INFECTION AND IMMUNITY, Jan. 1980, p. 175-180
0019-9567/80/01-0175/06$02.00/0

Vol. 27, No. 1

Dengue-2 Vaccine: Preparation from a Small-Plaque Virus Clone

KENNETH H. ECKELS,* VENTON R. HARRISON, PETER L. SUMMERS, AND PHILIP K. RUSSELL

Department of Hazardous Microorganisms, Walter Reed Army Institute of Research, Washington, D.C. 20012

The S-1 clone of dengue type 2 virus was used for the preparation of a live-attenuated vaccine after passage in DBS-FRhL-2 cell culture. The vaccine virus had a relatively higher replicative capacity at superoptimal temperatures than its precursor virus, S-1, passaged in primary green monkey kidney cells (S-1 PGMK). There was also a tendency for the S-1 vaccine virus to exhibit leakiness at increased temperatures. Another in vitro marker, replication in monkey peripheral blood leukocytes, indicated less host restriction for the S-1 vaccine in comparative assays with S-1 PGMK virus. Mouse virulence appeared to remain stable on passage in DBS-FRhL-2 cells, whereas monkey immunogenicity decreased. Cautious trials of the dengue type 2 S-1 vaccine in humans are indicated.

Dengue and dengue hemorrhagic fever occur in endemic or epidemic form throughout tropical areas of the world. In the Americas, dengue types 2 and 3 (DEN-2 and DEN-3) and, most recently, DEN-1, have been associated with widespread epidemics of dengue fever (2, 10, 18). In Southeast Asia, dengue hemorrhagic fever is a major public health problem. Widespread epidemics causing significant mortality in the pediatric age group have increased markedly in the last decade. Vector control is the only preventive measure available, but to date this has been ineffective in many areas.

The feasibility of producing live-attenuated vaccines for DEN-1 (7, 12) and DEN-2 (13) viruses has been investigated. These early vaccines used virus modified by passage in mouse brain. Modification was judged by the reduction in the severity or the complete loss of clinical signs of disease in humans. These vaccines, along with another mouse brain vaccine prepared by Wisseman et al. (19), were shown to be effective and to have low reactogenicity.

Present-day methodology has made it possible to select temperature-sensitive and host-restricted, conditional lethal mutants of many viruses. Several flavivirus mutants with reduced animal virulence have been studied on a limited scale. These include Japanese encephalitis (3, 5), tick-borne encephalitis (8), Langat (9), Kyasanur Forest (11), and DEN-2 (1a, 15, 16) viruses.

Growth of mutant viruses in cell culture substrates suitable for vaccine production and careful documentation of passage history of the original isolates may permit a potential virus clone to be used in human vaccination trials. Passage of a small-plaque clone of DEN-2 virus in fetal primate diploid cells and the preparation of a candidate human vaccine in these cells are described here. Animal and in vitro markers of attenuation of the S-1 vaccine are compared with those found using earlier-passaged virus.

MATERIALS AND METHODS

Cells and media. A diploid cell line of fetal rhesus monkey lung cells (DBS-FRhL-2) derived by Wallace et al. (17) was used for virus passage and the preparation of production seed and vaccine. Frozen ampoules of DBS-FRhL-2 cells were obtained from Merrell-National Drug Co., Swiftwater, Pa. These were frozen at the 17th passage level and extensively tested by the supplier for the presence of viral adventitious agents, mycoplasma, and bacteria. Karyological studies were done on cells one passage beyond the frozen state. Cells used for the preparation of seed virus and vaccine were at the 18th passage level. For other experiments, DBS-FRhL-2 cells between the 20th and 30th passages were used.

Fetal bovine serum (FBS) used in cell culture media was purchased from HEM Research, Inc., Rockville, Md. Serum was certified free of bovine adventitious agents, mycoplasma, and bacteria. Further testing for the presence of bacteriophage was done at the Walter Reed Army Institute of Research by Peter Gemski.

Cell culture medium for DBS-FRhL-2 cells was Eagle minimal essential medium (GIBCO, catalog no. F-15, Grand Island, N.Y.) containing 10% FBS and antibiotics for seeding and outgrowth of monolayers. Maintenance medium contained 2% FBS or 0.25% human serum albumin (Hyland, Costa Mesa, Calif.) for vaccine preparation.

Plaque assay. LLC-MK_2 cells were used to assay virus as described previously (1a). For assay at nonpermissive temperatures, plaque flasks were sub-

merged in water-tight boxes in constant-temperature, circulating water baths. Temperatures did not fluctuate more than 0.05°C in these baths. Plaque assays at 35°C were done in constant-temperature, forced-draft incubators.

