RESEARCH ON DENGUE DURING WORLD WAR II

ALBERT B. SABIN

Army Epidemiological Board, Preventive Medicine Division, Office of the Surgeon General

STATUS OF PROBLEM PRIOR TO WORLD WAR II

Most of the basic and significant contributions to our knowledge of dengue prior to World War II were made by honored members of the medical department of the U. S. Army. Ashburn and Craig (1) provided the evidence for the viral etiology of the disease. Siler, Hall and Hitchens (2) clearly established the period of infectivity of dengue patients for Aedes aegypti mosquitoes, the period required for the development of the virus in these mosquitoes before they could transmit the infection, as well as the very long period during which these mosquitoes were capable of transmitting dengue. Simmons, St. John and Reynolds (3) established (a) the role of Aedes albopictus in the transmission of dengue, (b) the occurrence of inapparent infection in certain monkeys under experimental and possibly also natural conditions, thus suggesting the existence of a "jungle" type of dengue fever exclusive of the human cycle, (c) the persistence of immunity to the homologous strain of virus for 13 months in human volunteers residing in an endemic region, and (d) many of the properties of the virus. It is necessary to recall, however, that the latter investigators completed their studies in 1930, before most of the important, newer virological techniques and procedures had been developed. In 1934, Snijders, Postmus and Schöffner (4) reported some immunity experiments on human beings in Holland with two different strains of virus which left the subject of immunity to dengue in a rather unsettled state. In 1936, Shortt, Rao and Swaminath (5) reported the successful cultivation of dengue virus on the chorioallantoic membrane of chick embryos, but their conclusions were not based on tests on human beings. Otherwise, little or no work was done on dengue during the period of 1930 to 1940.

Thus, while a good deal of fundamental information about dengue was available at the beginning of World War II, it was also apparent that most of the elementary requirements which would permit one to carry out systematic studies with the virus of dengue fever were lacking. No strains of the virus were anywhere available; there was neither a suitable laboratory animal for experimental work nor an established method of in vitro cultivation, and almost nothing was known regarding some of the basic physical and biological properties of the virus.

1 This article was prepared as part of the medical history program of the Army, and, with certain minor modifications, will appear in the forthcoming HISTORY OF PREVENTIVE MEDICINE, U. S. ARMY MEDICAL DEPARTMENT, WORLD WAR II. It is an honor and privilege to have this article published in a volume dedicated to Colonel Charles Franklin Craig, who, together with Ashburn, demonstrated the viral etiology of dengue and supplied a great deal of additional important fundamental information regarding this disease.

2 Lieutenant Colonel, Medical Corps, Army of the United States. Present address: The Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Cincinnati 29, Ohio.
PRIMARY OBJECTIVES OF RESEARCH OF MILITARY IMPORTANCE

From the point of view of military preventive medicine, there was a great need for an immunizing agent capable of protecting against dengue, preferably in a manner analogous to that achieved by the yellow fever vaccine. Another great need was for some practical, specific diagnostic test which would permit one to determine the precise role of dengue in the causation of the large number of “fevers of unknown origin” encountered in dengue endemic regions. It was clear that these needs could not be fulfilled until a great deal more was learned about the basic properties of the virus, the immunity which follows natural infection, and the immunological characteristics of strains from different parts of the world.

RESEARCH UNDER U. S. ARMY AUSPICES DURING WORLD WAR II

Organization of Dengue Research Unit

Two basic principles for the organization of a dengue research unit were apparent from the beginning: (a) that no satisfactory work could be done without a constant, fairly large supply of human volunteers located within a relatively short distance from the laboratory, and (b) that it was better to carry on the work in a dengue-free area of the United States, bringing the viruses to the research unit, than to bring the research unit to an area where dengue was occurring in our troops. The unit was organized by this writer early in 1944, at a time when he was already engaged in studies on sandfly fever in human volunteers. The work was begun in the writer’s laboratory at the Children’s Hospital Research Foundation, University of Cincinnati College of Medicine, utilizing patients in need of fever therapy at the Longview State Hospital in Cincinnati, Ohio. Since the number of such patients was small, and most of those requiring fever therapy had already been used for tests with sandfly fever, the unit was transferred to New Jersey. The human volunteers consisted of white Americans who were serving sentences for civilian offences at the New Jersey State Prison at Trenton, New Jersey. The authorities of the state prison very kindly donated a hospital unit of seventeen beds which was mosquito-proofed and used for housing the volunteers during specified periods of experimentation. Two nurses (one day and one night) and a clinical laboratory technician assisted in the work of the hospital unit. The laboratory facilities were located at the Rockefeller Institute for Medical Research, near Princeton, New Jersey, approximately 15

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*The great interest of Brig. Gen. James S. Simmons, MC, chief of preventive medicine, SGO, in the establishment of such a unit, the indefatigable help of Brig. Gen. S. Bayne-Jones, MC, deputy chief, preventive medicine, SGO and administrator of the Army Epidemiological Board, and the unfailing support of Dr. John R. Paul, director of the Commission on Neurotropic Viruses, were largely responsible for the creation and operation of the dengue research unit. The cooperation of the Longview State Hospital at Cincinnati, O. (Dr. Douglas Goldman), of The Rockefeller Institute at Princeton, N. J. (Dr. Carl Ten Broeck), and of the inmates and officials of the New Jersey State Prison at Trenton, N. J. was vital in the operations of this unit. The ASTP students of the University of Cincinnati College of Medicine, who volunteered for experimental inoculations in 1945, also rendered valuable service.*
miles from the hospital unit in Trenton. Lt. (later Capt.) William L. Jahnnes, Sn. C. was assigned to the unit as the entomological associate, and Lt. (later Capt.) R. Walter Schlesinger, M. C., joined the unit as a virological associate. Several civilian, technical aides were kindly made available by the Rockefeller Institute.

