

Title: Arbovirus Infections in Men and Experimental Animals

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Objectives:

- a. To develop improved virologic and serologic methods for diagnosis and study of arbovirus diseases in Thailand.
- b. To determine the antigenic relationships and study the biologic properties of arboviruses of medical importance.
- c. To obtain information on the sequential changes in the immunoglobulin response of man and experimental animals to single and multiple dengue infections and to relate these observations to disease processes.
- d. To study the etiology of human cases of encephalitis and determine the importance of arboviruses and other viruses as causative agents.

Description:

The plaque reduction neutralization test (PRNT) for dengue virus antibody described in the previous annual report was further investigated to determine the effect of varying conditions and to estimate the accuracy of the test. The rate of thermal inactivation of several dengue virus strains was determined. Serum from human and animal sources were tested to determine the amount of heat labile inhibitors present. A statistical analysis of tests performed with antiserum of known comparative potency was done to determine the accuracy of the test for estimating antibody levels.

A method of primary isolation of arboviruses from serum and mosquito pools was developed using a plaque assay in LLC-MK₂ tissue culture. The newly developed method was compared with intercerebral inoculation of suckling mice for sensitivity to unpassaged virus strains.

Dengue viruses isolated from a variety of sources including dengue fever cases, hemorrhagic fever cases, and mosquito pools were identified by the plaque reduction neutralization test method using reference monkey antiserum.

Antigenic relationships of several strains within each serotype were compared.

Experimental dengue infection in monkeys and gibbons were studied to determine susceptibility to subcutaneous infection, duration of viremia, and immune response.

Using density gradient centrifugation, ion-exchange chromatography and gel filtration, studies were done to determine the nature of the antibody response to both primary and secondary dengue infections in man. Immunoglobulins were identified by immunoelectrophoresis and immunoprecipitin methods; antibody activity was tested by hemagglutination-inhibition and complement-fixation.

Cases of human central nervous system disease referred from hospitals in various areas of Thailand were studied by serologic and virologic methods to determine the viral causes of human CNS disease in Thailand.

Progress:

Plaque Reduction Neutralization Test.

The method for performing the PRNT for dengue neutralizing (N) antibody in LLC-MK₂ cell culture was described in detail in the previous annual report. A serum-virus incubation time of 1 hour at 37°C is routinely used. Since heat lability of seed virus is an important factor which affects the amount of non-viable antigen in the test system, several experiments were performed to determine the effect of temperature on dengue virus seeds diluted in M-199 containing 5% inactivated calf serum buffered with NaHCO₃ to pH 8.2. This approximates closely the conditions of the neutralization test. Virus suspensions containing 50 to 100 pfu at zero time were heated to 37°C or 42°C in water baths and infectivity determined at various time intervals by plaque assay. The following strains were tested at indicated suckling mouse passage level:

Hawaii (dengue-1)	sm 125
TH-Sman "	sm 4
# 10572 "	sm 3
New Guin. "C" (dengue-2)	sm-26
TH-36 "	sm-14
# 10286 "	sm-4
H-87 (dengue-3)	sm-21
H-241 (dengue-4)	sm-25

Results expressed as percent survival are summarized in Table 8. A marked difference between dengue strains is apparent. TH-36 was the most heat labile of the strains tested showing only 28% survival after 1 hour at 37°C. Hawaii and No. 10572 were somewhat less labile, however, the reduction of infectivity after 1 hour at 37°C was significant. H-87 appeared least affected by heat and the remaining strains were moderately heat resistant.

Heat resistance varies markedly between strains of dengue viruses. It apparently is not related to mouse passage and probably is not related to antigenic type. Three of the 8 strains tested showed a 50% or greater loss of infectivity after 1 hour at 37°C.

The plaque reduction neutralization test is very sensitive to the effect of non-specific anti-viral substances in serum. Sera from several species were tested against dengue viruses by plaque reduction to measure non-specific anti-viral activity. The tests were performed in the identical manner as the PRNT except the sera were freshly collected, had never been frozen, and were not heat-inactivated. Heat

inactivated (56°C, 1 hour) sera were used as controls. Results, given in Table 9, show the presence of non specific inhibitors in all sera tested. In the majority of sera significant anti-viral activity was present at the 1:10 dilution. The common occurrence of heat labile non-specific anti-viral substances in fresh sera precluded the use of fresh sera as a source of "accessory substance" to enhance virus neutralization.

