One of the hypotheses invoked to explain the occurrence of different diseases associated with dengue virus infection is that dengue viruses differ in virulence properties. Observations on virologically studied dengue fever and dengue hemorrhagic fever (DHF) patients have reasonably established that both of these diseases are associated with dengue viruses of all known antigenic types. The possibility that virus strains within the same type differ in their virulence properties and that these differences might be genetically associated with surface antigen or other readily measured markers has not been examined. This hypothesis was the initiating motivation for this study. Using single lots of mouse immune ascitic fluids, we tested a large number of dengue viruses by a plaque reduction neutralization test and the results were compared with the disease response and geographic origin of virus strains. A few observations on biologic properties of the examined dengue virus strains are included.

**MATERIALS AND METHODS**

*Plaque reduction test*

The production of LLC-MK2 monolayers, the techniques of plaque assay and the plaque reduction neutralization test have been given elsewhere. In all tests, six replicate 2 oz. plaque bottles were used. All neutralization tests reported in this paper were performed by incubating serum and virus mixtures at 22-25°C. for exactly 60 minutes. Reactions were stopped by dilution of serum-virus mixtures to 1:100 in chilled 0.75% bovine albumin-phosphate buffered saline, pH 7.8-8.0 (BAPS) or 20% heat inactivated agamma calf serum in phosphate buffered saline, pH 7.8-8.9. Residual virus was incubated on cell sheets for 90 minutes at 37°C. before agar overlay.

*Viruses*

Thailand viruses studied were recovered in suckling mice or tissue culture by methods described previously. Also studied were 19 dengue virus strains that had been isolated in other Asian countries and in the 1963 Caribbean outbreak. Data on the year

---

* Presently, Professor of Medicine, Chairman of Section of Tropical Medicine and Medical Microbiology, School of Medicine, University of Hawaii, Honolulu, Hawaii 96816.

** Jr. Lecturer, Faculty of Public Health, Bangkok, Thailand.

† Supported by Grant No. DA-49-193-MD-2846 from the U.S. Army Medical Research and Development Command.

Received for publication 16 August 1969.
Table 1. Dengue Strains Originating Outside Thailand Selected for Antigenic Analysis. (d = dengue)

| Strain | Mouse passage | CF test identif. | Country of origin | Year isolated | Disease association |
|--------|---------------|------------------|-------------------|---------------|---------------------|
| d-1 (Hawaii) | 60+ | ? | Hawaii | 1945 | dengue |
| d-2 (TR 1751) | 56+ | 2 | Trinidad | 1952 | dengue |
| d-3 (H-87) | 23 | 1 | Philippines | 1956 | hemorrhagic fever |
| d-4 (H-241) | 15 | 2 | Philippines | 1956 | hemorrhagic fever |
| 23085a | ? | 2 | India | 1959 | dengue |
| 23086a | 29 | 1 | India | 1959 | dengue |
| 611337a | ? | 4 | India | 1961 | dengue |
| 62878a | ? | 1 | India | 1961 | dengue |
| 64412a | 16 | 2 | India | 1963 | dengue |
| 63491a | 6 | 4 | India | 1962 | dengue |
| PR 6b | 12 | 3 | Puerto Rico | 1963 | dengue |
| S-601c | 14 | 1 | Singapore | 1960 | dengue |
| S-389e | 10 | 1 | Singapore | 1962 | dengue |
| S-843c | 39 | 2 | Singapore | 1960 | dengue |
| S-378e | 3 | 2 | Singapore | 1961 | dengue |
| S-888c | 7 | 3 | Singapore | 1963 | dengue |
| S-1033c | 11 | 3 | Singapore | 1963 | dengue |
| S-132o | 10 | 4 | Singapore | 1961 | dengue |
| S-212c | 15 | 4 | Singapore | 1961 | dengue |
| 16572a | 3 | 4 | Philippines | 1964 | hemorrhagic fever |
| 16604a | 3 | 4 | Philippines | 1964 | hemorrhagic fever |
| 16562a | TC-3 | 4 | Philippines | 1964 | hemorrhagic fever |
| 4328-S* | SM | 4 | Philippines | 1966 | hemorrhagic fever |

a Sent through the kindness of Dr. Donald Carey, Vellore, India and the Virus Research Centre, Poona, India.

b Isolated by Dr. Charles Wiseman; strain received from Dr. J. Casals, Yale Arbovirus Research Unit (YARU), New Haven, Conn.

c Strains generously sent by Dr. Lim Kok An, University of Singapore, Singapore.

d Recovered in SMRL Virology Department, Bangkok by Dr. Virginia Basaca-Sevilla from sera collected in Manila, 1964.

* Strain recovered in Manila, 1966 by Dr. Lourdes Espiritu Campos; sent to Dr. Wilbur Downs, YARU.

f All strains shown were identified by CF or immuno-diffusion tests using prototype d 1-4 antisera; Philippine strains (except dengue 3 and 4 prototypes) were identified at the Virology Department, SEATO Medical Research Laboratory; Indian strains by Drs. Donald Carey and Jordi Casals and Singapore strains by Drs. Lim Kok An and Y. C. Chan.11
of isolation, disease association, country of origin, and donors of these latter strains are summarized in Table 1.

