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STUDIES ON ARTHROPOD-BORNE VIRUSES

FINAL SCIENTIFIC REPORT

by

Charles L. Wisseman, Jr., M.D.

October 1975
(For the period 1 Sept 1970 to 31 Aug 1973)

Supported by

U.S. ARMY MEDICAL RESEARCH & DEVELOPMENT COMMAND
Office of the Surgeon General, Washington, D. C. 20314
in cooperation with the Commission on Rickettsial Diseases
of the Armed Forces Epidemiological Board

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University of Maryland School of Medicine
Department of Microbiology
Baltimore, Maryland 21201

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SUMMARY

I. Dengue Virus Vaccines. Starting with the Sabin mouse adapted strain of type 1 dengue virus, four different passage series were developed. All four strains were characterized in mice, and in monkeys. Monkeys inoculated intracerebrally with these strains developed neutralizing antibodies but presented no clinical evidence of paralysis, encephalitis or weakness. Fever was minimal or absent.

A limited field trial was carried out on 16 young adults. One strain, the MD-1 strain, failed to cause any detectable undesirable reactions but did result in the production of neutralizing antibodies. A larger group of volunteers was tested with the vaccine. All volunteers developed neutralizing antibodies and clinical symptoms were minimal or absent.

A larger field trial was conducted on volunteer military personnel at Fort Bragg, North Carolina. As in the previous studies, no untoward reactions were seen in the vaccinated individuals although a transient rash was seen in 10-15% of the volunteers.

The opportunity for a larger field trial was afforded when a dengue epidemic occurred in Puerto Rico. Although subsequent studies revealed that the etiologic agent of the epidemic was a strain of type 3 dengue virus, the type 1 dengue vaccine afforded a partial protection for almost three months. The vaccine itself did not cause any significant reactions in the vaccinees.

Although the vaccine had been shown to cause minimal reactions in volunteers - it was at least 50% effective against homologous challenge, afforded significant heterologous cross immunity against type 3 dengue under epidemic conditions, and was free from extraneous passenger agents, - serious objections were raised against the use of a mouse brain propagated vaccine. It became necessary to explore tissue culture systems as possible substitutes for the production of the MD-1 vaccine virus.

Chick embryo cell cultures were tested but did not replicate significant levels of the vaccine virus. Hamster kidney and monkey kidney cell cultures replicated the MD-1 virus to $10^{5.0}$ - $10^{6.0}$ PFU/ml but the problem of extraneous viral agents and the problems attendant with the screening and testing programs made these systems prohibitive for live virus vaccine production.

The human diploid line, WI-38 was tested extensively. A variety of parameters was studied in attempts to increase

efficiency of adsorption and virus yield. Techniques were developed for serial passage of the vaccine virus in monolayer cultures, with yields of $10^{5.6}$ - $10^{6.1}$ PFU/ml. A stabilizing medium, devoid of whole serum was developed for production of the vaccine virus. Attempts to clone the vaccine virus, either by terminal dilution or by plaquing under agar or methylcellulose, were unsuccessful.

While the work with the attenuated type 1 dengue virus vaccine was in progress, similar studies were carried out on type 2 dengue virus strains. The type 2 vaccine strain obtained from Dr. Sabin's laboratory caused a marked febrile response in most of the experimental monkeys tested and 7 of 10 monkeys developed apparent weakness or paralysis. Additional type 2 strains were adapted to mice, and tested in monkeys. At least three of these strains showed some promise as vaccine candidates in that they did not cause paralysis or death in monkeys although a varied febrile response was noted. However, no further tests were conducted because the major thrust of the vaccine development and testing program was devoted to studies on the MD-1 strain.

During the early phases of the vaccine development program, some studies were carried out on formalinized dengue virus vaccines. Although the initial studies in mice were promising, subsequent testing in monkeys was unsatisfactory when we were unable to demonstrate an immunological response to the formalinized vaccines. Further work on killed vaccines was abandoned.

II. Studies with 17D Yellow Fever Vaccine. Since extensive cross reactions are found with the HI tests, the CF tests and to a lesser extent the neutralization test and, since repeated exposure of experimental animals to one virus appears to broaden and intensify serological cross reactions with other viruses, it was conceivable that immunization with two or three selected group B viruses, might collectively produce a broad range of protection against an appreciable proportion of group B viruses.

To test this hypothesis, volunteers with pre-existing Japanese encephalitis neutralizing antibodies were vaccinated with 17D yellow fever vaccine and the neutralizing antibody response monitored. The yellow fever antibody response was similar to that observed in individuals without prior group B exposure. There was, however, a broadening of the neutralizing antibody spectrum for other group B viruses. In a subsequent study, volunteers with Japanese encephalitis neutralizing antibodies were immunized with the 17D yellow fever vaccine and challenged one month later with type 1 dengue virus. No cross protection against dengue fever was detected.

Virologic and serologic studies were conducted on individuals receiving the 17D yellow fever vaccine. On primary vaccination, viremia peaked on about day four and declined thereafter. Upon revaccination, 14 months after primary vaccination, no viremia could be detected, and the neutralizing antibody response reached levels similar to those obtained after primary immunization. Yellow fever antibodies were shown to persist for at least 19 years without benefit of booster immunization or known group B exposure.

III. Pathogenesis of Arbovirus Infections. The pathogenesis of West Nile virus was studied in normal, passively immunized and actively immunized hamsters. In normal hamsters viremia reached a peak on the fourth day and decreased thereafter. This was about the same time that neutralizing antibodies were first detected. Subsequent to antibody appearance virus in most tissues declined with the exception of the inoculation site and the brain, where virus content increased until the animals died. Passive transfer of immune serum to hamster protected the animals only if the level of circulating antibodies was high. In immunized animals, most animals were protected from death from a standard dose of virus. Virus replication and/or spread was dependent upon the level of circulating neutralizing antibodies.

The pathogenic capabilities of three strains of type 1 dengue virus were studied in suckling, weanling and adult mice. All strains replicated well in suckling mice and to a lesser extent with the older mice. There seemed to be a direct relationship between serial mouse passage history of the virus and capacity to kill mice of increasing age. Hyperthermic mice tended to produce lower levels of low passage virus, and when surviving animals were returned to room temperature many tended to develop signs of neurologic disease. The high mouse passage virus was less affected by hyperthermic conditions.

Interferon levels in the brains of mice tended to be directly related to the degree of viral replication.

IV. Dengue Viruses in Rhesus Monkeys. It was thought that the rhesus monkey could be used as a primate model for dengue virus infections in Man. Although we could detect as little as 10 PFU/ml dengue virus in monkey plasma, we were unable to detect any viremia in monkeys infected with the 4 prototype dengue viruses. In subsequent studies monkeys inoculated with viremic human serum or low mouse passaged dengue virus, viremia could be detected but it was usually at a low level. In these studies only viremic monkeys subsequently developed neutralizing antibodies.

V. Sero-surveys. Sero-surveys were conducted on sera collected in Puerto Rico, Pakistan, Nepal, New Guinea, Australia and a number of Pacific islands. In most areas group B activity predominated followed by group A and then group C in activity.

VI. Isolation and Identification of Arboviruses. During the Puerto Rican dengue epidemic in 1963 four strains of virus were isolated from patients during the early acute phase of disease. Subsequent studies in tissue culture and in suckling mice revealed that all four strains were identical with one another but were not identical to any of the four prototype dengue viruses. In a collaborative study with WRAIR it was later determined that the virus causing the epidemic was a substrain of type 3 dengue virus.

A virus was isolated from the CSF of a Pakistani child during an epidemic of encephalitis. Extensive HI, CF and neutralization testing suggested that the agent was a group B arbovirus and most likely one of the dengue viruses. Since the encephalitis pattern of disease was not compatible with the classical features of dengue fever the available fragmentary clinico-epidemiological and virological data were re-evaluated. These data suggest that the epidemic was the result of two disease entities: a true encephalitis and hemorrhagic dengue.

In another study in West Pakistan, seven viral agents were isolated from hard ticks. Two new viruses, the Hazara virus and the D. G. Kahn were isolated in this study. Additional isolations included viruses similar to or identical with Semunya or Congo virus and two isolates similar to or identical with Crimean Hemorrhagic fever virus.

