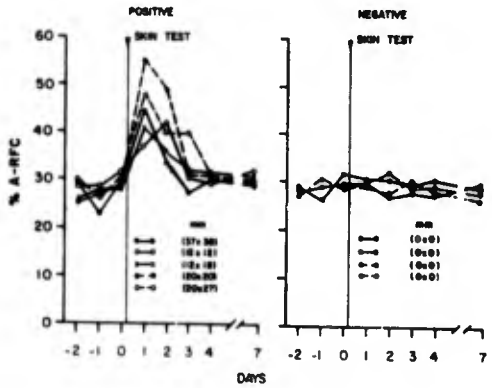

Figure 4. A-RFC response following second strength PPD skin test.

DCH reactions, and 4 were skin-test negative. A significant rise in A-RFC was observed during the first 3 days in only the skin-test positive group. No significant changes were observed in the percentages of total rosette-forming cells or in B cells in either group during the study.

A third study was conducted with tularemia antigen (Figure 5). Four

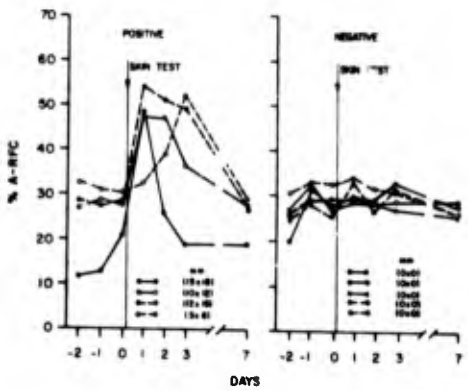

Figure 5. A-RFC response following tularemia skin test.

individuals previously vaccinated against tularemia exhibited a positive DCH reaction to the tularemia antigen, while the 5 not vaccinated were tularemia skin-test negative. The skin-test positive group showed a significant increase in their A-RFC, whereas the negative individuals did not. Again, no consistent changes were observed in the percentages of total T or B cells in either group.

I would like to make a few comments about these in vivo studies before moving on to describe in vitro experiments. First, the kinetics of the A-RFC response closely parallel the skin test response. Both reactions appeared to peak between 24 and 48 hr, except in one person in whom maximum skin test induration and peak A-RFC values occurred at 72 hr after the tularemia skin test. Studies of 2 tularemia-positive individuals bled at 6-hr intervals indicated that the number of active rosette forming cells begin to increase in the circulation between 12 and 24 hr after intradermal deposition of antigen. Secondly, 3 persons skin-test positive to 250 tuberculin units but negative to 5 units all showed a very brisk rise in A-RFC after challenge with the smaller dose of antigen. This would suggest that active rosette formation is highly sensitive. It may be a better index of cellular immunity to mycobacterial antigens than the delayed cutaneous hypersensitivity reaction when low doses of PPD are used.

Finally these results confirm the findings of Wybran[1] and Yu[2] and their co-workers who reported that the active rosetting cells are a subpopulation of total T lymphocytes, but may vary independently of total T lymphocytes. A recent report by Yu[3] showing different surface membrane sulfhydryl and sialic acid residues on A-RFC provides further support for the thesis that these cells comprise a distinct subpopulation of T lymphocytes.

We have worked under the assumption that skin test antigens somehow alter the lymphocyte membrane in vivo to permit the lymphocyte to more avidly bind SRBC. We have attempted to develop an in vitro system that would detect the same change.

Initially, purified lymphocytes from 3 tuberculin positive donors were incubated with and without PPD at 37 C. A PPD dose of 100 ng/ml was used, because this dose is similar to that employed in vitro assays of lymphocyte transformation. Quadruplicate samples were assayed for their ability to form active rosettes after 1, 2, 4 and 6 hr incubation with PPD.

As can be seen (Figure 6) in these 3 PPD positive donors, the lymphocytes incubated with PPD antigen increased in their ability to form active rosettes. No rise occurred in the absence of PPD. The optimum incubation time appeared to be $\sim$ 4 hr. We next performed dose-response experiments with PPD.

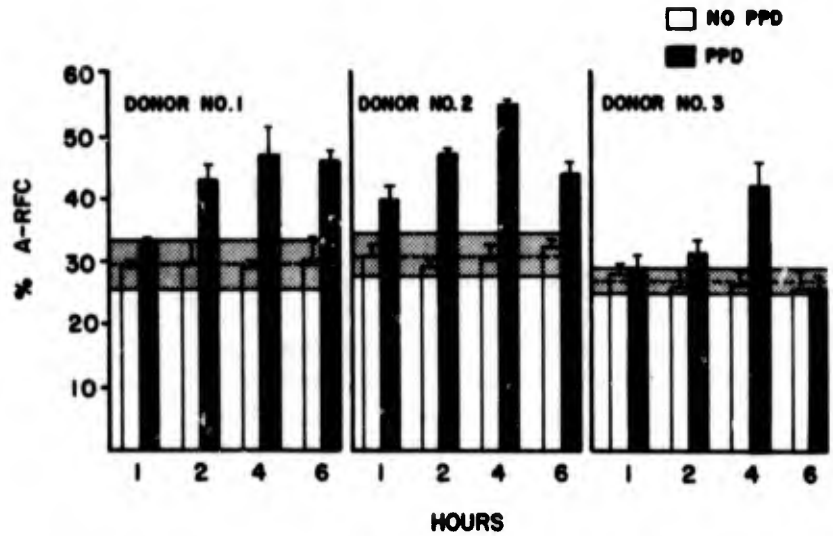

Figure 6. Effect of PPD on in vitro A-RFC.

We show the composite results of 7 skin-test positive and 3 skin-test negative donors in Figure 7. The dose of PPD giving maximum increase in active rosettes after 4 hr incubation in positive donors is 100 ng/ml, the highest dose tested. Lymphocytes from PPD-negative donors do not respond to any of the antigen concentrations used.

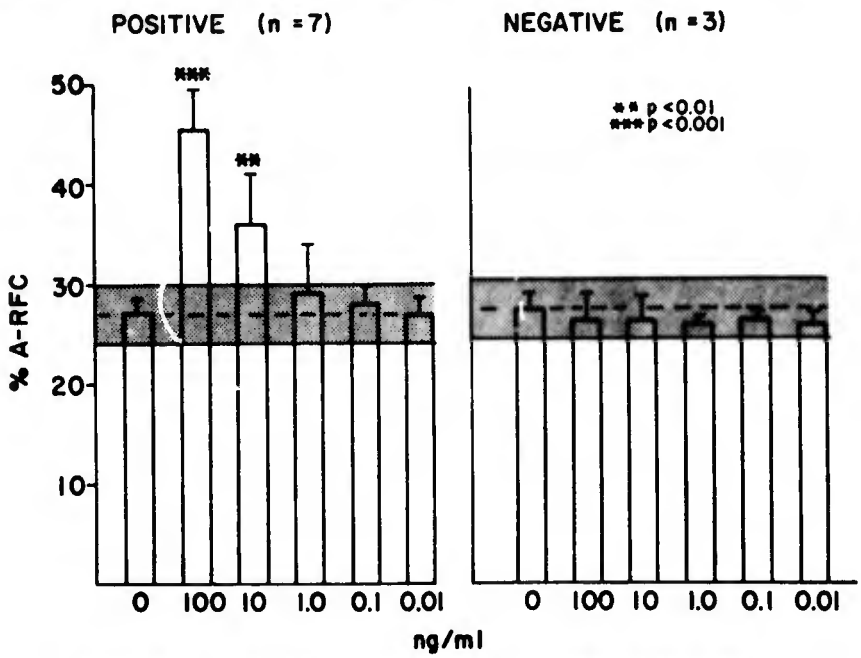

Figure 7. A-RFC in vitro PPD dose response.

Using lymphocytes of one tularemia-positive blood donor, (Figure 8) the maximum rise in percent of active rosettes occurs after 3-4 hr. This

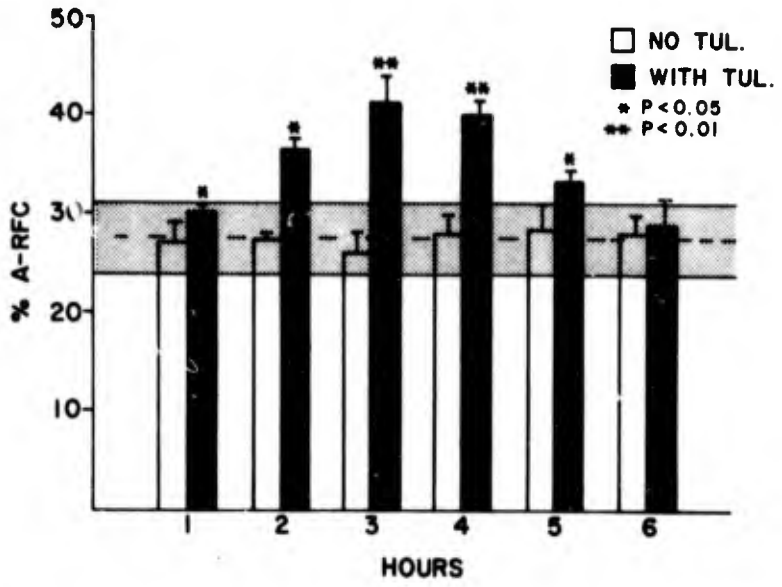

Figure 8. A-RFC temporal response in vitro to tularemia antigen.

is the same time required for a maximum PPD response. We used 160 ng/ml of tularemia antigen. The fall in numbers of active rosettes after 4 hr is interesting but unexplained.

The dose response in tularemia-sensitized cell cultures is illustrated in Figure 9. We obtained lymphocytes from 2 tularemia-positive

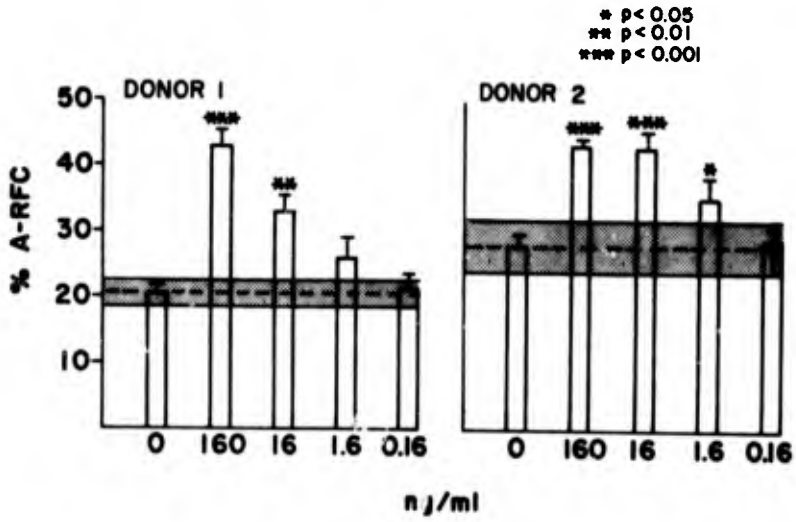

Figure 9. A-RFC in vitro tularemia dose response.

donors. The dose of antigen producing maximum rise in the percent of active rosettes after 4 hr incubation was 160 ng/ml in one donor, and between 16-160 ng/ml in the other. Concentrations > 160 ng/ml were cytotoxic.

We tested 2 tularemia-negative lymphocyte donors. Tularemia-negative donor cells treated with the optimal antigen dose and incubated 4 hr did not respond to the antigen (Figure 10, left side). We collected

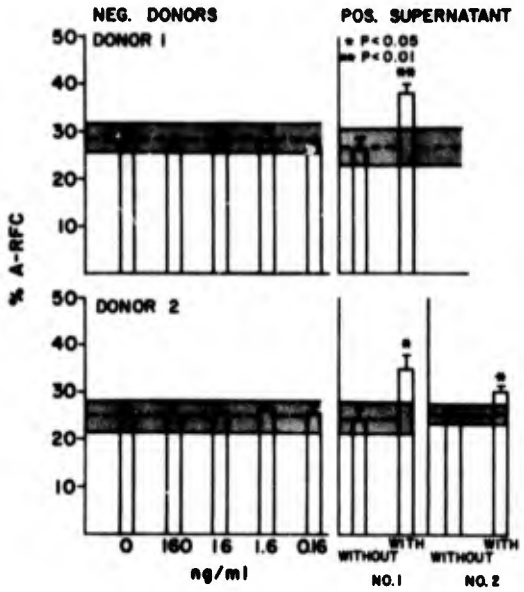

Figure 10. A-RFC *in vitro* tularemia dose response.

the supernatants from sensitized lymphocyte cultures incubated for 4 hr with the antigen. We then incubated non-responding lymphocytes with this supernatant; the percentage of active rosette-forming cells increased. It would appear that a soluble factor may be released upon incubation of sensitized lymphocytes with antigen. This factor or factors can convert a nonrosetting cell into an active rosetting cell.

Preliminary data suggest that this *in vitro* assay may provide a sensitive test for dealyed hypersensitivity to microbial antigens. The test is rapid, it can be done in one day while other *in vitro* tests of cell-immunity require many days. The *in vitro* test is somewhat fickle, and is as yet technically difficult to run.

Our *in vivo* results suggest that the increase in circulating A-RFC may represent a subpopulation of immunologically active T cells. An increase of these cells in the circulation would imply augmented T cell activity.

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DISCUSSION

Dr. Pappenheimer posed three questions: (1) Why did Dr. Wybran think that the percentage of rosetting cells in the blood reflected cellular immune competence in patients with cancer; (2) Have the effects of neuraminidase, trypsin and other enzymes been thoroughly investigated to determine their influence on the membrane of normal lymphocytes? Dr. Pappenheimer stated that such substances might be liberated following the antigen reaction with the T cell; and (3) Would the number of rosette-forming cells be increased if normal lymphocytes were broken up, then added to a fresh pool of lymphocytes?

Dr. Edelman replied that Wybran's data showed that patients with metastatic disease exhibited severe depression in their active rosetting ability. With treatment and favorable clinical responses, their active rosetting cells came back to normal levels, or even to above normal values in some patients. In addition, patients with immunodeficient syndromes, when treated with transfer factor, showed a rise in the rosetting cells and the rise was associated with the production of migration-inhibition factor in cultures and the appearance of lymphocytotoxic reactivity.

Dr. Edelman answered question (2) by stating that cells treated with trypsin lose their rosetting ability because the enzyme seems to cleave off a trypsin-sensitive receptor site on the surface of the lymphocyte.

In answer to question (3), LTC Edelman indicated that he had not added lysates of fractured lymphocytes to intact lymphocytes.

Dr. Nathanson posed several questions: (1) What happens when a mitogen(s) is introduced into the system; and (2) What is known about the chemistry of the reaction? Dr. Nathanson also stated that it was important to use purified B and T cell populations because, for example, the B cell could release a lymphokine which was able to convert some of the T cells to active rosetting cells. LTC Edelman responded that he had not investigated the effects of mitogens, that virtually nothing was known concerning the chemistry of these reactions and that Dr. Nathanson's last point was well taken.

Dr. Sawyer asked if the rosetting phenomenon could be caused by the mobilization from lymph node sites of that segment of the actively rosetting population which is normally there; i.e., mobilization vs. conversion. LTC Edelman indicated that nothing is known of the mechanism *in vivo* and that either mechanism is possible. Dr. Sawyer asked if pertussis lymphocytosis-promoting factor had been investigated, since it has been shown to flush-out the lymph nodes in mice. He suggested that pertussis-infected kittens might serve as a model to answer this question. LTC Edelman reported that very few people had worked in this area but that he hoped to study pertussis at some future time.

Dr. Benenson commented that the skin test may simply elicit recall, a memory phenomenon, and suggested that in future experiments, the strength of the antigen not be increased but held constant. LTC Edelman reemphasized the point that the skin test represents a very complex reaction and for some reason the active rosette test detects those tuberculin-sensitive individuals who do not have a normal skin test response to lower doses of tuberculin antigen.

MULTIPLE LEUKOCYTIC FACTORS THAT INDUCE REACTIONS CHARACTERISTIC OF THE INFLAMMATOPY RESPONSE

Carol A. Mapes, Ph.D.

Leukocyte-derived substances have been studied for nearly 30 yr. Interest in these substances has developed from the discovery of mediators that induce metabolic and physiologic alterations in experimental animals characteristic of those observed in nonspecific host inflammatory responses. Many of the leukocyte mediators currently being studied in this Institute and elsewhere are derived from stimulated rabbit peritoneal polymorphonuclear leukocytes (PMN).

The following method of preparing leukocytic mediators was adapted from the initial studies of King[1] and Gillman[2] and their associates. Cells of 2-4-kg New Zealand, white rabbits are stimulated and called forth by IP injection of 300-500 ml of 0.2% glycogen in physiological saline. Sixteen hr later the fluid is aspirated from the peritoneal cavity and passed through sterile gauze. The leukocytes, containing more than 95% PMN, are harvested and processed by a series of steps including: low speed centrifugation; washing with Krebs-Ringer phosphate (KRP) buffer, pH 7.0, containing PSH (2000 units penicillin, 0.4 mg streptomycin and 10 units heparin/ml); osmotic lysis of contaminating RBC; and repetition of the KRP wash. The washed PMN are resuspended in saline-PSH at a final concentration of 10^8 cells/ml. Following 2 hr incubation at 37 C in a shaking water bath, the cells are removed by centrifugation and the supernatant is filtered through 0.45-μ Nagle filters. The filtered solution is a crude preparation of leukocyte mediators.

Although little is known about the specific composition of this crude preparation, two areas of research have evolved utilizing the material. One area of research has concentrated exclusively on a mediator of febrile response termed endogenous pyrogen (EP), while the second area has described multiple inflammatory responses attributed to a material termed leukocytic endogenous mediator (LEM). The multiple responses elicited in rats by IP injection of LEM are listed in Table I with the optimal time of assay and the reference initially reporting each observation. IP administration of LEM causes a decrease in the concentrations of plasma Fe^3 and Zn^4 as well as depressed levels of plasma and portal vein glucose.[5] Coincident with the decreases in these parameters, there are: increased incorporation of amino acids into the liver,[6] elevated levels of peripheral neutrophils,[7] increased concentrations of plasma Cu,[8] ceruloplasmin,[8] acute-phase globulins,[9,10] glucagon,[5] and insulin;[5] and an increase in hepatic RNA synthesis.[11] In addition to these responses, LEM preparations engender a hypothermic response in rats[12] and a hyperthermic response in rabbits.[13]

Use of the dissimilar terms EP and LEM to denote the same material suggests a difference in molecular species; however, purified pyrogen[14] has not been studied with respect to any metabolic response other than fever induction. In addition, there exist few experiments in which either

TABLE I. BIOLOGICAL RESPONSES TO LEM

RESPONSE	OPTIMAL TIME OF ASSAY (HR)	REFERENCE
Decreased concentration of		
Plasma Fe	5 - 6	Kampschmidt et al.[3]
Plasma Zn	5 - 6	Kampschmidt et al.[4]
Plasma & portal vein glucose	4	George et al.[5]
Increased:		
Hepatic amino acid flux	3	Wannemacher et al.[6]
Neutrophil release	2	Kampschmidt et al.[7]
Serum Cu & ceruloplasmin	12 - 24	Pekarek et al.[8]
Acute-phase globulins	10 - 48	Kampschmidt et al.[9] Pekarek et al.[10]
Glucagon & insulin conc.	4	George et al.[5]
Hepatic RNA synthesis	10	Wannemacher et al.[11]

crude or partially purified preparations were used to demonstrate simultaneously similarities or differences between pyrogen and LEM. This situation coupled with the Institute's desire to characterize and purify LEM, necessitated answering the question of whether EP and LEM are different molecules.

One possible method of delineating differences between LEM and pyrogen was through studies designed to compare the release of multiple mediator activities from stimulated PMN, since information was available on substances inhibitory to release of pyrogen activity. One of the better studies in this area was that of Berlin and Wood[15] who in 1964 demonstrated that 5 mM K^+ inhibited release of pyrogen from stimulated rabbit peritoneal cells. Earlier this year the effect of K^+ on release of LEM and EP was compared in a collaborative study with LTC Sobocinski.

The inhibitory effect of K^+ was studied using two series of experimental incubations that contained specified concentrations of the ion. Incubations to test the inhibitory effect of K^+ consisted of 2.4×10^9 cells suspended in 6.0 ml saline-PSH + 6.0 ml of KCl solution having twice the desired molarity. This mixture was incubated 2 hr at 37 C in a shaking water bath, then diluted with an equal volume of saline-PSH. Control incubations containing 2.4×10^9 cells suspended in 12.0 ml saline-PSH were incubated 2 hr at 37 C, then diluted with 6.0 ml of the appropriate KCl solution and 6.0 ml of saline-PSH. This experimental design maintained equivalent dilutions of LEM and K^+ concentrations in all incubations.

The effect of K^+ on general protein release from stimulated PMN is shown in Figure 1. Between 0 and 15 mM added K^+, an increasing inhibition of protein release is noted. The addition of 15 mM potassium

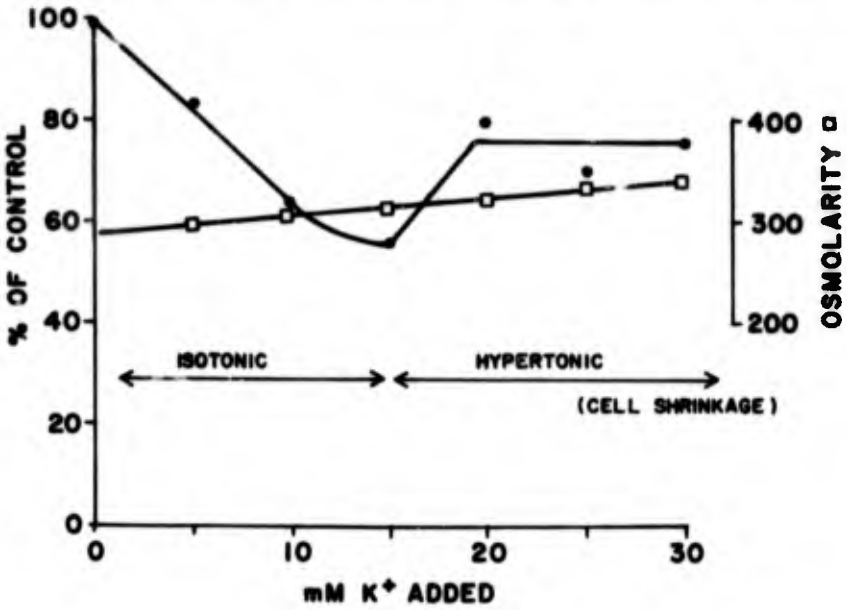

Figure 1. Effect of K^+ on protein release from stimulated PMN.

changes the solution from isotonic to hypertonic. Although studies were not undertaken to elucidate any deleterious effects of hypertonicity of the cellular system, it appears that inhibition of protein release is muted by hypertonic potassium solutions. The significance of this is unknown but the observation may aid in explaining some pecularities in the data to be presented.

The effect of potassium ion on release of pyrogenic activity is shown in Figure 2. Each point on the graph represents the mean and

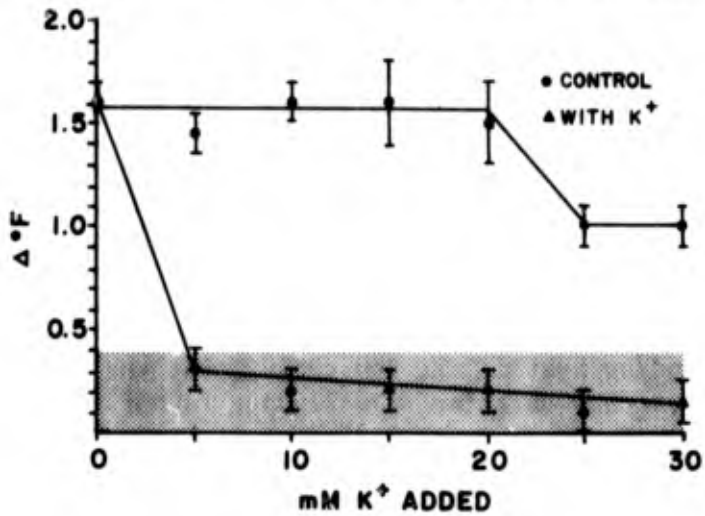

Figure 2. Effect of K^+ on EP release from stimulated rabbit PMN.

standard deviation for fever responses as obtained by the pyrogen assay method of Murphy.[16] Changes in temperature are defined as the difference between the mean temperature in the 60 min preceding injection and the maximum temperature attained 45 min following IV administration of a sample. As depicted by the circles, the release of pyrogenic activity in control incubations was not effected by addition of 5-20 mM K^+. Controls show some inhibition above 20 mM K^+, but still induce significant pyrogenic responses. The cause of this inhibition is unknown, however one can hypothesize either an inhibitory effect on preformed pyrogen or alterations in molecular conformation that render the molecule partially inactive. In contrast, the triangles depicting the release of pyrogenic activity in the presence of K^+, show that pyrogen release is inhibited by concentrations ranging from 5-30 mM. The pyrogenic activity released in the presence of 5 mM K^+ does not induce fever statistically different from the fever produced by an equivalent volume of the nonpyrogenic substance saline. The pyrogenic response to IV administration of saline is indicated by the open circle near the ordinate and is comparable to that produced by pyrogen released in the presence of 5 mM K^+. This indicates complete inhibition of pyrogen release from stimulated PMN.

In the same experiments we also studied the effect of K^+ on release of substances mediating three of the metabolic responses attributed to LEM (Figures 3-5).

As can be seen in Figure 3 there are no significant differences between the controls and LEM prepared in a total absence of K^+. In contrast, all of the LEM released in the presence of this ion has less activity than the normal LEM preparation, thereby indicating some perturbation of the system even in the absence of detectable inhibition. Comparison of each potassium incubation with its control demonstrates that 10 mM K^+ is required to achieve inhibition of LEM release. The inhibitory effect of potassium ion on release of Zn-depressing activity increases in a nonlinear fashion between 10 and 20 mM of added K^+. However, all LEM prepared in the presence of potassium ion has significantly more activity than either saline or the heated LEM control. This indicates < 100% of release.

Figure 4 shows the inhibitory effect of potassium ion on release of amino acid fluxing activity which is evaluated from the amount of ^{14}C-labeled nonmetabolizable amino acid (α-aminoisobutyric acid) incorporated into the liver. Again, none of the controls differed statistically from the normal LEM preparation. There is no detectable inhibition of release with potassium ion concentrations between 5 and 15 mM. At concentrations of $\geq$ 20 mM there is a significant difference between LEM released in the presence of K and the corresponding controls. Again, all of the LEM prepared in the presence of potassium ion has more activity than saline and/or heated LEM, indicating that the ion does not completely inhibit release of an amino acid fluxing factor.

The effect of potassium ion on release of a neutrophil-enhancing activity is shown in Figure 5. In this case, there is no significant difference between LEM released in the presence of 5-15 mM K^+ and that

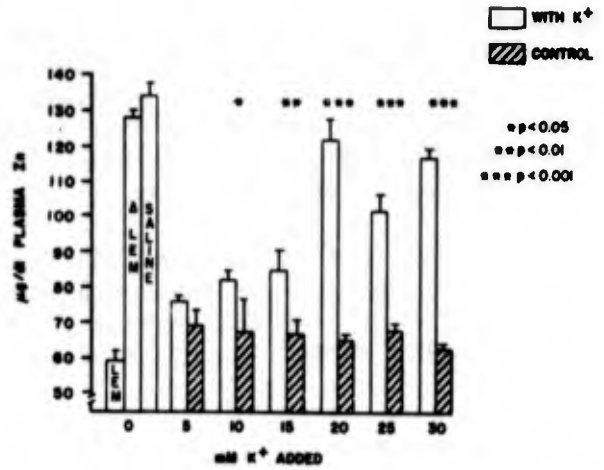

Figure 3. Effect of K^+ on release of a substance mediating plasma Zn depression.

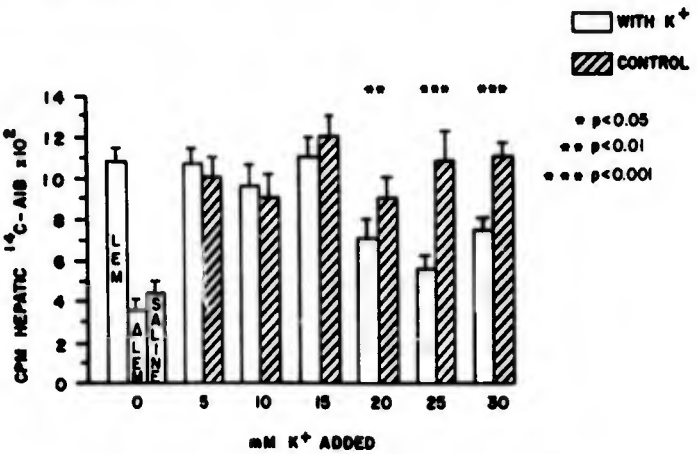

Figure 4. Effect of K^+ on release of an amino acid fluxing activity.

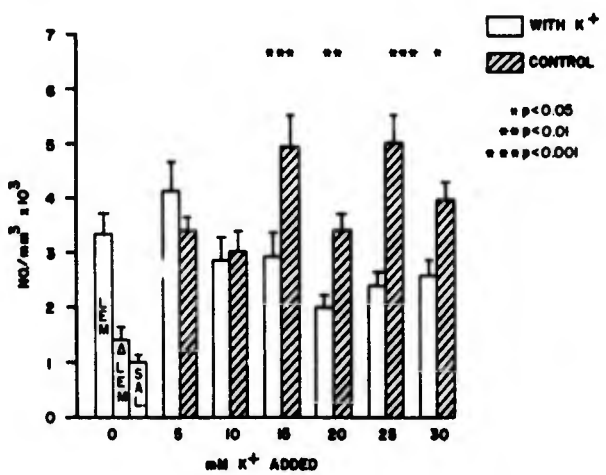

Figure 5. Effect of K^+ on release of a neutrophil elevating activity.

released in the absence of potassium ion. All preparations are significantly inhibited above 15 mM K^+. In addition, a significant increase of activity is observed in both the 15 and 25 mM K^+ controls in comparison to the normal LEM preparation. This results in statistical differences between the 15 mM K^+ preparation and its control even though inhibition of release was not detected at this concentration.

Based on these studies one can conclude that the factor(s) inducing LEM responses differs from pyrogen since release of pyrogenic activity is completely inhibited by addition of 5 mM K^+, whereas 10-20 mM K^+ is required before there is detectable inhibition of LEM release from stimulated PMN.

A second and perhaps more convincing method of demonstrating that FP and LEM are different molecular species comes from their physical separation. Numerous reports indicate that pyrogen purification is plagued by continuous loss of activity due to nonspecific binding to glass.[14] Therefore 75-150-μ glass beads were tested as a possible affinity adsorbent for leukocyte mediators. The recovery of protein and pyrogenic activity in two representative studies are summarized in Table II; ∿ 80% of the total protein in a LEM preparation binds to glass. In the unbound material, there is no detectable pyrogenic response although the untreated LEM preparations induce significant febrile responses. Attempted elution of pyrogen from the glass was unsuccessful, as might be anticipated from some reports that indicate either an irreversible binding, or perhaps denaturation, of the molecule. Since pyrogenic activity could not be recovered, the experimental conditions were assimilated to insure that they had no detrimental effect on activity. The results indicate an actual binding to glass rather than inactivation of pyrogenic activity.

Various LEM activities also were assayed in these experiments. Figure 6 shows a 3-point dose-response curve for a given LEM preparation before and after binding to glass beads. Comparison of the LEM activity in

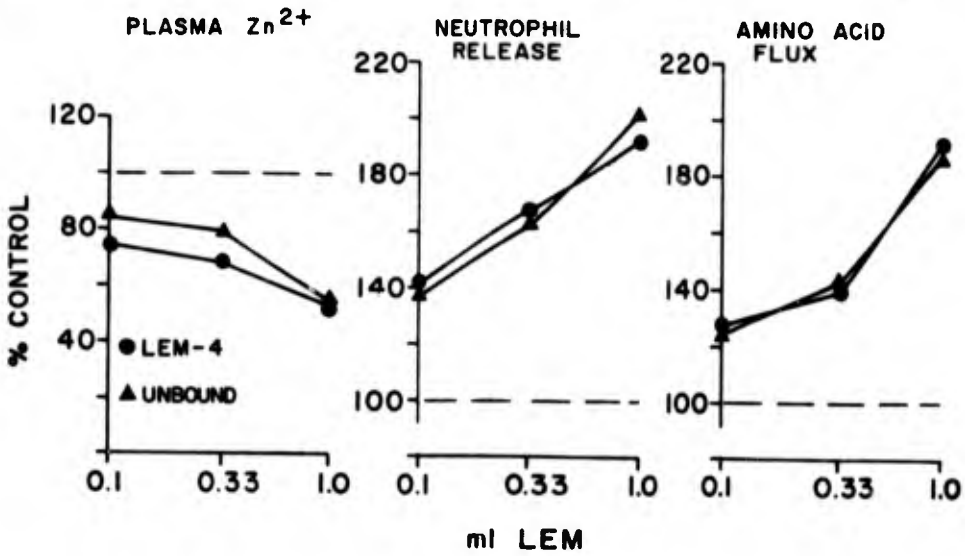

Figure 6. Binding of LEM to glass beads.

TABLE II. BINDING OF LEUKOCYTIC MEDIATORS TO GLASS BEADS

FRACTION	PROTEIN		FEVER (°F ± SE)		Δ°F
	Total mg	% Recovered	T_0	T_{45}	
Study 1					
LEM #4	41.5	100	101.1 ± 0.17	102.1 ± 0.21	1.0
Unbound	10.3	24	101.2 ± 0.1	101.3 ± 0.1	0.1
Study 2					
LEM #9	61.6	100	101.7 ± 0.2	102.9 ± 0.3	1.2
Unbound	12.3	20	103.8 ± 0.2	103.7 ± 0.1	-0.1
0.75 M NaCl eluate	30.8	50	103.3 ± 0.1	103.3 ± 0.1	0.0
LEM #9 - stirred 2 hr at 4 C			102.3 ± 0.2	103.3 ± 0.1	1.0

an untreated mediator preparation with that remaining unbound after adsorption to glass indicates that, in contrast to EP, Zn-depressing, neutrophil-releasing, and amino acid fluxing activities do not bind to glass beads.

