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1976 USAMRIID PLANNING SESSION WITH THE AD HOC STUDY GROUP FOR --ETC(U)
FEB 77 W R BEISEL, P B JAHRLING, N H LEVITT

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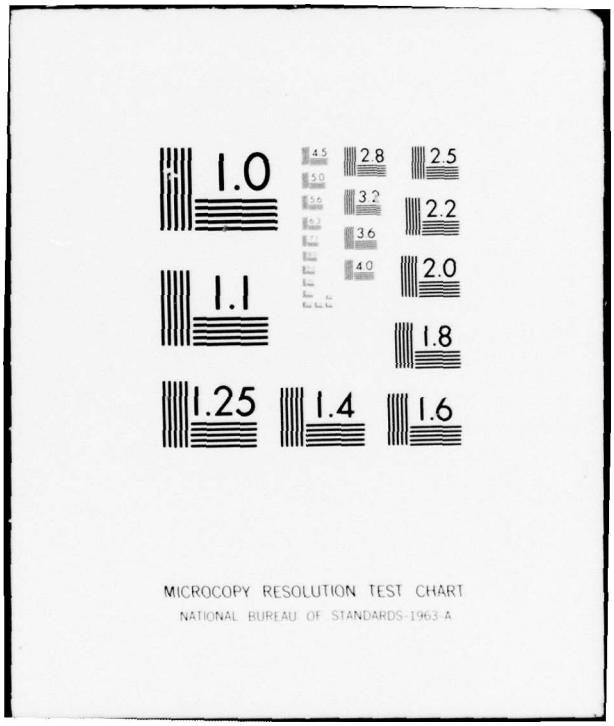
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1976 USAMRIID PLANNING SESSION

WITH THE

AD HOC STUDY GROUP

FOR

SPECIAL INFECTIOUS DISEASE PROBLEMS

18-19 NOVEMBER 1976

USA MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

FORT DETRICK

FREDERICK, MARYLAND 21701

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U. S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES

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Arboviruses	Leukocytic endogenous mediator	Venezuelan equine encephalitis
Early diagnosis	Phagocytosis	
Electron microscopy	Prostaglandins	
Influenza	Prophylaxis	

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.

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TABLE OF COMMENTS

Ad Hoc Study Group

Abram S. Benenson, M.D.*

Bennett L. Elisberg, M.D.

Ralph D. Feigin, M.D.

Karl M. Johnson, 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

Floyd W. Denny, M.D.

William S. Jordan, M.D.

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

Guests

COL Richard F. Barquist, MC

COL LeeRoy G. Jones, MC

COL Phillip K. Russell, MC

COL Phillip E. Winter, MC

MAJ Douglas Stutz, MSC

*Absent

COMMITTEE ON ARRANGEMENTS

Theodore E. Woodward Joseph F. Metzger William R. Beisel

The Eighth
Joseph E. Smadel
Memorial Lecture

CONFERENCE ROOM

UNITED STATES ARMY MEDICAL
RESEARCH INSTITUTE OF
INFECTIOUS DISEASES

Thursday, 18 November 1976 at 1800 hours

Fort Detrick Frederick, Maryland

The Committee on Arrangements cordially invites your attendance at the Smadel Memorial Lecture. This lecture is in honor of Joseph E. Smadel, M.D. and recognizes his distinguished career. It is the eighth of a series to be given by outstanding scientists from the United States and abroad.

Friends and colleagues of Dr. Smadel, by their contributions, made this memorial to him possible as a fitting tribute following his death on 23 July 1963.

FORMER LECTURES

1965 DR. JAMES H. S. GEAR
Walter Reed Army Institute of Research,
Washington

1967 DR. THOMAS FRANCIS, JR.
University of Maryland at Baltimore

1968 DR. THEODORE E. WOODWARD
South African Institute for Medical
Research, Johannesburg

1970 DR. MARCEL BALTAZARD
DR. PIERRE LEPINE
L'Institut Pasteur, Paris

1971 DR. COLIN M. MAC LEOD
University of Pennsylvania Hospital,
Philadelphia

1974 DR. DAVID BODIAN
The Rockefeller University,
New York

1975 DR. DOROTHY M. HORSTMANN
Yale University School of Medicine,
New Haven

DR. CHARLES H. RAMMELKAMP, JR.

Professor and Director, Department of Medicine,
Cleveland Metropolitan General Hospital,
Case Western Reserve University

will lecture on the subject of

EPIDEMIOLOGICAL STUDIES ON TRANSMISSION OF STREPTOCOCCAL AND STAPHYLOCOCCAL INFECTIONS

During and after World War II considerable emphasis was placed on the role of airborne bacteria in the transmission of respiratory and wound infections. Indeed, mysophobia became a characteristic feature of nurses, epidemiologists and surgeons. As a result prevention of these infections was attempted by the elimination or control of bacteria in the environment and in the air.

Since these procedures resulted in minimal or no reduction in disease, the Commission on Streptococcal Diseases of the Armed Forces Epidemiological Board undertook a series of studies in the military designed to dissect out the various pathways of transmission of streptococcal infections. Because of the success of this approach, Dr. Joseph E. Smadel urged that similar studies on transmission of staphylococcal wound infections be instituted. As a result, the Commission undertook such studies, but the major model employed now was the newborn infant whose umbilicus served as a wound which frequently becomes infected. These investigations were made possible because of the ability to identify precisely strains of these two bacteria and thereby to follow their pathways of transmission. The results have important implications in relation to the control of these infections in both military and civilian populations.

THE CURRENT STATUS OF USAMRIID AND ITS RESEARCH ACTIVITIES

William R. Beisel, MD
Scientific Advisor

It is a pleasure to welcome you again this year to our Institute and to initiate the business portion of this two-day meeting. We are continuing to try to improve these USAMRIID meetings to take full advantage of your consultative visits, and accordingly we have made some modifications in this year's format and agenda. Also, in addition to our regular sessions, we have a special highlight for the meeting as you know, with Dr. Rammelkamp's presentation this evening of The Eighth Joseph E. Smadel Memorial Lecture. USAMRIID is honored to host this unique event as part of this year's Ad Hoc Committee meeting.

The initial portion of our agenda has been planned to review for the entire consultant group broad aspects of current Institute functions and performance. Presentations this morning will illustrate several USAMRIID research approaches in various areas of interest. Speakers this afternoon will present a brief but comprehensive review of our progress during the last several months after USAMRIID was called upon by the National Institutes of Health to assist with studies related to swine influenza. We were asked primarily to employ our unique primate model using squirrel monkeys to determine the clinical response to the newly isolated swine virus, to determine through challenge experiments the efficacy of the vaccines being produced commercially, and to determine if antiviral chemotherapy had any prophylactic and therapeutic usefulness. USAMRIID was also asked to collaborate in the testing of the new swine vaccines for reactogenicity and immunogenicity in volunteers. Our work in this area has now been virtually completed and we will bring you up to date on details of our findings.

In the latter half of the afternoon, our research Division Chiefs will each present a brief synopsis of their on-going work. Although I reviewed this type of material for you in previous years, we believe that it would be more useful for the entire committee to hear presentations directly from the Division Chiefs themselves, since many of them are new to you.

Tomorrow morning we will ask each individual consultant to meet with the Chief of a single division and with individual researchers. Then, in the afternoon Executive Session, we will concentrate on the questions we have listed for you.

Now let me turn to a brief general review of research priorities and progress during the past year. Perhaps the only thing that has not changed has been the formal statement of the USAMRIID mission:

> 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 diseases of peculiar military importance, and on microorganisms the study of which requires special containment facilities.

Although the mission has remained constant, our approach to the performance must continually be reevaluated, refined, and updated in terms of establishing revised priorities in response to our awareness of the BW threat, the guidelines we receive from higher Headquarters, and Congress, and constraints with respect to funds and personnel.

At least 15 different microorganisms or their toxins have appeared on lists of "Potential BW Threats" published in the open literature. Syndicated columnists for local newspapers about the country also write that Soviet research constitutes a growing threat, that the microbiological warfare studies being carried out by the Russians include diseases which attack humans and combinations which are capable of inducing several infectious diseases at the same time.

As you recall from last year's meeting, constraints with regard to areas for research study now limit USAMRIID to problems of unique military importance. We must focus on infectious diseases which are of unique military importance, the inference being that the infections must be those which either occur rarely in the U.S. civilian population but frequently in the military or those that could result from the use of BW operations by an enemy force. As has already been mentioned, the House Armed Services Committee has indicated by the specific deletion of research funds that they do not consider a disease such as Rocky Mountain spotted fever to be of unique military importance. Their clear decision in this matter raises important questions concerning some of our major conceptual approaches.

We have believed that our work could proceed most successfully whenever we initiated studies in a new group of microorganisms by selecting a major, well-characterized representative of the group for initial detailed study. We tried to employ an organism that could be studied in relative safety and utilized as a model by which to develop laboratory techniques and methodologies that would ultimately allow us to investigate with greater ease other members of a group that were more difficult to study. We will share these problems of selecting the most appropriate organisms and establishing priorities with you and ask for ideas and guidance in our series of questions to be discussed during the Executive Session.

Fig. 1 depicts the year-by-year trends in the key population of USAMRIID staff members, our investigators. As shown clearly, we have experienced a decline in their numbers in the last several years. Losses were composed almost entirely of military personnel. The greatest decimation of our research staff came, as predicted, among the Medical Corps officers. This decline in physician strength was anticipated some years ago and has hit us severely. As projected by the dotted lines, we anticipate that our total professional strength will remain relatively steady during the present fiscal year.

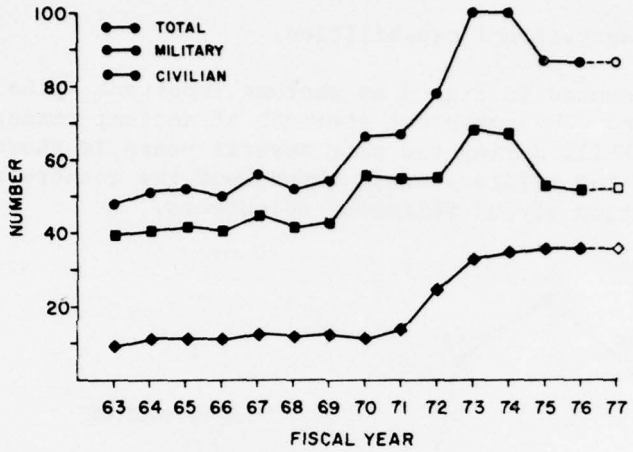

Fig. 1. Professional investigator population of USAMRIID by fiscal year.

Hopefully, the military programs initiated some years ago to fund medical school costs for potential military physicians should begin to pay off in the next several years. We anticipate that this will make available increased numbers of military physicians who wish to pursue a research career.

Despite the decline in investigator strength during the past several years, our production of published manuscripts has not fallen off (Fig. 2). Hopefully this progression will continue. At the present time we are

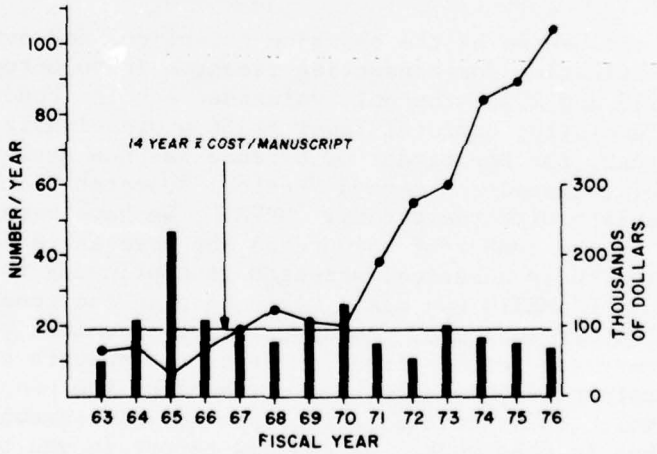

Fig. 2. USAMRIID senior author manuscripts by fiscal year.

averaging slightly more than one manuscript published each year for each individual staff member who can be classified as a primary investigator. This does not mean that each investigator has published one manuscript, although that is our goal. USAMRIID's major scientific output is the production of manuscripts and new vaccines. Since the total dollar amount in our budget has remained relatively fixed during the past several years, and since continuing inflation has reduced the buying power of those dollars, we believe that the upward trend in manuscript production does indicate a more efficient

use of our facilities and operational capabilities.

We view the topic presented in Fig. 3 as another important "plus" in terms of Institute function. The numerical strength of medical research volunteers assigned to USAMRIID during the past several years is shown. As you know, the cessation of the military draft throughout the country resulted in a virtually total depletion of our Whitecoat volunteers.

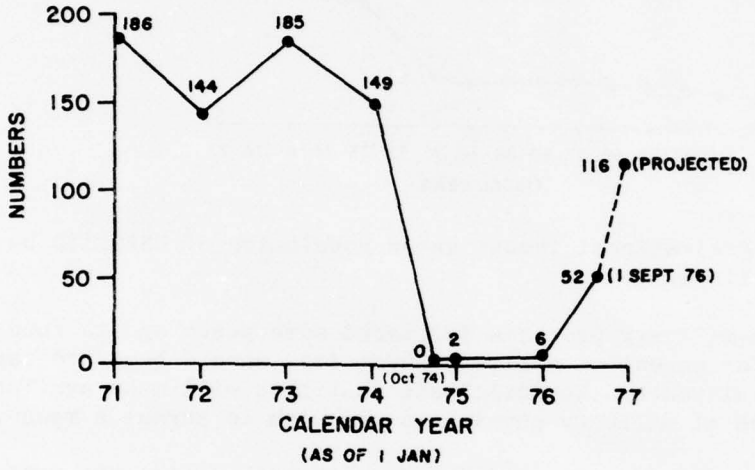

Fig. 3. Assigned strength of Medical Research Volunteer Subjects (MRVS).

The program was also influenced by the changing guidelines and new requirements for formal certification for conducting research in volunteers. During the calendar years 1975 and 1976, the only volunteer studies conducted at the Institute were those employing Doctoral level staff professionals. As described to you last year, the Department of Defense has now authorized a new assignment category of enlisted men termed "Medical Research Volunteer Subjects" which we abbreviate with the acronym "MRVS." We have been both surprised and gratified by the number of volunteers who have asked for a MRVS assignment to USAMRIID; their numerical strength is continuing to increase. This upswing means that USAMRIID can again begin to plan and conduct appropriate studies in volunteer subjects. As described to you last year, the administrative machinery was reestablished in order to evaluate any proposed research volunteer protocol and to have them reviewed by the proper committees to obtain approval. This administrative machinery is somewhat complex and time consuming, but it does work. We can now report to you that studies using the MRVS have been approved and completed during the past year. In addition, the new Army regulations make it possible to conduct studies in other groups of volunteers.

In this regard we can report the highly successful completion of a multi-institute volunteer study conducted here, at NIH, and at Walter Reed Army Institute of Research, in which several new influenza vaccines were tested in various combinations in large numbers of volunteers. This group, included active military duty personnel, civilian employees, and family members. We are pleased then, that our future research plans can once more include the important studies that can be conducted only in volunteer subjects.

Finally, let me turn to the series of questions that require your attention. Because of the very useful discussion period during last year's Executive Session, we wish again to pose a series of questions for your comments, advice and recommendations. As you can see, these involve both specific and conceptual questions in several areas. We are asking for specific advice concerning the development or testing of several vaccines. Other questions deal more closely with your perception of the reality of potential biological warfare threats. We are seeking advice and recommendations concerning both the direction of future USAMRIID research efforts as well as the priorities under which they should be conducted. For many of the questions, your responses must be based largely on judgement and experience factors, rather than on aspects of our on-going program that you can ask about during the meeting. Nevertheless, we wish to pose the questions to you at this time so that you can begin to think about them and consider them during the interval between now and the Executive Session. With that, let me close my formal presentation and throw the floor open for questions and discussions about these matters.

RELATION OF VIRION SURFACE CHARGES TO VIRULENCE OF ARBOVIRUSES

Peter B. Jahrling, PhD

Recently, we have established a new and useful marker to differentiate between lethal and benign strains of alphaviruses. This marker depends on the elution characteristics of the viruses on hydroxylapatite chromatography columns. We have applied this technique to examine populations of virus for virulence heterogeneity and to select candidate vaccine strains of virus from virulent virus stocks; conversely we can test vaccine virus stocks for the presence of virulent contaminants.

Our use of this technique evolved as a natural extension of some comparative pathogenesis studies. I would like to review these early studies with you briefly, since they will establish the rationale for initiating the chromatography studies.

Our long-term goal has been to define a structural determinant for virulence of group A, or alphaviruses. Alphaviruses, which include Semliki Forest and Eastern, Western, and Venezuelan encephalitis viruses (SF, EEE, WEE, VEE) have relatively simple biochemical structures. Yet within virus groups, strains exist which differ markedly with respect to virulence. In some cases, low virulence can be correlated with temperature sensitivity, small plaque size, inefficient replication, or with enhanced induction or sensitivity to interferon or antibody. However, these are not always reliable markers. For example, the vaccine strain of VEE, strain TC-83, cannot be differentiated from the lethal Trinidad strain using any of these markers. Yet TC-83 vaccine virus must be different from Trinidad virus; we have just not been looking at the right markers.

As an approach to defining new virulence markers, we initiated studies to compare the pathogenesis of lethal and benign virus strains for various laboratory animals. One objective was to identify a critical virus-to-cell interaction. We have concluded that in general the interaction of alphaviruses with hepatic macrophages is a critical determinant of virulence. In turn, viruses which adsorb efficiently to Kupffer cells also adsorb avidly to hydroxylapatite. Thus, the chromatographic behavior of alphaviruses may be useful as a marker functionally related to virulence.

For most of our comparative pathogenesis studies, we have used the adult golden Syrian hamster as the model animal. Table I lists some of the alphavirus strains studied, according to hamster lethality. The lethal strains kill essentially all adult hamsters inoculated subcutaneously with a low dose. The benign strains, when inoculated peripherally, kill practically no hamsters.

TABLE I. ALPHAVIRUS STRAINS STUDIED

LETHAL	BENIGN
VEE	
Trinidad (LP)	Trinidad (SP)
69Z1 (SP)	TC-83 vaccine (LP)
68U201 (LP)	Pixuna (LP)
WEE	
72V1880 (LP)	72V1880 (LP)
72V4768 (LP)	72V4768 (SP)
SFV	
LIO (LP)	A774 (LP)

Our initial approach was to describe the pathogenesis, for hamsters, of each of these virus strains, in the hope that we could make some generalization about the basis for differences in virulence. From these initial studies, we did reach one general conclusion. The benign alphaviruses all lacked the ability to produce high level viremia. In many cases however, the benign strain was fully capable of replicating in primary target tissues.

Fig. 1 compares viremias for the small and large plaque clones of VEE on their growth in the spleen. It is apparent that the benign SP clone

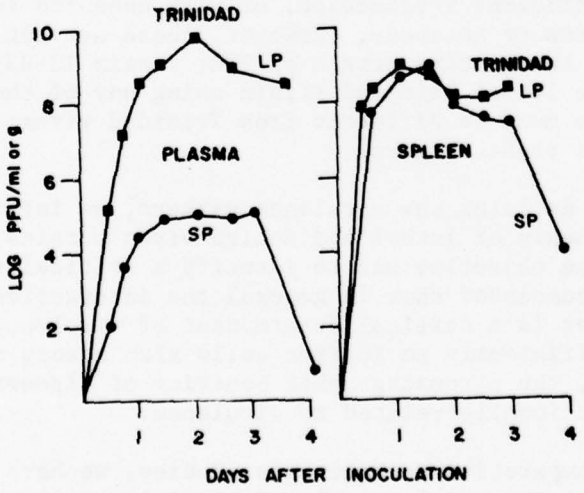

Fig. 1. Plasma viremia levels and virus growth in spleen for large and small plaque clones of Trindad strain VEE (1).

produced a significantly lower viremia than the lethal LP clone, 4-5 logs vs. 9-10 logs. This is no real surprise. What is a surprise is that the replication of the benign SP in target tissues, such as the spleen, is just as exuberant as the virulent strain.

How does one explain the low level viremia in the face of efficient replication in target tissues? One possibility is that benign virus is either inactivated or efficiently removed from the blood, while lethal strains are not cleared as efficiently. This is the hypothesis that we tested. It seems to be true. The mechanism for clearing benign but not lethal viruses from the blood involves preferential adsorption of benign strains to certain cells, including cells of the heptic RE system. The example of Trinidad LP and SP viruses will be used to support this conclusion. When a mixture of LP and SP plaque VEE virus is inoculated, via the intracardiac route into hamsters, SP is preferentially cleared. We have directly compared the rates at which SP and LP Trinidad VEE virus are cleared from the blood of hamsters.

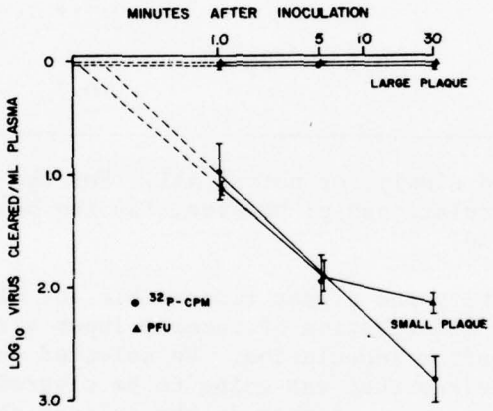

Fig. 2. Clearance of ^{32}P-labeled SP and LP VEE virus from blood of hamsters (n = 10).

To do this experiment, we inoculated 9 logs of ^{32}P-labeled virus directly into the hearts of anesthetized hamsters, and bled them from the orbital sinus at 1, 5, and 30 min after inoculation. We assayed the virus remaining in the blood in two ways, by counting plaque forming units (PFU) and by measuring the ^{32}P intrinsically associated with the viruses. We plotted logs of virus cleared vs. time after inoculation. The difference is dramatic. The LP virus was not detectably cleared within 30 min of inoculation. In contrast, the SP virus was rapidly cleared. Five minutes after inoculation >2 logs, or> 99%, of the input virus had been cleared. It is also important to note that the ^{32}P and the infectivity clearance curves are essentially identical. I interpret this to mean that virus was physically removed from the blood, and not simply inactivated.

Table II shows the efficiency of viral clearance for all of the viruses in the study. For simplicity we are looking only at the logs, or %, of virus cleared 10 min after inoculation. For all of the benign WEE and VEE strains, >2 logs, or 99% of the inoculum were cleared within 10 min. In contrast,

TABLE II. EFFICIENCY OF VIRAL CLEARANCE 10 MIN AFTER INOCULATION

VIRUS	STRAIN	LETHAL		BENIGN	
		Log_{10}PFU	%	Log_{10}PFU	%
WEE	LP	0.0	0		
	SP			2.4	99
VEE	LP	0.1	20		
	SP			2.2	99
	69Z1	0.4	60		
	TC-83			2.2	99
	68U201	0.3	50		
	Pixuna			2.3	99
SFV	LIO	0.1	20		
	A774			0.7	80

the lethal strains were cleared slowly, or not at all. For the Semliki strains we detected a similar relationship; however, for the benign strain, A774, clearance was not as rapid.

Our next goal was to identify the organs responsible for clearing virus. We did this by measuring the % distribution of labeled input virus in tissues of inoculated hamsters 30 min after inoculation. We selected this time, since by 30 min essentially all the virus that was going to be cleared had been. Presumably at 30 min, cleared virus was either in the eclipse phase of replication, or was being degraded; either way its distribution could not be determined by infectivity titration.

It can be seen in Table III that the liver contained 47.6% of the input SP virus. In contrast, all the LP virus can be accounted for in the plasma. We have similar data for all of the other viruses tested, and the pattern is the same. A significant proportion, usually better than half of the input benign virus can be found in the liver. From these data, it seemed that the liver was an appropriate place to attempt to localize SP virus by electron microscopy.

When Kupffer cells were examined one could see cytoplasmic vacuoles containing virions. By 15 min we were unable to detect virus, which we believe means that virus was degraded. We used the same procedure to hunt for LP virus in the liver; but failed to see it. Even when we inoculated 10 times as much LP virus it could not be visualized. Large plaque VEE virus apparently did not interact with these cells. We have identical data for WEE large and small plaque viruses. Western SP virus is easily visualized in the liver, while LP virus is not. Likewise, TC-83 vaccine virus can be found in the liver, while lethal VEE strain 69Z1 cannot.

TABLE III. DISTRIBUTION OF ^{32}P-LABELED SP AND LP VEE ENCEPHALITIS VIRUS IN ORGANS AND PLASMA OF HAMSTERS 30 MIN AFTER INTRACARDIAC INOCULATION (from ref. 1)

	SMALL-PLAQUE		LARGE-PLAQUE	
Site	Mean cpm/ organ	% of inoculum ± SE	Mean cpm/ organ	% of inoculum ± SE
Plasma	4,800	0.71 ± 0.06	126,580	99.2 ± 0.04
Liver	308,520	47.6 ± 2.19	25,310	19.8 ± 0.05
Kidneys	25,689	4.0 ± 0.21	11,140	8.7 ± 0.43
Spleen	7,160	1.1 ± 0.23	3,790	3.0 ± 0.54
Lungs	2,880	0.4 ± 0.05	7,590	5.9 ± 0.68
Brain	23	0.1 ± 0.01	1,210	0.9 ± 0.31

Thus we have a plausible explanation for why the benign viruses in general failed to produce a high level viremia: they were rapidly cleared from the circulation, in part by interaction with Kupffer cells. Also, we now have identified a virus-to-cell interaction which deserves to be studied in cell culture. The benign strains of alphaviruses, those which are cleared rapidly from the circulation, also adsorb rapidly and efficiently to Kupffer cells in culture. We would like to understand more about deficienices in adsorption of viruses to these cells. This adsorption depends at least in part on receptor interactions which in turn depend on charge. Therefore, it seemed reasonable to test the hypothesis that alphaviruses which differ with respect to virulence, also differ with respect to charge. One convenient way to detect differences in surface charge is to compare the elution profiles of these viruses on hydroxylapatite columns.

Adsorption and elution of viruses to hydroxylapatite, depend on charge, which in turn depends on two principal factors, pH and the ionic strength of the elution buffer. In most of our experiments, we held the pH constant, and determined the elution profiles for each virus using a linear gradient of increasing phosphate molarity. Usually, the gradient began at 0.05 and ran to 0.5 M. Fig. 3 illustrates the effect of pH on the elution profiles of 2 WEE viruses, the LP and SP clones. At pH 7, the virulent, LP virus is eluted immediatedly by low molarity phosphate buffer; that is it did not adsorb at all.

