OBSERVATIONS RELATED TO PATHOGENESIS OF DENGUE HEMORRHAGIC FEVER.

I. EXPERIENCE WITH CLASSIFICATION OF DENGUE VIRUSES:

Two distinct syndromes associated with dengue virus infection are prevalent in tropical Asia; dengue fever, a benign disease characterized by fever, myalgia, leucopenia and a maculo-papular rash and dengue hemorrhagic fever (DHF), accompanied by fever, shock, hemorrhagic diathesis and a significant mortality. The dengue viruses, a group of antigenically related members of the Group B arthropod-borne virus family, are transmitted from man to man by the mosquito, *Aedes aegypti*. Multiple members of the group are known to be simultaneously transmitted in large urban areas of tropical Asia. Dengue disease syndromes of both types may occur concurrently, DHF usually is restricted to the indigenous population, while dengue fever may occur in both indigenous and non-indigenous residents of an area.5–6

When dengue hemorrhagic fever was first described in the Philippines, two new dengue viruses, types 3 and 4 were recovered from patients.2 Shortly thereafter, dengue strains recovered from DHF patients in Thailand were tentatively designated types 5 and 6.5 As one explanation of the malignant type of dengue fever, it was suggested that dengue viruses had acquired virulence properties.6 A corollary to this hypothesis was that virulence and surface antigen were genetically linked and thus, the "newer" types of dengue viruses were the cause of the severe dengue syndrome.

The hypothesis that DHF is due to a self-destructive host response has been proposed by Halstead and associates.7 They suggested that some persons are sensitized by their first dengue infection. In such a host the course

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of a second infection with a virus of a different type may be altered adversely by an immune response. The following kinds of evidence support this hypothesis: a) almost all patients with severe hemorrhagic fever (dengue shock syndrome) exhibit a secondary (IgG) dengue antibody response; b) the dengue shock syndrome occurs only in areas of tropical Asia where two or more types of dengue viruses are simultaneously or sequentially endemic; c) no single strain of dengue virus has been associated with dengue shock syndrome, at least four types having been isolated from patients; d) at most, one dengue virus has been isolated from a single patient, suggesting that symptoms are precipitated by a single infection, and e) the shock syndrome is not seen in short term residents of dengue endemic areas who, if infected, have primary but only rarely, secondary dengue infections.

This series of communications4-13 extends our preliminary reports on dengue infections in man and summarizes extensive observations both on dengue viruses and the dengue infected host which appear to be related to pathogenetic mechanisms operating in this system.

The following paper presents an antigenic analysis of dengue virus strains and attempts to correlate these with the disease response in the host.8 Paper III summarizes investigations on a series of fatal hemorrhagic fever cases.9 The fourth paper analyses severity of host response by the type of antibody response and the type of dengue virus recovered.10 The fifth employs a mathematical model to analyze sequential infection phenomena and compares data derived with observed hemorrhagic fever hospitalization rates in Bangkok.11 The final paper discusses pertinent observations and proposes general hypotheses of the pathogenesis of dengue and DHF.12

This paper summarizes a variety of observations made during the course of identifying nearly 300 dengue virus strains recovered in several host systems in 1962-1964. Details of a highly successful single-overlay dengue plaque assay and a plaque reduction neutralization test that uses micro-quantities of reagents are included. Comparative observations on virus identification attempts by several methods are presented and the relation of these results to the question of whether the viruses isolated during this period differed from prototype dengue virus types 1-4 are considered. Some of the techniques evolved during this experience have been described.9,14

MATERIALS AND METHODS

Viruses. Thailand viruses were recovered in suckling mice or tissue culture by methods described previously.15 Attempts were made to keep laboratory host passage of virus strains as low as possible. Between January, 1962 and January, 1965, 45 dengue-like viruses were recovered from arthropods and 250 dengue-like viruses were recovered from human beings with various febrile illnesses. On initial recovery attempt,
140 were recovered in suckling mice, 145 in BS-C-1 (continuous grivet monkey kidney) cells, and 23 strains were recovered in both host systems.