Summation of the experimental data provides evidence that EP and LEM are different molecular species based on: (a) differences in their release from stimulated PMN in the presence of K^+ and (b) their physical separation using glass beads as a nonspecific affinity adsorbent for pyrogenic activity.

Having demonstrated that LEM preparations contain EP as an individual mediator, the next question was whether LEM itself was composed of multiple mediators. Various fractionation procedures have enabled us to conclude that LEM activity is due to at least 4 factors that induce specific metabolic alterations as assayed in rats. The methods used to obtain specific mediator fractions have been compiled into a theoretical flow diagram (Figure 7). The solid lines represent steps that have been carried

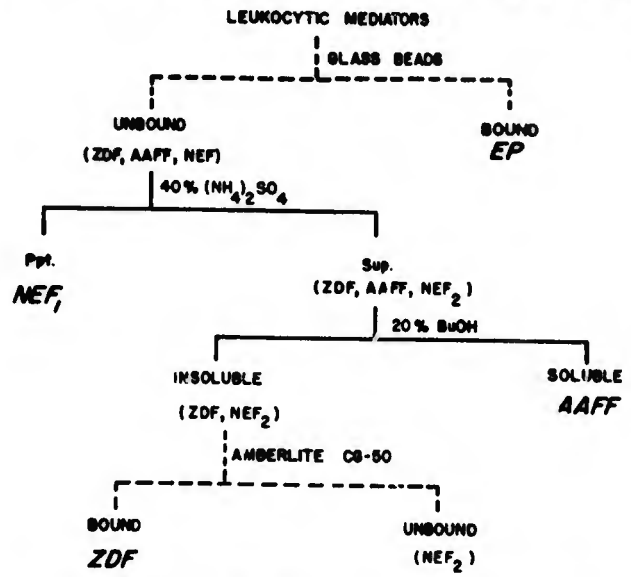

Figure 7. Theoretical flow diagram.

out sequentially while the dashed lines represent those procedures that have been used successfully but not necessarily at the point shown in the diagram.

As previously demonstrated, EP is selectively bound to glass beads. This step serves as an excellent purification step for other mediators since approximately 80% of the contaminating protein is removed without detectable loss of activity. Using a preparation that had no detectable EP, a classical $(NH_4)_2\ SO_4$ precipitation was carried out. The precipitate formed by 40% saturation with ammonium sulfate had detectable activity only for elevating total neutrophils (NEF_1). Zn-depressing activity, amino acid fluxing activity, and a second factor elevating total

neutrophils (NEF_2) remained in the supernatant. The two factors elevating total neutrophils differ, in that NEF_2 causes both an elevation of the circulating neutrophil population and a decrease in the total number of circulating lymphocytes. These alterations are not accompanied by significant changes in the total peripheral leukocyte count. Conversely, NEF_1 increases the total peripheral leukocyte count without causing detectable alterations in the differential count. Several leukopoietic factors having either one or both of these activities have been described in the literature. None of the leukopoietic factors have been well characterized, thus it is not known whether we are dealing with previously described substances.

Initial purification attempts utilizing the butanol-methanol (BuOH) method of Rafter et al.,[17] rapidly lead to the isolation of another factor. The addition of butanol to a final concentration of 20% (v/v) results in the formation of a biphasic solution. The amino acid fluxing factor (AAFF) was the only detectable activity found in the upper butanolic phase. Zn-depressing activity, neutrophil-enhancing activity, and endogenous pyrogen, if present, were in the lower aqueous phase.

A third method of separating mediators involved the use of Amberlite CG-50 which is a weakly acidic, carboxylic acid-type cation exchange resin. Utilizing a batchwise chromatographic separation, Zn-depressing factor (ZDF) binds strongly to the resin leaving neutrophil-elevating factors and the amino acid fluxing factor, if present, in solution.

Thus the factors indicated by slanted letters, have been physically separated from all other detectable activities. In addition to the physical separation of multiple factors, nature has provided a second method of demonstrating a multiplicity of factors. These same 4 factors have been singly missing or selectively produced by various PMN preparations. EP and NEF_1 are not detectable in some preparations; whereas, ZDF and AAFF have been the only detectable activities produced by other preparations.

In summary, we have demonstrated that stimulated PMN produce at least 5 mediators; EP, NEF_1, NEF_2, ZDF and AAFF. Of these, EP has been studied for nearly 30 yr during which time it has been well characterized and purified to electrophoretic homogeneity.[17] It thus does not seem profitable to continue or initiate additional studies concerning it. Instead we will focus on the mediators collectively termed LEM.

There are several experimental areas that one can develop in continuation of the project. As alluded to earlier, LEM production is not standardized. In addition to the fact that not all factors are consistently produced, the potency of each preparation differs and various factors within a given preparation often differ in their thermal lability. This, in part, is due to the fact that we do not know how to best stimulate the PMN or understand the mechanism by which they release various substances. This indicates that one needs to elucidate the mechanism for production and release of leukocyte factors.

A second area that has been initiated within the Institute is defining the physiology involved. Specifically, how the mediators interact in host metabolism, what systems they activate, or what target organs they act upon directly. This area might best be pursued with purified compounds and should lead into studies designed to elucidate any clinical efficacy that the leukocyte mediators may have.

The third and most obvious area of study is the purification and physiochemical characterization of the biologically active compounds. We are currently attempting to study the stimulation and release phenomena as well as to characterize the biologically active components released from stimulated PMN.

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DISCUSSION

Dr. Pappenheimer asked if more sodium is added to the system, is more potassium needed? Dr. Mapes replied that the inhibitory effect of K^+ on release of leukocyte mediators was not studied with respect to other electrolytes. The studies were based on publications of Berlin and Wood and were done solely for comparing the effect of K^+ on release of LEM and endogenous pyrogen. The initial work of Berlin and Wood indicated that Na^+ does not effect pyrogen release whereas Ca^{2+} appears to potentiate the inhibitory effect of potassium ion. PMNs used in these studies were suspended in physiological saline; the suspensions contained 3 mM potassium and 10-15 mM calcium prior to further addition of electrolytes.

ARENAVIRUS STUDIES AT USAMRIID

Gerald A. Eddy, LTC, VC

A research plan undertaken 3 yr ago has been largely completed in terms of the objectives set forth at that time. The first of our objectives was to define an animal model for Bolivian hemorrhagic fever (BHF) as it is seen in humans. Figure 1 shows viremia, deaths and neu-

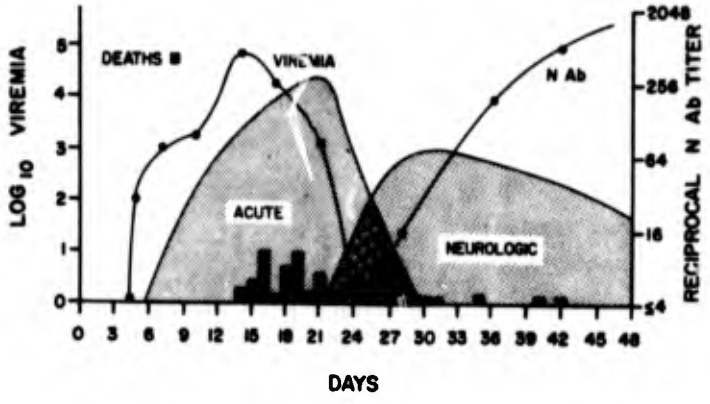

Figure 1. Pattern of disease in rhesus monkeys.

tralizing antibody responses of 45 rhesus monkeys. The deaths shown in solid blocks indicate that most of the monkeys died during the acute phase of disease which commenced on about day 6. Acute signs included fever, anorexia, depression, occasional hemorrhage, constipation changing to diarrhea, diminished water intake, dehydration, and occasional myoclonic spasms. These signs progressively increased in severity and terminated in death about 15-25 days after inoculation. As these signs subsided about 10-20% of the monkeys improved clinically, but then began to exhibit signs of neurologic involvement as shown in the next overlay. After days 25-30 the deaths of the monkeys were increasingly associated with neurologic manifestations and with fewer of the acute signs. Deaths due to late neurologic illness have occurred as late as 90 days. The monkeys dying with the late, subacute illness showed paresis, paralysis, intention tremors, ataxia, occasional blindness and convulsions, coma and death. The histologic lesions of the monkeys during the latter phase of illness typically included vasculitis throughout much of the CNS and frequently in many other organs as well. This pronounced perivascular cuffing was seen in virtually all monkeys that survived beyond 25-30 days including those which survived the disease entirely and developed neither acute nor neurologic signs. This late phase occurred in the presence of high levels of antibody.

Except for the late phase of disease this system provides us with a relatively good model of severe human illness insofar as acute disease is concerned. Late neurologic involvement is not seen in humans.

On the basis of this model we then studied passive prophylaxis of illness using homologous antibody in monkeys. In brief, we found that the passive administration of neutralizing antibody at titers in the recipient of ∿ 1:8 were sufficient to protect against acute clinical disease. As a result of this evidence that antibody would protect, we undertook in collaboration with others an effort to obtain BHF immune globulin by the method shown in Figure 2.

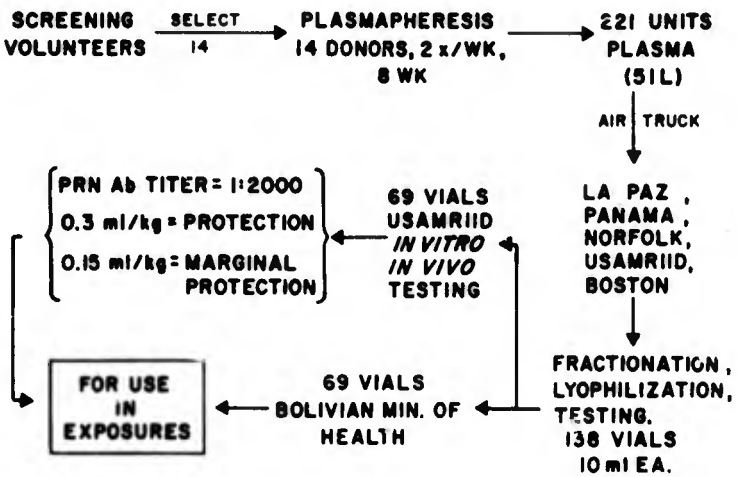

Figure 2. Production of BHF immune globulin.

We have also explored the use of antibody therapy in the treatment of acute illness in rhesus monkeys after the onset of clinical illness. We carried out 7 separate studies to assess antibody therapy after the onset of acute signs. In all instances shown, the monkeys were inoculated with 1000 PFU of virus and then given a single dose of antibody on a selected day. We varied the amount of antibody and the day of treatment. We treated a total of 62 monkeys and had 21 survivors; in the untreated controls there was only 1 survivor from 21 monkeys. Table I summarizes distribution of deaths and survival by treatment day.

These data show that almost all monkeys treated by day 7 survived the acute illness and that approximately 60% of the monkeys survived acute illness when treated on days 8, 9 or 10. The percentage of overall survivors progressively decreased as treatment was delayed.

We varied the dosage of neutralizing antibody used in these monkeys also; Table II shows the breakdown of this same group of monkeys by quan-

TABLE I. EFFECT OF DAY OF ANTIBODY THERAPY ON BHF IN MONKEYS

TREATMENT DAY	N	NO. OF DEATHS (%) Acute	Neurologic	% SURVIVAL
None	21	19 (90)	1 (5)	5
7	8	0	1 (12)	88
8	20	11 (55)	2 (10)	35
9	9	6 (67)	0	33
10	25	14 (56)	7 (28)	16

TABLE II. EFFECT OF ANTIBODY DOSE IN TREATMENT OF MONKEYS INFECTED WITH BHF

DOSE	N	NO. OF DEATHS (%) Acute	Neurologic	% SURVIVAL
Control	21	19 (90)	1 (5)	5
Low	18	10 (56)	5 (28)	16
Medium	15	11 (73)	1 (7)	20
High	29	10 (34)	4 (14)	52

tity of antibody. In general the doses ranged from a few 100ths mg/kg of immune globulin or serum to several 10ths ml. The data clearly suggest a dose-response relationship in terms of total survivors although there is a marginally significant suggestion that a small dose of antibody was more effective than a moderate dose in the monkeys surviving acute signs of disease. We conclude that antibody is an effective means of therapy and that efficacy is enhanced by early treatment in high dosages. Moreover, when we consider that the disease is far more severe in monkeys than in humans and that we should probably consider only the acute deaths in assessing efficacy, our evidence indicates a basis for the use of antibody in any dose at any stage of illness if it is available. In no instance was there a suggestion that Machupo neutralizing antibody enhanced the severity of illness or the magnitude of viremia when compared to controls.

Earlier I mentioned late neurologic involvement seen in most of the monkeys that survived the acute illness. We have seen a somewhat different type of neurologic illness in some monkeys. Table III is a composite of 2 separate studies in rhesus monkeys. In these experiments the monkeys were inoculated with 1000 PFU of Machupo virus and 4 hr later were treated

TABLE III. OCCURRENCE OF DISEASE IN PASSIVELY PROTECTED MACHUPO VIRUS-INOCULATED MONKEYS

IMMUNE GLOBULIN DOSE[a] ml/kg	ACUTE DISEASE/ TOTAL (DEATHS)		MEAN DAY OF DEATH	NEUROLOGIC DISEASE/ TOTAL (DEATHS)		MEAN DAY OF DEATH
1.5	0/8	(0)		7/8	(5)	50
0.5	0/3	(0)		1/3	(1)	42
0.15	4/8	(2)	26	0/6	(0)	

a. Monkeys inoculated with 1000 PFU Machupo virus: 4 hr later they were given indicated dose of immune globulin.

with immune globulin. Severe acute disease occurred in none of the monkeys receiving intermediate or high doses of antibody, but it occurred in 4 of 8 monkeys that received the low dose of immune globulin, and 2 died. Late neurologic illness occurred predominantly in the monkeys receiving the highest dosage of immune globulin (7 of 8 monkeys) and 5 died. There was 1 death in the intermediate dosage group and no neurologic disease or deaths in any of the monkeys from the low dose group. It appears that we can induce late encephalitis in monkeys fairly reproducibly by inoculating them with virus, giving them a high dose of antibody and waiting a couple of months.

I would like to point out certain differences between the monkeys with late encephalitis following severe acute illness as I described in the rhesus model and the induced neurologic disease shown here.

The former type is less acute and may take 7-10 days or more from onset to death whereas this type is very abrupt in onset and the monkeys which are going to die do so within 4-7 days. The former type occurs only after severe acute signs and the more severe the acute illness the more severe the late neurologic disease. The antibody-induced type occurs only in the monkeys that show little or no acute disease. The former type occurs in the presence of high levels of active neutralizing antibody whereas this type occurs in monkeys with little or none at the time of neurologic disease. We don't know whether the virologic mechanisms in the 2 entities are similar, but clearly overlying immunologic and clinical manifestations appear to be rather different. Virus isolation studies were equivocal, but all of our recent attempts to cultivate virus, short of co-cultivation have yielded nothing. In brief, we are puzzled by the occurrence of this late neurologic illness, and we emphasize it because of the possible association with high levels of passive antibody and what this may imply with respect to the use of vaccines, particularly killed vaccines.

I would next like to describe our efforts to immunize against BHF. We have explored 3 alternatives. These are (1) immunization with one or more of the apparently innocuous, related arenaviruses of the Tacaribe complex; (2) the development of an attenuated Machupo virus, and (3) the preparation of a killed vaccine. I would first like to briefly reacquaint you with the arenaviruses of the Tacaribe complex Table IV. All are

TABLE IV. ARENAVIRUSES OF THE TACARIBE COMPLEX

COUNTRY	VIRUS	VIRULENCE FOR HUMANS
Bolivia	Machupo	high
Bolivia	Latino	
Paraguay	Parana	
Argentina	Latino	high
Brazil	Amapari	
Colombia	Pichinde	
Trinidad	Tacaribe	
United States (Florida)	Tamiami	

closely related by complement fixation, but are clearly distinguishable and have little or no cross-reactivity by virus neutralization. Machupo and Junin viruses cause BHF and Argentine hemorrhagic fever, respectively, and are the only known human pathogens; all the others are enzootic in rodents. None are arthropod-borne; all are geographically isolated and frequently produce long term if not lifelong viremias in their respective rodent hosts infected as neonates. All are distantly related to LCM and Lassa viruses. We assessed the innocuous viruses for their ability to cross-protect monkeys against Machupo virus; we found that the two closest geographically did not protect at all and that two of the most distant, Tacaribe and Tamiami, protected best but not absolutely. We therefore set up a study to determine the cross-protective efficacy of certain virus combinations. Thus we see that whereas we had previously found that Latino and Parana viruses individually did not protect at all, when given in combination (Table V) monkeys challenged 2 mon later with Machupo virus were only moderately ill, had little viremia and survived. When we gave Tamiami and Tacaribe viruses in combination and challenged either 2 or 4-1/2 mon later monkeys were unaffected by challenge, showed no viremia and developed antibody by days 10-14 (earlier than control monkeys which, if they survive, develop antibody on days 24-28). Tacaribe virus alone protected against challenge at 2 mon, but not at 4, whereas Tamiami virus did not protect well at 2, but did at 4, months. This curious phenomenon may account in part for the efficacy of the combination.

TABLE V. PROTECTIVE EFFECT OF ARENAVIRUS COMBINATIONS AGAINST MACHUPO VIRUS CHALLENGE

VIRUS COMBINATION	NO.	MONTHS TO CHALLENGE	DAYS VIREMIA/ DAYS TESTED	SEVERITY OF ILLNESS	MACHUPO Ab BY DAY
Latino-Paraná	2	2	2/10	Moderate	14-17
Tacaribe-Tamiami	3	2	0/15	Nil	14
Tacaribe-Tamiami	3	4.5	0/15	Nil	10
Tacaribe	2	2	1/10	Mild	14
Tacaribe	2	4.5	2/ 8	Mild, severe	14, None
Tamiami	2	2	7/ 8	Severe	Died
Tamiami	2	4.5	1/10	Mild	17-21

We ran a control to see if this heterologous protection was mediated by antibody (Table VI); 4 groups of monkeys which received either

TABLE VI. EFFECT OF PASSIVELY ADMINISTERED PICHINDE OR TACARIBE VIRUS ANTIBODY ON RESPONSE TO MACHUPO VIRUS CHALLENGE

TREATMENT	NO.	NO. VIREMIC (TITER) ON DAY 4	7	11	FEBRILE BY DAY	DEATHS (MEAN DAY)
None	2	0	2 (600)	2 (1500)	6	2 (15)
Pichindé antiserum	2	0	2 (1500)	2 (16000)	5-6	2 (15)
Tacaribe antiserum	3	3 (130)	3 (10000)	3 (39000)	4	3 (18)
Machupo immune globulin	3	0	0	0	Nil	1 (53)

Pichindé, Tacaribe or Machupo antibody were challenged a few hours later with Machupo virus. Prior Tacaribe virus infection protected monkeys reasonably well, prior Pichindé infection did not. We see here that both the controls and the Pichindé antiserum recipients became febrile

on days 5-6, viremic by day 7 and died more or less as expected. The Tacaribe antiserum recipients became febrile and viremic by day 4. Moreover, they had significantly higher viremias on day 7 than any of the other monkeys. The recipients of Machupo antibody showed no acute illness, no viremia and 1 of 3 developed late neurologic signs and died on day 53.

The conclusion is that cross-protection is real and is not mediated by heterologous antibody; some heterologous antibody may even augment illness and viremia. This method of immunization is intriguing because it would probably protect against both Argentine and Bolivian hemorrhagic fever.

We have explored another potential means of immunizing against BHF by attenuating Machupo virus. The virus was passaged in COFAL chick embryo cell culture. Table VII summarizes this preliminary study. We

TABLE VII. PRELIMINARY STUDIES OF CHICK CELL PASSAGED MACHUPO VIRUS IN RHESUS MONKEYS

PASSAGE LEVEL	YIELD IN CHICK CELLS LOG_{10} PFU	TITER SH:PFU	ILLNESS IN MONKEYS	DAYS VIREMIC/ DAYS TESTED	SURVIVORS/ TOTAL
0	1-2	1:1	Severe	9/ 9	0/2
18	3-4	1:30	Severe	8/ 9	1/2
23	4-5	1:100	Moderate	6/10	2/2
32	4-5	1:3000	Mild	2/10	2/2

see that at the zero passage level the plaque assay titer and the suckling hamster SH titer were essentially the same, yield was very low, and the virus produced severe illness with typical viremia and deaths in monkeys. At the 18th passage level the yield was a little higher. The IC virulence for suckling hamsters was lower; it produced severe disease with atypical viremia and one monkey survived. Five passages later the yield in chick cells was higher, the IC virulence in hamsters was still less in relation to the plaque assay, the virus produced only moderate illness in monkeys with less viremia and no deaths. At passage 32 the virus was further attenuated for both hamsters and monkeys. All surviving monkeys developed Machupo virus neutralizing antibody.

From these data and other studies it appears that Machupo virus and arenaviruses generally can be rather easily adapted or attenuated. Perhaps a little too easily. In any event we appear to have attenuated the virus substantially and reduced the neurovirulence to fairly low levels at this intermediate passage level.

As a third possible immunogen we have prepared an experimental killed vaccine. We are in a rather early stage of testing this product; it can be said that it protects guinea pigs and monkeys. We are currently measuring its efficacy in monkeys and looking for the possible development of late encephalitis. The problems which we may encounter in monkeys with a killed vaccine may be similar to the problems with passively administered antibody. Are monkeys which develop high levels of antibody and are protected against the acute signs rendered susceptible to late encephalitis? Additional studies and more testing should ultimately answer this question.

An additional caveat with regard to arenaviruses is their apparent genetic instability and their ready adaptation to new substrates. The chick cell adaptation is but one example. Our prototype Machupo strain was not a suitable system for producing hemorrhagic deaths in guinea pigs, but after only 3 or 4 guinea pig passages the virus killed 100% of inoculated guinea pigs, most dying with hemorrhagic manifestations. The prototype Tacaribe virus would not kill adult mice by IC inoculation, but after 3 passages in adult mouse brain the virus would then kill not only adult mice but rats as well.

There are other examples of the adaptability of these viruses which suggest that they have unusual genetic mechanisms to yield such rapid alterations in phenotypic expression. The genetic mechanisms are important if we are contemplating the use of live attenuated vaccines.

In terms of our research objectives, the first question is whether we should be in this area of work at all. Does the risk justify the objectives. We believe that future research must be directed specifically toward vaccine development. Few other Institutes have the unique combination of both vaccine development facilities and expertise plus the background and hardware for containment of highly virulent viruses. It would appear from our preliminary view that we have a plethora of alternatives for immunizing against Bolivian hemorrhagic fever and probably against Argentine hemorrhagic fever as well. Using BHF as a model we would probably apply similar principles to Lassa fever. If we do so, we must decide which approach to follow. I do not think I have to enumerate the pitfalls attendant to each.

DISCUSSION

Dr. Woodward indicated that he was familar with the sequential sacrifice studies that had been performed in animals regarding vascular changes and was also aware of the argument as to whether the vasculitis in spotted fever was an immunopathogenic lesion. He wanted to know if sequential experiments would be accomplished to measure serial blood vessel changes in infected animals since BHF may or may not represent an aberrant antigen-antibody reaction. LTC Eddy replied that this type of experiment would receive high priority.

Dr. Jordan was pleased to learn that Machupo virus had been adapted to guinea pigs and felt that experiments could now be implemented more readily than with monkeys. He asked LTC Eddy to speculate about the cause, if the hemorrhagic phenomenon was not an immunopathologic response. LTC Eddy replied that acute deaths were apparently caused by direct virus damage of cells. He noted that attempts to delay death of rhesus monkeys by immunosuppression and decomplementation were not successful. Dr. Jordan asked about coagulopathy. LTC Eddy replied that experiments should be conducted in this area. He felt that a minimum level of knowledge on the pathogenesis of the disease needed to be established in order to make an intelligent decision on the type of vaccine to select.

Dr. Pappenheimer asked if the neurologic signs could be produced in guinea pigs. LTC Eddy replied that in the initial studies, the guinea pigs died only with late encephalitis but after a few virus passages in guinea pigs these rodents developed the hemorrhagic lesions and deaths that were observed in monkeys.

Dr. Rammelkamp asked if the disease could be produced from aerosols and if any virus reached the gastrointestinal tract. LTC Eddy reported that no aerosol experiments had been done with Machupo virus and none were anticipated. In regard to gut lesions, LTC Eddy stated that Major McLeod had evidence of severe gut lesions, but there was no evidence as to how the virus reached the gut. Colonel Metzger stated that we would not consider aerosol studies with the virus. He indicated that Class III systems imposed severe restrictions of the types of studies which could be undertaken. However, the recent modification to Building 1412 would increase the Institute's capability to conduct Class III studies.

Dr. Benenson commented that he was intrigued by the late neurological deaths in those animals which had received passive immunizations and asked if immunosuppressive agents had been used. LTC Eddy replied that cytoxan M and corticosteroids had been studied only minimally; they had been used when the test animals developed late encephalitis. Immune suppressors given to monkeys before infection resulted in earlier hemorrhagic deaths. Dr. Benenson asked if there was demyelinization. Major McLeod answered that demyelinization occurred only in the most severe neurologic cases in lesions of vasculitis, usually peripheral to the vessel involved. He concluded that myelin loss and necrosis were not major features of the disease.

Dr. Pappenheimer asked if Machupo virus was an RNA or DNA virus and wanted to know its size. LTC Eddy replied that it was an RNA virus; that the virus was highly pleomorphic and ranged between 90 and 250 nm. Recent studies provided data suggesting infectivity over the entire size range.

Dr. Sawyer asked if late encephalitis developed only in animals treated with neutralizing antibody after infection. LTC Eddy replied "No, late encephalitis occurs in animals which received treatment either before or following infection." All passively protected animals become infected regardless of antibody titer at time of virus inoculation.

Dr. Nathanson expressed an interest in the rapid adaptation of the virus in several animal hosts and asked the following questions: (1) If plaques are picked from a strain that has not been adapted, can you find within that population, some clones that behave like those that are adapted, i.e., is it a selection or a mutation? and (2) Have recombination studies been undertaken, i.e., coinfect with Tacaribe, Tamiami, and Machupo viruses, then plaque-purify in an attempt to obtain clones that show evidence of recombination? LTC Eddy replied that in general, no differences were noted in plaque size, morphology or plaquing characteristics between chick-cell adapted and the original virus strain with which work was initiated. In reference to the question (2), LTC Eddy replied that he had not performed recombination studies but had considered such an approach in the development of a vaccine strain; however, the ease with which a killed vaccine can probably be prepared may have eliminated the need for such an approach.

Dr. Pappenheimer asked if the rapid adaptation could be caused by the virus wrapping host material around itself. LTC Eddy indicated that there was little information regarding this matter. He reported that the ability to neutralize the virus was highly dependent on the origin of the virus. For example, monkey antibody would neutralize virus from a monkey much better than virus from a hamster. He noted that these viruses were unusual; they have the ability to incorporate host ribosomes and appear to possess segmented genomes.

The point was made that a survey was conducted in which many individuals were shown to have high levels of antibody to Lassa fever virus although they had not experienced serious illness; the question was posed if this situation existed for BHF. LTC Eddy noted that Dr. Karl Johnson had collected similar survey data which showed that people with high titers to BHF always recalled having been seriously ill.

THE AFRICAN GREEN MONKEY AS AN ALTERNATE PRIMATE HOST FOR STUDYING MACHUPO VIRUS INFECTIONS

Franklin S. Wagner, CPT, VC

A monkey model for the study of Bolivian hemorrhagic fever (BHF) has been described in both Macaca mulatta and Macaca vesicularis: respectively, the rhesus and cynomolgus monkeys. However, because of the current and anticipated shortages of Asian monkeys, an alternate primate model for BHF infection was considered desirable. An alternate model provides additional means for the evaluation and assessment of a severe Arenavirus infection in primates generally. I would like to describe the results of our initial studies with Machupo virus infection in Cercopithecus aethiops, the African green (AG) monkey.

Six fully conditioned, young-adult African green monkeys were placed in individual primate cages in the class III suite for 10 days before virus inoculation. All monkeys were inoculated SC with 1,000 PFU of the Carvallo strain of Machupo virus. The infection was allowed to follow its natural course; all monkeys were observed daily, clinical signs were recorded, and neutralizing antibody responses were measured. Body temperatures were monitored daily with a rectal thermometer. Hematocrits, total leukocyte values, and differential counts were determined. Blood samples for viremia and antibody assays were collected at 3 to 4 day intervals. Complete necropsies were performed shortly after death.

Figure 1 shows the clinical signs of Machupo-virus infected, African

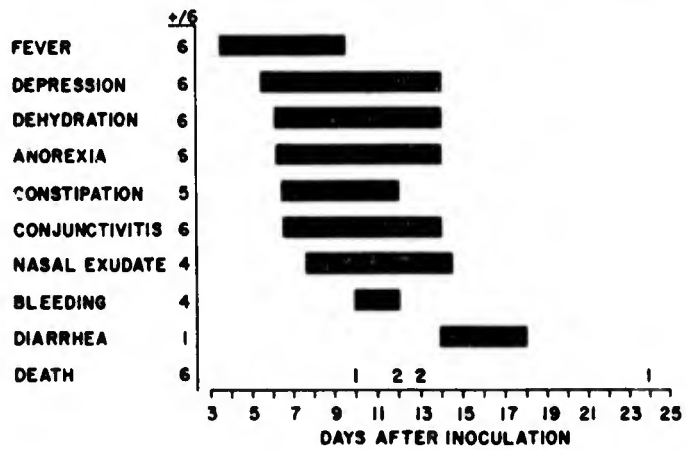

Figure 1. Clinical signs of Machupo-virus-infected AG monkeys.

green monkeys, the mean day of onset and duration of clinical signs. Fever was the first detectable clinical sign in all monkeys; the mean day of onset was 3.5 days, and it persisted for an average of 9.5 days. Depression was evaluated daily by visual observation of changes in behavioral activity and reduced response to stimuli. All monkeys showed some evidence of progressive depression beginning on day 5. The degree of skin elasticity was used to assess the level of dehydration; such changes correlated closely with anorexia. Type and amount of food intake were used to assess and grade anorexia. Monkeys were fed a commercial monkey chow diet, supplemented with oranges and apples. Constipation was a common clinical sign in 5 of 6 monkeys, starting between days 6 and 7. Infected monkeys often went 4 to 5 days without evidence of a bowel movement. Stools of constipated monkeys were hard, scant, and infrequent; the condition persisted until death. Conjunctivitis, typically manifested by red, watery eyes, was a prominent sign in all monkeys. A serous nasal exudate was found in 4 monkeys midway through the course of infection; 2 of these progressed to a serosanguinous discharge. Bleeding was a prominent feature in 4 monkeys; it occurred from the noses in 2 monkeys, the gums in 4 monkeys, and the rectum in 1 monkey. The site of oral bleeding was the area where canine teeth had been extracted some 10 days prior to initiation of the study. Milk rectal bleeding occurred in 1 monkey 24 hr prior to death. Diarrhea was observed in only 1 monkey; it was present intermittently over a 1-week period in the last surviving monkey during the latter part of the study. All monkeys died, with a mean time to death of 14 days.

The mean body temperature of the 6 monkeys during the clinical course of illness is shown in Figure 2. There was an increase in body temperature

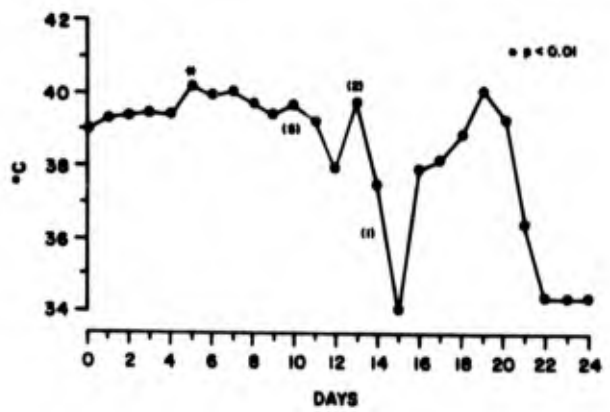

Figure 2. Mean body temperature in Machupo-virus-infected AG monkeys.

during the course of the disease followed by a rather rapid decline to hypothermic levels just prior to death in most monkeys. The last surviving monkey experienced a biphasic temperature pattern, with the first peak on day 7 and the second on day 19; both peaks were followed by several days of subnormal temperatures, the second of which terminated in death on day 24. Monkey survived for 4 days during hypothermic periods of < 35 C.

Figure 3 shows the mean values for lymphocytes and neutrophils.

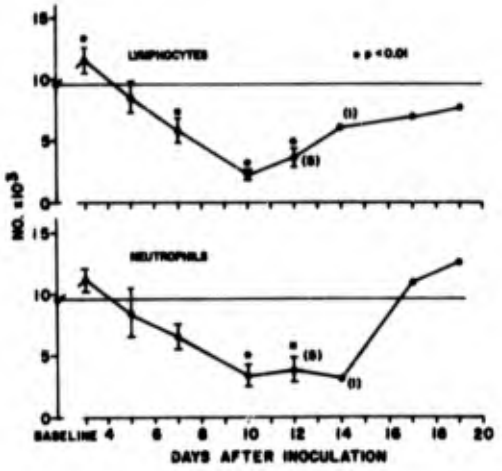

Figure 3. Leukocyte response in Machupo-virus-infected AG monkeys.

From normal preinfection base-line values, the mean lymphocyte counts were increased initially on day 3, and then decreased to minimum levels by day 10, at which time one monkey died. The mean counts in the 5 remaining monkeys increased slightly on day 12. Only one monkey's values are shown for the remaining times. It can be seen that the neutrophil count followed a similar pattern. The mean hematocrit is shown in Figure 4. Values initially decreased slightly by day 5; the minimum value was again reached on day 10.

Mean viremia levels are shown in Figure 5. There was no detectable viremia through day 5; on day 7, 4 of 6 monkeys were viremia and all 6 were viremic by day 10, with mean values of 2.8 and 3.2 logs of virus, respectively.