To determine the accuracy of the plaque reduction neutralization test when two or more sera are simultaneously tested against a single virus for purposes of determining comparative potency, the following experiments were performed: Dengue-1 (Hawaii) antiserum was diluted 1:4 and 1:8, the two dilutions were then treated as serum of unknown potency and tested against the homologous Virus. In a second test dengue (TH-Sman) antiserum was diluted 1:4 and 1:8 and tested in parallel with undiluted serum. The dilution factor was unknown to the technician performing the test who treated each one as a whole serum of unknown titer. The results obtained are presented graphically in figures 7 and 8. The 50% effective dose (ED₅₀), 95% confidence limits of the ED₅₀, relative potency of the two sera in each test, and the 95% confidence limits of the relative potency were calculated by method for parallel-line, graded response bioassay. Results of statistical analysis indicated that in both tests the 95% confidence limits of the ED₅₀ were exclusive, indicating that this technique can distinguish two fold differences in 50% plaque reduction titers. In addition the dose response curves were linear and parallel indicating that estimation of 50% plaque reduction end points by the method of Cutchins is in an appropriate way of describing potency of antisera.

Additional experience with the use of this test for comparing serum titers against different virus strains, and for comparing results of tests performed at different times with different lots of seed virus indicates that, under conditions where such additional variables are present, two fold differences may not be significant. This is especially true with early antiserum where the slope of the dose response curve is steep. In such cases, observed differences in 50% plaque reduction titers must be 4 fold or greater to be certain of biologic significance.

Dengue Virus Identification.

The PRNT used in conjunction with reference anti sera made by a single subcutaneous injection of live virus in Macaca irus monkeys has proven to be an excellent method for identification of dengue viruses. Dengue virus strains identified by this method are listed in Table 10 with host and passage level, country of origin, and year of isolation. The reference strains were originally obtained from Dr. Wm. McD. Hammon. The Thailand and South Vietnam strains were isolated in this laboratory from human serum. Twenty-five were isolated in suckling mice, nine were isolated in BS-C-1 tissue culture by the challenge virus resistance method and three were isolated by direct plaque method in LLC-MK₂ cell cultures.

The Thailand viruses from 1962, 1963 and 1964 are selected strains that could not be readily identified by complement fixation tests or neutralization tests in BS-C-1 tissue culture. Thus they are not representative strains from that period. After 1965, identification by other methods was not done.

The majority of the strains, including all four serotypes, were isolated from patients with the clinical diagnosis of hemorrhagic fever. Three strains came from patients with an undifferentiated febrile illness and thirteen strains were isolated from cases of dengue fever in caucasians.

The Pak-18 strain was obtained from WRAIR, and the Philippine strains were isolated in this laboratory by Dr. Basaca Sevilla.

The Tahiti strain was isolated from human serum by Dr. Leon Rosen, during the dengue epidemic in late 1964 and was sent to this laboratory for identification. This agent (T 502066) was isolated in mice by blind passage but produced no symptoms in mice up through 10 passages. Virus growth was identified by resistance of mice to challenge with virulent dengue virus and by production of challenge virus resistance on passage to BS-C-1 cell culture. BS-C-1 passage virus was propagated in this laboratory in LLC-MK₂ cells.

Antisera to all strains were made in Macaca irus monkeys by a single subcutaneous injection of 10^3 to 10^4 plaque forming units (pfu) or, in the case of the mouse adapted reference strains, 10^4 to 10^5 suckling mouse intracerebral LD₅₀. Serum was harvested approximately one year post inoculation for the reference strains and 2 months post inoculation for the other strains. Mouse antiserum to dengue-4 was made by three subcutaneous injection of 10^5 suckling mouse IC LD₅₀ one week apart and harvesting serum after 4 weeks.

The results of cross neutralization tests with reference dengue strains and antisera are given in Table 11. The neutralizing antibody titers clearly distinguished between types 1 through 4. TH-36 cannot be clearly differentiated from dengue-2 nor can TH-Sman be clearly distinguished from dengue-1 although small differences in neutralization titers are observed.

The type 1 and type 2 antisera reacted only within their serotypes with no detectable titer to types 3 or 4. Antisera to types 3 and 4, on the other hand, gave evidence of cross reaction with heterologous types. However, wide differences between heterologous and homologous titers were observed. The most notable cross reaction was the titer of 1:90 of dengue-3 antiserum against type 1 virus compared to a heterologous titer of 1:350. This cross reaction is not reciprocal and does not preclude differentiations between viruses within the 1 and 3 serotypes.

Results of testing 10 virus strains in the type 1 group, including strains from Thailand and South Vietnam against the reference antisera are given in Table 12. In addition, monkey antisera to four Thai strains was tested. All of these agents were neutralized by dengue-1 and TH-Sman antiserum. The titers obtained by testing these isolates with dengue-1 antiserum were uniformly lower than the homologous serum titer. The titers against the TH-Sman antiserum, however, were not significantly different from homologous titers. Three strains showed very low titered cross reactions, of doubtful significance, with dengue-2 or TH-36 antiserum. Seven of the ten strains were neutralized in low titer with the dengue-3 antiserum. Antisera made to the 4 Thai strains neutralized TH-Sman virus with titers not significantly different from homologous titers. There appeared to be some variation in neutralization of dengue-1 virus by the Thailand strain antisera. Antisera to No. 12900 and 22448 had similar titers against homologous virus dengue-1 and TH-Sman. However, antisera to strains Nos. 14580 and 18280 had somewhat lower titers to dengue-1 than to TH-Sman or homologous strains.