Production seed virus. The passage history of the DEN-2 PR-159 human isolate, preparation of a parent virus seed, and derivation of the S-1 clone have been described in detail (1a). For adaptation to DBS-FRhL-2 cells, the 19th passage of the S-1 clone in primary green monkey kidney cells (PGMK) (previously designated in Harrison et al. [6] as S-1 p-19a) was used to inoculate DBS-FRhL-2 monolayers. After two passages in 25-cm^2 flasks, the S-1 virus was inoculated into 700-cm^2 roller flasks. Viral inocula were diluted so that a low multiplicity of infection (MOI) was used to prevent reversion to large-plaque, temperature-resistant virus (1a). Supernatant fluids were harvested between days 7 and 10 and were assayed for infectious virus by plaquing at 35 and 39.3°C.

Tests for adventitious microbial agents in the production seed virus pool were performed as described (1a) and followed Public Health Service regulations for licensed, live-attenuated vaccines (*Code of Federal Regulations*, chapter 21, subchapter F, Biologics).

Vaccine. DEN-2 S-1 vaccine was prepared according to procedures established for the preparation of production seed. DBS-FRhL-2 roller flasks were inoculated with S-1 seed virus at an MOI of 5×10^{-4}. After adsorption for 1.5 h at 35°C, the inoculum was removed and flasks were washed three times with 100 ml of Hanks balanced salt solution (HBSS). Maintenance medium (200 ml per roller) consisted of Eagle minimal essential medium with 0.25% human serum albumin, 0.22% $NaHCO_3$, streptomycin (50 μg/ml), and neomycin (100 μg/ml). Medium on all flasks was changed on day 4, and supernatant fluids were harvested on day 6. Before centrifugation at $1,050 \times g$ for 20 min, human serum albumin was added to supernatant fluids, resulting in a final concentration of 2.75%. The pH of the albumin was adjusted to 7.6 before addition to the virus fluids. As a final step in clarification, fluids were filtered through a 0.45-μm membrane filter (Nalge, Rochester, N.Y.). After removal of samples for safety testing and plaque assay, the remaining volume was held in ice baths in a 4°C refrigerator for 7 days pending results of sterility and plaque assays. A final pool of vaccine was made by using virus fluids from flasks containing <5 plaque-forming units (PFU)/ml at 39.3°C and with no detectable large-plaque virus at 35°C. The titer of the vaccine pool was 5.5×10^4 PFU/ml. After freeze-drying in 3-ml amounts, the average titer for 10 vials was 8.5×10^4 PFU/ml, with no detectable large-plaque virus present. The infectivity of freeze-dried vaccine held at −20°C has remained stable for a period of approximately 2.5 years.

Safety tests on supernatant fluids before centrifugation were identical to those performed on the vaccine seed preparation. Final container tests for 25 vials of vaccine included bacterial sterility and the inoculation of two young adult mice and two guinea pigs. All tests were negative, indicating the absence of any adventitious agents in the DEN-2 vaccine, lot no. 1.

Neurovirulence testing of vaccine has been described in detail elsewhere (6). Eleven male rhesus monkeys were inoculated intracerebrally into each hemisphere and intraspinally, using 0.5 ml of vaccine per injection site. Two control monkeys received similar inoculations with virus-free cell culture fluids. All monkeys were held for 19 days and observed daily for evidence of central nervous system involvement or other physical abnormalities. Upon completion of the holding period, blood samples were taken from each animal for evidence of DEN-2 replication. Under deep pentobarbital sodium anesthesia, each monkey was perfused with 10% Formalin, and appropriate tissues were removed. Histological sections of the lumbar and cervical cord, lower and upper medulla, mesencephalon, and the motor cortex were made and examined for viral pathology. Lesions observed in both brain and spinal cord sections were due to needle insertion trauma, and none could be attributed to viral infection processes. No increase in the number or severity of lesions was seen in the vaccinated group of monkeys compared with the two control monkeys.

Monkey peripheral blood leukocyte (PBL) cultures. Heparinized blood from a cynomolgus monkey, immunized 9 months previously with DEN-3 virus, was mixed with an equal volume of Ca- and Mg-free HBSS. Fifteen milliliters of Ficoll-Hypaque was layered under 35 ml of the diluted, heparinized blood and centrifuged at $350 \times g$ for 40 min. The leukocyte band was removed and washed once in HBSS and once in RPMI 1640 medium containing 10% FBS, 2 mM glutamine, 0.01 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and antibiotics. Cells were resuspended in RPMI 1640 medium and adjusted to a concentration of 6.8×10^6 cells/ml before inoculation of the suspension with virus. Growth curves were performed at 35°C.