In summary, the clinical signs and hematologic data reported here, and the histopathologic lesions to be described next by Dr. McLeod, extend our previous studies of rhesus and cynomolgus monkeys as a model for BHF. The African green monkey was highly susceptible to Machupo virus. Although our experience with African green monkeys infected with the virus has been limited to 6 animals, we have observed a much more acute and

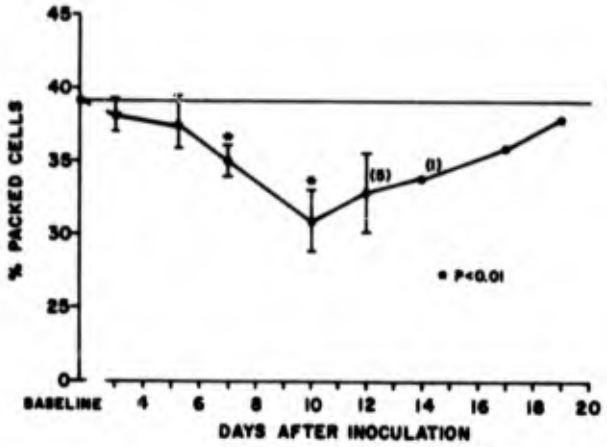

Figure 4. Packed cell volume of Machupo-virus-infected AG monkeys.

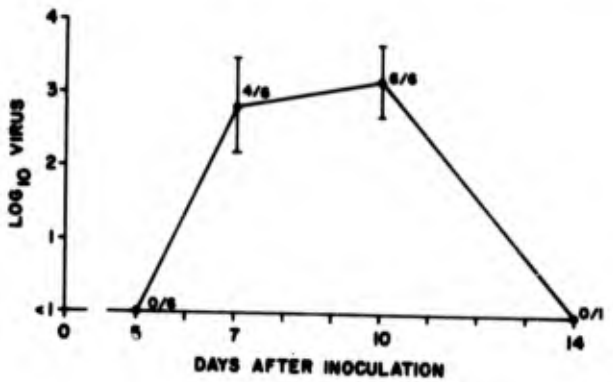

Figure 5. Mean viremia levels in Machupo-virus-infected AG monkeys.

severe clinical illness than that previously described in the genus Macaca. The infected African green monkey produced a more hemorrhagic model, with bleeding from the nose, gums, and rectum in 4 of 6 animals. Oral hemorrhage was thought to be a contributing factor in the death of the first monkey on day 10. The fall in hematocrit occurred during periods of severe dehydration, at a time when the packed cell volume would otherwise be expected to rise. This correlates with the necropsy and histopathology which revealed hemorrhage and free blood in the liver, lung, and other tissues. Diarrhea, although a prominent sign in the majority of Macaque monkeys, was only observed briefly in the last surviving monkey. There was good correlation between the magnitude of viremia on day 10 and the mean day of death, in that the monkeys with the higher viremias died earlier.

The first significant neutralizing antibody titer, 1:32, was detected in the last surviving monkey on day 14; no antibody was detectable on day 21, although a 1:32 titer was found again on day 24. This unusual, biphasic

pattern of antibody response has been seen numerous times in rhesus monkeys which have survived cross-protection experiments with other arenaviruses, and also in passive antibody experiments. In conclusion, the African green monkey was found to be a useful model for the study of BHF. This model is useful for further studies designed to develop a regimen for prophylaxis and for treatment of this disease entity. Also, the hemorrhagic component of Machupo virus infection may warrant the study of disseminated intravascular clotting in this species.

PATHOLOGY OF BOLIVIAN HEMORRHAGIC FEVER (BHF) IN THE AFRICAN GREEN MONKEY

Charles McLeod, Jr., MAJ, VC

Significant lesions of acute BHF in the African Green monkey were seen in the liver, gastrointestinal tract, skin, adrenal, lung, blood vessels, and lymphoid organs (Table I).

TABLE I. INCIDENCE AND SEVERITY OF MICROSCOPIC LESIONS OF BHF-INFECTED AFRICAN GREEN MONKEYS

LESION	SEVERITY BY MONKEY NO.					
	1	2	3	4	5	6
Necrosis						
liver	+++	+++	++++	+++	+++	-
intestine	+	+++	++++	*	+++	-
skin, oral mucosa	+	+++	++	++	++	-
adrenal cortex	++	++++	+++	+++	+++	-
lymphoid	+	++	-	-	-	-
Lymphoid depletion	-	+++	+++	-	++	-
Hemorrhage	-	+	+++	+++	+++	-
Thrombosis	-	+++	+++	++	+++	-
Pancreatitis	+	+++	++++	-	-	-
Pneumonia	++++	++++	+++	+++	+++	++++
Bacteremia	++++	++++	-	++++	+	-
Encephalomyelitis	-	-	-	-	-	++++
Day of death	10	12	12	13	13	24

*Autolysis

I would like first to describe the pathology observed in the 5 monkeys that died during the acute phase of the infection, between days 10 and 13. Lesions seen in the sixth monkey that died on day 24 were more typical of chronic BHF infection and will be discussed separately.

Hepatic necrosis was grossly evident in the 5 monkeys. Livers were yellow-tan, swollen and friable. The lobular pattern was accentuated in many cases. Microscopically there was hepatocellular swelling, fatty degeneration, and distortion of the normal lobular pattern. Many foci of necrosis were located randomly within the lobules; entire lobules were necrotic in some cases.

Intestinal lesions were most severe in the ileum. The small intestine contained fluid; mucosal discoloration was often seen through the serosa. Intestinal lymph nodes were swollen. The mucosa of the small intestine was often extensively eroded and inflamed. The earliest change recognized microscopically in the intestine was acute necrosis of mucosal epithelium. There was often acellular necrotic debris in the lamina propria. Later fusion and atrophy of villi were observed.

The skin and mucosa of the pharynx and esophagus often had small necrotic foci. Epithelium of hair follicles occasionally had basilar necrosis. Arenavirus particles have been demonstrated in a similar focus of necrosis in the pharynx of a rhesus monkey.

Necrosis was seen in the adrenal cortex of the 5 acutely infected monkeys. It varied in severity from very small foci of acute necrosis and hemorrhage to large confluent areas of poorly stained and sometimes necrotic cells that involved practically all cells of the zona fasciculata.

Lymphoid necrosis and depletion were seen in 4 of the 5 acute deaths. The spleen contained small lymphoid nodules and the periarticular lymphoid sheaths were reduced in size. Hemorrhage and fibrin were often seen around lymphoid nodules. Peripheral lymph nodes were often depleted of lymphocytes whereas many of the mesenteric nodes and intestinal lymphoid tissues were heavily populated with them.

Hemorrhages were observed in many organs but were most striking in the subcutis, lung, and submucosa of the intestine. Free blood was not observed in the lumen of the intestine except in the one case mentioned by CPT Wagner. That animal was found to have a severe ileocolic intussusception.

Small thrombi were associated with some of these hemorrhages in 4 monkeys. Most of these were seen in the lung or intestines but were observed in the choroid of one animal.

Pancreatic lesions were observed in only the first 3 monkeys, and consisted of mononuclear infiltration of the parenchyma and dilation of acinar glands and small ductules.

Hemorrhagic bronchopneumonia was seen in all 6 monkeys. All lobes were affected but in several cases the anterior ventral portion of the lung was most extensively involved. Masses of gram negative bacterial rods were present in some of the inflammatory foci.

Gram negative bacterial rods were seen in many other tissues of the 5 monkeys that died of acute BHF. There was usually no inflammation associated with these bacteria except when they were in the lung.

Monkey No. 6 that died 24 days following inoculation did not have the necrotic lesions observed in the other monkeys. Mild emaciation and bronchopneumonia were noted at necropsy. The only microscopic evidence of previous damage in the liver was a mild lymphoreticular infiltration. The important lesions in this monkey were in the CNS and lung.

The brain of monkey No. 6 had severe nonsuppurative lymphoreticular vasculitis and gliosis. Vessels contained both an intramural and perivascular infiltrate of lymphocytes and macrophages. The endothelial cells were hypertrophic. Vessels from the brain stem were similarly affected. Lesions were distributed in both white and grey matter and were most severe in the brain stem; the spinal cord was similarly affected. The adrenal and spinal ganglia of this monkey had a mild lymphoreticular infiltrate.

It should be emphasized that necrosis was consistently seen in the monkeys 1-5 but not in the monkey that lived until the 24th day (Table I). This pattern of chronic infection as seen in monkey No. 6 has been observed in more than 20 rhesus and cynomolgus monkeys surviving the acute stage of infection.

I would like to mention some of the differences between the rhesus and African Green and Machupo virus models. Hemorrhages were much more prevalent in the African Green monkeys. Thrombosis which was seen in 4 of the African Green monkeys is rarely observed in rhesus monkeys that die of BHF. The pneumonia present in all six of the African Green monkeys is uncommon in the rhesus.

For obvious safety reasons, the canine teeth had been extracted about 10 days prior to the beginning of the experiment. Inhalation of the suppurative material from the infected extraction wounds which developed in several monkeys may have contributed to the pneumonia observed.

I feel these lesions should be evaluated with caution because some of them, especially the hemorrhage and thrombosis, may be more of an effect of the gram negative bacteremia than of the Machupo virus infection. Future studies should include blood culturing and possibly assay for endotoxin to determine the role of bacteremia in this disease.

OVERVIEW OF RICKETTSIOLOGY RESEARCH

Carl E. Pedersen, Jr., MAJ

In recent years, following a drastic decline of interest in rickettsial microbiology, there has been a gradual reawakening spurred, no doubt, by increased numbers of cases of Rocky Mountain spotted fever (RMSF) in this country. As of 8 November there have been 789 cases of tick-borne typhus reported in the United States, 50 more than reported last year at this time. This far exceeds other cases of specified notifiable diseases such as brucellosis, diphtheria, malaria, tetanus, tularemia and typhoid fever. The lowest level was reached in 1959; since then there has been a gradual but constant increase in numbers every year, despite the fact that effective antibiotic therapy is readily available to any clinician who recognizes the disease.

Therein lies the problem. In general, the medical/scientific community has relegated the rickettsiae to a position in the back of the bus in favor of more exotic organisms. In fact, these obligate intracellular parasites have caused more human fatalities than any other form of illness except malaria. We still do not completely understand the chemical composition of the rickettsiae, the pathogenesis of the disease process, or the immunological interaction with the host defense mechanisms.

In September, 1974, plans were initiated to institute the formation of a separate division for studies in the biology of rickettsiae and of the diseases they cause. During the past year this division has undergone a metamorphosis to a cohesive unit with the broad continuing mission of conducting research in rickettsial diseases. This includes development and evaluation of rickettsial vaccines and diagnostic reagents; developing methods for the early specific diagnosis of rickettsial disease; studies of the immunologic response to rickettsiae and investigations in the pathogenesis; and pathophysiology of rickettsial disease. We are currently located in building 1412 in completely renovated and updated laboratory facilities. We perform all studies with live rickettsiae in Class II suites. The Division currently is staffed with 4 Ph.D. microbiologists, 2 MDs and one DVM in addition to technical and administrative support personnel.

While our primary efforts are devoted to the study of the spotted fever rickettsiae with emphasis on Rickettsia rickettsii, the etiological agent of RMSF, during the past year the scope of our research has been expanded to include Q fever rickettsiae and the agent of scrub typhus, R. tsutsugamushi. Active protocols are currently extant in the areas of the basic biology, biochemistry and immunology of these organisms. Efforts are underway to define the interrelationship between humoral and cell-mediated immune responses to rickettsial infection. We are involved in describing the pathogenesis and pathophysiology of the disease process to include studies in disseminated intravascular coagulation. We have

utilized animal models, such as the rhesus monkey, to describe the disease process; in future studies we will examine therapeutic and supportive measures which will be of potential value to the patient with spotted fever.

The interest in rickettsial disease is not limited to investigators within our Division, however. We are extensively involved in collaborative studies throughout the Institute. Utilizing some of the outstanding examples of scientific expertise found in other primary fields of interest we readily call upon the Virology Division for tissue culture support; the Bacteriology Division for immunologic and diagnostic capabilities; the Pathology Division for analysis of tissues by histologic and electron microscopic techniques; Physical Sciences Division for sophisticated biochemical studies; the Animal Assessment Division for expertise in the physiological parameters of animal models used in our studies and, of course, the Animal Resources Division which provides the animals used in our studies and the competent personnel who care for the animals. We are indeed fortunate to have such a wealth of resources from which to derive much needed assistance when developing a new program. In addition, as this field is rather small, we are fortunate in that we may readily call upon scientists employed in similar projects at governmental laboratories located at Walter Reed Army Institute of Research, Rocky Mountain Laboratory and the Center for Disease Control. We also maintain a close relationship with investigators at the university level.

In order to better understand why this Institute has undertaken to study rickettsiae, I should like to point out that the diseases rickettsiae cause vary enormously, from self-limiting to the most fulminating known. Competency to deal effectively with rickettsial disease is steadily decreasing while the incidence of diagnosed cases has been rising for the past 10 years.

Money and professional expertise devoted to the field of rickettsial disease have declined steadily over the past 20 years. The largest group of rickettsiologists averages 55 years of age, the second largest, 55, with only 4 scientists under 40 in career jobs at the beginning of this decade. It seems reasonable to conclude that biomedical competence in rickettsial disease in the United States will be largely lost within the next 10 years if present trends continue. In light of these facts, we feel that our program has a significant impact on the civilian as well as the military scientific community.

One example of the studies which have been performed during the past year includes the development, production and testing of a formalin-inactivated vaccine for RMSF. This vaccine is superior to the existing commercial product in that it contains 20-fold less total protein (primarily avian host material) and, in addition, contains intact rickettsial bodies. Our requirement for this vaccine is tied to the protection of laboratory workers who might be exposed to the agent, since RMSF is infectious either by parenteral injection or aerosol

exposure. We believe that this vaccine will be effective in protecting our laboratory workers and could have applications within the civilian community. The RMSF vaccine is currently in the first phase of human testing. This vaccine is a substantial improvement from that prepared in the 30's by Weigl, who infected lice by intrarectal inoculation and subsequently removed the intestines and ground them into an emulsion. The immunization of one individual required the yield from approximately 100 lice.

Dr. Kenyon will be presenting his data on immunological studies in conjunction with vaccine development for the spotted fever group rickettsiae. Also, we are interested in developing sensitive and rapid diagnostic tests for the early detection of rickettsial infection; MAJ Oster will present our efforts using a radioimmunoassay. We are involved in biochemical analyses of the organisms in a search for the antigens responsible for protection in both humoral and cell-mediated immune responses.

IMMUNOLOGICAL ASPECTS OF SPOTTED FEVER VACCINES

Richard H. Kenyon, Ph.D.

As MAJ Pedersen has just mentioned, Rocky Mountain spotted fever (RMSF) continues to be a problem in the United States. The commercially available vaccine has been shown to be quite unacceptable in regard to purity and efficacy. We have prepared an improved RMSF vaccine made from rickettsiae grown in chick embryo cells.

A large part of the material presented was done in cooperation with Captain Lenora Sammons. Preliminary experiments were performed to examine the various methods of RMSF antibody measurement available in our laboratory.

We examined the antibody response in infected rhesus monkeys. The antibody responses in a typical nonfatal infection are shown in figure 1.

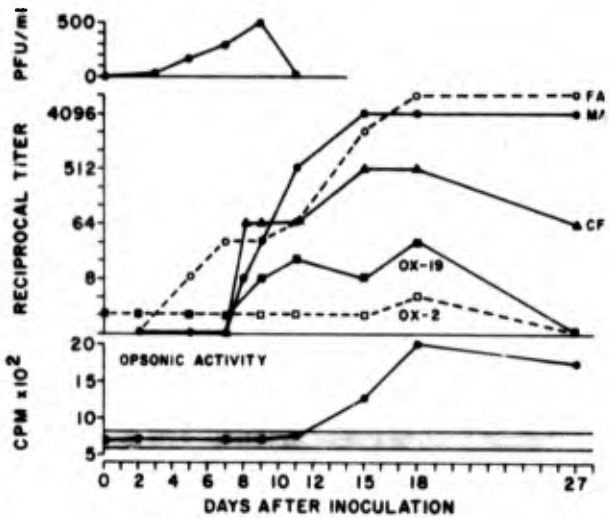

Figure 1. Antibody responses and viremia of a monkey infected with 50 R. rickettsii.

In general the earliest and highest titers were found in micro-agglutination (MA) and fluorescent antibodies (FA). Rickettsemia levels are also plotted and, as expected, preceded antibody responses. The antibody response using a larger infecting dose has the same pattern, but the entire spectrum is moved to the left.

For measurement of opsonic activity, serum was incubated with killed Rickettsia rickettsii; the antibody-coated rickettsiae were incubated with monkey leukocytes in the presence of ^{14}C-glucose. Energy is expended by the leukocytes in phagocytizing antibody-coated rickettsiae; with release of $^{14}CO_2$. However, vaccinated monkeys showed no enhanced opsonic activity. Therefore, at the time these vaccine studies were performed, we concluded we could best measure RMSF antibody by either FA or MA methods.

More recent developments suggest that a radioimmune assay or methods to detect cell-mediated immunity may be more sensitive. After preparation in chick embryo cells (CEC) of one lot of RMSF vaccine for potential human immunization, we examined the efficacy of this vaccine in rhesus monkeys and compared it with the Lederle vaccine. Monkeys were given one immunization and challenged 30 days later. Antibody titer at time of challenge was measured by the MA technique (Table I). Our CEC-grown vaccine stimulates MA antibody production to a greater extent than the Lederle vaccine. About all we can conclude from these data is that absence of MA titer is not indicative of lack of protection. However, in no case did a monkey with an MA titer die after challenge.

TABLE I. CLINICAL RESPONSES IN MONKEYS VACCINATED WITH VARIOUS VACCINES

			≥ 1:4 MA		< 1:4 MA		
VACCINE	NUMBER	RECIPROCAL GMT	Not ill	Ill (died)	Not ill	Ill	(died)
None	4	0				4	(4)
CEC							
Undiluted	6	32	2	4			
1:10	6	4.8		3	2	1	
1:100	6	3.2	1	2		3	(1)
Lederle							
Undiluted	6	1.7		1		5	(1)
1:10	6	1.0			2	4	(1)
1:100	6	1.6		2		4	(2)

Since our vaccine is a killed product, we presumed that a series of several immunizations would be necessary to afford optimal protection. A study was undertaken to determine the most efficacious vaccination schedule. Vaccination schedules used and geometric mean MA antibody responses are shown in figure 2. There were 4 monkeys in each group.

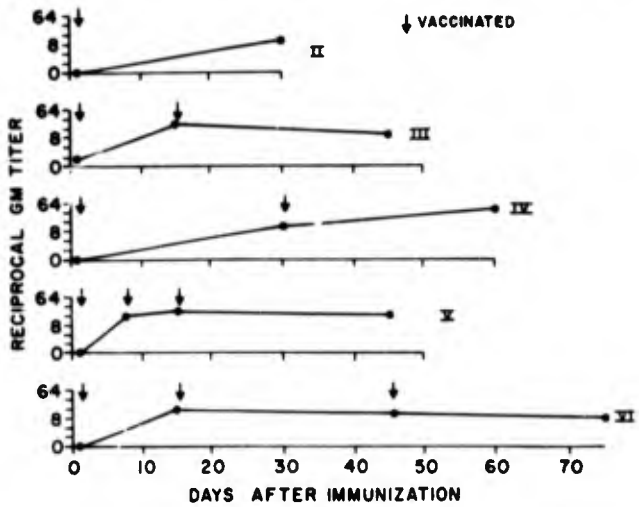

Figure 2. Comparative MA antibody titers for different immunization schedules.

All monkeys were challenged at 30 days after their final immunization. Our bleeding schedules were not designed to detect secondary antibody response. Arrows indicate days on which monkeys were vaccinated. It can be seen that a second immunizing dose given 8 to 15 days after the primary injection afforded no long-term advantage in antibody titer (groups III, V, VI). Likewise, a third injection given 7 or 30 days after the second appeared to have no additive effect. In fact, during the 30 days preceding challenge of groups III, V, and VI antibody titers decreased. In contrast, in group IV on a 30-day, 2-dose schedule, titers tended to increase during the 30-day prechallenge period. A rise is seen in group II on a 1-dose schedule.

Clinical response after challenge is seen in Table II. Lymphopenia, characteristically observed on days 4-10 in nonvaccinated infected monkeys occurred in the control groups, but was not observed in any of the vaccinated groups. There is no real significant between-group difference in MA titer of vaccinated monkeys at time of challenge, suggesting that although a MA titer of 1:8-1:64 is indicative of response to vaccine and possibly protection from fatal infection, it is not necessarily related to resistance to illness. It is interesting to note that no clinical response is seen in the group with the highest MA titer (group IV). It is also obvious that with the present experimental design, MA titer is ineffective as a guide for selection of the best schedule for immunizations.

TABLE II. CLINICAL RESPONSES TO VARIOUS VACCINATION SCHEDULES

GROUP	DAYS VACCINATED	GM MA TITER AT CHALLENGE	NO. ILL	MEAN DAYS		NO. WITH		
			4	Onset	Duration	Rash	Rick.	Died
I	None	2	4	4.0	7.5	3	3	1
II	1	13	2	5.0	4.7			
III	1,15	13	0					
IV	1,30	38	0					
V	1,8,15	16	1	6.0	2.0			
VI	1,15,45	8	3	4.7	5.3			

However, on the basis of both clinical response and MA antibody, the data suggest that 2 immunizations spaced 15 or 30 days apart is the immunization schedule of choice. Efforts are under way to determine if a cell-mediated immune response can be detected after immunization with our vaccine.

The balance of the report presents preliminary studies to examine the feasibility of an inactivated vaccine to protect against all spotted fever members. Based on the careful serological and cross-protection studies of Lackman et al.,[1] we decided that a likely combination might be R. rickettsii and R. australis. Since this experiment was initiated before we had evaluated the optimal immunization schedule, guinea pigs were immunized with 3 injections 1 week apart. Each injection contained 3 x 10^8 R. rickettsii and 5 x 10^8 R. australis. Guinea pigs were challenged with 1 of the 5 spotted fever members 30 days after the last injection. Mice, which are susceptible to R. akari, were injected by the identical schedule but with 1/2 the volume. Results after challenge are shown in Table III. Other than protection against infection with R. rickettsii and R. australis, mouse deaths indicate that the vaccine confers protection against R. akari. Rickettsemia data suggest some protection against R. siberica infection. R. conori appears to cause such a mild disease in guinea pigs that adequate evaluation of the vaccine is impossible. The greatest drawback to this study is the absence of suitable animal models for R. siberica, R. conori and R. australis. Studies are under way for more suitable animal models to evaluate spotted fever vaccines. We are presently attempting to establish a colony of voles which may serve this purpose.

TABLE III. PROTECTIVE EFFICACY OF COMBINED, KILLED R. AUSTRALIS - R. RICKETTSII VACCINE IN GUINEA PIGS

CHALLENGE RICKETTSIAE	RICKETTSEMIA	DEATHS (NO./TOTAL)	SCROTAL REACTION (0 to 4+)	NO. DAYS MEAN TEMP. 103.8 F
R. australis				
Vaccinated	-	0/6	1+	1
Control	-	0/6	2+	3
R. rickettsii				
Vaccinated	-	0/6	0	0
Control	+	4/6	4+	8
R. siberica				
Vaccinated	-	0/6	1+	1
Control	+	0/6	3+	1
R. conori				
Vaccinated	-	0/6	1+	0
Control	-	0/6	3+	1
R. akari				
Vaccinated	-	0/5	3+	2
Control	-	0/6	4+	3
R. akari (mice)				
Vaccinated	+	0/20		
Control	+	18/20		

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1. Lackman, D. B., E. J. Bell, H. G. Stoenner, and E. G. Pickens. 1965. The Rocky Mountain spotted fever group of rickettsias. Health Lab. Sci. 2:135-141.

DISCUSSION

Dr. Elisberg commented that it was important to characterize the nature of the immune globulins being generated and indicated that IgM was responsible for the rickettsial agglutination in murine typhus, for as long as 2 yr. He felt that the reason that no marked changes were shown in these microagglutination test data may be due to the fact that this test detects IgM but probably not IgG. Dr. Elisberg asked what the FA antibody response was in those animals which had received multiple doses.

Dr. Kenyon replied that the tests have been started but the data are not yet available. Dr. Benenson indicated that the range of data should be presented and not limited to the mean. Dr. Kenyon reported that a scatter diagram was available which would answer this point.

Dr. Woodward recalled that several years ago, if 3 doses of vaccine were combined into a single dose, the antibody titer was much higher. Then if revaccination occurred after another 6 mon, there was quite a boost in the antibody response. Dr. Sanford felt that definite conclusions regarding the vaccination schedule should not be based on results covering only 45-60 days and asked if animals will be available for testing 6-9 mon after inoculation. Dr. Kenyon answered that as yet there were no studies to answer this point. MAJ Pedersen indicated that the experimental vaccine schedule selected for these studies were based on two considerations: (1) the recommendation of Lederle Laboratories for administering their RMSF vaccine; and (2) the schedule that has been used for Q fever vaccination. He reported that they were still trying to develop a good, consistent serological test because complement fixation data were unreliable; the Weil-Felix test provided sporadic results; moreover, there was hope that the new radioimmunoassay procedure would provide an index of the IgG response. Thus far, however, the FA test yields the best results.

Dr. Elisberg described the work comparing the degree of heterologous protection afforded by administration of a killed vaccine to animals then challenged with various members of the spotted fever group of rickettsiae. He noted that these results were in line with what Dr. Kenyon reported. For example, a close relationship existed between R. siberica and R. rickettsii. R. conori is in a distinct subgroup; and R. australis and R. akari show a close relationship. The speculation was that if a polyvalent vaccine were formulated, the vaccine should include R. siberica, R. conori, and either R. akari or R. australis.

DIAGNOSIS OF ROCKY MOUNTAIN SPOTTED FEVER USING RADIOIMMUNOASSAY TECHNIQUES

Charles N. Oster, MAJ, MC

Rocky Mountain spotted fever is an illness with a case fatality rate of 5-7%, a rate which has not changed significantly since chloramphenicol and tetracycline were shown to be effective treatment some 30 years ago. The primary reason that this rate has not changed is because spotted fever is often misdiagnosed and, as a result, effective treatment is not given.

Spotted fever presents as an acute febrile illness characterized by severe headache and a rash which often involves the palms and soles; the usual case is easily confused with many diseases which present with similar clinical pictures. Laboratory tests are not distinctive enough to suggest spotted fever over other common febrile illnesses. Diagnosis can be established, with certainty, only by paired acute and convalescent serologic tests; diagnosis is, therefore, delayed long past the point of clinical importance and is often pursued only for interest. The clinician caring for an acutely ill person with suspected spotted fever must, therefore, make the decision to treat with appropriate antibiotics solely on the basis of his clinical knowledge. The unhappy consequence is that some cases are not treated; the mortality of untreated spotted fever ranges as high as 20-30%.

This absence of a clinically available test for spotted fever has given us the impetus to develop sensitive and specific assays for Rickettsia rickettsii antigens and anti-R. rickettsii antibody in clinical specimens. The latter assay has been developed. Technical aspects of the development of the assay and preliminary data comparing this assay to currently available serologic tests for spotted fever will be presented. All of this developmental work has been done using monkeys.

The assay is a solid phase radioimmunoassay employing antigen-coated microtiter plate wells. The antigen is formalinized R. rickettsii, Sheila Smith strain, grown in chick embryo cell culture and purified by rate zonal centrifugation; the controls are uninoculated chick embryo cell cultures, prepared by the same procedure as the antigen. Dilutions of serum to be assayed for anti-R. rickettsii antibody are incubated in rickettsial and control antigen wells, depicted as Ab_1 below.

	1st Incubation	2nd Incubation
R. rickettsii wells	Ag_R-Ab_1	Ab_2-^{125}I
Control wells	Ag_{CEC}-Ab_1	Ab_2-^{125}I

After washing, the bound antibody is detected by a second incubation with ^{125}I-labeled species specific anti-immunoglobulin, Ab_2. After a second

wash, the wells are separated and counted in an automatic γ-counter. Results are calculated as a binding ratio (BR), that is, the counts per minute of ^{125}I-anti-immunoglobulin bound to rickettsial antigen wells minus background, divided by the CPM bound to control antigen wells, minus background.

$$BR = \frac{CPM_{Rick.} - Background}{CPM_{CEC} - Background}$$

Final titers of a test serum by this procedure are given as the highest dilution which gives a binding ratio significantly different from a ratio of one, $P < 0.001$. Each step in this final radioimmunoassay procedure was investigated to determine procedures which maximized the binding ration. The effect of dilution of the stock antigen preparation, containing about 2×10^9 rickettsial particles/ml is shown in Figure 1. CPM bound to ric-

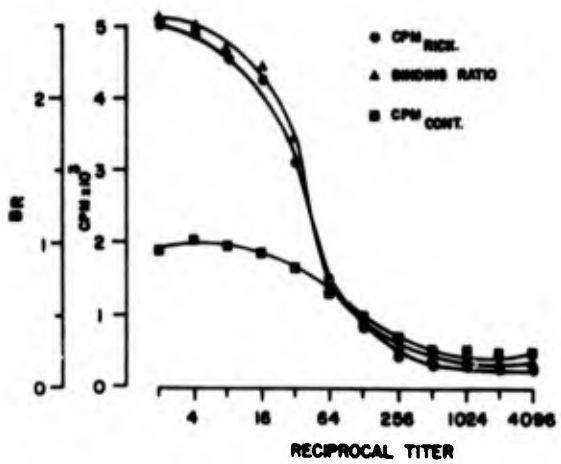

Figure 1. Antigen dilution curve.

kettsial antigen falls off rapidly after a dilution of 1:4-1:8, as does BR. Therefore, a working dilution for this antigen preparation would be 1:4-1:8. After antigen was coated to the walls of the microtiter wells, it was incubated with each of the reagents listed in Table I to investi-

TABLE I. EFFECT OF FIXATIVE ON BINDING RATIO

FIXATIVE	BINDING RATIO	P
None	6.526	
Diethylether	8.064	< 0.001
Acetone	4.517	
Methanol	2.781	
Ethanol	3.124	
Glutaraldehyde	1.142	

gate their property of fixing, or stabilizing, the antigen. Only diethylether significantly improved the binding ratio as compared to no fixation. The effect of different diluents for the first incubation, with test serum, is shown in Table II. The addition of protein to the dilu-

TABLE II. EFFECT OF DILUENTS IN FIRST INCUBATION ON BINDING RATIO

DILUENT	BR	P
PBS	2.214	
+ 2% BSA	2.215	< 0.001
+ 1% BSA	2.501	< 0.001
+ 0.5% BSA	2.323	
+10% calf serum	2.522	< 0.001
+ 5% calf serum	2.288	
EMEM	2.320	
+10% calf serum	2.723	< 0.001
+ 5% calf serum	2.694	< 0.001

ent (PBS - phosphate buffered saline, and EMEM - Eagles minimal essential medium) either as bovine serum albumin (BSA) or as calf serum, significantly improved the binding ratio when compared to PBS.

The effect of pH of the diluent on the binding ratio is seen in Figure 2. There is a broad pH optimum peaking about pH 7.8. Increas-

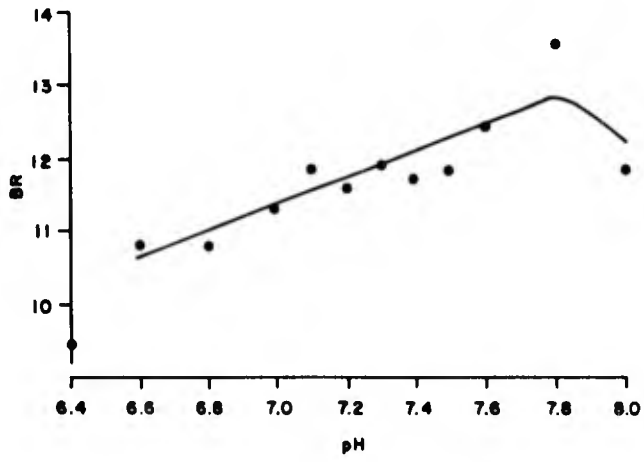

Figure 2. Effect of pH on binding ratio.

ing duration of the incubation period, up to 2-3 hr. increased the binding ratio. At times longer than 3 hr there was no additional significant improvement, Figure 3. The second antibody is prepared by labeling purified

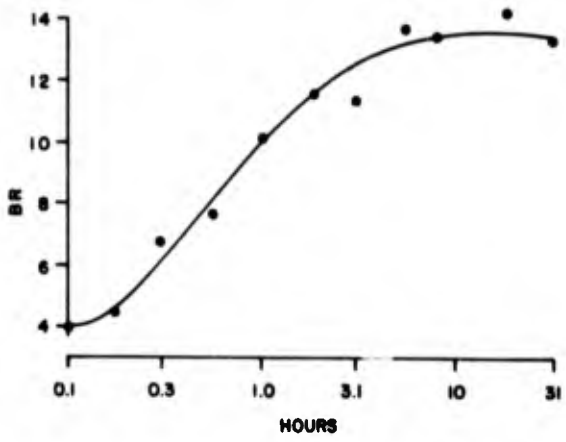

Figure 3. Effect of time of 1st incubation on BR.

rabbit anti-monkey γ-globulin with ^{125}I by the lactoperoxidase method. After separation from free ^{125}I, the labeled second antibody is used in serial 2-fold dilutions in an assay to determine a working dilution which will give maximal binding ratio, yet conserve second antibody as much as possible. An example of a second antibody dilution curve is shown in Figure 4. For this preparation of second antibody, a dilution of 1:20-1:80

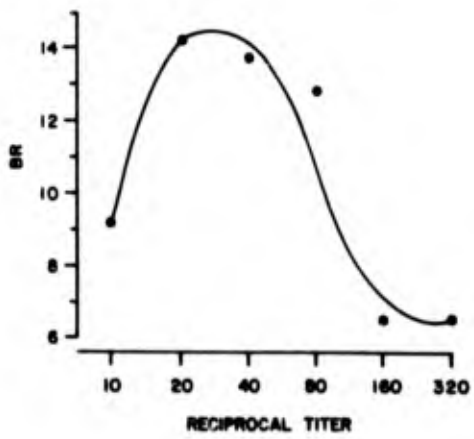

Figure 4. 2nd antibody dilution curve.

would be used. Various diluents for second antibody are compared in Table III; again, as seen for the diluent for the first incubation

TABLE III. EFFECT OF DILUENTS IN SECOND INCUBATION ON BINDING RATIO

DILUENT	BR	P
PBS	1.562	
+ 2% BSA	2.209	< 0.001
+ 1% BSA	2.255	< 0.001
+ 0.5% BSA		
+10% calf serum	2.588	< 0.001
+ 5% calf serum	2.469	< 0.001
EMEM	1.402	
+10% calf serum	3.480	< 0.001
+ 5% calf serum	3.209	< 0.001

period, the addition of protein significantly improved the binding ratios. The addition of 0.1 M EDTA to the diluent for the second incubation did not significantly improve the binding ratio. Binding ratios reach a maximum at 2-3 hr duration of the second incubation period (Figure 5).