Three different sets of prototype dengue viruses were employed: A. Dengue 1 (Hawaii) at 70th suckling mouse passage (smp), dengue 2 (TR 1751) at 70th smp, dengue 3 (H-87) at 36th smp, dengue 4 (H-241) at 36th smp, TH-36 at 17th smp and TH-Sman, 17th smp. These viruses were received in Thailand in 1960 or 1961, probably from the laboratory of Dr. W. McD. Hammon. B. Dengue 1 (Hawaii) at 124th smp, dengue 2 (New Guinea C) 25th smp, dengue 3 (H-87) 20th smp, dengue 4 (H-241) 24th smp, TH-36, 13th smp, and TH-Sman, 12th smp; each of these strains had been cloned by three terminal dilutions in mice in Hammon's laboratory. C. Dengue 1 (Hawaii) 70th smp, dengue 2 (TR 1751) 70th smp, dengue 3 (H-87) 23rd smp, dengue 4 (H-241) 15th smp, TH-36 15th smp and TH-Sman 15th smp. These were in the collection of arthropod-borne viruses at the Yale Arbovirus Research Unit having been received a varying number of years previously from laboratories in which the strains were originally isolated.

Antigens. Acetone-ether extracted mouse brain virus antigens prepared by the method of Clarke and Casals* were used for both hemagglutination-inhibition and complement-fixation tests.

Hemagglutination-inhibition (HI) test. HI tests were performed by the method of Clarke and Casals* with the modification for Microtiter equipment. Eight units of hemagglutinin of each virus antigen were used in the tests reported. The temperature of incubation of virus-cell mixtures was 22°C. The HI titer is expressed as the last dilution at which a complete inhibition of hemagglutination occurred.

Complement fixation (CF) test. Complement-fixation tests were done by the method described by Fulton and Dumbell** modified for Microtiter equipment. Two exact units of complement and three minimal hemolytic doses of antisheephemolysin were used in all tests. Serum and saline controls and known positive and negative sera were included in each test.

Immune Serum. Mouse immune sera. Technique 1. Suckling mice were inoculated intracerebrally (IC) with dengue seed virus. After mice became sick, a 10% suspension of mouse brain was prepared in normal saline. This suspension was inoculated immediately into 21-42 day old mice intraperitoneally (IP) in 0.2 ml. amounts. Five or more injections of fresh mouse brain-saline suspensions were inoculated at one week intervals. Trial bleedings were made to determine when satisfactory antibody titer had been obtained.

Technique 2. Three-to-four-week-old mice were inoculated IP with 0.3 ml. of 10% fresh mouse brain in saline harvested from mice infected with unidentified viruses at low mouse passage. Two weeks later mice were inoculated with 0.03 ml. of 10^-2 virus suspension intracerebrally. A trial bleeding was performed 10 days later. If titer was not satisfactory new groups of weanling mice were immunized using a second schedule. First, 0.3 ml. of 10^-3 virus suspension was inoculated IP; 10 days later 0.1 ml. of 10^-4 virus suspension was given IP and subcutaneously. Ten days later, 0.03 ml. of 10^-4 virus suspension was injected into mice intracerebrally. Trial bleeding was made two weeks later.*

Monkey antisera. Young adult Macaca mulatta free of group B hemagglutination-inhibition antibodies and plaque reduction antibody to dengue types 1-4 were inocu-
lated subcutaneously with 0.5 ml. of a 1:10 dilution of virus in 0.75% bovine albumin in phosphate buffered saline (BAPS). Virus was prepared by homogenizing a suspension of infected LLC-MK2 (continuous rhesus kidney) cell monolayers harvested on the sixth day after virus inoculation. Monkeys were bled six weeks or more after infection.

**Tissue culture neutralisation test.** The property of BS-C-1 cells infected with dengue viruses to resist challenge with a cytopathic virus has been described. Tube cultures of these cells were used to test for dengue neutralization antibodies using the virus dilution, serum-constant method. Virus-serum mixtures were incubated at 37°C for one hour. Equal amounts of normal mouse serum and virus dilutions were incubated similarly for estimation of virus titer. Chikungunya virus was used as a cytopathic challenge virus to test for dengue virus-induced cellular resistance. Results are expressed as log neutralization index (LNI). LNI of 1.7 or greater was considered significant neutralization.