Recovery of Strains of Dengue Virus from Hawaii, New Guinea and India, and Criteria for Identification

During the period of 1944 to 1945, seven strains of dengue virus were recovered from Americans stationed in Hawaii (one strain), New Guinea (4 strains) and India (2 strains) by subinoculation of serum specimens into human volunteers in the U. S. A. The strains were recovered from serum specimens which were transported from overseas frozen in dry ice as well as from specimens which were merely refrigerated by ordinary ice. No sera were ever used in transmission tests until the patients from whom they were derived had recovered and it was clear that the clinical course was compatible with dengue. The first strain of virus was recovered in March 1944, when a pool of sera obtained 24 to 48 hours after onset from 6 patients with a diagnosis of dengue during an epidemic in Hawaii was subinoculated intracutaneously in 6 patients at Longview Hospital in Cincinnati, Ohio. All the subinoculated patients developed a febrile illness with rash and leukopenia, from which they promptly recovered. A large amount of serum obtained within 24 hours after onset of fever and stored partly in dry ice and partly in the lyophilized state served as a source of virus for many subsequent studies. Although the incubation period and clinical manifestations of the experimental disease were in most respects compatible with dengue, there was some concern about the occurrence of a rather marked, petechial eruption over the feet, ankles, legs and in some instances over the hands and wrists in 3 of the 6 patients toward the end of the febrile period or after defervescence. Since petechial hemorrhages had not previously been stressed in the clinical picture of dengue, it appeared necessary to determine whether or not a rickettsial agent might have been responsible. However, the convalescent sera of the 6 patients yielded negative results in Weil-Felix agglutination (OX-2 and OX-19) and rickettsial complement fixation tests, and inoculation of chick embryos into the yolk sac and of guinea pigs and mice with acute phase serum also failed to yield any evidence of rickettsiae. However, the identification of the virus as dengue was not considered established until transmission by Aedes aegypti mosquitoes, after the characteristic extrinsic incubation period, was accomplished. The continued occurrence of petechial hemorrhages in human volunteers after passage of the virus through mosquitoes and also after filtration through gradocol membranes with pores too small to permit the passage of rickettsiae finally eliminated the suspicion that the petechial hemorrhages might have been due to a rickettsial agent.

Sera from several types of febrile illness were collected in New Guinea between 30 April 1944 and 13 May 1944 and forwarded by Lt. Col. Cornelius B. Philip, Sn. C. in the fluid state refrigerated by ordinary ice. The sera, which were
suitable for study, were derived from essentially 3 types of cases:
  a) Fevers of 2 to 3 days' duration followed by rapid recovery without rash, which were diagnosed as “Febricula” or “F. U. O.”
  b) Fevers of 4 to 6 days' duration without rash which were regarded as dengue.
  c) Fevers of 24 to 36 hours' duration, without rash, with leukocytosis of 10,000 to 19,000, diagnosed as F. U. O.

It was reported that the majority of cases in New Guinea were characterized by a short febrile course, and that very few exhibited rash or “classical saddle-back” fever. The results of the subinoculation tests in human volunteers in the U. S. A. were as follows:
  a) Four strains of virus were recovered which produced a febrile illness with rash, clinically compatible with dengue.
  b) Two of the strains were obtained from the serum of patients who exhibited fever of approximately 2 days' duration, and the other two strains from the serum of patients who had fever of 4 days' duration or longer.
  c) Although none of the 5 original patients in New Guinea, from whom these 4 strains of virus were recovered, was reported to have had a rash, all 8 volunteers inoculated either with the original serum or first passage serum exhibited a rash and febrile course, clinically compatible with dengue.
  d) The experimental disease produced in human volunteers by the New Guinea strains was generally less severe than that produced by the Hawaii strain.
  e) Cross resistance tests in human volunteers performed 4 to 8 weeks after the initial experimental attack served to identify the New Guinea viruses with the Aedes aegypti transmitted Hawaii dengue virus, although tests carried out after longer intervals subsequent to the original infection ultimately revealed that 3 of the 4 New Guinea strains were immunologically different from the Hawaii strain.
  f) No virus was recovered from a pool of serum obtained 32 to 36 hours after onset from 2 patients who exhibited fever not exceeding 36 hours in duration associated with leukocytosis but without rash. The two volunteers used in this test showed no signs of illness and subsequently were proved to be susceptible to inoculation with 2 of the New Guinea strains of dengue. Accordingly, it was concluded that the brief, febrile illness with leukocytosis which was seen in New Guinea was most likely not a manifestation of dengue.

A variety of febrile illnesses, some with and others without rash, pleocytosis, or other changes in the cerebrospinal fluid, was occurring among U. S. Army personnel in India. It was not clear whether one or several etiological agents were involved, and whether or not the viruses of sandfly fever, dengue or one of the neurotropic viruses were among these agents. Col. Herman L. Blumgart, M. C., medical consultant in the India-Burma theater, obtained serum and cerebrospinal fluid from a number of such patients and the specimens were transported frozen in dry ice by air to the dengue laboratory in the U. S. The clinical

4 Credit for stimulating interest in this group of patients is due to the many medical officers in the field, who in reporting these cases indicated their suspicion that the viruses of sandfly fever or dengue, or some other virus, might be responsible.
TABLE 1
Summary of Data on Isolation and Identification of Two Strains of Dengue Virus from "Fever" Among U. S. Army Personnel in India

<table>
<thead>
<tr>
<th>SOURCE</th>
<th>SPECIMENS TESTED</th>
<th>DATA ON ORIGINAL PATIENTS (DONORS)</th>
<th>RESULTS OF HUMAN TRANSMISSION TESTS</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Name</td>
<td>Duration of fever</td>
<td>Rash</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td></td>
<td>Kettrop</td>
<td>5 to 6</td>
<td>+</td>
<td>Protein</td>
</tr>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcutta 142nd General Hospital</td>
<td>Walker</td>
<td>4 to 5</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Ploof</td>
<td>4 to 5</td>
<td>+</td>
<td>Cells-0 Protein 60 mg %</td>
</tr>
<tr>
<td></td>
<td>Dillworth</td>
<td>4 to 5</td>
<td>+</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>Bennett</td>
<td>6 to 7</td>
<td>+</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>Dillworth + Bennett</td>
<td></td>
<td></td>
<td>See Above</td>
</tr>
<tr>
<td>New Delhi 100th</td>
<td></td>
<td></td>
<td></td>
<td>135 cells</td>
</tr>
</tbody>
</table>
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histories of some of the cases, particularly those occurring during the latter part of September 1945 in Calcutta, were compatible with dengue and it was considered worthwhile to attempt isolation of new strains of virus for immunological comparison with viruses already available from other parts of the world.