Eight dengue-2 strains were included in this series and all were neutralized in high titer by dengue-2 antisera and in very low titer by dengue-3 antisera as shown in Table 13. The titers of dengue-2 antisera against the Southeast Asian strains were significantly lower than against the homologous virus and against TH-36, both of which are highly mouse adapted.

Table 14 shows the results of neutralization tests with 14 strains which were neutralized by type 3 antisera, with one exception, a marked antigenic uniformity among dengue-3 group is apparent. None of the dengue-3 strains reacted with heterologous antiserum. Antisera made against strain No. 14670 and the Pakistan-18 strain reacted similarly with the homologous virus and with the reference dengue-3 strain. The Tahiti strain was neutralized only by type 3 antiserum but the serum titer was significantly lower than titers to all other strains.

The monkey antiserum made against the dengue-4 prototype strain had comparatively low titer compared to mouse antiserum to the same virus as seen in Table 15. The monkey antiserum to strain 14486 had a similar low titer, however, there was no cross reaction with the other serotypes. As shown in Table 16, 8 isolates were neutralized by dengue-4 mouse antiserum although five of the eight strains reacted with lower titers than the prototype strain. The cross reactions noted between dengue-4 prototype and 14486 indicate some degree of antigenic variation within the group.

The results described above indicate that the plaque reduction neutralization test is an extremely useful method for identification of newly isolated dengue virus strains. Of primary interest is the fact

that strains isolated in tissue culture could be identified without adaptation to mice and without extensive adaptation to a tissue culture system. It was possible to identify several strains of dengue-3 and dengue-4 which could not be identified by mouse neutralization test, complement fixation tests, or neutralization tests in BSC-1 tissue culture. In our experience a tissue culture seed with a titer of 500 plaque forming units per 0.15 ml is adequate for accurate typing by this method.

Monkey antisera prepared in Macaca irus monkeys by a single subcutaneous inoculation of virus were shown to have a high degree of specificity and this presently is considered to be the method of choice for producing dengue antiserum. When tested by the plaque reduction neutralization test sufficiently high titers are observed to identify viruses and measure antigenic relationships.

It is of interest that all viruses tested could be placed within one of the 4 major dengue serotypes by a neutralization test against antisera to reference strains. In all cases where antisera to new isolates were made, cross neutralization tests confirmed the results obtained by the typing test. These results suggest that the typing test using reference antisera is sufficient for routine epidemiologic studies. Antigenic variation within the major serotypes has been previously emphasized by Hammon, and the results described above indicate antigenic differences between dengue-1 and TH-Sman and dengue-2 and TH-36 as well as similar within the dengue-4 serotype. On the basis of these results, however, variation does not appear to be of sufficient magnitude to justify classification of other than 4 major serotypes.

There appeared to be no significant differences between virus strains isolated from hemorrhagic fever patients and those isolated from patients with undifferentiated fever and classical dengue. The two strains of dengue 4 isolated from patients in South Vietnam represent the first strains of this serotype found in that area.

Identification of Dengue Viruses from Koh Samui.

The identification of dengue viruses isolated from human sera and mosquitoes collected on Koh Samui are presented separately below because different lots of typing antisera were used and some special problems arose. All viruses were isolated in LLC-MK₂ cell culture and the mosquito strains were also reisolated in suckling mice.

Results of identification of 16 strains isolated from human sera are presented in Table 17. The six type 3 strains appear similar to previously identified type-3 strains from Bangkok and other areas. Several of the type 2 strains, especially 24367, 24453, 24464, 24742 and 25076 were neutralized only by low dilutions of the dengue-2 (New Guinea "C") antiserum.

Similar results were seen when tissue culture seeds of viruses isolated from mosquitoes were tested against reference antiserum. Table 18 gives the results of typing by plaque reduction neutralization test of 7 dengue strains all of which are dengue-2. Of interest is the fact that suckling mouse passage BKM 551-66 was neutralized by high dilutions of dengue-2 antiserum whereas the tissue culture line of the same virus was neutralized only by low dilutions of the same antiserum.

Antisera made in monkeys to suckling mouse and tissue culture strains of BKM 551-66 both had low neutralizing antibody titers when tested against the tissue culture propagated virus; however the antisera made to suckling mouse propagated virus neutralized prototype high mouse passage dengue-2 strains and the homologous virus at high titer.