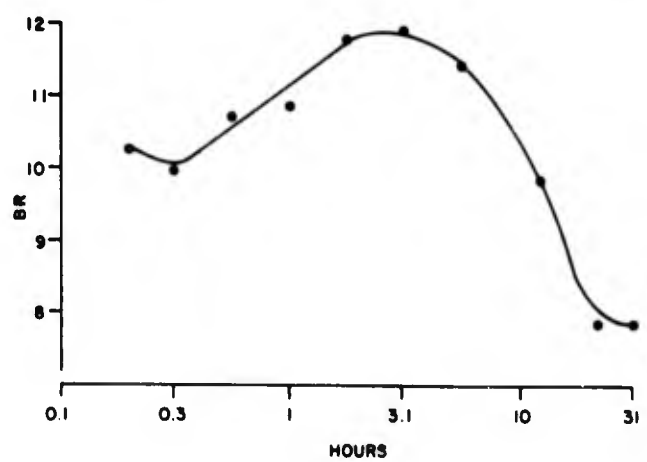

Figure 5. Effect of time of 2nd incubation on BR.

After the second incubation, the wells are washed prior to counting.

Washing with tap water was compared to washing with 2 detergents, 1% Brij-35 and 2% Isoclean, at 1:40-1:320 dilutions of second antibody. The Isoclean wash significantly improved the binding ratio as compared to water, with second antibody dilutions up to 1:320 and compared to Brij-35 at second antibody dilutions up to 1:160, Table IV.

TABLE IV. COMPARISON OF WASHES ON BINDING RATIO

Ab_2 titer	BR		
	Water	Isoclean	Brij
1:40	4.001	6.950	5.006
1:80	5.085	8.659	5.506
1:160	6.965	9.945	7.355
1:320	6.407	8.491	8.343

Utilizing these data, a procedure has been selected for radioimmunoassay of antibody to R. rickettsii. Microtiter plate wells are coated with dilutions of rickettsial and control antigens, fixed with ether, and stored for later use. Dilutions of test sera are then incubated in the rickettsial and control antigen wells for 2 hr. The wells are washed and an appropriate dilution of labeled rabbit anti-monkey γ-globulin is added prior to a second 2-hr incubation. The wells are then washed with 2% Isoclean, separated, and counted. The whole procedure can be performed within one working day. The results of one assay are shown in Figure 6;

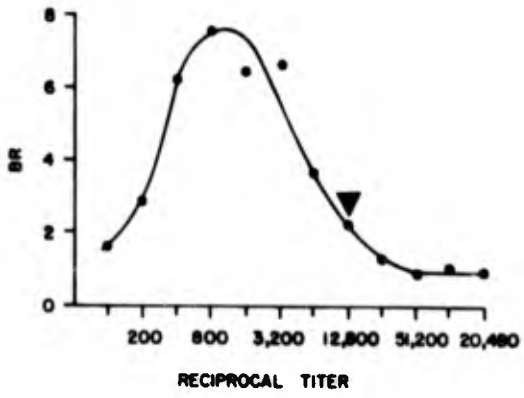

Figure 6. Typical dilution curve.

the arrow shows the last dilution of serum which gives a binding ratio significantly greater than one. This dilution, 1:12,800, represents the radioimmunoassay titer of this serum. Repeat assays on the same serum yielded titers which varied by no more than one 2-fold dilution. Table V shows data comparing radioimmunoassay titers to microagglutination titers

TABLE V. COMPARISON OF MICROAGGLUTININ AND RADIOIMMUNOASSAY TITERS

MA	RIA
0	< 100
0	< 100
512	3,200
128	3,200
64	12,800
256	6,400
256	6,400
16	3,200
32	1,600
128	12,800
64	12,800
GMT 102	5,486
P < 0.001	

on the same sera. As can be seen, the radioimmunoassay is 10-100 times more sensitive than microagglutination for measuring antibody to R. rickettsii.

Thus, we have developed an assay for Rocky Mountain spotted fever which offers several advantages over currently available techniques; it is sensitive and reproducible, yet simple to perform; it employs techniques and reagents which, except for the rickettsial antigen, are generally available. It offers versatility by substitution of other rickettsial antigens, or by substitution of antiglobulins specific for other animal species, and immunoglobulin classes.

Using this assay, we are currently investigating the production of specific R. rickettsii antibodies of the various immunoglobulin classes in response to vaccination and experimental infection of monkeys. Studies of the specificity of this radioimmunoassay are planned, using antigens prepared from other members of the genus and from the proteus strains used

in the Weil-Felix reaction. Finally, the assay will be modified for detection of R. rickettsii antigen in serum specimens. Hopefully, it will prove to be possible to detect antigen early in the course of acute Rocky Mountain spotted fever, before the appearance of specific antibody. If so, we may have a test that will aid the clinician planning a therapeutic regimen for his patient with an unexplained acute febrile illness.

DISCUSSION

Dr. Elisberg commented on the fixative and felt that it would either bind the rickettsiae to the plastic or influence the antigens present on the surface of the rickettsial coat. He indicated that he was impressed with the system but cautioned about collecting baseline data on the basis of sensitivity because of the Q fever experience with the radioimmunoassay (RIA). MAJ Oster replied that he had also come to the same conclusion, i.e., an original P value of 0.05 had been selected but the data required more stringent criteria. MAJ Oster reported that he could not explain the role of the fixative, that diethylether offers several advantages over the others tested. Dr. Nathanson also was concerned about antigens and their denaturation when complex microorganisms are fixed to a plastic surface.

Dr. Pappenheimer asked what accounted for the variable differences between the MA and RIA test data. MAJ Oster referenced a comment made by Dr. Elisberg earlier in which the MA test was thought to measure IgM, whereas, the RIA test measures IgG. MAJ Oster indicated that he wanted to collect information to account for the current differences. Dr. Canonico wanted to know how much background noise was present in the wells. MAJ Oster replied that the noise level was small and could be controlled; in the background data ranged from 50-200 or 300 CPM. He felt that this favorable level was due to: (1) adding protein to the diluent; and (2) vigorously washing with harsh detergent to eliminate nonspecific bound iodine from plastic wells.

SPECIFIC IN VITRO LYMPHOCYTE TRANSFORMATION TO ROCKY MOUNTAIN SPOTTED FEVER RICKETTSIAL ANTIGEN

Michael S. Ascher, MAJ, MC

Our work here at USAMRIID over the last 2 yr has focused on determining the role of delayed type hypersensitivity and its in vitro correlate cell-mediated immunity (CMI) in model infectious diseases. Initial observations of other workers led us to conclude that the time was right for direct application of the in vitro methods of CMI to human disease processes. Our first observations demonstrated in vitro lymphocyte transformation to specific antigen in individuals vaccinated with VEE virus or tularemia bacteria.

Having established the reliability and utility of this method in these model diseases, our next step was to attack the problem in rickettsial diseases. Available serology is of limited value in identifying those individuals with natural immunity, those who convert after vaccination to the immune state, or those who acquire immunity through apparent or inapparent laboratory infection. We have also addressed the corollary question as to whether individuals become immunized who take prophylactic antibiotics after overt laboratory exposure. It was our contention that lymphocyte transformation as a measure of CMI would be more useful in these situations than any serological procedure currently used. To test this hypothesis we began studies in collaboration with the Rickettsiology Division to measure lymphocyte transformation to RMSF rickettsial antigen in the population of laboratory workers here at USAMRIID. The antigen is derived from the vaccine recently prepared by Dr. Kenyon for human use.

Leukocytes are separated from heparinized peripheral blood by sedimentation of the erythrocytes and added to the wells of microtiter plates containing antigen. After 6 days, radioactive ^{14}C-thymidine is added to determine the degree of cellular proliferation that has occurred in response to the antigen. Figure 1 summarizes our observations. The numbers on the ordinate refer to counts of ^{14}C-thymidine incorporated. An exquisitely sensitive individual in the tuberculin system will incorporate around 8,000 CPM and this is roughly the ceiling of the system. A broad spectrum of response is obtained and there is no clear difference between the 19 unvaccinated and 9 vaccinated individuals. Further subgrouping according to degree of laboratory exposure to the rickettsiae, shows that in the unvaccinated group, individuals with no exposure did not differ from those with mild laboratory contact. Three unvaccinated individuals with heavy exposure; those who had clinical RMSF, had vigorous lymphocyte reactivity to the antigen. Another individual with equal exposure and tetracycline prophylaxis remained negative. The open symbols show the breakdown of vaccinated individuals. One individual

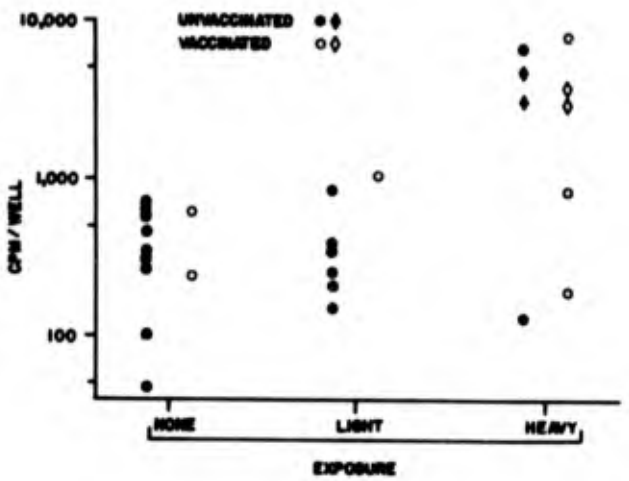

Figure 1. Effect of exposure to RMSF on lymphocyte response to RMSF antigen (clinical cases shown by diamonds).

worked intimately with the organism in preparation of the vaccine antigen material and had a vigorous response. Two recent clinical cases in the vaccine group had vigorous transformation. Thus, it would appear that almost all possible combinations of results are seen with no exception to the hypothesis that exposure to live organisms is necessary to acquire lymphocyte transformation whereas vaccination alone, mild exposure, or heavy exposure with prophylaxis does not induce lymphocyte transformation. Due to the apparent high degree of reliability of this test, we are conducting experiments in a prospective manner in people who receive the new tissue culture vaccine. We will thus settle the question definitively whether vaccination alone can induce conversion of lymphocyte reactivity. This test will be valuable from two other standpoints in that people who already naturally have lymphocyte transformation probably do not require vaccination and that monitoring of the rate of laboratory infection is critical and cannot be done by serology in vaccinated individuals.

It would certainly be very worthwhile to generate findings similar to these in an animal model so that chemical and immunologic manipulations could be applied and tested for their effects on the system. We therefore began studies using a guinea pig model and tested the effects of immunization vs. infection on the lymphocyte transformation response.

Table I shows the results using a guinea pig whole blood system and the nonspecific mitogen phytohemagglutinin. This is as high a level of stimulation as I have seen in any microsystem. This method has the advantage that animals can be repeatedly bled with no ill effects and followed sequentially for changes in lymphocyte transformation.

TABLE I. PHYTOHEMAGGLUTININ (PHA) STIMULATION OF GUINEA PIG LYMPHOCYTES

RELATIVE DOSE OF PHA	CPM/WELL
0	29
1	338
10	6647
100	12205

We then tested lymphocyte transformation to RMSF antigen in naive guinea pigs, recipients of killed vaccine and survivors of infection. Immunization with killed vaccine does not induce lymphocyte transformation (Table II); exposure to the live organism in the course of infec- does. This finding does not surprise us, since historically CMI usually cannot be induced by killed vaccines.

TABLE II. RMSF VACCINE ANTIGEN STIMULATION OF GUINEA PIG LYMPHOCYTES

ANTIGEN ADDED	CPM/WELL BY STATUS OF DONOR		
	Naive	Vaccinee	Convalescent
None	78	27	63
RMSF, 1:10	158	51	9749

Figure 2 shows results at 1 week of a group of 8 animals given live RMSF organisms. The left-hand side of the graph represents control cultures, showing that in the absence of antigen there is a slight, but consistent depression during the week after infection. In the presence of antigen, right panel, by week 2 there is a spectrum of responses which range from a 30-fold increase to very little. There is some crossing over and perhaps a gradual decline. Of interest, the animals in this group were rechallenged after the 6th week. The 3 lowest animals and one individual near the top died. This is a far as we have gone at present. Further work will concentrate on pharmacological manipulation of this response and determination of its correlation with protection. As an example we want to know the effect of protective vaccination with killed antigen, which is by itself not immunogenic as measured by lymphocyte transformation, on priming the subsequent lymphocyte transformation response to infection.

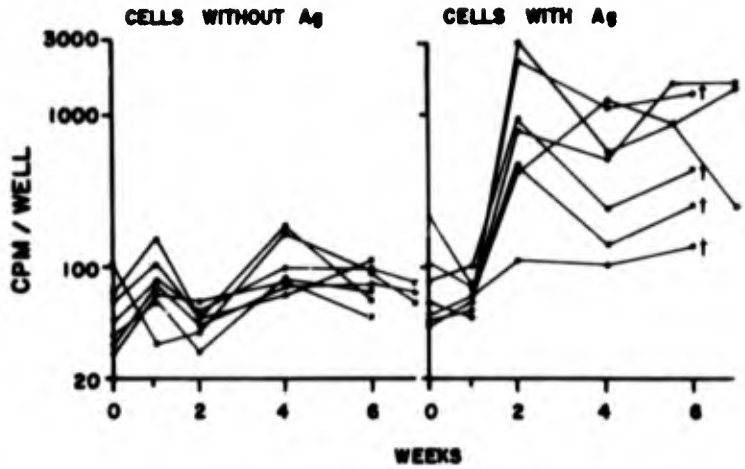

Figure 2. Effect of infection with RMSF on lymphocyte response to RMSF antigen (Ag) in guinea pigs.

Additionally, leukocyte extracts and certain drugs such as cytotoxan known to enhance CMI in other systems will be examined for their effects on modulating lymphocyte transformation and resistance to challenge in recipient pigs.

DISCUSSION

COL Metzger assured the group that the column of data for vaccinated individuals represented the Lederle vaccine, not the USAMRIID vaccine.

Dr. Elisberg cautioned about drawing the conclusion that lymphocyte transformation might be used to determine if an individual needs to be immunized. He felt that it may not be so specific, particularly with respect to allied and related antigenic microorganisms.

MAJ Oster asked why over half of the animals died following re-challenge. MAJ Ascher replied that the dose selected represented an LD_{50} challenge and speculated that there may be some correlation with resistance. The cause of death, however, was not determined to everyone's satisfaction.

Dr. Pappenheimer felt that people with cellular immunity, as detected by the skin test, should respond much better to a vaccine, as opposed to not vaccinating those with a positive skin test. He asked if there were any instances in which CMI would protect against a large challenge dose. COL Dangerfield responded by describing the tularemia mouse model, where mice are protected against a challenge of 100 lethal doses.

MAJ Ascher reemphasized the point that studies are designed to obviate the humoral response in order to gain insight into cell-mediated immunity.

Dr. Sanford recommended that it would be interesting to study people before they worked with virulent organisms.

IMMUNOLOGICAL POTENTIAL OF THE SOLUBLE ANTIGEN OF COXIELLA BURNETII

Ralph F. Wachter, Ph.D.

The soluble phase I antigen of Coxiella burnetii has been used as a vaccine for man. This was reported last year by Brezina et al.[1] Forty-six volunteers were given 1 ml of a dialyzed trichloracetic acid (TCA) extract of the phase I antigen. Serum samples taken 10-14 days later were tested for agglutinating antibodies; 16 individuals (35%) had phase I antibodies (13 of these also had phase II antibodies), 11 had phase II antibodies only and 19 had no antibody response. Fourteen days after the first dose, 23 of the 46 volunteers were given a second 1-ml dose; 10-14 days later 19 of the 23 (83%) possessed phase I antibodies, 2 had phase II only and 2 were negative for both.

Apparently the volunteers in this study worked in the laboratory in Bratislava. Brezina stated: "Volunteers highly exposed to infection under laboratory conditions did not contract Q-fever, whether they received one or two doses of the vaccine and whether they did or did not develop antibodies to C. burneti at the intervals examined. Exposure to infection was confirmed by 5 diagnosed cases of Q-fever in the control group not vaccinated with TCA-extract. None of the 46 vaccinated volunteers had any serious local or systemic reactions, except some tenderness, and redness (. . .) at the site of inoculation lasting for 24 to 48 hr."[1]

The phase I antigen is present in TCA extracts in low concentrations; use of such extracts requires the removal of TCA, generally by dialysis. We have used Na_2SO_3 as a precipitant to concentrate the phase I antigen and to circumvent the need for dialysis. Na_2SO_3 was employed by Cohen et al.[2] in 1949 to precipitate the so-called soluble fraction of typhus vaccines.

The phase I antigen can be dissolved from purified or partially purified preparations of phase I C. burnetii by cold 8% TCA. The extract used in this work had a CF titer of 1:512 and a protein content of 30 µg/ml. The addition of 20% Na_2SO_3 caused rapid precipitate formation. This precipitation, followed by low-speed centrifugation, eliminated CF antigen from the supernatant, which still contained about 50% of the protein and 30% of the carbohydrate of the original TCA extract. Recovery of antigenicity may depend upon the physical state of the precipitated antigen: the sulfite-antigen complex did not dissociate in water or phosphate buffer and recovery of antigenicity was 10%; suspension in 0.001 M EDTA resulted in the disappearance of particles, a decrease in optical density, and 50-100% recovery of antigenicity.

At the time the Brezina paper appeared, we were conducting an experiment to determine whether the sulfite-antigen complex was immunogenic. In this experiment samples of the antigen complex, both as a particulate sus-

pension in buffer and as a soluble product in EDTA, were compared for immunogenicity with dialyzed TCA extract from which they were derived. These samples were resuspended to original volume and, therefore, contained less protein than the TCA extract (12 µg/ml vs. 30 µg/ml).

Guinea pigs, in groups of 6, were given single, 1-ml, subcutaneous injections of undilute, 1:10, or 1:100 dilutions of the samples. Twenty-six days later the animals were bled, but neither phase I nor phase II antibodies were detected by CF or microagglutination (MA) tests. Challenge was accomplished one day later with 10^4 ID_{50} of the 4th yolk-sac passage of Henzerling strain C. burnetii injected IP. Temperatures of guinea pigs were taken daily for 2 weeks. Guinea pigs with temperatures > 40 C for 2 or more consecutive days were considered to lack protection. The results of the test are summarized in Table I.

TABLE I. PROTECTION OF GUINEA PIGS AGAINST CHALLENGE WITH COXIELLA BURNETII PHASE I ANTIGEN AND SULFITE-ANTIGEN COMPLEXES

PREPARATION	DILUTION	NO. WITH FEVER/TOTAL	RECIPROCAL GM TITER			
			CF-I	CF-II	MA-I	MA-II
TCA extract	Undilute	0/6	0	81	64	64
Complex-EDTA		0/6	5	161	32	57
Complex-PO_4		0/6	2	102	32	32
TCA extract	1:10	3/6	0	18	10	13
Complex-EDTA		5/5	0	13	81	100
Complex-PO_4		4/6	0	57	45	45
TCA extract	1:100	4/6	0	6	36	64
Complex-EDTA		5/5	0	37	7	37
Complex-PO_4		4/5	0	11	28	24
Controls		6/6	0	4	3	6

Undilute TCA extract and sulfite-precipitated antigen samples protected all animals while all nonimmunized controls showed a febrile response. Partial protection was afforded by 1:10 and 1:100 dilutions of the extract (3 and 0.3 µg of protein, respectively) and of the antigen complex in buffer but not in EDTA. (The antigen complex dilutions contained 1.2 and 0.12 µg protein, respectively.) Guinea pigs were bled at 15 days. Most control animals typically failed to develop antibodies, whereas postchallenge sera from immunized groups contained both phase I and II antibodies. By the MA reaction, 93% of the samples were positive for phase I antibodies and 87% for phase II; 87% of the sera contained phase II CF antibodies, but only 8% had phase I CF antibodies of low titer. For individual animals no correlation between number of fever days and antibody level was noted.

In a second experiment which is now in progress, groups of guinea pigs were injected with the same preparation of TCA extract used in the first test or with freshly-prepared sulfite complexes of this preparation. However, these animals were given 2 doses of vaccine instead of one.

Figure 1 indicates the overall antibody response following 2 doses

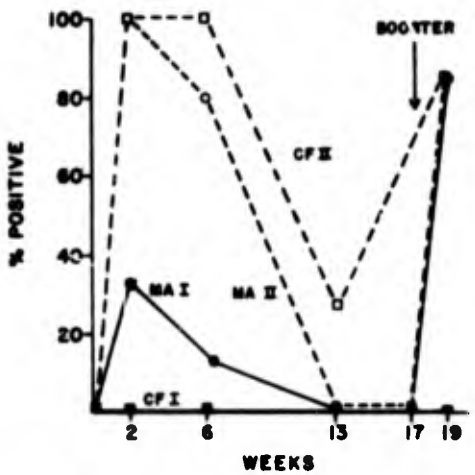

Figure 1. Antibody responses of guinea pigs.

of vaccine. It shows the percentage of animals positive 2, 6 and 13 weeks after the second dose and later, at 19 weeks, which was 2 weeks after a booster dose. We thought that phase I CF antibody (the so-called "late" antibody) might be present at the 6-week interval; none was detected at any time. However, at 2 weeks, all serum samples were positive for phase II CF and MA antibodies and 33% were positive for phase I agglutinating antibody. At 6 weeks, although titers were lower, 100% of the sera were still positive for phase II CF antibody and 85% for phase II MA antibody; only a low percentage showed any phase I agglutinins. At 13 weeks no agglutinating antibodies were detected and only 25% of the guinea pigs possessed any phase II CF antibodies. At 13 weeks no agglutinating antibodies were detected and only 25% of the guinea pigs possessed any phase II CF titer. At 17 weeks, groups of animals were given booster doses of the respective vaccine preparations. Two weeks later 85% of the serum samples contained phase II CF antibodies and both phase I and II agglutinating antibodies. Phase II CF antibody titers were at about the same level as at the original 2-week interval while agglutinating titers were about twice as high.

With the cooperation of Drs. Kenyon and Ascher, blood samples taken from guinea pigs before and after administration of the booster dose were tested for lymphocyte transformation. No indication of cell-mediated immunity was obtained.

In summary, Na_2SO_3 quantitatively precipitates phase I antigen, effecting some purification in the process. Since the resultant sulfite-antigen complex is immunogenic in both particulate and soluble forms, sulfite precipitation may provide a mechanism for comparing the immunogenic effectiveness of soluble and insoluble forms of the same antigen.

The demonstration of the use of phase I antigen as a vaccine in man has significance for us. In the Institute's experience the whole organism Q fever vaccine has caused some sterile abscess formation.

According to Brezina et al.,[1] the soluble antigen caused no serious skin reaction in man. According to Anacker et al.[3] of the Rocky Mountain Laboratory, it has 1/100 the skin reactivity of the whole organism. In our tests with sensitized guinea pigs it has given negative or borderline positive skin reactions.

The phase I antigen appears to be stable for years at 4 C either in TCA solution or after dialysis of the TCA, based on retention of CF titer.

Because of its soluble nature and immunogenic effectiveness in very low amounts, the phase I antigen should be ideal for aerosol studies and possibly as a suspending medium for other rickettsiae for multivalent rickettsial vaccines of the future.

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DISCUSSION

Dr. Benenson aksed if the yolk sac material represented phase I or phase II challenge. Dr. Wachter replied that it was phase I, i.e., Henzerling third egg passaged material.

INTERACTION BETWEEN GUINEA PIG PERITONEAL MACROPHAGES IN COXIELLA BURNETII IN VITRO

Richard A. Kishimoto, MAJ

A survey by Kaplan and Bertagna[1] showed that Q fever, which is caused by Coxiella burnetii exists in 51 countries on 5 continents. This disease is primarily a zoonosis, but many epidemics of Q fever involving thousands of soldiers in Europe, during World War II, gives evidence that this disease is a significant military problem. Stoker and Fiset[2] observed that C. burnetii undergoes a host-controlled variation. They demonstrated that rickettsia isolated from naturally-infected animals and ticks are in phase I, but that after 8-25 passages in embryonated eggs, the phase I strain could be converted to a phase II.

The pathogenesis of Q fever disease and the mechanisms of immunity are not well known. However, we know that the humoral immunity in man following vaccination with the current approved phase II vaccine is short lived; yet recent work by Jerrells et al.[3] has shown that years after infection blast cell transformation was observed in seronegative individuals. Therefore, we have recently initiated a program which asks certain questions given these facts: (1) Is cell-mediated immunity (CMI) involved in protection against Q fever? Is it the major or minor component? We are currently studying with the aid of light and transmission electron microscopy (TEM) this aspect using methodologies which include phagocytosis and the killing effects of normal and immune macrophages. In addition, macrophage inhibition factor (MIF) studies are being used to confirm our results. (2) What is the best antigen to stimulate CMI? This question is currently being studied by us, Dr. Ralph Wachter of Rickettsiology Division, and a contractor, Dr. David Hinrichs. (3) Should the antigen be administered as an aerosol? This question was asked since we know one of the routes of infection of C. burnetii is via the respiratory tract in nature. However, more data has to be obtained covering questions (1) and (2) before meaningful experiments can be designed for question (3).

Three different types of guinea pig peritoneal macrophages were used: normal macrophages obtained from nonimmunized guinea pigs, and macrophages obtained from phase I and II immunized guinea pigs. These cells were then interacted with either phase I or II C. burnetii previously treated with either normal or homologous antiserum. Immune serum was obtained by immunizing guinea pigs IP with whole cell phase I or II antigen at weekly intervals for 4 weeks.

Table I shows the difference in the phagocytosis of phase I rickettsiae by different types of macrophages in normal or immune serum after the 60-min interaction period. Percentage of macrophages containing phase I organisms are relatively low by normal, I and II macrophages. There is essentially no difference in the number of rickettsiae/macrophage. In contrast, in the presence of immune serum, phase I rickettsiae are more

TABLE I. PHAGOCYTOSIS OF C. BURNETII BY DIFFERENT TYPES OF MACROPHAGES IN SELECTED GUINEA PIG SERA

SERA	% PHAGOCYTOSIS BY			MEAN NO. OF RICKETTSIAE/MACROPHAGE		
	Normal	Phase I	Phase II	Normal	Phase I	Phase II
Normal						
Phase I	5	6	12	3	4	6
Phase II	80	96	84	20	29	30
Immune						
Phase I	87	95	88	10	32	24
Phase II	98	92	99	41	42	56

avidly phagocytized by all types of macrophages, and the number of rickettsiae/infected cell is also greatly increased. Phase II organisms are phagocytized by a greater percentage of macrophages than phase I, and the average number of rickettsiae/infected cell is also increased. The presence of immune serum, however, had little effect on the percentage of macrophages containing phase II rickettsiae; this is due to the fact that most of the normal macrophages phagocytized phase II organisms. However, the number of phase II rickettsiae/macrophage is increased in the presence of immune serum. In summary, phase I organisms were found to be more resistant to phagocytosis than phase II organisms by several orders of magnitude. Of equal or greater importance is the fate of the ingested organisms after phagocytosis.

Figure 1 shows a comparison of the fate of phase I rickettsiae

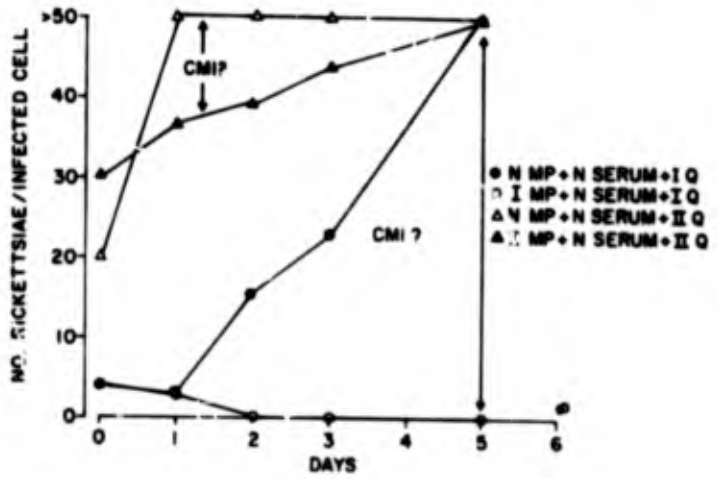

Figure 1. Role of phase I macrophage.

previously treated with normal serum when phagocytized by normal macrophages and macrophages obtained from phase I immunized guinea pigs. The baseline ( ) shows that the phase I rickettsiae grow readily in normal macrophages, but that phase I immune macrophages kill ingested microorganisms ( ). We wonder if this may be due to CMI? When normal macrophages ingest phase II organisms previously treated with normal serum, growth of rickettsiae occurs rapidly after 1 day ( ). However, in the heterologous system, after ingestion of phase II rickettsiae by phase I macrophages, there appears to be an inhibition of growth for 1-2 days, but eventually rickettsiae overwhelm the macrophages ( ).

Figure 2 shows the fate of phase II rickettsiae previously treated

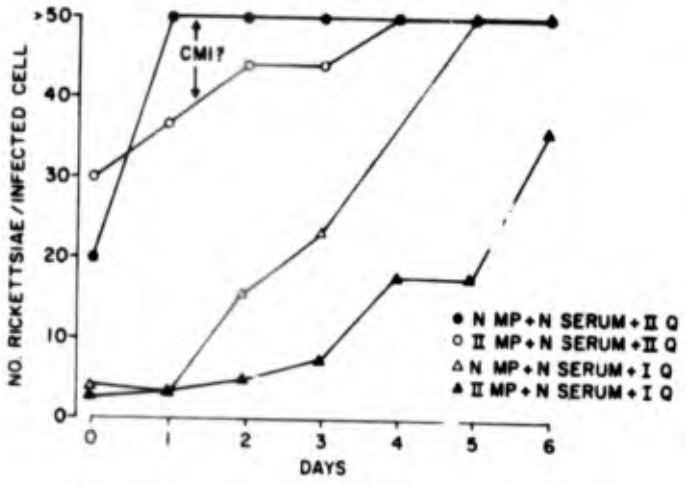

Figure 2. Role of phase II macrophage.

with normal serum when phagocytized by normal macrophages, and macrophages obtained from phase II immunized guinea pigs. Phase II rickettsiae grow very rapidly in normal macrophages, but some inhibition of growth in phase II immunized guinea pig macrophages occurred for 1-2 days, but eventually rickettsiae multiplied uninhibitedly within macrophages. After ingestion of phase I organisms by normal macrophages there are >50 organisms per macrophage after 3-5 days. However, it appears that there is a slight inhibition of phase I rickettsial growth in phase II macrophages, but not as dramatic as seen for phase I macrophages. Normally, antibody-sensitized phase I and II rickettsiae after ingestion were killed by all types of macrophages by 3-5 days. In summary, it appears that macrophages from phase I immunized animals are more rickettsiacidal than normal macrophages or those from phase II immunized hosts.

Because of the small size of rickettsiae, and difficulty in quantitating death or growth after ingestion, interaction studies were further explored by transmission electron microscopy. By this means direct visual

evidence concerning the fate of rickettsiae could be obtained. The following data represent only the interaction of rickettsiae with normal macrophages. Studies are currently underway with immune macrophages. All FM support was provided by COL Veltri and Mrs. Frances Shirey of Pathology Division.

Usually, when normal macrophages phagocytized phase I or II rickettsiae previously treated with normal serum, only small, rod-shaped, compact organisms with thick walls, and containing either a filamentous or dense nucleoid mass in the central region were seen within well-defined phagosomes. Most of the rickettsiae remained fairly intact after the interaction period. In general, both types of rickettsiae multiplied at about the same rate within macrophages after infection, with rickettsiae seen in well-defined phagosomes by 3 days; however, most of the macrophages at this time were disrupted, with liberation of rickettsiae. In rare instances, rickettsiae were observed multiplying by binary fission.

An interesting observation was made within macrophages initially observed 1 day after infection, which became more pronounced 2 to 3 days later. Round to oval forms, lacking thick walls, and whose nucleoid filaments were more dispersed were observed. Many of these larger forms were seen in various stages of degradation, or completely disrupted, indicating that they are degenerated forms. Suspending rickettsiae in homologous immune serum rendered the organisms more susceptible to phagocytosis, but more importantly, potentiated their destruction. After the 60-min interaction period, both rod-shaped as well as round to oval forms were seen. Most of the rod-shaped forms remained fairly intact, but many of the round to oval forms were observed which showed morphological alterations such as swelling or condensation of the cytoplasmic material or complete disruption. In some instances a triple-layered structure, representing cell-wall fragments remaining after enzymatic digestion of the organism was seen. Usually by 5 hr post-infection, there were very few intact rickettsiae, and by 1 day after infection, none.

In brief, our EM studies have confirmed the phagocytosis and rickettsiacidal observations. Inhibition of macrophage migration is the best characterized *in vitro* correlate of delayed hypersensitivity *in vivo*. Some workers have correlated a positive macrophage inhibition factor (MIF) as an *in vitro* correlate of cell-mediated immunity. However, we feel that the MIF test is a good correlate of CMI. Therefore, studies were initiated to determine whether peritoneal exudate cells obtained from guinea pigs immunized with whole cell rickettsial antigen would demonstrate MIF production. The direct MIF test using the agarose droplet technique of Harrington and Stastney[4] was employed. Peritoneal exudate cells were obtained from immunized guinea pigs 12-14 days after the last immunization. An inhibition of 20% or more was considered evidence for the presence of MIF.