**Plaque reduction neutralization test.** A preliminary communication has described the LLC-MK2 dengue virus plaque assay. A strain of LLC-MK2 propagated at Yale University School of Medicine for an unknown period was used in these studies. Test conditions and techniques for propagation of cells differed somewhat from those reported earlier. Cells were grown in a medium consisting of Eagle's Basal Medium, 5% agamma calf serum, 5% calf serum, final concentrations of glutamine of 200 mM per ml., bicarbonate of 0.45 mg/ml. and penicillin and streptomycin, of 100 units and 100 mg/ml., respectively. Seven days after inoculation of 2 oz. plaque bottles with 7 ml. containing 120,000 cells/ml., monolayers were ready to use. Virus was diluted either in 0.75% BAPS, 20% agamma calf serum in phosphate buffered saline, pH 7.8-8.0 (PBS), or acetone extracted normal mouse ascitic fluid in PBS. After incubation under conditions described in the text, neutralization reaction in virus-serum mixture was stopped by one hundred-fold dilution in chilled diluent. Growth medium was poured off cell sheet and 0.5 ml. of the diluted virus-serum mixture added without washing cells. Tubes were then incubated at 37°C for 90 minutes, next, virus inoculum was removed and in a darkened room, agar overlay added. Agar overlay contained a final concentration of 1:10,000 (millipore filtered) neutral red and 1% Noble agar; concentrations of other ingredients were identical to those in growth medium except that one half concentration of bicarbonate was used. The pH of agar overlay was approximately 7.9. Plaque bottles were held at 35-36°C for 7 days and then for two weeks at room temperature. All incident light was excluded during the period. Plaques continue to appear and enlarge for many days, necessitating readings at 7, 14, and 21 days.

Plaque reduction serum titers (50% end point) were estimated by the method of Russell, et al.

**RESULTS**

The earliest method employed for identification of dengue viruses was a virus dilution, serum constant neutralization test performed in BS-C-1 cells using the CVR method. Mouse antisera to prototype dengue viruses were prepared by technique 1. Table 1 shows a grid-type comparison of prototype dengue 1-4, TH-36 and TH-Sman virus strains (group A) and antisera. Although some degree of crossing was observed, most lots of serum were relatively specific to homologous virus. Significant cross neutralization
Table 1. Cross Neutralization Tests of Dengue 1-4, TH-36 and TH-Sman Viruses in BS-C-1 Cells

| Antiserum | Virus   | d1 | d2 | d3 | d4 | TH-36 | TH-Sman |
|-----------|---------|----|----|----|----|-------|---------|
| d1        | 3.0     | 1.0| 1.2| 0.5| 1.0| 2.5   |
| d2        | 1.0     | 3.0| 0.8| 0.7| 3.0| 1.2   |
| d3        | 1.5     | 1.0| 2.0| 0.5| 0.8| 1.0   |
| d4        | 0.5     | 0.5| 0.0| 4.0| 0.9| 0.5   |
| TH-36     | 1.0     | 3.5| 0.7| 1.0| 2.5| 0.3   |
| TH-Sman   | 2.0     | 1.2| 1.5| 0.3| 0.8| 3.4   |

*d* = dengue.

was observed between dengue 1 and TH-Sman and dengue 2 and TH-36 viruses and antisera. Less significant neutralization was noted between dengue 1 and 3 viruses. During three years of preparation of hyperimmune mouse serum many lots of dengue 3 and 4 antisera failed to neutralize significantly homologous virus. It was found that use of frozen virus-brain seeds was not as suitable as fresh material in producing a good antibody response in mice.

