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# NEUTRALIZATION CHARACTERISTICS OF DENGUE VIRUSES ISOLATED IN MOSQUITO CELL CULTURE AND SUCKLING MOUSE BRAIN FROM A CASE OF LABORATORY DENGUE INFECTION[1]

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SUMMARY One of the authors (Y. O.) of this paper was accidentally infected in the laboratory with dengue type 4 virus, isolated in Thailand in 1978 using the C6/36 clone of Singh's *Aedes albopictus* cells (SA). Two strains of the virus were isolated from Y. O.'s serum on the first day of illness using SA and suckling mouse brain (SMB). The SA-isolate and the infecting virus were neutralized with high efficiency by Y. O.'s convalescent sera, but the SMB-isolate was neutralized much less efficiently by the same sera. When the hosts of the isolates were exchanged (SA to SMB, and *vice versa*), the neutralization efficiencies were reversed.

For analysis of this phenomenon, prototype dengue type 4 virus exclusively passed in SMB and the same type of virus passaged 10 times in SA were compared with the initial SA- and SMB-isolates in neutralization tests with sera from patients with dengue hemorrhagic fever (DHF) obtained in Thailand. The two strains of the prototype virus and the SA-isolate had similar high reactivities, but the SMB-isolate had low reactivity with sera of the DHF patients.

## INTRODUCTION

In 1978, four strains of dengue (DEN) viruses were isolated from DHF patients in Thailand by use of the C6/36 clone (Igarashi, 1978) of Singh's *Aedes albopictus* cells (Singh, 1967). All the strains isolated and passaged in C6/36 cells (SA) gave ambiguous reactions in neutralization (N) tests with anti-DEN 1–4 prototype rabbit sera, but after several passages in suckling mouse brain (SMB), three of them showed DEN-2 specific reaction patterns in the N tests. The fourth strain (No. 124), which did not show a clear type-specific pattern even after SMB passages, was identified as DEN type 4 virus by complement fixation (CF) tests with anti-DEN 1–4 prototype mouse sera and antigens prepared from infected SMB and infected SA culture fluids

1 Part of this work was reported at the first ICMR Seminar held in Kobe in November 1980.

(Fukunaga et al., 1980). Because of this difference in neutralization of strain No. 124, this strain was studied further.

During these studies, Y. O. was accidentally infected with one of the clones. In this infection, the clone of infecting virus was clearly known and the virus was re-isolated from Y. O. in two kinds of hosts, SA and SMB. We compared the neutralization characteristics of these SA- and SMB-isolates from the same original source, because we have had problems in serotype-identifications of SA-isolates by N tests, as mentioned above.

As described in this paper, we found differences in the neutralization characteristics of the virus infecting Y. O. and SA- and SMB-isolates from Y. O..

## MATERIALS AND METHODS

### 1. *Cells*

1) For virus isolation

The C6/36 clone of *Aedes albopictus* cells (SA) was cultured in 2 ounce bottles or in tubes at 30 C in cell growth medium consisting of 10% fetal calf serum in Eagle's medium (Eagle, 1959) supplemented with 0.2 m*M* of non-essential amino acids.

2) For virus infectivity assay and neutralization (N) tests

BHK-21 cells were grown in growth medium consisting of 10% fetal calf serum in Eagle's medium at 37 C in Lab-Tek 8-chamber slides (Miles, Ill., U.S.A.) in a $CO_2$-incubator for one day before use.

### 2. *Anti-dengue virus (DEN) sera*

1) Reference anti-DEN rabbit sera

Prototype viruses of DEN 1 (Hawaii), DEN 2 (New Guinea B), DEN 3 (H-87) and DEN 4 (H-241) passaged in SMB in this laboratory were used as immunogens. Rabbits were given two intramuscular injections of the virus in Freund's complete adjuvant with an interval of one month between injections. The materials injected were partially purified virus samples prepared from infected SMB by protamine sulfate clarification and ultracentrifugation (Smith et al., 1970). Rabbits were bled 10 days after the last injection.