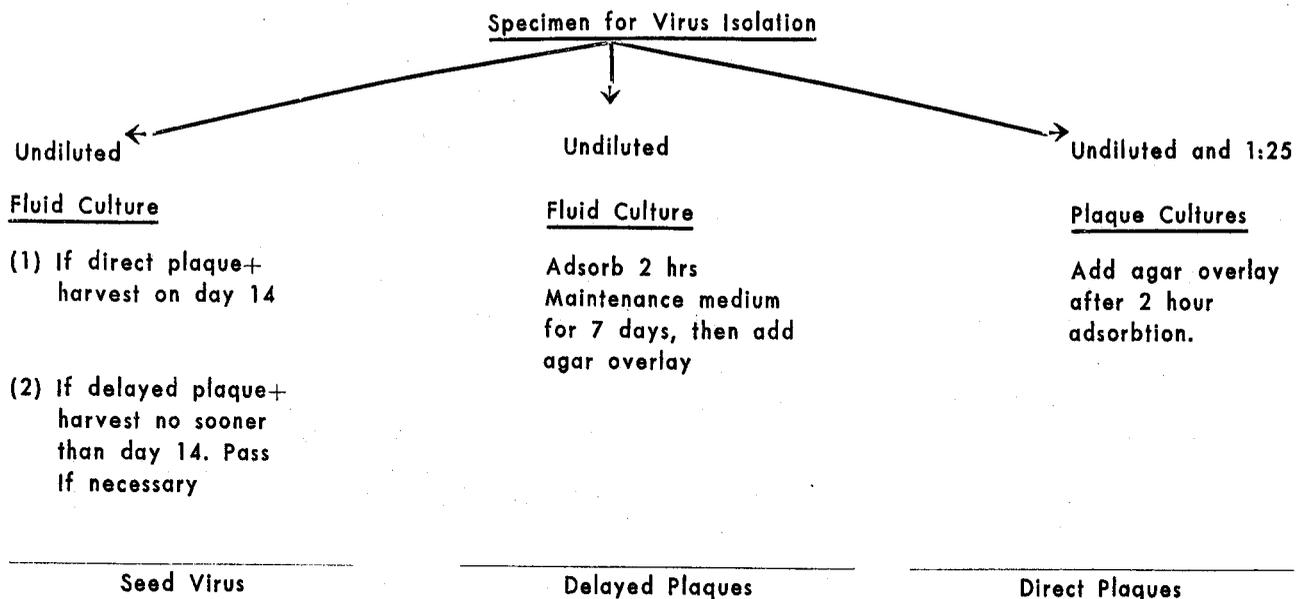
This phenomenon is unexplained and introduces additional difficulties in identification of some newly isolated dengue viruses. The effect of the host on the susceptibility of dengue virus strains to neutralization by antibody is presently being investigated.

Virus Isolation Methods.

Isolation of arboviruses had previously been attempted in suckling mice and BS-C-1 cell cultures. The challenge virus resistance technique using BS-C-1 cells proved to be extremely sensitive for dengue and Japanese encephalitis viruses. As with suckling mice, however, this method often requires up to three blind passages (with attendant dangers of cross contamination) before virus presence can be detected. A large number of tubes is also required, which limits the number of specimens which can be carefully tested. A system sensitive to all arboviruses known to be present in Thailand but which would be simple, direct and suitable for testing a large number of specimens was desired.

Because all known Thai arboviruses produce plaques in LLC-MK₂ cell cultures, this easily handled cell line was adopted for the isolation system. At present, isolation from all non-blood-engorged mosquitoes are being attempted by direct plaques. Mosquito suspensions undiluted and diluted 10 and 100 fold are adsorbed onto LLC-MK₂ cell monolayers in 1 oz bottles and overlaid with agar as previously described. In addition, two bottles are inoculated with undiluted mosquito suspensions and held as fluid cultures for virus seeds. Agar overlaid bottles are reoverlaid and stained at 7 days and examined on days 8 to 12. If plaques appear the fluid cultures are harvested on day 14 and titered. If the titer is not adequate for plaque reduction typing, additional passages are made.

For blood-engorged mosquitoes and tissue or serum, where antibody may be present, a direct and delayed plaque system was developed as follows:



The delayed plaque system allows time for (1) virus—early antibody dissociation; (2) adsorbition when very small numbers of viruses are present and (3) one cycle of replication to occur. The delayed plaque system is more sensitive than direct plaques alone, and has resulted in isolation of about one third more dengue viruses (Table 20).

For isolation of dengue virus from mosquito suspensions, however, the LLC-MK₂ plaque system appeared to be superior to suckling mice, the mice either failing to detect virus or being resistant to dengue 2 challenge but not becoming ill. (Table 22). It should be noted, however, that it has been difficult to prepare virus seeds of high titer from some mosquito isolates.

Suckling mice appear to be fairly sensitive for isolation of dengue viruses from human serum. Mice were successfully used to isolate viruses from 15 plaque positive viremic human sera from the Koh Samui and Vietnam dengue outbreaks. These sera contained dengue types 1, 2 or 3. In 9 of 15 cases, however, mortality did not occur until the third blind passage (Table 21), whereas in the direct-delayed plaque system virus is detectable in the first passage.