Group 3 prototype dengue viruses described in a previous communication were used.*

Preparation and standardization of immune mouse ascitic fluid (MAF)*

In preliminary studies it had been observed that neutralizing antibody to a variety of high and low mouse passage dengue strains of types 1, 3, or 4 viruses could not be consistently demonstrated at 1:10 or higher dilutions of mouse ascitic fluids (MAF) when only a single stimulus was given or two antigenic stimuli were given at an interval of one week. MAF with significant neutralizing activity at these dilutions could be produced by three weekly intraperitoneal inoculations of 0.5 ml. of a 10% saline suspension of mouse brain virus seed. Immune fluids were produced by mixing the third administered virus suspension with 0.5 ml. of ascitic fluid containing S-180 cells. Seven to 20 days later mouse ascitic fluids were tapped. Since other data had suggested the antigenic similarity of TH-Sman and TH-36 with dengue 1 and 2, respectively,* only dengue 1-4 viruses were used in the preparation of immune MAF. To standardize amount of antibody employed in tests, serial 2-fold dilutions of immune MAF were tested against homologous virus by incubating virus-serum mixture at 22-25°C. for 30 minutes. This incubation temperature was used because preliminary experiments had indicated that homologous reactions proceeded more quickly than heterologous at 22-25°C. while at 37°C. differences in neutralization kinetics were minimal. The dilution of ascitic fluid producing approximately 50% homologous plaque reduction was then used to test all examined dengue virus strains at twice the original incubation period (60 minutes).*

Reliability of plaque counts

The significance of the difference between two independent plaque counts (x₁ and x₂) was tested by Detre's formula:* the Null Hypothesis, H₀, is rejected when

\[
\frac{x₁ - x₂}{x₁ + x₂} \geq 1.96 \text{ (confidence level of 0.05).}
\]

As predicted by Lorenz and Zoeth,* the difference between estimated input plaque forming units (PFU) and observed plaque count increases in the LLC-MK2 dengue virus plaque system when observed plaque counts are 40 and above. Dengue viruses in LLC-MK2 cells have plaque diameters usually ranging between 2-5 mm. Overlap phenomena may reduce estimated plaque reduction from its true value. When control plaque counts are in excess of 40, the measured plaque reduction in test control bottles may be somewhat underestimated. No compensatory calculations of true input plaque forming units were attempted. All tests reported were repeated at least twice; results given are from a single test. Results in repeat tests were consistent with data given below.

Disease response in host

Clinical information regarding type of disease in the host was provided by persons supplying virus strains. Clinical diagnoses were those given by hospital physicians. Information regarding type of antibody response occurring during illness was not obtained, except for Thai cases. All Thai patients with designated diagnosis of hemorrhagic fever (HF) had secondary-type hemagglutination-inhibition (HI) antibody response. Criteria for classifying antibody response have been published.*

* The principles of laboratory animal care as promulgated by The National Society for Medical Research were observed.
RESULTS

Nonspecific inhibitors of dengue viruses

In early experiments it was observed that after incubation of virus with 1:20 or 1:40 dilutions of normal mouse ascitic fluids for one hour at 25°C. or 37°C. there was significant reduction in dengue plaque counts. Heating mouse ascitic fluids at 56°C. for 30 minutes only slightly reduced this dengue neutralizing effect. Acetone extraction removed nonspecific dengue neutralizing substances at ascitic fluid dilutions of 1:5 and above. Broadly reactive, acetone extractable dengue neutralizing substances were found in individual sera from Macaca mulatta, humans, rabbits, and guinea pigs. Significant variation with different individuals was observed. In the following studies, immune and control ascitic fluids were acetone extracted and diluted to at least 1:10 before use.

Study of strains

Tables 2-5 show percent plaque reduction obtained with identical lots and dilutions of immune ascitic fluids when incubated with a large number of dengue strains of diverse geographic or disease origin. Results are grouped by the typing result obtained in other tests or the "major" neutralization reaction obtained. Plaque reduction of prototype dengue 1-4 viruses with homologous MAF at 25°C. for 60 minutes are shown in Table 2. These data should be compared with results in other tables.

Table 2 shows that 8/14 dengue 1 strains were significantly neutralized by dengue 3 MAF. Less frequently (2/14), cross reactions occurred with dengue 4 MAF. In the limited number of strains examined there did not appear to be any particular antigenic pattern associated with disease response or area of origin of the strain. Prototype dengue 1 and TH-Sman differed in their reactions with dengue 4 MAF.

