# ISOLATION OF THREE STRAINS OF TYPE 1 DENGUE VIRUS FROM A LOCAL OUTBREAK OF THE DISEASE IN MALAYA*

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(With 3 Figures in the Text)

On clinical grounds, dengue has been believed to be endemic in Malaya for many years, but there was no proof that the dengue viruses occurred in Malaya because the clinical syndrome can be mimicked by other infections such as, for example, West Nile fever (Bernkopf, Levine & Nerson, 1953), and leptospirosis (Daniels & Grennan, 1943), the latter being common in Malaya (I.M.R. 1955*a*, *b*, 1956). The virus isolations reported here prove that some, at least, of the dengue-like fevers of Malaya are caused by type 1 dengue (dengue-1) virus.

Smithburn (1954) tested a small number of human sera from around Kuala Lumpur and found neutralizing antibodies to dengue-2 virus in a small proportion (1/18) of persons aged under 15 years of age, and in 44% (36/81) of adults. Sabin (1955) reported testing twenty human sera from Malaya for haemagglutinin-inhibiting antibodies to both types of dengue virus; he found a very high incidence of each, but as cross-reactions between them, and with a number of other viruses such as Japanese encephalitis (JE) virus, are very marked, the meaning of these findings is uncertain.

Recent surveys of 250 sera from five ecologically different rural communities in Malaya (I.M.R. 1956) have shown, broadly speaking, that neutralizing antibody to dengue-1 virus is present in 25% of people below the age of 10 years, in 50% aged 11–20 years, and in nearly 100% of persons over 30 years of age. Some sera have antibody only to dengue-1 virus; others only to dengue-2 virus, or to JE virus. Many sera have neutralizing antibodies to two and some to all three viruses. Although there are marked cross-reactions between the two types of dengue virus, it seems likely that dengue-2 virus also occurs in Malaya, but this can be proved only by its isolation.

Japanese encephalitis virus was first isolated from a human case in Malaya by Paterson *et al.* (1952), and subsequent studies in Malaya have shown infections with this virus to be widespread, although encephalitis is apparently uncommon. The occurrence of JE, and probably other arthropod-borne viruses, in Malaya increases the difficulties of the serological diagnosis of dengue because of the ability of other group B viruses (Casals & Brown, 1954) to cause cross-reactions and, probably, anamnestic antibody responses. Neutralizing antibody studies by Smithburn (1954) indicated the probable occurrence of Uganda S, Semliki Forest,

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Zika, and Ntaya viruses in Malaya. Pond *et al.* (1955) studied the incidence of neutralizing antibodies in a population of rubber estate workers near Kuala Lumpur, and found that 90 % of them had antibodies to JE and Ntaya viruses by the age of 13 years, and 35–75 % of them antibodies to Zika, Ilheus and Semliki Forest viruses by the age of 33 years. Of these viruses, Ilheus, Ntaya, Uganda S and Zika belong to Casals's group B.

## ISOLATION OF THE VIRUSES

### (1) *Methods and difficulties*

The original isolations of dengue viruses of both types 1 and 2 were made by Sabin and his co-workers (Sabin & Schlesinger, 1945; Sabin, 1952). They used acute-phase samples of blood from cases of dengue in American personnel in the Pacific area. The sera received were inoculated into human volunteers in the United States, and it was from them that the isolations were made. The earliest success was obtained with human serum concentrated by high-speed centrifugation and then inoculated intracerebrally into 10–12-day Swiss mice. 'Dilute brown non-agouti' (DBA) mice were found to be most susceptible. Sabin (1956) points out that at least one variety of white mice (PRI) is said to be genetically resistant to viruses of the dengue—yellow-fever—West-Nile—Japanese encephalitis group. He recommends the use of the Swiss mice 2–4 days old. Hotta (1952) achieved mouse adaptation of a strain of dengue-1 virus in 6–7 g. mice but did not observe paralysis until after two blind passages. Meiklejohn appears to have been the first to suggest the use of suckling mice for propagation of dengue viruses (Schlesinger & Frankel, 1952). It is because there have been so few successful isolations of dengue viruses and these with difficulty that the isolations reported here are described in detail. Further progress in investigation of the epidemiology of dengue and especially of its probable jungle reservoir (I.M.R. 1956) is going to depend very largely on finding improved isolation methods for these viruses. It is hoped that by a detailed study of isolations so far made, some clue may be found to a more reliable technique.

The mice used in the isolations described here were a mixture of at least three strains of Swiss mice obtained at different times from the Haffkine Institute in Bombay, from Australia, and from the United States. Later work, including the identification of the virus, was carried out using DDM mice which originated in Ehrlich's laboratory in Germany and were obtained from Japan through the 406th Medical General Laboratory, U.S. Army, Tokyo. This strain now meets all the requirements of virus research in the Institute.