Characteristics of dengue virus growth in LLC-MK₂ cells

Use of LLC-MK₂ cells for detection of dengue viruses in acute phase human sera and in suspensions of field-collected mosquitoes has been extremely successful. There has been some difficulty, however, in consistently making cell culture virus seeds with titers adequate for typing largely because the optimal time from inoculation until harvest was unknown. Experiments designed to determine this interval in LLC-MK₂ tube cultures of various ages, inoculated with minimal doses of low tissue culture passage dengue-2 virus, were carried out. At the same time, similar aged tube cultures of primary human embryonic lung and kidney cells were inoculated in an attempt to determine if diploid human cells supported dengue virus replication to higher titer than did the continuous monkey kidney cell line.

In addition to LLC-MK₂ monolayers from one through seven days old at the time inoculation, cell suspensions were also inoculated with virus. Cells were counted daily before new cultures were prepared so that on the day of virus inoculation the cell populations of each age group were approximately equal. Human primary cells were of limited availability and so only monolayer tube cultures seven days of age were used. The cells were washed once with Hank's balanced salt solution (HBSS) and inoculated with 15-20 pfu of dengue-2 virus, BKM-540 in LLC-MK₂ passage two (first experiment) and passage three (second experiment). The virus was allowed to adsorb at 37°C for 1 1/2 hours. The tubes were then maintained with M-199 with 5% heat inactivated calf serum, pH 8.3. Cell-associated virus was harvested in 1 cc per tube of medium 199 with 50% heat-inactivated calf serum. In the first experiment (cell suspensions and monolayers 1-4 days of age) the virus was harvested from randomly selected tubes on alternate days by mechanically lysing them in the micro-homogenizer attachment of the Omni-Mixer, with sterile sand, at about 30,000 rpm for three minutes. This method provided lysis superior to sand alone with vortex mixing or osmotically shocked cells followed by sand and vortex mixing, and equal to three cycles of rapid freezing and thawing. Since freezing and thawing was the simpler method, it was used for harvesting dengue virus in the second experiment. Cell lysates were centrifuged and stored at -70°C until assayed.

Ability to support virus replication increased with the age of the cell monolayer at time of inoculation up to four days of age, after which there was little difference. Virus titers after 19 days incubation, however, were higher for 7 day old cells than the younger 4 and 5 day old cells. Virus titers were independent of the slight differences in cell populations. Virus titers in LLC-MK₂ cells rose to a peak on day 6 (second experiment) and day 8 (first experiment) fell two days later and rose to a new, higher peak four to 8 days after the first peak (Figures 9 and 10).

The occurrence of both peak titers in the second experiment two days in advance of and higher than of the first experiment suggests that the one additional LLC-MK₂ cell passage may have resulted in further adaptation of the virus to the cell system.

The virus titers in human embryo kidney and lung cells (Figure 10) rose more slowly, the first peak occurring on day 8-10, the titer falling and then peaking again on day 16, and falling again on day 19, but at no time achieving the maximum titers of the LLC-MK₂ cells.

In both cell systems, the occurrence of two peaks in virus titers suggests that when the virus input is small, virus replication proceeds synchronously in the cells, mature virus appearing, reinfecting and eclipsing, and progeny virus maturing again, all at about the same time. It was not until after the second peak, 19 days post infection, that the synchrony broke down. This was most striking in the human

embryonic lung cells, when only one pfu was seen in three bottles on day 12, a drop in titer of over 10^3 pfu in 48 hours. These cycles point up the importance of allowing an adequate time interval between inoculation of virus and harvest.

Virus propagated in human embryonic lung cells produced plaques of uniform size and clarity. Both kidney cell types, human primary and monkey heteroploid, produced virus of mixed plaque size and morphology, small clear plaques and large hazy plaques in a ratio of about 3:1.

Dengue Viremia in Primates.

In the course of the studies on dengue antibody in monkeys, viremia was measured in 12 Macaca irus monkeys following a single subcutaneous inoculation of dengue virus. A variety of dengue viruses including high mouse passage prototype dengue-1 and low tissue culture passage local strains were used. The results, shown in Table 23, indicate that Macaca irus monkeys regularly developed viremia following inoculation with dengue viruses types 1, 3 and 4.

A preliminary experiment in March 1966 indicated that the white-handed gibbon (Hylobates lar) developed HI antibody following subcutaneous inoculation with dengue-1 virus. To determine the usefulness of this species for studies of cross immunity of dengue virus, a second experiment was done in which 7 gibbons were given a subcutaneous inoculation of approximately 100 plaque forming units of a local dengue-2 strain (No. 10044) in the 3rd BS-C-1 passage. Five gibbons had no previous exposure to dengue and two had had a dengue-1 infection 5 months previously. Viremia was estimated on days 2 through 10 following inoculation.