Serum from individual patients, rather than pools, were used for transmission studies to permit detection of multiple etiological agents and correlation of the clinical picture presented by the original patient with any virus that might be isolated. When pleocytosis was present, human transmission studies were postponed until it could be shown that neither the serum nor the cerebrospinal fluid contained an agent pathogenic for mice. The human transmission tests, summarized in Table 1, yielded two strains of virus. Both strains were recovered from the filtered serum (500 mg gradocol membrane) of two individual patients, who became ill in Calcutta on 25 September and 27 September 1945 respectively. It is noteworthy that the cerebrospinal fluid yielded no virus, although in one instance it was obtained 2 days after onset at a time when the blood of the same patient (Kettrop) contained virus. No virus was recovered from the serum of the patient in New Delhi who exhibited a pleocytosis, and it may be pointed out that his clinical history was also not compatible with a diagnosis of dengue because he had a moderate leukocytosis instead of a leukopenia. Both strains of virus from India were identified as dengue on the basis of the following properties:

a) The clinical manifestations of the experimentally transmitted disease included skin lesions, which appeared after a suitable incubation period at the sites of intracutaneous injection of the serum, a self-limited fever after an incubation period that is usual for dengue, transitory leukopenia with the usual changes in the leukocytic formula, generalized macular or maculopapular rash and terminal petechial eruption.

b) Transmission of a clinically similar illness by Aedes aegypti mosquitoes after an extrinsic incubation period of 14 days following feeding on human beings infected with this virus.

c) Resistance of human beings recovered from infection with known dengue virus to inoculation with these new strains.

d) Human beings infected with the Calcutta strains developed neutralizing antibodies for the Hawaii mouse-adapted virus.

Properties of Dengue Viruses Determined by Studies on Human Volunteers

Minimal Infective Dose and Pathogenic Effects of Minimal Amounts of Virus.—Serum obtained from experimentally infected persons within the first few hours after onset of fever was stored in a dry ice chest and constituted the source of virus. The determination of the amount of virus present in such serum was desirable not only to permit quantitative work with this virus but also to ascertain whether minimal doses of virus produced a modified clinical syndrome and whether "subclinical" doses were immunogenic. Such infected serum, even after varying periods of storage in dry ice and repeated freezing and thawing, was found to contain one million human minimal infective doses (M. I. D.) per ml.,
when the dilutions were made in 10 per cent normal human serum-saline and inoculated intracutaneously. Ten M. I. D. injected intracutaneously produced as severe an infection as did one million M. I. D. However, one M. I. D. of virus produced different results in different individuals: (1) a typical unmodified attack resulted in 2 of 4 volunteers; (2) a short febrile illness without rash, followed by solid immunity, occurred in one of 4 volunteers, and (3) no evidence of infection, i.e., neither symptoms, fever nor leukocytic changes, followed by a partial immunity to reinfection in the fourth volunteer.

Cutaneous Lesions and the Local Sparing Phenomenon.—Intracutaneous injection of 0.1 to 0.2 ml. of human serum, containing 10 or more M. I. D. of dengue virus, was regularly followed after an interval of 3 to 5 days by local edema and erythema, 1 to 4 cm. in diameter. This skin lesion invariably appeared one or more days before onset of fever and, as a rule, disappeared before the occurrence of the generalized maculopapular or scarlatiniform eruption. However, when the generalized rash did appear, a striking sparing phenomenon was observed at the sites of the original skin lesions which stood out as blanched zones surrounded by the diffuse rash. This sparing was specific in that normal human serum or other irritants did not give rise to the same phenomenon. The specificity of the dengue skin lesion, and its dependence on local viral multiplication, were further demonstrated by the fact that no lesion occurred when a suitable number of M. I. D. of dengue virus were mixed with homologous convalescent serum prior to inoculation, or when the virus was inoculated into dengue convalescents. Injection of convalescent serum into an established maculopapular or scarlatiniform rash failed to cause blanching.

It was of interest to note that the local irritation and small papules resulting from the bites of dengue-infected Aedes aegypti mosquitoes could not be distinguished from those resulting from the bites of uninfected Aedes aegypti mosquitoes. However, when the generalized rash occurred, it was more marked at the sites originally bitten by the normal mosquitoes, while each papule resulting from the bite of an infected mosquito was surrounded by a blanched halo. Biopsies performed on the local skin lesions showed that the epithelium was not involved and no inclusion bodies were found. The chief abnormality was found in and about the small blood vessels and consisted of endothelial swelling, perivascular edema and infiltration with mononuclear cells.

Particle Size of the Virus.—The diameter of the virus as determined by filtration of highly infectious human serum through gradocol membranes was estimated at 12 to 25 millimicrons (μm), because all the volunteers who received the filtrates from membranes with an average pore diameter (A. P. D.) of 75 μm or greater developed typically severe dengue, while 2 volunteers who received the filtrate from the membrane with an A. P. D. of 50 μm remained well. However, since the latter volunteers exhibited a partial immunity to reinoculation several months later, it is possible that approximately one M. I. D. of virus might have passed the 50 μm membrane, and that the virus may actually be somewhat smaller than 17 to 25 μm. The virus could be sedimented from human serum by centrifugation at 24,000 r.p.m. for 90 minutes in an 8-inch rotor of a vacuum ultracentrifuge. Examination with the electron microscope of preparations from
highly infectious human dengue serum, purified by differential centrifugation, revealed dumb-bell-shaped structures (700 m\( \mu \) x 20–40 m\( \mu \)), which were not found in similar preparations from normal human serum (studies carried out in association with Capt. R. W. Schlesinger, M. C. and Dr. Wendell M. Stanley of the Rockefeller Institute).

Cultivation in Various Media in Vitro.—Although successful cultivation of dengue virus on the chorioallantoic membrane of the developing chick had been claimed, the claims were either not substantiated by tests on human beings, or the human tests which were carried out yielded [in this writer's opinion] no conclusive evidence that the cultured material was dengue virus. Numerous attempts were made to propagate a variety of strains of the human, unmodified virus in embryonated eggs inoculated by various routes, or in tissue cultures containing minced chick embryo, minced mouse embryo, or human leukocytes. However, all of these attempts yielded negative results as judged by tests on human volunteers who failed to develop either disease or immunity. After the dengue virus was successfully adapted to propagation in mice, it proved possible to cultivate it in chick embryos.