VII. Basic Studies. A variety of studies was conducted with West Nile virus. A sensitive plaque assay was developed using chick embryo cells and an unusually high divalent cation requirement described. Neutralization kinetics were studied with and without the addition of "accessory factor". Accessory factor was investigated with respect to levels in different animal species sera, thermolability, concentrations required for virus neutralization and the role of different immunoglobulins in virus neutralizations.

When the laboratory studies with dengue viruses were initiated, the suckling mouse was the only satisfactory host system for the production and assay of these viruses. A number of cell culture systems were studied and essentially a sensitive plaque assay was developed using BHK-21/15 monolayer cultures. Later cell culture adapted type 1 dengue virus was routinely produced in quantities sufficient for biophysical characterization. The density of type 1 dengue virus was determined and the morphology determined by electron microscopy.

Optimal conditions for long term low temperature storage of type 1 dengue was determined. Methods for the growth of high titered dengue 1 virus, in a chemically defined medium, were developed.

The relative merits of various neutralization tests employed for the identification of dengue and dengue-like viruses were investigated. Either the mouse neutralization test or the 50% plaque reduction neutralization test could be employed to identify prototype or sub-type dengue viruses provided monkey serum is employed. When hyperimmune mouse serum is employed in the tests, it may not be possible to identify some variant strains of dengue viruses.

The thermostability of three strains of type 1 dengue virus was determined at temperatures ranging from 26 C to 56 C. All three strains were similar in their thermolability but thermal inactivation appeared to proceed by two different mechanisms: one operative above 41 C and the other below 40 C.

Type 2 dengue virus-induced modification of host cell membranes were studied. By the combined use of enzymatic markers, electron microscopy and polyacrylamide gel electrophoresis the virus-induced protein and RNA synthesis could be localized in the rough endoplasmic reticulum of infected cells. After actinomycin D and cyclohexamide treatment seven virus specified proteins were associated with all membrane fractions. The relative levels of the various virus specified proteins were determined for each of the cell membrane fractions.

VIII. Genetic Studies. In an attempt to produce temperature sensitive dengue virus mutants, several chemical mutagens were employed. Nitrous acid was found to be too toxic and no surviving virus could be found after one minute treatment. Because erratic results were obtained with reduced concentrations of nitrous acid other mutagenizing agents were investigated. All four dengue serotypes were mutagenized with 5-azocytedine but testing had just been initiated when this program was terminated.

FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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I. Dengue Virus Vaccines

A. Background

Dengue has been a disease of importance to American military medicine for many years. Prior to World War II it was a serious problem in the Philippines and to a lesser extent a problem in Panama. Over the years (from 1907 - 1931) much of the basic information on dengue, such as viral etiology, mosquito transmission, epidemiology and immunity was obtained in classical studies in the Phillipines by a small group of investigators. Dengue became prominent again in the Southwest Pacific during World War II. At that time, the virus had not been adapted to laboratory animals, no vaccines were available and no laboratory diagnostic procedures were available. To cope with this problem the Army sponsored a program, directed by Dr. Sabin to study dengue. Working with human volunteers he was able to show that all available strains of dengue virus fell into one of two immunologic types. Subsequent studies by Dr. Hammon demonstrated the existence of two additional immunologic types.

The early human volunteer studies also yielded valuable information about the occurrence and persistence of both type specific and heterologous immunity. Immunity to reinfection by the homologous serotype was found to persist for at least 18 months. Heterologous challenge failed to produce disease for two months after initial infection -- however, subsequent infection resulted in a mild disease, with viremia, unrecognizable on clinical grounds as dengue, although a transient cross immunity could be determined by challenge experiments, the demonstration of neutralizing antibodies to the heterologous type, even at low levels, was a rare occurrence.

The adaptation of dengue viruses to mice provided a useful laboratory tool for the study of dengue viruses and permitted the development of the neutralization test, complement fixation test and hemagglutination inhibition test. Subsequent studies indicated that dengue viruses could be isolated directly from clinical materials by use of the suckling mouse.

Concomitant with the adaptation of dengue viruses to infection in weanling mice, type 1 virus was found to undergo a change in virulence for man so that it produced a modified type of infection. Experiments with mouse adapted viruses were carried out in human volunteers by Dr. Sabin's group. Incubation period was prolonged, fever was absent or reduced and generalized symptoms were lessened although the modified virus retained the capacity to produce a rash. However, there was a strong immunity to back challenge with either virus containing acute phase human serum or by the bite of infected mosquitoes.

Subsequent studies with vaccine strains serially passaged in suckling or weanling mice resulted in a reduction in virulence for man and an increase in neurotropism for monkeys inoculated by the intracerebral route. Vaccination with a single type of virus usually resulted in the production of hemagglutination-inhibiting and type specific neutralizing antibodies. However, in studies employing suckling mouse passaged viruses, challenge with unadapted human viruses had not been performed.

It was at this stage of development that the dengue vaccine program was transferred from Dr. Sabin's laboratory to the University of Maryland. The immediate project was to develop the vaccine to a point where it could be applied satisfactorily to soldiers in the field. However, before this could be accomplished, it was concluded that serious consideration had to be given to (1) the matter of further attenuation of the living viruses, (2) the instability of the diluted virus during administration, (3) the complications introduced by interference and cross immunity between different serotypes of virus and (4) the lack of information on the capacity of these vaccine to protect against infection by unmodified virus.

B. Live Attenuated Type 1 Dengue Strains

Since it would be necessary to prepare and dilute the vaccine virus for use in humans and to store the virus at low temperatures for extended time periods, investigations were initiated in an attempt to find a diluent which would stabilize the virus and which would be suitable for human use.

Stability studies were conducted utilizing a variety of diluents, pH's and protein carriers. Optimal stability depended upon the presence of added protein. For human use in vaccination 5 - 10% human serum albumin diluted in Sucrose PG was the most useful, however, this was not as effective as bovine albumin or normal rabbit serum.

Although the strain of type 1 dengue virus developed in Dr. Sabin's laboratory could probably be used for immunizing a limited number of persons under unusual conditions, the relatively high reaction rate made it desirable to develop improved vaccines. Since the attempts at developing a killed vaccine were disappointing and since the early attempts in tissue culture systems were unsuccessful, attempts were made to improve the characteristics of the vaccine strain by use of the older method of terminal dilution. Use was made of the different selective pressures afforded by mice at various ages inoculated by various routes. Starting with the seed pool for the then current type 1 attenuated virus vaccine, at mouse passage 18, three different passage series were established. Additionally, a new series was started from the 1945 virus vaccine established by Dr. Sabin. This resulted in four new type 1 strains described below.

1) Suckling mouse intracerebral series (MD-1). Seven direct brain to brain transfers were made in 3-5 day old mice using 10% infected suckling mouse brain suspensions. This was followed by five limiting dilution passages and primary and secondary seed pools prepared from infected suckling mouse brain. The secondary seed pool material gave similar titers in suckling and weanling mice. In monkeys, 1,000 ID_{50} given by the intracerebral route resulted in questionable fever in two of four animals. All animals developed neutralizing antibodies and none presented clinical evidence of paralysis, encephalitis or weakness.

2) Weanling mouse intraspinal series (MD-2). This series was begun with weanling mouse passage 21 and was passaged only by the intraspinal route in weanling mice. After five terminal dilution passages in weanling mouse cord primary and secondary seed pools were established. The secondary pool was prepared as suckling mouse brain material at the 32nd mouse passage. This material had approximately the same titer for both weanling and suckling mice and had a longer incubation period than the MD-1 strain. When 100-10,000 suckling mouse ID_{50} were inoculated intracerebrally into monkeys, none of the animals developed fever or evidence of paralysis while all animals developed neutralizing antibodies.

3) Intracerebral weanling mouse series (MD-3). This material was serially passed in weanling mouse brain with five 'limiting' passages before a primary seed pool was prepared. The secondary seed pool, at the 32nd mouse passage, was prepared from suckling mouse brain material. When tested in mice it titered approximately 10 times higher in suckling than weanling mice. Monkeys inoculated intracerebrally with either 800 or 80,000 suckling mouse ID_{50} developed fever but no evidence of paralysis or weakness. This strain was characterized as having a low pathogenicity for weanling mice with a relatively long incubation period but still retaining the capacity to produce fever in monkeys.