### (2) *Isolation of three strains of virus*

A local outbreak of a dengue-like disease was reported in a Kuala Lumpur girls' school hostel in March 1954. The clinical and epidemiological aspects of this outbreak have been reported elsewhere (Smith, 1956). There were about forty cases, thirty-one of which were studied in detail. This paper reports successful isolation of virus in all of three attempts made from these patients.

*Chia.* Blood was obtained from a 17-year-old Chinese girl when she had felt unwell for only a few hours, her temperature being only 99·4° F. As soon as a small amount of serum had separated (about an hour after collection) it was inoculated in 0·01 ml. quantities intracerebrally (i.c.), and 0·03 ml. quantities intraperitoneally (i.p.), into the same family of 1-day-old suckling mice (Fig. 1). Two of this litter of six died, presumably from non-specific causes, on the 2nd day.

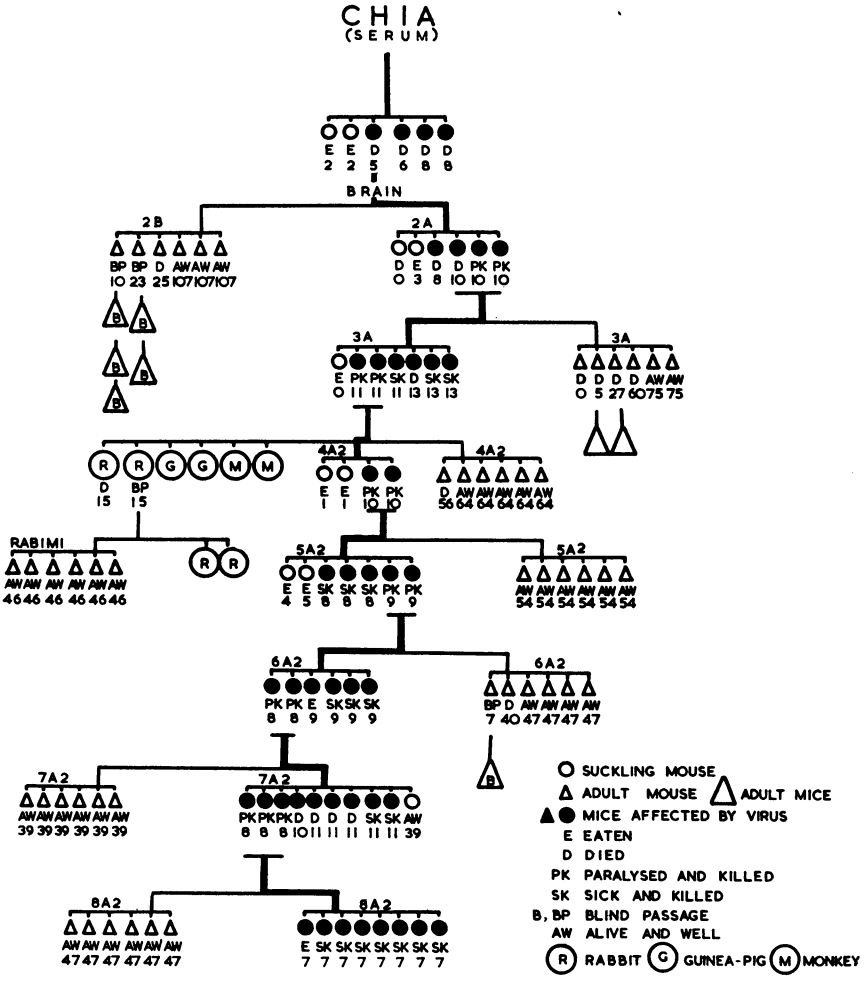


Fig. 1

A third died on the 5th day and a 10% suspension of its brain was prepared in 10% inactivated rabbit serum in saline (RSS). This suspension, after brief centrifugation, was inoculated i.c. and i.p. into another family of sucklings (2A) and a group of adult mice (2B). Again, there were two early deaths in the sucklings, followed by one on the 8th day. The mother mouse died on the 9th day, so that a suckling found dead on the 10th day, together with its two surviving littermates who appeared sick, were killed and their brains inoculated i.c. into both adult and suckling mice (3A). Of the 3rd passage sucklings, two were paralysed and

one sick on the 11th day, and the remaining three were sick or dead on the 13th day. Of the adult mice inoculated, one died on the 5th day and one on the 27th day. The further course of the establishment of Chia in suckling mice is shown in Fig. 1. At the 10th passage in sucklings, the titre ($LD_{50}$) in sucklings was $10^{-6 \cdot 6}$.