In contrast, the small plaque virus adsorbs to the column more avidly, requiring 0.25 M phosphate before it is eluted. At lower pH values, both viruses adsorb to the column more avidly. At pH 6.5 LP virus was retained until fraction 7 or 8, corresponding to a phosphate molarity of 0.18; SP virus was not completely eluted even by 0.5 M buffer. At pH 6.0, neither virus eluted from the column. In the future, it may prove useful to compare elution profiles of various viruses at different pH values, or to run pH

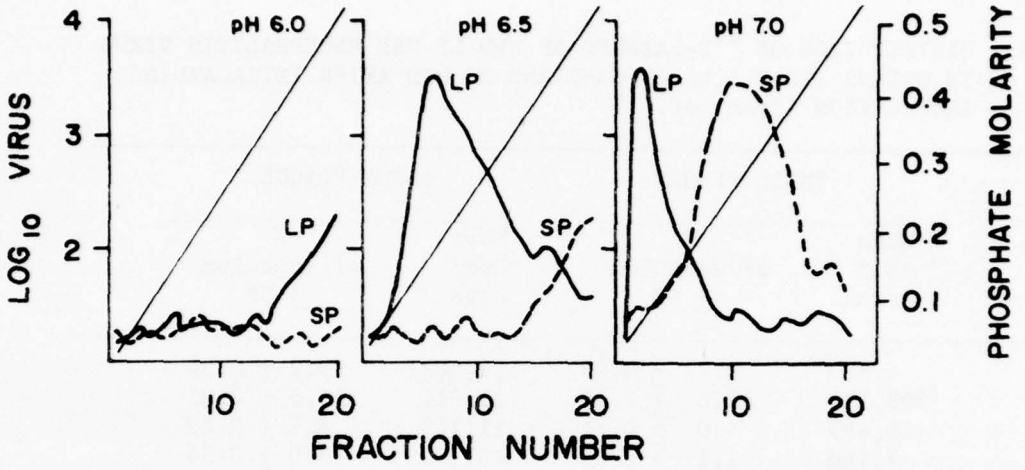

Fig. 3. Elution of Western LP and SP viruses on hydroxylapatite.

gradients at constant phosphate molarities. So far, however, we have looked only at elution profiles for viruses run at pH 7.0. When the elution peaks of two viruses are separated from each other by as many fractions as they are for Western LP vs. SP at pH 7, then the column becomes a useful tool for detecting heterogeneity among virus populations. Very low levels of large plaque contaminating a small plaque virus population can be detected.

Fig. 4 illustrates the elution profiles for 4 VEE viruses; the 2 benign strains, Trinidad SP and Pixuna, elute late, compared to the two lethal strains, Trinidad LP and 69Z1. Elution does not depend on plaque morphology. While in general small plaque viruses elute late, strain 69Z1 forms very small plaques but elutes early, relative to Pixuna, which forms large plaques. That is, elution profiles correlate with adsorption and clearance in the intact hamster, and not necessarily with plaque size.

The phosphate molarities of the elution peaks for the alphaviruses studied are shown in Table III. The general pattern is obvious. In all cases the lethal strains were eluted earlier than their benign counterparts. The benign viruses, which adsorb avidly to hydroxylapatite, are the same as those which adsorb avidly to hepatic Kupffer cells. In short there was a very close association between low virulence, low viremia, efficient clearance, efficient adsorption to cells in culture, and avid adsorption to the column. This association in turn suggests that a critical determinant of virulence is surface charge.

Therefore some of our future efforts will be directed at determining the structural bases for differences in surface charge. We are now setting up isoelectric focusing experiments to determine the isolectric points of intact virus particles and of isolated envelop proteins. I am optimistic that we will detect differences in isolectric points which will correlate with the chromatographic data and with the in vivo clearance data. If so, we will then be very

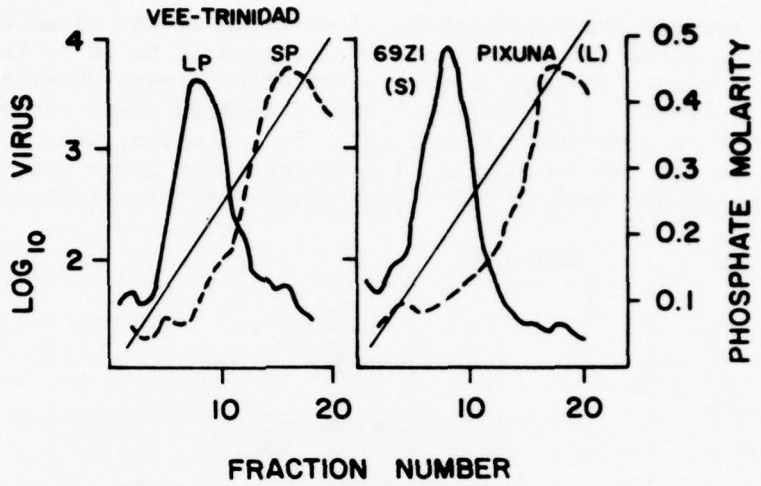

Fig. 4. Elution profiles of Trinidad SP and LP clones and Pixuna and 69Z1 strains.

TABLE IV. PHOSPHATE MOLARITY OF ELUTION PEAKS

VIRUS	STRAIN	MOLARITY	
		Lethal	Benign
WEE	LP	0.050	
	SP		0.251
VEE	LP	0.205	
	SP		0.400
	69Z1	0.200	
	TC-83		0.245
	68U201	0.050	
	Pixuna		0.415
SFU	LIO	0.326	
	A774		0.426

close to defining at least one structural determinant for virulence of alphaviruses. We will then also have an additional marker for identifying alphavirus strains of low virulence.

I would now like to conclude by briefly listing three recent and important applications of the hydroxylapatite column to the characterization of heterogeneous viral populations. Approximately a year ago, the CDC reported the isolation of what they were then calling an aberrant strain of WEE. What intrigued me was that when this strain was passed in low dilution in mice its

virulence increased rapidly and predictably. I wondered if the virus were really a mixture, so I asked Dr. Monath, at CDC, to send it to me so that I could apply it to a column. He sent me a representative strain CM4-146, and a virulent strain (72V4768) for comparison. When I applied these viruses to the column, the 2 elution profiles differed (Fig. 5). However, when I titrated the early fractions in the CM4-146 column, I detected a few large plaques, very similar in both elution profile and plaque morphology, to virulent WEE.

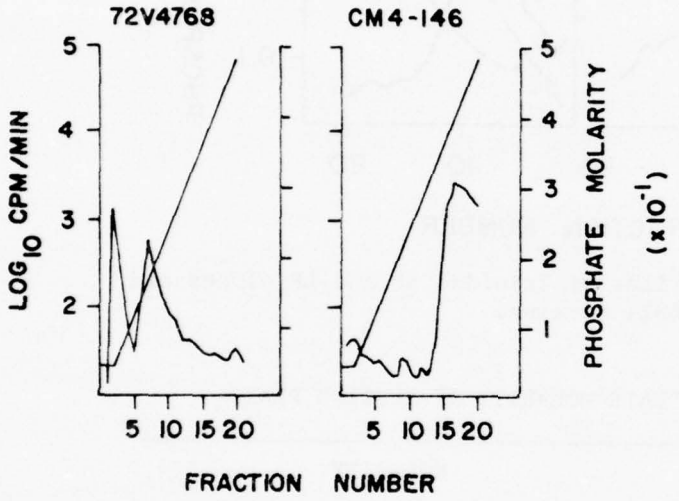

Fig. 5. Elution profiles of two strains of WEE.

When a dilution of the original virus suspension titrated on duck cells was plaqued it produced uniformly SP. However, the early fractions off the column contained the LP, unmistakably different from the predominant virus population. The reason that we don't see LP in the original population is that they are present in very low proportion. But the most important part of this story is that the LP virus has turned out to be serologically, most closely related to VEE virus, not Western. It thus represents the first isolation of VEE virus in the northwestern United States; it is the first isolate from which a blood sucking parasite that feeds on birds, Oeciacus; it raises a number of important epidemiological questions concerning VEE in the United States. We would never have seen this virus without the selective efficiency of the column.

The column is also useful in selecting for attenuated virus from a predominantly virulent virus population. We have recently isolated an attenuated strain from Trinidad strain VEE virus. This suggests that it might have been possible to derive an attenuated vaccine strain of VEE, not only by serial passage, as of course was done for TC-83, but more directly, in one step, by column chromatography. It also suggests that other candidate vaccine strains of alphaviruses might also be isolated using this technique.

Finally, we have used the column to characterize virus isolated from throat washings of laboratory personnel who became sick following immunization with TC-83 vaccine. Nine of 23 people, immunized with TC-83 vaccine, became ill; we isolated virus from the nasopharynx of 8 of these 9. In contrast, we failed to isolate virus from any of the 12 people who did not become sick. The viruses isolated were of intermediate virulence for adult hamsters, and the elution profiles on the column for 7 of the 8 isolates were clearly different from TC-83 vaccine. There seems to be a clear correlation between the development of a vaccine reaction and the isolation of a more virulent virus, for which we now have a marker. We are presently attempting to optimize the conditions for isolating this more virulent virus subpopulation, from the original TC-83 inoculum virus suspension. I suspect that the "revertant" virus is there to start with, and that we will isolate it using the column technique.

In short then, we are now a bit closer to understanding the biochemical basis for differences in virulence among alphaviruses. We have developed a useful marker which is functionally related to virulence and we now have a powerful tool for examining the heterogeneity of viral populations and for selecting candidate strains of vaccine virus.

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DISCUSSION

1. Considerable interest developed around why the benign strain of VEE is benign. Hamsters inoculated with virulent VEE virus do not die from encephalitis; rather, they die an acute death, the most striking feature being a necrotic intestional lymphoid lesion. WEE is better in the hamster model to study than VEE since the hamster dies with CNS lesions and the target tissue is well-defined. Benign strains of WEE when inoculated into the hamster cause encephalitis and death. This is not true of VEE; there is considerable doubt as to what the true target tissue is for this virus in various species. The theory is that benign strains of VEE are benign because they cannot get to the target tissue.

VEE infection in hamsters produces primarily, a plasma viremia supported by virus growth in lymphoid tissue, i.e., spleen, Peyer's patches, and bone marrow. Although the right experiment has not been designed yet, it was thought that the virus crossed the blood brain barrier by a passive mode, i.e., if a threshold concentration of virus in the blood is exceeded, then the virus gets into the brain. It was recalled that based on Dr. Claude Bradish's work with virulent and nonvirulent strains of Semliki Forest virus, the benign strain remained benign even when injected into the CNS target tissue. It was concluded that other factors were involved in the phenomenon of virulence.

2. It was suggested that hydroxylapatite column technology be used to study influenza virus in reference to 3 things: (a) charge differences occurring among human strains isolated from naturally-occurring infections; (b) strains responsible for the unusual syndrome in young children, in whom the liver may become infected with different influenza viruses, predominantly influenza B, and may lead to death; and (c) why human strains of influenza virus lose virulence for man when they become adapted to mice.

3. A question was raised as to whether the finding of a VEE isolate in a supposed strain of WEE virus was real; for example, could it merely be due to a laboratory mix-up. It does not appear to be a laboratory error; the Communicable Disease Center also confirmed this finding. The suggestion was made that mixing small plaques of WEE virus with large plaques of VEE virus would provide the viral characteristics observed in this study. Dr. Jahrling reported that there is a functional relationship between these 2 viruses; however, a simple mix-up was not an adequate explanation. Of 11 strains studied, 8 of them contained VEE. This frequency is too great to be a coincidence.

4. Virus adsorption and attachment were discussed in terms of loss of virulence. If the cell is permissive to the virus, it would enhance viral replication. The benign virus that adsorps avidly to a cell, e.g., a macrophage, would have the reverse effect. It was hypothesized that serial passage of virus in cell culture usually results in attenuation because the benign virus has a selective advantage. It adsorbs more avidly to any cell than its more virulent counterparts.

The question was asked if protein could be extracted from the virus that might block adsorption on the hydroxylapatite column. The answer was that it had not been tried, but would be of interest.

VIRUS REPLICATION IN PERIPHERAL HUMAN LEUKOCYTES

Neil H. Levitt, PhD

Since 1964, over 6,000 persons have been given the TC-83 VEE vaccine (1). Its use has been limited to persons at risk of infection because of their occupation or to those participating in clinical studies. Clinical reactions resulting from infection with the vaccine virus occur in approximately 25 - 30% of persons inoculated, and range from mild transient responses to those that may be of sufficient magnitude to cause the vaccinee to seek bed rest. Virus has been isolated 8 - 12 days postvaccination from the throats of individuals showing illness. The duration of symptoms varies, but recovery is rapid and sequelae have not been observed. No substantiated explanation for the varied clinical responses in man has been offered. In our laboratory, we are concerned with the role of the RE system during VEE vaccine virus infection since this system has been shown to be important in many generalized virus infections. The studies to be presented here are primarily concerned with one component of the RES, the peripheral blood leukocytes, and their subsequent interaction with VEE vaccine virus during the course of human infection. It is important that these cells in particular be examined because of their intrinsic role in humoral and cellular immunity.

Initial studies concerned with the measurement of in vitro propagation rates of VEE vaccine virus (TC-83) revealed that the virus did indeed replicate in mixed leukocyte cultures. Furthermore, when the individual leukocyte subpopulations were purified and tested, the virus replicated to the highest titer in the macrophage culture, as can be seen in fig. 1. The data show that macrophages support the growth of TC-83 virus in vitro to high titers, while there is little or no growth in lymphocyte or PMN cultures.

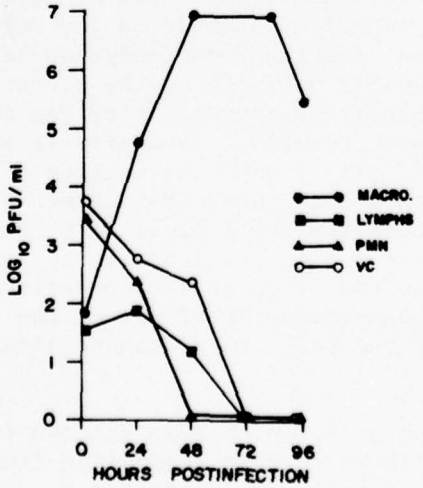

Fig. 1. Growth of TC-83 virus in peripheral blood leukocytes

Using a tube dilution infectious cell assay, it was determined that 1% of the lymphocytes become infected at an inoculum of 1 virus particle/cell,

while 30 - 50% of the macrophages became infected.

It was of interest to determine if the mononuclear cells (macrophages + lymphocytes) taken from vaccinated individuals would interact with vaccine virus in the same manner as those from nonvaccinated persons. Table I shows the comparative results of TC-83 virus growth in cells from nonimmune (NI) and immune (I) individuals. Four multiplicities of inoculum were tested. Growth in NI and I cells was comparable except at the lowest input multiplicity, 1:10,000 cells, where no growth was seen in NI cells.

TABLE I. EFFECT OF INOCULUM RATIO ON VIRUS GROWTH

CELLS	INOCULUM: CELLS	VIRUS GROWTH (LOG_{10} PFU/ml) BY DAYS		
		1	2	3
NI	100:1	6	7	6
I	100:1	7	6	5
NI	1:1	6	6	6
I	1:1	7	6	5
NI	1:100	4	7	7
I	1:100	5	7	5
NI	1:10,000	None	3	4
I	1:10,000	4	7	6

Results from an infectious cell assay showed that > 10-fold more immune cells become infected than nonimmune when infected with a low virus inoculum. These findings suggest that there is a possible difference between the cell surfaces of immune and nonimmune macrophages. This change seemed to impart to the cell a greater affinity for the virus. Several investigators have demonstrated the presence of cytophilic antibody on the surface of PMN and macrophages (2,3). The function of surface antibody has yet to be determined. The presence of TC-83 virus specific antibody on the surface of immune macrophages may provide these cells their greater affinity for the virus. With this in mind, an experiment was performed to determine if antibody or immune globulin could be absorbed artificially onto the surface of nonimmune cells and subsequently alter the virus growth rate. Nonimmune cells were incubated in pre- and post-TC-83 virus antiserum for 1 hr at 37°C, then infected with a virus inoculum shown previously not to replicate in nonimmune cells. The results shown in fig. 2 indicate that when nonimmune cells are pretreated with pre- or heat-inactivated post-immune TC-83 serum, the cells do not support virus growth. In contrast the cells do propagate virus after incubation in post-TC-83 serum.

These data show that immune globulin or some unknown factor in immune serum can convert nonimmune cells to a state resembling immune cells. Carrying this approach one step farther, we attempted to determine how macrophages would handle virus-antibody complexes used as inocula. Results from a representative experiment are seen in Table II.

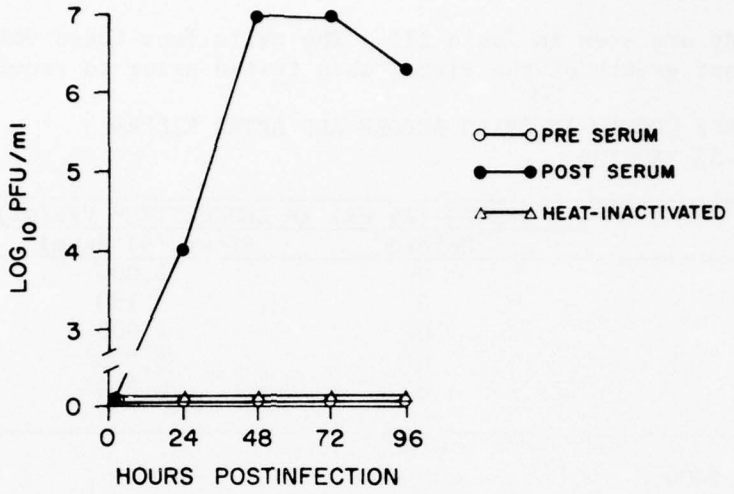

Fig. 2. Growth of virus in nonimmune peripheral blood leukocytes after incubation in pre- and post-TC-83 antiserum

TABLE II. PROPAGATION OF TC-83 VIRUS BY HUMAN LEUKOCYTES INFECTED WITH VIRUS-ANTIBODY COMPLEXES

INOCULUM	VIRUS GROWTH, PFU/ml LEUKOCYTES BY DAYS		
	1	2	3
Virus	0	0	0
Virus + ab[a]	3.5×10^3	3×10^7	1.1×10^7
Virus-ab complex[b]	1×10^4	1×10^7	1×10^6
Virus-ab complex[b] (heat-inactivated)	0	0	0

[a] Antibody.
[b] No free virus.

It can be seen from these results that free virus (500 PFU) did not grow in the nonimmune cells. However, when immune serum (ab) was added at the same time or as virus-immune complexes, virus growth was noted. Heat-inactivated serum neutralized the virus and formed-complexes, which were not taken up and propagated by the cells.

These results indicate that macrophages are able to take up antibody-neutralized virus and propagate the virus. In addition it appears that a heat-labile factor, possibly complement, is required for binding of the complex to the cell. We are presently examining this rather interesting phenomenon.

The next experiments utilized individuals in a volunteer project testing the efficacy of a killed TC-83 virus vaccine. To see if cell conversion occurs in vivo, mononuclear cells from 5 donors nonimmune to TC-83 virus were tested for virus growth before and after receiving killed vaccine.

Results of this study are seen in Table III. The cells from these volunteers, did not support growth of the virus, when tested prior to receiving

TABLE III. VIRUS GROWTH IN CELLS BEFORE AND AFTER KILLED TC-83 VACCINE

VOLUNTEER	GROWTH (24 HR) IN LEUKOCYTES[a] PFU/ml)	
	Before	After (37 days)
1	0	3,000
2	0	150
3	0	4,000
4	0	3,000
5	0	20,000

[a]Inoculum:cells = 1:2000.

the killed TC-83 virus vaccine. When the cells were tested 37 days after vaccination, the cells were capable of propagating virus. The sera from all 5 donors also possessed high neutralizing antibody titers to TC-83 virus. It appears that the macrophage surface changed in vivo sometime after exposure to TC-83 antigen.

In summary, we have demonstrated:

1. That the VEE vaccine virus (TC-83) replicates in vivo to high titers in human peripheral macrophages while in lymphocytes there is poor or no replication. Virus multiplication within circulating human monocytes has been demonstrated previously with certainty for 3 other viruses: vesicular stomatitis (4), 17-D yellow fever (5), and measles (6). Poliovirus can replicate in human peritoneal macrophages (7); whether circulating monocytes have a similar capability has not been reported.

2. Cells from TC-83-virus vaccinated donors show greater virus attachment and virus replication rates than cells from nonvaccinated donors.

3. Virus neutralized with antibody can still be absorbed to, and replicated in, macrophages.

The greater virus attachment rate of immune cells may be due in part to the presence of cytophilic antibody or specific immune globulin to the virus on their surface. The apparent in vitro anomaly of immune cells attaching and supporting growth of the virus more efficiently than nonimmune cells is puzzling; however, it is not unique. Halstead and co-workers have recently shown that dengue virus replicates in both human and monkey leukocytes from immune donors but not in nonimmune cells, which are refractive (8,9,10,11).

At this moment we are unsure of the true significance of this seemingly paradoxical phenomenon of immune cells (or macrophages plus antibody) supporting virus growth better than naive cells. However, we think it possible that this mechanism may play a role in biphasic virus disease. The mild illness which occurs in a fraction of vaccinees following inoculation of the attenuated VEE vaccine (TC-83) does not occur during the viremic

phase, days 2-5; rather, it occurs 8-12 days after vaccination. It is characterized by virus shedding in the throat but little or no viremia. It is possible that the phenomenon we have just described may aid in providing a whole new substrate for virus growth, the lymphoreticular organs, when augmented by a developing, but still low level, antibody response.

This mechanism may play a role not only in the mild illness following attenuated VEE vaccination but perhaps in the biphasic clinical response which characterizes many viral illnesses. The developing immunity may in fact contribute to the second phase of fever and disease.

The in vitro environment in which our experiments were conducted is obviously not the same as for the in vivo situation. Although vaccine virus attaches more efficiently to immune macrophages than to nonimmune macrophages, the resulting growth and release of the virus in the test tube, in fact, may not occur to a detrimental extent in vivo. That is, the TC-83 vaccine gives effective protection against VEE infection. Perhaps, in vivo the virus affinity for the immune macrophage is an immune mechanism which expedites the clearance of the virus from the circulation and the body. This phenomenon may be simply an in vitro artifact. The data we presented, involved complement only during the virus attachment phase. We have preliminary data which suggest that the continuous presence of complement in our macrophage cell culture medium during initial virus growth within the cell may result in cell lysis, provided sufficient cytophilic antibody is present on the macrophage.

At this point in our investigations, we do not know if an immune macrophage can be equated with protection. If this is the case, we may have an additional diagnostic tool for determining VEE immunity. Furthermore, the detection of immune macrophages could be used to determine protection in those individuals who never appear to convert using the conventional serological tests.

We feel our experiments are just beginning to give us a glimpse not only into the pathogenesis of VEE disease in man, but into the extremely complex picture of mans' immunological response to this virus.

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DISCUSSION

1. It was noted that 2 pathways were described by Dr. Levitt: one was concerned with replication of virus; the second was concerned with degradation of the virus by lysozymes. The question was asked if the macrophage could be manipulated or altered physiologically to change the pathway. This particular area had not been investigated. The closest study that would answer the question was concerned with the attachment of WEE and EEE virus to macrophages of immune and nonimmune individuals; however, these 2 viruses do not grow in culture to titers achieved by VEE virus.

2. Questions were asked regarding the percentage of the macrophages that were infected, i.e., what number were replicating VEE virus when examined by immunofluorescence and what was the fate of noninfected macrophages? About 30-50% of these cells were infected using a 1:1 multiplicity of infection (MOI). Although the fate of the noninfected macrophage had not been examined, it was Dr. Levitt's educated guess that these cells become secondarily infected, because virus titers remain exceptionally high for several days. It was suggested that both the noninfected and infected populations be examined by electron microscopy and by immunofluorescence. Another questioner asked if circulating macrophages became infected in other animals. The only other species studied was the monkey; TC-83 virus would not grow in cells from either immune or nonimmune monkeys at a low MOI. Attempts have been made in the monkey to duplicate results obtained in humans; however, the experiments have not been successful thus far.

3. It was suggested that in order to simulate the in vitro study, an animal without basic immunity should be administered antibody alone to determine if the in vivo response duplicated the in vitro response. This approach would be useful, because one component of the immune response could be studied at a time without other complicating factors. An experiment is planned in which immune antibody would be adsorbed to the macrophage in vitro then injected back into the same animal. The animal would then be challenged

to determine if the cytophylic-antibody-coated macrophage would protect the animal in the absence of circulating humoral antibody. It was felt, however, that administering antibody to a completely naive animal would be the best approach. This type of experiment could lead to the explanation for the presence or absence of passive immunity.

In this regard, Dr. Jahrling described an experiment in which he had passively immunized hamsters, then challenged them with the virulent strain of VEE. The passively-immunized hamster survived; on this basis it could be concluded that the hamster did not become sensitized, and did not die when exposed to the virulent virus.

4. A description was requested for an immune macrophage. It is one that has changed in response to an immunogen as opposed to one that has adsorbed cytophylic or opsonizing antibody. The macrophage from an immune individual attaches and grows the virus better than one from a nonimmune individual. Attempts were made to define the type of antibody, γG or γM, on the surface of the macrophage. These efforts were unsuccessful, because antiglobulins that were purchased were preserved in sodium azide. Current efforts must be devoted to removing the azide and trying to define the type of antibody.

5. Based on his current hypothesis, one consultant interpreted the work as follows: the type of response observed is due to humoral mechanisms, i.e., antibody that attaches is either cytophylic in the absence of antigen or promotes attachment of the antigen as an antigen-antibody-complement complex. Dr. Levitt reviewed his concept as follows: the immune macrophage has either cytophylic antibody or some immune globulin on its surface which aids in the attachment and removal of VEE virus from the circulation. Preliminary data indicate that if all the components are present, i.e., antigen, antibody, and complement, no virus growth is observed. If complement is not present, the macrophages propagate virus. Hopefully, future experiments will show that although an individual has no circulating antibody he may still be protected against VEE virus. Previously, one individual was described who had no demonstrable neutralizing VEE antibody. When tested in this system, his macrophages behaved as immune macrophages and grew VEE virus to titer of $\simeq$ 8 logs.