Cells from phase I immunized guinea pigs were inhibited from migration when exposed to phase I and II antigens (Table II). However, cells

TABLE II. INHIBITION OF MACROPHAGE MIGRATION FROM GUINEA PIGS IMMUNIZED WITH PHASE I OR II C. BURNETII ANTIGEN

TYPE CELLS	% INHIBITION	
	Phase I	Phase II
Phase I	+ (42)	+ (21)
Phase II	- (0)	+ (34)

obtained from phase II immunized guinea pigs were inhibited only with phase II antigen, but not phase I. Studies are currently underway to determine the role of MIF on the phagocytosis and destruction of rickettsiae by macrophages. It is interesting that the MIF data demonstrated that phase I immunity results in specific immunity against both phase I and phase II organisms as antigen while phase II immunity appeared to be type specific.

In summary although we realize that much work remains to be done, we feel that certain tentative conclusions and implications can be drawn: (1) CMI is involved in immunity to C. burnetii based on the rickettsiacidal effects of phase I immune macrophages and the MIF test; (2) coupling these results with the data of others, that humoral immunity is short lived following either infection or immunization, we tentatively conclude that CMI is the major component, and (3) our results demonstrate that phase I antigen is a much more potent stimulator of CMI than phase II.

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EFFECTS OF POLY (ICLC) ON YELLOW FEVER, MACHUPO, AND VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS DISEASES IN MONKEYS

Edward L. Stephen, MAJ, VC

We have recently undertaken the problem of prophylaxis and treatment of diseases caused by viruses, for which we do not presently have effective vaccines, and of refractory respiratory infections caused principally by gram negative bacteria. We have chosen to move into the area of antiviral chemotherapy for several reasons. The drug industry and various research organizations have been attempting to identify potential antiviral compounds for a number of years. While their efforts have not produced the armamentarium required to prevent diseases successfully, the basic tools are now at hand. Numerous compounds have been synthesized in recent years that possess potent antiviral properties in vitro. Several reports indicate that less than 1% of the compounds possessing significant antiviral activity in vitro are also active in vivo. For this reason, and because we did not want to get into the testing business, only those agents possessing in vitro and in vivo antiviral activity have been selected for initial evaluation. None of these compounds would ever be tested against viruses of importance to our Institute by the drug companies.

We selected VEE virus and yellow fever virus as representative of group A and group B arboviruses, respectively. While we have safe, effective vaccines for use against both of these viruses, they lend themselves well as disease models since the pathogenesis of each has been studied extensively in both mice and monkeys. After we identified several compounds from the literature and through personal communication with investigators heavily involved in viral chemotherapy, we evaluated these compounds in the laboratory mouse. Mr. Kuehne will present this information as an overview of the testing to date in the various systems, with special emphasis on one approach to in vivo testing that appears to offer advantages over the traditional all-or-none, live-dead models.

Most of you are aware that USAMRIID has a unique capability with regard to aerosol technology. While administration of vaccines and drugs as small-particle aerosols for prophylactic or therapeutic purposes is not a completely new concept, few laboratories have conbined the biology and engineering in an attempt to understand or control what is being done. We have completed experiments which indicate that the aerosol route of drug administration is a highly efficacious means of treating influenza virus infections in mice. Major Walker will describe these results and a recent experiment using an isotopically labeled compound. The experiments with labeled drug were done to evaluate drug concentrations in various organs and to determine clearance rates.

In addition to our experiments using aerosolized antiviral drugs, we have also initiated some studies incorporating antibiotics. The use of aerosolized antibiotics in the treatment of unmanageable respiratory infections has been reevaluated, primarily in Europe and the Soviet Union. These studies report improved efficacy of various antibiotics and decreased fatality rates. For this reason, we have directed our studies toward describing why one might expect these agents to be more effective and less toxic when given in a small-particle aerosol. These experiments will be described by Dr. Berendt.

Under our present plan, compounds having potent antiviral activity in mice are evaluated in monkeys. The ultimate goal, of course, is to define drugs that might be used in man. One such compound is lysine-stabilized polyriboinosinic-polyribocytidylic acid (poly I·C). This compound was developed at the National Institutes of Health and was given to people on a limited basis in the recent outbreak of Saint Louis encephalitis. The circumstances for its use were not optimum for an interferon inducer, since the complex was only given to comatose patients. The only comatose patients who survived were those given the stabilized poly I·C. The studies I am reporting today are the results of collaborative work with Doctors Baron and Levy of NIH.

All of the interferon assays were performed at NIH in Dr. Baron's laboratory. While animal studies initially indicated that poly I·C might play a key role in the therapy of viral diseases through induction and release of endogenous interferon, experience in man and primates has proven otherwise. Poly I·C in man, subhuman primates, and some animals other than rodents, is susceptible to rapid enzymatic degradation in body fluids to an inactive state, perhaps, accounting for its unsuccessful use. Poly I·C stabilized with low molecular weight poly-l-lysine and carboxymethyl cellulose (PIC-L) is 4-10 times more resistant to hydrolysis by pancreatic ribonuclease and human serum than the parent compound. The successful prophylaxis of simian hemorrhagic fever in rhesus monkeys was the first report of control of a systemic virus disease in monkeys by an interferon inducer.

Levy et al.[1] reported serum interferon levels as high as 6,000 units/ml in rhesus monkeys following IV injection of 3.0 mg/kg of the complex (Figure 1). In studies done at USAMRIID, 2 lots of PIC-L were similar in that significant levels of interferon were detected in the serum following IV injection of 3.0 mg/kg. The poly I·C complex used in all 3 cases was prepared by Dr. Levy at NIH; lots 33 and 37 were prepared recently, however. The reasons for the apparent loss of inducing capacity of the complex are currently being investigated. Since 25 units/ml of interferon are capable of inducing *in vivo* antiviral effects against an interferon-sensitive virus, the 50-100 units shown here are significant, but certainly not as impressive as the previously reported mean value of 3,000 units/ml.

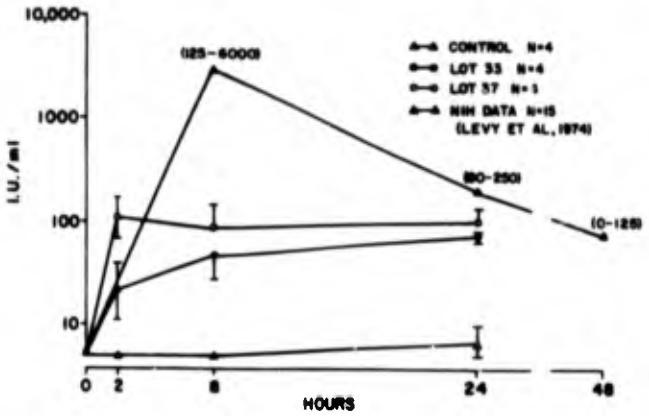

Figure 1. Serum interferon response of monkeys given PIC-L, 3 mg/kg iv.

The design of the first experiment with Asibi strain yellow fever (YF) virus was adapted from the original study with simian hemorrhagic fever. Since peak interferon levels were expected to occur 8 hr after the complex was administered, we injected the first dose of PIC-L 8 hr prior to challenge. Additional injections were given on days 1-4, 7, 9, 11, 15 and 17. Treated animals received 3.0 mg/kg of stabilized poly I·C in each injection. The monkeys were bled daily for viremia, antibody and interferon assays. Challenge was with 1000 PFU of YF. Treated monkeys were challenged 8 hr after the first injection of PIC-L. Previous experiments have shown this dose of virus to be uniformly lethal in 4 and 6 days. YF infection in rhesus monkeys has been carefully studied at our Institute, only 1 monkey was used as the virus control. Four monkeys were treated and challenged. Two additional monkeys served as drug controls. The viremia response of each monkey is shown in Figure 2 as it relates to treatment and virus challenge. The days of treat-

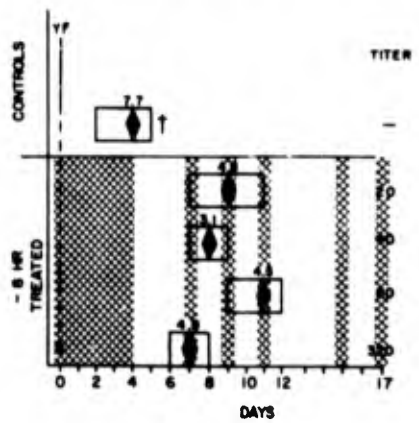

Figure 2. Responses of 4 monkeys given PIC-L and challenged with yellow fever (Asibi).

ment are shown by cross-hatching. PIC-L was administered 8 hr prior to virus challenge and again on days 1-4, 7, 9, 11, 15 and 17. The rectangle shows the time of onset and duration of viremia and the diamond indicates the day of peak titer, with the log titer on that day shown above. The control monkey's response is representative of the response of monkeys infected with Asibi strain yellow fever virus. Note that virus was detectable in the serum beginning on day 2 postinfection and reached a peak of $10^{7.7}$ PFU/ml 1 day prior to death.

In treated monkeys, the onset of viremia was delayed 4-7 days; and peak viremia was at least 1,000-fold lower than that of the control monkey. All of the treated monkeys survived. Previous consideration of yellow fever virus disease in rhesus monkeys indicated that all monkeys with detectable viremia subsequently died, thus making the present observations even more interesting. The monkeys all demonstrated detectable neutralizing antibody on day 17. On day 37 postinfection, the monkeys were backchallenged with 1,000 PFU of virulent virus. The antibody titers of the monkeys were > 1:320 by this time. No viremia was detected following this challenge and all of the monkeys were protected by the previous nonlethal infection. The serum interferon response of monkeys is shown in Table I as it relates to treatment. Where numbers

TABLE I. SERUM INTERFERON RESPONSE OF 7 MONKEYS GIVEN PIC-L IV AND SUBSEQUENTLY CHALLENGED WITH ASIBI STRAIN YF VIRUS

GROUP	IU/ML BY DAY											
	0	1	2	3	4	5	7	8	9	11	12	15
PIC-L control	32	16	-	-	-	-	400	-	400	-	-	
	-	200	-	-	-	-	-	-	-	-	-	
Virus control	-	-	-	16	400	400†						
Treated & challenged	50	500	-	-	-	-	-	-	25	32	16	-
	125	125	-	-	-	-	-	-	32	-	25	-
	16	80	16	-	-	-	-	-	-	-	-	-
	-	100	25	20	-	-	125	125	16	-	-	-

are not shown interferon was not detected. The interferon responses of the drug control monkeys was erratic and not predictable. One of the monkeys responded to the stabilized poly I·C injection only on day 1. The other monkey demonstrated low levels of interferon on days 0 and 1 and responded to PIC-L on days 7 and 9 with interferon levels of 400 units/ml. Interferon was detected in the serum of the virus control monkey beginning on days 3-5, when he died. The peak interferon level for this monkey was as high as the peak response of the drug control monkey.

The interferon response of the 4 treated and challenged monkeys is shown on the lower portion of the slide. The most consistent interferon response occurred on day 1, 8 hr after the second injection of PIC-L. Very little interferon was detectable in the serum in response to injections of the complex after the onset of viremia.

In a second study, designed similarly had 2 groups added to which the complex was given (Figure 3). One group was treated 8 hr postinfec-

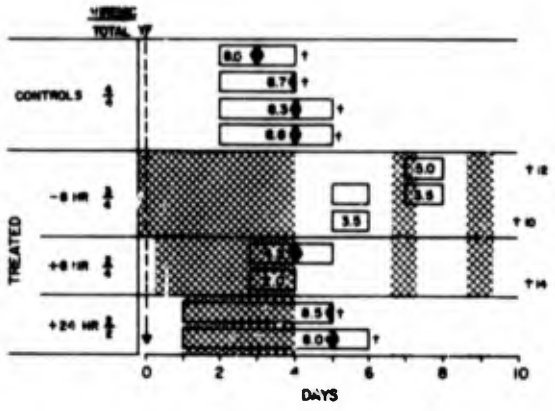

Figure 3. Responses of monkeys given PIC-L at various times and challenged with yellow fever virus.

tion and one, 24 hr postinfection. The control monkeys all died within 4-6 days as before. Again, for monkeys given the complex 8 hr prior to challenge, the onset of viremia was delayed. In addition, the peak virus titer was 1,000- to 10,000-fold lower than for control monkeys. One of 4 monkeys did not become detectably viremic and is not shown.

Two of 4 monkeys treated with PIC-L 8 hr after injection of virus were not detectably viremic and are not shown. The peak titer of the other monkeys was greatly reduced compared to the titer of control monkeys, but the delay in onset of viremia was not apparent.

For monkeys treated 24 hr after injection of virus, the time to onset of viremia was 24 hr earlier and the duration was greater. There was no difference in peak virus titer compared to control values except that the peak was delayed 24 hr. Both monkeys died.

In this experiment, 2 of 4 monkeys given stabilized poly I·C 8 hr prior to infection died on either day 10 or 12 postinfection. One of 4 monkeys given the complex initially 8 hr after infection died on day 14 postinfection. Since these monkeys were not viremic prior to death and did not have histopathological liver involvement similar in degree to control monkeys infected with this strain of YF virus, it seems unlikely

that these monkeys died as a direct consequence of the yellow fever virus infection. Other possibilities, such as toxicity of the inducer or fatal septicemia, are currently being investigated to determine the cause of death.

In order to clarify the question of whether the deaths in our treated group resulted from toxicity of PIC-L, we undertook a dose-response study using 0.3, 1.0, 3.0 and 6.0 mg/kg injected IV for 10 consecutive days. The total number of injections in the previous experiments was 10, given on an interrupted schedule. These results are shown in Figure 4. Each

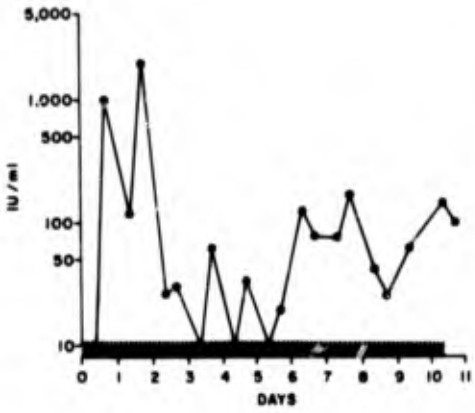

Figure 4. Mean serum interferon responses of rhesus monkeys following repeated iv injection of 3 mg/kg PIC-L.

data point represents the geometric mean interferon response of 3 monkeys. The hashed bar indicates the consecutive days of treatment. The interferon response to 3.0 mg/kg was 1,000 IU/ml. The serum concentration declined to 100 units 24 hr later. The response 8 hr after the second injection was 2,000 units, with a more rapid decline so that only 1 of the 3 monkeys had detectable interferon 24 hr after the second injection. Note that the hyporesponsiveness expected from previous studies in mice became evident after only 2 injections and persisted for 4 additional injections. The interesting finding is that the response appeared to return, or the monkeys appeared to escape the hyporesponsive state. The reasons for this are not clear, but it may represent a shift from the pure release of preformed interferon to de novo synthesis and release more typical of viruses than poly I·C. When 1.0 or 0.3 mg/kg was injected, the responses were essentially the same as for 3.0 mg/kg; 6.0 mg/kg appeared to offer some advantages since the hyporesponsive state could be overcome and the late responses were more predictable. We hope to use this information to establish the optimum regimen of therapy. None of the monkeys died as a result of this intensive therapy.

In a study with yellow fever in rhesus monkeys, we showed that 1.0 mg/kg was indeed as effective as 3.0 mg/kg in terms of protection. These findings were compatible with the results of the dose-response study. Having shows that lysine-stabilized poly I·C was an effective prophylactic and therapeutic agent, when used early, against yellow fever virus infection of monkeys.

We extended our studies to explore the range of effectiveness by using another model virus infection. For these studies, we used VEE-infected rhesus monkeys. Again, this model has been carefully studied at USAMRIID and was therefore easily adapted to chemotherapy studies. The design of this experiment was the same as for the yellow fever experiments. Briefly, monkeys were given the complex on day 0, but 8 hr prior to virus challenge. The treatment was repeated on days 1-4, 7, 9, 11, 15 and 17. In order to circumvent the numerous manipulations of the monkeys to treat and obtain blood samples, the monkeys in this experiment were restrained in chairs and the femoral vein was cannulated for injections and sampling. As shown in Figure 5, untreated virus control monkeys were

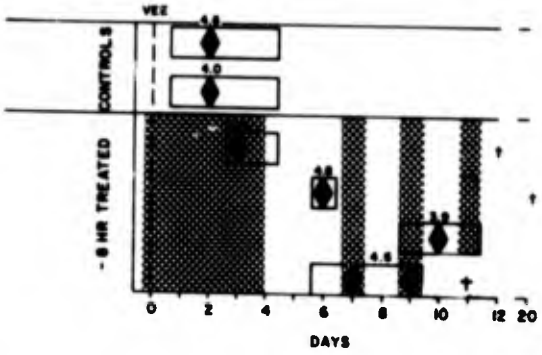

Figure 5. Responses of individual monkeys given PIC-L and subsequently challenged with Trinidad strain VEE virus (1000 PFU).

detectably viremic on day 1 and had a peak viremia of approximately $10^{4.0}$ PFU/ml on day 2. The viremia persisted to day 4. For monkeys treated with the complex and infected, the onset of viremia was delayed 2-8 days. Unlike monkeys infected with YF virus, these did not have lower peak virus titers compared to control values; 3 of 4 treated monkeys died. This finding was particularly disturbing, since VEE virus infection in rhesus monkeys is not normally a lethal infection. It is possible that the combined stress of VEE-virus infection, chair-restraint, vigorous bleeding, emboli from the catheters, and toxicity of the complex combined to make this VEE experiment unrealistic. In order to clarify the question of whether PIC-L potentiated an otherwise nonlethal disease, we repeated the previous experiment except that the monkeys were restrained in cages and no catheters were implanted. The challenge dose of virus in this experi-

ment was 50 PFU/monkey. Replicate results of 2 experiments are shown in Figure 6. The days of treatment are again shown by the cross-hatched

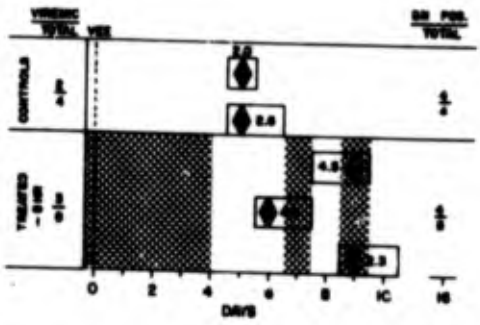

Figure 6. Responses of individual monkeys given PIC-L and challenged with Trinidad strain VEE virus (50 PFU).

bars. Only 2 of 4 virus control monkeys became detectably viremic. There was a 4-day delay in the onset of viremia compared to the previous study. All of the control monkeys developed serum-neutralizing antibody indicating that all of the monkeys were infected. For treated monkeys, approximately the same percentage was detectably viremic. Of the monkeys that were viremic, the time to onset of viremia was delayed 1 to 4 days compared to controls. Only 4 of the 8 monkeys had detectable neutralizing antibody , of these contributed by the monkeys with detectable viremias. No monkeys died. These data support the conclusion that the conditions of the first experiment were not realistic. It is not possible at this time to say whether the lower percentage of seroconversions in the treated group represents an antiviral effect or merely a delay in conversion related to the delay in onset of viremia. When our serological data are complete, we intend to repeat this experiment. Again, using the 1,000 PFU challenge dose.

LTC Eddy and CPT Wagner conducted a preliminary experiment in Machupo virus-infected monkeys to expand the spectrum of virus diseases that might be favorably affected by treatment with the complex. The results of this study are shown in Figure 7. The untreated virus control monkeys were detectably viremic on day 7 and reached a peak viremia 10^4 PFU/ml on day 14. The treatment schedule was varied in this experiment to encompass prophylaxis and late treatment. There was no indication that the complex could be used to alter favorably the pathogenesis of Machupo virus infection in monkeys. While the numbers of monkeys were small in each group, there appeared to be an earlier onset of viremia in the treated monkeys as well as higher peak titers. In addition, treated monkeys died earlier.

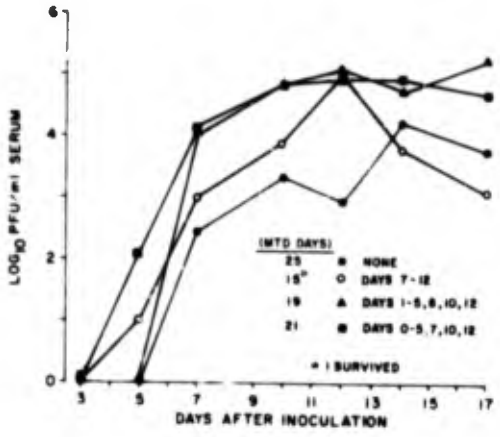

Figure 7. Effect of PIC-L (3 mg/kg) given iv on 3 schedules to Machupo-virus-infected rhesus monkeys.

The results of this experiment might have been predicted based on the in vitro sensitivity of the arenaviruses to interferon. Experiments conducted in the Virology Division have shown that the arenaviruses are relatively insensitive to the effects of interferon. By contrast, VEE virus appears to be only slightly less sensitive to the effects of interferon than vesicular stomatitis virus (VSV). VSV is considered very sensitive and is commonly used to assay for interferon in cell culture systems. In our studies, yellow fever virus appears to lie between VEE and Machupo viruses in terms of sensitivity to interferon, while other investigators have shown it to be more sensitive than VEE virus. We are now attempting to resolve this disparity.

In summary, we have shown that lysine-stabilized poly I·C is an effective prophylactic and therapeutic agent against yellow fever virus infection of monkeys. There appears to be a definite time postinfection when initiation of poly I·C therapy is of diminished value, since both monkeys treated initially 24 hr after infection died. YF-infected monkeys treated with stabilized poly I·C developed antibodies and resisted subsequent challenge. This is important, since many antiviral agents do not permit sufficient antigenic stimulation for development of host resistance. In addition, it appears that while interferon inducers have a broad spectrum of antiviral activity, their use can not be expected to meet with uniformly favorable results since viruses vary widely in their sensitivity to interferon.

Yellow fever and VEE viruses are more sensitive to the effects of interferon in tissue culture than Machupo virus. The time to onset of viremia is delayed in monkeys challenged with yellow fever and VEE viruses, but earlier in Machupo virus-infected monkeys. The peak vire-

mia is much lower in the yellow fever-infected monkeys, unchanged in the VEE-infected monkeys, and significantly greater in Machupo-infected monkeys. Increased survival was apparent only in yellow fever-infected monkeys. The equivocal results in VEE-infected monkeys and the potentiation of Machupo virus disease suggest that poly I·C should only be used in the prophylaxis and treatment of virus diseases previously shown to be favorably affected by its use.

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DISCUSSION

Dr. Rammelkamp asked the following questions: (1) Is not yellow fever a good interferon inducer and how does this infection compare to poly I·C as an inducer; and (2) Is it correct to assume that the hyporesponsiveness of poly I·C is caused by its inhibiting the interferon induced by the yellow fever virus itself. MAJ Stephen replied that he was not sure of the amounts of interferon measured in the 17-D experiments; however, with VEE- and YF-infected animals he noted that high levels of interferon were always observed during viremia and just before death.

Dr. Sanford commented that there was extreme strain variability within the arbovirus group and asked if these particular strains were interferon sensitive. MAJ Stephen indicated that the tissue culture data indicated that the strains were very sensitive to interferon.

Dr. Nathanson discussed the relationship between the refractory period and the inducer and asked what would happen if the stabilized inducers were administered on a different schedule; i.e., can you get around the refractory periods, and can the system be manipulated to better advantage? MAJ Stephen replied that experiments are now underway to answer these questions.

Dr. Woodward wanted to know if yellow fever-infected monkeys have about the same neutralizing antibody levels as observed in humans; that is, did the titers rise and follow the same patterns? Dr. Lennette replied that the antibody titers came up on about the fifth or sixth day.

Dr. Sanford asked if the stabilized poly I·C produced lymphocyte depletion in the nodes of germinal follicles. He recalled that both VEE and YF infections produced this effect in the test animals. MAJ Stephen replied that they had observed a transient fall in peripheral lymphocyte populations after the first injection; however, specific investigations are now being conducted to clarify lymphocyte depletion.

MOUSE MODELS FOR EVALUATING POTENTIAL ANTIVIRAL COMPOUNDS: A NEW "INDIRECT" EVALUATION MODEL

Ralph W. Kuehne, M.S.

Probably the most important problem confronting the viral chemotherapist is the selection of appropriate model systems to test the antiviral properties of potential compounds. Numerous reports can be found in the literature identifying compounds that reportedly have antiviral activity against one or more viruses in cell culture or laboratory animal test systems. The serious problem seems to be that of demonstrating sufficient antiviral activity in vivo to justify clinical trials in man. In attempting to develop a usable experimental laboratory virus system with a human disease-producing virus, investigators have tended to select a virus strain with a short incubation period and high mortality. Administering high dosages often inoculated by an unnatural route into a host selected for maximum susceptibility. The use of such unrealistically demanding models to screen candidate antiviral compounds might be expected to yield essentially negative results.

We decided when we began this project to look first at a number of these compounds in the laboratory mouse. We selected this animal for initial screening for several reasons: availability, ease of handling, minimal space requirements, general susceptibility to a wide range of viruses, the ability to use large numbers and, of course, cost. Initial mouse studies were performed using a group A arbovirus, Venezuelan equine encephalomyelitis (VEE), which has an incubation period of 5-7 days in the weanling Swiss mouse. Mice were inoculated with the Trinidad strain of VEE by the subcutaneous (SC) route and treated with a battery of reported antiviral compounds. These compounds shown in Table I have all been reported to have exerted antiviral activity in some animal-virus test system, not merely in tissue culture, and were selected to cover a broad range of activities. Results are given in the first column of Table II. All of the interferon inducers, that is tilorone and its analogs and stabilized poly I·C, showed significant antiviral activity in this model; the other compounds were inactive

We next wanted to expand our areas of investigation of all of these compounds in the mouse by measuring their effectiveness against a group B arbovirus and a member of the arenavirus group, because of the increasing interest at USAMRIID in these 2 groups. The Asibi strain of yellow fever (YF) virus was selected as the group B arbovirus mainly because the disease model has been carefully worked out. Since the use of Machupo virus requires class III system containment facilities which are very limited here, Tacaribe virus was selected as the representative of the arenavirus group. This virus was available and could be handled with relatively low risk. The problem in trying to duplicate the VEE study with either of these 2 viruses is that, unlike Trinidad VEE, neither is fatal for weanling mice by the SC route, and both produce lethality only when given intracerebrally. Since we considered the intra-

TABLE I. ANTIVIRAL COMPOUNDS

ACTION	NAME
Interferon inducers	Tilorone 11,002 11,567 11,877 Stabilized poly I·C
Inhibits penetration and/or uncoating	Amantadine Rimantadine
Virucidal	Kethoxal
Inhibits intracellular events	Ribavirin Bis-benzimidazole
Unknown	Mepacrine WR-2721 (Radioprotectant) SM-1213

TABLE II. ANTIVIRAL ACTIVITY OF VARIOUS COMPOUNDS IN MICE

COMPOUND	ACTIVITY				
	VEE		YF	Tacaribe	JE
	Direct	Indirect	Indirect	Indirect	Direct
Tilorone	+	+	+		
11,002	+	+	+		
11,567	+	+	+	-	
11,877	+	+	+		
Stabilized poly I·C	+	+	+	-	+
Kethoxal	-	-	+	-	-
Mepacrine	-	-	+	-	
SM-1213	-	-			
Amantadine	-		+		
Rimantadine	-		+		
Bis-benzimidazole			+	-	
WR-2721	-		+		
Ribavirin	-			-	

+ $P < 0.005$ compared to untreated control mice by χ^2 analysis.

cerebral route to be somewhat unrealistic and presented possible obstacles to absorption of the administered compounds, we decided to look at the effect of these compounds on the 2 viruses in an indirect manner. In the direct VEE model, the antiviral compound acts directly on an otherwise lethal virus, limits its replication, and prevents death. The theory of the indirect model depends on the assumption that mice will not be immunized by a live virus if an antiviral compound prevents replication to critical antigenic mass; thus, successfully treated mice would be susceptible to backchallenge. In other words, an effective drug limits replication of the initial dose of virus so that immunity is not attained. In this model, death of the animal indicates successful treatment.

In order to test the validity of the indirect concept, an indirect mouse model using the attenuated TC-83 strain of VEE virus was designed and evaluated (see Table II, VEE Indirect). Weanling mice were given TC-83 by the SC route and treated with the drugs shown; they were challenged 21 days later with Trinidad VEE by the SC route. Of the compounds utilized, those showing significant antiviral activity when compared to untreated controls were the same as those determined by the direct model, i.e., the interferon inducers. On the basis of these results, it was concluded that the concept of an indirect mouse model for use in chemotherapy studies appeared to be valid, since no discrepancies occurred between the direct and indirect VEE models with the compounds tested.

The effectiveness of these compounds in the prophylaxis of yellow fever in mice was evaluated using the indirect method with the Asibi strain, and results are presented in Table II. The compounds were given at the time of the immunizing inoculation of virus except in the case of the interferon inducers, where the compounds were given 24 hr prior. Appropriate controls were, of course, incorporated into the study, including identically challenged, untreated, immunized mice and untreated, unimmunized mice. All compounds tested showed antiviral activity against YF, since all treated mice were susceptible to backchallenge by the intracerebral route 21 days after subcutaneous inoculation of the virus, whereas untreated controls survived. These effects were dependent upon the dose of initial virus, in that they were effective against an initial dose of 10^3 PFU, but not against 10^5 PFU. We cannot say with certainty that these results reflect true antiviral activity, or nonspecific effects such as immunosuppression. We suspect, however, that we are looking at specific antiviral effects, since these compounds are not uniformly effective in the indirect VEE mouse model.

While these results look encouraging, we are suspicious of the across-the-board activity of all the compounds tested. In order to determine directly whether these compounds were indeed antiviral, we challenged mice SC and treated them with the compounds as in the previous study, except that mice were bled sequentially for viremia determinations. Since no infected, untreated mice became detectably viremic, this approach did not clarify the dilemma. We plan to inoculate this serum intracerebrally into mice in an attempt to determine whether virus is actually present in the serum but undetectable due to insensitivity of the tissue culture assay.

The results of the indirect Tacaribe virus-mouse model developed to assess the potential of several compounds to alter arenavirus infections are also given in Table II. We determined earlier that 42-day-old mice can be killed with Tacaribe virus if they are injected intracerebrally; virus is not lethal by peripheral routes. Furthermore, mice given the virus subcutaneously 21 days prior to intracerebral challenge are protected. None of the 6 compounds administered were sufficiently active to prevent the immunizing challenge from protecting the animals, including ribavirin, which appears to be effective against Machupo virus in vitro. The findings again support the hypothesis that these compounds are not immunosuppressive. Since subcutaneous administration of Tacaribe virus effectively immunizes mice, it is conceivable that a marginally effective drug might be overlooked. In any event, we will investigate this model further, using Paraná virus to immunize, since the resulting cross-protection is not complete and may be a more realistic means to evaluate potential compounds.

Since Japanese encephalitis (JE) virus kills mice by the SC route and is a group B arbovirus, a preliminary experiment to evaluate several compounds in a direct mouse model similar to the direct VEE mouse model was undertaken. Most antiviral compounds are noted for specificity related to a particular virus or group of viruses. Our thinking in this regard is that many of the compounds found effective in the indirect YF mouse model should be effective in the direct JE mouse model if they are truly antiviral. The Peking strain of JE was inoculated SC into mice. Three drugs were tested. Results are shown in Table II. This study is still in progress; we intend to rechallenge survivors on day 28 by the intracerebral route to measure immunity. Poly I·C was reported by Postic and Sather[1] in 1970 to be effective in reducing the mortality of the Peking strain of Japanese encephalitis in mice. They rechallenged survivors intracerebrally with a different strain; 50% survived the rechallenge. These findings, coupled with the inactivity seen here with rimantadine and kethoxal, also add weight to the argument that these compounds are not immunosuppressive. To summarize the effectiveness of these compounds, the interferon inducers tested were effective against VEE, Yellow fever, and Japanese encephalitis viruses and were ineffective against Tacaribe virus. The other compounds utilized showed activity only in the indirect yellow fever model.

In summary, an indirect method for the evaluation of antiviral compounds has been developed in mice which should prove to be useful with certain viruses that are only lethal by unrealistic routes. The indirect method appears to be a valid one, based on the similarity of results when it is compared to a direct method using VEE virus.

The effect of those compounds showing antiviral activity in the mouse models studied is suggested to be antiviral and not due to immunosuppression. Evidence for this is that activity is seen against some viruses, but not others; that activity is dependent upon the dose of the virus; and that, as reported by others, a significant number of survivors are immune to rechallenge.

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THE USE OF SMALL-PARTICLE AEROSOLS OF ANTIVIRAL COMPOUNDS FOR TREATMENT OF TYPE A INFLUENZA IN ANIMAL MODELS

Jerry S. Walker, MAJ, VC

The studies to be reported were designed to determine the therapeutic effect of 3 antiviral compounds, amantadine, rimantadine, and ribavirin in mice infected with small-particle aerosols of influenza virus. The first objective was to compare conventional parenteral administration of the antiviral drugs with their administration in small-particle aerosols. The concept for this investigation was based on the type of viral pneumonia seen in man and mouse as a result of influenza infection. This pneumonia is characterized by degeneration of the ciliated epithelium of the trachea and bronchi early in the disease and later by metaplasia of the epithelium and fetalization of the alveolar type 1 pneumocytes. It seemed logical to us and to others that this type of pneumonia could be best treated by administration of an antiviral compound to the epithelial surface of the respiratory tract.

The second opjective was to determine the effects of drug treatment on developing lung pathology during the infection and on lung virus titers. Hopefully, this would give us some insight into the therapeutic effect of antiviral compounds in contrast to their prophylactic effects.

The third objective was to determine if therapy could be delayed until the onset of pneumonia. While chemoprophylaxis of influenza would be desirable, the major clinical requirement is for effective therapy after infection is established.

The type A strain used in these studies was a mouse-adapted variant of Aichi/2/68, an H3N2 strain of influenza virus. The inhaled virus doses ranges from $10^{4.7}$-$10^{5.6}$ EID_{50} which were aimed at achieving an LD_{90} in the untreated virus control mice. For aerosol therapy, mice were placed in an aerosol chamber equipped with a modified Collison atomizer which allows us to expose them for 24 hr/day to small-particle aerosols (SPA) of antiviral compounds. The mass median diameter (MMD) of the aerosol particles produced with the modified Collison atomizer was 1.5 μm and 95% of the particles were < 4 μm in diameter. Based on published work with aerosols of this size, the therapeutic agents were deposited deep in the bronchial tree and in the alveolar spaces.