*Schleman.* During the same outbreak, blood was obtained from an adult American on the 2nd day of the disease. The blood was defibrinated by shaking with glass beads and inoculated i.c. and i.p. within about an hour into a family of sucklings aged 1 day (1A in Fig. 2) and another aged 6 days (1B). In each, half the litter was inoculated with neat defibrinated blood and the other half with

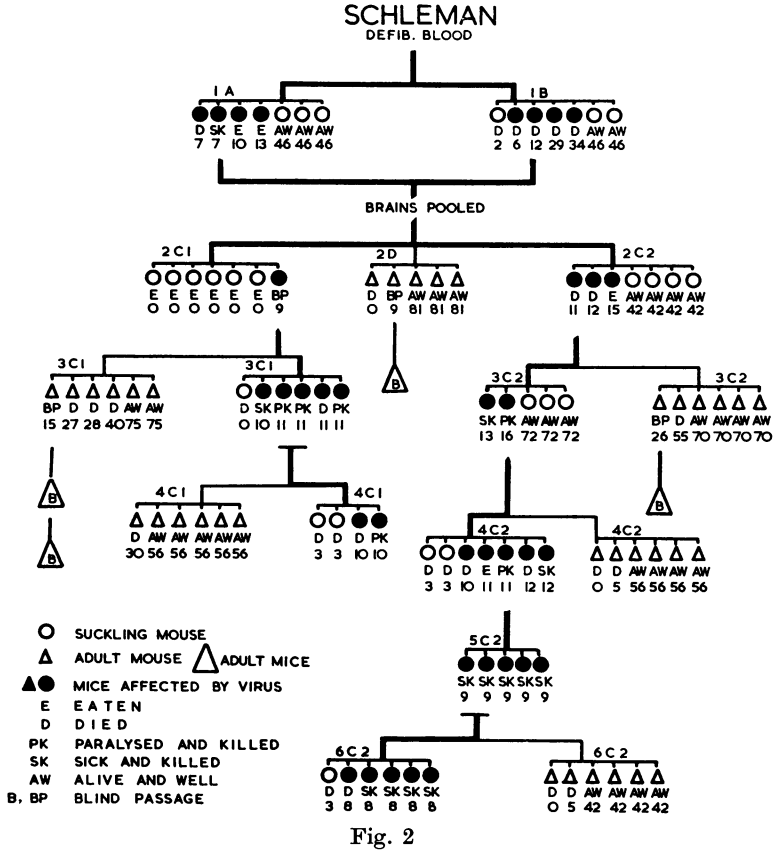


Fig. 2

a 1/1000 dilution of it in RSS. Only one of the mice inoculated with the 1/1000 dilution (3 on right of 1A and 1B) succumbed and that on the 34th day. Of the 1-day-old sucklings inoculated with neat material there was one dead and one sick on the 7th day. The latter was used for passage. Of the other two mice in this group inoculated with neat material, one was eaten on the 10th and the other on the 13th day. Of the 6-day-old sucklings inoculated with neat material, deaths occurred on the 6th, 12th and 29th days after inoculation. Brains from these two groups of sucklings were pooled and inoculated into two groups of sucklings (2C2, 2C1) and one of adults (2D). Fig. 2 shows that at the 5th passage (5C2) suckling mice were uniformly sick on the 9th day.

*Smith.* Defibrinated blood was obtained as described above from another American adult in the same outbreak on the 1st day of the disease (temperature 102·6° F.). It was inoculated i.c. and i.p. into a family of 3-day-old sucklings. One was eaten on the 5th day, and one was paralysed and killed on the 10th day. Its brain was inoculated into sucklings and adults (2A, 2B, Fig. 3) and its carcass into sucklings (2D). Another suckling died on the 16th day and its brain was also passaged (2C). At the second passage (2A; using 1-day-old mice) deaths occurred on the 11th, 12th and 14th days, while one mouse with severe ataxia on the 13th day was killed. In the 3rd suckling passage all the mice were paralysed or died between the 9th and 12th days. The 2C line of adaptation gave uniform paralysis of sucklings on the 8th day at the 7th passage (7C1).