2) Anti-DEN mouse sera

Prototype viruses were used for immunizations. Adult mice were injected with 0.5 ml of the supernatants of 10% homogenates of infected SMB. Injections were given intraperitoneally 5 times at weekly intervals.

### 3. *Neutralization (N) test*

Neutralizing antibody titers were determined by 50% focus reduction tests with the peroxidase-anti-peroxidase (PAP) staining method (Okuno et al., 1977; Okuno et al., 1978).

### 4. *Complement fixation (CF) test*

CF tests were performed by the microtiter method (Sever, 1962) with antigens prepared from infected SMB by sucrose-acetone extraction, and anti-DEN 1–4 mouse sera as antibodies, with two units of complement of fresh guinea pig sera.

## RESULTS

### 1. *Laboratory infection and virus isolation from the case*

The original strain of the infecting virus in the laboratory infection was isolated from a Thai DHF patient in the C6/36 clone (SA) in 1978. As shown in Fig. 1, the strain (No. 124) had been passaged 9 times in SA and then 18 clones had been obtained.

1) Laboratory infection

When Y. O. was distributing the C15 clone (C15/124: SA-9), with an infectivity titer of $1.9\times10^6$ FFU/ml on BHK-21 cells, into ampoules, he happened to prick his finger with a syringe-needle containing the virus. The prick was so slight that no one thought of his having been infected at that time (May 2, 1980). In the evening of May 8, he developed malaise and fever (38.0 C), and other typical symptoms of dengue fever followed. Samples of blood were taken from Y. O. daily after the onset of the disease starting from May 9, which is designated as day 1 of illness in this paper, until day 14 and the series of serum samples obtained were used for studies.

2) Virus isolation

Virus isolation from Y. O. serum was attempted using SA cells and SMB inoculations.

a) Isolation in SA cells: Ten fold diluted

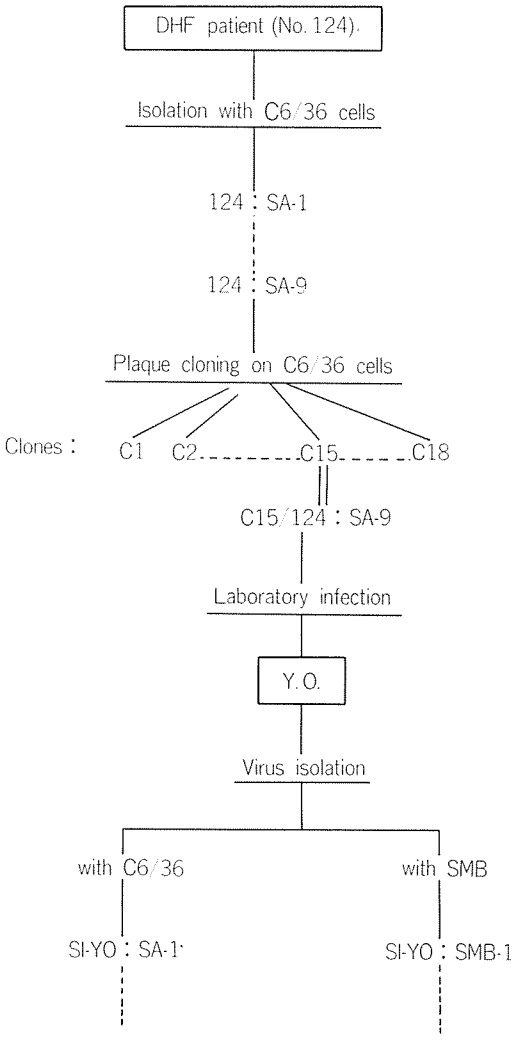


FIGURE 1. History of the infecting virus and the isolates.