Results of testing dengue 2 strains are presented in Table 3. The majority of recent isolates differed in their reactivity pattern from the prototype strain (TR 1751); 4/17, 5/17 and 11/17 were significantly neutralized by dengue 1, 3, and 4 MAF, respectively. It was of interest that all four dengue 2 strains from patients with diseases described as dengue fever showed no antigenic crossing with dengue 4 antiserum. Of 13 viruses recovered from patients clinically diagnosed as hemorrhagic fever (all from Thailand) and two viruses recovered from Thailand mosquitoes, all but two strains were significantly neutralized by dengue 4 MAF. A variety of cross reactions with dengue 1 and 3 MAF were also noted. These did not appear to occur in any consistent pattern.
### Table 2. Plaque Reduction Studies of Dengue 1 Strains*

| Virus strain       | Host passage | Orig. iden. | Disease response | Year and country of origin | Control PFU (mean) | % Plaque reduction vs. indicated antisera** | Ratio PFU† 35°C-39°C. |
|--------------------|--------------|-------------|------------------|----------------------------|--------------------|-------------------------------------------|---------------------|
| dengue 1 (Hawaii)  | smp 60+      | 1           | dengue           | 1944? Hawaii               | 31.7               | 68                                        | 2                   |
|                    |              |             |                  |                            | 2                  | 51                                        | 49                  | .73                  |
| dengue 2 (TR1751)  | smp 57+      | 2           | dengue           | 1952 Trinidad              | 82.3               | 0                                        | 8                   |
|                    |              |             |                  |                            | 86                 | 8                                        | 8                   | .25                  |
| dengue 3 (H-87)    | smp 24       | 3           | HF? (?)          | 1956 Philippines           | 19.3               | 3                                        | 16                  |
|                    |              |             |                  |                            | 60                 | 31                                       | .21                 |
| dengue 4 (H 241)   | smp 16       | 4           | HF (?)           | 1956 Philippines           | 26.8               | 2                                        | 16                  |
|                    |              |             |                  |                            | 10                 | 51                                        | 0                   |
| BKM 336            | smp 5        | 1           | isol. A. aegypti | 1963 Thailand              | 12.0               | 41                                       | 10                  |
|                    |              |             |                  |                            | 38                 | 10                                       | .38                 |
| BKM 427            | smp 5        | ?           | isol. A. aegypti | 1963 Thailand              | 14.3               | 51                                       | 17                  |
|                    |              |             |                  |                            | 27                 | 25                                       | 0                   |
| S-601              | smp 15       | 1           | dengue           | 1960 Singapore             | 17.3               | 45                                       | 4                   |
|                    |              |             |                  |                            | 0                  | 23                                       | .70                 |
| 62878              | smp ?        | 1           | dengue           | 1961 India                 | 22.0               | 56                                       | 14                  |
|                    |              |             |                  |                            | 18                 | 0                                        | 1.0                 |
| S-389              | smp 11       | 1           | dengue           | 1962 Singapore             | 22.8               | 51                                       | 4                   |
|                    |              |             |                  |                            | 2                  | 48                                       | .71                 |
| 1645-63            | smp 4        | 1           | dengue           | 1963 Thailand              | 36.0               | 55                                       | 10                  |
|                    |              |             |                  |                            | 48                 | 0                                        | .94                 |
| 13287              | smp 5        | 1           | dengue           | 1964 Thailand              | N.D.               |                                          | .76                 |
Table 2. (Continued)

| Virus strain | Host passage | Orig. iden. | Disease response | Year and country of origin | Control PFU (mean) | % Plaque reduction vs. indicated antiserum** | Ratio PFU† 35°C-39°C. |
|--------------|--------------|------------|------------------|---------------------------|--------------------|------------------------------------------|----------------------|
|              |              |            |                  |                           |                    | d1 (1:20) | d2 (1:80) | d3 (1:10) | d4 (1:10) |                  |                      |
| 13505        | smp 6        | 1          | dengue           | 1964 Thailand            | N.D.               |           |           |           |           | .67               |                      |
| 13284        | smp 4        | 1          | dengue           | 1964 Thailand            | 13.0               | 44         | 11        | 13        | 0         | .63               |                      |
| TH-Sman      | smp 16       | 1          | HF ?             | 1958 Thailand            | 22.8               | 58         | 20        | 38        | 3         | .60               |                      |
| 3157-62      | smp 4        | 1          | HF               | 1962 Thailand            | 14.3               | 65         | 13        | 37        | 0         | .89               |                      |
| 3890-62      | smp 4        | 1          | HF               | 1962 Thailand            | 5.0                | 87         | 3         | 57        | 23        | .88               |                      |
| 13703        | smp 5        | 1          | HF               | 1964 Thailand            | 22.5               | 44         | 13        | 0         | 15        | .94               |                      |
| 13825        | smp 5        | 1          | HF               | 1964 Thailand            | 6.7                | 53         | 10        | 47        | 0         | .83               |                      |
| 16007        | TC4†         | 1          | HF/shock         | 1964 Thailand            | 9.4                | 48         | 0         | 28        | 5         | N.D.              |                      |

* Virus strains incubated with identical lots and dilutions of prototype dengue 1-4 immune mouse ascitic fluids at 22-25°C for 60 minutes. Control virus incubated with 1:10 dilution acetone extracted normal mouse ascitic fluid.

** Numbers underlined indicate significant plaque reduction (see text).

† Indicates fractional reduction in plaque count after incubation of plaque bottles containing indicated viruses at 39°C for 7 days compared with replicate dilutions incubated at 35°C.