6. Another participant noted that there were striking similarities with dengue virus, but there were also some interesting differences. With dengue virus, the enhancement of macrophage infection requires either heterologous nonneutralizing antibody or neutralizing antibody which is highly diluted beyond the neutralization point. Moreover, dengue virus does not require complement. The question was raised as to whether different populations of macrophages were involved. For example, in the dengue system, macrophages that appear to be infected are low in number. They seem to be the weakly adherent fraction and become even less adherent following infection. The question was asked if one could differentiate the subpopulations that become infected in the VEE system . Dr. Levitt indicated that the strongly adherent macrophage grows the virus to high titer, while the weakly adherent macrophage grows the virus only to low titer. This difference may be due to a smaller population of weakly adherent macrophages.

SYSTEMIC METABOLIC ALTERATIONS ASSOCIATED WITH PHAGOCYTOSIS: AN INTEGRATED SYSTEM OF NONSPECIFIC HOST DEFENSE RESPONSES

Michael C. Powanda, PhD

In the event of overt or covert BW in this era of genetic recombination studies when microorganisms may be developed to specific actions, many antibiotics may become less effective, if not totally ineffective. The chore of preparing and distributing the appropriate vaccines under these circumstances will become immensely more difficult. The lack of satisfactory antiviral agents will be more deeply felt. Defense against, or more realistically, treatment of, infections arising out of BW may have to rely heavily on supplemental, supportive therapy. In order to provide this therapy we will have to understand the pathogenesis of each disease and be aware of the component parts of the host's own defense system so as not to compromise it by our intervention.

I will try to show that many of the metabolic alterations which occur subsequent to phagocytosis and prior to the development of immunity are a vital, integral part of the host's defense systems. I will put forth evidence that a number of these metabolic alterations stem from a common event, the stimulation of phagocytic cells, with the consequent release of endogenous mediators. I will show data that certain of these metabolic alterations appear to have value not only as indicators of exposure to infection, but also as indices both of the severity and prognosis of the disease. Finally I will put forth a concept which outlines the potential interactions between various metabolic alterations and the classic aspects of host defense against infection, i.e., phagocytosis and immunity.

The idea that metabolic alterations during the acute phase of an infection might play a role in host defense arose out of an attempt to explain the apparent differences between prolonged fasting or starvation and the anorectic state which accompanies severe infection, as well as a belief in teleology. Although fasting and infection-induced anorexia are alike in that there is a marked reduction in nutrient intake in both, they differ qualitatively and quantitatively in regard to metabolism. First, an infected patient continues to catabolize protein and amino acids (1-3) in excess of that which can be accounted for by anorexia (3,4). Second, infected animals and septic patients have significantly lower plasma ketone bodies (5,6). Third, septic patients and animals have a seemingly inappropriate elevation of plasma insulin levels (5-8), instead of the decreased levels as expected during fasting (9). Fourth, there are profound differences in the pattern of amino acid metabolism between the 2 states (10). Fifth, there is markedly increased synthesis of the so-called plasma acute-phase globulins during infection which is absent in fasting (11). Sixth, there is the synthesis of proteins associated with the development of humoral and cell-mediated immunity peculiar to specific infections (12,13).

To explain these differences I propose that the host, during the acute phase of an infection, is redirecting his metabolism to provide nitrogen for use in

various aspects of host defense, in particular for the synthesis of plasma acute-phase globulins. The modus operandi by which the pattern of nitrogen metabolism in a healthy person is transformed into that of a septic individual appears to be by the direct and/or indirect action of mediators derived from granulocytes and fixed RE cells, as a consequence of phagocytic cell activation. In the case of bacterial illnesses the stimulus may be provided by phagocytosis of the invading microorganisms and/or of tissue damaged either by the microorganisms, or by phagocytes themselves during their attempt to destroy the microorganisms. Evidence for a relationship between phagocytosis and the onset of a wide range of metabolic alterations, which I have chosen to term induced metabolic sequence (IMS), arose out of our studies of tularemia (14,15), one of the likely candidates for use as a BW agent.

Fig. 1 shows some of the metabolic alterations which occur in a rat following the IP injection of 10^4 live vaccine strain (LVS) Francisella tularensis in relationship to the bacterial concentration in liver and the development of liver lesions. The presence of bacteria does not itself trigger the onset

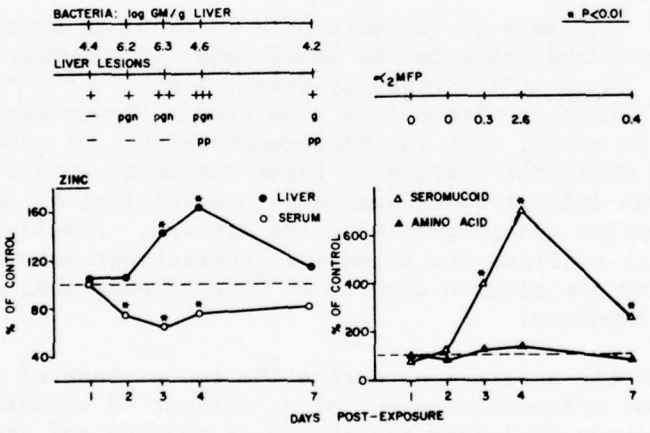

Figure 1. Response of unvaccinated rats to 10^4 live F. tularensis vaccine strain (15).

of metabolic sequelae and this may be related to the fact that F. tularensis is not readily phagocytized in the absence of immune sera. Rather IMS await the development of pyogranulomatous (PGN) liver lesions. Moreover, it is only when these lesions become moderately severe (day 3) that there is an influx of zinc into the liver and an outpouring of acute-phase globulins from the liver (signified by the increase in seromucoid). Plasma lysozyme activity (not shown) increases significantly on days 3 and 4, indicative of enhanced phagocytic activity and/or phagocyte destruction. Note that in this nonlethal illness there is little evidence of increased amino acid movement into liver and plasma α_2-macrofetoprotein (α_2-MFP) levels are barely above background.

Fig. 2 demonstrates some of the metabolic alterations which occur in rats injected IP with 10^4 SCHU S4 strain F. tularensis. Again, the onset of IMS awaits the development of pyogranulomatous lesions. But note that in this fatal illness there is a marked increase in amino acid uptake into liver and high levels of α_2-MFP.

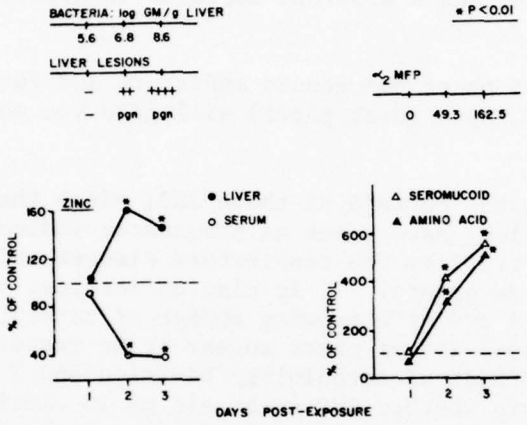

Fig. 2. Response of unvaccinated rats to 10^4 SCHU S4 F. tularensis (from ref. 15).

Evidence that endogenous mediator-like substances are indeed active during tularemia in the rat is presented in Fig. 3. Donor rats were inoculated

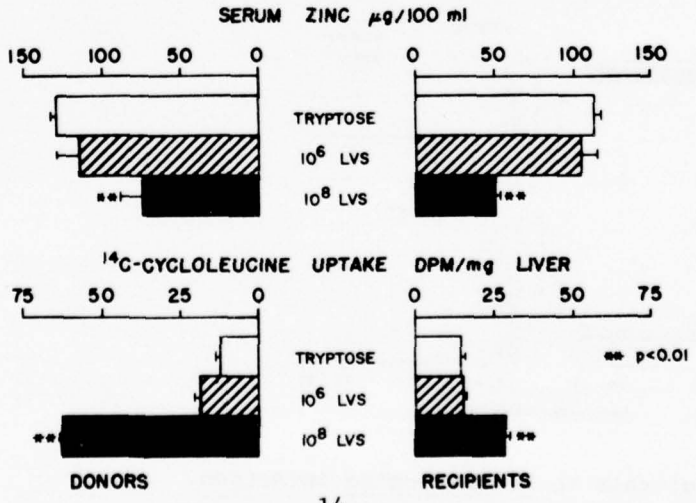

Fig. 3. Serum zinc and [^{14}C]cycloleucine uptake in donor and recipient rats.

IP with tryptose broth or 10^6 or 10^8 LVS F. tularensis; 24 hr later the donors were bled and streptomycin sulfate was added to the blood; the plasma was collected and filtered. Sterile plasma (2 ml) was then injected into recipient rats which were killed 5 hr later. Plasma zinc concentration and amino acid

uptake into liver were measured in both donors and recipients. Tryptose broth alone or 10^6 LVS bacteria caused no significant changes in donor rats at 24 hr, nor did the plasma from these donor animals induce changes in normal recipient rats. In contrast, 10^8 LVS induced a significant depression in serum zinc concentration and a 5-fold increase in the hepatic amino acid uptake in donor rats. Plasma from these rats, when injected IP into normal recipient rats, elicited a similar depression in serum zinc and a 2.5-fold increase in hepatic amino acid uptake.

In regard to the nature of these endogenous mediators and factors which may regulate their metabolism, Dr. Mapes (next paper) will give you some of our current thoughts (16).

Now I will show evidence that certain of these IMS, since they stem from a prime host-parasite interaction, have value as prognostic indicators. Klebsiella pneumoniae in rats typifies the respiratory disease which may arise from aerosol dissemination of BW agents. It is also of interest because it is a disease which is likely to affect a growing number of patients hospitalized with severe wounds, and some 25% of cases appear to be resistant to antibiotics. Recently, Dr. Berendt of Aerobiology Division and I began a series of studies to ascertain whether IMS might aid us in elucidating the pathogenesis of this disease and whether selected sequelae might have prognostic value (17). We found upon serial sacrifice that certain parameters exhibited a threshold in regard to the severity of the illness, as judged by bacterial concentrations in the lung. We postulated that this threshold was related to the onset of lung damage. We then set up an experiment wherein, after infection, we repeatedly bled rats from the orbital sinuses. Fig. 4 shows our findings.

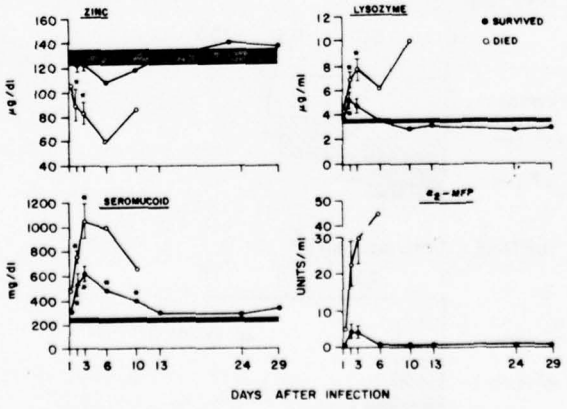

Fig. 4. Response of rats to K. pneumoniae infection.

The data were grouped according to whether the rats died or survived following infection. As can be seen rats which ultimately died from the infection had lower plasma zinc levels and higher plasma seromucoid, lysozyme

and α_2-MFP levels than both survivors and controls. Plasma lyxozyme activity and α_2-MFP concentration proved to be the most sensitive prognostic indicators. We are presently carrying out studies to see whether they will also function as prognostic indicators in antibiotic-treated animals.

My most recent studies have concerned the relationship between the host-parasite interaction and the panoply of metabolic alterations which occur during the acute phase of infection. In order to dissect out this interrelationship, a system was devised in which polymorphonuclear leukocytes (PMN) were induced to migrate to the peritoneal cavity by an injection of glycogen and then heat-killed staphylococcus either aureus or epidermidis, was repeatedly injected. Rats were killed after single or multiple injections of killed organisms (18). A comparison of the systemic metabolic alterations which occur in a host during infection with those produced by leukocytic derived factors [LEM (EP)] or which appear to be associated with phagocytosis is summarized in Table I.

TABLE I. ALTERATIONS IN METABOLISM ASSOCIATED WITH INFECTION, LEM(EP), PHAGOCYTOSIS IN NATURALLY ACQUIRED OR EXPERIMENTALLY INDUCED INFECTIONS OF MAN AND ANIMALS.

METABOLIC ALTERATION	INFECTION	LEM(EP)	PHAGOCYTOSIS	REF.
Fever	+	+	±	
Plasma Zn concentration	+	+	+	11,19-22
Altered plasma aminograms, PHE/TYR	+	+	+	9,19,23-26
Amino acid flux to liver	+	+	+	14,15,26
Muscle protein synthesis	+	+	NM	27-29
Plasma albumin	+	+	+	11
Plasma Cu/ceruloplasmin	+	+	+	11,19,20,30
Acute-phase globulins	+	+	+	11
Haptoglobin	+	+	+	11
Plasma glucagon	+	+	+	7,8
Plasma insulin	+	+	+	5,7,8

INCREASED; + = OBSERVED, DECREASED NM = NOT MEASURED

Leukocyte derived fators, commonly called LEM, have been able to induce in healthy animals, metabolic alterations similar in magnitude to those observed during infection (31-37). These metabolic alterations also arise in response to phagocytosis (14,18,38).

These data do not prove that phagocytosis is the trigger for this panoply of systemic metabolic sequelae, since perturbation of the phagocyte surface membrane may be sufficient to initiate mediator release. For example, release of pyrogen and LEM from phagocytes can be triggered by either endotoxin or poly(I)·poly(C) (39,40). These data are, however, presumptive evidence that the profound alterations in host metabolism stem from a host defence

response, i.e., are intrinsic to the host, and are not produced by the invading microorganism but rather, occur in an attempt to combat the invading microorganism.

The postulated interactions which may exist between various alterations in host metabolism during the acute phase of illness and the other more classic aspects of host defense, phagocytosis and immunity, are summarized in fig. 5.

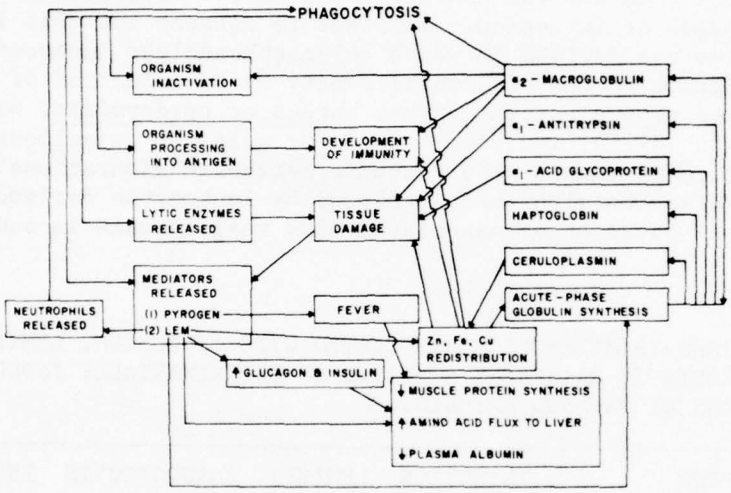

Fig. 5. Diagrammatic scheme of interactions of metabolic alterations with phagocytosis and immunity.

The key triggering reaction is phagocytic cell activation. During a bacterial infection this is typically initiated by phagocytosis of the microorganism or of tissue damaged by the microorganism, by granulocytes, macrophages and/or localized RE cells. This often but not always, results in activation of the microorganism (41-44); the processing of the microorganisms into a suitable antigen with the subsequent development of immunity (45); the release of lytic enzymes which may lead to tissue damage (46) and the release of mediators such as pyrogen and LEM. Pyrogen produces fever which may function to enhance the catabolism of peripheral nitrogen stores. LEM has many possible functions: it increases plasma glucagon (37), which may in part be responsible for the enhanced amino acid uptake by liver; it also elicits a paradoxical increase in plasma insulin (37). LEM alters the plasma concentration of Zn, Fe and Cu (31,32) which may aid phagocytosis (47), the development of immunity (48-52) the repair of tissue damage and influence the synthesis of acute-phase globulins (14,56,57). LEM produces a decrease in muscle protein synthesis and in plasma albumin (34) as well as increased movement of amino acids (58) into liver to provide essential nitrogen for the synthesis of acute-phase globulins. Those globulins which are synthesized may redistribute copper (59,60); maintain a balance between circulating levels of vasodilators and vasoconstrictors (61) (ceruloplasmin); prevent free hemoglobin from inducing renal damage as well as scavenging iron (62,63) (haptoglobin), aid in wound healing (64) (α_1-acid glycoprotein), minimize tissue damage and clotting (62,65-70) ($\alpha_1$antitrypsin and α_2-macroglobulin), promote granulopoiesis (71) so as to provide more cells to phagocytize invading microorganisms, activate macrophages (72) to kill microorganisms, induce humoral immunity (73) and further stimulate acute phase globulin synthesis (74) (α_2-macroglobulin). LEM also stimulates the release of neutrophils from bone marrow (31), again pro-

viding more phagocytic capability. Viewed in this overall fashion one may discern the possibility of positive and negative feedback affecting almost all aspects of host defense, thus enabling this "system" to vary its response over a wide range to meet a variety of challenges. Moreover, one may perceive that the acute-phase globulins synthesized during infection play a critical role in regulating both the primary host defense, i.e., phagocytosis, and the development of long lasting protection and that they form, as it were, a bridge between the two processes.

This panoply of host metabolic alterations which occur usually quite early in the course of an infection may be construed as part of a generalized system of host defense responses adapted over the ages to deal with a wide range of inflammatory stimuli. Thus it is possible that specific aspects of this system may be inappropriate in responding to a particular stimulus. It is conceivable that the array of metabolic alterations, in the event that adequate immunity fails to develop, may continue for so long a period as to deplete the host and thus, eventually become deleterious. Clearly it is a matter of clinical experience whether this array of defenses can be overwhelmed.

It is possible that not all the metabolic alterations noted are of benefit to the host. However, I would like to suggest that a thorough knowledge of host-parasite interactions and their metabolic conquences, in conjunction with an understanding of the pathogenesis of each disease, will help us to formulate effective supplemental, supportive therapy to be used in combination with antimicrobial drugs. This approach should also help us to treat effectively, not merely symptomatically, individuals with viral or antibiotic-resistant diseases. The evidence to support this contention is scanty, yet tantalizing. For example, zinc, which appears to be purposefully redistributed during infection, has been used to protect rats and mice against endotoxin administered IP (75,76). The zinc appears to diminish uptake of endotoxin from the peritoneal cavity. It is tempting to speculate that zinc given in conjunction with antibiotics might minimize the risk of shock and toxic damage to the liver during treatment of gram-negative peritonitis.

Glucagon and insulin, which increase during severe infection, may function not only to redistribute amino acids, glucose and ketone bodies but also to protect the liver from damage or aid in its repair. Recent studies by Dr. Bucher at Howard University indicate that infusions of glucagon and insulin increase survival time and even protect about 40% of mice exposed to a normally lethal, murine hepatitis virus (77).

It is conceivable that administration of protease inhibitors such as trasylol might decrease the severity of diseases such as respiratory K. pneumoniae infection, in which tissue damage appears to be related to, if not the cause of death (17), by inhibiting proteases released from leukocytes or damaged cells.

Another approach would be to look for drugs which modify selectively the IMS, either to bolster the host's own defenses during the acute phase of the illness or, once the acute phase is over, to hasten convalescence. In this regard we have found that clofibrate, nominally an antihyperlipidemic drug, appears to mute selectively the synthesis and/or release of acute-phase

globulins during inflammation (78) and protects against pneumococcal sepsis in the rat (79), although it is neither bactericidal nor bacteriastatic. The mechanism of this protection is unknown but the plasma levels of clofibrate are such that one would expect to inhibit the appearance of α_2-macrofetoprotein but not of the other acute-phase globulins. The significance of this remains to be determined; but it is clear that host resistance can be enhanced by drugs which of themselves have no antimicrobial activity. It is tempting to think that clofibrate might be of use, in conjunction with antibiotics, in treating previously antibiotic-resistant infections.

Finally, knowledge of the metabolic alterations which occur during infection will help us to formulate the appropriate parenteral and hyperalimentation to speed tissue repair and convalescence without compromising host defense mechanisms, since it is clear that nutrition markedly affects host resistance.

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DISCUSSION

1. It was noted that a number of categories were discussed during the presentation as if they represented one component only; e.g., amino acids include a multitude of substances. Individual amino acids may not all react the same way to each type of infection. Also, LEM is probably more than one substance. In response, Major Powanda indicated that there was much evidence that LEM constitutes a multiplicity of endogenous mediators derived from phagocytic cells. A question was then asked whether specific differences, either qualitative or quantitative, could be observed in the responses of the host when different organisms were used in the system. In reply, it was noted that some of the data presented suggest that there are differences, depending upon whether a nonlethal or lethal infection is used. A major difference is clear from data obtained from the live vaccine strain of tularemia vs. the SCHU 4 strain. It is also probably true for viral disease, although viruses have not been studied extensively. Studies are

planned to use a strain of VEE which induces lethal encephalitis. These host responses must represent a fundamental phenomenon since they occur in patients who are protein-deficient and in laboratory animals that are zinc-deficient, and malnourished. It suggests that the responses observed are almost "primeval", and may represent one of the first host defensive systems devised. These responses are probably beneficial and allow the host to deal with many types of infections. An understanding of these responses and their manipulation might prove to be very valuable. For example, simply measuring what substances are excreted in the urine, and then replacing these substances cannot be considered adequate supportive therapy today. An understanding of the metabolism and the alterations of it must be achieved.

2. Two examples in human medicine were described which might be considered counterparts of this laboratory study. One was the Cleveland Family Study directed by Dr. Rammelkamp which showed that the cumulative curve for disease patterns, year after year, was about the same. Even when epidemics of Asian flu occurred and half of the population became sick, the basic curve remained stable. The second study concerned the observations of children at Chapel Hill over several years. With a few notable exceptions, when a particular agent infected a community of children, all other disease agents seemed to disappear. It appeared that the epidemic shut off or stopped other infections before they could become established. Major Powanda was asked to comment on these phenomena. His response was not in terms of clinical but laboratory experience. If turpentine is administered to an animal 24 hr before Diplococcus pneumoniae, the majority of the animals are protected. The turpentine apparently stimulates some defensive system. It is not certain whether the number of phagocytic cells are increased and clear the bacteria before they multiply. Since part of the LEM feed-back system would also enhance the number of circulating phagocytes, nonspecific stimulation should decrease the severity of a subsequent disease at some point in time. The mechanisms of the nonspecific defensive action seem to require a proper time relationship with one another: If they do not occur at the right time, they may well be deleterious to the host.

3. It was asked if the Ehrlich urinary diazo reaction, a measure of tryptophan metabolism, would be helpful. It was noted years ago that patients who were going to die of typhoid fever excreted more diazo reactant than those who survived. The magnitude of the reaction also had a relationship to the degree of susceptibility. Dr. Powanda recalled that those people who had an outpouring of tryptophan metabolites into the urine were going to contract typhoid fever. Malnutrition appears to reduce the ability of the host to mount a good defense.

4. Another questioner asked if there were major differences in these parameters between heat-stroke victims and those with artificial fevers. Heat-stroke studies indicated that death occurred after tissue damage, but metabolic changes were not defined. It was pointed out that artificially induced fever can cause nitrogen loss via the urine. What causes these changes in metabolism are currently under investigation. Fever is a part of the host's response but is probably not the most compelling aspect of the response.

5. The question was asked if turpentine abscesses caused fever. The answer was, yes. A consultant reported that he had measured amino acids in febrile children who were not infected; the serum amino acids were normal or elevated. There are differences between infected and noninfected patients that are not accounted for by the presence of fever.

6. Another consultant commended the investigator for having undertaken such a large study. However, it would be possible to use another 125 tests to measure additional parameters, and more changes would undoubtedly be noted. These changes would require an entire career to sort out in order to arrive at a reasonable position. Years ago another investigator measured a limited number of variables, electrolytes and fluid volumes, during acute pneumococcal pneumonia; he found that if the patient died quickly of pneumonia, there was an almost normal electrolyte balance. In other words, the patient died before metabolic abnormalities developed. If the laboratory data were examined, one would conclude that the patient was healthy when, in fact, he was dead. The time sequence and the severity of the disease are important variables which significantly influence the changes that could occur.

PROSTAGLANDINS AND THE HOST'S METABOLIC RESPONSE TO INFECTIOUS DISEASE

Carol A. Mapes, PhD

Rabbit peritoneal exudate cells produce a presumably proteinaceous substance that historically has been termed LEM or endogenous pyrogen. As shown in Table I, this crude material induces a number of metabolic and physiologic alterations at various times after administration to experimental animals. The alterations, as recently reviewed, decreased concentrations of plasma iron, zinc, and glucose and increased hepatic amino acid flux, release of neutrophils from bone marrow, serum concentrations of copper, ceruloplasmin, acute-phase globulins, glucagon, and insulin, hepatic RNA synthesis, and body temperature (11).

TABLE I. BIOLOGICAL RESPONSES TO LEM

RESPONSE	OPTIMAL TIME OF ASSAY (HR)	REFERENCE
Decreased:		
Plasma Fe	5-6	1
Plasma Zn	5-6	2
Plasma and portal vein glucose	4	3
Increased:		
Hepatic amino acid flux	3	4
Neutrophil release	2	5
Serum Cu and ceruloplasmin	12-24	6
"Acute-phase" globulins	10-48	7-8
Glucagon and insulin	4	3
Hepatic RNA synthesis	10	9
Fever	1	10

Although several theories have evolved to integrate these responses and explain their value, a basic problem in LEM research has been a lack of identified compounds or mediators for determining either the value of a given response or the mechanism by which it occurs. Therefore, our early studies were directed toward mediator purification. Last year we discussed the fact that LEM and endogenous pyrogen (EP) are different molecular species based on: 1) physical separation of pyrogenic activity from activities attributed to LEM; 2) preferential production of LEM activities by some stimulated cell preparations; and 3) differences in the production of LEM and endogenous pyrogen by cells treated with various drugs and chemicals (12). It also was

reported that LEM itself consists of multiple activities that are separable by standard protein fractionation techniques (13). Activities separated include those that: depress plasma zinc, increase hepatic amino acid flux, and alter circulating levels of peripheral neutrophils.