serum from Y. O. on day 1 of illness was inoculated onto SA grown in 2 ounce bottles. The cells started to show cytopathic changes (syncytium formation) after 3 days and about 50% of the cells showed cytopathic changes after 4 days. Then the culture fluids were harvested and stored in ampoules at −70 C (SI-YO: SA-1 in Fig. 1).

b) Isolation in SMB: Six litters of suckling mice were injected intracerebrally with

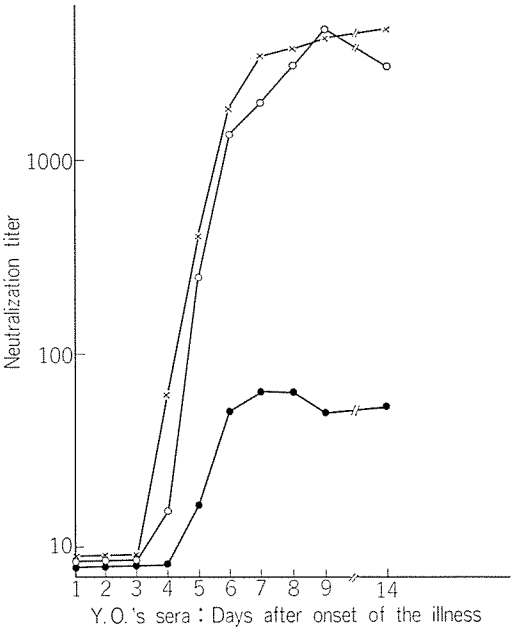


FIGURE 2. Neutralization of the infecting virus, the SA-isolate and the SMB-isolate with Y. O.'s sera. Symbols: ×, the infecting virus (C15/124: SA-9); ○, the SA-isolate (SI-YO: SA-1); ●, the SMB-isolate (SI-YO: SMB-1).

0.025 ml of diluted Y. O. serum obtained on day 1 of illness. Some of the suckling mice in three litters developed slight neurological symptoms 12 days after the injection but none in the other three litters showed any symptoms. The brains of the suckling mice with symptoms were harvested and stored at −70 C (SI-YO: SMB-1 in Fig. 1).

The SA-isolate (SI-YO: SA-1) and the SMB-isolate (SI-YO: SMB-1) showed infectivity titers of $1.2\times10^7$ and $5.5\times10^5$ FFU/ml, respectively, on BHK 21 cells.

### 2. *Neutralization of the viruses by Y. O.'s sera*

The infecting virus, the SA- isolate and the SMB-isolate were neutralized with Y. O.'s sera obtained on day 1 to 14 of the illness. The results are shown in Fig. 2. The N titer curve of the SA-isolate coincided well with