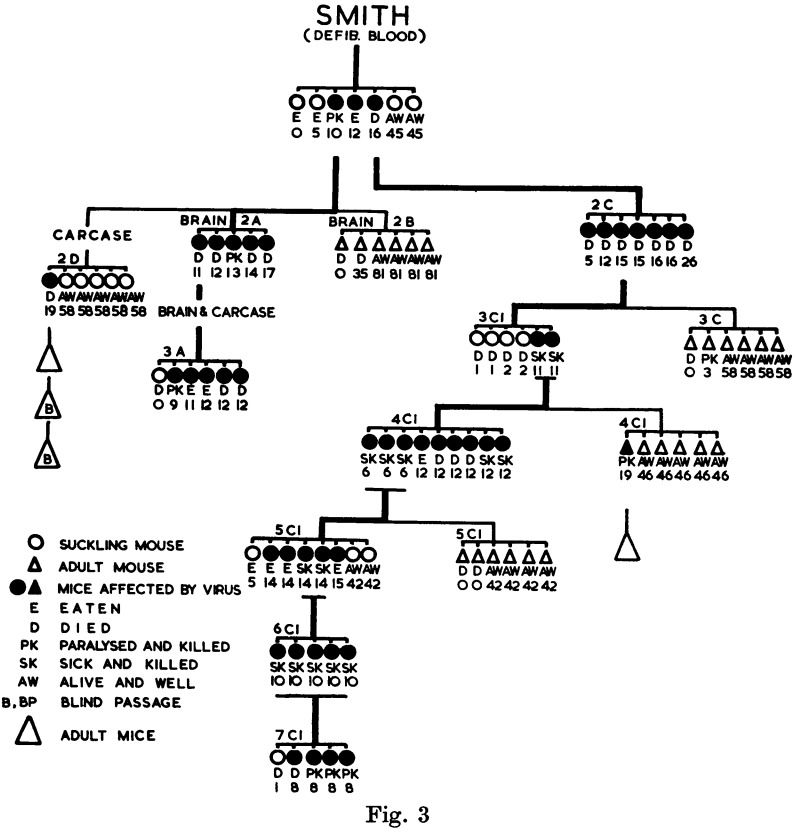


Fig. 3

## CHARACTERIZATION OF CHIA VIRUS

### (1) *Adaptation of Chia to adult mice*

Sabin & Schlesinger's (1945) original Hawaii strain of dengue-1 virus became fully adapted to adult mice after about fifteen passages. That is, the virus uniformly affected all the mice inoculated with lowest dilution (largest amount) of virus. It is not clear at what level Hotta (1952) achieved full adaptation of his strain, but it was apparently incompletely adapted at the 50th passage. Meiklejohn, England

& Lennette (1952) achieved adaptation of the New Guinea 'C' and 'D' strains of dengue-2 virus to suckling mice after four blind and two other passages. They failed to adapt strain 'C' directly to adult mice after nine blind passages. Schlesinger & Frankel (1952) adapted the New Guinea 'B' strain of type 2 virus to suckling mice after four passages in DBA mice and two in suckling Swiss mice. Nineteen further passages in sucklings were necessary to adapt the virus to adult mice.

Chia was adapted to adult mice by serial passage of 10% brain suspensions through suckling mice; as a check on the degree of adaptation, six adult mice were inoculated with the 10% suspension at each passage. Schlesinger & Frankel (1952) found that during adaptation, the mortality ratio is paradoxically lower in mice inoculated with the largest amounts of virus than with higher dilutions such as for example 1/1000. The same was found to apply to Chia. The phenomenon is probably due to interference with the adult-pathogenic virus by preponderant amounts of suckling-pathogenic virus which does not affect adult mice. It was therefore possible to assess the degree of adaptation of Chia by inoculation of adults with 10% brain only, full adaptation being indicated by sustained 100% mortality. Adaptation was completed at the 33rd passage in sucklings at which the titre in adults ($LD_{50}$) was $10^{-5.5}$. The process of adaptation has been further studied and will be discussed in detail in a later paper. Dengue seed virus must always be prepared from suckling mouse brain, as repeated passage in adult mice, even with fully adult-adapted virus, leads to rapid diminution in titre.

### (2) *Demonstration that Chia was the cause of the illness in the patient from whom it was isolated*

It should first be stated that no dengue viruses were imported to Malaya until after these isolations had been effected. Subsequently dengue-1 (Hawaii) and dengue-2 (New Guinea 'C') viruses were obtained at their 115th and 20th mouse passages respectively from the Walter Reed Army Institute of Research, Washington, which had received them from Dr Sabin.