The results of additional purification studies, however, suggested that the essential components of LEM might not be protein in nature. Perhaps the earliest clue was that chloroform-methanol extractions could be modified in a manner that would deplete mediator preparations of all detectable protein without a resultant loss of LEM activities. As one might predict, all of the detectable material remaining in the active chloroform-methanol upper phase was lipid in nature. Additional studies further demonstrated that LEM contains milligram quantities of extractable lipid which can be separated into numerous compounds by thin-layer chromatography using several different lipid-solvent systems. This indicates that the lipid is composed of numerous subclasses rather than a single compound. Lipids normally found in this type of extract include: prostaglandins, endoperoxides, thromboxanes, hydroxy fatty acids, gangliosides, and possibly sulfatides, lysophosphatides, and glycerolphosphatides.

Of these compounds the prostaglandins (PG) and their by-products seemed the most likely candidates for investigation since they are present in LEM (14) and because other laboratories have implicated them in the development and possibly mediation of inflammatory responses.

Although much information is available concerning the prostaglandins (15-17), only a few pertinent topics will be discussed today. Prostaglandings arise spontaneously through enzymatic transformations of polyunsaturated fatty acids that are generated primarily from membrane phospholipids by the action of phospholipase. For the purpose of this discussion, we consider families of compounds that arise from arachidonic acid, a 20-carbon, tetra-unsaturate. Fatty acid cyclo-oxygenase will convert some of the fatty acids into endoperoxides. Unlike arachidonic acid, these compounds are substituted with oxygenated cyclopentane rings and possess functional groups at C-15.

The endoperoxides are modified further by several enzymatic reactions. One synthetic route yields malonaldehyde, C-17 hydroxy fatty acids, and thromboxanes. The thromboxanes have an oxane ring structure and are essentially hemiacetal derivatives of the endoperoxides. Alternatively, endoperoxides can be converted into the more familiar prostaglandins F, E, A, and D. These compounds all contain substituted cyclopentane rings and differ structurally only in the functional group substitutions at C-9 and C-11.

Second, if lipoxygenase is present in the synthetic system, fatty acids also are converted into hydroperoxy derivatives which form the corresponsing hydroperoxy fatty acids and unidentified products. In addition to these reactions, 3 other primary reactions occur in most synthetic systems. First, some of the prostaglandins undergo degradation as they are formed. One of the first degradation products and the best measure of in vivo PG synthesis is the 15-keto-13,14-dihydro derivatives. Second, other long-chain polyunsaturated fatty acids undergo similar reactions. Third, in the presence of oxygen, autocatalytic lipid peroxidations occur via free radical mechanisms and alter the enzymatically formed products. The net result is formation of about 150 identified compounds and a large number of compounds that have not been characterized.

Of the identified compounds, the majority have documented physiologic activities. A few have "mediator-type" activities. For example, one of the endoperoxides (PGG) causes platelet aggregation and thromboxane A, the unstable precursor to thromboxane B, is rabbit-aorta contracting substance. Thus far, only one of these compounds has been implicated in the action of a leukocytic mediator. Administration of EP to animals causes de novo synthesis of PGE which probably mediates fever through its ability to alter cyclic AMP levels. In addition, the total PG profile has been implicated in cellular migration and manifestation of pain, injury, and swelling that accompanies tissue injury.

A starting point for studying the involvement of prostaglandins in an in vitro system is the use of anti-inflammatory drugs such as indomethacin and aspirin since they inhibit the action of lipoxygenase, fatty acid cyclo-oxygenase, and phospholipase depending upon its source and the drug's concentration.

Fig. 1 shows the effect of indomethacin on LEM release. Three metabolic responses were assayed 5 hr after IP administration of a test sample to rats.

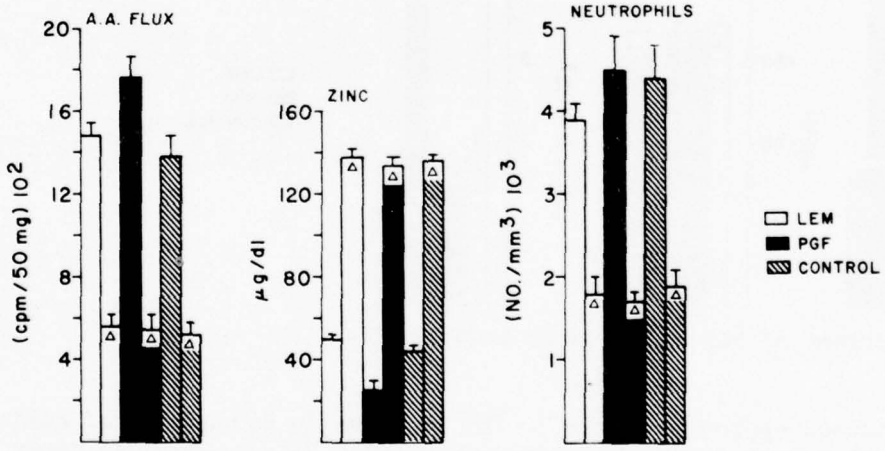


Fig. 1. Effect of indomethacin on mediator release.

Amino-acid fluxing activity is expressed as cpm [^{14}C]AIB/50 mg wet wt liver The open bars represent activity produced from a normal LEM preparation; the closed bars represent the activity produced in the presence of indomethacin; and the cross-hatched bars represent the activity obtained from control PMN to which an equivalent concentration of indomethacin was added at the termination of incubation. Each corresponding bar marked with a Δ represents the bioassay control obtained by administering a heat-inactivated sample. In most cases, this value represents the basal level for each parameter since it does not differ from a saline control.

Comparing the closed bars or indomethacin-treated samples with any heat-inactivated sample, we see that the amino acid-fluxing, zinc-depressing and neutrophil-releasing activities produced in the presence of indomethacin do not differ significantly from heat-inactivated preparations, thereby indicating

nearly complete inhibition of mediator production. In contrast, the activities of controls to which indomethacin was added at the termination of incubation, do not differ significantly from LEM prepared in the normal manner.

Additional studies showed that aspirin affected the synthetic system in the same manner, whereas, morphine, a drug normally used as a control in this type of experiment, had no detrimental effect on mediator production. Due to the possible nonspecific actions of these drugs complementary experiments were performed using available prostaglandins.

Fig. 2 shows the effect of PGE on mediator release, closed bars represent the activity produced in the presence of PGE; and cross-hatched bars represent the activity of controls to which PGE was added subsequent to the incubation period.

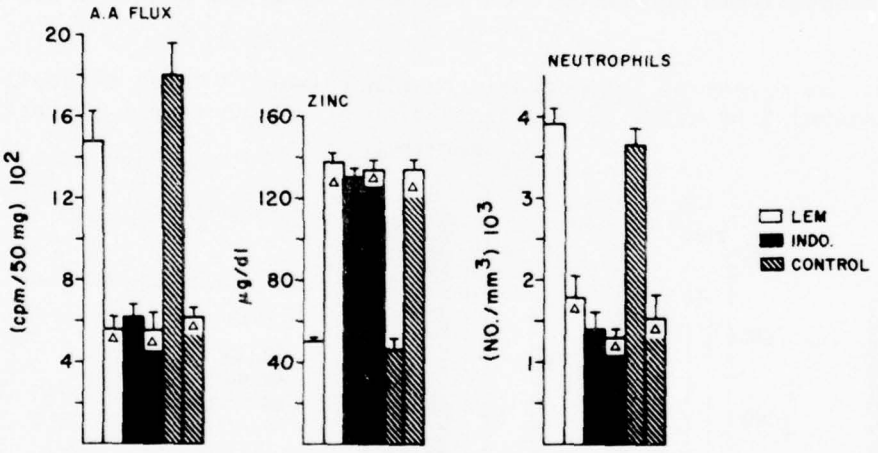


Fig. 2. Effect of PGE on mediator release

As shown, PGE caused approximately a 2-fold increase in production of amino acid fluxing-activity, but did not effect production of zinc-depressing or neutrophil-releasing activities. In contrast, addition of PGE at the termination of incubation did not significantly alter mediator activities.

As shown in fig. 3, addition of PGF had no effect on production of amino acid-fluxing or neutrophil releasing activity. However, it caused an approximate doubling in production of zinc-depressing activity as reflected by decreased plasma zinc concentration obtained with PGF-treated incubations as compared to the corresponding controls. These results suggest that prostaglandin synthesis is required for production of endogenous mediators. These results combined with additional studies demonstrating that: 1) the prostaglandin's effects do not result from cyclic nucleotide alterations, and 2) that prostaglandin-like material is not present in inactive mediator preparations, which further implies that prostaglandins, their metabolites, or by-products are the biologically active components of LEM. However, bioassays employing pure prostaglandin E, F, and A were equivocal at best, suggesting that we should look more closely at some of the related products.

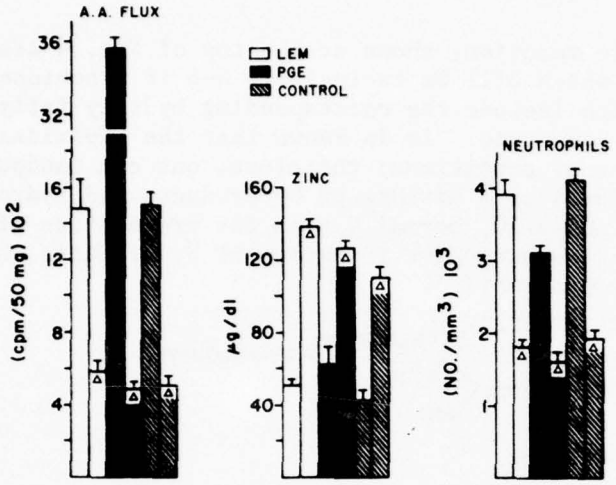


Fig. 3. Effect of PGF on mediator release

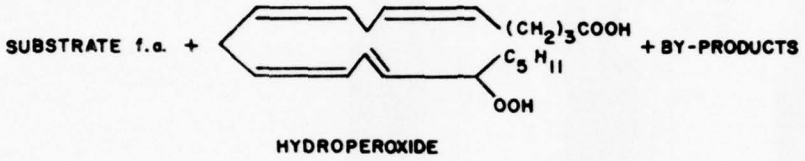
Products related to the prostaglandins include: endoperoxides, hydroperoxides, hydroxy fatty acids, and thromboxanes. Soybean lipoxidase, a commercially available enzyme, mimics mammalian lipoxygenase, converting free fatty acids into products similar to those produced by this system. The first approach was to add lipoxidase to peritoneal cell preparations to determine whether it could either enhance or induce repetitive mediator production. Although successful, the most interesting aspect of these experiments was the control incubation.

As shown in Table II, cell-free incubations consisting of lipoxidase, arachidonic acid, and saline synthesize a substance which causes approximately 45% depression of plasma zinc when compared to a saline control. This depression is comparable to that obtained with many active mediator preparations. Although the most active synthetic product is obtained with arachidonic acid as substrate, the reaction can utilize most long-chain, unsaturated fatty acids as demonstrated by formation of an active product from linoleic acid. It should be noted that neither the substrate fatty acids nor lipoxidase induce detectable biological responses when equivalent concentrations are administered to rats.

TABLE II. CELL-FREE SYNTHESIS OF MEDIATOR ACTIVITY

INCUBATION	PLASMA Zn (µg/dl)
Saline	130 ± 4
Lipoxidase	137 ± 3
$C_{20:4}$	136 ± 5
$C_{18:2}$	135 ± 4
Lipoxidase + $C_{20:4}$	69 ± 3
Lipoxidase + $C_{18:2}$	84 ± 2

The products of the enzymatic reaction, shown at the top of Fig. 4 are unused substrate, hydroperoxides which will be exclusively Ω-6 if arachidonic acid is used, and by-products which include the corresponding hydroxy fatty acids as well as uncharacterized compounds. It is known that the lipoxidase reaction is influenced by a number of conditions; therefore, one can manipulate the reaction to form only by-products or a mixture of by-products and hydroperoxides. Conditions of pH 7.0 and 37°C, normally used for preparation of cell-derived mediators, result in the exclusive formation of by-products which appear to be the active synthetic mediator(s).



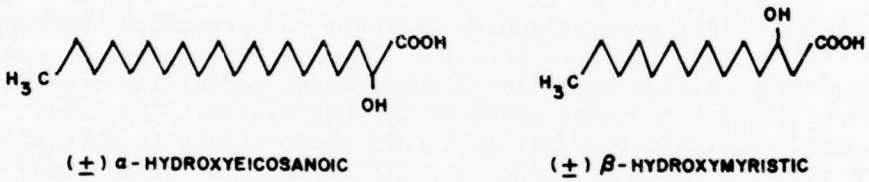


Fig. 4. Enzymatic reaction (above) and 2 synthetic hydroxy fatty acids.

Thus, one suspects that hydroxy fatty acids may be one class of compounds that can mediate plasma zinc depression. This hypothesis is supported, in part, by the observation that the synthetically prepared hydroxy fatty acids, α-hydroxyeicosanoic and α-hydroxymyristic acid (fig. 4 bottom) also induce zinc depression. Both are fully saturated which indicates that they will only transiently assume the configuration of their unsaturated, naturally-occurring analogs and the hydroxyl groups are repostitioned near the carbonyl group which may interfere with ionic interactions and possibly recognition of receptor sites. Nevertheless, microgram quantities of either compound induce zinc depressions which are statistically significant ($P < 0.001$).

Two additional pieces of information further indicate that endogenous mediators are lipids. First, studies which we have not discussed indicate that other mediator activities are attributed to lipids that are not hydroxy fatty acids. Second, a number of lipids that do not induce metabolic responses, when administered as pure compounds, have been shown to do so when provided an appropriate carrier system.

Numerous questions obviously remain concerning both the implications and the specificity of lipids in control of the inflammatory response. One of the primary questions is: what are the potential applications of lipid mediators?

We are dealing with a series of compounds that are rapidly synthesized in vivo, presumably forming lipid mediators as well as stable degradation products and by-products. The majority of these compounds are detectable in microgram quantities using mass spectral peak matching analyses. Thus, one may be able to utilize the appearance or enhanced concentration of some lipids as an early indicator of infection. Preliminary studies in this area have shown that at least 2 lipid components, not present in appropriate controls, appear in the plasma of rats 6 hr after exposure to live bacteria. The exposed animals have no detectable signs of illness at this time. It is obviously too early to assess the practical application of these observations; however, future studies will endeavor to determine: 1) the relevance of this observation to the infectious process and 2) the relation of these lipids to specific mediators.

A second area of research involves elucidation of mediator mechanisms. This is envisioned as an essential element in our research program since the value of early detection of inflammation, resulting from identified or unidentified agents, would be greatly enhanced if we had the knowledge to either prevent clinical onset of illness or promote rapid recovery. Thus, purified compounds also will be used to determine the value of specific biological responses as well as the mechanisms by which they occur. It is hoped that these studies will yield information applicable to development of methods for enhancing or diminishing metabolic responses to the host's benefit, thereby, leading to treatment and possibly control of the infectious process.

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RADIOMETRIC METHODS FOR RAPID DIAGNOSIS OF VIRAL INFECTION

M.-F. Tsan, MD, and H. N. Wagner,* MD

Our studies are based on the following hypotheses: 1) early metabolic effects of virus on cell culture can be used as an indication of the presence of virus; 2) specificity can be achieved by neutralization of the viral effects with specific antiserum; and 3) radiometric technique can be used to measure these metabolic effects. The test model consists of herpes simplex viruses (HSV) and human lung fibroblasts (WI-38). Two systems were studied: 1) effect of HSV on glucose oxidation by WI-38 cells and 2) effect of the virus on the DNA synthesis by WI-38 cells as measured by [^{3}H]thymidine incorporation, or [^{125}I]5-iododeoxyuridine (^{125}IdU) incorporation.

In the glucose oxidation experiments, WI-38 cells formed a monolayer in a 10-ml serum vial, then infected with HSV-1 and 4 Ci of glucose-1-[^{14}C] were added. Glucose oxidation was measured by $^{14}CO_2$ output using an ionization chamber as described previously (1). As shown in Fig. 1, glucose oxidation was inhibited by HSV-1. The titer of virus used was 10^6 $TCID_{50}$ units. There was significant inhibition 6 hr after infection. It approached 50% at 24 hr. However, by this time cytopathic effect (CPE) was also apparent.

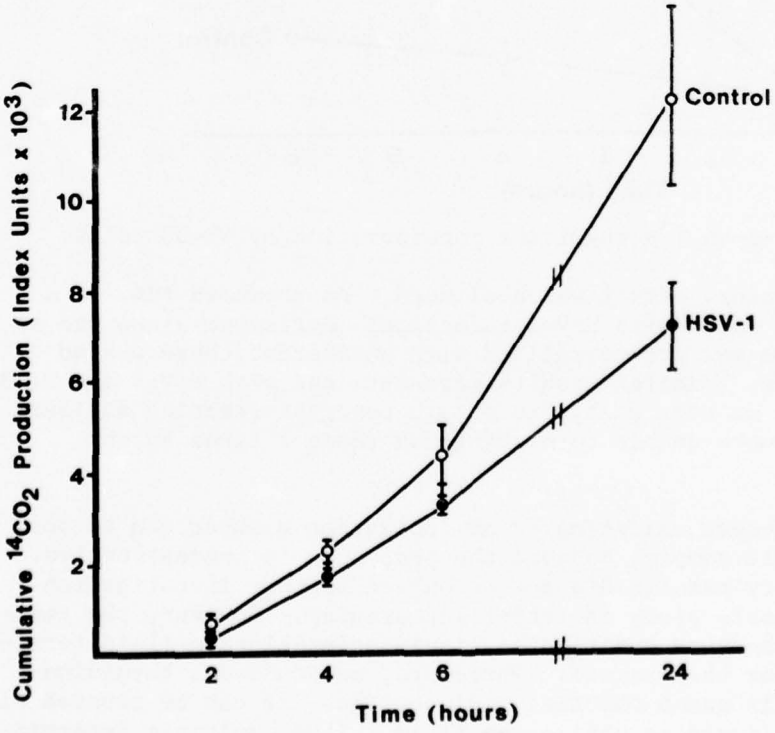

Fig. 1 Effect of HSV-1 on glucose-1-[^{14}C] oxidation by WI-38 cells

*Contract DAMD-17-75,C-5041, Johns Hopkins University.

In the nucleic acid synthesis experiments, cells formed a monolayer in a liquid scintillation counting vial and were infected with HSV-1. For the purpose of studying DNA synthesis, [^{3}H]thymidine was used, for RNA synthesis, [^{3}H]uridine was used. The radioactivity was then quantitated by liquid scintillation counting. There was essentially no effect on the RNA synthesis by WI-38 cells. In contrast, a marked stimulation of DNA synthesis was noted 4 hr after infection with HSV-1 (Fig. 2). When HSV-1 was neutralized with

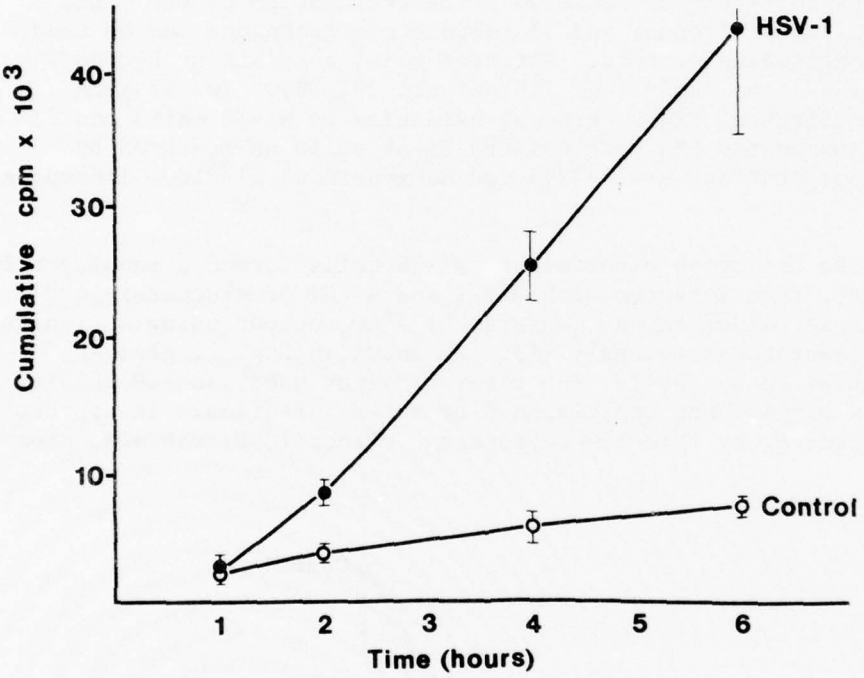

Fig. 2. Effect of HSV-1 on [^{3}H]thymidine incorporation by WI-38 cells

antiserum, this stimulatory effect was abolished. As shown in Fig. 3, a marked stimulation was noted with HSV-1 infection. Antiserum alone had no effect. When the virus was preneutralized with antiserum, there was no stimulatory effect seen. Similar results were obtained with HSV-2 and WI-38 cells. However, since we were unable to obtain noncross-reacting antisera for types 1 and 2, we were unable to distinguish these 2 types by our technique.

Measurement of glucose oxidation by an ionization chamber can be done repeatedly from a single sample, because the procedure is nondestructive. However, our preliminary results did not encourage further investigation. The nucleic acid synthesis study is rather encouraging. However, the technique is destructive, because addition of liquid scintillation fluid terminates the experiment for that sample. Therefore, we studied a thymidine analog, ^{125}IdU, which is gamma-emitting radionuclide. It can be counted with a gamma counter using liquid scintillation fluid. Thus, multiple determinations can be performed on a single sample. Fig. 4 shows the results using ^{125}IdU. In this study WI-38 cells were infected with different numbers of virions ranging from 10^1 to 10^6 $TCID_{50}$ units. The results are expressed as % of control. As can be seen, at high titers, ^{125}IdU incorporation was markedly stimulated within a few hours. At low titers, stimulation was noted 2 - 3 days after infection.

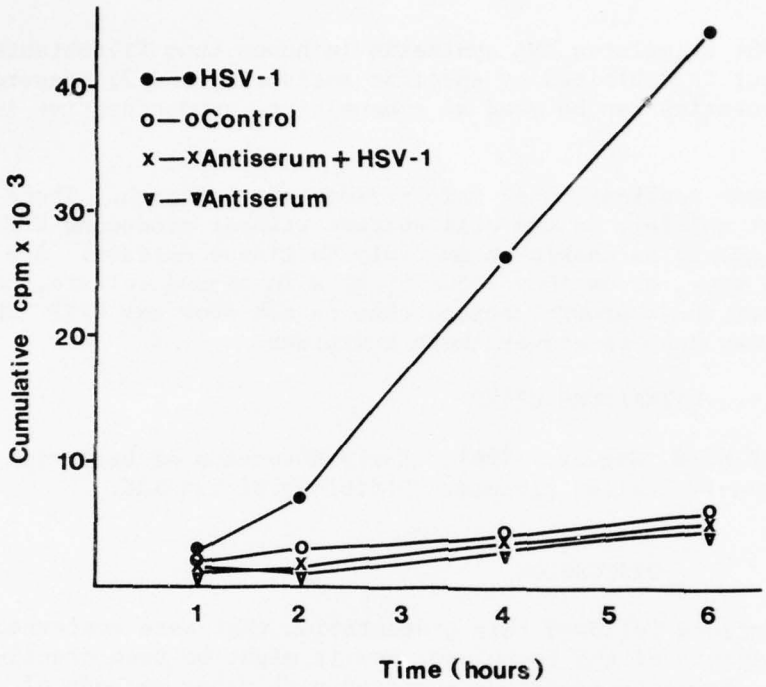

Fig. 3. Effect of HSV-1 neutralization on [^{3}H]thymidine incorporation by WI-38 cells

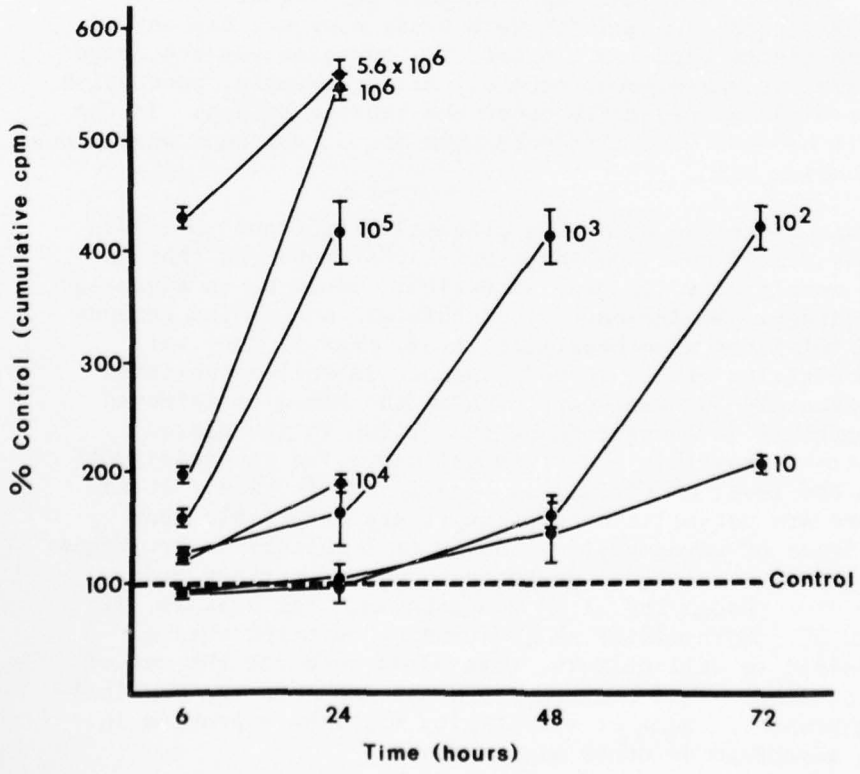

Fig. 4. Effect of varying numbers of HSV-1 on ^{125}IdU incorporation by WI-38 cells

In summary, 1) HSV stimulates DNA synthesis in human lung fibroblasts (WI-38); 2) this effect is inhibited by specific antiserum; and 3) measurement of ^{125}IdU incorporation can be used as a sensitive, nondestructive index of HSV activity.