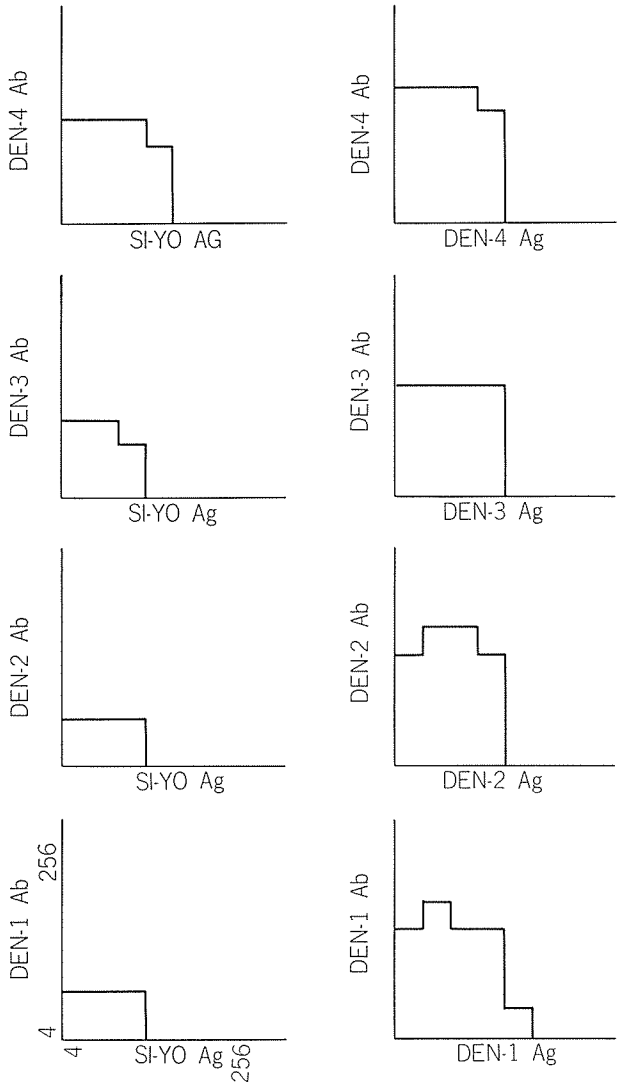


FIGURE 3. Complement fixation tests on the SMB-isolate (SI-YO: SMB-7) with dengue type 1–4 antisera.

the curve for the infecting virus and both curves reached a plateau (1: 3000–1: 4000) on day 7 of the illness. The SMB-isolate gave a different curve and the plateau of the curve was much lower (<1: 100) than those of the infecting virus and the SA-isolate.

As these two types of curves appeared so different, the serotype of the three virus strains were confirmed by N tests using reference anit-DEN 1–4 rabbit sera. Judging from the N titers shown in Table 1, all three strains seemed to react most with anti-DEN 4 serum, though the titers observed were low for serotype determination when compared with the N titers of the reference anti-sera to homologous prototype viruses.

For confirmation of the serotype, further typing was carried out by CF tests using an antigen prepared from the SMB-isolate passaged in SMB (SI-YO: SMB-7) and anti-

TABLE 1. *Neutralization tests on the infecting virus and the isolates with reference anti-dengue rabbit sera*

| Strain | Anti-dengue reference sera: | | | |
|---|---|---|---|---|
| | DEN-1 | DEN-2 | DEN-3 | DEN-4 |
| C15/124: SA-9 | <20 | <20 | 30 | 58 |
| SI-Y. O: SA-1 | <20 | <20 | 20 | 54 |
| SI-Y. O: SMB-1 | <20 | <20 | <20 | 23 |
| Homologous vlrus | 780 | 1600 | 1500 | 880 |

TABLE 2. *Comparison of the first and the second isolates by neutralization tests*

| Strain | Anti-DEN sera | | | | |
|---|---|---|---|---|---|
| | DEN-1 | DEN-2 | DEN-3 | DEN-4 | YO-serum (9 th day) |
| SI-YO: SA-1 | <20 | <20 | 20 | 54 | 3700 |
| SI-YO-(2): SA-1 | <20 | 60 | 52 | 84 | 4000 |
| SI-YO: SMB-1 | <20 | <20 | <20 | 23 | 140 |
| SI-YO-(2): SMB-1 | <20 | <20 | <20 | 30 | 51 |

DEN 1–4 mouse sera. The checker board CF titration patterns shown in Fig. 3 indicate that the isolate was a DEN 4 virus. Furthermore, DEN 4 virus-specific IgM antibody was detected by both HI and N tests in a fraction obtained by sucrose gradient centrifugation of Y. O.'s serum obtained on day 9.

3. *Reproducibility of the difference observed in N tests*

As the SA- and SMB-isolates gave such different N antibody titers against Y. O.'s sera, the reproducibility of the phenomenon was confirmed by repeated virus isolation. Two virus strains were also isolated in the second isolation experiment from the same source using SA and SMB. Table 2 shows a comparison of results with the first and second isolates by N tests on reference anti-DEN 1–4 sera and on Y. O.'s serum on day 9 of illness. The second SA-isolate (SI-YO-(2): SA-1) and the second SMB-isolate (SI-YO-(2): SMB-1) gave similar results to those in the first experiment; namely, they showed high reactivity of the SA-isolate and low reactivity of the SMB-isolate.