Table 2 illustrates the results of attempted identification of the 121 dengue viruses recovered in suckling mice. Of the total tested, 71 viruses were neutralized significantly by one or more antisera. Of 50 viruses neutralized either by dengue 2 or TH-36 antisera, 23 were significantly

Table 2. Summary of Attempted Identification of 121 Suckling Mouse Adapted Viruses. Virus Isolates Were Tested Against Hyperimmune Serum to Dengue 1-4, TH-36 and TH-Sman by a Neutralization Test in BS-C-1 Cells

| No. isolates neutralized significantly by indicated antisera | No. isolates not neutralized by sera | Low or no virus titer | Results contradictory |
|------------------------------------------------------------|-------------------------------------|-----------------------|----------------------|
| d1                                                         | 7                                   |                       |                      |
| TH-Sman                                                   | 3                                   |                       |                      |
| d1 + TH-Sman                                              | 3                                   |                       |                      |
| d2                                                         | 4                                   |                       |                      |
| TH-36                                                     | 13                                  |                       |                      |
| d2 + TH-36                                                | 33                                  |                       |                      |
| d3                                                         | 7                                   |                       |                      |
| d4                                                         | 1                                   |                       |                      |
| Total                                                     | 71                                  | 28                    | 18                   | 4                    |

*d* = dengue.
neutralized by both. Only 3 of 13 viruses in the dengue 1-TH-Sman group were neutralized by both antisera. Significant neutralization by more than one dengue antiserum (types 1-4) was observed with four viruses. Identification could not be established in these instances. Twenty-eight viruses were not significantly neutralized by any of six dengue antisera. To cope with these viruses, other identification methods were investigated.

**Preparation of immune serum to isolates.** Hyperimmune sera prepared by technique 1 to low mouse passage isolates were tested by HI and CF. Representative results are shown in Tables 3 and 4. By HI, hyperimmune

### Table 3. Identification of Dengue Viruses. Hemagglutination-Inhibition Test of Antiserum Prepared to Isolates

| Bangkok virus mouse immune serum | Reciprocal of antibody titer vs. indicated dengue antigens |
|----------------------------------|----------------------------------------------------------|
|                                  | d1  | d2  | d3  | d4  | TH-36 | TH-Sman | Homol |
| 2358-62                          | 40  | 160 | 40  | 80  | 160   | 80      | 160   |
| 3149-62                          | 80  | 40  | 80  | 80  | 80    | 160     | 320   |
| 5031-62                          | 160 | 160 | 80  | 160 | 80    | 320     | 320   |
| BKM-60-62                        | 80  | 320 | 80  | 160 | 640   | 80      | 1,280 |
| BKM-331-62                       | 40  | 160 | 80  | 80  | 20    | 20      | 160   |
| BKM-418-62                       | 160 | 640 | 40  | 80  | 160   | 80      | 320   |

Homol. = Homologous antigen.

### Table 4. Identification of Dengue Viruses. Complement-Fixation Test of Hyperimmune Serum Prepared to Isolates by Technique I

| Bangkok virus hyperimmune serum | Reciprocal of CF antibody titer vs. two units of indicated prototype dengue antigen |
|---------------------------------|-----------------------------------------------------------------------------------|
|                                 | d1  | d2  | d3  | d4  | TH-36 | TH-Sman | Homologous virus |
| 2358-62                         | 4*  | 32  | 0** | 4   | 4     | 4       | 64/128†         |
| 3149-62                         | 16  | 8   | 8   | 4   | 16    | 16/32   |
| 5031-62                         | 16  | 4   | 4   | 8   | 8     | 16/32   |
| BKM-60-62                       | 0   | 16  | 0   | 8   | 0     | 16/32   |
| BKM-331-62                      | 16  | 8   | 64  | 8   | 0     | 32/32   |
| BKM-418-62                      | 16  | 32  | 16  | 8   | 32    | 64/128   |
| BKM-475-62                      | 16  | 0   | 0   | 0   | 8     | 8/16    |

* Signifies reciprocal of highest dilution of serum which gives 2+ or more fixation of complement.