‡ 3 passages in BS-C-1 cells and 1 passage in LLC-MK2 cells.
| Virus strain | Host passage | Orig. iden. | Disease response | Year and country of origin | Control PFU (mean) | % Plaque reduction vs. indicated antisera** | Ratio† | 35°C-39°C. PFU |
|--------------|--------------|------------|------------------|---------------------------|-------------------|------------------------------------------|--------|----------------|
| BKM 58       | samp 5       | 2          | isol. *A. aegypti* | 1962 Thailand             | 70.8              | 6                                       | 94     | 21             | 28   | .06            |
| BKM 59       | samp 4       | 2          | isol. *A. aegypti* | 1962 Thailand             | 55.8              | 0                                       | 86     | 24             | 27   | .29            |
| 64412        | samp 17      | 2          | dengue           | 1963 India                | 31.8              | 0                                       | 88     | 38             | 0    | .48            |
| 23085        | samp ?       | 2          | dengue           | 1959 India                | 89.0              | 4                                       | 85     | 5              | 0    | .12            |
| S-843        | samp 40      | 2          | dengue           | 1960 Singapore            | 12.3              | 0                                       | 76     | 9              | 0    | .31            |
| S-378        | samp 4       | 2          | dengue           | 1962 Singapore            | 43.0              | 27                                      | 83     | 16             | 6    | .57            |
| TH-36        | samp 16      | 2          | HF               | 1958 Thailand             | 30.0              | —                                       | —      | —              | —    | —              |
| 913-62       | samp 4       | 2          | HF               | 1962 Thailand             | 26.2              | 0                                       | 82     | 6              | 0    | .22            |
| 3360-62      | samp 4       | 2          | HF               | 1962 Thailand             | 9.2               | 27                                      | 95     | 38             | 24   | .43            |
| 6141-62      | samp 5       | 2          | HF               | 1962 Thailand             | 18.7              | 38                                      | 72     | 37             | 40   | .21            |
Table 3. (Continued)

| Virus strain | Host passage | Orig. iden. | Disease response | Year and country of origin | Control PFU (mean) | % Plaque reduction vs. indicated antisera\(*\) | Ratio† PFU 35°C-39°C. |
|--------------|--------------|------------|-----------------|---------------------------|-------------------|---------------------------------|-----------------|
| 1537-63      | smp 5        | 2          | HF              | 1963 Thailand             | 33.7              | 0                               | 26              |
| 10312        | smp 4        | 2          | HF              | 1964 Thailand             | 12.7              | 0                               | 33              |
| 16770        | smp 5        | 2          | HF              | 1964 Thailand             | 33.2              | 14                              | 18              |
| 4942-62      | smp 4        | 2          | HF/shock        | 1962 Thailand             | 15.8              | 42                              | 51              |
| 4989-62      | smp 5        | 2          | HF/shock        | 1962 Thailand             | 133.8             | 0                               | 42              |
| 10937        | smp 4        | 2          | HF/shock        | 1964 Thailand             | 27.3              | 2                               | 50              |
| 16681        | TC 4$\ddagger$| 2          | HF/shock        | 1964 Thailand             | 11.0              | 7                               | 35              |

* Virus strains incubated with identical lots and dilutions of prototype dengue 1-4 immune mouse ascitic fluids at 22-25°C for 60 minutes. Control virus incubated with 1:10 dilution of acetone extracted normal mouse ascitic fluid.
** Numbers underlined indicate significant plaque reduction (see text).
† Fractional reduction in plaque count after incubation of plaque bottles containing indicated viruses at 39°C for 7 days compared with replicate dilutions incubated at 35°C.
$\ddagger$ 3 passages in BS-C-1 cells, 1 passage in LLC-MK2 cells.
### Table 4. Plaque Reduction Studies of Dengue 3 Strains*

| Virus strain | Host passage | Orig. iden. | Disease response | Year and country of origin | Control PFU (mean) | % Plaque reduction vs. indicated antisera** | Ratio† PFU | 35°C - 39°C.
|--------------|--------------|------------|-----------------|--------------------------|-------------------|-------------------------------------------|-----------|----------------|---|----------------|---|----------------|---|----------------|---|----------------|---|
| 1482-63      | smp 4        | 2          | dengue          | 1963 Thailand          | 42.7              | 45 44 67                                    | 13        | .55             |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |
| 1490-63      | smp 6        | 3          | dengue          | 1963 Thailand          | 4.3               | 10 51                                      | 1         | .24             |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |
| S-1033       | smp 11       | 3          | dengue          | 1963 Singapore         | 36.5              | 15 66                                      | 28        | N.D.            |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |
| PR 6         | smp 13       | 3?         | dengue          | 1963 Puerto Rico       | 7.7               | 22 50                                      | 37        | .90             |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |
| 4473-62      | smp 5        | 3          | HF              | 1962 Thailand          | 37.4              | 34 49                                      | 15        | .86             |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |
| 16562        | TC 4**       | 4          | HF              | 1964 Philippines       | 38.0              | 34 0                                       | 55 50     | N.D.            |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |                |   |

* Virus strains incubated with identical lots and dilutions of prototype dengue 1-4 immune mouse ascitic fluids at 22-25°C for 60 minutes. Control virus incubated with 1:10 dilution of acetone extracted normal mouse ascitic fluid.