TABLE 3. *Comparison of initial isolates with their low descendants by neutralization tests*

| Strain | Anti-DEN sera | | | | |
|---|---|---|---|---|---|
| | DEN-1 | DEN-2 | DEN-3 | DEN-4 | YO-serum (9 th day) |
| SI-YO: SA-1 | <20 | <20 | 20 | 54 | 3700 |
| SI-YO: SA-5 | <20 | 62 | 58 | 110 | 2500 |
| SI-YO: SMB-1 | <20 | <20 | <20 | 23 | 140 |
| SI-YO: SMB-7 | <20 | <20 | 21 | 42 | 130 |

4. *Dependence of the difference observed in N tests on the host*

As the difference observed in N tests seemed to depend on the host from which the isolate was obtained, we carried out the following experiments on the effect of the host.

1) Successive passages of the isolates in their initial hosts

The SA-isolate (SI-YO: SA-1) was passaged 5 times in SA cells and the SMB-isolate (SI-YO: SMB-1) was passaged 7 times in SMB. The resulting viruses, SI-YO: SA-5 and SI-YO: SMB-7, were neutralized with reference anti-DEN 1–4 sera and Y. O.'s serum obtained on day 9. These N titers were compared with those of the same sera against the viruses from the first passages of the isolates (SI-YO: SA-1 and SI-YO: SMB-1). Table 3 shows that there were no significant differences between the neutralization

TABLE 4. *Reactivity of isolated strains passaged in exchanged hosts in neutralization tests with Y.O.'s serum of day 14 of illness*

| SA-strain: | | SMB-strain: | |
|---|---|---|---|
| Passage level | Neutralization titer | Passage level | Neutralization titer |
| SI-YO: SA-1: | | SI-YO: SMB-1: | |
| SMB-0 | 2800 | SA-0 | 100 |
| SMB-1 | 150 | SA-1 | 2600 |
| SMB-2 | 130 | SA-2 | 2600 |
| SMB-3 | 100 | SA-3 | 2900 |
| SMB-4 | 88 | SA-4 | 4300 |
| SMB-5 | 32 | SA-5 | 4600 |
| SMB-6 | 94 | SA-6 | 5000 |
| SMB-7 | 60 | SA-7 | 4100 |
| SMB-8 | 88 | SA-8 | 5600 |
| SMB-9 | 82 | SA-9 | 4000 |
| SMB-10 | 94 | SA-10 | 5100 |

characteristics of the virus after one and several passages. Moreover, the difference in results of N tests on the SA-isolate and SMB-isolate was still seen after these passages.

2) Effect of host exchange

The SA-isolate was passaged in SMB and the SMB-isolate in SA cells up to 10th passage level. When the SA-isolate was first transferred to SMB, its infectivity titer decreased to less than 1/100 of the titer in preceding passage. Conversely when the SMB-isolate was passaged in SA cells its titer increased more than 10 times. As shown in Fig. 4, the infectivity level of the former remained rather low in following passages, while that of the latter fluctuated in following passages, but after 9 to 10 passages the titers of both strains reached similar levels.