4) Weanling mouse brain intracerebral series, Sabin - 1945 (MD-4). Direct brain to brain passage was carried out in weanling mice followed by terminal dilution series and the preparation of primary and secondary seed pools. This strain appeared to be more pathogenic for suckling than adult mice although the incubation periods were similar to that seen with MD-1. However, it was shorter than that of MD-2 or MD-3. When the 34th passage material was inoculated intracerebrally into monkeys it produced minimal fever in three of eight animals but there was no evidence of paralysis or weakness in any of the animals.

Prior to use in human volunteers, seed materials were tested and found to be free from detectable bacteriological and

mycological agents. Additional tests indicated that the seed materials were devoid of LCM virus.

Histological examination of the brains of the intracerebrally inoculated monkeys was performed in tissue obtained from various levels of the brain, brain stem and spinal cord. These were compared with sections from monkeys that had shown paralytic signs as a result of infection with an encephalitogenic strain of type 2 dengue. All vaccines produced a mild inflammatory response. When neuronal degeneration was detected it was minimal. None of the candidate strains of type 1 dengue showed as much neurological involvement as is permitted with the 17D strain of yellow fever. It was concluded that there was no contraindication to their trial in human subjects, as far as could be determined by any laboratory criteria available at that time.

A limited trial was carried out on 16 young healthy adult male prisoner volunteers. An estimated 10,000 suckling mouse intracerebral LD_{50} doses of the vaccine were given intradermally into the skin of the arm. Clinical evaluations were made daily and a variety of parameters followed to determine the effects of the vaccine.

The group which received the MD-1 vaccine strain failed to exhibit any reactions of significance while the other three groups developed mild reactions to varying degrees. Fever was not a prominent part of the reaction and when it did occur, it was usually of short duration and under 101 F. All volunteers, except those in the MD-1 group, developed a rash which was generally of moderate intensity. In all instances, neutralization indices of 1.0 or greater and HI titers of 1:5 or greater were developed between 21 and 28 days after vaccination. The HI antibody titers, although strikingly low, tended to follow patterns comparable to neutralizing antibodies.

Because the MD-1 strain failed to cause any detectable undesirable reactions but resulted in the production of neutralizing antibodies in all four subjects inoculated with it, it was selected for further detailed study in volunteers. Two groups of ten prisoner volunteers each were inoculated intradermally with either $10^{4.3}$ or $10^{5.3}$ suckling mouse i.c. LD_{50}. The negligible reaction of human subjects to the MD-1 strain noted in the first small group was confirmed in this study. Even a ten-fold greater dose than formerly used failed to induce significant clinical reactions. Febrile response was virtually absent and none of the subjects was incapacitated. A mild retrobulbar pain was reported by a few individuals and a moderate lymphadenopathy was detected in most subjects. There was no detectable rash and true leukopenia was not observed.

All volunteers developed neutralizing antibodies for type 1 dengue virus with maximum titers occurring about 23 days after vaccination. Low levels of HI antibodies were detected in 17 of the 20 volunteers. No volunteer in either group developed complement fixing antibodies, detectable at a 1:2 dilution, in the 42 days following vaccination.

Although the two vaccine trials had indicated that the MD-1 strain resulted in minimal clinical symptoms while stimulating the formation of type specific neutralizing antibodies, the efficacy of the vaccine had not been established. Approximately 14 months after the second vaccine trial had been initiated, 6 of the volunteers and two non-vaccinated control subjects were inoculated intradermally with an estimated 30-100 human infectious doses of unmodified, second mouse passage, Hawaiian strain type 1 dengue virus. The volunteers were observed carefully under hospital conditions. In this study, both control subjects developed typical dengue fever while half of the vaccinated subjects completely resisted challenge. In those vaccinated individuals who did not resist challenge the incubation period was prolonged by one or more days, the rash was not as intense as in the controls, the HI response was greatly increased over the controls but no significant differences were seen in the level of neutralizing antibodies between those who resisted challenge and those who did not.

In a collaborative study, a larger field trial was conducted on volunteer military personnel of the XVIII Airborne Corps at Ft. Bragg, N. C. A total of 140 individuals were inoculated with the vaccine using doses ranging from 0.4 to 100,000 suckling mouse i.c. LD_{50}. An additional 81 subjects served as controls. As in the previous two tests, no untoward reactions were seen in vaccinated individuals although approximately 10-15% of vaccinated individuals exhibited a sparse uncomplicated transient rash. As little as 4-10 suckling mouse i.c. LD_{50} was capable of eliciting a HI response in approximately 70% of the volunteers.

At about the same time the Ft. Bragg study was being completed, the opportunity for a larger field trial was afforded when a dengue epidemic occurred in Puerto Rico. Although the exact etiology of the agent of the 1963-64 Puerto Rican epidemic was unknown, it was decided that to wait for a definitive laboratory identification would be tantamount to losing all opportunity for a reasonable field trial. It was felt that, if the etiologic agent belonged to the dengue virus complex, a test of either homologous or heterologous immunity could be expected. Approximately 1100 people were selected for this study, of which about 50% received vaccine and the remainder received diluent only.

Evaluation of the data obtained from this study indicated that vaccinees experienced a significantly lower overall

attack rate of clinical dengue than did the controls. Since the attack rate in the vaccine group did not reach a minimum until about three weeks after vaccination, a time interval compatible with that required for the vaccine to induce antibodies, it was believed that the reduced attack rate in the vaccine group resulted from partial protection afforded by the vaccine. The protective effect lasted for at least 85 days after vaccination and since it was subsequently established that the virus causing the epidemic was a type 3 dengue the protective effect afforded by the vaccine was based on acquired heterologous or cross immunity. The vaccine itself did not cause any significant reactions simulating a modified dengue and the partial protection afforded by the vaccine did not cause a significant shift of clinical disease from overt dengue to a higher modified dengue.

Concurrent with the vaccine field trials, additional testing was carried out on the mouse brain propagated vaccine pools to insure their freedom from unwanted passenger agents. Exhaustive testing has shown the vaccine seed pools to be free from bacterial, mycotic and mycoplasma agents as well as the murine viruses, REO 3, PVM, GD VII, K, Polyoma, Sendai, mouse adenovirus, mouse hepatitis (3 serotypes), LCM, lactic dehydrogenase and murine leukemia virus.

Thus, at this point in time, a vaccine had been developed with the following characteristics. It was attenuated to the point that only a small number of recipients exhibited minor, non-debilitating symptoms. It was at least 50% effective against homologous challenge and a significant heterologous cross immunity was afforded against type 3 dengue under epidemic conditions. Safety testing has indicated that it was free from the extraneous passenger agents one might expect to find in a vaccine derived from murine tissue. However, serious objections were raised against the use of a mouse brain propagated vaccine and despite the development of logical procedures to overcome these objections and the apparent safety and efficacy of the vaccine, it became necessary to explore tissue culture systems as possible substitutes for growing the MD-1 vaccine virus.

In initial studies we utilized chick embryo cell cultures since this cell culture system had already been accepted for the production of yellow fever and measles virus vaccines. Primary chick embryo cell culture replicated MD-1 virus to a very low level. Seldom did peak titers exceed $10^{4.0}$ PFU/ml. In a collaborative study with Dr. Zwartouw, attempts were made to grow the vaccine virus in concentrated suspended chick embryo cell cultures. Although a variety of experimental conditions were employed, the peak titers seldom exceeded $10^{3.0}$ - $10^{4.0}$ PFU/ml. Thus, chick embryo cells either as monolayer cultures or as concentrated suspended cells did not replicate significant levels of the vaccine virus and this cell system was discontinued.

Primary fetal and newborn hamster kidney and primary monkey kidney cell cultures were also tested. The MD-1 strain replicated to $10^{5.0}$-$10^{6.0}$ PFU/ml and although it was believed that this represented a practical level for the production of vaccine virus, the problem of extraneous viral agents in apparently normal hamster and monkey tissues, the screening and testing programs necessary to exclude such agents from a vaccine pool posed problems that could make those systems prohibitive.

The human diploid line WI-38 was selected for study as the one cell system with the greatest chance of being accepted for the production of a living attenuated arbovirus vaccine.

Initial studies with the MD-1 virus in WI-38 cell cultures were promising, with peak titers of $10^{5.6}$ to $10^{6.1}$ PFU/ml produced in 4 to 5 days. Subsequent studies gave quite variable titers indicating that unknown factors were influencing some aspect of the overall replicative cycle.