(*a*) A neutralization test was carried out in suckling mice using 10th passage Chia virus and sera obtained from the patient Chia on the 1st day of her illness and 66 days later. Tenfold dilutions of virus were mixed with equal volumes of undiluted serum, using normal rabbit serum which had been heated at 56° C. for 30 min. (NRS) as a control. The mixtures were incubated at 4° C. for 4 hr. (Meiklejohn, England & Lennette, 1952), then each was inoculated into a family of suckling mice i.c. The mice were observed for death or paralysis for 21 days. For calculation of mortality ratios, mice paralysed on the 21st day were regarded as having died, and mice dying before the 5th day were disregarded. The difficulties in the use of suckling mice for titration procedures due to cannibalism, traumatic deaths, etc., were recently discussed by Beswick (1955). The $LD_{50}$ titre of the virus after incubation with each serum was determined graphically by plotting the probits (Finney, 1952) of the percentage mortality of each group of mice against the corresponding log virus-dilution and drawing the straight line of best fit. The log dilution at which this line cuts the probit value 5 is taken as the $LD_{50}$ titre. This

computation method is used in preference to the more conventional Reed-Muench method (Reed & Muench, 1938), which gives roughly the same answer; because the former is applicable to irregularly sized groups of mice, it does not employ cumulative mortality totals which can readily be abused, and a good estimate of the standard error and confidence limits of the probit $LD_{50}$ can easily be computed by the method described by Miller & Tainter (1944).

The log neutralizing index of a serum sample is the difference between the log $LD_{50}$ of the virus after incubation with it, and the log $LD_{50}$ of the virus after similar incubation with a control (non-immune serum); in other words, the neutralizing index of a serum sample is the number of times it is more potent in neutralizing power than a control serum. For the purposes of diagnosis, however, the neutralizing index of a serum sample obtained as early as possible in the disease is compared with that of a sample obtained in convalescence. It is the magnitude of the increase in antibody content of the patient's serum during the

Table 1. *Increase in neutralizing antibody to 10th passage Chia virus during the course of the illness of the patient Chia (using suckling mice)*

| Serum | Log titre | Neutralizing index | Rise in titre |
|---|---|---|---|
| NRS | 6·6 | — | — |
| 1st day | 6·3 | 2 | 1 |
| 67th day | 4·8 | 63 | 31 |

Table 2. *HI antibody titres of the patient Chia at intervals after onset of the disease against 16 units of Chia and the two types of dengue antigen*

| Day after onset | Inhibition titre* against | | | Number-fold rises in titre | | |
|---|---|---|---|---|---|---|
| | Chia | Dengue-1 | Dengue-2 | Chia | Dengue-1 | Dengue-2 |
| 1 | <10 | <10 | <10 | 1 | 1 | 1 |
| 19 | 30,720 | 30,720 | 10,240 | 6,144 | 6,144 | 2,048 |
| 67 | 7,680 | 7,680 | 3,840 | 1,536 | 1,536 | 768 |
| 209 | 960 | 640 | 320 | 192 | 128 | 64 |

* In this paper HI titres are expressed as the reciprocal of the highest serum dilution (before addition of antigen and cells) giving partial inhibition.

course of the illness which permits diagnosis of an infection with the virus used in the test.

The results of the neutralization test are shown in Table 1, where it can be seen that there was a 30-fold rise in neutralizing antibody to Chia during the course of the illness in the corresponding patient. This indicates that infection with this or a closely related virus coincided with the occurrence of the illness.

(*b*) The sera of the patient Chia, obtained at intervals during and after her illness, were tested in the haemagglutinin-inhibition (HI) test by the method of Sabin (1956). Sixteen units of antigens prepared from Chia, dengue-1 (Hawaii) and dengue-2 were used. The results are shown in Table 2 and demonstrate that large increases in HI antibody to all three viruses occurred and that there was no difference in the HI response to Chia and Hawaii.

These two experiments provide evidence that the virus isolated was the cause of the illness in the patient from whom it came.

(3) *Identification of Chia*

(*a*) Adult-mouse-adapted Chia (35th mouse passage) was set up in a neutralization test against an immune serum pool prepared against Chia, and against immune sera for a number of other viruses. The other immune sera (marked* in Table 3) were very kindly given to me by Dr William L. Pond of the Walter Reed Army Institute of Research in Washington. The Chia immune serum was prepared as follows: two guinea-pigs, which had been inoculated i.c. and i.p. respectively with Chia passage 3A (Fig. 1) and had shown no signs of sickness, were later given two i.p. doses each of 1 ml. of a 10% suspension of Chia suckling mouse passage 7A2 at a 7-day interval and were bled out 19 days after the last dose. Like all other sera used in this study, they were stored frozen at −20° C.