Viruses of the first to 10th passage of both strains were neutralized with Y. O.'s serum obtained on day 14 of illness. The results of the neutralization experiments in Table 4 show that there were marked changes in the N titer when one isolate was transferred to another host: that is, when SI-YO: SA-1 (passage level: SMB-0) was transferred to SMB, the N titer of the virus in the next generation, SI-YO: SA-1: SMB-1 (passage level: SMB-1)

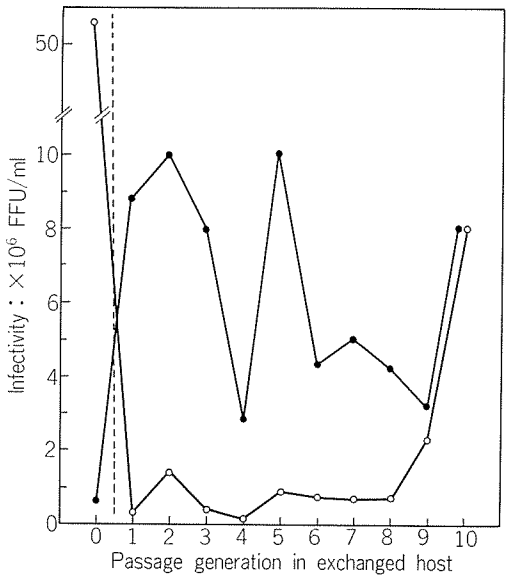


FIGURE 4. Infectivity titers of isolates passaged in exchanged hosts. Symbols: ○, SA-isolate in SMB; ●, SMB-isolate in SA.

TABLE 5. *Neutralization titers of reference anti-dengue rabbit sera against SA cell passaged dengue prototype viruses and their original strains*

| Viruses | anti-DEN(SMB) rabbit sera | | | |
|---|---|---|---|---|
| | DEN-1 | DEN-2 | DEN-3 | DEN-4 |
| DEN-1 (SMB) | 1300 | 150 | 160 | 13 |
| DEN-1 (SA-10) | 1900 | — | — | — |
| DEN-2 (SMB) | 15 | 950 | 12 | 16 |
| DEN-2 (SA-9) | — | 790 | — | — |
| DEN-3 (SMB) | 38 | 100 | 960 | 33 |
| DEN-3 (SA-10) | — | — | 1600 | — |
| DEN-4 (SMB) | 10 | 24 | 12 | 400 |
| DEN-4 (SA-10) | — | — | — | 750 |

was decreased to that of SI-YO: SMB-1 (passage level: SA-0); similarly, when SI-YO: SMB-1 was transferred to SA, the N titer of the virus in the next generation, SI-YO: SMB-1: SA-1 (passage level: SA-1), was increased to the same order as that of SI-YO: SA-1 (passage level: SMB-0). It is noteworthy that the N titers of these serially passaged strains in each series remained steady after these marked changes, regardless of changes in infectivity, or changes in growth capacity caused by exchange of hosts.

The results suggest that the difference between SA- and SMB-isolate observed in neutralization tests is a reflection of the host in which the viruses multiplied, and not related to genetically stable characteristics of the isolates.

5. *Comparison of neutralization characteristics of SMB-passaged and SA-adapted prototype viruses, the SA-isolate, and SMB-isolate*

Prototype DEN 1–4 viruses have been maintained by SMB-passage in this laboratory (DEN 1–4 (SMB)). Each of these SMB-passaged prototype viruses was passaged 10 times in SA cells to obtain SA-adapted prototype strains (DEN 1–4 (SA-10)). The neutralization titers of these 4 pairs of prototype viruses were determined with reference anti-DEN 1–4 rabbit sera, which had been prepared by immunization with the SMB-passaged prototype viruses. As shown in Table 5, there was no significant difference between titers of SMB-passaged virus and its SA-adapted strains with any of the prototype viruses.

The neutralization characteristics of the SMB-passaged and SA-adapted prototype DEN 4 viruses were compared with those of SMB- and SA-isolates with DHF patient sera obtained in Thailand. The results are shown in Table 6. The sera gave similar N titers against the SMB-passaged and SA-adapted strains of the prototype virus. However, the N titers against SMB- and SA-isolates with convalescent sera from the patients were very different: the N titers with the SA-isolate were very higher, as in the case with Y. O.'s sera shown in Fig. 2.