Experimental Illness Following Infection with Dengue Virus by Scarification of Skin, Conjunctival or Nasal Instillation.—The investigation of the results of infection by "unnatural" routes was part of a search for some means of producing immunity without disease. Undiluted, infectious serum rubbed into the scarified skin produced unmodified dengue. Nasal instillation of one million or 100,000 M. I. D. (based on infectivity by intracutaneous route) resulted in a very mild or negligible illness with rash in 4 of 6 human volunteers, while 2 others suffered from a typical, unmodified attack. Nasal instillation of 10,000 M. I. D. produced neither disease nor immunity. Instillation of 200,000 M. I. D. into the conjunctival sac produced typical dengue in one volunteer, while 10,000 M. I. D. produced neither disease nor immunity in another. Mosquitoes feeding on a volunteer with the modified, negligible disease following nasal instillation of the virus, at the time the rash first appeared (in absence of fever), developed the capacity to transmit the unmodified disease. About 5 weeks after nasal instillation of the virus, 3 of the volunteers, who exhibited the mildest, symptomatic reaction, were exposed to the bites of dengue-infected mosquitoes and were found to be immune.

Effect of Atabrine and Penicillin.—The effect of atabrine on the course of dengue was investigated in order to determine whether or not this drug might have been responsible for the mild forms of dengue encountered in our troops in New Guinea. Three volunteers were given 0.1 gram of atabrine daily for a period of 12 days prior to infection by exposure to the bites of 22 to 24 Aedes aegypti mosquitoes. The same dose of atabrine was then continued throughout the incubation and febrile periods. All 3 volunteers, who were daily observed to swallow the drug, developed the disease without modification of the incubation period, clinical severity, rash or duration of fever. Penicillin administered at the onset of fever (25,000 units every 3 hours, day and night) had no effect on the course of the experimental disease.

Capacity of Certain American Mosquitoes to Transmit Dengue.—This investiga-
tion was carried out to determine to what extent mosquitoes prevalent in the U. S., other than *Aedes aegypti*, might be potential vectors. The feeding of any species on human volunteers, although occurring at the onset of fever, was always checked by allowing *Aedes aegypti* to feed simultaneously. The extrinsic incubation periods varied from 2 weeks to over a month. The following species of mosquitoes were found not to transmit the infection under conditions which permitted *Aedes aegypti* to act as an effective vector: *Aedes vexans, Aedes solicitans, Aedes taeniorhynchus, Aedes cantator, Anopheles punctipennis, Anopheles quadrimaculatus*, and *Culex pipiens*.

**Preservation of Virus by Freezing and Lyophilization.**—Dengue virus, in the form of human serum, has been found to be remarkably stable on storage in the frozen state in a dry ice chest, or in the lyophilized state in an ordinary refrigerator. Preparations have remained infective for a period of 5 years, the longest interval tested thus far (7).

**Effect of Formalin and Ultraviolet Light on Infectivity and Immunogenic Capacity.**—The virus in human serum or in mosquito suspensions was inactivated by ultraviolet light (an exposure of 0.47 sec. in the Oppenheimer-Levinson apparatus was sufficient) or by 0.05 per cent formalin, and when so inactivated, it failed to produce immunity in human volunteers.

**Effect of Dengue Virus on Laboratory Animals**

Infant mice and hamsters, newborn and adult guinea pigs, cotton rats, rabbits and rhesus monkeys inoculated intracerebrally or intra-abdominally, or both, with serum or whole blood of proved infectivity for human beings exhibited no clinical signs of infection. Tests with guinea pig brain tissue in a human volunteer revealed no evidence of inapparent infection in the guinea pig. On the other hand, subinoculation of serum obtained from rhesus monkeys 6 days after inoculation of human serum produced typical dengue in a human volunteer, thus confirming the occurrence of inapparent infection in rhesus monkeys. The ultimate adaptation of the virus in mice will be described subsequently.

**Immunologic Studies on Human Volunteers**

**Immunity to Reinfection with Homologous and Heterologous Strains of Virus.**—Human volunteers reinoculated with the same strain of virus proved to be completely immune for as long as 18 months after a single infection—the longest period tested thus far. These tests are especially significant because they were carried out on human beings residing in nondengue areas and there can be no question of the immunity having been reenforced by intercurrent, inapparent reinfection. The results of reinoculation with a heterologous strain were found to depend on the interval after the original attack. Active immunity to heterologous strains was, as a rule, demonstrable during the first 2 months after an attack. That this cross-immunity is most likely due to a group specific antigenic stimulus and not to nonspecific resistance resulting from a preceding febrile illness was confirmed by the fact that phlebotomus fever convalescents exhibited no such immunity to dengue. Reinfection with a different immunologic
type of dengue virus approximately 2 to 3 months after a primary attack had been found to give rise to malaise and slight fever for less than 24 hours, and mosquitoes which fed on such patients acquired the capacity to transmit the unmodified disease. Group immunity was still evident for as long as 9 months after the primary attack, since volunteers who were then shown to be resistant to the homologous type reacted with a rash-free, febrile illness of 2 to 3 days' duration upon inoculation with a heterologous type of dengue virus. These modified attacks, clinically not recognizable as dengue, were proved to be dengue by both mosquito and blood transmission tests. By this method of comparison it was found that 4 of the 7 human strains studied, i.e., the Hawaii, New Guinea "A", and 2 strains from India, belonged to one immunological type or group, while the other 3, all from New Guinea, belonged to another. Since more than one immunological type of dengue virus was thus found to be present in New Guinea at the same time, it is possible that reinfection with a heterologous type of virus may have been one of the causes for the many atypical febrile illnesses which were diagnosed as fever of unknown origin, but which were shown to be dengue by recovery of the virus.