Monkey immunogenicity studies. Details of monkey inoculation experiments and procedures for testing complement-fixing, hemagglutination inhibition, and neutralizing antibodies have been described elsewhere (6, 14).

RESULTS

S-1 replication in cell culture. DEN-2 S-1 vaccine was prepared at the fourth passage level in DBS-FRhL-2 cells. Virus used to initiate this line of passages was S-1 at the 19th passage level in PGMK cells (S-1 PGMK). Experiments to characterize the vaccine virus were initiated to detect any changes in the virus that may have occurred during passage in DBS-FRhL-2 cells. Previous data for S-1 passaged in PGMK cells indicated that restriction of replication of this virus occurred at temperatures of 37°C and higher. Growth kinetics for the S-1 vaccine and S-1 PGMK viruses were compared at the permissive temperature of 35°C and also at 37 and 38.5°C. This was accomplished by inoculation of $LLC-MK_2$ cells at an MOI of approximately 0.01 and removal of supernatant culture fluid samples for days 2, 3, and 4 post-inoculation. Results of this experiment (Fig. 1) revealed that very

little restriction of replication occurred for S-1 vaccine at 37°C, whereas S-1 PGMK virus was significantly restricted at the same temperature. At 38.5°C no detectable virus could be found in culture fluids from S-1 vaccine- or S-1 PGMK-inoculated flasks. Parent virus (not shown) replicated with reduced yields at 38.5°C (1a).

Efficiency of plating (EOP), i.e., the ability to form plaques at superoptimal temperatures, was calculated for both the S-1 PGMK virus and the S-1 vaccine. For comparison, the DEN-2 parent (PGMK-6) virus was also included in the assay. To calculate the EOP, plaque flasks were incubated at permissive temperature (35°C) and also at 38.5 and 39.3°C. The ratios of plaque titers at 38.5 or 39.3°C to the plaque titer at 35°C are listed in Table 1. The twofold-higher EOP for the S-1 vaccine at 38.5°C could be repeated in three separate comparative assays. The higher EOP indicated a greater efficiency of the S-1 vaccine virus to form plaques. Together with data from the growth curves of Fig. 1, a higher replicative capacity for the S-1 vaccine was found at superoptimal temperatures.

Effect of temperature on virus phenotype. Maintenance of small-plaque, temperature-sensitive virus on further passage of S-1 vaccine virus could be achieved by incubation at

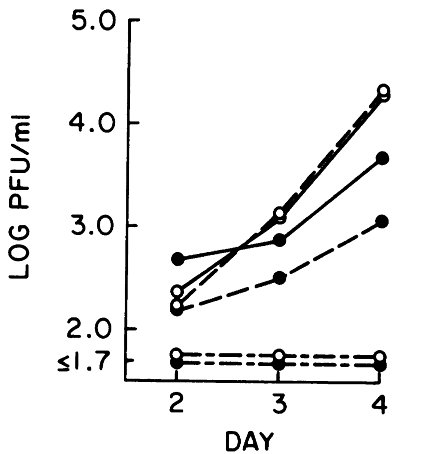

FIG. 1. *Replication of S-1 vaccine* (○) *and S-1 PGMK* (●) *viruses at 35°C* (——), *37°C* (- - -), *and 38.5°C* (-·-·-·-) *in LLC-MK₂ cells.*

31°C. The effect of temperature on the emergence of temperature-resistant virus was assayed for both the S-1 vaccine and S-1 PGMK viruses by growth at 31°C, 35°C, and 37°C. Table 2 lists the 7-day harvest titers of these viruses plaqued at 35°C as well as the number of cell culture flasks containing virus which plaqued at 39.3°C. The S-1 vaccine-inoculated flasks showed an increase in the percentage of flasks with temperature-resistant virus as a function of increased temperature. At 37°C, 65% of these flasks contained virus in supernatant culture fluids that formed plaques at 39.3°C, whereas no temperature-resistant virus was found in any of the infected flasks at 31°C. None of the S-1 PGMK virus-inoculated cell culture flasks at 31, 35, or 37°C was found to contain temperature-resistant virus; however, virus titers were appreciably lower at all temperatures than virus from flasks inoculated with S-1 vaccine virus. Lower virus titers may account for the inability to demonstrate this phenomenon with the S-1 PGMK virus.