Viremia developed in all gibbons (Table 24). The five animals which had had no previous exposure to dengue to virus all had between 4 and 6 days of viremia with maximum titers of approximately 10^2 . The two gibbons which had previous dengue-1 infections had a shorter viremia with lower titers.

It appears that both the cynomolgous monkeys and white-handed gibbons will be useful for future studies on cross protection between dengue strains. With the virus strains tested, all inoculated animals developed viremia. It is anticipated that detailed studies on cross protection between dengue virus strains will be carried out using these animals as experimental hosts.

Dengue Antibody and Monkeys.

Studies reported in the previous annual report demonstrated that experimental infection of Macaca irus monkeys with dengue-2 virus resulted in an early 19S antibody response followed by production of 7S antibody. The 19S antibody was identified by immunoelectrophoresis as Ig-M and the 7S antibody as predominately Ig-G. Ig-M antibody fell to undetectable levels within 2 months after infection. Further studies were carried out to determine the nature of the immunoglobulin response following primary and secondary dengue infections in monkeys. Two monkeys (A-21 and A-37), free of B group arbovirus antibody, were inoculated subcutaneously with 120 pfu of a local strain of dengue-2 virus in the 3rd tissue culture passage. Approximately one year later the same monkeys were inoculated subcutaneously with 1000 pfu of dengue-1 virus in 2nd tissue culture passage.

Table 25 presents the results of density gradient centrifugation of serum collected at frequent intervals following these experimental infections. In both animals the primary dengue-2 infection caused a 19S antibody response (measured by HI test) beginning on day 10 and disappearing prior to day 42, the 7S response began on the 12th day following infection and 7S antibody was present one year later. The second dengue infection with dengue-1 virus did not result in production of detectable amounts of 19S antibody; however, 7S antibody titers rose rapidly by the 12th day post inoculation.

The virus neutralizing properties of mercapto-ethanol sensitive 19S antibodies against dengue viruses had been previously demonstrated. The specificity of such antibody was compared with the specificity of 7S antibodies in sera from 2 monkeys 14 days following dengue-2 infection. Pooled fractions from 19S and 7S zones of the density gradient were extracted with acetone and tested by plaque reduction neutralization test against prototype dengue viruses. Results are given in Table 26. Monkey A-39 had a very high 7S antibody titer. The apparent minor differences in specificity between 19S and 7S fractions are probably due to relative amounts of antibody rather than actual differences in specificity.

Neutralizing antibody in whole serum of monkeys A-21 and A-37 was measured before and after the secondary dengue-1 infection by plaque reduction neutralization test. The results, given in Table 27, show type specific neutralization one year after the dengue-2 infection, but 21 day after the dengue-1 infection broad cross reactivity was apparent. The rise of neutralizing antibody (Table 28) roughly parallels in time the rise of 7S HI antibody in these monkeys, reaching high titers by 14 days post inoculation.

Immunologic Response to Dengue Infection in Man.

Preliminary studies reported in the previous annual report demonstrated a short period (2 to 4 weeks) of 19S antibody production following primary dengue infections in Thai children. The cases of secondary dengue infection studied, however, had little or no 19S antibody and a marked 7S antibody response. These studies were continued and a total of 23 sera from 8 cases of mild dengue infections exhibiting a primary type of antibody response were fractionated by sucrose density gradient ultra-centrifugation. The fractions were tested for anti-dengue HI activity and for sensitivity to reduction by 2-mercapto ethanol. A typical pattern of primary response is shown in Table 29. The 19S mercaptoethanol-sensitive, antibody appeared on the 6th day of illness and 7S antibody appeared on the 10th day. Similar patterns were seen in the other 7 cases studied with 19S antibody appearing as early as the 4th day of illness and persisting as long as the 30th day. In 3 cases, 19S antibody was no longer detectable in the serum after 18 days.

Forty-one sera from 14 cases of secondary dengue infections were fractionated. Table 10 shows a typical pattern seen in a secondary dengue infection associated with the dengue shock syndrome. A marked 7S antibody rise preceded 19S antibody formation, and the 19S response was markedly suppressed. In 5 of 14 cases, small amounts of 19S antibody were detected between the 4th and 18th day of illness, however, in the remaining 9 cases, no 19S antibody was found.

These studies utilizing density gradient ultra-centrifugation technique indicated that, in the case of primary dengue infections, the first anti-dengue immunoglobulins detectable are 19S globulins, and 7S antibodies appear 1-3 days after the appearance of the 19S antibodies. In such cases if antigen-antibody complexes are formed within the host the complexes would consist predominately of 19S antibody and viral antigen.

In the case of secondary dengue infections, the first detectable antibodies are 7S globulins which rise rapidly in titer; the 19S globulins in most cases cannot be detected or, are produced in small amounts. In the secondary cases, therefore, antigen-antibody complexes, if formed, would contain predominately 7S antibody.