_Demonstration of Type-Specific Neutralizing Antibodies._—Previous attempts by other investigators to demonstrate a neutralizing antibody in convalescent dengue serum were unsuccessful. It became apparent that the previous lack of success was at least in part due to the fact that unknown quantities of virus were used in the tests. Thus, it proved possible to demonstrate that the serum of volunteers convalescent from infection with the Hawaiian strain of dengue virus was capable of neutralizing 1,000 but not 100,000 M. I. D. of virus. This means that when 0.9 ml. of convalescent serum was mixed with 0.1 ml. of acute dengue serum (with one million M. I. D. per 1 ml.) diluted 1:100, and the mixture, after in vitro incubation, was injected intracutaneously in 0.2 ml. amounts in a human volunteer, neither local lesions nor illness developed. Utilizing the skin surface of the arms, abdomen, and back, 20 to 40 different sera, including normal controls, could be tested simultaneously in a single volunteer, the presence or absence of neutralizing antibody in a given specimen being determined by the appearance or nonappearance of a local lesion. Neutralizing antibody against 1,000 M. I. D. of virus was demonstrated in the serum of Hawaii dengue convalescents obtained 1 week, 1, 2, 3, and 8 months after defervescence. The dermal neutralization tests which established the type-specificity of the antibody were performed with approximately ten minimal skin-lesion-producing doses. The results obtained in tests with 5 human strains of virus, shown in Table 2, indicate only 2 distinct immunologic types among them—the Hawaii and New Guinea "A" belonging to one type, and the New Guinea "B", "C", and "D" strains to another. Convalescent serum obtained during the first 2 months after an attack, when active immunity to heterologous types was readily demonstrable, contained only type-specific antibody in the dermal neutralization test.

_Interference between 17-D Strain of Yellow Fever and Dengue Viruses._—The investigation on the relationship between the yellow fever and dengue viruses was undertaken to determine whether or not vaccination with the 17-D strain
of yellow fever virus can modify the clinical course and severity of dengue. This was carried out in search for an explanation for the mild form which dengue assumed in American troops in New Guinea. Thirty volunteers were used in this study and the following results were obtained:

1. When dengue virus, in amounts of 10 to 1 million human M. I. D., was inoculated simultaneously with, or 3 days after, yellow fever vaccine, the onset of dengue was delayed for 3 to 6 days, and the resulting disease was milder and of shorter duration.

2. When the dengue virus was injected 1 week after the yellow fever vaccine, the incubation period was unaffected, but the resulting disease was milder and of shorter duration.

3. When infection with dengue virus (either by the bites of infected mosquitoes or the inoculation of 10 human M. I. D. of infectious serum) was postponed for 5 weeks after the yellow fever vaccine, neither the incubation period nor the duration or severity of the resulting dengue were modified.

It was concluded, therefore, that immunity to yellow fever, resulting from vaccination of human beings with the 17-D strain of yellow fever virus neither protects against nor modifies the disease resulting from infection with dengue virus. While a definite interference phenomenon was demonstrable during the period of propagation of the two viruses, and while the simultaneous injection of yellow fever vaccine and dengue virus resulted in a rather mild and modified form of dengue, it could not be regarded as a feasible method for the simultaneous immunization against both diseases.

**Interference Between Dengue and Viscerotropic Yellow Fever Virus in Rhesus Monkeys and Mosquitoes**

One of the reasons the problem of interference between these two viruses was pursued further is the peculiarity in the epidemiology of yellow fever that it has apparently spared many parts of the world (e.g., India, Indonesia, Australia,
etc.) where dengue has been endemic. The study on rhesus monkeys and mosquitoes was carried out in association with Dr. Max Theiler of the International Health Division of the Rockefeller Foundation. In the tests on human volunteers, the yellow fever virus (17-D) was the agent which produced the inapparent infection while dengue produced the clinically apparent disease. In the tests on rhesus monkeys, the dengue virus (highly infectious human serum injected intra-abdominally or intracerebrally) produced the inapparent infection while the viscerotropic yellow fever virus (Asibi strain) produced the clinically severe, fatal disease. When the dengue virus was injected in rhesus monkeys 2 or 3 days before the viscerotropic yellow fever virus, it interfered with the multiplication of the latter virus and 6 of 7 monkeys survived, while all 9 control monkeys, inoculated with yellow fever virus only, died. When the interval between the dengue and yellow fever inoculations was 4 to 7 days, there was still demonstrable interference with the multiplication of the yellow fever virus, but death of the monkeys, while postponed, was not prevented. When the yellow fever virus (100 M.L.D.) was injected one month after the dengue virus, 6 of 8 monkeys died in a manner which suggested that no significant cross immunity existed between the two viruses.

Since the *Aedes aegypti* mosquitoes serve as natural vectors for both the dengue and yellow fever viruses, and since available evidence indicated that mosquitoes remain infected for life, the possible occurrence of interference between these two viruses in mosquitoes was of special interest and possible epidemiologic significance. *Aedes aegypti* mosquitoes which first were proved to have become infected with dengue virus (by tests on human volunteers) were allowed to feed on monkeys infected with the highly virulent Asibi strain of yellow fever virus. After a suitable interval these mosquitoes were tested for their capacity to transmit yellow fever, and 2 of the 3 monkeys bitten by them died of yellow fever. However, tests in which extracts of individual mosquitoes were tested in mice suggested that some of the dengue-infected mosquitoes did not become infected with the yellow fever virus, while all the normal mosquitoes did. In view of the fact that in feeding on monkeys infected with the Asibi strain of yellow fever virus, each mosquito acquires about 10 million M.L.D. of virus, a degree of infection which may not obtain in nature, other experiments were performed in which normal and dengue-infected mosquitoes were allowed to feed on artificial mixtures containing varying amounts of yellow fever virus, and the multiplication of virus in them determined by tests in mice. These experiments strongly suggested interference with multiplication of smaller doses (about $10^5$ LD$_{50}$, or less) of yellow fever in dengue-infected mosquitoes, and in another biting test in monkeys, the normal mosquitoes which ingested $10^5$ LD$_{50}$ of virus transmitted yellow fever, while a similar number of dengue-infected mosquitoes which fed on the same mixture did not. These results suggested the possibility that the introduction of yellow fever virus in a dengue-endemic area may find enough mosquitoes relatively refractory to the yellow fever virus to prevent the establishment of yellow fever in the same area.
Adaptation and Propagation of Dengue Virus in Mice

The following quotation from this writer's report to the Fourth International Congresses on Tropical Medicine and Malaria (8) summarizes the experiences encountered in the adaptation of dengue virus to mice:

"...the many similarities between the viruses of yellow fever and dengue and the available knowledge of the varying behavior of yellow fever virus in mice, were, in large measure, responsible for the persistence with which my associate, Dr. R. W. Schlesinger, and I continued the work on adaptation of dengue virus in mice in the face of many failures. Ultimately it appeared that in the primary adaptation of human dengue virus in mice, the important factors were the breed and age of the mice, the strain of virus, and the proportional concentration of virus and inhibitory factor apparently present in infectious human serum. The best results were obtained with the Hawaii virus, either concentrated by ultracentrifugation at 25,000 revolutions per minute for 90 minutes, or with highly infectious human serum diluted 1:100. The so-called Webster Swiss mice were better than other albino mice, and the 'dba' mice bred at the Jackson Memorial Laboratory at Bar Harbor, Maine, appeared to be better than any of the albino and colored (C-57 black, C-57 brown) mice that were tested. Two-weeks-old or younger mice were needed for the initial passages, and it was not until the virus was thoroughly adapted after many serial passages in young mice, that older mice would succumb with regularity. The diagram in the accompanying chart (fig. 1) shows only that portion of the passage-tree which yielded successful consecutive passages and not the hundreds of mice which in the early passages exhibited nothing or yielded nothing on further passage. Only 10 to 20 per cent of the inoculated mice at first exhibited clinical signs of the infection (slight weakness of the extremities detectable only by special tests in some and distinct flaccid paralysis or encephalitic signs in others), and the incubation period was frequently 3 to 4 weeks. It took 15 passages before 100 per cent of the mice inoculated with a 10 per cent brain and cord suspension succumbed, and the incubation period was reduced to 9 to 14 days. At this stage the 50-per cent morbidity and mortality endpoint in mice did not exceed 10^{-5}, but continued serial passages in young mice gradually increased the titer and shortened the incubation period until now, after more than 80 such passages, the intracerebral titer for the 0.03 cubic centimeters dose in mice is 10^{-5.8}, and the incubation period for the highest concentration is approximately 6 days. We could not be certain that this virus in mice was indeed dengue virus, until, after appropriate preliminary tests in laboratory animals, the early passage material was inoculated in human volunteers and produced in them solid immunity to unmodified human dengue virus. Similarly, we know that the virus of higher potency which is now being passed in mice is still dengue virus, because it is neutralized specifically by the human convalescent sera and by the sera of rhesus monkeys and chimpanzees inoculated with human virus that has never been through mice. This mouse-adapted dengue virus produces neither apparent nor inapparent infection in cotton rats, hamsters, guinea pigs, or rabbits..."
Effect on Human Volunteers of Mouse-Propagated Dengue Virus at Different Stages of Adaptation

Extracts of the brain and spinal cord of paralyzed mice, derived from the first 6 consecutive passages in mice, upon inoculation in human volunteers produced clinical manifestations of varying severity — relatively mild in some and fully severe and unmodified in others (9). Local skin lesions appeared at the sites of intracutaneous injection after an incubation period of 5 to 7 days, and marked generalized maculo-papular and ultimately petechial eruptions appeared in all. Leukopenia with the qualitative and quantitative changes in the leukocytic formula usually seen in dengue were also present. Blood serum obtained from the above volunteers at the onset of the generalized eruption produced typical dengue in another volunteer.

Beginning with the seventh passage in mice, however, the virus had lost its capacity to produce the severe illness and protracted fever, characteristic of the unmodified disease in human beings, but retained its capacity to produce a rash and solid immunity to the unmodified virus. Fever either did not occur,
or was low-grade, lasting 24 hours, or less. Blood taken from such persons at the time the rash first appeared produced only the same type of modified reaction in other volunteers. Immunity to infection with the unmodified virus was already present 12 days after inoculation, the shortest interval tested.

Use of Mouse-Adapted Dengue Virus as a Vaccine

The results of tests on 9 human volunteers inoculated with the seventh to the tenth mouse-passage virus indicated that the virus had undergone sufficient attenuation to permit its consideration for use as a vaccine against dengue. Accordingly, two vaccines were prepared from fifteenth and nineteenth mouse-passage virus and tested both in medical student volunteers of the Army Specialized Training Program and in schizophrenic patients. The fifteenth mouse-passage vaccine, consisting of a centrifuged 1:10 mouse-brain extract in 10 per cent human serum-saline solution, was tested in 16 individuals in doses of 0.5 ml. of the 1:100, 1:1,000 and 1:10,000 dilutions. While the titer of this virus in mice was only 1:100 for the 0.03 ml. dose, all inoculated persons (including those receiving 0.5 ml. of the 1:10,000 dilution) developed a maculo-papular eruption of varying extent, while systemic symptoms were either absent, negligible or very mild. Twenty-one to 38 days after vaccination, all were found to be immune upon exposure to Aedes aegypti mosquitoes of proved infectivity. Thus, it became apparent that the extract from a single mouse brain and cord, preserved in the frozen state, had enough antigen in it to immunize at least 10,000 people. When some of the same fifteenth mouse-passage vaccine was lyophilized together with yellow fever vaccine and tested in 10 students (the inoculum contained 1:100 or 1:1,000 dengue and the standard dose of yellow fever vaccine), 5 of 7 students subsequently exposed to dengue-infected mosquitoes were not immune. It was not clear whether the chick embryo yellow fever vaccine did not constitute a suitable protective agent for the lyophilization of the diluted, modified dengue virus, or whether the proportions of the two viruses in the inoculum were such that the multiplication of the smaller dose of modified dengue virus was suppressed by the larger dose of yellow fever virus.

Since it was desirable to get away from human serum as a constituent of the vaccine, crystalline bovine albumin was selected as the protein to be used for both extraction and lyophilization in the tests with the nineteenth mouse-passage vaccine. A relatively large amount of vaccine (enough for more than 50,000 men) was prepared both as a model for production on a still larger scale and for the purpose of having enough available for a field trial if the preliminary human tests were satisfactory. With the aid of the facilities of the laboratories of the International Health Division of the Rockefeller Foundation, the vaccine was lyophilized in 2 forms—as 10 per cent and as 1 per cent extract of mouse brain and cord in 10 per cent crystalline bovine albumin. Simultaneous titrations in mice of the frozen and lyophilized 10 per cent vaccines revealed that the lyophilized material had only \( \frac{1}{10} \) as much infective virus as the frozen preparation, i.e., that 90 per cent of the infectivity was lost on lyophilization. However, tests on 15 persons revealed that the 10 per cent lyophilized vaccine produced
the same type of reactions (i.e., predominantly rash) as the fifteenth mouse-
passage frozen vaccine, except that in the 1:10,000 dilution, the lyophilized
preparation produced rash in only 1 of 3 individuals, and immunity to a large
dose of unmodified dengue virus (probably as much as 1 million M. I. D.) in 2
of the 3 individuals. The 1 per cent lyophilized vaccine was apparently ineffective
(probably more deterioration on lyophilization) since neither rash nor immunity
resulted from the 1:1,000 or the 1:10,000 doses.