For this test, the virus was diluted in fresh normal guinea-pig serum (NGPS) in order to supply the heat-labile factor of normal serum (Sabin, 1950). Tenfold virus

Table 3. *Neutralization of Chia virus by antisera to a number of serologically related viruses*

| Sera | Log titre after incubation | Neutralizing index | Neutralizing index against homologous virus |
|---|---|---|---|
| NRS | 4·2 } Mean 4·4 | — | — |
| NGPS | 4·6 } | | |
| Chia, guinea-pig | 1·9 | 320 | — |
| Dengue 1* | 1·7 | 500 | — |
| Dengue 2* | 4·2 | 2 | — |
| JE* | 3·8 | 4 | 50,000 |
| Murray Valley encephalitis* | 3·5 | 8 | 4,000 |
| West Nile* | 3·7 | 5 | 3,160 |
| St Louis encephalitis* | 3·6 | 6 | 250 |

* Sera supplied by Dr W. L. Pond.

dilutions were mixed with equal volumes of the sera to be tested. Each mixture was then incubated at 37° C. for exactly 2 hr. and inoculated in 0·03 ml. quantities i.c. into eight 6–10 g. mice. This is the standard neutralization test for the dengue viruses in my laboratory except that groups of six mice are usually employed. The mice were observed for 21 days and the results computed as described above. Table 3 shows that significant neutralization of Chia virus was obtained only with homologous antiserum and with dengue-1 antiserum.

(*b*) Using the same method with groups of six mice, serial specimens of serum from the patient Chia were tested against both types of dengue virus with the results shown in Table 4. It can be seen that the greatest rise in titre was against dengue-1 virus and that it was maintained for 6 months, while the lesser rise in titre against dengue-2 virus had fallen to an insignificant level by the 209th day. It is evident that while cross-reactions between homologous and heterologous viruses were marked 3 weeks after onset, the neutralizing antibody against heterologous virus diminished fairly rapidly thereafter.

(*c*) Chia virus of the 5th mouse passage was freeze-dried and sent to Dr Pond in Washington. He tested it in a neutralization test using suckling mice against

immune sera for both types of dengue virus, JE virus and phlebotomus virus. His results are shown in Table 5. It is clear that the closest relation is again with dengue-1. Pond also tested a Chia mouse immune-serum pool against haemagglutinins of dengue-1 and dengue-2 virus. The inhibition titre was 160 against dengue-1 and only 40 against dengue-2 virus.

Table 4. *Neutralizing antibody in serial sera from Chia patient against homologous and heterologous dengue virus*

| Sera (days after onset of disease) | Dengue-1 | | Dengue-2 | |
|---|---|---|---|---|
| | Titre | Number-fold rise | Titre | Number-fold rise |
| 1 | 4·5 | 1 | 4·8 | 1 |
| 19 | 1·0 | 3,160 | 1·5 | 1,995 |
| 67 | 1·0 | 3,160 | 2·6 | 158 |
| 209 | 1·4 | 1,260 | 3·6 | 16 |

Table 5. *Neutralization of Chia virus by specific antisera*

| Immune monkey serum | Log titre of virus serum mixtures | Neutralizing index |
|---|---|---|
| Control | 5·8 | — |
| JE | 4·9 | 8 |
| Dengue 1 | 1·7 | 12,590 |
| Dengue 2 | 2·3 | 3,160 |
| Phlebotomus | 5·4 | 3 |

Table 6. pH *and temperature requirements of freshly prepared Chia and type* 1 *dengue haemagglutinins*

| Virus | Sedimentation temperature (° C.) | pH range | Optimum pH : titre |
|---|---|---|---|
| Chia | 25 | 6·3–6·9 | 6·6 : > 5,120 |
| Faber (type 1) | 25 | 6·3–6·9 | 6·5 : > 5,120 |
| Chia | 4 | 6·3–7·1 | 6·5–6·7 : > 5,120 |
| Faber (type 1) | 4 | 6·3–7·1 | 6·5–6·9 : > 5,120 |

(*d*) Freeze-dried 5th passage Chia was also sent to Dr B. H. Sweet at Cincinnati, Ohio. He prepared haemagglutinins by the KCl-borate procedure (Sabin, 1956) and tested them, when freshly prepared, for haemagglutinating activity under different conditions of pH and sedimentation temperature. His results are shown in Table 6. Chia resembled type 1 dengue in causing haemagglutination at room temperature and over a wide pH range, while freshly prepared type 2 haemagglutinin produced no reaction at room temperature, reaction only at a narrow pH range (6·5–6·7) at 4° C., and a maximum titre of only 640.

From the above four experiments it can be concluded that Chia virus is a strain of dengue-1 virus.

## PROBABLE IDENTIFICATION OF SMITH AND SCHLEMAN AS DENGUE-1 VIRUSES

Smith and Schleman viruses have not been adapted to adult mice so that no attempt has been made to set them up against a variety of antisera as was done with Chia.