There might be some application of this radiometric approach. There are some viruses which multiply in the cell culture without producing CPE. Some viruses are thought to be unable to multiply in tissue culture. Are they really unable to grow, or do they actually grow in tissue culture, but we are unable to detect their growth because they do not show any CPE? The radiometric approach may help to answer these questions.

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DISCUSSION

1. Numerous questions followed this presentation that were concerned with the quantative aspects of the technique, how it might be used practically in a laboratory, how this technique compared with other methods of rapid diagnosis, how the technique could be used with viruses of an unknown nature, etc. Answers were not provided to all questions but those that were answered follow. Dr. Tsan reported that cytopathic effects in culture were compared to his method. He found the 2 methods were equal in time requirements when high titers of organisms were used; however, his method was not as useful when titers were low. A specific question was concerned with the fact that some viruses depress metabolism; for example, production of ^{14}H and O_2. Other viruses frequently cause the reverse effect. In the clinical situation, it becomes more difficult when you do not know what group of viruses you are dealing with.

2. How would this system be used in a clinical situation? Dr. Tsan reported that this was a difficult problem. Dr. Wagner reported that he thought that lack of specificity, in many situations, could be an advantage rather than a disadvantage. He indicated that this was one of the reasons the program had been initiated with hepatitis virus. First, they were encouraged by the sensitivity of the method since it is well-established that there are approximately 10^5 Dane particles in the blood of infected persons. The Dane particle is thought to be the virion in the plasma of people who demonstrate hepatitis B surface antigen. The sensitivity of this method is below the level of viremia in hepatitis infection. It is conceivable that there are metabolic changes which are detectable even when there is no evidence of virus replication in cell cultures. Dr. Wagner concluded that it could be possible to demonstrate that a patient had viremia in the serum even though the virus was unknown. For example, if marked incorportation of [^{3}H]thymidine or [^{3}H]uridine occurred when a patient's serum was added to cell culture, this would rule out the use of this blood in a transfusion. This observation would indicate the possibility of a virus being present. Lack of specificity might be a problem in certain cases and an advantage in other situations.

3. A member of the USAMRIID staff indicated that it might be useful to combine cell-mediated immunity technology with this procedure. The combined technology could be used to characterize the reactivity of human immune lymphocytes. This concept would require large numbers of cell banks similar to the tissue types of Fritzbark. This approach would eliminate the current problem of specificity. The contractor was asked to comment on this approach but there was no response. The final question concerned the use of clinical samples as opposed to purified test samples of virus. To date, purified samples of virus have been used for developing the procedure because it has been difficult to obtain sufficient amounts of clinical material. Plans are underway to use clinical specimens.

SEQUENTIAL IMMUNIZATION OF "AT-RISK" PERSONNEL WITH SPLIT AND WHOLE VIRUS A/SWINE INFLUENZA VACCINES.

Robert Edelman, MD

About 6 months ago, USAMRIID was requested by the National Institute of Allergy and Infectious Diseases to investigate the protective efficacy of swine influenza vaccines in laboratory animals. USAMRIID personnel at risk of acquiring swine influenza in the laboratory where immunized in order to prevent disease and transmission of the virus to the community. One hundred thirty-three laboratory workers were initially immunized with 400 chick cell agglutinating (CCA) units of the split-virus vaccine made by Wyeth Laboratories; 31 of these persons, whose antibody titers did not reach 1:40, were later given a booster dose of the Wyeth product. Fourteen persons required a third dose, but this time we used Merck, Sharpe and Dohme whole virus vaccine. I would like to summarize for you the side reactions and antibody responses after the sequential use of these experimental influenza vaccines.

Although 133 persons were considered to be at risk to swine influenza infection, only 16 persons actually entered the room where the virus was contained. The chance of infection, was limited further by strict safety precautions. Thus, we feel reasonably confident that the responses to the vaccines were not confounded by laboratory infections. Careful clinical surveillance failed to uncover any laboratory-acquired influenza illness.

Informed consent was obtained from the laboratory workers. They were in good health at the time of immunization and ranged in age from 19 - 62 years.

Oral temperatures were taken just before vaccination that morning, that same evening, and at 24 and 48 hr. Local reactions were measured immediately, and at 24 and 48 hr; they were graded as absent, slight, moderate or marked and were based on the subjective impressions of the participants.

Another group of 173 healthy adults at Fort Detrick, not at risk to influenza, participated in a placebo-controlled, double-blind trial of trivalent influenza vaccines. Thirty-three of these persons were sham-vaccinated in both arms with 0.5 ml of vaccine diluent which was buffered saline. Their local and systemic reactions were graded. I've included their results for purposes of comparison (Table I). After primary inoculation of Wyeth vaccine, 2 persons developed low grade fevers of 100.2° and 101.2°F that lasted less than 24 hr. Systemic and local reactions were similar in placebo and Wyeth vaccine groups, while the Merck vaccine was more reactogenic.

We obtained sera before vaccination and at 3 wk. Sera were first titrated by the hemagglutination-inhibition (HAI) test by Col. Frank Top (WRAIR). Dr. Top used the swine strain A/Mayo Clinic/74. A more complete set of sera were later tested by Dr. Gary Noble (CDC) who used A/Victoria/75 and the A/New Jersey/76 swine influenza antigens. The WRAIR results were obtained first to determine who should receive booster immunizations, but the results to be presented are from CDC, because they represent simultaneous titrations on complete sets of sera.

TABLE I. REACTIONS TO MONOVALENT A/SWINE INFLUENZA VACCINES AND TO VACCINE DILUENT AT FORT DETRICK

STUDY GROUP	NO. OF PERSONS	% WITH REACTIONS					
		Fever	Systemic*		Local**		Temp. ≥100 F or
		> 100°	Mild	Mod.	Mild	Mod.	Moderate
Placebo	33	0	19	6	58	6	7
Primary (Wyeth)	133	2	21	2	38	1	5
Booster 1 (Wyeth)	31	0	19	0	16	0	0
Booster 2 (Merck)	14	0	36	14	71	0	14

*Malaise, myalgia, headache, feverish, or nausea
**Tenderness, redness, or induration

The chronology of type A influenza infections since 1918 is presented in Table II. Major antigenic changes occurred in the virus surface hemagglutins after 1918. Each virus bearing a new hemagglutin circulated for many years before it was replaced by an antigenically different strain. Persons 52 years and older who were living during the era of prevalence of HSwN1 swine strains were first infected (primed) during 1918 - 1928. If infected again during a later era when another type A virus circulated, the person would respond by producing antibody against the original priming strain in addition to the new infecting strain. Also, younger people (age 35-51 yr) never primed by swine virus often produced swine antibody when infected by HoN1 strains because HSW- and HO-bearing viruses are closely related antigenically. With this chronology in mind we can better interpret the age distribution and pre-existing antibody status of vaccinees at Fort Detrick (Table III).

TABLE II. CHRONOLOGY OF TYPE A INFLUENZA INFECTIONS SINCE 1918

Era of prevalence	1918-1928	1929-1945	1946-1956	1957-1967	1968-1976
Hemagglutinin-neuraminidase surface antigen	HSwN1	HON1	H1N1	H2N2	H3N2
Influenza virus strains	Swine	PR8	-	Asian	Hong Kong
Age group	≥52	35-51	25-34	14-24	≤13

TABLE III. AGE DISTRIBUTION AND PRE-EXISTING ANTIBODY STATUS OF VACCINEES IN THE WYETH A/SWINE INFLUENZA VACCINE STUDY

AGE GROUP	NO. VACCINEES	%	% WITH PRE-EXISTING HAI ANYIBODY	
			A/NJ/76	A/Victoria/3/75
19-24	29	22	0	66
25-34	23	17	17	57
35-51	58	44	53	52
52-62	23	17	96	39
Total	133	100	43	53

The serological results agree with the published national data showing no significant difference among age groups of A/Victoria/75 antibody, but striking age-related prevalence of A/NJ/76 swine antibody. These data suggest that A/Victoria strain has recently circulated in our entire study population, but that A/swine virus or closely related serotypes have circulated in older persons before 1957. Many of our 35-51-year olds were vaccinated with military vaccines between 1955 and 1969; these vaccines contained A/swine antigen, thus accounting for the high prevalence (53%) of A/swine antibody in this age group.

The serological response after split-virus vaccine was poor in the 19-24-year olds, but good in persons 25 years and older (Table IV). Only 24% of persons 19-24 years old had post vaccination titers of $\geq$ 1:40; by contrast 83 100% of persons aged 25 - 62 yr achieved this titer. Although the geometric mean pre-vaccination titers in the 2 youngest age groups were similar (1:5 and 1:6), the geometric mean post vaccination titers of those 2 age groups were not (1:12 vs. 1:101), suggesting that the older group had been previously primed by swine-like antigens.

TABLE IV. HAI ANTIBODY RESPONSE AFTER PRIMARY IMMUNIZATION WITH WYETH A/SWINE INFLUENZA VACCINE

AGE GROUP	NO.	VACCINE DAY	GMT	CUMULATIVE %			
				< 1:10	$\geq$ 1:10	$\geq$ 1:20	$\geq$ 1:40
19-24	29	Pre	$\leq$1: 5	100			
		Post	1: 12	62	38	28	24
25-34	23	Pre	$\leq$1: 6	83	17	4	
		Post	1:101	4	96	91	83
35-51	58	Pre	1: 31	47	53	38	28
		Post	1:277	2	98	97	95
52-62	23	Pre	1: 52	4	96	87	70
		Post	1:414	0	100	100	100

The data in Table V show that even though some older persons may lack detectable A/swine antibody before vaccination, they respond better than younger persons to the split-virus vaccine, and develop higher swine-antibody conversion rates and progressively higher geometric mean titers after a first inoculation with the A/swine vaccine.

TABLE V. EFFECT OF WYETH A/SWINE INFLUENZA VACCINE ON GMT AND HAI ANTIBODY RESPONSES OF PERSONS WITHOUT PRE-EXISTING A/SWINE ANTIBODY

AGE GROUP	NO.	GMT	CUMULATIVE %			
			<1:10	≥1:10	≥1:20	≥ 1:40
19-24	29	1: 11	62	38	28	24
25-34	19	1: 69	5	95	89	79
35-51	27	1:123	0	96	93	89
52-62	1	1: 80	0	100	100	100

In subjects circulating swine antibodies before vaccination the fold-increase in titers did not increase with advancing age, but rather tended to decrease (Table VI). The differences noted, however, were not statistically significant.

TABLE VI. EFFECT OF WYETH A/SWINE INFLUENZA VACCINE ON HAI ANTIBODY RESPONSES OF PERSONS WITH PRE-EXISTING A/SWINE ANTIBODY

AGE GROUP	NO.	CUMULATIVE % WITH FOLD-INCREASE OVER PRE-TITERS							
		<1:2	≥1:2	≥1:4	≥1:8	≥1:16	≥1:32	≥1:64	≥1:128
19-24	0	0	0						
25-34	4	0	100	100	75	75	75	50	50
35-51	31	0	100	90	84	68	39	19	13
52-62	22	14	86	64	59	50	23	9	5

The effect of a Wyeth booster inoculation was disappointing in those 19-24-year olds who had a poor response to the primary inoculation (Table VII). Only 26% developed antibody titers of ≥1:40. The post-booster titers in 25-49-year olds was not much better, for only 42% of this group had antibody of ≥ 1:40 after the booster inoculation, and except for 1 or 2 persons titers did not respond to the booster dose at all.

TABLE VII. HAI ANTIBODY RESPONSES AND GMT AFTER PRIMARY AND BOOSTER VACCINATIONS OF 31 PERSONS WITH WYETH A/SWINE INFLUENZA VACCINE

AGE GROUP	NO.	BOOSTER	GMT	CUMULATIVE % <1:10	≥1:10	≥1:20	≥1:40
19-24	19	Pre	1: 5	100			
		Post 1	1: 7	79	21	11	5
			P < 0.001				
		Post 2	1:17	16	84	58	26
25-49	12	Pre	1: 5	92	8		
			P < 0.005				
		Post 1	1:25	17	83	67	42
		Post 2	1:27	8	92	67	42

Fourteen persons, with low-titered antibody after 2 inoculations of split-vaccine, responded more favorably to a 3rd inoculation using a whole virus vaccine (Table VIII). Two doses of the split virus vaccine may have

TABLE VIII. HAI ANTIBODY RESPONSES AFTER A PRIMARY AND 2 BOOSTER VACCINATIONS OF 14 PERSONS WITH WYETH AND MERCK A/SWINE INFLUENZA VACCINES

AGE GROUP	NO.	BOOSTER	GMT	CUMULATIVE % <1:10	≥1:10	≥1:20	≥1:40
19-24	9	Pre	1: 5	100			
		Post 1 (Wyeth)	1: 5	100			
			P < 0.001				
		Post 2 (Wyeth)	1:13	11	89	33	11
			P < 0.001				
		Post 3 (Merck)	1:29	0	100	89	67
25-46	5	Pre	1: 5	100			
		Post 1 (Wyeth)	1:10	20	80	20	
		Post 2 (Wyeth)	1:15	20	80	40	40
			P < 0.05				
		Post 3	1:35	0	100	100	40

primed them sufficiently to respond to this 3rd dose of whole virus vaccine. Alternately, the whole virus vaccine could be more antigenic. Because the persons re-inoculated were preselected on the basis of their poor serological response to a primary or booster inoculation, their serological responses cannot be readily compared with vaccinees studied elsewhere and boosted regardless of their primary antibody response.

In summary, the Wyeth split-virus vaccine was safe given as primary and booster doses of 400 CCA units. The Merck vaccine given as a booster was safe but slightly more reactogenic. The split-virus vaccine was poorly antigenic when used as a primary vaccine in 19-24-year olds. This vaccine was also poorly antigenic given as a booster dose to persons who were poor antibody responders regardless of age.

DISCUSSION

The point was made that if base-line antibody titers were small, a 10-fold rise in titer would not be much; however, a titer of 1:20,000 is a substantial titer and any increase above this baseline may or may not be significant. In reply, it was noted that the higher the base-line titer, the less the change observed in titer following administration of swine flu vaccines.

2. A very large number of reactions occurred in the placebo group; a question was raised as to what the placebo was. It was buffered saline. It was agreed that there were many adverse reactions in this group. In fact, one of the largest erythemas with induration and fever occurred in the control group. It was suggested that perhaps some of these adverse reactions were caused by the preservative; this point was not resolved. The placebo group was composed of people from all over the Post, some of whom were not used to receiving vaccine antigens. The experimental group, all members of this Institute, are experienced. Attitudes between the 2 groups may influence the observed responses.

3. A rapid response was observed in the 25-35-year-old age group; the presumption was that these people had never experienced the antigen. It was mentioned that the neuraminidase antigen on the swine virus may be the same as was on the PR-8 strain of influenza.

IMMUNO- AND CHEMOPROPHYLAXIS OF A/NEW JERSEY/76 (SWINE) INFLUENZA IN SQUIRREL MONKEYS

Richard F. Berendt, PhD

In February 1976, influenza virus bearing the antigenic determinants of swine influenza was isolated from recruits at Fort Dix, N. J. Because the great majority of the American population was considered to be susceptible to this strain of virus, a massive campaign was initiated to immunize as many individuals as possible. Most of the experimental and clinical reports associated with this program have dealt with reactogenicity and immunogenicity of candidate vaccines in selected groups of volunteers (1). Immunity has usually been measured in terms of titer of serum hemagglutination-inhibiting (HAI) antibody.

Because the virulence of the New Jersey strain of virus is unknown, experimental infection of human volunteers with it has not been attempted in this country, although Beare and Craig have inoculated volunteers with it in England (2). Animal experimentation has also been limited to avoid accidental spread of the virus.

During the past several years, we have developed mouse and squirrel monkey models for experimental influenza infections. In addition, we have containment facilities available and demonstrable competence in their use. Therefore, we were requested to carry out evaluations in mice and monkeys of the protective efficacy of both candidate vaccines and selected chemotherapeutic products. The research with mice was carried out by Dr. Scott whose paper follows.

Our initial experiments were carried out to determine the reaction of squirrel monkeys to intratracheal (IT) challenge with the New Jersey strain of virus. The monkeys were all males and ranged in weight from 500 - 900 gm. After careful examination, our veterinary staff concluded that they ranged in developmental status from pre-adolescence to young adulthood. Virus used was obtained from the Bureau of Biologics, Food and Drug Administration, as a 5th egg passage. We passed it once by intra-allantoic inoculation of eggs to grow a pool for routine use. We confirmed antigenic purity of the hemagglutinin using reference materials supplied by CDC.

The technique that we employed for IT inoculation of virus is diagrammed in fig. 1. Monkeys were anesthetized by intramuscular (IM) injection of 10 mg of ketamine; a plastic tube was inserted in the trachea and 0.5 ml of virus suspension was inoculated. In all experiments, at least 2 days were devoted to obtaining base-line data prior to infection; observations of infected monkeys were carried out for 10 days in initial experiments. Subsequently we shortened the observation period to 7 days. The determinations carried out included pharyngeal virus shedding, rectal temperature, body weight, total and differential leukocyte concentrations, hematocrit, respiratory rate, appetite, water consumption, presence of nasal discharge, coughing and sneezing, labored breathing and degree of activity. Some of the measurements made on 9 monkeys infected with 10^7 EID_{50} of the New Jersey strain are presented in fig. 2 for the first 7 days of illness; after this time there was considerable variation in the rate of convalescence, but all values had either returned to normal or were tending in that direction by the 10th day.

Temperature is presented in terms of % change from baseline. The curves for virus isolation, coughing, and nasal discharge, show the % of monkeys

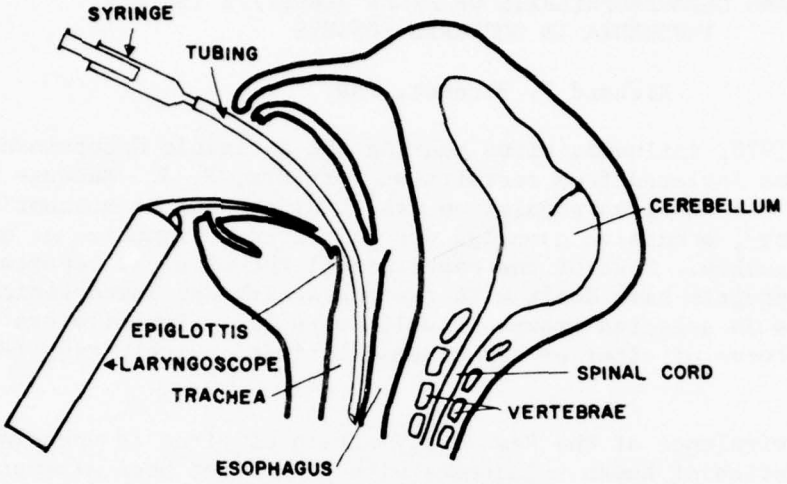

Fig. 1. Intratracheal inoculation technique (Diagrammatic representation)

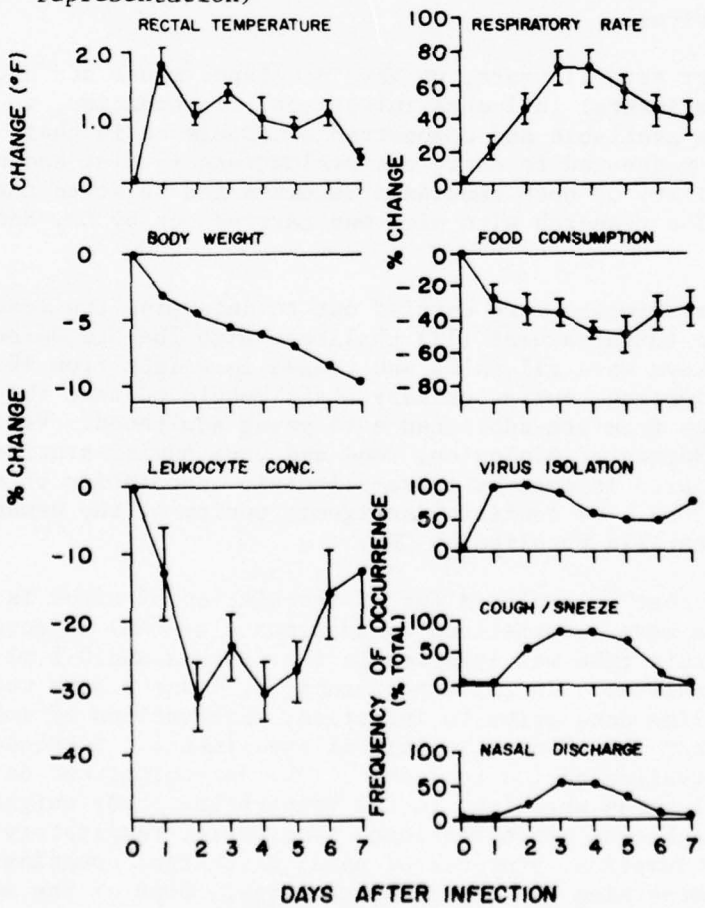

Fig. 2. Effect of IT inoculation of type A/NJ/76 influenza virus on squirrel monkeys (10^7 EID_{50})

responding. Activity and dyspnea were observed in all monkeys for 7 days and are not shown. These data indicated that IT instillation of the New Jersey strain of virus caused mild illness in monkeys. This observation is consistent with the findings of Beare and Craig, who inoculated volunteers and noted only mild illness, although all were infected (2). Since the model was intended for use in vaccine and drug evaluation experiments, a means of comparing results was required. For this purpose, an illness scoring system was devised. The mean illness scores calculated from the previously presented data from 9 monkeys are presented in Table I as an example of the system. These scores give the greatest weight to those parameters that were considered

TABLE I. SEVEN-DAY ILLNESS SCORE FOR SQUIRREL MONKEYS INFECTED WITH SWINE INFLUENZA VIRUS (A/NEW JERSEY/76)

PARAMETER AND SCORING PROCEDURE	MEAN SCORE ± SE FOR 9 INFECTED MONKEYS
1. Virus Shedding 1 for each day that virus was detected, plus 1 additional for each day that ≥ 66% eggs were positive	8.75 ± 1.25
2. *Temperature* 2 for each day that rectal temperature was ≥ 1 F above baseline	8.0 ± 1.31
3. Body weight 1 for each % loss of body weight at 7 days	9.9 ± 1.94
4. Leukocyte Concentration 1 for each day that total leukocyte count was ≤ 1500 than baseline	4.4 ± 0.84
5. Respiratory Rate 1 for each day that rate was ≥ 40% of baseline	3.5 ± 0.78
6. Appetite 1 for each day that biscuit consumption was < 33% of baseline	5.9 ± 0.58
7. Nasal Discharge 2 if nasal discharge observed for ≥ 2 days	2.0 ± 0
8. Coughing/Sneezing 2 if coughing was observed for ≥ 2 days	1.25 ± 0.37
9. Dyspnea 2 if labored breathing was observed for ≥ 2 days	2.0 ± 0
10. Activity 2 if activity was reduced for ≥ 2 days	2.0 ± 0
Overall mean illness score	47.6 ± 2.89

the most important and which could be objectively measured. Note that the first parameter, virus shedding, is an index of infection; the balance of the parameters represent signs of illness. Because we considered that virus shedding was very important, we have scored one for every day that virus was detected and have added an extra point for high concentrations. We have arbitrarily scored the balance of the signs of illness as given. A maximum score of 2 was assigned to each of the last 4 parameters, either because of increasing subjectivity of measurement, as in dyspnea and activity. Note that the mean score was 47.6; a critically-ill monkey would score $\geq$ 70, sham-inoculated monkeys would usually score $\leq$ 5.

After we were satisfied that a useful model had been developed, we initiated vaccine studies. The protocol for these studies is given in Table II. Vaccines were prepared from a recombinant of the PR-8 strain of virus with the New Jersey strain. Surface antigens of the recombinant, namely hemagglutinin and neuraminidase, were those of the New Jersey strain. The recombinant, designated X-53, was recently described in the literature by Palese and coworkers (3). The hemagglutinin of the X-53 was tested and confirmed as identical to that of the New Jersey strain by methods and materials provided by CDC. The 15 prior-illness controls were used at least 30 days after recovery from influenza. The nonvaccinated, sham-infected monkeys were

TABLE II. PROTOCOL FOR DETERMINATION OF THE EFFICACY OF SELECTED VACCINES AGAINST INFLUENZA A/NEW JERSEY/76 VIRUS

TREATMENT	NO. MONKEYS	DOSE OF VACCINE INOCULATED (CCA units)
Nonvaccinated sham-infected	3	0
Placebo controls	19	0
Prior-illness controls	15	0
Split-virus vaccine (1 injection)	7	400
Split-virus vaccine (2 injections)	7	400 (each inoculation)
Whole-virus vaccine	7	200
Combination vaccine (1 injection)	7	400 split-virus 25 whole-virus

inoculated IT with uninfected allantoic fluid at the same time as the other animals were inoculated with virus. Prior to vaccination, base-line serum samples were obtained, and a site for injection was prepared by shaving the leg; after vaccination, all monkeys were observed for 3 days for fever, anorexia, lethargy and localized reactions. Placebo-vaccinated monkeys were injected IM with 0.7 ml of saline; the split-virus vaccine groups were injected once or twice as indicated. The split-virus vaccine was manufactured by Wyeth Laboratories. The whole-virus vaccine was manufactured by Merck, Sharpe and Dohme. No attempt was made to re-assay either vaccine for

potency. Dilutions were based on the label claims of the manufacturer. The rationale for the use of a combination of vaccines was based on the report of Laver and Webster (4) that the immune response of hamsters to a subunit vaccine could be potentiated by simultaneous injection of an intact heterologous influenza virus. We altered the techique by using the homologous virus and mixing the vaccines prior to injection. In every case the dose of vaccine was diluted to a final volume of 0.7 ml and injected IM. All monkeys, except the placebo-controls, were febrile, with 1-2° of fever, for 24-48 hr after vaccination. No other systemic or local reactions were noted. Twenty-five days after vaccination, serum for HAI determinations was obtained; 5 days later all monkeys were challenged with virulent virus. Serum was then obtained 7 and 14 days after virulent virus exposure. HAI titers are given in fig. 3. For the purpose of calculation, seronegative monkeys were

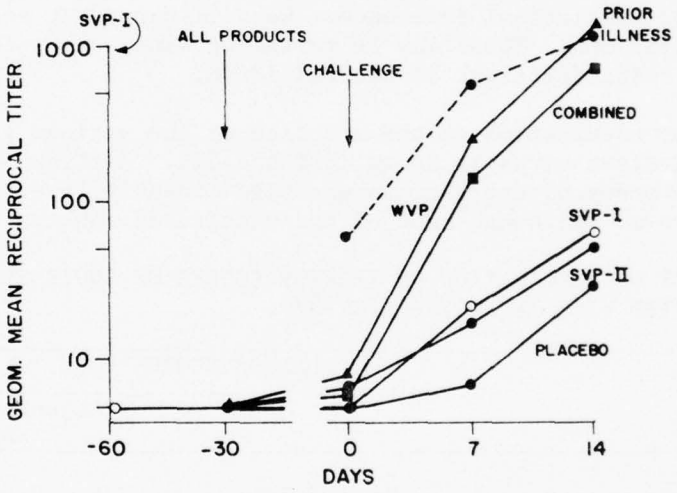

Fig. 3. HAI response of squirrel monkeys to vaccination with all products and challenged

arbitrarily assigned a titer of 1:5. Few of the monkeys showed increased titers after vaccination on day -30. Just prior to virulent-virus challenge on day 0, only 3 monkeys that received whole virus had titers, 2 vaccinated with 2 injections of split virus and one from the combination group. Hence, the very low or negative responses seen here on day 0. The titers that developed subsequent to challenge are interesting in that they provide a clue to immunologic memory in the various groups. The top curve shows the increase in titer in the prior-illness controls. Had we obtained daily samples, this curve probably would have had the characteristic shape of an anamnestic reaction. The increase shown by the placebo-controls was as expected; they had no titer prior to challenge, and only moderate amounts of antibody by the 7th day after virulent virus challenge. The increase in titer following challenge of the monkeys that received a single injection of split virus (SVP-I) was very similar to that seen in the controls. An exception to this was one monkey that had a 1:160 titer on day 7 and 1:1280 at 14 days. This suggests that most of this group did not receive sufficient stimulation from the vaccine to develop an immunologic memory. The reaction of the monkeys receiving 2 vaccinations with split-virus vaccine (SVP-II) was similar. At 14 days, the geometric mean titer of both split-virus groups did not differ significantly from the controls; however, considerable variation

was observed in the 2-injection group. One monkey had a 1:1280 titer at this time, while 3 had titers of $\leq$ 1:20. Although only 3 monkeys in the whole-virus group (WVP) and one in the combined group had titers 25 days after vaccination, all rapidly developed serum antibody after challenge. Again, had we obtained daily serum samples, we probably would have seen typical anamnestic reactions. At this point, it appeared that at least small amounts of whole-virus were necessary for the development of immunity to the New Jersey strain. With few exceptions, even 2 injections of split-virus were insufficient to stimulate serum HAI antibody production. It is of interest to note here that the serological reactions of these monkeys seem to resemble those that have been reported for human children and adolescents (1).