Each vial of the 10 per cent lyophilized vaccine contained 1 ml. of the centri-
fuged extract of mouse brain and cord, and was to be reconstituted in 20 ml.
of saline. Thus 0.1 ml. of this diluted material (the projected dose for subcutane-
ous injection) contained the mouse brain and cord extract as well as the crystal-
line bovalbumin in a final dilution of 1:200, and one vial would have supplied
enough vaccine for approximately 200 men. This vaccine was to be used in a
field trial among troops who were being transferred from Europe to the Pacific,
bu the war fortunately came to an end before this eventualty occurred.

*Infectivity of Aedes Aegypti Mosquitoes Feeding on Human Beings Inoculated
with Mouse-Adapted Dengue Virus*

This investigation was prompted by two practical questions: (a) is it possible
that the virus might revert to its original, unmodified virulence after passage
through mosquitoes and (b) could *Aedes aegypti* mosquitoes transmit the modified
infection after feeding on vaccinated individuals at certain periods after inoculation? The following results were obtained: (a) *Aedes aegypti* mosquitoes which fed on the human volunteer inoculated with the second and third mouse-
passage virus (i.e., before fixed modification had occurred) during the period
of low-grade fever produced unmodified dengue in another volunteer after an
extrinsic incubation period of 22 days, but not after 15 days, even though 40
mosquitoes fully engorged. (b) *Aedes aegypti* mosquitoes which fed on people
inoculated with the tenth mouse-passage virus acquired the capacity to transmit
the modified infection, i.e., the bitten volunteer developed only the rash and
leukopenia without fever or other significant symptoms. (c) Extensive tests
carried out on students, inoculated with the fifteenth mouse-passage virus, indicated
that several lots of *Aedes aegypti* mosquitoes, which had fed daily on the
experimental subjects for 14 days after vaccination, were unable to transmit the
infection even after prolonged extrinsic incubation periods of 29 to 33 days.
With 22 to 42 mosquitoes engorging in the tests on each volunteer, there was no
rash or other clinical evidence of infection in any of the 14 men who were ex-
posed in these tests.

*Cultivation of Mouse-Adapted Dengue Virus in Chick Embryos*

No success was achieved in the cultivation of dengue virus in chick embryos
until the virus had undergone approximately 16 consecutive passages in mice.
Attempts to propagate the mouse-adapted virus, using passages IV, V and XIII,
in fluid or plasma clot cultures containing mouse embryo brain tissue were un-
successful in that no virus pathogenic for mice was demonstrable after one, two
or three passages. When the 13th passage mouse-adapted virus was used for inoculation of 6 or 10 day old chick embryos which were subsequently incubated for 8 or 4 days, respectively, at 37°C, no mouse-pathogenic virus was demonstrable after one to four serial passages. The results were the same when the inocula were introduced into the yolk sac or embryo, allantoic or amniotic sacs. When the 13th passage mouse-adapted virus was inoculated directly into the brain of 10 day old embryos which were subsequently incubated for 7 days at 37°C, mouse-pathogenic virus was demonstrable in the chick embryo brain tissue in the first passage, but not in the second. Repetition of this procedure with 16th passage mouse-adapted virus yielded negative results even in the first passage. When the 16th or 18th passage mouse-adapted virus was used for inoculation (toward the embryo) of 5 day old chick embryos which were subsequently incubated for 8 days at 37°C, mouse-pathogenic virus was demonstrable in whole embryo extract of the first passage in 3 separate series. In only one of these series, however, was virus demonstrable in the second passage, but even in this series it was no longer present in the 3rd passage when the incubation was at 37°C. It was found, however, that incubation at 35°C was more suitable for the serial propagation of the virus, so that it has been possible to demonstrate appreciable amounts of virus in the 3rd passage of at least 2 series. There was some indication that 4 or 5 days of incubation was not as good as 8 days, and that while the virus was present in the amniotic membrane as well as in the whole embryo, little or no virus was found in the amniotic fluid.

It finally proved possible to obtain continuous cultivation of the virus in embryonated eggs when 5-day embryonated eggs were used for inoculation and a period of from 8 to 10 days at 35°C for incubation. Tests in 4 human beings with extracts of the 3rd passage whole chick embryo or amniotic membrane revealed that the virus had remained in its modified form, producing the characteristic macular or maculopapular eruption after an incubation period of 9 to 11 days, without fever (except for elevation of 1°F for 24 hours in one subject) or other clinical manifestations.  

Serological Tests with Mouse-Adapted Dengue Virus

After the Hawaii dengue virus became sufficiently adapted to mice to yield an intracerebral titer of $10^{-3}$ to $10^{-4}$, it was possible to develop a mouse test for the detection of neutralizing antibody. At that stage no neutralization of the virus could be demonstrated without preliminary incubation of the mixtures at 37°C for 2 hours, or if the serum was heated at 56°C for 30 minutes. Neutralization tests performed under optimal conditions (i.e., with sera transported and stored in dry ice, and serum-virus mixtures incubated at 37°C for 2 hours) on sera from human volunteers infected with various strains of virus and from individuals with histories of naturally acquired infections in various parts of the world yielded considerable interesting information. Thus, it was established that the neutralizing antibody was type-specific and appeared as early as 1 week.

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after onset of illness and persisted for at least 2 years (more recently, at least 4 years) in individuals residing in nondengue areas. Neutralization tests on sera from people with a diagnosis of dengue during the Hawaii epidemic of 1943–1944, or the Japanese epidemics of 1944–1945, revealed that the Hawaii type of virus was probably predominant in those outbreaks, while similar tests on the sera of Americans who had the disease on Guam in 1944–1945, and from Americans and Panamanians in the Panama Canal Zone indicated that another type or types of dengue were probably more prevalent there.