** No fixation at 1:4 dilution of serum.

† The numerator represents the maximum dilution of serum fixing complement in the presence of antigen and the denominator the maximum dilution of an antigen fixing complement in the presence of antiserum.
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serum titers to each of six prototype antigens varied from each other by
two-to fourfold. Highest titers to prototype antigens often were within two-
fold of titers to homologous virus. Results by CF were sometimes quite
specific. Usually there was a marked similarity in serum titers with dengue
1 and TH-Sman or dengue 2 and TH-36 antigens, respectively. In some
instances, CF titers to two or more antigens (dl-4) differed by at most
only twofold, making test results difficult to interpret. For this reason, a
modification in the antiserum preparation method was introduced. Table 5

Table 5. Identification of Dengue Viruses. Complement-Fixation Test
Using Immune Serum Prepared by Two Inoculations of Indicated
Isolates (Technique II)

| Immune mouse serum | Antigen | Reciprocal of antibody titer vs. two units of indicated prototype dengue antigen |
|--------------------|---------|--------------------------------------------------------------------------------|
|                    | d1      | d2      | d3      | d4      | TH-36 | TH-Sman |
| 2358-62            | 0*      | 32      | 0       | 0       | 16    | 0       |
| 3149-62            | 16      | 0       | 0       | 0       | 0     | 8       |
| 5031-62            | 4       | 0       | 0       | 0       | 8     | 0       |
| BKM-60-62          | 0       | 8       | 0       | 0       | 8     | 0       |
| BKM-331-62         | 0       | 0       | 16      | 0       | 0     | 0       |
| BKM-418-62         | 0       | 4       | 0       | 0       | 4     | 0       |

* No fixation at a 1:4 serum dilution.

shows representative CF results obtained with mouse antiserum prepared
by technique 2. In this system little heterologous reaction was observed.
There was a consistently close correlation between serum titers to dengue 1
or TH-Sman and dengue 2 or TH-36 antigens, respectively. Results are
illustrated in Tables 6 and 7.

Table 6. Correlation Table Showing CF Antibody Titers of 26 Bangkok
Dengue Mouse Sera to Dengue 1 (Hawaii) and Dengue TH-Sman
Antigens

| Reciprocal CF titer vs. two units of dengue 1 (Hawaii) antigen |
|---------------------------------------------------------------|
| Reciprocal CF titer vs. two units of TH-Sman antigen         |
| Reciprocal CF titer                                         |
| <4                                                      | 4 | 8 | 16 | 32 |
| 4                                                      | 2 | 2 | 1 |
| 8                                                      | 4 | 5 | 1 |
| 16                                                     | 2 | 4 | 1 |
| 32                                                     | 1 | 1 | 2 |

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Table 7. Correlation Table Showing CF Antibody Titters in 44 Bangkok Dengue Virus Mouse Immune Sera to Dengue 2 (New Guinea C) and TH-36

| Reciprocal titer vs. two units of dengue 2 (New Guinea C) antigen | Reciprocal titer | <4 | 4 | 8 | 16 | 32 |
|---------------------------------------------------------------|-----------------|----|---|---|----|----|
| <4                                                            | 1               | 2  | 3 |
| 4                                                             | 3               | 3  | 1 |
| 8                                                             | 3               | 1  | 4 | 4  | 2  |
| 16                                                            | 2               | 4  | 5 |
| 32                                                            | 1               | 1  | 3 |

A good correspondence was observed between results in the neutralization test and the CF test of serum prepared to isolates (Table 8). The CF identification results obtained for the 50 viruses for which contradictory neutralization indices or no neutralization occurred are not shown. Of 28 strains to which detectable dengue antibodies developed in mice, 21 fixed complement only with dengue 3 virus. Of the 28 viruses not significantly neutralized by any prototype dengue antiserum, 14 were subsequently identified as dengue 3. It appeared that many "wild" dengue 3 strains were poorly neutralized by antiserum prepared to H-87 strain of dengue 3 virus.