We have carried out some serum neutralization titrations. Results seem to correlate highly with those of the HAI tests, but too few determinations have been made for statistical inference. We will carry out serum anti-neuraminidase titrations. These may be revealing since the vaccines have been reported as being deficient in neuraminidase.

The effect of vaccination on the reaction of the various groups to challenge with virulent virus is shown in Table III. Analyses of variance revealed that the score of every group was significantly lower than that of the placebo controls. Although none of the vaccinated monkeys were as well

TABLE III. EFFECT OF VACCINATION ON ILLNESS SCORES OF SQUIRREL MONKEYS INFECTED WITH INFLUENZA A/NJ/8/76

TREATMENT	ILLNESS SCORES ± SE	
		Adjusted for virus shedding
Placebo controls	42.5 ± 2.15	33.5 ± 2.11
Noninfected sham-inoculated	4.7 ± 0.35**	
Prior-illness controls	17.0 ± 1.75**	13.9 ± 1.48*
Split-virus vaccine (1 injection)	24.9 ± 1.92**	15.7 + 1.66*
Split-virus vaccine (2 injections)	26.9 ± 2.48**	20.1 ± 1.94*
Whole-virus vaccine	26.0 ± 2.36**	17.0 ± 2.71*
Combination vaccine (1 injection)	29.9 ± 2.06**	20.1 ± 1.88**

*$P < 0.005$
**$P < 0.001$

protected as the prior-illness controls, there was no significant difference in the degree of protection afforded by the various vaccines or vaccination

schedules. Thus, although there were differences in serum antibody titers, we have been unable to detect any correlation between those titers and illness scores ($r = -0.2$ to $+0.4$). We must conclude, therefore, that serum HAI antibody is not operative in protecting squirrel monkeys against influenza due to the New Jersey strain of virus. It was of interest to us to determine where the effect of the vaccines was manifested. After analysis of the data, it appeared that the primary change was that of reduced severity of illness rather than lessened degree of infection. If we adjusted the illness scores by subtracting the effect of virus shedding, we could see that the scores of all groups of monkeys were still significantly different from the controls, indicating that the primary effect of vaccination had been to reduce the severity of illness (Table III).

To determine whether vaccination had any effect on infection and thereby on virus shedding, we calculated the mean duration of virus shedding as shown in Table IV. Only one of the vaccination procedures resulted in significantly less shedding of virus than controls, the 2-injection sequence of split-virus vaccine. Prior-illness controls, however, shed even less virus.

TABLE IV. VIRUS SHEDDING BY VACCINATED MONKEYS

TREATMENT	MEAN DAYS VIRUS SHEDDING $\pm$ SE	P
Placebo control	6.5 ± 0.29	
Prior-illness controls	2.7 ± 0.30	0.001
Split-virus vaccine (1 injection)	5.7 ± 0.61	NS
Split-virus vaccine (2 injections)	3.7 ± 0.57	0.001
Whole-virus vaccine	5.6 ± 0.37	NS
Combination vaccine (1 injection)	6.0 ± 0.44	NS

Analyses of the various illness parameters to determine which were most affected by prior vaccination, revealed that almost all were affected to some degree. Histograms that present the effects of the various vaccines on 4 selected illness parameters are shown in fig. 4. On the upper part are 2 common signs of influenza, nasal discharge and coughing. With one exception, all groups were significantly lower than the placebo controls. The exception, in the case of nasal discharge, was the group that received the combined vaccine. This group did not differ significantly from the controls in 3 of the 4 parameters shown. The loss of weight seen on the lower left generally seemed to be caused by anorexia, but there were some cases of loss of weight in the presence of normal appetite. The respiratory rate data provide even more evidence of lessened disease in vaccinated monkeys. It is pertinent to speculate as to why the prior-illness controls were not

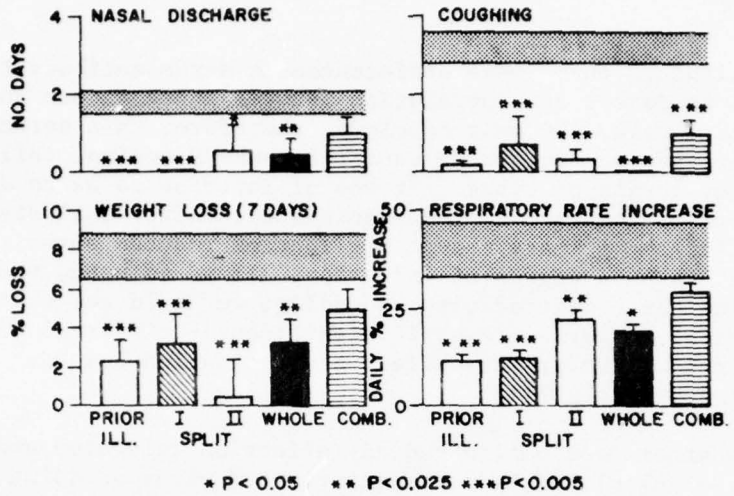

Fig. 4. Analysis of 4 illness parameters compared to placebo-vaccinated, infected controls (dotted band)

completely protected against subsequent challenge. The most plausible reason is the high dose of virus used for challenge. Whether lower challenge doses would result in altered responses in this and the subsequently-discussed studies would require additional investigation.

In summary of the vaccination studies, 1) all vaccines and vaccination procedures effectively reduced illness in squirrel monkeys, but not to the extent afforded by recovery from prior illness; 2) virus shedding was reduced only in the case of the 2-injection sequence of the split-virus vaccine, but not to the degree seen in the prior-illness controls; and 3) serum HAI antibody did not appear to play a significant role in protection against this disease in squirrel monkeys. It is possible that serum neutralizing antibody is also without effect. We have no information on either local or cell-mediated immunity. We hope that we will have information concerning concentrations of antibody against neuraminidase in the near future.

Finally, the results of experiments on chemoprophylaxis and chemotherapy of type A/New Jersey virus in monkeys are presented. We investigated the activity of both amantadine HCl and its analogue, rimantadine HCl for their effect on swine influenza. Preliminary studies had showed that the activity of rimantadine did not differ from that of amantadine. We therefore concentrated on the latter, especially because its use against influenza has already been approved by the Food and Drug Administration. In all experiments the drug was dissolved in water and given orally; 0.5 ml containing one-half of the prescribed dose was given in the morning and the balance in the late afternoon. Control monkeys were given the same volume of sterile water. We combined the data from 2 experiments, after statistical analysis had shown that there was no difference between the responses of the control monkeys in the 2 experiments. The illness scores are given in Table V. The monkeys treated prophylactically were given amantadine 24 hr prior to virus challenge. The drug was then given twice daily through day 7. For therapy experiments, amantadine was given 48 hr after challenge, a time when overt illness had developed, and was continued through day 7. The illness scores given here were all significantly lower than those of the water-control monkeys, indicating that amantadine was effective both prophylactically and therapeutically.

TABLE V. EFFECT OF ORALLY-ADMINISTERED AMANTADINE UPON ILLNESS SCORES OF SQUIRREL MONKEYS INFECTED WITH INFLUENZA A/NJ/8/76

TREATMENT	NO.	DOSE mg/kg/day	ILLNESS SCORE ± SE	
				Adjusted for virus shedding
Water control	8	0	45.9 ± 2.9	38.9 ± 3.2
Prophylactic-therapeutic	4	7.5	21.2 ± 4.9*	15.4 ± 3.7*
	4	15.0	23.4 ± 3.8*	16.4 ± 2.8*
Therapeutic beginning at 48 hr	4	7.5	24.9 ± 2.5*	20.9 ± 4.6*
	4	15.0	18.2 ± 1.4*	14.7 ± 1.3*

*$P < 0.005$

As with the vaccinated monkeys studies, we attempted to separate infection, as exemplified by virus shedding, from illness. It can be seen that after subtracting the effect of virus shedding from the illness scores, the resulting values still differ significantly, indicating that amantadine significantly reduced the severity of illness (Table V). Table VI shows the totally unexpected observation that only therapeutically administered amantadine significantly reduced virus shedding. It should be remembered that therapy was not initiated until the second day; thus, it is obvious that virus shedding was almost totally inhibited by amantadine as soon as it was given. Analysis of the other parameters that constitute the illness score revealed a lessening in all of them after prophylaxis or therapy, rather than an effect on any particular ones. No clear-cut effect of dose was discernible. In summary, 1) oral administration of 7.5-15 mg/kg of amantadine HCl to squirrel monkeys reduced the degree of illness caused by influenza A/New Jersey/8/76 virus. The drug was effective prophylactically (starting 24 hr prior to infection) and therapeutically (48 hr postinfection), and 2) therapeutic administration reduced virus shedding; prophylactic administration did not.

In conclusion, it is of interest to speculate upon the relationship of our findings to the national swine influenza program. If we assume that data obtained from monkeys will apply to man, then we can say that immunization will be only partially effective. Individuals who are infected will have only minimal signs of disease, but will be capable of disseminating the virus to others. However, amantadine will successfully inhibit shedding of virus. Only with additional research can we determine whether vaccination procedures can be developed that will effectively inhibit infection as well as illness.

TABLE VI. EFFECT OF AMANTADINE UPON PHARYNGEAL VIRUS SHEDDING

TREATMENT	NO.	DOSE mg/kg/day	MEAN DAYS VIRUS SHEDDING ± SE
Water control	8	0	5.75 ± 0.67
Prophylatic-therapeutic	4	7.5	6.25 ± 0.75
	4	15.0	4.50 ± 0.65
Therapeutic beginning at 48 hr	4	7.5	2.75 ± 0.85*
	4	15.0	2.0 ± 0**

*P 0.025
**P 0.005

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DISCUSSION

The question was asked about how the doses of amantadine given to to the monkeys in this study compared to the dose level suitable for use in man. The answer was that the human dose was 1/3 the lowest dose used in the study. Another question concerned whether this primate model allowed graduations in the level of virulence among various strains of influenza, i.e., A/Victoria, Hong Kong (B), and swine. This model may provide information on differences in virulence but has not been employed in this manner.

IMMUNO-AND CHEMOPROPHYLAXIS OF A/NEW JERSEY/76 (SWINE) INFLUENZA IN MICE

George H. Scott, PhD

We examined the effects of amantadine, rimantadine and ribavirin on type A/New Jersey influenza infections and evaluated the whole-virus vaccine on the basis of antibody titers elicited by, and resistance of, vaccinated mice to subsequent infections. The mice used were Swiss females obtained from the "Sendai-free" Montreal colony of Charles River Laboratories.

The mouse-virulent virus used was derived from the 6th egg passage material described by Dr. Berendt (preceding paper). Its virulence was enhanced through 9 serial passages in mice by intranasal (IN) challenge with infected lung homogenates. A working virus stock was then prepared in 10-day-old embryonated eggs and stored in 5 ml volumes at -60 C. The titer of the infected allantoic fluid was $10^{7.7}$ EID_{50}/ml. The antigenic identity of the virus after passage was confirmed using serologic reagents provided by CDC.

Marked differences in survival were observed between young mice (21 days) and adult mice (6-9 weeks) following infection with this virus. Intranasal doses of $10^{3.8}$ EID_{50} killed half of the 21-day mice inoculated and the mean time to death was < 6 days. The LD_{50} for older mice was estimated to be > $10^{5.8}$ EID_{50}, and even higher doses usually resulted in fewer deaths, suggesting that viral interference was occurring. High virus titers in the lung, > 10^{7} EID_{50}, were observed at 1 and 3 days postinfection in both age groups of mice. Virus levels gradually declined and disappeared by 9-11 days postinfection; this is typical for influenza viruses in mice. Marked consolidation of the lung was seen in both young and old mice at 6 days; with the older mice, increased lung weight served as a useful index of the degree of the infection. The utility of lung weight as an index was corroborated by densitometric measurements made on infected lung sections by a procedure described in the next paper by Major Hall.

Our initial study employed adult mice and tested the prophylactic and therapeutic efficacies of the 3 drugs when administered in drinking water. Based on an estimated consumption of 6 ml of water daily, the dose of drug in each case was 60 mg/kg body wt/day. For prophylaxis, drug administration commenced 24 hr prior to virus challenge and continued through 14 days. For therapy, drug administration commenced 15 hr after challenge and was continued through 14 days. The criteria for evaluating drug efficacy included: lung virus titers based upon virus recovered on days 3 and 7, the extent of lung lesions (scored 0 - 4, i.e., negative to total consolidation), and mean lung weights at 7 days.

Virus replicated in the lungs of infected, untreated mice to $10^{7.1}$ EID_{50}/lung within 3 days, but had diminished by 1.6 logs on day 7 (Table I). On the average, 40% of each infected lung had typical influenza lesions, and lung weights increased almost 3-fold as compared to lungs of noninfected mice. None of the drugs significantly affected lung virus titers at 3 days. By 7 days, however, titers were significantly lower in mice treated therapeutically with ribavirin. The development of lung pathology, as reflected by lung weight, was diminished only by rimantadine given prophylactically

and by ribavirin given therapeutically. Variations were too large in lung lesion scores in these adult mice to permit discrimination among treatments; mortality rates were too low (0-10%) for meaningful comparisons.

TABLE I. EFFECT OF DRUGS GIVEN ORALLY TO 8-WK-OLD MICE INFECTED WITH TYPE A/NJ INFLUENZA VIRUS

DRUG AND TREATMENT	GM LUNG VIRUS TITERS (Log_{10} EID_{50}/lung)		MEAN LUNG LESION SCORES	MEAN LUNG WEIGHT (mg)
	Day after infection			
	3	7	7	7
Noninfected controls	-	-	0	142*
Prophylactic				
Amantadine	7.7	5.8	0.9	310
Rimantadine	7.3	5.2	0.6	272
Ribavirin	7.0	4.8	2.0	332
Therapeutic				
Amantadine	6.8	4.2	1.2	324
Rimantadine	6.8	5.0	1.7	352
Ribavirin	7.1	3.6*	1.0	266*
Infected controls	7.1	5.5	1.7	390

*P < 0.05 compared to infected controls

Similar studies have been completed using younger (3-week-old), more susceptible mice and are summarized in Table II. The drugs did not affect

TABLE II. EFFECT OF DRUGS GIVEN ORALLY TO 3-WK-OLD MICE (N = 5) INFECTED WITH TYPE A/NJ INFLUENZA VIRUS

DRUG AND TREATMENT	GM LUNG VIRUS TITER (Log_{10} EID_{50}/lung)		MEAN LUNG LESION SCORE, DAY 7	% SURVIVAL (n = 30)	MEAN DAY OF DEATH
	3	7			
Prophylactic					
Amantadine	7.7	6.0	1.4	83	10.6
Rimantadine	7.5	5.8	1.0	93	10.5
Ribavirin	7.4	6.0	2.6	90	9.0
Therapeutic					
Amantadine	7.2	4.1*	1.0	63	7.4
Rimantadine	7.3	4.2*	1.5	83	6.0
Ribavirin	6.6	5.2	2.8	43	5.3
Infected controls	7.4	5.8	3.0	0	5.8

*P < 0.05 compared to controls

lung virus titers measured on day 3. But by 7 days, virus titers and lung lesion scores were reduced by therapeutic treatment with amantadine and rimantadine, but not with ribavirin. All 3 drugs, administered prophylactically, extended the survival time and effected 80-90% survival compared with a uniformally lethal response in untreated controls. Survival rates were lower when amantadine and ribavirin treatment was delayed for 15 hr postinfection, but were significantly higher than for untreated mice. Rimantadine was equally effective by both treatment schedules.

Another study in young mice was conducted to test the efficacy of the 3 drugs as a function of the time when treatment was initiated. The drugs were given to groups of mice beginning 48 hr before infection and 6, 24, or 96 hr after infection. In all cases treatment was discontinued at 14 days postinfection. Survival data, summarized in Fig. 1, clearly indicate that early treatment is desirable. Eighty-seven to 100% of the mice survived when the drugs were given either before or within 6 hr after infection; survival declined as treatment was delayed, but each of the drugs effectively reduced mortality even when treatment was delayed for as long as 96 hr.

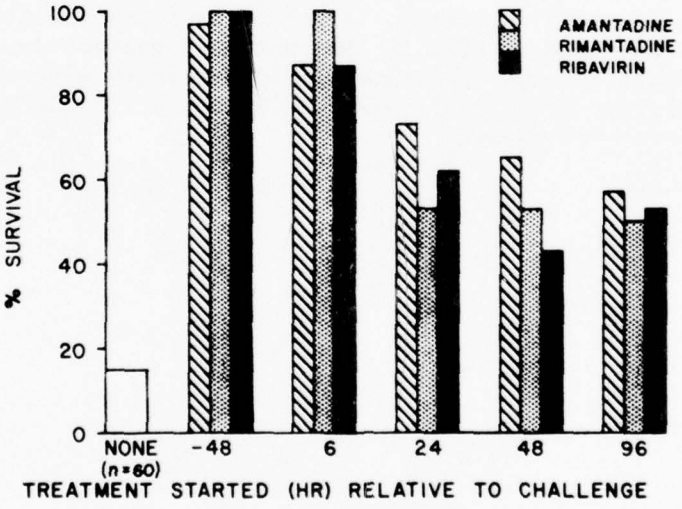

Fig. 1. Effects of initiation time on drug efficacy for the treatment of type A/NJ influenza infection in mice

To review, the prophylactic and therapeutic efficacy of all 3 drugs has been demonstrated on the basis of one or more criteria. In the less susceptible adult mice that did not succumb to infection, therapeutic treatment with ribavirin reduced lung virus titers and lung pathologic changes measured 7 days after challenge; prophylactic treatment with rimantadine reduced the lung lesion scores. Lung virus titers in younger, more susceptible mice were reduced following therapy with both amantadine and rimantadine, but not with ribavirin. All 3 drugs given either prophylactically or therapeutically effectively increased the survival rate of young mice following lethal virus challenge.

The second portion of this paper presents evaluation of the whole-virus vaccine prepared by Merck, Sharpe & Dohme in mice. Only a limited amount (18 ml) of this vaccine was available for use in mice. Most of this, and all of the split-virus vaccine, was preempted by the higher priority studies conducted in monkeys. It was necessary to employ adult mice in this study

we were interested in measuring antibody levels and resistance several weeks after vaccination. As indicated earlier most of these older mice could be expected to survive infection with swine influenza so our evaluation had to be based on parameters other than survival.

We compared IN and IP vaccination with the whole-virus vaccine on the basis of local and circulating antibody responses in adult mice, and protection against challenge with mouse-virulent homologous virus. We also attempted to obtain preliminary information on the duration of immunity. Approximately 90 mice were vaccinated by each route with 80 CCA units of the whole-virus vaccine. After collection of sera and bronchoalveolar wash fluids to test for antibody levels 17 days postvaccination, one-half of the mice were challenged with virulent virus. Sham-vaccinated control mice, and mice which had recovered from previous infection, were also challenged. At 60 days postvaccination, the remaining mice and additional sham-treated and convalescent mice were challenged. Mice from group were killed 3 and 7 days after virus challenge. Their lungs were scored for gross pathology, weighed, then homogenized in infusion broth and assayed for virus. Serum HAI titers obtained at the time of virus challenge, 17 and 60 days postimmunization, for each group are summarized in Fig. 2. HAI antibody was not detected in bronchoalveolar washes from mice vaccinated by either route with the killed vaccine. Low HAI activity (1:2) was present in broncheoalveolar washes of

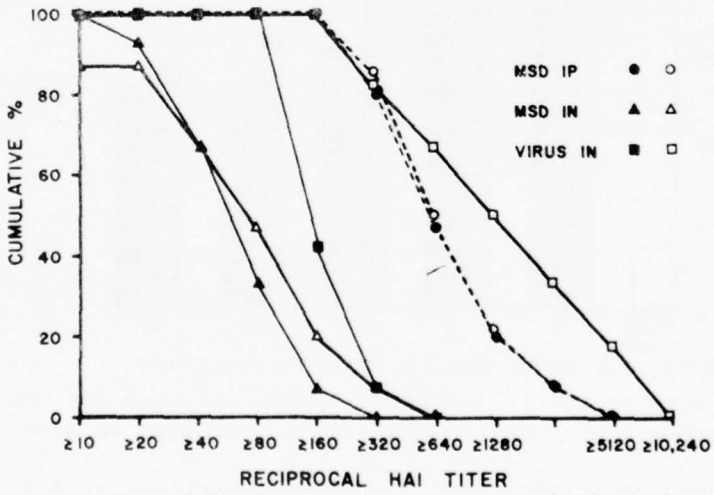

Fig. 2. Serum HAI antibody in mice vaccinated 17 (solid) or 60 (open) days before with Merck, Sharpe & Dohme type A/NJ influenza virus vaccine

previously-infected mice after 17 days, but was not detectable after 60 days. However, all of the vaccinated mice seroconverted. All IP vaccinated mice had titers of $\leq$ 1:160 within 17 days. Although all of the IN vaccinated mice had seroconverted by 17 days, titers were lower (1:40) than in IP vaccinated or previously infected mice. After 2 months, antibody could not be detected in 13% of the IN vaccinated mice; antibody in IP vaccinated mice was not diminished and patterns were similar to those observed 17 days postvaccination. By 60 days postvaccination, the number of mice in our convalescent group had been diminished through sampling, and a higher than expected mortality rate. Consequently none were available for challenge at this time,

and the plotted antibody titers represent only 7 mice. The apparent increase in antibody titers between 17 and 60 days postvaccination suggests that antigenic stimulus resulting from infection persisted longer than expected and antigen processing had not been completed at 17 days in these surviving mice.

The incidence of infection and lung pathologic changes as indicated by gross lung lesions and lung weights following virus challenge of each group are presented in Table III. These data are consistent with the antibody patterns. All of the IP immunized mice were solidly protected against infection. Virus was not detected in their lungs, nor was there evidence of lung lesions following IN challenge with $10^{6.0}$ EID_{50} of virulent virus, a dose that infected 100% of the nonimmunized mice. Mice vaccinated by the IN route appeared less resistant, especially when tested 60 days postvaccination. Only 1 of 5 became infected when challenged after 17 days, but they all became infected when challenged at 60 days. Although lungs from the IN

TABLE III. RESISTANCE TO TYPE A/NJ INFLUENZA VIRUS INFECTION BY PREVIOUSLY INFECTED MICE AND MICE VACCINATED 17 OR 60 DAYS BEFORE WITH MSD WHOLE VIRUS VACCINE

VACCINE AND ROUTE	LUNG VIRUS ISOLATIONS (No. positive/5)		LUNG LESIONS AT 7 DAYS		MEAN LUNG WEIGHT (mg)
	DAY 3	Day 7	No. pos./10	Score	
17 Days					
HIB-IN	5	5	10	2.1	307
MSD-IP	0	0	0	-	202*
MSD-IN	1	0	2	0.7	245*
Live virus-IN	0	0	6	0.8	268
60 Days					
HIB-IN	5	5	10	1.6	301
MSD-IP	0	0	0	-	206*
MSD-IN	5	1	4	0.5	247*

*$P < 0.05$ compared to nonimmunized controls

immunized mice weighed significantly less than those from nonimmunized mice following challenge at 17 or 60 days, lesions had developed in 20-40% of the lungs within 7 days of challenge.

We attempted to administer the same amount of antigen by the IP and IN routes, but it is probable that a portion of that given IN was swallowed or expelled from the nose so that a smaller amount reached the respiratory tract than was deposited in the peritoneal cavity. This fortuitous difference in antigenic mass may in part account for the observed differences in immune response achieved by the 2 routes, but at the same time it affords an opportunity to note that resistance to infection appears to correlate well with the level of circulating antibody in mice.

The importance of antibody was emphasized further by demonstrating that passively transferred immune serum conferred protection on recipient mice.