Since patients with dengue shock syndrome have a secondary type antibody response which may be associated with the pathogenesis of this syndrome, studies were carried out to determine the identity of the 7S anti-dengue antibodies present in the serum during and after the shock phase.

Patients with hemorrhagic fever who were admitted to the Children's Hospital in the early stages of dengue shock syndrome, were carefully followed clinically, and blood specimens obtained at frequent intervals. Sera were quick frozen immediately after separation and stored at -70°C. Sera were tested for dengue HI antibody and pooled for fractionation by DEAE-cellulose chromatography.

Pooled sera (4 to 8 ml) were applied to a 2.2 x 40 cm column of Sephadex G-25 and eluted with 0.03 M phosphate buffer, pH 6.4. The protein containing eluate from the Sephadex column was applied to a 2.5 x 80 cm column of DEAE-cellulose previously equilibrated with 0.03 M phosphate buffer, pH 6.4. The protein was eluted from the column with 0.03 M buffer at a flow rate of 5 ml/minute. Protein concentration (optical density at 280 m μ) was measured continuously and the protein containing eluate pooled as fraction I. Subsequent elutions were made in a similar manner with 0.1 M and 0.3 M phosphate buffers also at pH 6.4. Three fractions were thus obtained and the globulins in each fraction were concentrated by precipitation in 50% ammonium sulphate. Precipitates were sedimented by centrifugation, dissolved in normal saline, and dialysed against normal saline for 24 hours.

The immunoglobulins contained in the concentrated fractions were assayed by two methods. Single radial diffusion in agar containing specific antibody against Ig-M, Ig-G or Ig-A was done using commercial reagents (Immunoplate, Hyland Laboratories). Immunoelectrophoresis was done using goat antiserum against Ig-G, Ig-A, and Ig-M (Hyland Laboratories), and antihuman globulin serum prepared in rabbits.

Each fraction was tested for HI antibody against 4 dengue antigens and the sensitivity of the HI antibody to reduction by 2-mercaptoethanol was determined.

The HI antibody titers of the sera and the serum pools are given in Table 31. In all cases high antibody titers were present at the time shock was observed. Table 32 presents the results of immunoglobulin and antibody assays of the fractions from the DEAE chromatography.

The radial diffusion method proved superior to immunoelectrophoresis for detection of low concentrations of immunoglobulins, and in addition, allowed measurement of concentrations.

The method used for fractionation was found to have two major disadvantages. First, the yield of partially purified immunoglobulin was somewhat low; due in part to loss on the DEAE column and in part to the method of concentration. Second, and more importantly, fraction II and III, containing Ig-A and Ig-M, respectively, always contained measurable amounts of Ig-G as well.

Fraction I, eluted with 0.03 M phosphate buffer, contained >95% of the total Ig-G, and no Ig-A or Ig-M was detectable in this fraction in any case. It is apparent from table 32 that most of the antibody activity was also found in fraction I in each case. HI antibody titers in fraction I are 40 to 1000 fold greater than the titers seen in fraction II or III.

Ig-A was found in fraction II although traces were present in fraction III in cases HFI-747 and HFI-749. Ig-M was detected only in fraction III.

It is apparent that the HI antibody activity in fraction I is related primarily to the Ig-G content. In every case, the HI antibody titers of fraction III were low and were not affected by treatment with 2-mercaptoethanol. It is reasonable to conclude, therefore, that the HI antibody activity in fraction III was due to the small amounts of Ig-G present and not due to Ig-M, which is sensitive to the action of 2-mercaptoethanol. This was confirmed in case HFI-773 by sucrose density gradient ultracentrifugation of the serum pool. The results, given in Table 33 show that no HI antibody activity was found in the Ig-M containing fractions (2, 3 and 4), and all HI antibody was 2-mercaptoethanol resistant. The findings in these cases are consistent with previous findings that little or no anti-dengue Ig-M antibody is present in the sera of patients with dengue shock syndrome.

The results obtained with the Ig-A containing fraction II were more difficult to interpret. The amount of Ig-G present in fraction II in each case was of the same order of magnitude as the amount of Ig-G in fraction III. The HI antibody titers are likewise in the same range. Allowing for the inaccuracies of measurement of both immunoglobulin concentration, and of antibody titers it appears that all HI antibody activity could be ascribed to the Ig-G content of fraction II in each case. However, it is impossible from these results to state with assurance that Ig-A did not contribute to the antibody activity measured. The amount of HI antibody activity due to Ig-A in the original serum pools, if present at all, must be very small in comparison with Ig-G antibody activity.

Additional studies of this nature are in progress as well as experiments to improve the techniques of purifying immunoglobulins.

Complement fixation by 19S and 7S antibodies to Arboviruses.