From August, 1963, BS-C-1 cells were used for the majority of dengue virus isolation attempts. Tissue culture passage viruses brought new problems in identification. While the neutralization test employing mouse hyperimmune serum could be used, the percentage of untyped viruses was about the same as reported above. Attempts to produce immune or hyperimmune serum by IP and IC challenge in mice to tissue culture viruses failed in most instances. An exception to this observation occurred with the dengue 2-TH 36 complex viruses, which did stimulate antibody production in mice. As evidence of their low pathogenicity, undiluted tissue culture viruses rarely resulted in sickness or death following intracerebral inoculation of

Table 8. Comparative Results Between Complement-Fixation* and Neutralization** Techniques for Identification of Dengue Virus Isolates

| Total viruses identified by Nt | = 71 |
| CF results identical to Nt† | = 59 |
| CF results different | = 7 |
| No CF antibody detected | = 5 |

* Mouse immune serum prepared by two or three injections of dengue virus antigens.
** Low mouse passage dengue virus isolates tested against hyperimmune (five antigen injections) dengue prototype mouse serum in BS-C-1 cells.
† Considered identical if Nt results were positive for d1 and/or TH-Sman and CF results positive for both d1 and TH-Sman. Same conditions apply for d2-TH-36.
weanling mice. A process of blind intracerebral passage of viruses in suckling mice, similar to that used to recover dengue viruses from human serum, had to be employed to adapt tissue culture passage strains to mice. For a large number of tissue culture viruses this was done and antiserum prepared in mice and virus identified as described above.

**Plaque reduction test.** The inadequacies apparent in each of the identification systems described led to a search for a more discriminating and yet versatile technique. A plaque reduction test using the LLC-MK2 plaque assay system fulfilled these criteria. Studies of the neutralization characteristics of *Macaca irus* immune serum by some of us had led to a highly reproducible dengue virus identification technique applicable to virus from any host. In the Yale laboratory these results have been extended to antisera prepared in *Macaca mulatta*. There appears to be an important advantage in using the latter species. More than 80 young adults of this species, purchased in four separate lots in 1966-1967, have been found to be free of neutralizing antibody to dengue types 1-4 viruses. Sera from 16/20 *Macaca irus* of unknown geographic origin, obtained in 1967, contained high titered plaque reducing antibodies to dengue 1 and/or dengue 2 viruses.

Cross neutralization tests between dengue types 1-4 and TH-36 and TH-Sman strains using monkey antisera are illustrated in Table 9. Also included are neutralization data for dengue strain 13711 isolated from a hemorrhagic fever patient in the 1964 Ubol, Thailand outbreak. These data show nearly identical 50 percent serum plaque reduction titers between dengue 1, TH-Sman and 13711 viruses and antisera and between dengue 2 and TH-36 viruses and antisera, respectively. Table 10 shows further application of *Macaca mulatta* antisera to the typing of tissue culture adapted dengue strains. It was of interest that 1950-63 dengue strain was poorly neutralized by homologous as well as two different heterologous dengue 2 immune sera. In contrast, the antibody produced in two monkeys following infection with 1950-63 strain was nearly equal in titer to that produced by animals infected with 16681 dengue 2 strain.

Of 22 viruses not identified by any other technique, 17 were typed by plaque reduction neutralization test (courtesy of P. K. Russell). Only five virus strains were untyped. These were dengue-like in their biologic behavior, but after 1966 no further identification attempts could be made and the strains are now lost. Of all the 295 dengue-like agents, 207 were identified in two or more systems.

**DISCUSSION**

Several important observations were made: (1.) Using as assay system fluid-covered cell monolayers, neutralization of some dengue viruses by
### Table 9. Cross Neutralization Studies of Mouse Passaged Dengue Viruses Using 6 Week Post-Infection Macaca mulatta Sera

Reciprocal of dilution of monkey serum producing 50% plaque reduction vs. indicated viruses*