Immune sera from previously infected mice with a neutralizing titer of 1:160 was administered in 0.1-ml amounts by the IP or IN routes to groups of mice. Two and 24 hr after receiving the antisera, mice from each group were challenged IN with 10^6 EID_{50} of virulent virus. Lungs from these mice were assayed for virus 3 days after challenge; all groups were essentially unchanged at 21 days.

As shown in Table IV, all of the control mice became infected and > 70% died. By contrast, virus was detected only in 1 of 5 of the mice that received antibody IN prior to virus challenge; at 7 days none had died.

TABLE IV. PASSIVE IMMUNIZATION OF MICE AGAINST TYPE A/NEW JERSEY INFECTION

TIME OF CHALLENGE (hr)	CHALLENGE ROUTE	LUNG VIRUS (No. pos./5)	GEOM. MEAN TITER (Log_{10}/lung)	% SURVIVAL AT 7 DAYS (n=35)
2	None	5	6.6	29
	IP	4	5.4	77
	IN	1	5.5	100
24	IP	5	6.5	86
	IN	1	6.1	100

Antiserum injected by the IP route had little effect on the incidence of infection, but substantially reduced mortality resulting from virus challenge at either 2 or 24 hr.

Unlike squirrel monkeys, which did not consistently produce antibody but were protected, vaccination invariably elicited HAI antibody in mice; the degree of protection appeared to be correlated with the magnitude of this response. The experiments in 2 widely different animal models strongly suggest a role for more than one mechanism of protection. Regardless of the immune mechanism involved, the efficacy of the whole-virus vaccine has been demonstrated; it ameliorated illness in monkeys and protected mice against lethal infections due to the New Jersey strain.

Using the mouse model we also demonstrated that amantadine, rimantadine, and ribavirin have significant prophylactic and therapeutic effect in the treatment of respiratory infections induced by type A/New Jersey influenza virus. Although none of the drugs prevented infection, the severity of the resulting illness was diminished, and treatment increased survival even when initiated after the onset of bronchopneumonia. If it can be assumed that results from these experiments in mice reflect the situation in the human population, then all 3 drugs would be about equally active against type A influenza infections. Of the 3 drugs, amantadine has been more thoroughly studied, and it currently enjoys FDA approval for use against influenza A infections in humans. However, the spectrum of organisms susceptible to amantadine is fairly limited. It does not include type B influenza virus,

nor many of the DNA viruses.

Ribavirin, on the other hand, is reported to have much broader antiviral activity encompassing a number of both RNA and DNA viruses. Previous studies by Walker and Stephen (1) of this Institute have indicated that ribavirin is especially efficacious for viral respiratory infections when administered as an aerosol. Hopefully, continuing studies on the toxicology, mutagenicity, teratology and other areas related to its safe clinical use will eventually permit its addition to the list of approved antiviral drugs.

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THE USE OF A PHOTODENSITOMETRIC TECHNIQUE TO EVALUATE THE EFFICACY OF ANTIVIRAL COMPOUNDS AGAINST SWINE INFLUENZA (INFLUENZA A/NEW JERSEY) INFECTION IN MICE

William C. Hall, VMD

Animal models for evaluation of potential antiviral compounds have frequently been developed using human disease pathogens adapted to produce high mortality. The efficacy of the compounds is thus measured by survival rate of treated, infected animals compared with untreated controls. These model systems are unnatural and unrealistic when the purpose intended for the drug is to treat diseases of man and animals characterized by high morbidity and low mortality (1). During studies of swine influenza (influenza A/New Jersey), it was observed that the mouse-adapted virus had reduced virulence for adult mice and thus resembled natural influenza infections of man (2). It was necessary to seek another end-point to measure the effectiveness of antiviral compounds on the respiratory disease in mice.

Numerous systems have been proposed to grade morphologic changes occurring in the lungs of animals infected with various respiratory disease-producing agents. Many are not completely objective. The majority of the reliable procedures are morphometric where various measurements can be made of pulmonary structure to obtain consistent quantitative values (3). These techniques require considerable time to perform and thus are inefficient when numerous specimens must be examined.

A technique, described for studying emphysema in pigs and rats, was based on the postulate that emphysematous lung sections are less optically dense than normal lung and that differences could be quantified by densitometry (4). A similar procedure has been employed to determine degree of erythrocyte aggregation in vitro (5). Likewise, pulmonary disease characterized by edema and cell infiltration into alveolar spaces and the interstitium should increase optical density. If this premise were correct, the effect of drugs, toxins, or chemicals, on the pulmonary response could be quantitatively assessed and compared in an unbiased, completely objective manner. This technique was utilized to compare the efficacy of antiviral chemotherapeutic agents in mice infected with swine influenza virus. Three groups of mice were prophylactically treated with amantadine, rimantadine or ribavirin in of 0.25 mg/ml (≈60 mg/kg/mouse/day) 24 hr prior to intranasal (IN) instillation of $10^{6.1}$ EID_{50} of influenza virus in 0.05 ml. Three other groups were treated therapeutically with each of the drugs beginning 15 hr after virus inoculation. In all groups, treatment was continued for 7 days after inoculation of the virus. Two control groups of mice were utilized, one was infected but not treated and the other was neither infected nor treated.

Seven days after infection, all mice were killed by cervical dislocation. Lungs of half the mice from each group, were dissected from surrounding tissue and weighed to the nearest milligram. The cutaneous tissue of the neck was dissected free from the trachea and the thorax was opened in the remaining mice. A 21-gauge hypodermic needle attached to a flask containing 2% glutaraldehyde, pH 7.2, was inserted into the trachea and the lungs were

perfused in situ for 30 sec at a pressure 20 cm H_2O. To maintain pulmonary distension a small vascular clamp was placed on the trachea. Lungs were then dissected free and suspended in 2% glutaraldehyde.

Perfused lungs were subsequently fixed in 10% buffered neutral formalin and embedded whole in paraffin. Paraffin blocks were sectioned horizontally through the lung lobes and three 6-µm sections were taken at 1.5-mm intervals (Fig. 1). The sections were deparaffinized and stained with hematoxylin and eosin (6). Staining was controlled so that variation was not observed among

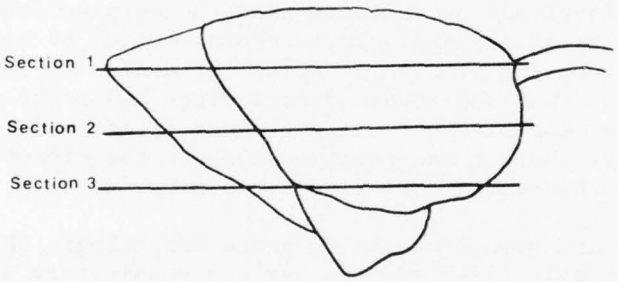

Fig. 1. Schematic drawing of lateral view of mouse lung showing the level of sectioning.

The sections of lung were placed on the mechanical stage of a Zeiss Ultraphot II and voltage was adjusted to 15; a filter with peak absorption at 580 and 645 nm was placed between the light source and specimen, and the image was projected onto a viewing screen using the 2.5 X objective; total magnification was 27 X. Twenty to 35 measurements were made on each section directly from the same location on the viewing screen using a photodensitometer. Optical densities were measured through 0.5-mm diameter holes spaced 0.85 mm apart in a rectangle of brass foil which was placed on the microscope slide. The photovolt meter was adjusted to 0 optical density at a place on the slide where there was no tissue. The lung at each perforation was viewed independently for artifacts or extraneous tissue and, if present, the field was eliminated from the sample. Essentially, only alveoli and small bronchioles were measured. The above procedure was repeated for each of the 3 lung sections and the mean optical density of each lung was calculated.

Data on lung weight and optical density were analyzed by one-way analysis of variance using the least significant difference test to determine differences between groups. A linear regression analysis was used to determine correlation of mean optical density and mean lung weight of each group (7).

Lesions of swine influenza in untreated infected controls consisted of multifocal areas of bronchiolitis surrounded by thickening of alveoli, minimal to moderate inflammatory cell infiltration, and edema in alveolar spaces. In contrast, lungs of uninfected control mice were free of lesions.

These morphologic changes were reflected by an almost 3-fold increase in pulmonary optical density in the untreated, infected control group compared to the uninfected controls (Table 1. Fig. 2). Consistent readings were obtained by repeating the procedure. In general, with the exception of mice treated with rimantadine 15 hr after infection, the mean optical density of treated mice was midway between the values of uninfected and infected, untreated controls and differed significantly from the latter ($P < 0.01$). By analyzing lung weights of the various groups, there was significant reduction of lung weight only for mice given rimantadine prior to virus inoculation and mice give ribavirin 15 hr subsequent to infection compared with infected-untreated ocntrols ($P < 0.01$). However, the trend was the same as that observed by densitometry, with the lung weights of infected-treated animals falling between values observed for both control groups. The linear regression analysis of the means of pulmonary optical density against means of lung weight gave a coefficient of correlation of 0.92.

TABLE 1. PROPHYLACTIC AND THERAPEUTIC EFFECTS OF ANAMTADINE, RIMANTADINE, OR RIBAVIRIN ON LUNG OPTICAL DENSITY AnD WEIGHT IN INFLUENZA A/NEW JERSEY-INFECTED MICE.

	MEAN VALUE ± SE	
GROUP	Optical density	Lung weight (mg)
Prophylaxis + Therapy		
Amantadine	0.069 ± 0.004[a,b]	310 ± 14[b]
Rimantadine	0.068 ± 0.005[a,b]	272 ± 17[a,b]
Ribavirin	0.071 ± 0.008[a,b]	332 ± 19[a,b]
Therapy		
Amantadine	0.063 ± 0.002[a]	324 ± 24[b]
Rimantadine	0.084 ± 0.011[b]	352 ± 19[a,b]
Ribavirin	0.056 ± 0.003[a]	266 ± 19[a,b]
Controls		
Infected-untreated	0.098 ± 0.011[b]	390 ± 36[b]
Uninfected	0.038 ± 0.001	142 ± 2

[a]Differs significantly from infected-untreated control ($P < 0.01$).
[b]Differs significantly from uninfected control ($P < 0.01$).

With influenza A/New Jersey infection, good correlation between mean optical density and mean lung weight for the various groups ($r = 0.92$) suggests that both methods have merit in the assessment of antiviral activity. The advantage of the optical density technique over lung weight in this experiment is the increased sensitivity observed with the former method. Reasons for this are: 1) the variability of optical density measurements were independent of lung size; and 3) structures not contributing to the pathologic process such as blood, large airways, tissue artifacts, etc., were selectively eliminated by viewing each field prior to obtaining an optical density measurement. In addition, this technique utilized photomicrography equipment already

found in most laboratories, thus precluding the expense of more elaborate apparatus.

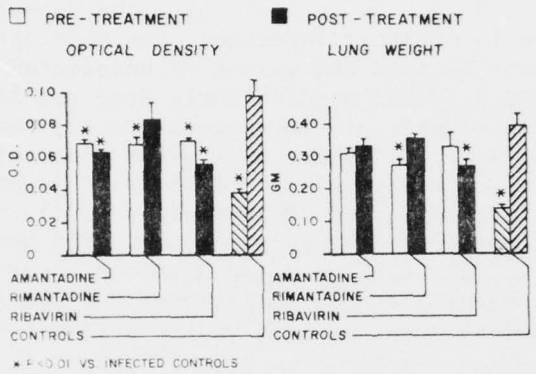

Fig. 2. Effect of antiviral agents on lungs of mice infected with swine influenza.

We feel this technique offers promise as a means of evaluating the response of lungs to a variety of infectious and toxic agents and will provide an objective, unbiased measure of the therapeutic value of various drugs for treating various respiratory diseases.

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ELECTRON MICROSCOPIC STUDIES OF INFLUENZA VIRUS

John D. White, PhD

Images produced by scanning electron microscopy (SEM) relate closely to our visual experiences in the real world because they have a 3-dimensional quality. It is easy to recognize structures in a SEM micrograph of lung. With assurance, we can identify alveoli, septa, capillaries, and cells within capillaries. Previous experiences with gross appearance, light microscopy and transmission electron microscopy (TEM) provided the bases for these recognitions. When we looked at the scanning image of influenza virions prepared for SEM in the usual manner, i.e., fixed in glutaraldehyde, osmicated, dehydrated in alcohol, dried, and sputter-coated with gold, there were many pleomorphic structures within the size range of 70-150 nm. Negative staining of the same infected allantoic fluid confirmed the pleomorphic nature of this virus and illustrated the basic structure of an influenza virion. Each virion is covered with a fringe of 2 morphologically distinct glycoprotein projections or spikes which are attached to a bilayered shell or envelope. Hemagglutinin and neuraminidase activity are localized in these spikes. Segments of double helical nucleocapsids can be seen in virions, the interior of which has been penetrated by the negative stain. Pleomorphism was most striking in the influenza strain, A/NJ/8/76, which had been adapted as described and used by Dr. Scott in mice. Bizarre shapes were frequent, although rods and spherical forms were commonly seen. The A/NJ/8/76 strain used by Dr. Berendt in monkeys was also pleomorphic but, not to the degree that the mouse strain was.

The use of labeled immunoglobulins as antigen markers in microscopy is well established. Since Coons et al. (1) description of fluoresceinated immunoglobulins in 1941, the use of heavy metals, enzymes, other complex molecules, and most recently bacteriophage, has captured the interest of microscopists. We have modified the procedure of Kumen et al. (2) to prepare an immunologic marker for the present studies. A goat immune serum against human γG immunoglobulin was purified by immunoadsorption with purified homologous antigen and coupled to T4 bacteriophage with glutaraldehyde. This reagent was then used in an indirect technique to detect specific antibody precipitated by influenza antigen in a manner similar to indirect immunofluorescence staining. The antibody used in the intermediate layer was obtained from the serum of a subject immunized with an experimental vaccine. The HA titer was > 1:1280.

Bacteriophage T4 could be recognized by SEM. The tail piece and hexagonal shape of the head were clearly shown. A sample of infected allantoic fluid was treated sequentially with specific immunoglobulin and the phage-labeled antiglobulin reagent, and then prepared for SEM examination. In the control, normal serum was substituted for the anti-influenza serum. In some slides phage particles surrounded the virion. Having established the efficacy of the reagents, tissues from mice infected with aerosols of the A/NJ strain were examined. These mice were killed between 1 and 3 days after exposure.

Islands of ciliated cells were separated by patches of nonciliated cells whose surface was covered by short microvilli. Distribution and numbers of

ciliated cells may vary with location but line the nasal cavities, trachea, bronchi, and major orders of bronchioles. The most frequent changes seen in these tissues were: desquamation and reduction in area of ciliated surfaces; increased numbers of cells on epithelial surfaces; and, at higher magnification, the presence of pleomorphic structures on the surface of cilia. These measured ≃ 100 nm and were specifically tagged by the immunolabeling. No immunolabeling was seen in tissues from normal animals. Several bacteriophage particles could be seen attached to particles on cilia. When comparable tissue was examined by TEM virions were found attached to cilia.

The terminal bronchiole was lined with ciliated and nonciliated cells. The latter, called Clara cells, are predominant in the mouse. The terminal bronchiole branches into a respiratory bronchiole lined by simple cuboidal epithelium. Alveolar ducts and alveoli are the functional unit of the lung where gas exchange occurs. Alveoli and the delicate walls which separate them were distended. When the lesion which occurs at this site was examined by SEM, the wall of the bronchiole was greatly thickened, as were the alveolar septa. There were plump necrotic cells lining the bronchiole and surrounding alveoli. A bronchiole might be lined with ciliated pseudostratified columnar epithelium, which became necrotic, died and sloughed away leaving the nonciliated basal cells. Subsequently mild pneumonitis developed which was characterized by septal thickening and proliferation of macrophages, lymphocytes, and edema. In addition, there was cellular necrosis; virus could be found admixed with the cellular debris.

In summary, features of the swine influenza virus and the disease caused in mice as seen by electron microscopy were presented with slides. This virus does not appear to be unique with respect to morphology or the disease elicited in mice. Morphologically the virus is identical to other influenza A strains and it produces a necrotizing lesion of the respiratory tract which is confined primarily to ciliated epithelium and secondarily involves portions of the lung, causing edema, septal thickening and infiltration with inflammatory cells. The use of a morphologic immunolabel in SEM was illustrated.

ACKNOWLEDGEMENT

The viruses used in these studies were provided by Drs. Berendt and Scott who also exposed animals to the viruses. I am also grateful to MAJ Hall, Frances Shirey and Mary Mullen for their patience and assistance as well as to Dr. Stephen Leppla for his work in the preparation of immunoglobulins and bacteriophage used here.

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DISCUSSION
OF REVIEW OF RESEARCH DIVISIONAL ACTIVITIES BY THE
DIVISION CHIEFS

Dr. Woodward stated that the Armed Forces Epidemiological Board had met at Edgewood Arsenal a week earlier. Four members were now present (Doctors Denny, Jordan, Rammelkamp, and Woodward). The Board was briefed comprehensively by the Preventive Medicine Officers of the 3 services. The briefings included, but were not limited to, such diseases as Marburg, plague, RMSF, other rickettsial diseases, and botulinum toxemias. Many of these diseases have been studied by this Institute for years. The data from these briefings could be used as a reference for the meetings with Division Chiefs and perhaps at the Executive Session. Colonel Metzger indicated that a meeting was scheduled with members of USAMRDC and that there was a possibility that USAMRIID would become involved in Marburg virus studies.

A consultant stated that he was intrigued by the studies on the VEE vaccine and wondered if the group of nonresponding individuals was studied clinically at the time they were given the original vaccine. Perhaps they constituted a large percentage of the group (5%) that got sick. He noted that the original VEE vaccine produced profound leukopenia, and for this reason live VEE vaccine had been used in one trial in an attempt to treat leukemia. In response to this question, it was reported that the available data, although not complete, suggested that these nonresponding individuals did not become ill following immunization with the killed vaccine. In one case, one individual received 12 immunizations, others as many as 5, 7, or 11 immunizations respectively and none were ill.

1. A dangerous but unproved hypothesis was proposed. Since VEE has the ability to replicate in peripheral blood leukocytes of certain people, this type of person, if primed with a killed VEE vaccine and then given the live vaccine later, might be in for a very bad response. This concept has many implications. Colonel Metzger stated that the new killed vaccine had already created confusion. As soon as the preliminary data were reported, he received several phone calls asking why should the live, attenuated vaccine be administered at all. He emphasized that these killed vaccine studies are preliminary; moreover, the killed vaccine may not confer immunity against other members of the VEE group and the degree of cross-protection needs to be established.

2. A consultant stated that he knew the Institute was interested in early and reliable diagnosis and listed a number of diseases which are being identified by counterimmunoelectrophoresis in civilian medicine. He asked if the Institute were developing specific antisera to some of the more prominent diseases of military importance. Colonel Metzger indicated that the Institute was using this procedure, but that the basic problem was: "with any agent which requires specific antiserum, how much storage capacity in a REVCO do you allot to that antiserum." Storage capacity could become a problem since you may not know with what disease entity you are dealing. A more effective approach, if technically feasible, is to employ methods which do not depend upon specific antisera, e.g., the techniques of Dr. Wagner's contract at The Johns Hopkins University, the laser beam, etc. However, we do have specific antisera for a number of agents.

3. A consultant recalled that in a previous meeting of this group, a procedure was described for obtaining a profile of chemical changes, early in the disease process, with this information fed into a computer. He asked about the status of this program. The Scientific Advisor replied that the Institute was still interested in this approach, conceptually; however, the program has been unavoidably delayed. The contractor left the University of West Virginia before we had a chance to get more than a preliminary number of samples. Moreover, the computer capability supporting the Institute has been transferred 3 times in the past few years to 3 different computer groups. Each transfer has required a rewrite of the program. This approach is not dead and the program is hopefully at a stage where it soon can be evaluated. The extent of metabolic responses in an infectious disease numbers in the thousands, and there remain unexplored possibilities that some combination of responses may have diagnostic value. A computerized method must be available for a period of time to demonstrate functioning feasibility with this concept or lack thereof.

4. A consultant made the point that the respiratory tract is the origin of a large number of infections and asked if this Institute had explored the impact of "psychology" of infection. The psychology of respiratory infections is receiving considerable attention in Europe. Colonel Metzger replied that we have had no experience in this area.

Another consultant felt that skin specimens should not be overlooked in arriving at a rapid diagnosis. For example, the herpes group can be identified rapidly using pharyngeal scrapings which can be examined by direct and indirect immunofluorescence. The work performed at this Institute on RMSF skin specimens clearly showed promise.

The subject of serum banks was raised once again when a consultant reconfirmed the fact that the size of the bank was certainly the major problem. Colonel Metzger recalled that many years ago, he had written a paper in which he indicated that rapid immunofluorescence diagnosis was no longer a problem. He and others tried to get commercial firms to stock antisera; these firms conjugated whatever sera they had on hand with fluorescein, but this type of product obviously did not work. Good antisera are available today in limited quantities and this approach is effective when there is a hint as to what group of organisms are involved. In nondescript, unrecognized disease, the problem becomes more significant.

One consultant asked Dr. Woodward if, since Congress had decided that RMSF was not a military problem, some other group was going to pick up this important work. Dr. Woodward replied that RMSF is both a military and civilian problem. The disease caused about 7% mortality this year, such a death rate is inexcusable. It is due to physician failure in recognizing the disease, or the patient not getting to the physician in time. He felt that the Armed Forces Epidemiological Board must give all the support it can to The Surgeon General to continue an important and vital program such as RMSF. If Congress could arbitrarily cut off funds like this, all research programs, military and civilian, would be in trouble. Colonel Metzger reported that about 3 years ago, when the Institute's program was

just starting, we had 90% of all the rickettsiologists of the country in one room. They reviewed our plans and agreed that the overall program was a good one. Building modifications delayed the implementation of the rickettsia program for at least 6 months. Major Pedersen was drafted to head the new division and the 2-year-old program has provided some interesting and exciting results. The orders that came down were specific for RMSF investigations.

A consultant noted that Dr. Woodward and several others in the room would have the opportunity to speak effectively on the subject. Perhaps logic would prevail after these discussions. There are many people who do not see each disease compartmentalized with the parsimonious distribution of monies. Another consultant expressed the opinion that once Congress chops one area they may indiscriminately chop other areas of medicine of critical importance.

This discussion ended on a brief review of Legionaire's disease which stressed current medical technology of diagnosis.

RESUME OF EXECUTIVE SESSION

Colonel Metzger introduced the executive session by stating that, like last year, several specific questions have been posed for which this Institute needs advice. The questions are most difficult and may not have clear-cut answers; however, if the questions were simple, the staff of this Institute would have already provided the necessary answers.

QUESTION 1. In view of the apparent efficacy of our new inactivated TC-83 Venezuelan equine encephalomyelitis vaccine, is a major field study in northwest Venezuela advisable? Such a study could assess vaccine efficacy in large numbers of humans prior to an anticipated epidemic or at the outset of an epidemic.

This question may now be somewhat premature. From the data presented yesterday, preexisting antibody resulting from the old killed VEE vaccine may predispose the individual to a "wipe-out" of his subpopulation of anti-VEE specific T cells, provided the individual subsequently becomes infected with the "hot" virus. The new killed product may not produce results similar to the older, more crude vaccine. The new killed vaccine probably represents an entirely different antigenic substance. Moreover, the current problem is entirely different; that is, in the past, those people who received the attenuated vaccine and did not respond serologically, were administered the vaccine again and again. This explains why a small group received 5 to 10 injections.

The question was raised as to how these possible problems concerning an altered immunopathologic status could be tested. Studies could be implemented in an effort to determine if the "apparent" T cell nonreactivity (reactivity of noncirculating lymphocytes) might cause either some sort of B cell suppression or the specific "wipe-out" of T cells that are turned on at the time the individual is administered the live vaccine virus.

One consultant recalled that attempts to develop a killed vaccine for *Mycoplasma pneumoniae* were unsuccessful. Prevention of disease with a killed vaccine could not be achieved because a large portion of disease was found to be immunologically mediated. Use of the killed vaccine seemed to cause cell-mediated, humoral and local antibody responses to get "out of kilter." Based on this type of experience, the question was raised if the USAMRIID staff felt comfortable with a killed vaccine. The reply was that we felt more comfortable with killed vaccines for the Group A arboviruses (alpha group) than with killed vaccines for the arenaviruses. Pathogenesis for this latter group is not well established. There had been no indication of immunopathologic problems for VEE until quite recently when 4 repeatedly immunized people were unable to "turn on" circulating lymphocytes in response to *in vitro* stimulation by VEE antigen.

The cost of conducting field trials with the new, killed vaccine was questioned since the natural disease in man is very benign. Apparently the disease is more severe if the patient is under 6 months or > 60-70 years of age. It was emphasized that the USAMRIID experience with TC-83 has been

primarily in 18-20-year-old males, with no experience in young people and little experience in the older population. The Institute also has had some frightening experiences with female vaccinees, including fetal teratogenesis and abortogenesis. These occurrences are too few to have statistical significance; however, the live, attenuated vaccine should not be considered for wide-spread use in females of child-bearing age.

It was also pointed out that the killed USAMRIID vaccine was developed not to protect women and children in Venezuela but to protect military personnel who might be obliged to enter an area where the disease is either endemic or epizootic. The live attenuated vaccine, on occasion, produces reactions that are almost as severe as the natural disease. A consultant made the point that there was a good reason to develop the vaccine and did not see why the program should not proceed. To stop now would leave a lot of hard work hanging. If an epidemic of VEE occurred in the United States, we would want the killed vaccine even though mosquito control may be effective. Another consultant felt that if the TC-83 vaccine (live attenuated) were widely used to control an epidemic, cancer induction must be considered.

Summary of Question 1. VEE vaccines have been studied in great detail. The purposes of the USAMRIID studies have been made clear. Field test trials may not represent the highest priority work of USAMRIID but they should be pursued depending upon other research priorities and pressures.

QUESTION 2. Is there a valid requirement for investigation into the subunit nature of the antigens possessed by rickettsiae. The ultimate goal of definition of these antigens would be to extract identifiable components in order to prepare vaccines free from undesirable extraneous material of host (growth substrate) or rickettsial (nucleic acids, etc.) origin.