TABLE 6. *Neutralization of SMB-passaged prototype dengue type 4 virus, its SA-adapted strain, and the SA-isolate (SI-YO: SA-1) and the SMB-isolate (SI-YO: SMB-1) from Y.O.'s serum, with DHF patient sera*

| Patient sera | Prototype DEN-4 virus | | Isolates from Y.O. | |
|---|---|---|---|---|
| | SMB-passaged | SA-adapted | SI-YO: SMB-1 | SI-YO: SA-1 |
| No. 118 A[a] | 130 | 220 | <20 | 80 |
| C[b] | 2300 | 2400 | <100 | 1800 |
| No. 124 A | <20 | <20 | <20 | <20 |
| C | 5900 | 4200 | <100 | 2700 |

[a] A: acute phase serum.
[b] C: convalescence phase serum.

## DISCUSSION

Two isolates in SA cells and SMB, respectively, were obtained from one of the authors (Y. O.) when he was infected with a known clone of DEN 4 virus in the laboratory. These isolates showed marked differences in neutralization characteristics: the SA-isolate was neutralized as efficiently as the infecting virus, but the SMB-isolate was not neutralized efficiently by convalescent sera from Y. O., from whom the virus had been isolated. Possible explanations for this phenomenon are as follows:

1) The virus population that can grow in one host is selected by the host and the observed difference reflects the antigenic difference between the selected virus populations.

2) Both SA and SMB permit growth of the same virus population, but in SMB, dengue virus that is less neurotropic than for instance Japanese encephalitis virus produces a considerable amount of defective interfering (DI) particle-like antigen(s) that consumes N antibodies, resulting in lower expression of neutralization antibody titers.

3) The virus population grown in SMB is readily modified by so-called host-controled variation, which makes the virus less reactive with neutralizing antibodies induced by other kinds of host-controled variants.

In the host-exchange experiments, marked changes in the N titers were observed, as shown in Table 4. These results suggest that the difference in the neutralization characteristics of the isolates from different hosts does not reflect genetic characteristics. Thus the first explanation seems unlikely. In preliminary experiments, we found that the SMB-isolate became more reactive in N tests with Y. O.'s serum when it had been partially purified by sucrose density gradient centrifugation. This finding seems to support the second explanation. From the data obtained so far, the second and/or third explanation seems most likely. However, the possibility that the characteristics of the isolates were changed by mutations occurring immediately after the host-exchanges cannot be excluded, as it has been shown that the effects of host passage of plant viruses are due to mutations (Yarwood, 1979).

In the N tests on the patient's sera the SMB-passaged prototype virus showed similar behavior to the SA-adapted prototype virus and the SA-isolates, whereas the SMB-isolate behaved differently. Though this finding seems to be rather contradictory to the explanation in the preceding paragraph, it could be explained as follows. The prototype virus has been passaged in SMB many times, and this may have resulted in its complete adaptation with regard to growth characteristics. On the other hand, a newly isolated virus had not yet become fully adapted to growth in SMB. Actually, suppressed growth, which might be caused by auto-interference, is frequently observed when a DEN virus strain newly isolated in SMB is passaged in SMB at higher concentration. Therefore, the difference observed in N tests between the SMB-passaged prototype virus and the SMB-isolate may due to the different contents of viral products, such as DI particles, related to differences in degrees of adaptation of the virus to SMB.

Some workers have discussed the disadvantages of use of SMB for DEN virus isolation as compared with use of laboratory-reared mosquitoes or mosquito cell cultures (Rosen and Gubler, 1974; Race et al., 1978; Tesh, 1978). Their interests, however, were mostly focused on the efficiency and technical convenience of isolation of virus from natural sources. The remarkable difference between results with SA- and SMB-isolates in N tests demonstrated in this paper indicates another advantage of use of mosquito cells for DEN virus isolation: more information on the neutralizing antibodies in dengue infections may be obtained by use of mosquito cell isolates (SA-isolates) than by use of SMB-isolates.

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