| Test virus | Serum | Mean control PFU** | Dengue 1 (Hawaii, smp 60+) | Dengue 2 (TR 1751, smp 57) | Dengue 3 (H-87, smp 24) | Dengue 4 (H 241, smp 16) | TH-36 (smp 16) | TH-Sman (smp 16) | 13711 (smp 4) |
|------------|-------|-------------------|-----------------------------|-----------------------------|-------------------------|--------------------------|----------------|----------------|-------------|
| dengue 1   |       | 7.0               | 125                         | <20                         | <20                     | <20                      | 120            | 85             |             |
| (Hawaii, smp 60+) |     |                   |                             |                             |                         |                          |                 |                |              |
| dengue 2   |       | 50.0              | <20                         | 79                          | <20                     | 22                       | 140            | <20            | <20         |
| (TR 1751, smp 57) |    |                   |                             |                             |                         |                          |                 |                |              |
| dengue 3   |       | 52.0              | <20                         | <20                         | 50                      | <20                      | <20            | <20            | <20         |
| (H-87, smp 24) |      |                   |                             |                             |                         |                          |                 |                |              |
| dengue 4   |       | 51.2              | <20                         | <20                         | <20                     | 35                       | <20            | <20            | <20         |
| (H 241, smp 16) |     |                   |                             |                             |                         |                          |                 |                |              |
| TH-36, smp 16 |     | 32.1              | <20                         | 85                          | <20                     | 25                       | 135            | <20            | <20         |
| TH-Sman, smp 16 |   | 43.2              | 120                         | <20                         | <20                     | <20                      | <20            | 125            | 95          |
| 13711, smp 4 |       | 23.7              | 95                          | <20                         | <20                     | <20                      | <20            | 110            | 110         |

* Virus-serum incubated for 1 hr. at 25°C. then diluted 1:100 before incubation on cell sheets. Monkey serum acetone treated. Titer calculated by the method of Russell and Nisalak.**

** Virus diluted in 20% heat-inactivated agamma calf serum.
Table 10. Cross Neutralization Studies of Dengue Viruses Using Monkey Antisera*

| Test virus | Monkey no. | 13014 | 13015 | 13020 | 13021 | 13016 | 13017 | 13018 | 13019 |
|------------|------------|-------|-------|-------|-------|-------|-------|-------|-------|
|            | 16007 Mean PFU (d1) | 16681 Mean PFU (d2) | 1950-63 Mean PFU (d2) | 16562 Mean PFU (d3) |
| Dengue 1   | 35.6       | >160   | 158   | <10   | 22    | <10   | 15    | <10   | <10   |
| Dengue 2   | 20.3       | 35     | 30    | 74    | 106   | >160  | >160  | 25    | 35    |
| Dengue 3   | 33.8       | 15     | 24    | 18    | 12    | <10   | 15    | 55    | 58    |
| Dengue 4   | 17.8       | <10    | <10   | 22    | <10   | <10   | <10   | <10   | <10   |
| 16007, TC4 | 27.5       | >160   | 155   | <10   | <10   | 10    | <10   | <10   | <10   |
| 16681, TC4 | 21.6       | <10    | <10   | 60    | 95    | 150   | >160  | <10   | <10   |
| 1950-63, TC4 | 12.0      | <10    | <10   | 35    | 40    | 25    | 55    | <10   | <10   |
| 16562, TC4 | 12.0       | <10    | <10   | <10   | <10   | <10   | 15    | >160  | >160  |

* Test conditions as in Table 9. Sera not heat-inactivated or acetone treated.
** Antisera obtained 6 weeks following subcutaneous inoculation of live dengue viruses.
hyperimmune mouse serum was either reversible or incomplete. This was frequently observed with strains of dengue 3 virus. Whether this is due to dissociation of virus-antibody complex, incomplete neutralization, lack of accessory factor(s), competition between cell and antibody for virus or other phenomena requires study. Dengue 2 viruses, on the other hand, quite regularly stimulated production of antibody in the mouse which could neutralize 50 or more InD$_{50}$ of homologous virus in this assay system.

(2.) When a course of only two or three antigenic stimuli with low mouse passage Thailand dengue viruses was used for antibody production in the mouse, resultant sera when tested by CF had titers to dengue 1 and TH-Sman or dengue 2 and TH-36 antigens which were closely similar. Although this identification system was useful for mouse passaged material it was of little value in identifying tissue culture passage viruses because of their low virulence and/or low immunogenicity for mice.

(3.) Dengue infection in susceptible monkeys from a single subcutaneous inoculation of live virus resulted in production of neutralizing antibody which, in a 50% plaque reduction end point test, had a high titer to the homologous virus and one of the dengue 1-4 prototypes. Immune monkey sera did not appear to distinguish between dengue 1 and TH-Sman or dengue 2 and TH-36 viruses. It was noted that two strains of tissue culture passage dengue type 2 differed in their neutralization properties with three dengue 2 antisera. A reason for the poor neutralization observed with the 1950-63 dengue strain might be that a large portion of this virus population exists in aggregates that break down and liberate virus under the agar overlay.