A series of studies was initiated in an attempt to define optimal conditions for the replication of the vaccine virus in WI-38 cells. The serum content of the growth medium was not critical provided at least 10% serum was employed. There was no advantage to using 20% serum and although calf or fetal calf serum would support virus replication, fetal calf serum was slightly but not significantly better. When 5% serum was employed, reduced virus titers were obtained. Human serum albumin could replace calf serum without significant loss in virus titers. Within a short temperature span, the highest titers of virus were obtained at 34 C with reduced virus production at 26 C and 37 C. The addition of 1 to 5 µg/ml poly L-ornithine to the adsorption medium did not significantly increase total virus yield although it apparently increased initial adsorption by a factor of ten. Alterations in the vitamin and amino acid composition of the growth medium did not appreciably alter virus yield.

Multiplicity of infection studies indicated that between 0.001 and 10 PFU/cell input virus did not significantly alter the peak level produced although there appeared to be an inverse relationship between the infecting dose and time required for peak titer. Once the input level was reduced below 10-50 PFU per culture, little or no virus was produced. Numerous attempts were made at terminal dilution of the vaccine virus in WI-38 cultures and at no time was there evidence of virus replication when the input level dropped below 10 PFU/culture. It was never determined whether this was a function of virus, cell or poor attachment kinetics. However, observations made during these studies indicated that virus yields could be increased if relatively low passage WI-38 cultures were employed as substrate and provided that the cells were infected before they became confluent. Under these conditions 1-7 X 10^5 PFU/ml virus was produced and it was possible to perform a limited number of

serial passages in WI-38 cultures. When optimal conditions were employed, it was possible to replicate 10^6 PFU/ml in roller bottle cultures.

Additional attempts to plaque purify MD-1 in monolayer cells under agarose or methyl cellulose were unsuccessful as were attempts to form plaques in suspended cells in agarose.

C. Live Attenuated Type 2 Dengue Strains

The material received from Dr. Sabin's laboratory designated as type 2 dengue vaccine had been tested in rhesus monkeys with some histologic evidence of neuronai damage and produced fever of 102 F or more for several days. This material had also been serially passaged in tissue culture and when tested in monkeys, resulted in weakness and flaccid paralysis of the lower limbs. In view of the above it was decided to attempt to purify and stabilize the virus by terminal dilution in suckling mice. After five terminal dilutions and the preparation of primary and secondary pools, the intracerebral inoculation of $10^{2.6}$ - $10^{7.6}$ suckling mouse LD_{50} caused a marked febrile response and 7 of 10 monkeys developed apparent weakness or paralysis. When additional studies carried out with the mouse adapted Trinidad 1751 strain of DEN-2 produced similar results in experimental monkeys, other DEN-2 strains were selected for characterization. These strains (New Guinea B, Senoi, Trinidad 5786, New Guinea D and C.S. Keum) were passed serially in suckling mice by the intracerebral route to the 19th passage, at which level seed pools were made and the viruses characterized in mice and monkeys. At least three strains showed good adaptation to suckling mice and varying degrees of adaptation to weanling mice. They did not cause paralysis or death in monkeys upon intracerebral inoculation but did show differences with regard to febrile response in monkeys. It was believed that the strains were ready for preliminary testing in human volunteers but because the MD-1 strain of type 1 dengue appeared so promising the major thrust of vaccine development and testing was directed to studies on the MD-1 strain.

D. Inactivated Dengue Vaccines

During the early phase of live virus vaccine development within this department, concurrent studies were carried out on formalinized dengue virus vaccines. Methodology for formalinization and testing was established and initial tests in mice with the formalinized vaccine indicated that a sporatic low level HI response did occur and there was some evidence that a small proportion of vaccinated mice resisted challenge with virulent virus.

Subsequent tests in mice indicated that both dengue-1 and dengue-2 formalinized vaccines afforded good protection against the homotypic virus. There was also evidence that some

cross protection was afforded, although to a lesser degree than the homotypic protection. Additional studies were conducted with the formalinized dengue-1 vaccine in normal rhesus monkeys and in monkeys which had received 17D yellow fever vaccine. Neither group of these animals responded with the production of a significant level of neutralizing antibodies, even when monthly booster immunizations were administered. Because of the lack of immunological response, even after previous preparation with the 17D yellow fever vaccine, further work on killed vaccines was abandoned.

II. Studies With 17D Yellow Fever Vaccine

A. Background

Immune prophylaxis against certain members of the group B arboviruses has been the object of concerted effort by both military and civilian groups. The pattern of approach to immune prophylaxis, for the most part, has been to develop a vaccine for each kind of virus as the need arises. Nevertheless, it might be predicted that many problems would probably arise if it were necessary to immunize a large group of individuals against any appreciable segment of the group B arboviruses. Hence, it would appear that the entire approach to the problem of immunization under these circumstances bears re-evaluation with the objective of simplifying immunization procedures while at the same time increasing the protective spectrum.

From serological studies it is known that extensive cross-reactions are found with the hemagglutination inhibition tests, the complement fixation tests, and, to a lesser degree, with the neutralization test. Repeated exposure of experimental animals to one virus appears to broaden and intensify the cross-reactions with other viruses in these laboratory tests. Additionally, there is some evidence that infection with attenuated dengue viruses causes a recall type of antibody response in persons previously vaccinated with yellow fever. Thus, it is conceivable that immunization with two or three carefully selected members of this group might collectively produce a sufficiently broad range of protection against an appreciable proportion of the whole group.

B. Cross Immunity Studies

A two phase study in U. S. and Japanese volunteers was planned and carried out on this following basis. A very satisfactory attenuated living vaccine was available in the form of the 17D strain vaccine for yellow fever. In the Tokyo region of Japan, a large proportion of young adults possessed JE virus antibodies acquired through inapparent natural infection.

The first phase of the study was to determine the effect of 17D vaccine on JE positive subjects with regard to the spectrum of neutralizing antibodies for heterologous group B viruses. Appropriate control groups were represented by U. S. and J. E. negative Japanese medical students. This study was carried out in collaboration with Dr. Masami Kitaoka and Dr. Takeo Tomuja of the Japanese National Institute of Health in Tokyo.

The 17D YF vaccine given to human volunteers, shown to be negative for group B antibodies, elicits the production of neutralizing antibodies highly specific for YF viruses.

When the 17D YF vaccine was given to human subjects who had previously experienced inapparent JE virus infection and who possessed JE neutralizing antibodies, the YF antibody response was similar to that observed in group B virgins. Additionally, there was a broadening of the neutralizing antibody spectrum for other group B viruses to which the subjects presumably had never been exposed (most subjects developed neutralizing antibodies detectable with type 1 dengue virus, many developed antibodies detectable with West Nile virus and a few developed antibodies detectable with type 2 dengue virus).

YF viremia following vaccination was detectable to an equal degree in group B "virgins" and subjects who possessed JE neutralizing antibodies at the time of vaccination.

The broadening of the neutralizing antibody spectrum gave hope to the idea that a broad cross-immunity could be induced by the administration of a few carefully selected group B virus vaccines. It was necessary, however, to prove that the serological cross reactions observed reflected cross immunity by the capacity to protect against disease.

Accordingly, the second phase of the study was carried out. In this study, subjects with pre-existing JE neutralizing antibodies were vaccinated with the 17D YF vaccine. One month later, when cross reacting neutralizing antibodies should be near their maximum the subjects, with appropriate controls, were challenged with unmodified type 1 dengue virus. The results were very disappointing for the future of cross-immunity because all of the volunteers developed clinical disease within the limits of variation of classical dengue fever. No cross protection against disease was detected.

While these results do not exclude the possibility that some combinations on group B agents may afford some degree of cross protection, they indicate that each system must be tested before any degree of reliance can be placed on laboratory results. No further attempts at producing cross-immunity were made.

C. Virologic and Serologic Studies in Humans

A great deal remains to be learned about arthropod-borne virus immunity, especially in Man. When the cross-immunity studies were initiated, concurrent studies were conducted to determine the extent of viremia and serologic response.

In U. S. medical student volunteers receiving YF vaccine for the first time, viremia reached a peak about 4 days after vaccination and declined thereafter, disappearing about the time neutralizing antibodies first appeared. Revaccination 14 months after primary vaccination resulted in no detectable viremia, but the antibody response elevated neutralizing antibodies to levels similar to those observed after primary immunization. It was not possible to determine if local viral replication occurred to initiate the immune response or if antibody stimulation was the result of antigenic mass in the inoculum. The nature of the immune response (primary or secondary) was not determined.