Treatment was initiated at either 6 or 72 hr after infection. Six hr was selected for early therapy since virus titers had started increasing in the lungs of mice by that time and continued to increase at the rate of $10^{0.2}$ EID_{50}/hr through 24 hr. The total lung virus titer increase was approximately $10^{4.2}$ EID_{50} during the period of 6-24 hr. The drugs were administered either IP in 0.2 ml of saline once daily through day 4 or as continuously disseminated aerosols for 4 days. Virus titers and pathology were determined at 4 and 7 days postinfection.

The second treatment schedule involved initiation of therapy at 72 hr postinfection continued through day 7. Histopathology at 72 hr is characterized by necrotic bronchitis with limited infiltration of inflammatory cells in the bronchial walls and surrounding alveoli; lung virus titers are $\geq 10^7$ EID_{50} per lung.

Lungs of 5 individual mice from these groups were examined for lung virus titer and pathology at 7 days. All percentage survival rates were calculated on deaths from 5-21 days postinfection. The IP dose of rimantadine and amantadine was 40 mg/kg/day of body weight, while that of ribavirin was 16 mg/kg/day. Quantitative assay of aerosol samples indicated that the effective retained aerosol dose of rimantadine was ∿ 4 mg/kg/day while that of ribavirin was 26 mg/kg/day based on the calculated inhaled dose and the retention properties of SPA of 1.5 µg MMD. For comparative purposes the aerosol dosage of amantadine was equal to that of rimantadine, ∿ 4 mg/kg/day.

In Figure 1 the 21-day percentage survival rates of mice given aman-

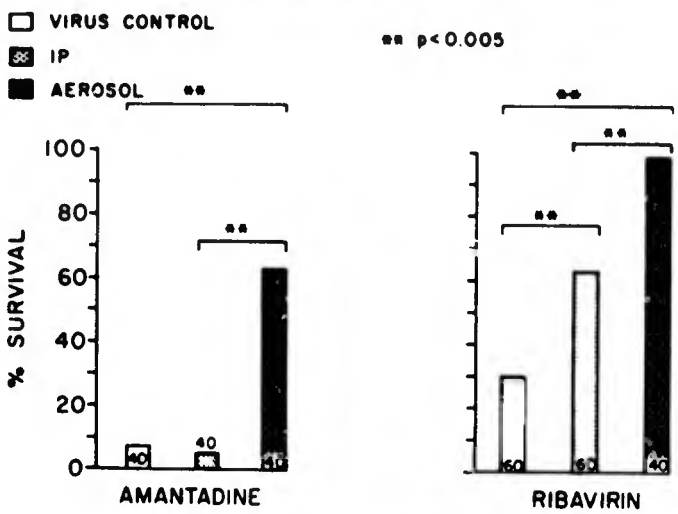

Figure 1. Effect on 21-day survival when therapy is initiated 6 hr after aerosol challenge with influenza virus.

tadine and ribavirin initially 6 hr postinfection are shown from representative experiments. Sixty-three percent of mice given amantadine as a continuously-disseminated aerosol survived, in contrast to less than 10% of virus controls. Treatment by the IP route was not effective. By contrast, ribavirin given IP resulted in 63% survival and the continuously-disseminated aerosols, 98% survival. The survival rate of mice treated IP with ribavirin was significantly different from virus controls. With both drugs the aerosol method of therapy was superior to IP.

Table I contains the data for the lung virus titers and lung lesion

TABLE I. EFFECT OF LUNG VIRUS TITERS AND PATHOLOGY WHEN THERAPY IS INITIATED 6 HR AFTER AEROSOL CHALLENGE WITH INFLUENZA VIRUS

DRUG & ROUTE (% SURVIVAL)	LUNG VIRUS TITERS GMT LOG_{10} EID_{50}/LUNG		LUNG LESION SCORES
	4 Days	7 Days	7 Days
Amantadine			
Virus control (8)	7.9	6.1	3.8
IP (5)	7.8	6.5	3.8
Aerosol (63)	7.8	6.4	3.2
Ribavirin			
Virus control (30)	7.8	5.3	3.4
IP (63)	7.4	5.4	2.8
Aerosol (98)	6.2**	4.3**	0.3***

** $P < 0.025$
***$P < 0.001$

scores for the 6-hr group of mice. Like rimantadine, amantadine did not alter virus titers or lung lesion scores. With ribavirin a different pattern was noted. Small-particle aerosols of ribavirin significantly lowered lung virus titers at 4 and 7 days and dramatically affected the development of lung pathology. Histologically, the lungs from the mice treated with small-particle aerosols of ribavirin were essentially normal at 7 days and remained so. Thus, ribavirin when administered as a small-particle aerosol commencing at 6 hr postexposure not only gives a survival rate of 98%, but essentially prevents the development of any pathologic changes in the bronchi or alveoli.

The second part of our experimental design involved initiation of therapy at 72 hr after the onset of bronchopneumonia, at a time when lung virus titers are $\geq 10^7$ EID_{50}/lung. Figure 2 shows the comparative 21-day survival rates for mice treated at 72 hr with rimantadine, amantadine, and ribavirin when therapy. Rimantadine was effective by both intraperitoneal and aerosol routes, but the aerosol method was more effective. As before, amantadine was effective only when administered as a small-particle aerosol. Again, ribavirin effectively increased survival when administered IP and as small-particle aerosols. There were no significant differences among the survival rates of aerosol-treated animals for the 3 compounds when therapy was initiated at 72 hr.

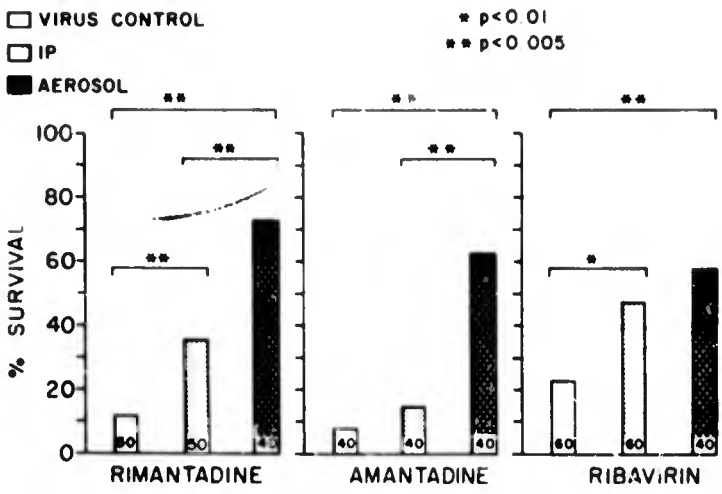

Figure 2. Effect on 21-day survival when therapy is initiated 72 hr after aerosol challenge with influenza virus (No./group indicated).

Table II compares the 3 antiviral compounds on the basis of lung virus titers and lung lesion scores. As before, rimantadine produced no

TABLE II. EFFECT ON LUNG VIRUS TITERS AND PATHOLOGY AT 7 DAYS WHEN THERAPY IS INITIATED AT 72 HR AFTER AEROSOL CHALLENGE WITH INFLUENZA VIRUS

DRUG & ROUTE (% SURVIVAL)	LUNG VIRUS TITERS GMT LOG_{10} EID_{50}/LUNG	LUNG LESION SCORES
Rimantadine		
Virus control (12)	5.6	3.2
IP (36)	5.9	3.4
Aerosol (73)	5.4	2.8
Amantadine		
Virus control (8)	6.7	3.8
IP (15)	6.4	3.7
Aerosol (63)	5.4**	2.0*
Ribavirin		
Virus control (23)	5.6	3.2
IP (48)	6.1	3.6
Aerosol (58)	4.3**	2.6

* $P < 0.05$
** $P < 0.025$

significant changes in either lung virus titers or lung lestion scores. Amantadine therapy by aerosol significantly lowered both the lung virus titers and lung lesion scores in contrast to the previous data for therapy initiated at 6 hr. As before, ribavirin therapy by aerosol significantly decreased lung virus titers.

At this point we expanded our studies using ribavirin as a model antiviral drug to include investigations of the antiviral effect of a metabolic breakdown product and a derivative and pharmacokinetic studies. The ICN Nucleic Acid Research Institute has reported that 5 derivatives of ribavirin exert an antiviral effect in tissue cultures. They kindly furnished them for aerosol studies. We administered the compounds as small-particle aerosols at a dosage of 47.5 mg/kg/day one time starting at 6 hr and continuing through 24 hr postexposure for a total of 18 hr of therapy. Ribavirin produced a 21-day survival rate of 48% compared to 8% for virus controls. Three of the main metabolic breakdown products are 5'-monophosphate and the di- and triphosphate forms. The 5'-monophosphate produced a survival rate of 57% compared to 8% for the virus controls. The ethoxyethyl form gave us a survival rate comparable to the other compounds of 43%. One of the things that always is desirable in therapy is delayed breakdown or inactivation of the active forms of the drugs. The ICN Nucleic Acid Research Institute, in collaboration with us, has compounded a prototype form of ribavirin to achieve this. The tri-O-acetyl form of ribavirin is inactive itself and must be deacetylated by an esterase to become active. The product looks promising, as we obtained a survival rate of 80%. In conclusion, it appears that at least 2 derivatives of ribavirin are as effective *in vivo* in the treatment of influenza infections as is the base compound. In addition, the results obtained with the prototype tri-acetyl form of ribavirin appear promising; it deserves further study. One of the basic questions that must be asked with aerosol therapy involves pharmacokinetics; that is, what is the immediate tissue distribution of drugs administered by SPA and what are the half-lives? Table III and Figure 3 illustrate some very

TABLE III. DISTRIBUTION OF RADIOACTIVITY IN MICE AFTER IP AND AEROSOL ADMINISTRATION OF EQUAL DOSES OF [^{3}H]-RIBAVIRIN

	% DISTRIBUTION			
	IP		Aerosol	
ORGAN	0.5 hr	8 hr	0.5 hr	8 hr
Liver	83	68	76	61
Spleen	6	5	4	6
Brain	5	10	5	22
Kidney	4	10	7	7
Lung	2	7	8	5

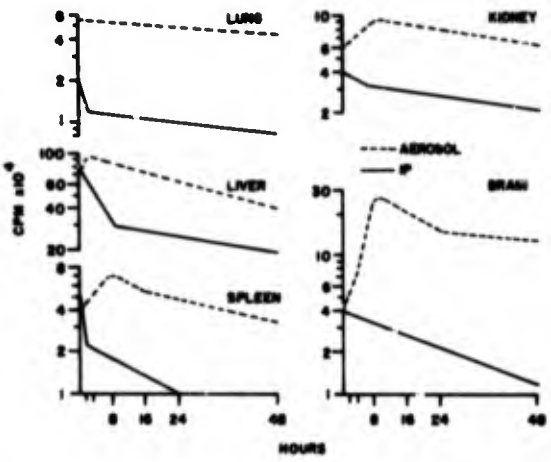

Figure 3. Pharmacokinetics of ribavirin.

preliminary data obtained with intrinsically labeled [^{3}H]-ribavirin. Tissue distribution of the drug at 30 min and 8 hr is shown in Table III. We were able to account for 67% of the total dose injected IP in these 5 tissues and 85% of the calculated effective retained dose by aerosol. At 30 min, most of the drug is in the liver as evidenced by values of 83% and 76% for IP and aerosol, respectively. As would be expected there is more drug in the lung following aerosol administration than IP injection. In fact, we predicted even greater differences than were observed. We did not do blood levels, as it has been reported that at 1 hr, < 0.5% of the total drug is found in the blood and a rapid decrease follows. At 8 hr the distribution pattern has shifted because of different clearance rates in the various tissues. Note that a greater percentage of the remaining dosage is now in the brain, particularly when administered as an aerosol.

Figure 3 shows the data plotted through 48 hr for all 5 tissues. All the plots are reported here as CPM per tissue and are on the same scale except for the liver which is 10x to allow direct comparisons. It should be noted that the mathematical form of the clearance kinetics are completely different for IP and aerosol administration. For IP administration they follow a form expressed as the sum of 2 exponentials, which is the classic form. For aerosol administration they are best expressed as a 3-order polynomial equation except for the lung where the relationship is essentially log-linear. We achieved a level in the lung following aerosol administration that is 3 times that found following IP injection. However, the differences do not stop there, since the higher levels obtained in the lung are essentially maintained through at least 48 hr, while the counts drop rapidly following IP injection. In the other 4 tissues the counts, or drug level per tissue, increased fol-

lowing aerosol administration through 8 hr before starting to decrease; rapid clearance was the order following IP injection. The high levels of the labeled drug detected in the brain tissue following aerosol administration are of particular interest. At the current time we have no answer for these increasing levels in tissues, particularly the brain, following aerosol administration. However, this was only a preliminary experiment and the data now allows us to design the definitive experiments. In brief, we feel that this experiment has answered our basic question: that is, the pharmacokinetics of the model antiviral drug, ribavirin, are different following aerosol administration.

In summary, we found that the most effective method of treatment was continuous aerosol administration regardless of the antiviral compound studied or the time of initial therapy. However, parenteral administration of rimantadine and ribavirin was also effective, even at 3 days. The most effective time of initial treatment varied among the 3 drugs; rimantadine was more efficacious when begun at 72 hr as compared to published data for 6-hr therapy initiation. Amantadine-HCl was equally effective when begun at 6 or 72 hr. Ribavirin was most effective when therapy was initiated at 6 hr, resulting in a 98% survival although it was partly effective at 72 hr as evidenced by the 58% survival.

The effect on lung pathology and virus titers varied depending on the antiviral compound and time of initiation of therapy. Rimantadine demonstrated no effect; amantadine had an effect only when therapy was initiated at 72 hr. Aerosols of ribavirin significantly lowered lung virus titers when therapy was initiated at 6 or 72 hr. While we did not demonstrate a significant reduction in lung virus titers with rimantadine, it did significantly reduce mortality even when therapy was first initiated at the time of peak lung virus titers, thus confirming previous results. This effect of rimantadine is virus-specific because aerosols of rimantadine are not effective in treating influenza B infections of mice at either 6 or 72 hr.

Amantadine exerts its antiviral effects *in vitro* by inhibiting penetration and uncoating processes of the virus; rimantadine is reported to have the same antiviral spectrum and proposed mode of action. These effects appear not to explain totally the *in vivo* results reported here for rimantadine and amantadine. Obviously, further studies are needed to determine the *in vivo* mechanisms involved. Small-particle aerosol therapy with rimantadine and amantadine apparently can be delayed until 3 days postexposure or at the onset of pneumonia as evidenced by histological lesions, with no loss in effectiveness. The dramatic increase in survival rates observed with all 3 drugs given as delayed continuously disseminated small-particle aerosols is encouraging. It suggests that antiviral aerosol therapy may have practical application in treating influenza infection and its pneumonia, and perhaps other respiratory infections as well.

DISCUSSION

Dr. Sanford noted that MAJ Walker had presented some very exciting observations and asked for more information on the schedule of IP therapy using ribavirin. MAJ Walker replied that ribavirin, as well as all other test compounds, was administered once a day. He wanted to employ the test compounds two or three times/day schedule but the mouse was too sick at 3 days and the extra handling caused severe trauma and death in itself. Dr. Sanford explained the logic of his question; i.e., with aerosol therapy the treatment is continuous, as opposed to discontinuous treatment, with a drug having a relatively short half-life. MAJ Walker replied that continuous aerosol therapy had been compared to either intermittent aerosol therapy or injection therapy administered 3 times a day starting at 6 hr and continuing through 4 days. Even under these conditions, continuous aerosol therapy was the best. Dr. Sanford asked if ribavirin had any immunosuppressive activity. MAJ Walker replied that this question was asked at every antiviral meeting and that based on his data, ribavirin has no suppressive activity. Dr. Sanford asked if animals had been challenged with large-particle aerosols as well as small-particle aerosols. He was concerned that small-particles probably create a diffuse pneumonia rather than one at the level of the respiratory bronchiole or just proximal to it. Under the latter conditions, the bronchiole is occluded, and if the animal is then exposed to aerosols, the drug will not reach all the ventilating areas; as in the infected area there would be very little air movement (occluded bronchiole). MAJ Walker replied that in the model reported, there is bronchopneumonia and occlusion as early as 3 days, but aerosol therapy still works. He agreed that getting drugs into an occluded bronchus was a major theoretical concern. He also stated that large- and small-particle aerosols had not been compared directly in his study; however, there was sufficient information available to show that large-particle aerosols (MMD 8 μm) did not kill the mice; i.e., large-particle aerosols of influenza caused only nonlethal disease in mice up to a presented dose of $\sim 10^5$ EID_{50}.

Dr. Sanford commented that things (drugs) that are put into the lungs eventually reach the brain and wondered if MAJ Walker's model might not be a good one for studying the transport mechanism from lung to brain. MAJ Walker agreed with him and stated that these studies are proceeding. Dr. Woodward recalled the early experiments in which tuberculosis was treated with aerosols of streptomycin; these experiments provided some good results as well as some dangerous reactions. MAJ Walker agreed with Dr. Woodward's concern and felt that aerosol therapy experiments could and should proceed only by careful selection of nonreactant drugs and the exercise of extreme caution. Of extreme importance also is the type of atomizer used (for control of particle size) and the inhaled dose of the drug(s). In addition, pharmacokinetic studies must be carried out before proceeding too much further as it appears that the pharmacokinetics of drugs administered as small-particle aerosols are completely different from the parenteral route.

FATE OF AEROSOLS OF KANAMYCIN IN NORMAL AND RESPIRATORY KLEBSIELLA PNEUMONIAE-INFECTED RATS

Richard F. Berendt, Ph.D.

Aerosol therapy of respiratory infection has been investigated in both laboratory and clinical situations. In recent years aerosol antibiotic therapy has been extensively studied because of the increasing incidence and high mortality of pneumonias of gram-negative etiology. We have undertaken to investigate the feasibility of aerosol therapy of acute bacterial infections by treating Klebsiella pneumoniae infections with kanamycin. Table I lists our objectives. In completing the first

TABLE I. RESEARCH OBJECTIVES OF AEROSOL THERAPY

1. Preliminary studies: compare aerosol and intramuscular administration
   - A. Time of administration
   - B. Dose administered
   - C. Pathology, pathogenesis
2. Pharmacokinetics:
   - A. Rate of antibiotic accumulation in selected tissues
   - B. Rate of antibiotic clearance
   - C. Site of deposition, particle size
3. Toxicity and hypersensitivity:
   - A. Local respiratory
     1. Bronchospasm
     2. Hypersensitivity
   - B. Systemic
     1. Nephrotoxicity
     2. Endotoxic effects
4. Effect of therapy on induced metabolic sequelae with Dr. Powanda: monitor efficacy
5. Formulate dosage, schedules and routes

objective, we have studied the treatment of respiratory K. pneumoniae infection in mice and have determined that aerosols of kanamycin led to higher survival rates of infected mice than did the more-commonly employed intramuscular administration.[1] We currently have a number of experiments underway on induced metabolic sequelae, the category 4. No research has been carried out yet on the third and fifth objectives.

The presentation will be concerned solely with the second category. The studies of Teske and Miller[2] and Prokhorova[3] have shown persistence of kanamycin in lungs and kidneys of rats and mice after aerosol treatment; several reports have been published on rapid disappearance of this antibiotic from blood of human patients. Our objectives have been to study distribution and clearance of kanamycin and to determine whether infection alters distribution in important tissues.

Fischer-344 rats were lightly anesthetized with ether and inoculated intranasally with 0.1 ml of culture containing 10^6 K. pneumoniae, type 1. Evaluation of therapeutic efficacy with these infected rats was based upon quantitative bacteriological examination. In order to determine the stage of disease with respect to time after infection, we carried out pathological examinations of infected rats. The most striking observations were bronchopneumonia and splenitis seen at 24 hr, which seemed to be resolving by day 6. Aerosols of kanamycin were disseminated into a modified Henderson apparatus with a Collison atomizer. Rats were exposed to aerosols for 40 min in a plastic box attached to the apparatus. Analysis of the kanamycin aerosol indicated that 50% of the antibiotic was located in particles < 3.5 μm in diameter. Samples of kanamycin for determination of aerosol concentration were obtained with impingers containing 20 ml of distilled water. All kanamycin assays were carried out by a modification of the microbiological method of Winters and co-workers.[4] All treatment schedules consisted of a single administration of antibiotic.

A preliminary experiment was carried out in which normal, uninfected, rats were given doses of 10 mg/kg B.W. of antibiotic by aerosol or by IM inoculation. At selected times, groups of 5 rats were bled and killed; homogenates were prepared from lungs and kidneys. A plot of results is shown in Figure 1. Concentration of antibiotic in lungs after aerosol

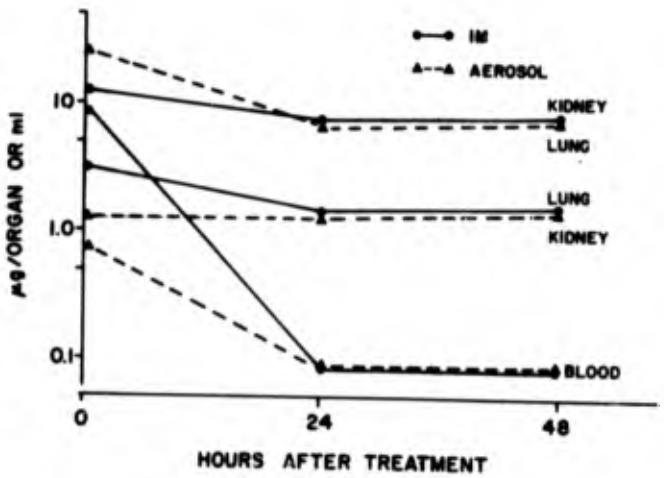

Figure 1. Kanamycin distribution in normal rats.

treatment was approximately 10 times that after IM inoculation; whereas levels in kidney were much higher after IM inoculation. Noteworthy is the rapid disappearance of antibiotic from blood regardless of the route of administration and persistence in lungs and kidneys after an initial rapid decline. Most important, the level of kanamycin in the lungs was significantly higher after aerosol therapy; the IM route provided significantly greater amounts in the kidneys. The previous experiments were carried out with normal rats.

Since numerous changes in physiology occur as a consequence of infection, we carried out an experiment similar to the one just described in which groups of 5 K. pneumoniae-infected rats were killed at periods ranging from 6-72 hr after aerosol or IM treatment. Therapy was administered 24 hr after organism inoculation at a time when a severe bronchopneumonia accompanied by splenitis was present. Bacterial levels at this time were approximately 10^5 in the lung and 10^2 in the spleen (Figure 2).

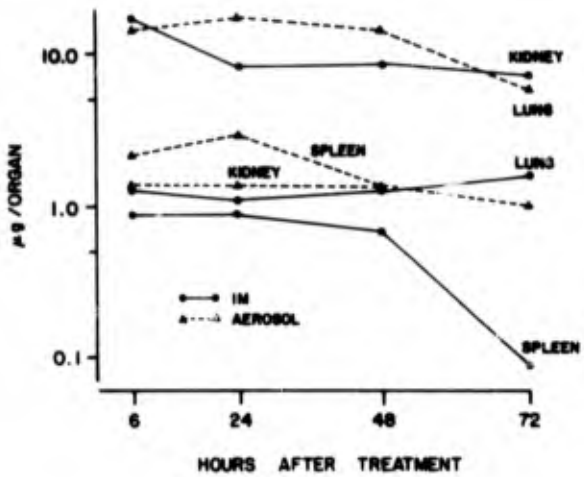

Figure 2. Kanamycin distribution in K. pneumoniae-infected rats.

Again, persistence was noted in lungs and kidney, and significantly higher kanamycin levels were detected in lungs after aerosol therapy than after IM; the reverse was true for the kidneys. The levels of antibiotic in the spleen were about 3-fold higher after aerosol treatment. Blood levels are not shown here because no kanamycin could be detected at 6 hr regardless of route of administration.

In subsequent experiments we found that kanamycin persisted for at least 10 days in the lungs of aerosol-treated rats and for 8 days in IM-treated rats. Antibiotic levels were significantly higher at all times in lungs of aerosol-treated animals. In the kidneys, however, kanamycin levels were higher after IM therapy, but the antibiotic was found for 10 days regardless of route of therapy.

The next experiment was designed both to determine the patterns of clearance during the first 6 hr after treatment and to compare the distribution of antibiotic in tissues in both infected and normal rats during this early period. The distribution of kanamycin in lung, spleen, urine and kidneys of both infected and normal rats after intramuscular or aerosol treatment is shown in Figure 3. Again, the infected rats

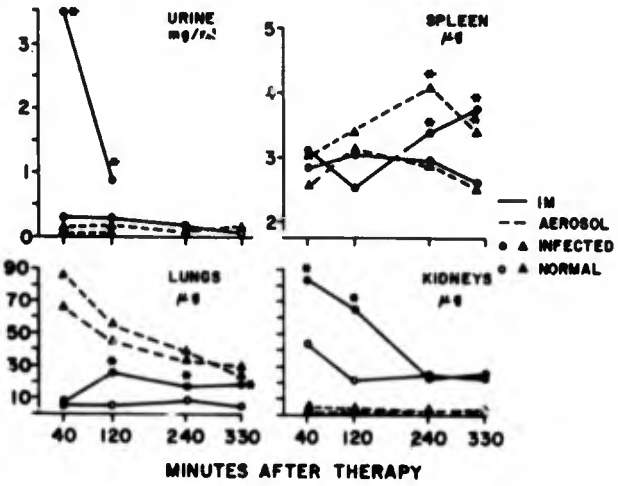

Figure 3. Effect of infection on kanamycin levels.

were treated 24 hr after inoculation with K. pneumoniae. Concentrations in blood are not shown because no antibiotic was detected after 40 min. Significantly more kanamycin was found in urine of infected rats treated by the IM route than controls. We conclude that infected rats excrete most of the IM-administered kanamycin within 120 min, while most of the aerosol dose is retained. Significantly more antibiotic was detected in the lungs of aerosol-treated rats than in IM-treated animals, but only in the latter did the presence of infection cause a significant difference. At 240 and 330 min, significantly more antibiotic was present in the spleens of infected than normal animals regardless of route of administration. These animals had a splenitis at this time. Kidney levels were significantly higher in infected animals given IM treatment only at 40 and 120 min. Kidney levels following aerosol therapy were too low at these early time periods to distinguish differences due to infection.

The persistence of kanamycin in tissues suggested that the antibiotic may be bound to cellular components. To test this theory in vitro, homogenates of various tissues were prepared; 12 mg/ml of kanamycin were added; and the homogenate incubated for 60 min at 37 C. A sample was removed for assay and the balance centrifuged and washed 4 times with phosphate-buffered saline (PBS). The pellet was resuspended in the original volume of PBS; antibiotic activity was measured. The ratio of pellet activity to whole homogenate activity expressed as percent

was considered as the amount bound. Results are given in Table II.

TABLE II. *IN VITRO* BINDING OF KANAMYCIN

TISSUE	% BOUND
Blood	0
Lung	14.5
Liver	38.7
Spleen	9.8
Kidney	14.7

Binding occurred with all tissues except blood, but was not extensive in any case. To determine the extent of binding of antibiotic within infected animals, 5 rats were exposed to aerosol doses of 12 mg/kg 24 hr after infection with *K. pneumoniae*. Twenty-four hr later the rats were killed, tissue homogenates were prepared and both whole homogenate and the washed pellet assayed. The results are presented in Table III.

TABLE III. *IN VIVO* BINDING OF KANAMYCIN* BY INFECTED RAT TISSUE

	μg OF ANTIBIOTIC/gm(ml) OF TISSUE		
TISSUE	Whole homogenate	Washed pellets	% BOUND
Blood	0	0	0
Lung	14.1	2.2	15.6
Liver	0	0	0
Spleen	7.3	Trace	0
Kidney	1.5	Trace	0

*Aerosol dose of 12 mg/kg administered 24 hr postinfection. Kanamycin assayed 24 hr after therapy.

The lack of response in whole blood is consistent with the data already presented. It is apparent that a relatively small fraction is bound *in vivo*; the bulk of antibiotic may either be contained in spaces such as the airways or is so loosely bound that it is eluted by the homogenization procedure.

Finally, we have carried out an experiment to determine the effect of therapy on viable Klebsiella concentration in vivo. Rats were treated with a single dose of 10 mg/kg of kanamycin by aerosol or IM routes 24 hr after intranasal inoculation of K. pneumoniae. One group of infected animals was not treated and served as controls. Ten animals in each group were killed at selected times from 30 min-96 hr. The log geometric mean colony forming units (CFU) in the lungs are shown in Figure 4. A highly

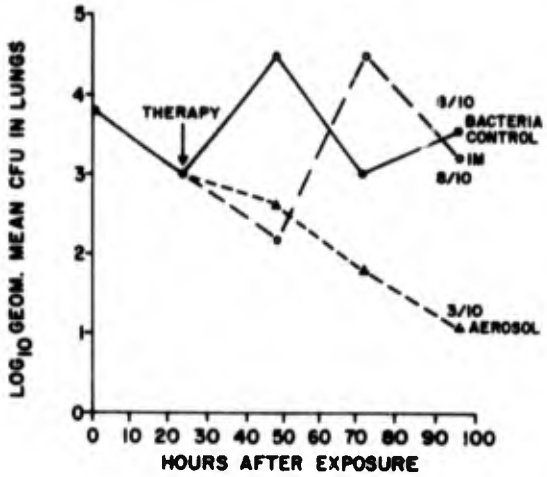

Figure 4. Viable K. pneumoniae concentration in lungs.

significant effect of aerosol treatment is seen, with significant reduction in numbers of bacteria, particularly at 96 hr. The fractions refer to the number of rats that had bacteria in their lungs.

The viable concentration in spleens is shown in Figure 5. Both IM

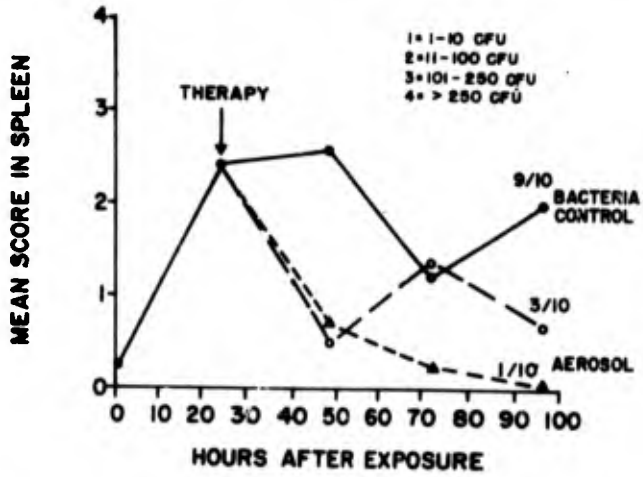

Figure 5. Viable K. pneumoniae concentration in spleen.

and aerosol therapy were significantly effective. Only one rat had bacteria in the spleen at 96 hr following aerosolized kanamycin therapy, in contrast to 9 of 10 of the untreated controls.

Although not shown, aerosol therapy was also more effective in controlling bacteremia than the IM route. We believe that this control resulted from the efficacy of aerosol therapy in reducing viable counts in 2 major foci of infection, namely, lungs and spleen, confirming similar findings with mice.

In summary, we have shown that kanamycin persists at significant levels in the lungs of infected rats for at least 10 days, and that aerosol therapy results in higher lung and lower kidney levels of antibiotic than IM administration. The presence of infection results in increased levels of kanamycin in IM-treated rats in several critical tissues, and higher levels in the spleens of aerosol-treated animals. Our data also show that blood and urine concentrations are extremely poor indicators of tissue levels of this aminoglycoside, regardless of route of administration in normal, and more importantly, in infected rats.

Furthermore, because of lower kidney levels of antibiotic produced, the aerosol route is probably far less prone to cause toxic reactions in patients with kidney disease than the intramuscular, particularly if they have a gram-negative infection. Future plans for this project involve a comparison of the relative toxicity of antibiotic administered by the aerosol route with IM administration, and determination of therapeutic and pharmacokinetic reactions after multiple doses of kanamycin. If monkeys are available, the therapeutic effect of aerosols will be evaluated in a primate host. Finally, a study of aerosols of gentamicin in the treatment of respiratory Pseudomonas infection will be initiated.

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DISCUSSION

Dr. Sanford stated that the aminoglycoside, gentamicin, was one of the best examples of antibiotic persistence in renal tissue. He surmised that kanamycin, another aminoglycoside, would have less nephrotoxicity given as small-particle aerosols than by the IV or IM routes. This has been shown by German workers who used gentamicin. He also stated that tissue binding was not reflected by blood levels and, in consequence, blood levels have limited value. Dr. Berendt thought that the major reason for examining blood levels was to check for kidney toxicity. If the kidneys failed to excrete the drug and blood levels remained high, then the patient would have serious problems. Dr. Sanford commented upon the many arguments about peak and valley relationships and the toxicity related to them. He thought that blood was tested frequently as it now is simply because it is the tissue most readily available.

Dr. Pappenheimer asked if kanamycin accumulated in the brain of the test animal like the antiviral compounds. Dr. Berendt did not know.

OVERVIEW OF TOXIN RESEARCH

Dr. Leonard Spero

The study of bacterial exotoxins has been an important part of the program of this Institute almost from its inception. Most of this work has been concerned with the enterotoxins produced by certain strains of Staphylococcus aureus. Six antigenic variants have been reported, identified as A through F,[1] but only three of the toxins, SEA, SEB, and SEC, have ever been isolated in significant quantities. We now produce these materials in the greatest yield and highest purity of any laboratory in the world. From single 50-L fermentations of the appropriate strains, COL Metzger obtains approximately 0.5-1 gm of SEA and SEC and 10-12 gm of SEB. Each product is virtually homogeneous with no detectable hemolysin and contains 0.1 µg of endotoxin/mg toxin.