** Numbers underlined indicate significant plaque reduction (see text).

† Fractional reduction in plaque count after incubation of plaque bottles containing indicated viruses at 39°C for 7 days compared with replicate dilutions incubated at 35°C.

‡ 3 passages in BS-C-1, 1 passage in LLC-MK2 cells.
Table 5. Plaque Reduction Studies of Dengue 4 Strains*

| Virus strain | Host passage | Orig. iden. | Year and response | Disease country of origin | Control PFU (mean) | % Plaque reduction vs. indicated antiserum** | Ratio† PFU 35°C-39°C. |
|--------------|--------------|-------------|-------------------|--------------------------|-------------------|---------------------------------------------|------------------------|
| 611337       | samp ?       | 4           | dengue            | India 1961               | 3.2               | 21                                          | 26                     | 42                      | 51                | .94               |
| S-132        | samp 11      | 4           | dengue            | Singapore 1961           | 13.5              | 3                                           | 2                      | 5                      | 63                | .66               |
| S-212        | samp 16      | 4           | dengue            | Singapore 1963           | 47.5              | 1                                           | 1                      | 1                      | 44                | .54               |
| 2543-63      | samp 5       | 4           | dengue            | Thailand 1964            | 5.5               | 35                                          | 28                     | 10                     | 50                | 1.0               |
| 13291        | samp 6       | ?           | dengue            | Thailand 1964            | 20.8              | 10                                          | 10                     | 13                     | 35                | .35               |
| 16572        | samp 6       | 4           | HF (?)            | Philippines 1964         | 15.5              | 14                                          | 14                     | 10                     | 36                | N.D.              |
| 16604        | samp 4       | 4           | HF (?)            | Philippines 1961         | 29.2              | 0                                           | 22                     | 17                     | 39                | .05               |

* Virus strains incubated with identical lots and dilutions of prototype dengue 1-4 immune mouse ascitic fluids at 22-25°C. for 60 minutes. Control virus incubated with 1:10 dilution of acetone extracted normal mouse ascitic fluid.

** Numbers underlined indicate significant plaque reduction (see text).

† Fractional reduction in plaque count after incubation of plaque bottles containing indicated viruses at 39°C. for 7 days compared with replicate dilutions incubated at 35°C.
As indicated in Table 4, four of six dengue type 3 strains were significantly neutralized by dengue 1 MAF. Neutralization by dengue 2 and 4 immune MAF were noted with two strains each.

Despite frequent neutralization of other viruses by dengue 4 MAF, of seven dengue 4 strains analyzed, only once was significant neutralization noted (by dengue 2 immune MAF). The strains tested were not always well neutralized by prototype dengue 4 MAF. While the homologous neutralizing activity of dengue 4 MAF was not high, it frequently neutralized an equal proportion of strains of other dengue virus types.

Further experience suggested that the patterns of neutralization observed in examined dengue virus strains might vary in different virus seed preparations. In experimental data described in Table 6, mouse brain seeds were made from washings of isolated plaques (only one plaque per bottle) from a number of terminal dilutions of dengue 2 strain 64412. These viruses were recovered from plaques even after two weeks incubation of infected cells at 25°C. Selected strains were inoculated intracerebrally into suckling mice. Brains were harvested and seeds tested against the standard dilutions of dengue 1-4 antisera. Marked differences in antigenic reaction patterns were noted in virus preparations obtained from different "clones." These data were reproducible.

In a second experiment, three passage levels of 4989-62 dengue 2 strain were tested against dengue 1-4 antisera. Again, each preparation showed different reaction patterns with dengue prototype immune MAF.

**Biologic properties of dengue viruses**

Among the virus strains examined there appeared to be a consistent relationship between plaque size, plaque definition, and host passage. Thus, nine days after inoculation of LLC-MK2 bottles, high mouse passage dengue 1 (Hawaii) produced indistinct plaques with irregular margins averaging 1-2 mm. in diameter. Dengue 3 (H-87) at 26th mouse passage frequently produced hazy, ill defined plaques, 2-3 mm. in diameter. Tiny "satellite" plaques were frequently seen. Low mouse passage dengue viruses, regardless of type, produced plaques with fairly sharp and regular margins. Plaques were mixtures of large and small variants, mean plaque size was 3-5 mm. Four dengue strains isolated in BS-C-1 cells at 4th and 5th tissue culture passage produced uniformly large plaques; with 16007 (dengue 1) and 1950-63 (dengue 2) and 16681 (dengue 2) strains, plaques were 4-5 mm., sharply margined with fairly regular edges. Strain 16562 (dengue 3), recovered in BS-C-1 cells from a patient with a clinical diagnosis of hemorrhagic fever in the Philippines, produced plaques which were 10-12 mm. in diameter. Of particular interest was strain 4328 S, a Philip-
Table 6. Plaque Reduction Studies on Different Subpopulations of 64412 and 4989-62 Dengue 2 Strains