TESTS FOR DENGUE IN THE PHILIPPINES AND OKINAWA DURING WORLD WAR II

Large numbers of cases of a febrile illness (or febrile illnesses) clinically similar to dengue, with the important exception that there was no rash, occurred in 1945 in the Philippine Islands and on Okinawa. In view of the success that was encountered in the recovery of several strains of dengue virus from clinically atypical cases in New Guinea, repeated attempts were made to demonstrate the presence of dengue virus in patients with this atypical illness in the Philippines and Okinawa. The serological tests for dengue were not yet adequately developed at the time, and reliance had to be placed on the inoculation of serum, obtained within 24 to 48 hours after onset of fever, into human beings in the U. S. Three pools of sera derived from 11 patients in Leyte, P. I. (February–March 1945) were subinoculated into 6 human beings in the U. S., and 2 sera from 2 patients on Mindoro, P. I. (April 1945) were subinoculated into 2 human beings in the U. S., but all the recipients remained well. Although the donor patients were selected because they remained free of jaundice, there was, nevertheless, a great deal of hepatitis in those regions at the time. It is noteworthy, therefore, that the recipients were observed for many months but did not develop jaundice. The sera of 5 patients from Okinawa (June–August 1945) were similarly inoculated into 4 human beings in the U. S. with completely negative results.6

DENGUE IN PANAMA CANAL ZONE DURING WORLD WAR II

An investigation into the occurrence of dengue in Panama during World War II was carried out in January and February 1946. It was found that in 1941 and early 1942, at a time when a great deal of new activity was in progress in the Canal Zone and when there was an influx not only of military personnel but also of laborers from adjacent countries, there was a considerable outbreak in Americans of a disease with clinical manifestations entirely compatible with a diagnosis of dengue. However, the diagnosis of dengue was not made and practically all of these cases were found in the files of the Gorgas Hospital under the diagnoses of “nasopharyngitis,” “nasopharyngitis with rash,” or occasionally

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6 Recent work (Gauld, R. et al., Leptospiiral meningitis: report of outbreak among American troops on Okinawa, J. A. M. A., in press) suggests that these illnesses might have been due to leptospira; the negative human transmission tests could be accounted for by the fact that the sera were frozen in dry ice and leptospira are not well preserved in the frozen state.
"X-Y-Z" fever. However, Major L. McCarty Fairchild, (1945) M. C., of the Gorgas Hospital reported a selected series of these cases as "Dengue-like Fever in Panama." (10) My own analysis of the charts of these patients indicated that clinically they represented classical forms of dengue. It is of interest that *Aedes aegypti* breeding in Ancon, Balboa and Panama City were sufficiently high (indexes of 10 to 17 per cent) to justify warnings regarding intensified mosquito control. It is furthermore of interest that during this same period the "great majority" (variously estimated as 80 per cent) of the native Panamanian population in Panama City was attacked by a disease which was diagnosed for the most part as measles, German measles, or glandular fever. However, some of the Panamanian physicians (11), suspected dengue at the time and my own examination of many of the clinical records revealed syndromes clinically characteristic of dengue. There were apparently no further outbreaks after 1942, but sporadic cases probably continued to occur since I observed four clinically characteristic cases in Americans during my visit early in 1946. Neutralization tests in mice yielded positive results with 3 of 8 convalescent sera from the 1941–1942 outbreak, and 1 of 6 sera from recent convalescents. It would appear that a type of dengue virus, immunologically distinct from the Hawaiian strain, may be prevalent in Panama. This investigation also suggested the possibility (a) that the interior of Panama may be an endemic focus of dengue, and (b) that the *Haemagogus* mosquitoes and monkeys in the jungles of Panama should be investigated as a possible source of "jungle dengue fever."

**Dengue in Japan During World War II**

According to an inquiry carried out by the writer in Japan in 1946, it would appear that approximately 2 million cases of dengue may have occurred in the port cities of Nagasaki, Kure, Sasebo, Kobe and Osaka between 1942 and 1945 (8). Dengue was said to have been unknown in Japan before 1942, and the epidemics were correlated with two facts: (a) These cities were the ports of entry for people from Shanghai, Singapore, and the Malay States where dengue was prevalent at the time, and (b) the water shortage and the later bombing led to the storage of water in all sorts of containers which became the breeding grounds for *Aedes albopictus*. The city of Osaka with a population of about 2 million had about 5,000 cases in 1942; 3,000 to 4,000 cases in 1943; 400,000 to 600,000 cases in 1944, and an unknown number in 1945, when, because of the air raids, the population dispersed to the surrounding villages. The situation was said to be similar in other port cities. Examination of the clinical charts of cases which occurred among the hospital personnel in Osaka revealed the classical clinical picture of dengue, and blood obtained from many of these patients in 1946 neutralized the mouse-adapted dengue virus.

Many Japanese investigators carried out experimental studies on dengue during the war, and many strains of virus were reported to have been adapted to a variety of experimental animals. Some of these animal-adapted viruses were no longer available in 1946. However, of 5 strains of "dengue" virus which were submitted by several investigators for comparative study, only the 3 mouse-
adapted strains recovered by Doctors S. Hotta and R. Kimura of Kyoto turned out to possess the properties of dengue virus; the other 2 “dengue” viruses submitted by others turned out to be Rift Valley Fever in one instance and fixed rabies virus in another. The neutralization tests performed with the Japanese convalescent sera indicated that the Hawaii type of virus was either predominant in, or exclusively responsible for, the epidemics in Japan.

**SUMMARY**

Research on dengue in the U. S. during World War II provided the following new information of special interest to military preventive medicine:

1. Proof of the existence of multiple immunological types of dengue.
2. The long persistence of immunity to homologous types of virus under conditions precluding reenforcement of immunity by subclinical reinfection.
3. The modifications of the clinical manifestations of the disease which result from reinfection with a heterologous type of virus at various periods after the primary attack.
4. The demonstration that in areas (e.g., New Guinea) where more than one immunological type of virus is present, fevers of unknown origin, clinically not recognizable as dengue, are actually caused by the dengue viruses.
5. The demonstration that type-specific immunity to dengue is associated with neutralizing antibodies for the virus, which can be used for diagnostic and epidemiologic survey purposes.
6. The propagation of dengue virus in mice with the resulting appearance of a mutant or variant strain which could be used for active immunization.

In addition to the discoveries listed above, a great deal more was learned about the basic properties of the dengue viruses. Thus was dengue research brought from the field into the laboratory and further progress has been made possible by work on experimental animals instead of on human volunteers.

**REFERENCES**

In future issues of this Journal, references will be cited by date and not by number.