Largely because of its greater availability, more work has been carried out on SEB than on any of the other antigenic types and it is now a well-characterized protein. It consists of a single polypeptide chain of 239 amino acids with one disulfide loop and has a molecular weight of 28,500.[2,3] Its amino acid sequence has been determined in Bergdoll's laboratory at the University of Wisconsin.[3] It would be very helpful to our studies of the 3-dimensional structure of the enterotoxins were known, but in the absence of crystalline toxin this cannot be determined. However, several methods have recently been developed to predict the secondary structure of proteins based on their amino acid sequence,[4-7] and we have applied one of these to SEB.[4] The results are shown in figure 1.

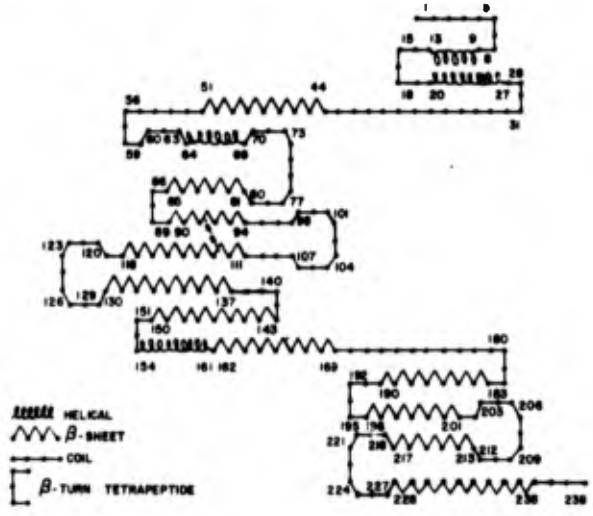

Figure 1. Schematic secondary structure of SEB.

The entire molecule is shown with the numbers representing those positions in the chain at the beginning and end of structural elements. Three types of structural features are predicted: α-helix, shown as loops, β-pleated sheet, shown as saw teeth, and β-turn or chain reversal as indicated. α-Helices and β-turns are stabilized by intramolecular hydrogen bonds, i.e., bonds between monomer units of the same structure, while β-pleated sheets are stabilized by intermolecular hydrogen bonds, i.e., between segments of adjoining parts of the polypeptide chain. It is clear that there is very little α-helix and a fairly high level of β-structure; this has been confirmed experimentally by circular dichroism.[8] In addition, the extensive β-sheet found on both sides of the disulfide bridge suggests that this entire region of the molecule exists in its tertiary folding as depicted here. The results from the laboratories doing sequence work on SEA and SEC should be very informative of features common to all 3 antigenic variants.

Much of the early work here was aimed at an elucidation of the biological responses of susceptible hosts to pharmacological doses of the enterotoxin. Physiological studies on enterotoxemia are continuing and one provocative recent finding by CPT Pettit of Animal Assessment Division suggests that endotoxin release may be involved in the lethality observed at high IV and respiratory dose levels. We have also been carrying out studies on the biochemical, biophysical, and immunological properties of the enterotoxins and some of these data have previously been reported to this Committee. For example, conditions for the preparation of an efficacious toxoid of SEB by formalinization were described.[9] These conditions have been successfully applied to SEC[10] but it must be noted that SEA, while detoxified and polymerized by this treatment, does not become an effective immunogen.[11] We have also described to you the kinetics of the isothermal denaturation of SEB by guanidine and urea.[12] From another study we described our use of limited proteolysis to produce large polypeptide fragments of SEB with the goal of defining the regions of the enterotoxin molecule possessing antigenic determinants.[12] Serological activity has since been successfully demonstrated on the amino terminal half of SEB[13] and on an even smaller amino terminal fragment of SEC.[14] Reaction with antibody has also been shown for the carboxyl terminal portions of SEB and SEC.[13,14]

Within the last year or two we have greatly broadened our areas of interest among the bacterial exotoxins. There appears to be a general renewal of interest in these substances in the scientific community both because of an increased concern over their medical importance and because the tools to study mechanisms of action at the cellular level have progressed so remarkably within the last decade. The work of Pappenheimer et al. on diphtheria toxin[15] and others[16,17] on cholera toxin illustrate the possibilities very well. Our current efforts are concerned with 3 exotoxins. The first of these is the heat-labile enterotoxin produced by some strains of Escherichia coli. This toxin produces a cholera-like syndrome and is thought to be the causative agent for much non-Vibrio diarrhea. There are at least a half a dozen laboratories working on the

isolation of the toxin and just about as many proposed methodologies.[18-21] Molecular weights varying from 20,000-100,000 have been claimed. However, in COL Metzger's laboratory only trace amounts of activity have ever been found in anything but a very high molecular weight complex material. Our best preparations are paritially purified fractions of this, but they possess a specific activity at least as high as any of the putative purified products.

The other 2 toxins provide the subject for our presentations this morning. They are the lethal exotoxin from Pseudomonas aeruginosa and an exfoliative toxin produced by S. aureus. There will be two papers on the Pseudomonas exotoxin.

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PSEUDOMONAS EXOTOXIN---PROPERTIES AND ROLE IN PATHOGENESIS

Stephen H. Leppla, Ph.D.

Infection by Pseudomonas aeruginosa is a frequent and serious complication in several classes of debilitated patients. For example, Pseudomonas sepsis has been responsible for up to 60% of the mortality among severely burned patients. The factors which make Pseudomonas particularly virulent in these situations are unknown. However it was shown several years ago, by Liu,[1] that P. aeruginosa makes a potent protein exotoxin, called exotoxin A. This protein was first recognized as a factor in bacterial culture supernatants which was lethal for mice. Attempts to purify the factor proved futile until Liu recognized that the toxin was being destroyed by the extracellular proteases which the bacterium also excretes into the medium. By growing a protease-deficient strain in a special medium which represses protease production Liu was able to obtain modest amounts of exotoxin A.[2]

A number of studies designed to determine the mode of action of exotoxin A were performed, but these were rendered largely academic by the very recent report of Iglewski and Kabat[3] showing that this toxin acts at the molecular level by blocking protein synthesis in the same way that diphtheria toxin does. Figure 1 provides a reminder of the mode of

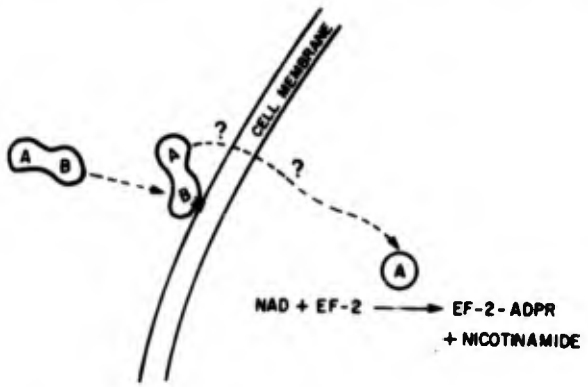

Figure 1. Mechanism of action of diphtheria toxin.

action of diphtheria toxin, as worked out by Pappenheimer and others. Diphtheria toxin contains 2 peptide regions, denoted A and B. B is responsible for binding to the cell. A is then separated from B by a proteolytic cleavage and reduction of a disulfide bond and A becomes internalized. It is fragment A which catalyzes the combination of NAD to EF-2. Iglewski and Kabat's contribution[3] was to show that the Pseudomonas toxin acts like diphtheria toxin at this last step. Essentially nothing

is known about the attachment, fragmentation, and internalization of the Pseudomonas toxin.

We began studies on the Pseudomonas toxin because it seemed the best candidate for explaining the special virulence of the organism. We have focused on defining the chemical properties of the toxin and determining its role in disease. After learning of the similarity to diphtheria toxin, many of our experiments have been designed as comparisons of these 2.

Our studies required first that we obtain substantial quantities of Pseudomonas toxin. We grow 50-L cultures, concentrate the toxin from the supernatants by precipitation with $ZnCl_2$ and $(NH_4)_2SO_4$ and purify the toxin by chromatography on DEAE-cellulose and hydroxylapatite. Fifty-liter cultures commonly yield 100 mg of essentially pure toxin.

Amino acid analyses showed that Pseudomonas toxin has a relatively normal composition, notable only for unusually low lysine and high proline levels. Analyses for carbohydrate with the anthrone reagent or by gas chromatography were negative.

We determined the molecular weight of this protein by electrophoresis on polyacrylamide gels containing SDS. The gel of Pseudomonas toxin alone demonstrated the purity. When run in combination with a mixture of fibrinogen peptide markers, the toxin migrated between peptides of molecular weights 57,000 and 70,000. To narrow the molecular weight range, the Pseudomonas toxin was run in combination with bovine albumin and diphtheria toxin, which have molecular weights of 68,000 and 63,000 respectively. Pseudomonas toxin ran between these markers. We conclude that Pseudomonas toxin has a molecular weight of 65,000.

From what has been said one might imagine that Pseudomonas toxin is a trivial variant of diphtheria toxin which has somehow been transferred into Pseudomonas. We excluded this possibility by a simple serological test. Each of the toxins was placed opposite its respective antitoxin and allowed to diffuse in agar. The resulting precipitin lines were completely independent and non-interfering, showing that the toxins were antigenically distinct. We have also shown with the cell culture technique to be described by Dr. Middlebrook that neither toxin is neutralized by the heterologous antitoxin.

In experiments which cannot be detailed in full, we have confirmed that the Pseudomonas toxin blocks protein synthesis in a cell-free system and catalyzes the ribosylation of elongation factor 2. We are now attempting to determine whether the ribosylation activity of the Pseudomonas toxin resides in a peptide analogous to fragment A of diphtheria toxin. Nearly all preparations of diphtheria toxin contain a fraction of nicked molecules which are dissociated by the reducing agent used in these gels into A and B. Our preparation contained about 10% nicked molecules. In the ribosylation assay only fragment A, and not the unnicked molecule, was active. Gentle treatment with trypsin converted all the molecules to A and B and a large increase in ribosylation activity resulted, consistent with a 10-fold increase in the amount of A fragment. The situation with Pseudomonas toxin appears quite

different. The original preparation has significant activity but appears to be free of fragments. However, it should be noted that the activity of the sample could be attributed to fragments present at the 1% level which might not be detectable on these gels.

We tried to generate increased amounts of these presumptive fragments by gentle treatment with several different proteases. The gels showed that both collagenase and trypsin effect specific cleavages, generating large peptides. However, the activity of the treated samples doesn't increase, and in fact decreases in the case of trypsin. We conclude that either the intact toxin possesses ribosylation activity or the generation of active fragments proceeds through a mechanism unlike that seen with diphtheria toxin.

One may then ask whether the analogy between these 2 toxins extends to their role in pathogenesis. As is well known, the pathology associated with diphtheria results entirely from the action of the diphtheria toxin. Immunity to diphtheria toxin, which is readily induced by vaccination with the toxoid, fully protects against the disease. We have taken several approaches to determining whether the Pseudomonas toxin also plays a major role in pathogenesis.

First we sought to determine whether production of Pseudomonas toxin was a common property of clinical isolates. If the toxin is an important virulence factor, then most clinical isolates should be toxinogenic. We, therefore, set out to screen a group of isolates collected at the Walter Reed Army Medical Center. Results are shown in Figure 2. Initial

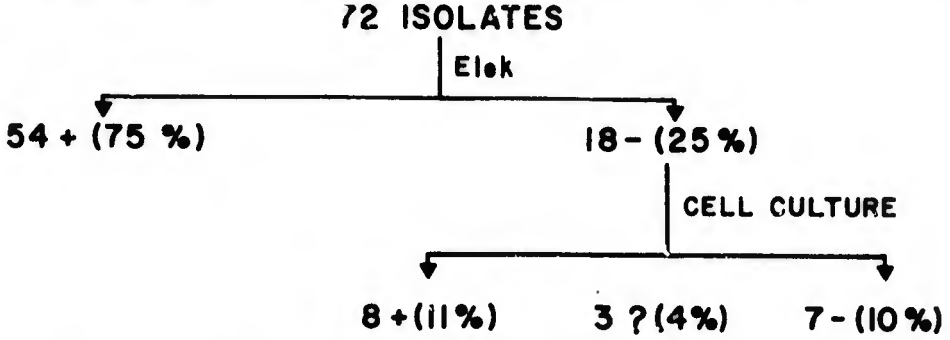

Figure 2. Protocol for screening clinical isolates.

screening was by a simple test procedure, designated Elek, which consists of streaking an isolate on solid growth media adjacent to a paper strip saturated with antitoxin. Seventy-five percent of the isolates produced a material which precipitated with the antiserum.

We suspected that some of the apparently negative strains produced levels of Pseudomonas toxin too low to be detected in the Elek test. Therefore, we tested these isolates in a more sensitive assay, the cell culture cytotoxicity method to be described shortly. This identified an additional 8 toxinogenic strains. We concluded that nearly all clinical isolates of Pseudomonas make exotoxin. This is consistent with the hypothesis that Pseudomonas toxin is an important virulence factor.

If the exotoxin is in fact produced *in vivo* during infection of animals and humans and is an adequate immunogen, then we would expect to find antibodies to toxin in appropriate sera. To detect antibody we developed a sensitive hemagglutination assay. Table I shows the result obtained when a

TABLE I. ANTITOXIN TITERS OF NORMAL AND IMMUNE SERA

SERUM		RECIPROCAL HA TITER
Rabbit, normal		Neg
immune		1280
Pony, normal		10
immune		> 10,000
Horse, normal		20
Calf, normal		20
Fetal calf		Neg
Human (25)	n = 20	Neg
	n = 3	20
	n = 2	10

number of sera were tested. Rabbits and a pony were injected with a series of increasing doses of *Pseudomonas* toxin. Their immune sera had high antibody titers, indicating the validity and sensitivity of the method. Commercial horse and calf sera and serum from the pony prior to immunization showed low titers of antibody. We also tested a group of 25 normal human sera and found 20% to have low titers. We believe that these low levels of antibody demonstrate that these serum donors have been exposed to *Pseudomonas* exotoxin during subclinical infections. To extend the work on human sera we are now collecting sera of patients having diagnosed *Pseudomonas* infections with the expectation that the sera will have significantly elevated antibody levels. Such a finding would prove that *Pseudomonas* toxin is made *in vivo* during infection, and would support the view that *Pseudomonas* toxin is an important virulence factor.

Finally, we have undertaken to establish whether immunity to *Pseudomonas* toxin can be induced in mice and whether that immunity would protect against infection by live bacteria. To induce immunity we prepared toxoids by reaction of the purified protein with formaldehyde and glutaraldehyde at several pHs. All the treatments used caused a polymerization of the protein as is common with these regents. In preliminary trials the glutaraldehyde toxoid seemed to be a better immunogen, so subsequent tests have used only this material.

The toxoid, administered to mice in two doses of 10 or 100 µg, induced resistance to subsequent challenge with at lease 10 LD_{50} of

Pseudomonas toxin (Table II). These mice were later rechallenged with

TABLE II. PROTECTIVE IMMUNITY INDUCED BY GLUTARALDEHYDE TOXOID

GROUP	TOXOID DOSE (μg)	% SURVIVAL FOLLOWING CHALLENGE DOSE (LD_{50}) 0.3	1	3	10
1	(PBS)	100	0	0	0
2	2 x 10	100	100	75	75
3	2 x 100	100	100	100	100

n = 3-4/point

50 LD_{50} and most were immune. Analysis of sera of the immune mice by Ouchterlony gel diffusion showed high levels of precipitating antibody.

We are now testing whether mice made immune to Pseudomonas toxin are more resistant to infection by live Pseudomonas. We intend to infect normal and immune mice by injecting bacteria in one of several sites or by applying bacteria to burn wounds. If these trials show that immunization has a protective effect we would be encouraged to proceed toward clinical trials of the toxoid.

In summary, we have shown that Pseudomonas exotoxin can be prepared in significant quantities and purified to homogeneity. The Pseudomonas exotoxin resembles diphtheria toxin in some of its chemical properties and acts in the same manner at the molecular level, but is clearly unique in its mode of activation. Most clinical isolates of Pseudomonas produce exotoxin, and the exotoxin appears to induce antibody during infection. Immunity to toxin can be induced in mice and may protect against infection by live bacteria. We feel that the results presented support the belief that exotoxin A is an important virulence factor of Pseudomonas.

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RESPONSE OF MAMMALIAN CELLS TO THE EXOTOXINS OF CORYNEBACTERIUM DIPHTHERIAE AND PSEUDOMONAS AERUGINOSA: DIFFERENTIAL CYTOTOXICITY

John L. Middlebrook, Ph.D.

Our laboratory is also studying Pseudomonas exotoxin (PE) but from another viewpoint. We are trying to understand the sequence of events which ultimately lead to its potent toxicity, i.e., its mechanism of action. Although we recognize that expression of toxicity in an animal usually results from a complex interaction of a toxin with various organs and systems, we have chosen to begin our work by examining the effects of the toxin on cultured cell lines. We have adopted this methodology first because of its relative ease, reliability, and simplicity when compared to animal studies, and secondly, because there is a wealth of information available about the effects of diphtheria toxin on cultured cells. Since there is evidence in the literature that Pseudomonas and diphtheria exotoxins have similar enzymatic activities, we felt that valuable insight might be gained by repeating some of these earlier experiments using both Pseudomonas and diphtheria toxins in a comparative fashion.

The basic experimental protocol is as follows. Cells, grown as recommended by The American Type Culture Collection, are trypsinized, counted, and suspended to the desired density in medium with 10% serum. The cells are aliquoted into multiwell tissue culture plates with Medium 199, Earle's salt plus 10% fetal calf serum and allowed to grow for 24 hr. The toxin is added to the concentration desired and incubation is continued for 48 hr. Each well is then washed to remove disattached cells and those cells remaining are solubilized for a Lowry protein assay. Figure 1 shows the results of just such an experiment using Pseudomonas exotoxin and mouse fibroblasts. In this experiment we also measured the effect of toxin on the ability of cells to incorporate ^{3}H-thymidine after 48 hr. As one can see, the effect of toxin on remaining cell protein (squares) parallels the effect on the ability to synthesize DNA (circles). We believe this experiment demonstrates that our protein-cytotoxic assay is a good measure of cell survival and viability.

We next investigated many factors we thought might have an influence on our cytotoxic assay. We found that only one variable was significant, namely the type of serum used to seed and maintain the cells for the duration of the assay. Figure 2 shows the effect of the toxin on Vero cells which were plated using 4 different types of serum. It is evident that the type of serum employed can profoundly shift the dose-response curve. Indeed, the difference between fetal calf and calf is almost 2 orders of magnitude. We observe exactly the same effect if diphtheria toxin is used instead of Pseudomonas. Moreover, this effect has been observed with every cell line examined.

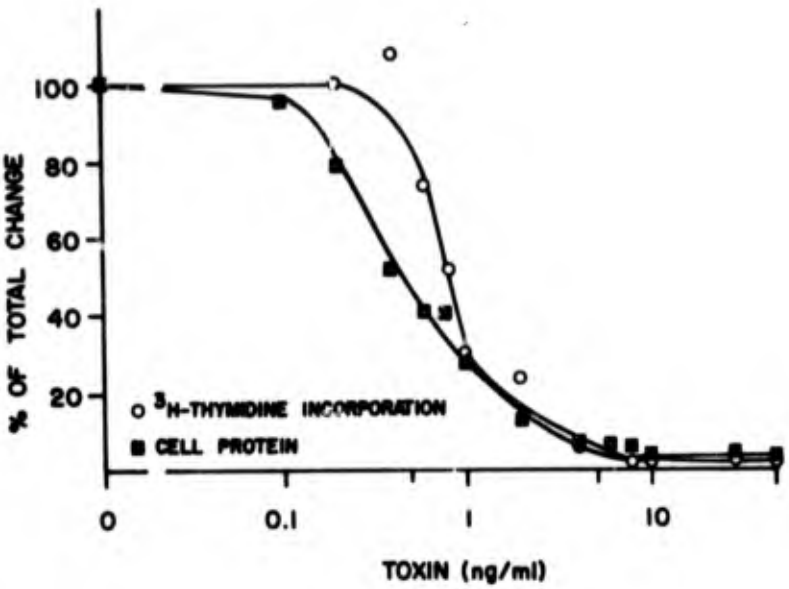

Figure 1. Measurement of cytotoxic response: cell protein assay vs. thymidine incorporation.

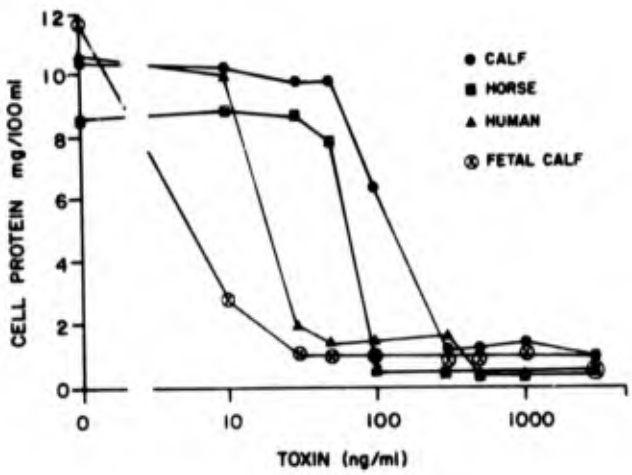

Figure 2. Effect of different sera on Pseudomonas exotoxin cytotoxic responses.

There are cells which can be maintained without any serum. When we examined the effect of Pseudomonas exotoxin on one of these cell types (chick fibroblasts) we found that the cytotoxicity to cells without serum was indistinguishible from cells in fetal calf serum. We interpreted this result to indicate that a number of sera other than fetal calf could, in various degrees, protect cells from the cytotoxic effects of both Pseudomonas and diphtheria exotoxins. Thus, only fetal calf serum was used in the comparative studies to be described.

The dose-response curves for the effect of diphtheria and Pseudomonas toxins on mouse L cells are shown in figure 3. It is reported in the

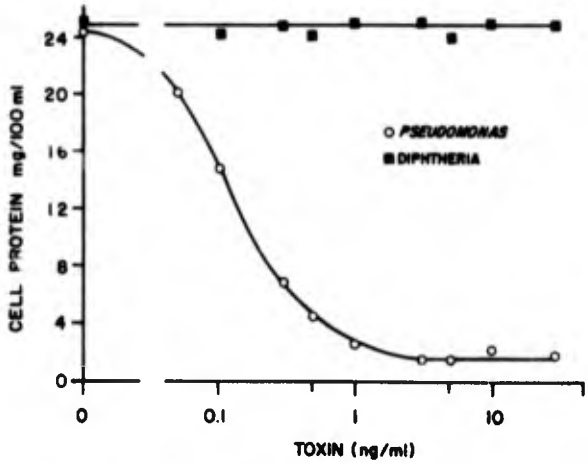

Figure 3. Dose response of mouse L929 cells to diphtheria and Pseudomonas exotoxins.

literature that mouse L cells are very resistant to the cytotoxic action of diphtheria toxin; our results are in agreement with this observation. On the other hand we found that mouse L cells are very sensitive to Pseudomonas exotoxin. We have defined an LD_{50} in our system to be that concentration of toxin which reduces the remaining cell protein to 50% of control level. Applying this definition to L cells we find that Pseudomonas exotoxin is at least 3 logs more potent than is diphtheria.

The effects of these two toxins on Vero cells are presented in figure 4. This cell line responds to Pseudomonas and diphtheria exotoxins in an opposite fashion to mouse L cells. Here diphtheria is approximately 3 logs more potent than Pseudomonas exotoxin. It should be emphasized that our comparative experiments are run with the same cell preparation on the same day and everything is identical except for the toxin that is added.

We have examined the sensitivities of many cell lines to the cytotoxic effects of both toxins. We ranked each cell line in order of its sensitivity to toxin (most to least). The left hand column of Table I gives an LD_{50} range in log steps while the middle and right columns list

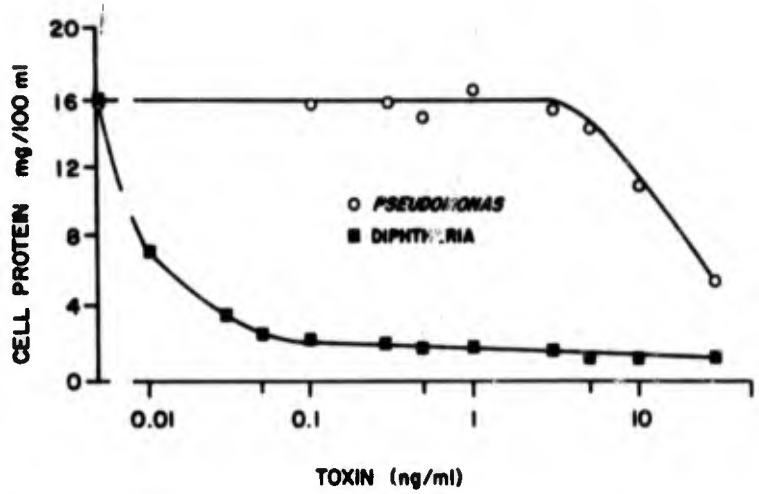

Figure 4. Dose response of Vero cells to diphtheria and Pseudomonas exotoxins.

TABLE I. SENSITIVITY OF CELLS TO PSEUDOMONAS AND DIPHTHERIA EXOTOXINS

LD_{50} CONCENTRATION RANGE (ng/ml)	PSEUDOMONAS	DIPHTHERIA
< 0.001		BSC-1 (monkey) Vero (monkey)
0.01 - 0.09		MK-2 (monkey) HAK (hamster)
0.1 - 0.9	L-929 (mouse) 3T3 (mouse) CC1-6 (human)	Chick fibroblasts CC1-6 (human) BHK-21 (hamster) WI-38 (human) KB (human) HeP-2 (human) WISH (human)
1 - 9	WI-38 (human) Chick fibroblasts KB (human) MK-2 (monkey) CC1-107 (rat) BHK-21 (hamster) HeP-2 (human) WISH (human)	HeLa (human)
10 - 90	Neuroblastoma (mouse) HAK (hamster) Vero (monkey) HeLa (human) BSC-1 (monkey)	
100 - 900		CC1-107 (rat)
> 1000		L-929 (mouse) 3T3 (mouse) Neuroblastoma (mouse)

those cells having an LD_{50} in the indicated range for either Pseudomonas or diphtheria toxins. We cannot see any correlation in sensitivities of cells to the two toxins either on an absolute or a relative scale. For example, it turns out that 2 of the cell lines most sensitive to Pseudomonas toxin (L-929 and 3T3) are virtually unaffected by diphtheria while the opposite is seen for cells very sensitive to diphtheria toxin (BSC-1 and Vero). Thus, there appear to be significant differences in the effects of thses 2 toxins upon cells. The next obvious question is what might be the source of these differences.

Figure 5 which Dr. Leppla used is a diagrammatic representation of the mechanism of action of diphtheria toxin. We have chosen to view the

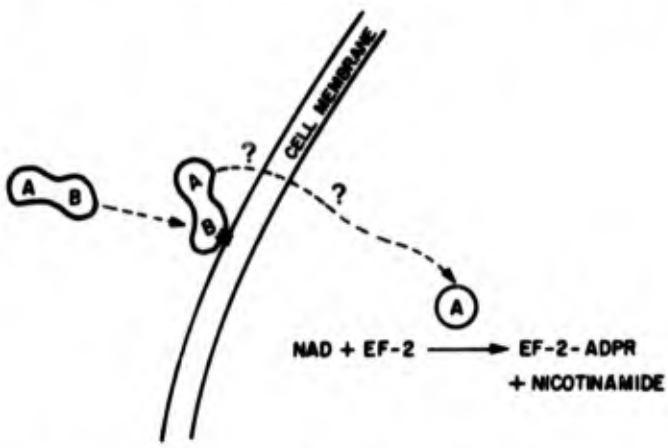

Figure 5. Mechanism of action of diphtheria toxin.

mechanism as consisting of 3 parts. First, there is the attachment of toxin to a cellular receptor. Secondly, there is an internalization of the toxin (with an activation in the case of diphtheria). Finally, there is the ribosylation of EF-2 catalyzed by the toxin. We have devised a number of experiments to determine whether Pseudomonas exotoxin behaves similarly to diphtheria toxin during each of these stages. Because of the time limitation, only one representative experiment designed to probe each stage will be discussed.

Concerning the ribosylation stage, Iglewski and Kabat[1] demonstrated that Pseudomonas exotoxin appears to have a ribosylating activity very similar to diphtheria toxin. It is possible then, that the different cytotoxicities of the toxins reside in their relative efficiencies at ribosylating EF-2 from different cell lines. To examine this possibility we titrated the enzymatic ribosylating activity of Pseudomonas and diphtheria exotoxins using partially purified EF-2 from mouse L-929 (a cell line sensitive to Pseudomonas but insensitive to diphtheria toxin) and from BSC-1 cells (a cell line insensitive to Pseudomonas

toxin but sensitive to diphtheria). Figure 6 shows that the ribosylating

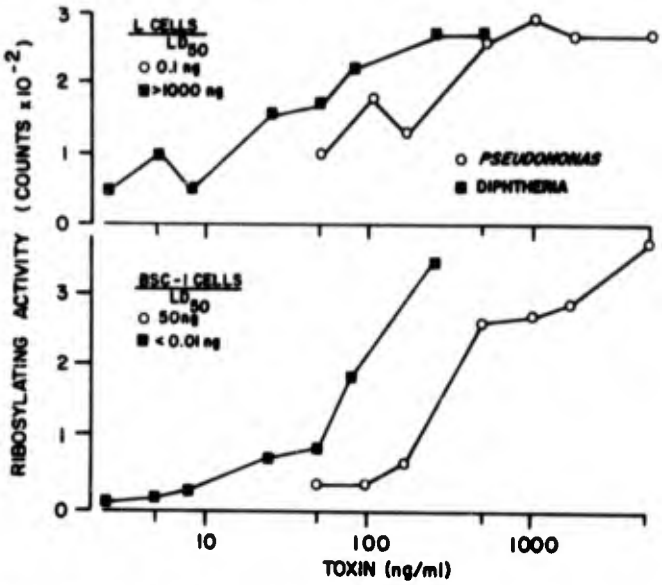

Figure 6. Ribosylation activity of Pseudomonas and diphtheria exotoxins.

activity of diphtheria toxin (squares) is not different in L or BSC-1 cells; a similar situation is observed for Pseudomonas exotoxin. We did not observe any reversal in the relative ribosylating activities of the 2 toxins, much less that required to explain the very marked differences in intact cell sensitivities.

Concerning the internalization stage, it has been demonstrated that a number of drugs or compounds can protect cells from the cytotoxic action of diphtheria toxin. One of these agents, NaF, is a potent inhibitor of glycolysis. Since much of the energy required for membrane transport is provided by ATP resulting from glycolysis, glycolytic inhibition should lead to a reduction in intracellular transport. We examined the ability of NaF to protect HeLa cells from both toxins (figure 7). Cells were incubated for 1 hr with the concentration of NaF indicated. Toxin was added and incubation continued for 2 hr followed by a thorough wash of the cells and 48 hr of further incubation. Controls indicated that cells could completely recover from the temporary inhibition of glycolysis induced by NaF over this concentration range. In agreement with the literature, we find that NaF can protect cells in a dose-dependent manner from concentrations of diphtheria toxin which are uniformally lethal (squares). On the other hand, NaF actually seems to potentiate the effect of Pseudomonas toxin (circles). We are not yet in a position to explain this remarkable finding. However, we present this experiment because we believe it is strong evidence that there are differences in the mechanism of action of Pseudomonas and diphtheria exotoxins. Furthermore, this particular experiment raises the possibility that different internalization systems are involved for these 2 toxins.

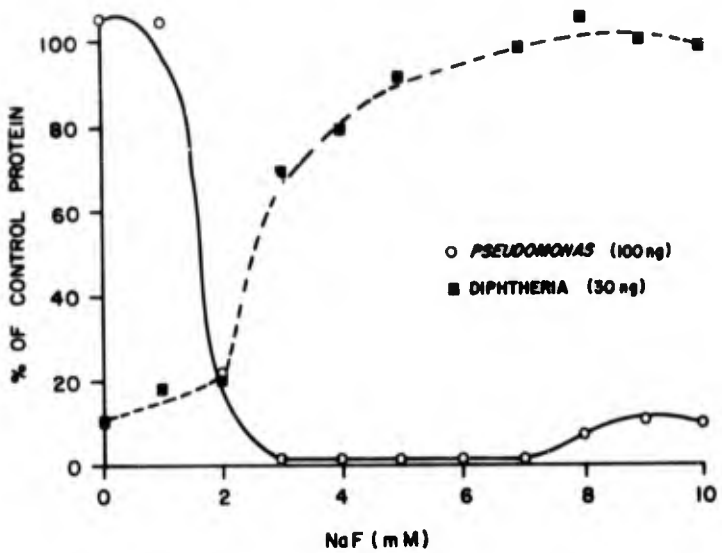

Figure 7. Protective effect of NaF on HeLa cells intoxicated with Pseudomonas and diphtheria exotoxins.

Concerning the attachment stage, if both toxins bind to the same cellular receptor but differ at some other stage of their mechanism of action it should be possible to choose a cell line sensitive to one toxin and insensitive to the other and then compete for, or block, the effects of the sensitive toxin with a large excess of the insensitive. In figure 8 we show measurement of protein synthesis in Vero cells, a

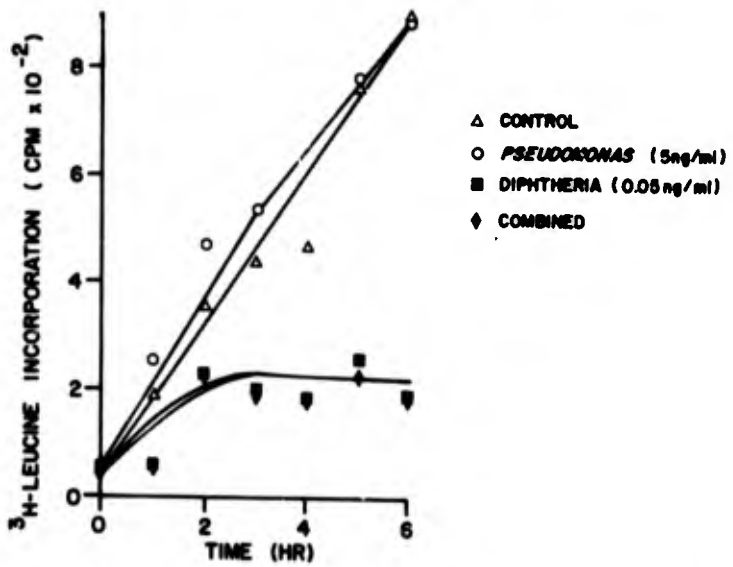

Figure 8. Inhibition of protein synthesis by diphtheria exotoxin: Pseudomonas exotoxin competition.

cell line 1000 times more sensitive to diphtheria than Pseudomonas exotoxin. The control level of protein synthesis (triangles) appears to be increasing linearly with time. Addition of a 0.05 ng/ml dose of diphtheria toxin will arrest protein synthesis (squares). The addition of a 100 molar excess of Pseudomonas toxin has no effect on protein synthesis relative to control (circles) nor does this excess toxin appear to block or even retard the diphtheria effect (diamonds). We have observed the same result when we performed the inverse experiment with a cell line sensitive to Pseudomonas toxin and tried to block its effect with large excesses of diphtheria toxin. These data do not prove, but are consistent with, the notion that Pseudomonas and diphtheria toxins have different cellular receptors.