Studies reported above have shown that both 19S and 7S anti-dengue immunoglobulins react in the hemagglutination-inhibition (HI) test and neutralize dengue viruses in vitro. Little information is available however on the complement fixing (CF) properties of human immunoglobulins from sera containing antibodies against arboviruses. The fact that CF activity is low or absent during very early convalescence from a primary dengue or a chikungunya infection when both HI and N antibody activity are present and 19S globulins comprise the major portion of active antibody in the serum, suggests that the CF activity of 19S globulins is low. Studies were done to determine the CF activity of 19S globulins in vitro.

Using sucrose density gradient methods, partially purified preparations of 19S and 7S globulins were obtained from early convalescent sera from cases of dengue, Japanese encephalitis and chikungunya infections in man. Fractions from the 19S and 7S zones of the density gradient were pooled, dialyzed against phosphate buffered saline and concentrated by lyophilization. HI and CF antibody titers of the original serum and the pooled fractions were measured against homologous and heterologous viral antigens. Antigens used were prepared by sucrose-acetone treatment of infected suckling mouse brain.

Results summarized in Table 34 indicate that the 19S pools have HI activity but no CF activity. The 7S pools, on the other hand have both HI and CF activity. The tests done with the Japanese encephalitis and chikungunya antisera indicate that the CF activity of the 7S fraction was specific for the homologous antigen.

The 19S pools were tested by single radial diffusion and immunoelectrophoresis to determine the content of specific immunoglobulins. Results indicated that the 19S pools contained Ig-M in concentrations of 25 to 65 mg% and traces of Ig-G. The 7S pools contained Ig-G and Ig-A with no detectable Ig-M. The HI antibody activity of the 19S pools was entirely 2-mercapto-ethanol sensitive and the HI activity of the 19S pools was entirely 2-mercapto-ethanol sensitive and the HI activity of the 7S pools was resistant to 2-mercapto-ethanol. Therefore, the antibody activity of the 19S pools was due to the Ig-M content.

Experiments were carried out to determine if 19S globulin known to have HI activity, but not CF activity, could block the complement fixing action of whole serum in vitro. Several methods were tried and the most effective was as follows: The CF antigen to be used in the test was incubated with 19S antibody pool (diluted 1:10 or 1:20) for 18 hours at 4°C. Following incubation with the 19S antibody, the antigen was added to the diluted serum in microtiter plates, complement was added, the mixture incubated again for 18 hrs. at 4°C prior to addition of the hemolytic system. The results of these experiments indicate that 19S antibody effectively blocks complement fixation by whole antiserum under the conditions described.

To determine the specificity of this blocking reaction 19S globulin pools from cases of dengue, chikungunya, and Japanese encephalitis were tested for blocking activity against homologous and heterologous antiserum. The results tabulated in Table 35 clearly showed that anti-dengue 19S globulin blocked the CF reaction between dengue antigen, and dengue antiserum, but failed to block the CF reaction between chikungunya antigen and antiserum, or Japanese encephalitis antigen and antiserum. Similarly anti-chikungunya 19S globulin and anti-JE 19S globulin blocked the homologous CF reaction between antigen and whole antiserum, and failed to block heterologous CF reaction.

The results obtained indicate that Ig-M antibodies bind specifically to the combining sites of CF antigen without fixing complement. The antibody binding is effective in preventing subsequent reaction of complement fixing antibody with the antigen.

Summary:

Factors effecting the plaque reduction neutralization test for dengue virus antibody were investigated. Several dengue virus strains were found to have a significant loss of infectivity when incubated at 37°C for one hour. Sera from several mammalian species contained non-specific heat labile virus inhibitors at dilutions of 1:10. Statistical evaluation of the plaque reduction neutralization test indicated that it was possible to distinguish 2 fold differences in comparative potency of antisera.

A method for identification and serologic classification of dengue viruses using the plaque reduction test with monkey immune serum is described. Results of typing 40 dengue strains confirmed the usefulness of this method for classification of low passage dengue strains.

A new and highly sensitive method for isolation of dengue viruses by direct and delayed plaque methods in LLC-MK₂ cell culture is described, and evidence is presented which confirms the usefulness of the method.

Studies on experimental dengue infections indicated that Macaca irus and Hylobates lar regularly developed viremia when infected subcutaneously with low passage dengue viruses. Primary dengue infections in Macaca irus results in an early 19S antibody response. Secondary infections however, are characterized by very small amounts of 19S and a marked rise in titer of 7S antibody components.

Fractionation of serum collected from patients with dengue shock syndrome using DEAE-cellulose chromatography indicated that most, if not all, of antibody activity present in the serum during the shock phase is Ig-G. No antibody activity due to Ig-M or Ig-A was detected.

Ig-M antibody present in the early convalescent sera of patients with primary dengue, chikungunya, or JE infections was found to lack complement fixing activity. This Ig-M antibody had HI activity and under experimental conditions was able to block complement fixation by whole antiserum.