| Virus strain | Host passage | Orig. iden. | Disease response | Year and country of origin | Control PFU (mean) | % Plaque reduction vs. indicated antiserum** | Ratio† PFU
|---------------|--------------|-------------|------------------|--------------------------|-------------------|---------------------------------------------|-----------------|
| 64412         | smp 17       | 2           | dengue           | 1963, India             | 31.8              | 0 88 38 0                                 | 0.48            |
|               | smp 18       | 2           |                   | 1963, India             | 83                | 10 88 21 35                              | 0.14            |
|               | " B          | 2           |                   | 1963, India             | 47                | 0 89 10 45                               | 0.18            |
|               | " D          | 2           |                   | 1963, India             | 36                | 0 88 0 12                                | 0.12            |
|               | " E          | 2           |                   | 1963, India             | 40                | 12 95 22 49                              | 0.18            |
|               | " F          | 2           |                   | 1963, India             | 72                | 51 88 67 22                              | 0.20            |
|               | " G          | 2           |                   | 1963, India             | 67                | 35 88 0 12                               | 0.19            |
|               | " H          | 2           |                   | 1963, India             | 16                | 0 77 59 21                              | 0.23            |
|               | " I          | 2           |                   | 1963, India             | 58                | 12 87 17 19                              | 0.22            |
|               | " J          | 2           |                   | 1963, India             | 73                | 18 88 10 22                              | 0.30            |
| 4989-62       | smp 5        | 2           | HF/shock          | 1962, Thailand          | 133.8             | 0 82 0 42                               | 0.09            |
|               | smp 9        | 2           |                   | 1962, Thailand          | 15.8              | 34 80 0 0                               | 0.24            |
|               | smp 17       | 2           |                   | 1962, Thailand          | 34.0              | 34 82 54 5                               | 0.26            |

* Single plaque derived populations of 64412 virus and varying mouse passage levels of 4989-62 viruses were incubated with identical lots and dilutions of prototype dengue 1-4 immune mouse ascitic fluids at 22-25°C for 60 minutes. Control viruses incubated with 1:10 dilution of acetone extracted normal mouse ascitic fluid.

** Numbers underlined indicate significant plaque reduction (see text).

† Fractional reduction in plaque count after incubation of plaque bottles containing indicated viruses at 39°C for 7 days compared with replicate dilutions incubated at 35°C.
pine dengue 4 virus recovered in suckling mice. This virus received five mouse passages, two passages in LLC-MK2 cells, then was plaqued. Plaques were predominantly large, 3-4 mm. in diameter. The virus was then inoculated subcutaneously into a dengue neutralizing antibody-free adult *Macaca mulatta*. During a 4-day period of viremia all plaques observed were very large, averaging 10-12 mm. in diameter.

Limited observations were made on the susceptibility of dengue viruses to nonspecific inhibitors. Dengue 2 (TR 1751 strain) at 70+ mouse passage was highly susceptible to acetone-extractable inhibitors found at 1:10-1:30 dilutions of normal monkey serum or plasma. Two tissue culture passaged dengue 2 strains (1950-63 and 16681) recovered from hemorrhagic fever patients in Thailand were not significantly neutralized by normal monkey serum or plasma at dilutions of 1:10 or higher. Although a large number of low mouse passage dengue viruses were found to be inhibited by normal mouse ascitic fluid at dilution of 1:10 or higher, a systematic study of this phenomenon has not been made.

Dengue virus temperature sensitivity was studied with a large number of dengue strains. Results are summarized in Tables 2-6. Twelve bottles each were inoculated with serial tenfold dilutions of dengue virus. One half of inoculated bottles at each dilution, chosen randomly, were incubated at 39°C for seven days and the other half at 35°C. Differences in plaque counts observed were always in the direction of lower counts in bottles incubated at higher temperature. Results are expressed as percentile plaque reduction in bottles incubated at 39°C compared with 35°C. Dengue strains showing .5 or greater plaque reduction at 39°C arbitrarily are defined as temperature sensitive. Eleven of 13 dengue 1 strains, three of four dengue 3 strains, four of six dengue 4 strains, but only four of 18 dengue 2 strains, were "temperature sensitive." It was of interest that three of four temperature sensitive dengue 2 strains were recovered from patients with a diagnosis of hemorrhagic fever. Several "clones" of a dengue 2 virus and different mouse passage levels of another dengue 2 virus were studied (Table 6). Temperature sensitivity characteristics were retained by all subpopulations.

**DISCUSSION**

Antibody obtained from mice receiving multiple separate antigenic stimuli with the same virus when tested against strains of dengue viruses in a plaque reduction neutralization test (PRNT) did not separate dengue viruses into clearly distinct types with little cross reaction as have other methods. Cross reactions between dengue viruses and heterologous antisera were minimal when antibody to low mouse passage dengue strains
prepared by two injections of mice was tested by complement-fixation against prototype dengue antigens, when dengue viruses were tested by PRNT against monkey antisera, or when dengue viruses were identified by micro precipitin in agar or immunoelectrophoresis. The use of multiple antigenic stimuli for production of antibody in our study appears to have increased the amount of heterotypic reaction. Heterotypic neutralization, in some instances, was stronger than that in the homologous system.