One consultant expressed the opinion that the answer in the long run of things is yes, but thought that there were more immediate problems. The current trend is for the development of subunit vaccine products. With current technology to do amino acid and key functional protein analyses, some anticipate that we should be able to synthesize vaccine antigens in the laboratory, and make them commercially. Notwithstanding, an inspection of rickettsial diseases, with the possible exception of Q fever, indicates we do not have any commercially available vaccines that are even moderately effective for epidemic typhus, RMSF, or scrub typhus. Within this frame of reference, vaccine studies should move back to "square one" and proceed initially with the development of killed whole-organism vaccines rather than attempt to make subunit vaccines. Once security has been achieved with a good safe, antigenic, killed, whole-organism vaccine, then studies can be initiated to define their antigenic components and determine what component is responsible for protective immunity. Another consultant confirmed this approach to rickettsial vaccine development and observed that interest in this area is declining. He warned that if vaccines are not prepared now for the major rickettsial diseases, they may never be developed. It would be heresy if USAMRIID did not use its expertise to do the job.

Another consultant reaffirmed the importance of conducting studies on the pathogenesis of the disease concomitantly with vaccine development; otherwise, 5 or 10 years from now, the Institute may have an ineffective vaccine and not know why.

Colonel Metzger noted at this point in the discussion that Questions 2, 3 and 4 were closely related. He briefly described the history of RMSF reasearch at USAMRIID. First, a model for RMSF was developed. The model was improved and all divisions of the Institute were encouraged to use the model in order to learn as much as possible regarding RMSF. A killed vaccine was prepared and tested against the base of information generated by the model. The vaccine looked good, and only then did USAMRIID start taking the vaccine apart. Essentially Question 3 is, "Should USAMRIID use the RMSF technology to prepare a typhus vaccine?"

QUESTION 3. The Rickettsiology Division has the technological capability to produce a typhus vaccine, using organisms propagated in tissue culture, killed with formaldehyde, concentrated and purified by ultracentrifugation and gradient procedures analogous to those employed for the preparation of our RMSF vaccine. Should such a vaccine be prepared? Are there any suggestions or recommendations to either improve on the proposed methodology or the final vaccine efficacy?

QUESTION 4. Should investigations be directed toward development of newer diagnostic techniques for rickettsial disease? In what areas would you recommend emphasis: humoral immunology, cell-mediated immunity, isolation and identification of antigens, soluble substances or organisms? Upon which rickettsiae would you recommend we concentrate our efforts in this regard?

A consultant noted that if Question 3 was answered in the affirmative, Questions 2 and 4 were also being answered in the affirmative. Given the history and importance of typhus in military campaigns, its association with major disasters, and the lack of efficacy in the present vaccine, the answer to Question 3 becomes a simple "yes."

The observation was made that the rickettsiae, with perhaps the exception of scrub tuphus and Q fever, are naturally adjuvanted vaccines if the whole rickettsial organism is used. Although it remained to be confirmed with the epidemic typhus model, the data obtained with the recently developed RMSF vaccine suggest that immunization with the entire organism induces both humoral and cellular immunity. Work at the University of Maryland indicates that cellular immunity is an important part of the protection mechanism. Again the point was made that it is important to define whatever host immune response is responsible for protection before proceeding to studies on subparticles. In rickettsial diseases, an immunological portion of the host reaction seems to be associated with perhaps the most severe aspects of the illness.

A member of the USAMRIID staff felt that the questions were written or phrased to "entrap"; for example, if a yes answer is provided for one question, a yes answer to another must follow. The Institute is looking for affirmation: "Are these desirable vaccine products?" Vaccine development does not proceed without ancillary studies and all divisions contribute to the total effort. More monies are involved than one might suspect.

A concern was raised why the live E strain typhus vaccine had not been included among the questions for discussion. Data from trials in Africa indicate it is a highly effective vaccine, providing 95% protection. Colonel Metzger responded by stating that we have discussed and considered living attenuated strains of rickettsiae for vaccine development. He used Institute studies on the M-44 strain of Coxiella burnetii as an example. If pathology studies are undertaken, the situation becomes more complex. Live rickettsiae can be recovered from immunized animals 6 months after vaccination, if cortisone is administered and the animal stressed. Many scientists worry about the widespread use of living attenuated rickettsial vaccines. Notwithstanding, if we really needed good rickettsial vaccines for an emergency, we could utilize strain E typhus, or M-44 Q fever. One of the consultants asked if the M-44 strain reverted to virulence following cortisone and stress. Colonel Metzger replied that the organisms recovered appeared to be an unaltered M-44 strain. With strain E, in contrast, organisms have been recovered that were different.

Another consultant briefly described the status of strain E as follows. World Health Organization has approved the use of strain E vaccine to control epidemic disease, but has not approved it for use in areas where the disease is endemic. The Russians have produced data in which the strain reverts to virulence; that is, when strain E was passed sequentially by intranasal inoculation in mice, virulent organisms were observed. Also, virulent organisms were observed 1 hr after IP inoculation. No one else, including the Russians, have been able to reproduce these results.

QUESTION 5. At least 15 different microorganisms or their toxins have appeared on lists of "Potential BW Threats" published in the open literature. Vaccines are available for some, vaccine production methods are known for others. Should a program of vaccine stockpiling be initiated for all possible BW threat candidates? If so, how big a stockpile? Should broad immunization programs be initiated for any?

One consultant felt that the answer to Question 5 could be answered either way. He recommended that the administrative experts that have made laboratory decisions for the Institute, should have a chance to answer this question. A long list of agents could be prepared which would keep USAMRIID busy for years to come; however, it would be quite possible that none of those agents would ever constitute an actual threat. Five years from now, a Congressional committee could say, "Those people at USAMRIID are fooling around making products to be stored forever and will never be used." He felt Question 5 required a major decision in consultation with higher levels of authority.

Colonel Metzger's response was to describe current philosophy, the contract with Merrell-National Laboratories. An industrial set of procedures that describe every aspect of vaccine production are developed, a "cookbook" of directions. The cookbook can be used by USAMRIID or by others to respond rapidly to an emergency. The Canadians used our VEE cookbook to prepare an acceptable vaccine in 6 weeks; a Latin American country used the same cookbook and required 4 months. So, although the contract with Merrell-National does not now represent a stockpile, it does represent a source of information on which vaccines can be produced relatively quickly.

A representative of USAMRDC indicated that we could develop a list of agents and assign priorities based on our best estimates of what organisms would most likely be used against us; for example, botulinum toxin is easy to make, disseminates effectively, and is very potent. There are other agents that are easily produced. This provides a clue as to what defensive measures we should be studying to protect ourselves. Colonel Metzger replied that there are about 18 - 20 agents people talk about in terms of BW, and perhaps others could be added to the list. Our approach has been to develop vaccine protocols and hope that we have selected the right protocol as well as having sufficient time to go into vaccine production.

Further, in the last few years, the Institute has produced a large stock of frozen cell substrates that are approved by the Bureau of Biologics for vaccines. These cell stocks reduce the time required for vaccine production, perhaps to as little as 2 weeks; however, several additional weeks are still required in order to get a vaccine tested for safety and potency.

A consultant made the point that even though you develop a list of potential BW agent candidates, there is no assurance that any one on the list would ever be used.

The Scientific Advisor pointed out that this question was formulated to stimulate the types of discussion and consideration being offered. It is a most difficult question and has an important impact on our mission. For years, the USAMRIID philosophy has been to study various prototype organisms which hopefully will include the major classes of potential BW agent threats against us to learn how to study and handle these organisms and to maintain a continuing level of familiarity and competence with their methodological technology. We would thus have the expertise to respond to any new developments in relatively short order. This question is also relevant whenever we must formulate a response to Congressional committees which will be believed and accepted.

In the past, we have downgraded the concept of stockpiling vaccines A, B, C, D, etc., in a warehouse somewhere. But is this a position that should be maintained? If our approach involving the study of model infections with "prototype" organisms is not being believed, should we augment or replace this approach with the stockpiling of vaccines? A comment was made during recent national influenza vaccine planning sessions that a vaccine on the shelf does no good, it is a vaccine in someone's arm that is important. Another aspect of the question becomes, if we do manufacture and stockpile vaccines, how much do we need? Should we actually immunize military forces now on the possibility that some day they may face one of these organisms?

One of the consultants replied that he could not provide a specific answer to these questions but that based on the VEE experience, the stockpile of TC-83 vaccine proved to be of great assistance in the Texas epidemic. He felt that the Institute did not get the credit it deserved in controlling the spread of the disease in horses. He further reported that there is not a single manufacturer in the United States for botulinum antitoxin. Supplies of the antitoxin are precariously low; the only supply left is material the Biological Laboratories, Fort Detrick, turned over to CDC. Unless more antitoxin is made, there isn't going to be any left in a few years. The Canadians might provide us with antitoxin but this is an assumption. The United States should be in a position of self-sufficiency for this and other biologics it needs. Colonel Metzger stated that the antitoxin supply is quite small and CDC had placed a contract to make more; the procedures did not work. He reported that the supply of toxoid was also quite low. Another consultant observed that if large quantities of vaccine are required, production is much more difficult than making laboratory amounts. Experience has shown that there are many problems. He then asked about the "state of the art" for those vaccines which USAMRIID has studied. Colonel Metzger reported that most of the vaccines were initially made "in-house" in laboratory quantities. Then the vaccines were converted to industrial protocols. The Bureau of Biologics requires at least 5 production lots to satisfy homogeneity of the product. However, only VEE had been tested extensively.

It was stated that the Armed Forces Epidemiological Board had discussed the problem of protection against botulism. It would be tragic if we did not maintain a position of strength and readiness. The Surgeon General feels we must have a posture of readiness; he thinks in terms of readiness.

A staff member of USAMRIID emphasized a compromise position regarding the size of a stockpile of vaccine. The minimum size of the stock should be sufficient to immunize troops in a non-wartime situation.

A representative of USAMRDC felt that the approach to the problem was backwards. We are used to thinking of specific agents and antigens. In the context of BW, possibilities are that our defensive measures would never be in the right place at the right time. Since we cannot make predictions, general methodologies must be developed; for example, DNA recombinants might dictate new general approaches that have a wide application. Several people voiced the opinion that DNA recombinants might provide generalized answers.

One consultant voiced the opinion that one area to review is the data accumulating for J5 E. coli mutant and Salmonella minnesota RH535 which not only cross from Enterobacteriaciae but into Pseudomoniaciae. If the core of the organism is studied, our concepts may be broadened, at least for the bacteria. He recommended, for example, studying what the J5 vaccine would do against some nonconventional agent.

Another consultant felt that interferon inducers, while not feasible in everyday clinical practice because of problems with toxicity or immunogenicity, might be appropriate in face of a massive BW attack of unknown specificity. He hoped that this approach was being considered.

One of the consultants reported that investigators were close to identifying the specific piece of protein responsible for attachment of M. pneumoniae to cells. Antibody against that piece of protein would probably prevent the disease; it is conceivable that this protein is common to a wide variety of bacteria. Prevent attachment through the use of this common protein and you have a nice method for preventing diseases.

Dr. Jordan returned to the problem of stockpiling and noted that the country was losing its ability to produce many biologics in addition to botulinum toxoid. Before we stockpile, we must be able to make the product. He reported that he had recently attended the National Immunization Conference which focused on some of these issues: for example, (a) production and supply; and (b) research and development, which are closely related. Industrial groups are motivated by profit and must answer to their stockholders. Dr. Jordan felt that it was becoming increasingly difficult for these concerns to stay in the business of making biological products. A task force of the Conference is studying how to meet and maintain the national capacity for production of biologics. Only one firm is now making polio vaccine, only one is making measles vaccine. Research and development also needs to be maintained in order to support the production capability. Perhaps this country needs a national facility similar to the Serum Institute of Denmark. He noted that in the past AFEB board meetings, the problem of getting a new vaccine from the experimental laboratory stage into production was discussed.

Colonel Metzger reported that for years USAMRIID has had a contract with National Drug, which is now Merrell-National Laboratories. They not only make our vaccines, but can produce any product we ask them to make since they are paid with our funds. With their containment facilities and personnel, USAMRIID has its own small drug house. Personnel from USAMRIID visit and work with these people to produce our industrial cookbooks. A 5-year contract period is coming to an end and we hope to be able to renew the contract for another 5 years.

One consultant felt that this arrangement could serve as a prototype of what is needed for the entire country. Another consultant noted that this contract represented an important national resource which should be strengthened. He noted that details of the contract, its cost, etc. could not be worked out at this meeting; however, the basic principle should be endorsed by the committee. Colonel Metzger replied that the Institute had been fighting hard to maintain and retain this contract. The attitude of the Merrell personnel was not too helpful. Another consultant asked why Merrell-National was not interested in contract renewal, was it profits or was the endeavor too risky? Colonel Metzger replied that these people told him that they could make twice as much money making aspirin in a plant 1/3 the size of the Swiftwater facility. There is no financial risk to Merrell-National. Another consultant made the point that the Government built this plant and stocked it with equipment. A member of the USAMRIID staff cited the NCI contract with Litton Bionetics as a possible prototype. The contract approach remains competitive for a variety of research needs and should be considered by Dr. Jordan. Colonel Metzger ended this discussion by describing the economic bind Merrell has been in regarding the cost of fuel. At the last negotiation, they were given only a 2% increase per year for fuel and over the past 5 years this has amounted to a shortfall of about $70,000.

AD-A038 648 ARMY MEDICAL RESEARCH INST OF INFECTIOUS DISEASES FR--ETC F/G 6/5
1976 USAMRIID PLANNING SESSION WITH THE AD HOC STUDY GROUP FOR --ETC(U)
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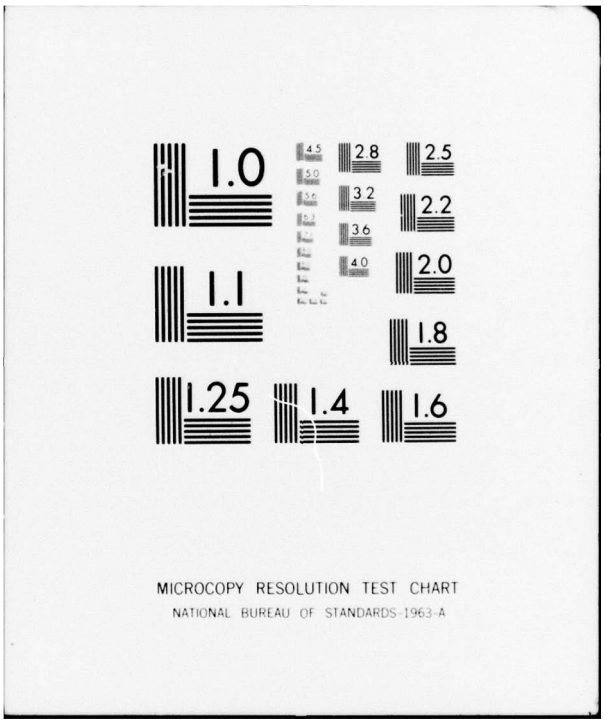
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QUESTION 6. Our guidance from Army through USAMRDC is that we should focus research on infectious diseases which are of unique military importance, the inference being that the infections must be those which either occur rarely in the U.S. civilian population but frequently in the military or those that could result from the use of BW operations by an enemy force.

a. What other infections deserve research because of BW potential?

b. In the absence of preexisting information, should we study the properties of the causative organisms with respect to aerosol stability and respiratory infectivity (dose response) for these infections before proceeding with research on such host-related factors as pathogenesis, early diagnosis, immunogenesis and therapy?

c. What is the relative priority of continuing studies of immunoprophylaxis and therapy of the respiratory form of infections whether or not the infections occur naturally as respiratory diseases?

A staff member of USAMRIID observed that some of the afternoon discussion has been concerned with the acceptability of studying agent models as opposed to studying specific agents. He thought the concept should be extended to include the assignment of relative priorities to agent candidates. Agent criteria were briefly described because the organism must have certain characteristics if it is to be considered a BW threat. Agent criteria included: (1) must be economically produced in high concentration and in large quantities; (2) must be stored economically for relatively long periods of time; (3) stable in aerosol; (4) infectious dose for man compatible with the produced concentration; and (5) capable of inflicting or imposing a significant military impact on enemy forces. Somehow, we should be able to get information from Combat Development Command on those agent characteristics they believe would be useful in a military situation. Having this information would permit us to establish a more intelligent order or priority. Another aspect of this question concerns information gaps that exist for our current list of potential BW agents. For example, do we know anything about the aerosol stability of a particular agent? If not, is it this Institute's responsibility to obtain this information? Is the information worth getting at all?

One of the consultants amplified these remarks by stating that since Question 6 is open-ended in regard to potential threats, a system of selecting threat agents needs to be devised. He felt the above comments described one approach to such a system. A small group of experts, through thoughtful consideration, could select potential threat agents and perhaps arrange them in 4 or 5 classes or priorities, or whatever number of defensive priorities that would be necessary. Colonel Metzger felt that much of our data was generated on the basis of organism availability; however, technology has improved significantly in the past 10 years. Small fermentors equipped with proper agitation, pH control, dissolved-0_2 monitoring, and other important controls can produce as much material as the older but much larger vessels. From the

standpoint of improved technology, the threat is much greater now and is less obvious. A few small fermentors hidden in a closet are not as easily detected as a 7-story building housing large fermentors.

Another consultant agreed with the approach of agent selection based on agent characteristics; however, he did not feel that people at Combat Development Command could provide specific information on agent properties. They could tell you what an enemy would like to have happen to our troops in terms of incapacitation, morbidity or mortality. The scientific community must be consulted in order to know what diseases produce incapacitation, morbidity and mortality. The question is important and must be presented to the right people. He felt that in addition to methods of production which Colonel Metzger had referred to, there have also been major changes in potential enemy delivery systems. Much of the original thinking was associated with specific methods of delivery which today may not even exist. For example, what impact would multiple-targeted missiles have on current thinking?

A member of the USAMRIID staff felt that a defensive priority list of threat agents appealed to man's logic, but asked how does one devise a priority list of agents for defense that correlates well with an unknown priority list of agents for offense: with 18 - 20 agents in combination the permutations are enormous. Moreover, once a defensive list is devised, and approved by committee, the list becomes locked in concrete and all flexibility is lost. Finally, he asked what happens to the priority list when a new agent like Ebola virus comes along.

Colonel Metzger felt that one unfortunate aspect of the past was that we devised defensive measures to counteract our own offensive capability. For example, we studied small-particle aerosol defense when at that time the Russians were dropping whole eggs and producing an entirely different situation. He agreed with the point that we provided defense against how we would conduct offensive BW.

A member of the USAMRIID staff felt that aerosol dissemination still influenced current thinking and proposed an alternative method of delivery which would be effective yet difficult to defend against: the introduction of RMSF via a vector, provided the vector was present in the target area.

A representative of USAMRDC noted that operations groups are quite concerned about such questions as: what agents are expected; what constitutes a creditable threat? The answers are endless; almost anything constitutes a creditable threat. Contaminated blankets may be the appropriate system of delivery for an infantry company in the field.

A representative of the Biodefense group at Edgewood Arsenal reported that agent priority lists have existed and were based on U. S. development over 30 years. These priority lists have been dormant for at least 5 years. Other aspects of the question were discussed.

QUESTION 7. How much of a threat to the USA does smallpox pose as a BW weapon? Now? In five years? What should the Army's defensive stance concerning this threat be? Now? In five years?

Colonel Metzger opened the discussion by stating that if the World Health Organization is correct and the earth is free of smallpox, and everyone stops being immunized, smallpox becomes a viable threat. He commented that it probably was the first BW weapon.

A staff member of USAMRIID indicated that Colonel Philip Russell of WRAIR was interested in smallpox for other reasons and would convene a meeting soon to discuss such things as:

(a) How will military personnel respond to vaccination 10 - 15 years from now who have never received a primary vaccination?

(b) Will the Bureau of Biologics allow the Army to use a vaccine which has not been used in 15 years?

(c) What will be the effect of that vaccine on troop effectiveness? [illegible] morbidity.

(d) Should the Army consider the possibility [illegible] a new vaccine with longer effectiveness, longer duration of immunity, and less reactogenicity?

These will be problems 15 years from now unless the Army decides it can afford to have troops in the field that are susceptible to smallpox.

One consultant felt that the first question may not be too serious a problem. For example, 3,000 hospital personnel were immunized in 2 days, many of the recipients were in their sixties, and few consequences were observed. Very few people lost any time from work and there were no problems of secondary transmission. He reported that a similar experience had occurred at the Johns Hopkins where 30% of the recipient population consisted of older adults who were receiving primary vaccination.

Another consultant felt that the question was: "What is the vaccination risk in an adult who has never experienced smallpox?" The answer is that it is an innocuous vaccine based on data collected in one of the Scandinavian countries. The problems seems to be in children. Smallpox vaccination is unfortunately a good method for picking out children with defective defense mechanisms. In the absence of smallpox vaccination, some other infections will identify them long before they reach 18 years of age. Almost all the smallpox problems seen in pediatrics are the result of defective defense mechanisms.

A consultant reported that CDC had performed surveillance studies with a large number of vaccinees. These data clearly identified the infant group as high risk. However, trying to develop a safer, less reactogenic vaccine would not be worth the effort. Another consultant felt that any list of BW agents would have to include smallpox. It seems highly unlikely that the Army would abandon smallpox in the near future. In a few years, large numbers of unvaccinated people will be entering the Army.

Colonel Metzger raised the question concerning the current and future availability of smallpox vaccine. A consultant thought that there should be

plans to maintain a large supply of vaccine for civilian and military use even in the absence of a BW threat. Another consultant stated that he knew of no plan to maintain a national stockpile of this vaccine. Another consultant voiced concern about current availability; in particular, there is no commercial market for it. He described a recent episode in the Dallas area where they were unable to get the vaccine. They turned to CDC for help but the Center had no supply and was unable to provide help. There was a 3-month period before an adequate stock of smallpox vaccine was collected. At the present rate of usage, it will not be long before the present national supply is exhausted.

Colonel Metzger provided another illustration of the problem of biological and vaccine supplies. About 8 months ago, our sole supplier of plague vaccine had an "equal-opportunity problem" within its organization. The Department of Defense made the decision that plague vaccine could no longer be purchased from this particular supplier. Alternative sources were sought but no one could be found to make the vaccine. The commercial laboratories are not interested in making vaccines any longer because there is no money in it for them. This type of experience makes us fight even harder to maintain our contract with Merrell-National. A consultant noted that this contract may serve as a prototype but does not meet all of the needs which have been addressed earlier. Someone in Government is going to have to underwrite the cost of a broad program to manufacture vaccines. Another consultant did not understand who in Government was responsible for such things. He noted that apparently little progress had been made since he talked to the President on this subject at the time the Institute of Medicine was founded. Dr. Jordan noted that it became apparent during the swine flu discussions that there were too many people, or no one, responsible.

A consultant noted that CDC is trying to document where smallpox virus is located throughout the country. What CDC will do with this information needs to be determined. Some have suggested that all stocks of smallpox virus be destroyed. Colonel Metzger replied that he had received a questionaire from CDC but had forwarded it to higher headquarters for answering. He felt that the free world should not destroy its stocks while the enemy sits back with a supply. Colonel Metzger felt that this was a real possibility. There are laboratories which are safety-engineered to work with dangerous microorganisms and they should be permitted to do so.

A consultant closed the discussion on Question 7 by stating that the answer to the problem is very simple. The military should be immunized. Moreover, smallpox constitutes a BW threat and will pose a more serious threat in time. We should maintain our seed stocks. There is a monkey pox in Africa which is almost identical to the human disease. He could not visualize the United States not taking appropriate steps to have vaccine available.

QUESTION 8. A BW attack could use a combination of agents. The University of Maryland group, several years ago, did a study to investigate the combined effects of simultaneous C. burnetii and F. tularensis exposure. Should this type of problem be studied as part of the USAMRIID program? At what priority level? With what organisms?

One consultant stated that he had been interested in agent interaction for a long time and he would encourage this type of study even though it represented a very tough problem. There seemed to be no effective way of coming to grips with the problem. In the laboratory, a model can be developed to show almost anything you want. There have been many such models developed; however, when one steps into the arena of natural disease, there are not many cases of dual infections. When one considers the number of human and animal diseases and their possible interactions, why more combinations of diseases do not occur, remains a vast area of ignorance. Another consultant felt that combinations of agents should be studied, but could not define the magnitude of effort. He did feel, however, that such studies should not go beyond a preliminary stage; he had observed advanced cases of leprosy and tuberculosis in the same patient. He felt that one would not expect to find these 2 diseases collaborating with one another. Another consultant added that there are some who feel that Burkitt's lymphoma represents an interaction of disease: tuberculosis plus malaria, or something of this sort.

QUESTION 9. In view of the lack of hard information about which specific organisms to defend against, how much USAMRIID research effort should be placed upon preventive or therapeutic approaches that are possibly useful against a broad spectrum of infections, i.e., stimulation of nonspecific host defenses, value of transfer factor, development of improved supportive therapy to maintain body homeostasis and organ functions?

A consultant felt that this question is like the previous question, in that it is open-ended and defies a precise answer. There has been a pronounced lack of success in achieving successful therapy or effective disease prevention. However, this Institute should continue studies since there may be a secret way of approaching these problems that hasn't been discovered. This type of study, however, would not be worth any large amount of research effort.

A member of the USAMRIID staff felt that some of the approaches were hard to justify because they involved unrealistic amounts of material. For example, 30 or 40 ml of pertussis vaccine is required to stimulate nonspecific defense mechanisms in man.

Colonel Metzger reported that we have studied broad mechanisms of immunization since there is a limit to the number of vaccines a person can receive. The Institute has studied interferon inducers with mixed success, sometimes curing, sometimes killing the animal. The problem really becomes how much of our research effort should be devoted to these types of studies.

One consultant replied that knowledge in these areas is in a preliminary stage and will probably become useful in a decade. Another consultant felt that some of the expertise for these questions resided at this Institute and studies should be continued.

Still another consultant expressed the opinion that this type of study should be encouraged. The nonconventional routes of administering products as a means of altering pharmacology represented an extremely interesting area of research. He recalled the aerosol administration of kanomycin in the Klebsiella model and reported that he was still impressed with the high levels of the antibiotic in the brain as contrasted to the levels achieved by intramuscular or intravenous administration. This Institute should continue to study agents that may be delivered by peculiar routes since it is an important part of the mission. He also felt that one should keep an open mind regarding the potential routes of administering therapy.

Colonel Metzger, in closing the executive session, reported that this meeting represented the fourth time he has had the pleasure of hosting the group. He expressed his thanks to all the members for the help and assistance each had provided to him personally and to the Institute.

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