In collaboration with Dr. R. W. Babione, it was possible to determine that YF antibodies persisted for at least 19 years without benefit of booster or known group B exposure. This study suggested that a single 17D YF vaccination may provide adequate protection to an individual for upwards to 20 years.

Additional studies were conducted to determine the nature of the immunoglobulins associated with primary and booster immunization with 17D YF virus. In the antibody response to YF virus, IgM antibodies consistently appeared between 6 and 14 days after injection while IgG antibodies represented the major immunoglobulins by the end of the second month. The booster response at one year after primary immunization gave rise to IgG antibodies only.

III. Pathogenesis of Arbovirus Infections

A. Pathogenesis of West Nile Virus in Hamsters

The golden Syrian hamster succumbs with great regularity to an acute encephalitis after peripheral inoculation of a minute dose of West Nile virus (WN). The virus presumably passes from the site of inoculation through the various natural barriers and the blood and finally involves the central nervous system. Hence, this virus-host system might serve as a convenient model of an arbovirus infection in which one could follow the course of virus infection in the various tissues, the development of antibodies and in which one could ascertain the influence of active and passive immunity on the multiplication and spread of the virus.

By 24 hours after inoculation of the hind foot pad with 30-100 mouse LD_{50} of WN virus, virus proliferation is detectable at the site of inoculation and in the popliteal lymph node draining the inoculation site. Viremia is demonstrable by 48 hours after inoculation. Presumably, other tissues are infected by the blood borne virus and additional secondary multiplication takes place in remote lymph nodes, spleen and lung. Virus is not detectable in the brain until the fourth day after inoculation, at which time virus was also found in the local draining lymph nodes, spleen, bone marrow, lung and liver. At this time, antibodies appear in the blood and virus titer in the blood begins decreasing in level. Subsequent to antibody appearance virus titer in the blood and most tissues declines with the exception of the inoculated foot pad and the brain where virus content increases rapidly until the animals die in an encephalitic coma.

The immunological response to infection appears to be a complex phenomenon with the production of a sequence of antibody species. The influence of humoral antibodies on the course of WN virus infection was studied by the passive transfer of homologous hyperimmune serum to normal animals. Animals could be protected from death only if sufficient antibody had been administered to raise the circulating level to a log neutralization index of 2.0 or greater. Lesser amounts merely prolonged the incubation period before the animals died. In passively immunized animals only a small amount of virus multiplication occurred at the site of inoculation and less was found in the regional lymph node. Viremia and invasion of other tissues was apparently prevented by the high level of circulating antibody. No change in antibody levels was detected.

Animals actively immunized with multiple doses of vaccine and virus developed high titers of circulating antibody and were solidly immune to death from standard challenge. In actively immunized animals, there was no evidence of virus multiplication, even at the inoculation site.

In animals immunized with a single dose of killed vaccine a very different response occurred. Although these animals did not possess detectable neutralizing antibodies at the time of challenge, they were protected against death from the standard dose. However, a widespread, sublethal infection was detected with virus appearing in many tissues, including the brain. Virus spread was not inhibited but the virus level in the various tissues was reduced. The antibody response was not detectably accelerated in these animals. The collective results suggest that in the actively immunized hamster some factor(s) other than conventional humoral antibody may participate in restricting virus multiplication.

B. Pathogenesis of DEN-1 Infections in Mice

A series of studies was initiated in an attempt to define some of the mechanisms involved in the pathogenic capabilities of several strains of DEN-1 virus in mice. The three strains selected for this study originated from a single strain maintained by serial human passage. The low passage (MP-3) Duff strain underwent 3 serial i.c. passages in suckling mice. The intermediate passage (MP-33) strain was identical to the MD-1 attenuated vaccine strain and the high passage (MP-125) strain was initially inoculated into 10-17 day old mice then passaged 91 times in weanlings after which suckling mice were used for the remaining passages. Attempts were made to describe the temporal relationships between virus replication and interferon production in the brain, the appearance of the immune response, the clinical course of disease in mice, the effect of hyperthermia and to study the infectious process in the CNS by immunofluorescence and pathological observations.

Each virus strain replicated to the highest titer in suckling mice with a rapidly fatal infection occurring before circulating antibodies could be detected. Peak virus titers were lower in older mice and marked dissimilarities appeared in the neurological manifestations of disease as well as in mortality patterns. Weanling mice were uniformly susceptible to the high passage strain and the MP-33 strain but only 40% of the mice died after inoculation with the Duff strain. In adult mice, the MP-125 strain was uniformly lethal, there was a 90% mortality with the MP-33 strain and less than 10% died after infection with the Duff strain. In these cases recovery could be correlated with development of circulating antibodies with concurrent elimination of virus from the brain.

When the mean body temperature of weanling mice was elevated to 39 C the MP-33 strain was markedly temperature sensitive showing a reduction in titer of greater than 2.5 logs. By comparison MP-125 was much less affected although the onset of disease produced by MP-125 was delayed by 1-2 days. When hyperthermic mice, surviving the initial incubation period, were placed at a lower temperature, many developed signs of neurologic disease and died in patterns similar to inoculated controls housed at the lower temperatures. In the analogous MP-125 infected mice, only a few virus specific deaths occurred during the same period.

If weanling mice were inoculated with 1000 LD_{50} MP-33 up to 50% survived for 35 days although some did exhibit nonfatal neurologic disease. No additional deaths occurred when these animals were returned to room temperature.

Interferon levels in the brains of mice infected with both viruses were, irrespective of temperature, directly related to the degree of viral replication and usually were not detectable until virus titers reached about $10^{5.0}$/g of brain. In hyperthermic animals infected with MP-33, interferon production was minimal and delayed by a week as compared to control animals. In MP-125 infected hyperthermic mice, interferon production was neither delayed nor significantly reduced.

Permanent recovery from MP-33 infection in hyperthermic animals was correlated with the appearance of the immune response.

When MP-3, MP-33 and MP-125 viruses were inoculated into suckling, weanling and adult mice and the course of the infections followed by sequential immunofluorescent and histological observations, the 9 host-virus combinations produced infections which ranged from consistently fatal to regularly benign.

Lethal host-virus combinations were characterized by a rapid spread of infection involving over 50% of the neuronal population by about 10 days after inoculation and resulted in necrosis, particularly marked in the cerebral cortex and hippocampus. In benign infections virus spread more slowly and no more than 25% of the cells were involved.

Even in the most susceptible CNS nuclei there was little or no apparent necrosis. After a marked infiltration of the CNS with inflammatory cells fluorescent viral antigen disappeared by 15-20 days post infection. Thus, these results support the hypothesis that in slowly spreading infections, the immune response arrests the spread of virus and ultimately clears the CNS of infectious virus.

IV. Dengue Viruses in Rhesus Monkeys

Since there are no laboratory animals that are uniformly susceptible to clinically recognizable dengue virus infection, the demonstration of viremia and/or production of neutralizing antibodies may be the only measurable parameters of dengue virus infection. The monkey was selected for study since it was believed to be as sensitive as any animal to infection by peripherally inoculated dengue virus.

Initial studies, with the 4 prototype dengue viruses, indicated that as little as 10 PFU/ml of dengue virus could be detected in artificially infected monkey plasma indicating that the assay system was sufficiently sensitive to detect low levels of virus in monkey plasma.

We were unable to demonstrate consistent viremia in monkeys inoculated peripherally with mouse passaged prototype

virus. Viremia was detected in monkeys inoculated with NG "C" dengue-2 virus, and mouse passaged types 1 and 3 dengue virus isolated in Puerto Rico and Pakistan. Immune response as measured by neutralizing antibodies occurred in most of the monkeys regardless of the level or extent of viremia. Of interest was the observation that in the monkeys inoculated with various strains of Puerto Rican Dengue Viruses, significant levels of neutralizing antibodies occurred only in the viremic monkeys.

V. Sero-surveys

The serological survey technique has proven to be an extremely useful initial approach to the problem of describing the distribution and ecology of arbovirus infections when its limitations are taken into account. While it is true that isolation of viruses from an area is an additional essential feature, this alone is insufficient to describe the total situation.

In some instances, no other kinds of information concerning arbovirus activity may be available. Additionally, a survey technique permits the selection of areas for the maximum chance for isolating viruses or where to study a given agent without the complication of infection by other arboviruses. Finally, no other technique will uncover the evidence of past epidemics, an important part of gaining perspective into the significance of arbovirus infections.