In conclusion, we believe we have demonstrated that there are differences in the mechanism of action of diphtheria and Pseudomonas exotoxins. Our strongest evidence for this contention is the widely varying sensitivities of cell lines for these 2 toxins. We would concur with the findings of Iglewski and Kabat[1] that Pseudomonas exotoxin is a potent inhibitor of protein synthesis and that the inhibition results from inactivation of elongation factor 2 in an analogous fashion to diphtheria toxin. However, we have shown you one experiment which indicates that Pseudomonas and diphtheria exotoxins probably have different cellular receptors. In addition, other data indicate that Pseudomonas exotoxin appears to enter the cell and/or become activated by the cell in a markedly different fashion than does diphtheria toxin. Further experiments are underway to determine the exact nature of these differences.

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DISCUSSION

Dr. Pappenheimer asked if the fast band that appeared in both sets of gels had been cut out and tested for enzymatic activity. Dr. Leppla replied that SDS gels were run and slices analyzed for ribosylating activity. The activity was always associated with the parent molecule. Dr. Pappenheimer remarked that it appeared that Pseudomonas exotoxin was 2 orders of magnitude less active in the in vitro system than diphtheria toxin on a weight basis. Dr. Leppla replied that Pseudomonas toxin can be activated by treating it simultaneously with a reducing agent and 8 M urea. The treatment increases enzymatic activity 10-fold and makes it comparable to diphtheria toxin activity on a weight basis. When the activated Pseudomonas toxin is run on gels, all of the activity is associated with the large molecules.

Dr. Sanford described a Pseudomonas-animal model system developed by Dr. Elizabeth Zeigler and Dr. Brodie which closely resembles human disease. The model uses either rabbits or guinea pigs, treatment with

nitrogen mustard and the introduction of Pseudomonas organisms into the conjuctival sac. Dr. Leppla was not aware of this particular model and thanked Dr. Sanford.

Major Pedersen reported that investigators at Fort Sam Houston had developed a burned rat model. He asked if Dr. Leppla were suggesting the development of a toxoid for burned rats to protect them from Pseudomonas sepsis. Dr. Leppla replied that first it must be demonstrated that the toxin is active in rats; however, the preliminary data are ambiguous. Dr. Pappenheimer recalled that an English investigator had performed some interesting studies with Pseudomonas in a burn model; i.e., pyocyanine prevented the tissue from healing by blocking the polymorphonuclear leukocytes from entering the area where the organism was growing.

Dr. Nathanson asked if the Pseudomonas toxin molecule were internalized in the cells. Dr. Middlebrook replied that it was presumed to be internalized since the ribosylating activity was internalized; however this has not been actually demonstrated using a radiolabeled toxin.

Dr. Elisberg recalled some studies whereby E. coli endotoxin would protect against not only E. coli infection but also against Pseudomonas infection in a burned animal model. The studies were derived from the burn model described previously by Dr. Sanford. Dr. Elisberg asked if contaminants of exotoxins could play a key role in the protection against infection. Dr. Leppla did not know since the purified toxin had not been assayed for endotoxin.

Dr. Sanford discussed the logic for and the need to study the safety and immunogenicity of toxoids by the aerosol route. The need for these studies resulted from a high percentage of patients developing Pseudomonas pneumonia when complications arose during elective surgery. He felt that much of the "shock-lung entity" is nothing more than pneumonia produced by gram negative organisms from contaminated equipment. If an effective toxoid could be developed for Pseudomonas, it should be studied within the aerosol frame of reference.

Dr. Benenson noted that there were differences in the lag time between diphtheria and Pseudomonas toxins. Dr. Middlebrook agreed and reported that the lag period of Pseudomonas exotoxin is much shorter than that of diphtheria toxin as measured by protein synthesis inhibition or by cell death in culture.

A short discussion arose on the role that Fe plays in toxin synthesis. Dr. Leppla stated that addition of Fe to the culture medium will prevent Pseudomonas exotoxin production.

STAPHYLOCOCCAL EXFOLIATIVE TOXIN

Anna D. Johnson

An exoprotein of S. aureus, phage type II, has been implicated as the etiologic agent of toxic epidermal necrolysis.[1] The clinical manifestations range from localized dermal abscesses to a wide-spread scalded skin syndrome frequently seen as hospital infections of newborn babies. The toxic substance produced by the bacteria has been purified and has been named exfoliatin. Melish et al.[2] developed an animal model for the study of this protein using newborn mice 1-2 days after birth. Test substances are injected IP or subcutaneously. A positive reaction is evidenced by wrinkling of the skin within 2-3 hr; higher doses of the toxin (≈ 10 µg) produce a blistering effect and eventual peeling off of the skin induced by slight rubbing.

Previous investigators have used limited methods for production of the toxin. Melish et al.[2] used dialysis sacs filled with medium 199, implanted into the peritoneum of rabbits. Other methods include the use of semi-solid agar in an atmosphere of 10% CO_2.[1] High titers of toxin are achieved by these methods, but the volume of material recovered is small. We have successfully used a liquid medium consisting of 1.7% trypticase, 1% yeast extract, 0.5% NaCl, and 0.25% K_2HPO_4 at pH 7.1. Using a stainless steel fermentor vessel, we can produce 50 L of culture supernatant containing 16 gm of crude toxin.

Cultures are routinely grown 20 hr at 37 C, then centrifuged to remove the bacteria. The supernatant is concentrated at 4 C, using an Amicon ultrafiltration system with 3 UM-10 membranes, having a molecular weight cut-off of 10,000; 10-fold concentration requires 24 hr. The concentrate is dialyzed against distilled water, lyophilized and stored for future use.

We have achieved purification using 2 chromatographic steps. The first, ion-exchange, is done on a column of CM-cellulose, equilibrated in 0.01 M phosphate buffer, pH 6.0. The lyophilized toxin is reconstituted in the same buffer, then run slowly onto the column. A small amount of unabsorbed material passes through the column, while a major peak containing the exfoliative activity is eluted with 0.05 M phosphate, pH 6.8 (Figure 1). At this stage, the material also contains α-hemolysin, another exoprotein of S. aureus. Titers of α-hemolysin were determined in microtiter plates, using equal volumes of toxin and 2% rabbit erythrocytes, incubated at 37 C for 30 min. Separation of the exfoliatin from the hemolysin has been a major problem.[3] Complete separation was achieved by hydroxylapatite column chromatography. A column 1.5 x 45 cm was equilibrated in 0.03 M phosphate buffer, pH 5.7; the toxin material from the previous step was dialyzed against the buffer, then run slowly onto the column. The exfoliatin was eluted with a gradient of increasing molarity from 0.2-0.4 M phosphate. Two separate peaks were eluted: the first major one contained the exfoliative activity, and the second smaller peak contained the hemolytic activity (Figure 2). The median ED_{50} of the pure toxin was 0.5 µg/mouse.

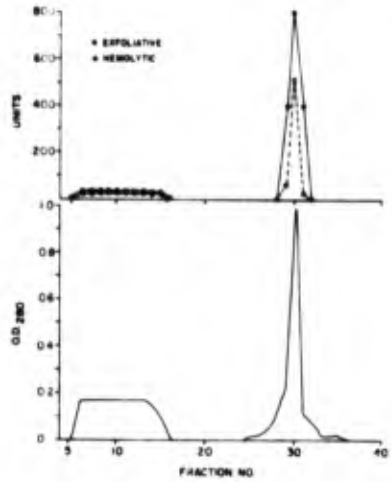

Figure 1. Ion exchange chromatographic separation of exfoliatin.

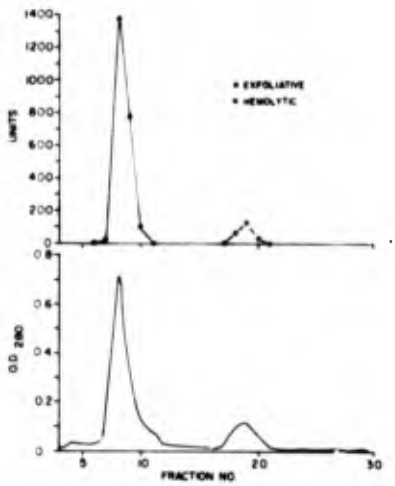

Figure 2. Chromatographic separation on hydroxylapatite.

Purification by this method gave an overall yield of 35%, stepwise results are shown in Table I.

TABLE I. EXFOLIATIVE ACTIVITY DURING PURIFICATION PROCEDURE

PURIFICATION STEP	PROTEIN mg	TOXIN mg	ACTIVITY U/mg	RECOVERY %
Lyophilized crude toxin	2000	200	200	-
Cm-cellulose	132	110	1000	55
Hydroxylapatite	68	68	2000	62
			Overall yield	34

The molecular weight of the exfoliatin was determined by electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulfate and β-mercaptoethanol. The toxin was homogeneous; by mobility in comparison with standard markers, the molecular weight was estimated to be 26,000. The pure toxin was negative for α- and β-hemolysin using rabbit and sheep erythrocytes respectively. Carbohydrate tests were performed using phenol-sulfuric acid,[4] and by gas chromatography,[5] both results were negative. We found, however, nonspecific binding to concanavalin A (con A) when large amounts of it were incubated with exfoliatin. This result is in agreement with previously reported findings of Rogolsky et al.[6] However, they erroneously stated that the exfoliatin contains 9% carbohydrate and used the con A data to substantiate this observation. In our experiments this interaction was observed between bovine serum albumin and con A as well, supporting our postulation that the binding is nonspecific.

Amino acid analysis was performed using a Technicon automated amino acid analyzer.[7] All common amino acids except half-cystine and methionine were present in the molecule. Lysine was the carboxyl terminal amino acid, but no free amino terminal residue was found.

Isoelectric focusing was performed in acrylamide gels and in a sucrose density gradient column using pH 3-10 carrier ampholines. A distinct microheterogeneity of the exfoliatin was revealed by both methods. The major component has a pI of 7.4 and the minor one, a pI of about 5.0. All components were immunologically identical, and all had approximately the same ED_{50} in mice. Studies are in progress to determine the mechanisms of producing this microheterogeneity and to investigate the instabilities of the various components.

In summary, we have developed culture conditions using liquid medium in a 50-L fermentor for producing large lots of crude staphylococcal exfoliatin. We have simplified purification procedures to two chromatographic steps: CM-cellulose and hydroxylapatite. The second step completely eliminated the contaminating hemolysin. Previously, separation of exfoliatin and hemolysin had only been accomplished by isoelectric focusing or starch-block electrophoresis, techniques which are restricted to small sample size. Column chromatography on hydroxylapatite is virtually unlimited in sample size, allowing us to produce large amounts of purified exfoliatin in a single lot. We have defined certain characteristics of the pure toxin. The molecular weight is 26,000 and it has a relatively normal amino acid composition. The median ED_{50} of the toxin is 0.5 µg/mouse. The toxin appears homogeneous by every method except isoelectric focusing. We have demonstrated microheterogeneity by this technique and are currently studying this phenomenon.

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DISCUSSION

Dr. Pappenheimer asked if this toxin is blocked by α-methyl mannoside. Mrs. Johnson replied that it has not been tried.

RESUME OF EXECUTIVE SESSION

Colonel Metzger expressed his appreciation to the Ad Hoc Study Group and AFEB members for their active participation in the review sessions and noted that no distinction was made between Ad Hoc and AFEB member assignments. The Institute needed help and suggestions from both groups. He indicated that the format of the executive session should consist of free and full discussion for each of the six questions asked. Colonel Metzger acknowledged that the members had relatively little time to consider each question, but he solicited their help, based on the mission of USAMRIID and their perception of military medical needs for medical defense against biological agents.

QUESTION 1. Concerning Bolivian hemorrhagic fever:

a) Do you consider a BHF vaccine feasible?
b) Which of three approaches (live attenuated, killed or heterologous live vaccine) might be best?
c) What additional studies may be required to better answer these questions?

One consultant favored the development of an inactivated vaccine as the most logical way to proceed. This type of vaccine was unique in that it was required to protect a small number of high-risk individuals; therefore, its development should be considered within this narrow context and not the normal context of most vaccines which must proceed through the Bureau of Biologics (FDA). The point was made that the Bureau of Biologics probably would not authorize a vaccine, even a killed one, based on the present passive antibody data, i.e., high-dose challenged monkeys developed delayed encephalitis of greater severity than seen in other groups.

Colonel Metzger pointed out that the delayed encephalitis might be unique to the monkey model and reported that at least four people have been treated with immune globulin without problems. Three of these individuals had received the globulin late in the course of the illness which should have increased the opportunity for the development of delayed encephalitis but, fortunately, this was not the case. He also indicated that to the best of his knowledge there was no clinical evidence of delayed encephalitis among the hemorrhagic fever patients in Bolivia. He described his deep concern when the recently obtained immune globulin was required to treat the USAMRIID investigator who had accidently exposed himself.

The initial point was reemphasized that this was a unique situation, and that the option of receiving a killed vaccine as opposed to no vaccine was a better one, a better benefit versus risk factor. It

was postulated that such a vaccine would not interfere with the administration of passive antibody should such a need arise. Colonel Metzger reported that even "USAMRIID limited use" vaccines and gamma globulin were processed through FDA. The recent incident with BHF, which necessitated emergency administration of globulin, occurred while the paperwork was being processed.

It was agreed that a pool of human gamma globulin should be developed for Lassa fever virus, as was the case with Machupo virus before (and if) USAMRIID instituted studies with Lassa fever.

Another consultant stated that the development of a killed vaccine appeared feasible; moreover, he indicated that if he were working with Machupo virus he would prefer to be vaccinated, even with the risk of delayed hypersensitivity. He noted with interest the ease by which Machupo virus adapts to a new host. If the virus wraps host material around itself, this represents a phenotypic change and not a genetic change and he urged that guinea pig studies be pursued vigorously to determine if they also develop hypersensitivity. It was noted that host specificity also seemed to change during virus growth in cultured cells. It was hypothesized that the facility by which the virus protects itself may be why it produces such a serious disease. Another viewpoint expressed was that Machupo virus may have a split genome with the possibility of recombinations. If so, genetic studies would be pertinent to vaccine development.

The point was made that USAMRIID would be criticized if a killed vaccine were not developed. In response to a comment that the recent patient receive follow-up electroencephalograms, Colonel Metzger assured the group that this individual was scheduled for a complete neurological work-up. He reported that three other cases were also followed up with neurologic studies.

Although Tacaribe and Tamiami viruses were considered relatively innocuous for man, it was generally agreed that there really wasn't much information available to confirm this point of view. If a vaccine program were initiated with these strains, the problems of residual live virus would still remain and it was therefore recommended that a killed vaccine for Machupo virus be developed.

Confidence was expressed in LTC Eddy's future program of investigations. Colonel Metzger explained the difficulties in performing research within a Class III system. The logistics of the system frequently dictated what could and could not be accomplished. He explained that the Institute would like to perform many more experiments, particularly sophisticated experiments, but this was not always possible. He indicated that even if an effective vaccine became available, it would not change the basic patterns of work within the Class III context. The vaccine would provide added insurance against development of the disease if an accident occurred.

SUMMARY OF QUESTION 1. The following general conclusions can be drawn: (1) There is a unique but critical requirement to develop a vaccine for BHF to protect a small number of "at-risk" laboratory workers; and once developed, the vaccine should be used based on current risk factors; (2) The primary approach to successful development is represented by a killed Machupo virus vaccine; and (3) USAMRIID should concentrate a significant portion of its total resources to the development of vaccines and the mechanisms by which they protect.

QUESTION 2. Should USAMRIID initiate attempts to develop a Lassa fever vaccine concurrently with ongoing Machupo virus studies?

Colonel Metzger introduced this question by stating that it was really an extension of Question 1. The Institute had presented USAMRDC with an integrated program document summarizing the important studies with Machupo virus and proposed studies with Lassa fever virus. He felt that these two viruses could be studied effectively at the same time. Moreover, there was the distinct possibility of a contractual arrangement for obtaining Lassa fever γ-globulin. If the USAMRIID program is approved, the contractual arrangements would be pursued; the Commander expressed the hope that specific immune plasma could be obtained in order to prepare immune globulin for emergency use before Lassa fever studies were initiated.

Although work was started with BHF before a large pool of immune globulin was available, the Institute had collected several units of plasma from immune donors. The plasma was frozen and was available in case it was needed.

A question was raised regarding USAMRIID rationale for these studies; was it because Lassa fever is unique to the military or was it due to the unique facilities? Colonel Metzger stated that Lassa fever is more of a military problem than Machupo virus, but that both studies could be justified on the basis of the special containment facilities. In addition to the Class III laboratory and animal areas, USAMRIID has excellent isolation facilities for patient care. It affords complete segregation of the patient, is well equipped for treatment and even includes operating room facilities.

The point was made that Lassa fever infection poses enormous problems, one of the most critical being, Where does one take care of sick patients? Could this capability be made available to these patients regardless of where they are from? Colonel Metzger reported that this question has been raised several times during the past three years, not only for Lassa fever but for other serious infections but that no formal request for use of the suite has been received. If USAMRIID were asked to accept this type of patient, he thought that approval would probably be given.

SUMMARY OF QUESTION 2. It was concluded that if approval is granted to USAMRIID to initiate Lassa fever vaccine development studies, specific experiments should not be undertaken until a pool of high titer antibody is acquired.

QUESTION 3. How much emphasis should be placed on the study of aerosol vaccines?

Colonel Metzger reported that we have excellent facilities and a well trained staff to perform unique aerosol studies; however, one of the problems was not letting technology interfere with the pursuit of science. He posed several questions: (a) Are we beating a "dead horse"?; (b) Is it worth adding three decimal points to existing data?; (c) Is it essential to define the exact size of the aerosol particle? and (d) Does this group feel that the aerosol vaccine approach is dead?

It was noted that aerosol vaccines were used in the late 1930's and were discontinued because of adverse reactions among people who were sensitized. Since a large part of the Institute's present Q fever program, i.e., the study of Phase I and Phase II particles, was based on the problem of sensitivity, aerosol vaccination seems to be contraindicated. The point was made that an adverse reaction in the arm is bad enough but in the pulmonary tissue, an adverse reaction could become very dangerous. Aerosol therapy might be justified but high dose levels of kanamycin were not feasible since they could become deleterious to the brain. Several members of the group objected to the aerosolization of any antigenic material.

Colonel Metzger asked if these objections would continue if the antigenic materials were purified of extraneous material. One consultant felt that this was still a problem because some people might become sensitized to the active ingredient itself. He agreed that most adverse reactions are caused by contaminants, but for the sake of the few people who respond adversely to the specific antigenic ingredient, aerosol administration of antigenic material is not warranted.

Another consultant disagreed with the viewpoint of discontinuing all aerosol vaccine studies since this was an area where much needed to be learned, i.e., the influence of influenza on the respiratory tract; also, the current push by some investigators to use temperature-sensitive mutants in which the work is confined entirely to the intranasal route. It was recommended that collaborative studies be continued with the National Institute of Allergy and Infectious Diseases. Several consultants felt that the major problem was not one of continuing or discontinuing aerosol vaccine studies but whether this Institute could study influenza at all since it is defined as a "civilian disease." Colonel Metzger explained that a limited number of studies could be undertaken as long as the work was directed toward the development of an effective model. He agreed that the influenza program had been the most

difficult to defend but as long as the Aerobiology Division building was a "clean" area, influenza could be defended on the basis of using it as a fill-in until the building modifications were completed. The building became functional for highly infectious work in July, 1975. Notwithstanding, Colonel Metzger hoped to continue to use influenza as a model because if these studies could determine where immune globulins were made after aerosol dissemination, and what classes they were, this was contributing to the general pool of knowledge that would be of direct benefit if, for example, VEE were disseminated as a BW agent. Colonel Metzger emphasized the point that it is much easier to collect this type of information using influenza than with the highly pathogenic Trinidad strain of VEE.

It was noted that preimmunization with parental vaccine seems to inhibit the immunizing capacity of small-particle aerosols of live vaccine.

Another member of the Study Group supported the view that aerosol immunization and aerosol therapy would never prove to be practical procedures adapted to routine use in the United States for a variety of reasons. He felt, however, that a level of competence should be maintained; or a level of alertness to what is going on and what might develop in the future. He felt that some serendipitous observations might change the complexion of what has been stated today. The point was made that these studies should not be discontinued even though they are technically oriented and are of short duration.

It was noted that more information was needed to determine what constitutes an infectious dose for various diseases; the literature was very sparse in this regard; some investigators believe that one virus in drinking water was bad and that this point of view was rapidly becoming dogma. No one really has information which answers the question. This consultant believed that this is the type of ancillary information which Aerobiology Division could acquire. Another member replied that the key question to be answered is how much emphasis should be given to the study of vaccines in aerosols and he felt that the answer must be "very little." The problem of aerosols created while centrifuging (Sharples) tuberculin was described.

A panel member asked if the immunology of pulmonary sensitization were clearly understood and felt that perhaps approaches could be developed that would get around this problem. Another member recalled that the aerosolization of BCG provides the best protection to monkeys when rechallenged. However, if the BCG was coupled to talcum, the monkeys developed tubercles and active infection. This member felt that it would be a shame to discontinue Institute studies as a model system, but that he was not impressed by the use of highly toxic antibiotics administered by the aerosol route to control Klebsiella. He felt that if we are worried about Klebsiella infections, and we are, particularly Klebsiella in alcoholics, diabetics and other compromised hosts, a good vaccine needed to be developed. He asked if such a vaccine would protect an intoxicated, immunosuppressed or a diabetic rat. He emphasized this was the type of study to pursue and perhaps would extend this type

of study to include Pseudomonas and perhaps Escherichia coli since these were the clinical problems the Army has. He again made the point that the Institute would not get to "first base" aerosolizing anything; that is, as far as antibiotics were concerned.

SUMMARY OF QUESTION 3. Question 3 received the greatest amount of discussion and these general conclusions can be made: (a) Aerosol vaccination studies, pursued on the basis of developing a practical procedure for use in the United States, were not warranted; (b) Aerosol therapy studies, pursued on the basis of providing antibiotic therapy, were not warranted; (c) Aerosol vaccination and aerosol treatment studies were justifiable on the basis of learning more about the immune responses and general mechanisms of action within the respiratory system; and (d) The development of appropriate aerosol-animal-disease models should be continued because they generate needed information concerning the respiratory tract.

QUESTION 4. Based on current concepts, is it worthwhile to place any emphasis on developing live rickettsial vaccines? Is it likely that any could ever be approved for use in man?

Colonel Metzger asked the group for their comments regarding the development of live rickettsial vaccines.

A member discussed the long-term adverse effect on the host following natural rickettsial infections. With the possible exception of a few Q fever and epidemic typhus cases, there were no documented instances of long-term adverse reactions following these diseases. He described a 20-year follow-up study of scrub typhus infections among soldiers in World War II. There were no long-term sequelae involving cardiovascular, hepatic, immunologic or psychologic functions. He felt that although some of the rickettsiae remain viable for long periods of time, this should not discourage the development of live rickettsial vaccines.

Colonel Metzger indicated that long-term viability of certain rickettsiae was of concern to him and cited the persistence of the M-44 vaccine strain of Coxiella burnetii in the guinea pig model some 18 months following vaccination. He felt that if attenuated strains were developed for the spotted fever group of rickettsiae, an artificial disease with late sequelae might be developed. The panel member could not deny this possibility but considered it quite remote. He stated that it was generally accepted that persistence of rickettsial antibody following natural infection was due to the persistence of the organism. He discussed the relationship between immunosuppressed patients and rickettsial diseases and concluded that although not much has been documented, it did not appear to be a critical clinical problem.

Another consultant pointed out that immunosuppressed patients received antibiotic therapy which also aborted (cured) the rickettsial group of diseases. He questioned the importance of the problem since the Q fever vaccine tested by USAMRIID was excellent. The initial vaccines contained significant amounts of extraneous material but this should no longer constitute a real problem. Colonel Metzger asked if 30% sterile abscesses did not constitute a problem. The consultant recalled that he and Smadel had published a paper on the intradermal skin test which was used to screen for hypersensitive individuals. If a person were hypersensitive, the vaccine was not administered. A consultant reported that he was at Fort Detrick when fairly large numbers of people were receiving Q fever vaccine. He recalled having seen two or perhaps three positive responses to the skin test. These people did not receive the vaccine.

It was reported that sterile abscesses could be related to the number of doses administered and that primary immunity may persist for much longer periods of time than is generally believed. If true, this would reduce the frequency of revaccination. This point of view was reinforced when the history of epidemic typhus was reviewed. At one time, people were immunized every 6 months; however one dose of vaccine was shown to provide a good serological response 16 years later. Q fever vaccine might be analogous to the epidemic typhus situation. Dr. Beisel reported that only a few people required immunization now, as a reflection of the limited work being done with Q fever. The Institute's policy was not to reimmunize anyone with a history of sensitization or a positive skin test.

SUMMARY OF QUESTION 4. The discussion of Question 4 permits the following conclusions to be made: (a) The employment of the skin test to screen for hypersensitive individuals has reduced the number of sterile abscesses associated with Q fever vaccine; (b) USAMRIID, because of its professional staff and facilities, was uniquely qualified to develop rickettsial vaccines; and (c) Live rickettsial vaccines would not currently receive the highest priority; however, this might change as a result of new information being generated by this Institute. It was recalled that the Q fever vaccine was so good that volunteers exposed to virulent C. burnetii, then vaccinated, were protected before the virulent challenge could cause illness. The vaccine developed for Rickettsia rickettsii, while good, could be improved and refined further. The question was asked why the Institute did not undertake to make a more effective vaccine for epidemic typhus since no one else was working on this problem. It was believed that live rickettsial vaccines would not enjoy the highest priority. This consultant then described how four U. S. congressmen became infected with South African tick bite fever. He felt that with its "model systems" and "personnel," USAMRIID was ideally suited to study the immune relationships among the rickettsiae. Whether live or killed vaccines should be developed was something that could be decided as studies progressed.

QUESTION 5. Should the work of any division be redirected to better serve our mission?

Colonel Metzger asked the panel members if they had any suggestions or recommendations regarding this question since they had visited the various suites and had talked briefly with division chiefs and their staffs.

A panel member responded with two observations: (1) routine data processing seemed to be handled quite efficiently but felt that there may be deficiencies with unusually sophisticated statistical analyses. He wondered if it might not be beneficial to USAMRIID to have an expert statistician as an active member of this review panel. (2) He reported that he was fascinated with the vaccine development programs that this Institute had undertaken and felt that mutual benefit would accrue if Institute investigators and epidemiologists from the Army Medical Department were to talk more. The investigators would be exposed to field problems and would obtain a better concept of large-scale immunization problems.

Another consultant raised two questions: (1) Some USAMRIID immunological studies appeared to be disjointed and lacked continuity; if true, was this caused by an individual investigator bringing a certain competence to a particular area, then losing the competence when the investigator left? (2) During the past year technology has been developed to begin to answer certain basic questions concerning suppressor T cells. For years, vaccine investigations were based on the premise that the biggest antigenic mass that did not create sterile abscesses was the way to proceed. He felt that if he interpreted recent data correctly, this may be the wrong approach because it turned on suppressor cells differentially. He sensed that the immunological programs of USAMRIID were not concerned with these central concepts, but rather with the immunology of specifics, i.e., cells, virus, etc. Should there be a new immunobiology division?

Colonel Metzger replied that the name, Bacteriology Division has been a misnomer for several years. He explained that although this division performed limited numbers of studies with bacteria, most studies were concerned with immunology. He described the emphasis being given to the immunology of vaccines. The vaccines produced by Merrell National Laboratory (MNL), using the USAMRIID cookbook, rarely performed in people as well as those vaccines produced at the Institute. He stated that we were unable to define the problem for MNL and that obviously we frequently measured the wrong immunologic parameter. All tests available to USAMRIID were centered on this vaccine problem in an effort to provide more quantitation and more meaningful criteria. He felt that although we may be overdoing the number of immunological tests, this was the right approach to correct this serious problem.

In answering part two of the question, Colonel Metzger explained that one prime source of immunological competence had been the Berry Plan physicians and they were no longer available. The consultant replied that he was impressed with the young toxin researchers and asked if several civilian immunologists could be hired. Colonel Metzger replied that the problem was not in finding good, young scientists since it was a buyer's market, but in money to pay salaries.

Another panel member asked for more information concerning the relationship between USAMRIID and Merrell-National Laboratories. Colonel Metzger replied that it was an exclusive and rather unique contract in that the Government paid for the building, currently pays for all salaries and equipment purchases. The contract is negotiated for periods of five years, the value is currently frozen at approximately $1,400,000 per year. Declining USAMRDC monies and the contractor wanting a larger budget would create a serious situation in two years when the present contract expires. Colonel Metzger felt that we needed this production capability, i.e., that MNL was really an extension of this Institute. The contractor provided volume of whatever product was requested, and produced the product by following Institute protocols.

A panel member asked Colonel Metzger to discuss "uniformed medical personnel" and specifically asked if Berry Plan physicians were eligible for incentive pay. Colonel Metzger replied that they received $350 professional pay per month and became eligible for the bonus the final year of the three-year tour. Colonel Metzger reported that USAMRIID was competing for physicians who were being shuttled to staff various Army hospitals as a first priority military need. He thought that the physician shortage might have bottomed out since two physicians have been recruited for USAMRIID next year. Colonel Metzger stated that a physician shortage had never really existed per se; there was an abundance of psychiatrists, pathologists and anesthesiologists. Filling existing research vacancies with these people would not be worth the time, effort and training to make them good researchers. The panel member commented on the need to improve the mechanism for obtaining good young researchers, not only here at USAMRIID but for the Public Health Service and also for the Bureau of Biologics. He believed the most successful recruitment was achieved by person-to-person contact. Colonel Metzger indicated that USAMRIID also recruited by this technique. He, Dr. Beisel and Dr. Spero participated in various NIH study sessions which were links to academia. He emphasized, however, that he was not able to successfully recruit civilian physicians, even on a part-time basis for ward duty. Another panelist said that we should advertise more, inform young people of what is being done here. He stated that he had had no concept of the extent of the research performed at USAMRIID, although he should have known because he is in a position to know. He felt that it is very important to talk to young people and assure them that if they joined the staff, they would be able to finish the tour of duty without being moved. Colonel Metzger replied that the tour of duty was not a problem.

Another consultant raised the point about bridging the gap between research and the clinical situation. He asked about carrying the BHF model to Bolivia and into the clinical area. Colonel Metzger replied that the clinical area of USAMRIID research constitutes a real problem. This mission is assigned to WRAIR; USAMRIID is not in the chain of clinical investigations. He reported that this creates a morale problem, particularly when USAMRIID has a clinically oriented physician. He thought that this problem might be improved with the possible realignment of WRAIR and USAMRIID missions. Dr. Beisel felt that the new military medical school, because of its close location to USAMRIID, should provide significant clinical opportunities for the USAMRIID staff. Dr. Woodward confirmed that this was an excellent potential source and one which should be followed.

SUMMARY OF QUESTION 5. The nature of question 5 elicited the discussion of many different subjects which defies a succinct summary. Notwithstanding, the following conclusions may be the most pertinent: (1) The apparent physician shortage was caused by the types and not numbers of physicians in the Army; (2) The shortage of research physicians may be over; (3) An "expert" statistician, if added to the panel, might provide some good suggestions; and (4) USAMRIID needed more clinical interaction which might be achieved with the soon-to-be completed military medical school.

QUESTION 6. In what direction should future volunteer studies proceed other than for testing vaccine safety and immunological responsiveness?

Colonel Metzger reported that some of the roadblocks for recruiting volunteers had been removed. The Institute currently has 5 and by next year should have 25 to 50. He noted that USAMRIID, historically, had employed its volunteers two ways: (a) as investigational controls; and (b) for the administration of bacterial or viral agents in which there was no significant risk to the volunteer. In the latter group, pathogenesis, immunological changes, metabolic changes and vaccine efficacy were studied. He asked the panel members if this approach could be improved. One panelist replied that this Institute should limit itself to vaccine development and all aspects of its testing, i.e., does the vaccine work and what is its immunology? He felt that within this framework, metabolic studies could be conducted but as ancillary experiments. The question was raised if this approach excluded human challenge because in order to define vaccine efficacy, the infectious dose must be known. The panel member replied that it did not exclude human challenge.

The same panel member discussed the need for collaboration and felt that medical people had been to rigid in the past. He viewed the ward facilities of this Institute as being an excellent place

to study cholera in volunteers. He stated that cholera was not completely a military or civilian or public health problem but a common problem for all. He felt that the mission of USAMRIID was flexible enough to study cholera since it was a toxigenic disease. He reported that E. coli toxins were most important to the military and that much of what was known of these toxins had been derived from the basic pool of cholera information. This constituted additional justification for USAMRIID to utilize its excellent facilities to study cholera.

SUMMARY TO QUESTION 6. The discussion of question 6 permits the following conclusions to be made: (1) The number of USAMRIID volunteers should increase during the next 6-12 months; (2) The isolation ward of USAMRIID was suggested as being ideal for cholera studies; and (3) The point was stressed again that USAMRIID should concentrate its studies on vaccine development and all phases of testing.

Colonel Metzger thanked the AFEB and Ad Hoc members for their active participation during the two days of review. He expressed the hope of bringing in individual members during the year to address specific problems as they arose if travel monies became available. Dr. Woodward, on behalf of all the members, thanked Colonel Metzger for providing an interesting and informative program. He announced that the Eighth Smadel Lecture would be held at the USAMRIID Planning Session next year.

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