(*a*) Haemagglutinins were prepared from the two strains and tested when freshly prepared in the pH and temperature conditions characteristic of dengue-1 and Chia. It is seen in Table 7 that titres greater than 640 were obtained, and that the reaction was as strong at room temperature as at 4° C. (see above).

(*b*) Serial serum specimens from the patient Chia, together with a human serum known to have an HI antibody titre of 160 against type 2 dengue and 10 or < 10 against dengue-1, and a bovine serum which had a titre of 160 against JE antigen and 10 against dengue-1, were tested against Smith, Schleman and Hawaii antigens with the results shown in Table 8. On this basis, the three antigens can be seen to be indistinguishable.

Table 7. *Properties of freshly prepared Smith and Schleman haemagglutinins*

| Virus | Suckling mouse passage | pH of reaction | Sedimentation temperature (° C.) | Titre |
|---|---|---|---|---|
| Smith | 8 | 6·5 | 27 | 960 |
| Schleman | 10 | 6·5 | 27 | 1,920 |
| Smith | 8 | 6·5 | 4 | 1,920 |
| Schleman | 10 | 6·5 | 4 | 2,560 |

Table 8. *Comparison of the inhibition titres of sera against Smith, Schleman and Hawaii type* 1 *dengue antigens*

| Serum | Inhibition titre against | | | Inhibition titre against the homologous antigen |
|---|---|---|---|---|
| | Smith | Schleman | Hawaii | |
| Chia, 1st day | < 10 | < 10 | < 10 | < 10 |
| Chia, 9th day | 10,240 | 10,240 | 7,680 | 15,360 |
| Chia, 67th day | 2,560 | 2,560 | 2,560 | 3,840 |
| Chia, 209th day | 160 | 160 | 120 | 480 |
| Dengue-2 serum | < 10 | < 10 | < 10 | 160 |
| JE serum | 10 | 10 | 10 | 160 |

Table 9. *Rises in neutralizing antibody content of serial sera from the patients Smith and Schleman against types* 1 *and* 2 *dengue viruses*

| Patient | Days after onset | Rises in titre against dengue-1 | Rises in titre against dengue-2 |
|---|---|---|---|
| Smith | 1 | 1 | 1 |
| | 19 | 25 | 2 |
| Schleman | 2 | 1 | 1 |
| | 16 | 160 | 25 |
| | 96 | 100 | 25 |

(*c*) Serial specimens of serum from the patients Smith and Schleman were

tested in a standard neutralization test against dengue-1 and dengue-2 viruses with the increases in neutralizing index shown in Table 9.

These three experiments strongly suggest that Smith and Schleman are also strains of dengue-1 virus and the fact that the patients they were isolated from were undoubtedly infected in the same outbreak as Chia (Smith, 1956) confirms this view.

## DISCUSSION

The successful isolation of dengue-1 virus in all of three attempts as reported here is perhaps surprising in comparison with the difficulties experienced by others. These attempts were, however, made during an outbreak, at which time one is able to recognize probable cases early, when the viraemia is most marked, and this greatly increases the chances of success. The use of suckling mice as recommended by Sabin (1956) undoubtedly greatly facilitates mouse adaptation, but it may be that, as with adults, different strains vary considerably in their susceptibility, and it would be worth while to attempt isolation from the same material in parallel in a variety of different strains of suckling mice, in the hope of finding some very highly susceptible strain. This would have to be done where a large range of mouse strains is available.

Further progress in the study of the epidemiology of dengue and especially of its transmission and probable jungle reservoir will be greatly dependent on the development of a more sensitive technique for isolation of the virus. Cortisone-treated adults were used in this study, without success, and, although they may be worthy of further trial, they are unlikely to be more susceptible than sucklings of the same strain. The inoculation of material suspected of containing virus into mice, and subsequent challenge of them with dengue virus to discover whether they have been immunized (Sabin, 1956), has been tried only in a small way in this laboratory, and there is no information about what degree of cross-reaction may be met with. This technique and the problem of analysis of results when the dose of challenge virus varies from test to test, as is inevitable, are very much worth study.

Other questions such as whether to dilute suspected material so as to diminish possible interference by non-mouse-pathogenic virus particles; whether, when and how often blind passage should be carried out; and whether a tissue culture can be found which will respond to infection with dengue virus with cytopathic changes, are all possibly profitable lines of approach.

## SUMMARY

1. Three strains of virus were isolated in suckling mice from the blood of three patients suffering from dengue during a local outbreak of the disease.

2. One strain was adapted to adult mice and identified as a dengue virus of type 1. The identification was confirmed by workers in America.