Over the course of eight years serosurveys were conducted on sera collected in New Guinea, the Cape York Peninsula of Australia, a number of the Pacific Islands, Puerto Rico, East and West Pakistan, and Nepal. The results and significance of the findings are far too extensive for summarization; however, in most areas studied group B arbovirus activity predominated, group A activity was detected to a lesser extent and group C activity was minimal or absent.

VI. Isolation and Identification of Arboviruses

A. Dengue Outbreak in Puerto Rico, 1963

In connection with the field trials of MD-1 dengue virus vaccine in Puerto Rico, a number of people were studied virologically in order to help establish the nature of the causative agent(s) of the epidemic. A total of 54 individuals were examined less than 24 hours after the onset of symptoms and whose clinical picture was compatible with dengue fever. Acute blood samples were obtained from 34 cases.

Acute phase sera was inoculated into newborn suckling mice and into a variety of tissue culture systems. To these systems was applied the fluorescent antibody technique as a

means for early screening for presence of a dengue agent and of selecting the best material for passage. Mice inoculated with material below the 6th passage showed only occasional and irregular pathogenic signs, such as lethargy, weakness or stiffness of the hind limbs and instability. By the 6th passage signs became more prominent with complete hind leg paralysis and convulsions. Autointerference to some degree was noted when several dilutions were inoculated. Nine specimens were found to contain virus by back-challenge and fluorescent antibody studies and four strains were adapted to the point that they could be used for characterization.

By cell culture plaque assay the virus titer in the whole blood was quite low, approximately 1000 PFU/ml. Although virus was capable of inducing plaque formation in a number of cell types, attempts at cell culture adaptation of the virus were unsuccessful. The combined results of fluorescent antibody studies and cell culture passage indicated that there was only a limited multiplication in the cells in fluid culture.

The four strains, isolated in mice, were subsequently identified as dengue virus in the CF and HI tests. These results were in agreement with serology performed on paired sera collected from Puerto Rican patients.

When the standard mouse neutralization test was employed to serotype the isolates somewhat conflicting results were obtained. When hyperimmune mouse serum was employed in the test, there were clear-cut differences between the Puerto Rican strains and the prototype dengue viruses. When convalescent human serum was employed in the test, no significant differences were demonstrated between Dengue 3 and the Puerto Rican isolates.

Subsequent to these studies, it was determined that the virus causing the 1963 Puerto Rican epidemic was a substrain of type 3 dengue virus. These results are in agreement with those obtained in other laboratories studying this virus.

B. Lahore Pakistan Encephalitis Agent ESP-7

During the summer of 1968 an epidemic of cases of encephalitis occurred in Lahore, West Pakistan, primarily in infants and young children. A virus isolate, ESP-7, was obtained from the CSF of a child during the outbreak. The virus was adapted to suckling mouse and after 19 passages was characterized. It was ether sensitive, filterable through a 0.22 millipore filter and lethal for suckling mice. In the HA test it reacted with most group B antisera but failed to react with group A or group C sera.

The strongest reactions were seen with antisera to JBE virus and to MVE virus in both the HI and CF tests. A series of cross-neutralization tests were performed and the results suggested that the virus was a member of group B and could not be specifically identified.

Since it seemed impractical to continue the cross-neutralization tests, arrangements were made with Dr. J. Casals at Yale to test ESP-7 virus against all of the known group B arboviruses in the CF test. The results of his testing suggested that the virus was more closely related to one of the dengue viruses than to any other group B arboviruses.

With this information we attempted to identify ESP-7 by use of the cross neutralization test using both hyperimmune mouse sera and convalescent monkey sera. Although the isolate appears to be a dengue virus it could not be serotyped because of the nature of the cross reactions.

Although the disease from which ESP-7 was isolated was occurring in epidemic proportions and was widely discussed in the Pakistan medical community as an encephalitis, there were significant gaps in our knowledge of the "encephalitis cases". No U. S. clinicians were present to examine the cases, hospital records were not available and paired sera were not collected and indeed, detailed knowledge about the clinical picture was almost non-existent. However, when it became evident that the agent was most likely a dengue virus we inquired in more detail of Pakistani physicians in one of the children's hospitals at one of the medical schools about the clinical picture. The results of this inquiry suggest that at least two disease entities were occurring during the epidemic: (1) a true encephalitis and (2) an acute febrile disease, often with hemorrhagic phenomena.

In view of these fragmentary clinico-epidemiological data and virological data, the most reasonable interpretation of the epidemic would be that there was an annual outbreak of viral encephaliti and simultaneously a dengue outbreak, frequently expressed as hemorrhagic dengue, the first recognized (retrospectively) outbreak of this syndrome in West Pakistan.

Unfortunately it has not been possible to conduct a systematic study of the mosquito-borne viruses in the area to determine the possible multiplicity of such viruses there.

C. Tick Viruses from West Pakistan

The field work conducted in West Pakistan provided an opportunity to study the distribution of arboviruses in ixodid ticks collected in diverse habitats and ecological situations.

Attempts were made to isolate in mice viruses from 200 pools of hard ticks collected from 22 genera of mammals. Seven genera of hard ticks were represented in pools tested.

A total of 7 viral agents was isolated as a result of this study. All were filterable through a 0.22 cc millipore filter and were sensitive to varying degrees to treatment with ether and deoxycholate. They showed varying degrees of pathogenicity for suckling, weanling and adult mice by different inoculation routes. Three produced hemagglutinins and when compared among themselves by CF, HI and neutralization tests, 6 of the 7 could be classified into 4 serological groups. An extensive study was then made comparing representative strains with a very large number of arboviruses.

The five representative strains tested were shown to be distinct from one another and to include two new viruses, the Hazara virus and the D. G. Kahn virus, and three viruses similar to or identical with previously isolated viruses. Of special interest was the finding that two additional viruses were found to be similar to, or identical with, Semunya or Congo virus, a known cause of human disease in Africa and Wad Medani virus, another African isolate. Two isolates from the Chonga Monga forest near Lahore were identified as similar to, or identical with Crimean Hemorrhagic fever virus. Since the Pakistan virus isolate Hazara virus is serologically related to but distinguishable from Crimean Hemorrhagic fever virus it may be that a new group of tick-borne viruses is being established.

VII. Basic Studies with Arboviruses

A. Studies with West Nile Virus

In 1959 when this program began there was no adequate *in vitro* system for the growth or assay of dengue viruses. Rather than apply the trial and error technique in an attempt to establish such a system, it was decided that a more productive approach to this end would be establishment of a satisfactory technique for assay and characterization of a group B agent known to multiply in cells *in vitro*. The WN-chick embryo (CE) system represented a virus-host system with many advantages and one which might be applied as a prototype system for future studies on the dengue viruses.

A plaque assay for WN virus in CE cells was developed which was as sensitive as the suckling mouse for virus quantitation. In this system a biphasic pH curve for adsorption was noted with optimal adsorption occurring at pH 8.3 - 8.5. The incorporation of 0.1m Mg^{++} into the virus diluent enhanced adsorption. To a certain extent Ca^{++} or Ba^{++} could substitute for Mg^{++}. Approximately 85 per cent of detectable virus was adsorbed

after 60 to 90 minutes. Although the CE system compared favorably with mouse titrations there was a fraction of virus which did not adsorb although this virus population was capable of attachment and subsequent infection of the cells.

With the development of a sensitive tissue culture assay system it was possible to study arbovirus neutralization under controlled conditions. Earlier investigations had shown that "accessory factor" (AF) enhanced the specific neutralization of Japanese encephalitis virus, yellow fever virus and West Nile virus. Using WN virus and a single lot of hyperimmune rabbit serum a series of investigations was carried out on the influence of AF on virus neutralization rate kinetics.

When AF, in the form of fresh, normal human serum, was added to the neutralization mixtures, the rate of neutralization increased three-fold. AF at 25% - 50%, in the absence of antiserum, had no measurable effect on the virus population. When WN virus neutralization was tested with antiserum diluted 1/500, 1/1000 or more, first order kinetic curves were obtained through at least 90% neutralization. At more concentrated levels of antiserum, a biphasic neutralization curve was obtained with a maximal rate through the first 70% virus neutralization. This phenomenon was most likely due to the relative concentrations of 7S and 19S antibody in the antiserum pool.