Strains within the major dengue serotype groups differed markedly in their reactions with heterotypic dengue antisera. In demonstrating antigenic heterogeneity of dengue strains our results are in accord with those of Ibrahim and Hammon who showed that dengue 1 or dengue 2 viruses and antisera when tested reciprocally with TH-Sman or TH-36 viruses and antisera contained distinguishing precipitin reaction both by Ochterlony and immunoelectrophoresis methods. The variation in antigenic patterns observed in our studies can be explained in a number of different ways. A simple hypothesis is that dengue virions contain “major” and “minor” antigens. Minor antigens might be either less reactive, distributed in less accessible sites or present at lower concentrations on virions than “major” antigens. Dengue viruses of one type contain “minor” antigens that are similar or identical to “major” antigens on other dengue virus types.

Between dengue 1 and 3 viruses and antisera, reciprocal cross reactions were observed with high frequency. This suggests a similarity of 1 and 3 “major” antigens. There was a marked inhomogeneity in neutralization of dengue 2 strains by antibodies to dengue 1, 3 and 4 viruses. By our hypothesis this could be explained by variation in the occurrence of “minor” antigens on dengue virions. A one way cross between dengue 4 antisera and dengue 2 viruses was observed frequently.

The original purpose of this study was to examine for variations between dengue virus strains of the same serotype that could be correlated with geographic origin or disease response in the host. Insufficient virus strains from various areas or diseased hosts were studied to attain this goal. From the heterogeneity of patterns observed, it is unlikely that consistent antigenic differences would have been detected had a larger collection been examined. Russell, et al. also examined dengue strains from Thailand, Vietnam, Pakistan, and Puerto Rico and failed to find major antigenic differences between dengue strains of the same virus type occurring in different areas. An observation made that may be worthy of further study is that dengue 2 viruses neutralized by dengue 4 antibody were recovered only from hemorrhagic fever patients and not from patients with a dengue fever response during infection.
The heterogeneity of neutralization reactions obtained with "clones" of 64412 strain of dengue 2 virus and several different mouse passages of 4989-62 dengue 2 virus strongly suggests that the antigenic pattern for any particular lot of virus seed may not be representative of the antigenic composition of the original virus population that caused human infection. It is known that when human adapted dengue viruses are passaged in laboratory hosts, biological properties change. For example, Sabin has shown that several strains of dengue viruses rapidly lost pathogenicity for humans following intracerebral passage of virus in mice.9 We have presented data indicating that high mouse passage dengue viruses produce small and ill defined plaques in monkey kidney cells while other strains of the same type of virus passaged only in primate cells produced uniformly larger plaques. A dengue 4 mouse passage strain isolated in mice on subsequent passage into a monkey changed from a medium to a large plaque variant. The work of Henderson, et al.9 suggests that antigenic variation during host passage may be a general phenomenon with arboviruses.

Although the "original" antigenic pattern of the dengue viruses that we have studied apparently cannot be known, it is not improbable that the antigenic heterogeneity observed most frequently in dengue 2, 3 and 1 viruses also occurs in natural virus populations. It is interesting to speculate on the possible creation of dengue antigenic heterogeneity in nature by a sharing of genetic material between dengue viruses during the simultaneous dengue infections that almost certainly occur in highly endemic areas or, contrarily, by the selection that may take place when a dengue virus infection occurs in an individual with pre-existing group B arbovirus antibody. Some important system of antigen sharing must exist to account for the cross protection induced in humans by dengue infection.9 Shared antigens may also result in sensitization phenomena. This has received fuller discussion elsewhere.9

Once it is determined whether antigenic patterns stabilize in populations derived from a single viable virus or whether antigenic patterns change in some regular fashion on passage in laboratory hosts, it should be possible to use antigenic studies of dengue viruses as a rational method for study of cross protection and cross sensitization phenomena. Since the described system of antigen analysis requires very small quantities of reagents, it may be possible to use viremic human serum itself for antigenic comparisons.

SUMMARY

Forty-nine strains of dengue viruses, types 1-4, of diverse geographic origin recovered from hosts with disease of varying severity were studied.
Antigenic properties of dengue

HALSTEAD, SIMASTHIER

for plaque reduction produced by identical lots of dengue type 1-4 mouse immune ascitic fluids (MAF). Patterns of neutralization varied markedly among dengue strains. Of the two types (dengue 1 and 2) with the largest number of strains available for analysis, dengue 2 viruses were more frequently neutralized singly or in combination by heterologous dengue immune MAF. Dengue 4 viruses were rarely neutralized by heterologous antibody. Reciprocal cross neutralization between strains of dengue 1 and dengue 3 viruses and immune MAF was frequent. Dengue 4 immune MAF significantly neutralized 11/18 dengue 2 strains; this pattern was absent in 4 dengue strains recovered from classic dengue fever cases.