(4.) A virus-dilution serum-constant neutralization test using dengue mouse immune serum indicated antigenic dissimilarity between the Hawaii and TH-Sman dengue 1 strains and between New Guinea C and TH-36 dengue 2 strains but two other test systems (complement fixation test of mouse dengue immune sera to Thailand isolates and plaque reduction neutralization test using serum obtained from infected monkeys) suggested that these two pairs of viruses are closely identical. These observations are similar to conclusions obtained in an immuno-electrophoretic study of dengue viruses, and an analysis of dengue viruses by plaque reduction using monkey immune serum.

No viruses were recovered from patients or mosquitoes in Thailand from 1962-4 that consistently resembled TH-36 or TH-Sman in more than one identification system. Evidence obtained in the virus dilution neutralization test in BS-C-1 cells supporting the antigenic distinctness of these viruses from dengue 2 and 1, respectively, must be qualified. Results obtained in this system were not always reproducible; further, many lots of immune
sera neutralized dengue viruses only poorly, or at threshold LNI values. We believe the variations observed may result from poorly understood factors that influence the completeness or reversibility of antibody-virus complexing. These factors could be extraneous to the antigenic composition of studied virus strains.

In a separate study we present results from an antigenic analysis of a large number of dengue strains. These studies suggest that the antigenic composition of dengue virus strains of the same type do differ. It is quite apparent that in some systems dengue viruses are poorly neutralized by antibody. Under these circumstances, immune sera containing antibodies to the greatest percentage of the antigenic mosaic of a test strain may neutralize that virus better than another virus with a less complete spectrum of antigenic sites. Does this imply that these strains are different enough to produce sequential infection in the same individual (i.e., are they different types)? We believe not. Based upon antibody responses to infection, the monkey and the human* distinguish in dengue viruses a major antigenic determinant of which there are, apparently, only four. At this stage in the technology of arboviruses, the most biologically relevant answer to the question of how many distinct dengue types there are might be derived from cross protection studies in susceptible hosts and not from in vitro antigenic analyses; dengue cross protection studies in primates are in progress in our laboratory.

SUMMARY

Two syndromes, a severe and sometimes fatal hemorrhagic fever (DHF), and benign dengue fever (DF), are associated with dengue infection in the human being. This series of papers describes studies of the properties of dengue viruses and aspects of the disease response in the host in an attempt to explain why these divergent syndromes occur. It has been postulated that virulent dengue disease is associated with infection by dengue types 3 and 4 and proposed types 5 and 6, while classic dengue fever is caused by types 1 and 2. This paper, the largest experience reported to date, summarizes comparative results obtained in typing 295 dengue viruses recovered from arthropods, DHF and DF cases in Thailand during a 3-year period, 1962-64. When dengue isolates were neutralized by antisera prepared in mice to prototype dengue types 1-4, TH 36 (type 5?) and TH-Sman (type 6?), and assayed in a continuous grivet monkey kidney cell tube test, some more nearly resembled the latter two Thai strains than dengue types 1-4. When antibodies to dengue isolates were produced in mice by a small number of antigenic stimuli, when tested by complement fixation, these reacted with only one of the four prototype
dengue antigens; TH-Sman and TH-36 antigens produced the same titers as dengue types 1 and 2, respectively. Similar results were obtained when isolates were neutralized by prototype dengue immune sera made in monkeys and assayed in a plaque reduction neutralization test. The latter two identification methods were more reproducible than the former. These data support classification of the studied dengue viruses into four, not six, types and are not compatible with the hypothesis that associates DHF with "newer" dengue virus types. Commercially purchased North Indian *Macaca mulatta* were found to be satisfactory for production of specific dengue typing antisera. A single agar overlay dengue plaque assay in LLC MK2 cells and a dengue plaque reduction neutralization test that utilizes microquantities of serum are described.

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