The AF activity of various species of animals was determined and was found to occur in the serum of man, monkeys, rats, guinea pigs and rabbits. AF activity was not detected in the serum of mice, hamsters and chickens.

The nature of AF was investigated and its characteristics were found to be compatible with complement. As described many times in the literature, AF was inactivated by 56 C for 30 minutes. Also, AF deteriorated at 37 C, losing about 30% of its activity in two hours.

Adsorption of fresh serum with an immune precipitate removed most of the AF activity. The cations magneseum and calcium were found to enhance AF activity in human serum. Pooling of human sera, as well as rat and guinea pig sera, yielded a higher titered AF. However, the complement titers of the various species did not correlate with AF levels. The lack of correlation may be due to the variability in the reactivity of various species of complement in different antigen-antibody systems.

The concentration of AF utilized in a neutralization rate experiment was found to be critical. As the concentration of AF was increased, a signoidal curve was described. The steepness of the central portion of the curve indicated that

potentiation of neutralization due to AF is sensitive to small changes in either quantity or quality of the fresh serum.

Early 7S and 19S anti-WN virus antibodies, separated on a sucrose gradient, were found to be totally dependent on the presence of AF for the demonstration of neutralizing activity. Neutralizing activities of hyperimmune 7S and 19S antibodies were potentiated by, but not totally dependent on the presence of AF in the reaction mixture.

AF, when added to diluted persistent fraction of WN virus antiserum mixtures, rapidly furthered the neutralization of antibody sensitized virus in the absence of free antibody. The use of 100 times more virus than usual caused a lag period, in the absence of AF, suggesting that the neutralization of WN virus in this system may not be a first order reaction.

The influence of AF on the neutralization of cell adsorbed virus was studied. There was potentiation of neutralization prior to penetration but the period of susceptibility was not extended. Additionally, it was found that agar exhibits an inhibitory action on WN virus for a short period after initial adsorption to CE cells which is not related to antibody sensitization of the virus.

B. Studies with Dengue Viruses

When the laboratory studies with dengue viruses were initiated the suckling mouse was the only system available for production and quantitation of this group of viruses. Attempts were made to develop a cell culture which could be used in place of the mouse for assay, and, if possible, production of viruses. A great deal of effort was expended in testing primary as well as established cell culture systems in an attempt to develop a sensitive assay system. Primary cultures of chick embryo, monkey kidney, baby hamster kidney and duck kidney were tested but demonstrated little or no cytopathic effect when infected with virus. Plaque formation was inconsistent or absent although a variety of overlay media were utilized.

HeLa, EE, mouse L, Hep-2, KB, VERO, LLC-MK-2, BSC-1, BHK-21/13 and BHK-21/15 cells were tested for their capability to replicate and/or plaque dengue viruses. Of the cell systems tested we had the greatest success with BHK-21/15 cells. This line not only replicated D-1 and D-2 viruses, as well as any other cell line tested, but also was as sensitive as the suckling mouse for quantitation for all dengue viruses tested. We therefore selected this cell system for <u>in vitro</u> studies with the dengue viruses.

With the development of a sensitive cell culture assay system, a variety of studies was undertaken in an attempt to produce type 1 dengue virus in sufficient quantities for characterization. High mouse passage Hawaiian strain was serially passed in BHK-21/15 cultures through 45 passages at which time it was well adapted to this line.

Virus yields of $10^{8.0}$ - $10^{8.6}$ PFU/ml were routinely obtained after 4-5 days growth. By growing viruses in roller bottle cultures with subsequent sedimentation, we were able to produce virus at $10^{9.5}$ - $10^{10.4}$ PFU/ml.

Although a great deal of information has been procurred about the physical and biological properties of type 2 dengue virus, similar information concerning type 1 dengue virus is not available. When high titered type 1 dengue virus was made available it became practical to attempt characterization of dengue 1 virus. The initial studies were designed to establish the interrelationship between bouyant density, HA activity, infectivity and appearance of the virion in electron micrographs.

After subjecting concentrated dengue 1 virus to equilibrium centrifugation in CsCl, it was found that the maximum HA activity occurred midway in the gradient and had a density of 1.22 gm/cm^3. Peak infectivity preceded the HA and had a density of 1.24 - 1.25 gm/cm^3. These figures agree quite well with those published for type 2 dengue virus. Electron microscopy of this material revealed some intact virions, however, numerous fragmented virus particles were observed. That the major proportion of input virus failed to survive centrifugation in CsCl was confirmed by infectivity titrations.

When infectious virus from the CsCl gradient was subjected to sucrose gradient centrifugation, a better separation of infectious virus (and intact virions) was achieved. Although only 1.1% of input virus was recovered in the peak fraction, it was relatively free from extraneous material as determined by electron microscopy and HA testing of all fractions. The particles recovered by this method were spherical and 45-50 nm in diameter, but no obvious envelope could be observed in these preparations.

With increased requirements for tissue culture derived type 1 dengue virus for use in a variety of programs, it was practical to determine the relative stability of cell culture virus in -70 C storage and necessary to define a diluent which would provide maximum infectivity after low temperature storage. A variety of buffers, protein stabilizers and DMSO were tested. Most diluents were satisfactory for 6-12 month storage provided that they were supplemented with 0.1% BSA, 20% serum or 10% DMSO singly or in combination.

In view of projected programs involving antigenic analysis and production of an attenuated living virus vaccine in cell culture studies were undertaken to determine if type 1 dengue virus could be propagated in media with low protein levels. It was found that significant virus titers could be produced in a defined medium in which bovine serum albumin at 0.4 - 0.8% was the only added protein. Additionally, it was found that if a 20% serum growth medium was employed, a continued high production of type 1 dengue virus occurred in spite of the daily replacement of the growth medium. These findings opened the possibility of producing significant levels of virus in a medium with a very low protein content and provided a means of producing large quantities of virus from a limited number of culture bottles.

The results of neutralization rate kinetic studies suggested that the seed virus preparations contained significant quantities of non-infectious virus, HA and viral components which could result in apparently low values for neutralization rate constants. Since we were attempting to utilize neutralization rate kinetics as a means of identification of dengue viruses, it was necessary to obtain virus preparations which were free from extraneous material and which were neutralized following first order kinetics. A variety of methods were employed in an attempt to prepare infectious virus free from extraneous but antibody reactive material.

Attempts at separation by passing the crude virus preparation through sepharose 2B and 4B columns were unsatisfactory since both HA and infectious virus were spread throughout the fractions collected.

Attempts at separation by linear sucrose gradient centrifugation were only partially successful, as were attempts at batch type centrifugation using a variety of sucrose concentrations and centrifugation conditions. Discontinuous sucrose gradients were also employed but without significant separation of infectious virus from HA.

Batch type centrifugation in 20, 25, and 30% ficoll resulted in a cleaner separation of infectious virus from extraneous HA but not sufficient enough for the critical neutralization studies. These investigations were discontinued.

During the early 1960's the list of group B arboviruses was rapidly increasing and a number of dengue serotypes had increased from 2 to 4 and possibly 6. A study was undertaken to characterize the dengue viruses and other selected group B agents by the HI, CF and neutralization tests. The results of these tests indicated that no single test alone

reveals the complete serological relationships, either differences or similarities. The dengue viruses available at that time could be differentiated into four serotypes. The dengue viruses are more closely related to one another than they are to JBE, MVE, WN or YF viruses, indicating that they may form a distinct subgroup within group B. Finally, strains of dengue virus tentatively classified as type 2, collected under conditions widely separated in time and place elicit the production of antibodies in hyperimmunized mice which cannot readily be distinguished from one another when tested with antigens prepared from the New Guinea C strain of type 2 dengue virus, suggesting little antigenic spread among them.

The relative merits of various neutralization tests employed for the identification of dengue and dengue-like viruses were investigated. These tests included the mouse neutralization test, the 50% plaque reduction neutralization test and neutralization rate kinetic studies. This investigation was stimulated by the results of various neutralization tests performed on isolates from the 1963 Puerto Rican dengue outbreak. Multiple isolates from this outbreak were shown to be identical by a variety of technics. By use of the tissue culture neutralization test with convalescent monkey serum one representative isolate was identified as a type 3 dengue virus. When isolates were tested in the mouse neutralization test, using hyperimmune mouse antisera, the results of reciprocal cross neutralization tests suggested significant differences between the Puerto Rican strains and the classical type 3 strain. In a similar manner, a dengue isolate from Dacca, Pakistan gave a one way cross in the mouse neutralization test.