Because the pattern of neutralization by heterologous antibody varied in virus populations derived from a single strain and in the same virus strain studied at different mouse passage levels, it was unknown whether the observed neutralization results represented the antigenic pattern of the original virus population in the diseased host. No conclusions could be reached concerning correlation of specific antigenic pattern and type of response of host to dengue infection.

The plaque forming property of dengue 2 viruses was stable at 39°C. more frequently than it was in other dengue types. Dengue strains at high mouse passage produced smaller plaques than viruses of the same type at lower mouse passage or when recovered in tissue culture. Reversion from small to large plaque variants was observed by single passage of a suckling mouse brain adapted dengue 4 strain into a sub-human primate.

ACKNOWLEDGMENTS

The technical assistance of Voranat Ritchie and Diane Doss is gratefully acknowledged.

REFERENCES

1. Carey, D. E., Myers, R. M., and Rodrigues, F. M.: Two episodes of dengue fever, caused by types 4 and 1 viruses, in an individual previously immunized against yellow fever. Amer. J. trop. Med. Hyg., 1965, 14, 448-450.
2. Deller, J. J., Jr. and Russell, P. K.: An analysis of fevers of unknown origin in American soldiers in Vietnam. Ann. intern. Med., 1967, 66, 1129-1143.
3. Russell, P. K. and Nisalak, A.: Dengue virus identification by the plaque reduction neutralization test. J. Immunol., 1967, 99, 291-296.
4. Halstead, S. B., Udomsakdi, S., Singharaj, P., and Nisalak, A.: Dengue and chikungunya virus infection in man in Thailand, 1962-1964. III. Clinical, epidemiologic, and virologic observations on disease in non-indigenous white persons. Amer. J. trop. Med. Hyg. 1969, 18, 984-996.
5. Neff, J. M., Morris, L., Gonzalez-Alcover, R., Coleman, P. H., Lyss, S. B., and Negron, H.: Dengue fever in a Puerto Rican community. Amer. J. Epid., 1967, 86, 162-184.
6. Johnson, K. M., Halstead, S. B., and Cohen, S. N.: Hemorrhagic fevers of Southeast Asia and South America. A comparative appraisal. Prog. med. Virol., 1967, 9, 105-158.
7. Goldsmith, R. S., Wong, H. B., Paul, F. M., Chan, K. Y., Loh, T. F., and Chan, Y. C.: Hemorrhagic fever in Singapore. A changing syndrome. Lancet, 1965, 1, 333-336.
8. Basaca-Sevilla, V. and Halstead, S. B.: Recent virological studies of hemorrhagic fever and other arthropod-borne virus infections in the Philippines. J. trop. Med. Hyg., 1966, 69, 203-208.

9. Halstead, S. B., Udomsakdi, S., Simasthien, P., Singharaj, P., Sukhavachana, P., and Nisalak, A.: Observations related to pathogenesis of dengue hemorrhagic fever. I. Experience with classification of dengue viruses. Yale J. Biol. Med., this issue.

10. Nimmannitya, S., Halstead, S. B., Cohen, S. N., and Margiotta, M. R.: Dengue and chikungunya virus infection in man in Thailand, 1962-1964. I. Observations on hospitalized patients with hemorrhagic fever. Amer. J. trop. Med. Hyg., 1969, 18, 954-971.

11. Chan, Y. C.: Rapid typing of dengue viruses by the micro-precipitin agar-gel diffusion technique. Nature, 1965, 206, 116-117.

12. Sartorelli, A. C., Fischer, D. S., and Downs, W. G.: Use of Sarcoma 180/TG to prepare hyperimmune ascitic fluid in the mouse. J. Immunol., 1966, 96, 676-682.

13. Detre, K.: Biometric aspects of virus assays. Dr. P. H. Thesis. Yale University School of Medicine, New Haven, Connecticut, 1967.

14. Lorenz, R. J. and Zoeth, B.: An estimation of the overlap bias in plaque assay. Virology, 1966, 28, 379-385.

15. Cuadrado, R. R. and Casals, J.: Differentiation of arboviruses by immunoelectrophoresis. J. Immunol., 1967, 98, 314-320.

16. Ibrahim, A. N. and Hammon, W. McD.: Application of immuno-diffusion methods to the antigenic analysis of dengue viruses. I. Precipitin-in-gel double diffusion in two dimensions. J. Immunol., 1968, 100, 86-92.

17. Ibrahim, A. N. and Hammon, W. McD.: Application of immuno-diffusion methods to the antigenic analysis of dengue viruses. II. Immunoelectrophoresis. J. Immunol., 1968, 100, 93-98.

18. Sabin, A. B.: “Dengue” in Viral and Rickettsial Diseases of Man, 3rd ed. Edited by T. M. Rivers and F. L. Horsfall, Jr. Philadelphia, Lippincott, 1959, p. 361.

19. Henderson, J. R., Shah, H. H., and Wallis, R. C.: Antigenic variants of arboviruses. I. The host as a determinant in the evolution of strain variants. Virology, 1965, 26, 326-332.

20. Halstead, S. B.: Observations related to pathogenesis of dengue hemorrhagic fever. VI. Hypotheses and discussion. Yale J. Biol. Med., this issue.