3. Evidence is presented that the other two strains of virus are similar to the one fully identified.

4. Attention is drawn to the need for detailed work to find a more sensitive method of isolation for dengue viruses.

I am very much indebted to Dr W. L. Pond, of the Walter Reed Army Institute for Research (now stationed at the Institute for Medical Research, Kuala Lumpur, with the U.S. Army Medical Research Unit), for his help with the identification of the virus, and for providing the necessary immune sera; and also to Dr B. H. Sweet, of the Children's Hospital Research Foundation, Cincinnati, for studying the haemagglutinins.

## REFERENCES

BERNKOPF, H., LEVINE, S. & NERSON, R. (1953). Isolation of West Nile virus in Israel. *J. infect. Dis.* **93**, 207–18.

BESWICK, T. S. L. (1955). The titration of viruses in baby mice. *J. Hyg., Camb.*, **53**, 339–56.

CASALS, J. & BROWN, L. V. (1954). Haemagglutination with arthropod-borne viruses. *J. exp. Med.* **99**, 429–49.

DANIELS, W. B. & GRENNAN, H. A. (1943). Pretibial fever: an obscure disease. *J. Amer. med. Ass.* **122**, 361–5.

FINNEY, D. J. (1952). *Statistical Method in Biological Assay.* London: Griffin.

HOTTA, S. (1952). Experimental studies on dengue: I. Isolation, identification and modification of the virus. *J. infect. Dis.* **90**, 1–9.

I.M.R. (1955*a*). Leptospirosis. *Rep. Inst. med. Res., Malaya, for* 1954, p. 67.

I.M.R. (1955*b*). Pyrexias of unknown origin. *Rep. Inst. med. Res., Malaya, for* 1954, p. 96.

I.M.R. (1956). *Rep. Inst. med. Res., Malaya, for* 1955 (in the Press).

MEIKLEJOHN, G., ENGLAND, B. & LENNETTE, E. H. (1952). Propagation of dengue virus strains in unweaned mice. *Amer. J. trop. Med. Hyg.* **1**, 51–8.

MILLER, L. C. & TAINTER, M. L. (1944). Estimation of the $ED_{50}$ and its error by means of logarithmic-probit graph paper. *Proc. Soc. exp. Biol., N.Y.*, **57**, 261–4.

PATERSON, P. Y., LEY, H. L., JR., WISSEMAN, C. L., JR., POND, W. L., SMADEL, J. E., DIERCKS, F. H., HETHERINGTON, H. D. G., SNEATH, P. H. A., WITHERTON, D. H. & LANCASTER, W. E. (1952). Japanese encephalitis in Malaya. I. Isolation of virus and serologic evidence of human and equine infections. *Amer. J. Hyg.* **56**, 320–30.

POND, W. L., RUSS, S. B., MCCRUMB, F. R., DINGLE, J. H., LEY, H. L. & SCHMIDT, J. R. (1955). Antibodies to arthropod-borne viral diseases in sera from residents of Southeast Asia. *Bact. Proc.* pp. 66–7.

REED, L. J. & MUENCH, H. (1938). A simple method of estimating 50 per cent endpoints. *Amer. J. Hyg.* **27**, 493–7.

SABIN, A. B. (1950). The dengue group of viruses and its family relationships. *Bact. Rev.* **14**, 225–32.

SABIN, A. B. (1952). Research on dengue during World War II. *Amer. J. trop. Med. Hyg.* **1**, 30–50.

SABIN, A. B. (1955). Recent advances in our knowledge of dengue and sandfly fever. *Amer. J. trop. Med. Hyg.* **4**, 198–207.

SABIN, A. B. (1956). Chapter on Dengue in *Diagnostic procedures for virus and rickettsial diseases*, 2nd ed. (in the Press). New York: American Public Health Association.

SABIN, A. B. & SCHLESINGER, R. W. (1945). Production of immunity to dengue with virus modified by propagation in mice. *Science*, **101**, 640–2.

SCHLESINGER, R. W. & FRANKEL, J. W. (1952). Adaptation of the 'New Guinea B' strain of dengue virus to suckling and to adult Swiss mice. *Amer. J. trop. Med. Hyg.* **1**, 66–77.

SMITH, C. E. G. (1956). A localized outbreak of dengue fever in Kuala Lumpur: epidemiological and clinical aspects. *Med. J. Malaya*, **10**, 289–303.

SMITHBURN, K. C. (1954). Neutralizing antibodies against arthropod-borne viruses in the sera of long-time residents of Malaya and Borneo. *Amer. J. Hyg.* **59**, 157–63.

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