From these and other results it appeared as though methodology as well as the type of test employed influenced the results obtained in neutralization tests and therefore the serotyping of new isolates. A comprehensive study was initiated to test the four prototype dengue viruses, a Puerto Rican isolate and Pakistan isolates in the mouse neutralization test, the 50% plaque reduction neutralization test. These tests employed single pools of hyperimmune mouse antisera, convalescent monkey serum and where available, convalescent human serum. In the cross neutralization tests using hyperimmune mouse serum the prototype dengue viruses are readily identifiable in the mouse neutralization test.

The PR-6 virus, isolated during the 1963 Puerto Rican dengue epidemic and tentatively identified as a type 3 dengue virus could not be classified as a type 3 dengue virus utilizing the results of these tests. Type 3 mouse antiserum did not neutralize PR-6 virus to a significant level and in a like manner PR-6 mouse antiserum did not neutralize the type 3 prototype virus. In a similar manner, the PAK-14 virus, which had been

provisionally classified as a type 3-like dengue virus, could not be assigned a dengue serotype on the basis of these tests. In the cross neutralization tests with type 3 dengue virus and antiserum it behaved like the PR-6 virus. In the mouse neutralization tests using convalescent monkey serum the prototype dengue viruses could be readily distinguished one from the other.

Both the PR-6 and PAK-14 viruses were neutralized by type 3 antiserum and conversely Puerto Rican antiserum and PAK-14 antiserum neutralized type 3 dengue virus to a high degree. The results of the cross neutralization tests with the monkey sera identify PAK-14 and the 1963 PR strains as type 3 or type 3-like dengue viruses.

Plaque reduction neutralization tests employing hyperimmune mouse sera were performed using the "standardized" procedure recommended at the March 1971 Dengue Task Force Meeting.

The prototype dengue viruses can be readily distinguished in this system since the homologous titers are significantly higher than the heterologous cross reactions. Both dengue 1 and dengue 2 mouse antisera neutralized the other dengue prototype viruses at least to a limited extent. Type 3 and type 4 dengue antisera, although low in titer in the homologous tests, did not cross react with the other prototype dengue viruses.

PAK-14 and PR-6 viruses could not be definitely serotyped on the basis of the tests with hyperimmune mouse sera. Type 3 dengue serum neutralized PR-6 virus but did not neutralize PAK-14 virus. PR-6 serum neutralized both dengue 3 and PAK-14 to a limited extent. PAK-14 serum, which was low titered in this test, neutralized PR-6 virus to the same extent as it did PAK-14 virus and also neutralized dengue 3 virus to a limited extent. The nature and extent of the cross reactions, especially with type 1 dengue viruses, make it difficult to assign a serotype to PR-6 and PAK-14 viruses.

In the plaque reduction neutralization tests with convalescent monkey sera antisera to dengue 1, 2 and 4 viruses readily identified the homologous prototype viruses in the cross neutralization tests. The type 3 monkey antiserum, although it had a neutralization index of 2.9 in the mouse neutralization test, failed to neutralize dengue 3 viruses in the plaque reduction test.

Convalescent sera to the Puerto Rican virus and PAK-14 virus reacted with type 3 dengue, PAK-14 and the Puerto Rican viruses and the results of the cross neutralization tests indicate that both PAK-14 and the Puerto Rican viruses are type 3 dengue viruses. However, in view of the differences in degree of cross neutralization, the Puerto Rican isolate and perhaps PAK-14 virus should be considered as variants of the classical type 3 dengue virus rather than identical to the prototype virus.

Thus, it appears that either the mouse neutralization test or the 50% plaque reduction test may be employed to identify field isolates such as type 1 or type 3-like dengue viruses provided that monkey serum is employed as the test reagent. The use of hyperimmune mouse serum in either neutralization test would be appropriate only when the isolates are identical to the prototype dengue viruses. The use of mouse antiserum for identification of serological variants of dengue viruses, as the 1963 Puerto Rican strain and PAK-14 strain appear to be, may not provide sufficient information for positive serotyping.

VIII. Genetic Studies

The first step in our program to produce temperature sensitive mutants of dengue virus was to select a model virus, capable of rapid growth, with which to develop technical procedures. Chikungunya virus was selected as the model, with the expectation that techniques developed with this virus would be useful in dengue investigations.

Isolation of temperature sensitive or host range mutants necessitated picking large members of isolated plaques. In order to facilitate this operation, a suspended cell-agarose overlay technique was adapted for use with selected arboviruses. This technique involves overlaying an infected cell suspension in agarose on a pre-formed solidified base of agarose. It results in plaque formation on the surface of a petri plate rather than on the bottom under the nutrient overlay. Plaques were therefore more accessible, and plaque picking was both less tedious and more reliable. This procedure has been shown to work well with CHIK virus, D-1, D-2, and D-3. Dengue-4 was not tested.

Nitrous acid was initially selected as a virus mutagen because of its proven effectiveness in producing temperature sensitive mutants of Sindbis virus. Equal volumes of crude CHIK virus and 1 molar K phosphate buffer (pH 6.7) were combined, then mixed with 2 volumes of freshly prepared 4 molar sodium nitrite and incubated at room temperature. At various times during incubation, aliquots were removed, diluted and plaque assays performed on monolayers of BHK 21/15 cells. The virus was inactivated at a rate of approximately 1×10^1 PFU/30 minutes incubation over the range of 1-90 minutes. No attempt was made to isolate temperature sensitive mutants in this model system, but other investigators have reported that a reduction in titer of 1×10^4 PFU yields survivors composed of approximately 1% temperature sensitive mutants.

When this technique was employed with D-1 virus, the virus was completely inactivated in one minute. Reduction of

the molarity of sodium nitrite to 0.5M, 0.25M or 0.10M prolonged the survival of dengue virus, but results were eratic from one experiment to the next and it was very difficult to predict the remaining titer after mutagenesis with nitrous acid. For these reasons, other mutagenizing agents for dengue virus were investigated.

5-azacytidine was tested as a virus mutagen and used in CHIK infected cells at a concentration of 25 micrograms/ml. The progeny virus showed a one log decrease in titer when plaqued at 37 C rather than the permissive temperature of 32 C. Stocks of mutagenized CHIK virus were plaqued at 32 C and individual plaques tested for their ability to replicate at 37 C. When a temperature sensitive mutant was identified (growth at 32 C, but no growth at 37 C) it was propagated in small petri plates of BHK 21/15 cells and frozen at -70 C in 10% DMSO) while awaiting chemical characterization. Two temperature sensitive mutants have been obtained from 275 plaques tested. All dengue serotypes have been mutagenized by 5-azacytidine. Their relative plaque titers at 32 C and 37 C were under investigation when this project was terminated.

IX. Personnel

Professional Staff

Barry Adels
Hideo Aoki, M.D.
Fatima Begum, Ph.D.
Ollie R. Eylar, Ph.D.
Eugene J. Gangarosa, M.D.
Henry W. D. Holljes, M.D.
Jose V. Ordonez, M.D.

Fellows

Robert W. Chandler, Ph.D. - 1967
Gerald A. Cole, Ph.D. - 1966
Yehia El Batawi, Ph.D. - 1963
John N. Hatgi, M.S. - 1963
Antonio R. Noriega, Ph.D. - 1964
Victoria Plaster
Anita N. Rudin
Stephen A. Stohlman, Ph.D. - 1975
William A. Wood, Jr., Ph.D. - 1965

Technical Assistants

William Brown	Animal Assistant
Vernon Burnett, B.S.	Laboratory Scientist I
Frederick D. Chapple	Laboratory Assistant
Rudolph Chapple	Laboratory Assistant I
Bertram R. Harrington	Laboratory Technician I
Mary Ann Lane	Laboratory Scientist II
Reginald Lindsay	
Thomas Mason	Animal Room Attendant
Marjorie D. Morrison	Secretary II (part time)
Patricia McKown	Laboratory Assistant II
Roger Saurusiter	Laboratory Technician II
Frances L. Segel	Laboratory Scientist I
Richard Steffens	Laboratory Assistant
George Sweeney	Laboratory Scientist II
Herman Toulson	Animal Attendant
Martha Jane Wood	Laboratory Technician I

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