Individual plaque isolates were made from plaques formed at 39.3°C in four S-1 vaccine-inoculated flasks incubated originally at 37°C. The plaque picks were inoculated into LLC-MK_2 flasks incubated at 35°C, and the supernatant fluids were harvested after 14 days. Assays of the clone harvests from seven plaque picks listed in Table 3 indicated that six of seven did not form plaques at 39.3°C. This indicates that these clones are not revertant, and the one clone which plaqued at 39.3°C appeared to contain leaky virus.

TABLE 2. *Emergence of temperature-resistant S-1 virus as a function of increasing temperature*

Virus	Temp (°C)	No. of cultures with temperature-resistant virus/total no. of cultures
S-1 PGMK	31	0/21 ($9.9 \times 10^4 \pm 0.1$)[a]
	35	0/23 ($8.6 \times 10^4 \pm 0.1$)
	37	0/22 ($2.1 \times 10^4 \pm 0.2$)
S-1 vaccine	31	0/23 ($3.4 \times 10^5 \pm 0.3$)
	35	3/23 ($3.0 \times 10^5 \pm 0.2$)
	37	15/23 ($1.8 \times 10^5 \pm 0.2$)

[a] Numbers in parentheses are 7-day harvest mean titers ± standard error of the mean.

TABLE 1. *EOP for S-1 and parent viruses at 38.5 and 39.3°C*

Virus	PFU/ml			EOP	
	35°C	38.5°C	39.3°C	38.5/35°C	39.3/35°C
S-1 PGMK	7.0×10^6	8.0×10^4	<5.0	1.1×10^{-2}	$<7 \times 10^{-7}$
S-1 vaccine	7.5×10^5	1.6×10^4	<5.0	2.1×10^{-2}	$<7 \times 10^{-6}$
Parent	6.5×10^6	Not done	5.0×10^5		7.7×10^{-2}

TABLE 3. *Plaque formation by clones of S-1 virus grown at 37°C*

Clone	PFU/ml	
	35°C	39.3°C
1	1.5×10^{5} [a]	<5
2	7.5×10^{5}	<5
3	6.0×10^{5}	<5
4	4.3×10^{5}	<5
5	3.7×10^{5}	<5
6	2.0×10^{5}	<5
7	8.0×10^{5}	6.0×10^{2}

[a] Harvest of clones derived by plaque picking and amplification for 14 days in LLC-MK_2 cell cultures. Plaques selected for cloning were from a 39.3°C assay of S-1 vaccine virus grown at 37°C.

Replication in monkey PBL cultures. Halstead and O'Rourke (4) demonstrated that replication of dengue virus occurred in monkey PBL cultures when the PBL were from a dengue-immune monkey or when a nonimmune cell donor was used and diluted dengue antibody was added to the culture system. Cells from a cynomolgus monkey, previously immunized with DEN-3, were inoculated with S-1 vaccine and S-1 PGMK viruses at an MOI of approximately 0.015. Human antiserum with a DEN-2 neutralization titer of 1:80, previously shown to enhance dengue virus replication in human monocytes (1), was added to cell culture medium at a final dilution of 1:500. Both dengue-immune monkey PBL and addition of diluted homologous antibody were used for maximal enhancement of growth of the S-1 viruses. On days 2, 3, and 4 postinfection, triplicate vials were frozen at −70°C and later assayed in LLC-MK_2 cells. Growth curves plotted in Fig. 2 indicate that the S-1 vaccine virus grew to higher titer than the S-1 PGMK virus in monkey PBL cultures. In addition to temperature restriction, which was previously seen for S-1 PGMK virus, host restriction of this virus was demonstrated in the PBL growth curves.

Mouse neurovirulence. Suckling mouse neurovirulence for the S-1 clone is less than that of the parent virus (1a). Death of mice after intracerebral inoculation of S-1 virus is used as a measure of virulence and compared with the plaque titer of the S-1 virus assayed in LLC-MK_2 cells. The virulence ratios for S-1 PGMK and S-1 vaccine viruses are listed in Table 4. Mouse virulence appears not to have changed after passage of S-1 in DBS-FRhL-2 cells, and further attenuation, which would result in a lower virulence ratio, has not occurred. With this marker, the parent virus is approximately 20 times more virulent than the S-1 clone.

Monkey immunogenicity. Rhesus monkey antibody response to the S-1 vaccine was compared with responses in earlier monkey inoculation experiments, using approximately the same amount of virus in the inoculum (Table 5). Data shown in this table were compiled over a period of 3 years; however the complement-fixing, hemagglutination inhibition, and neutralization test procedures did not change over this period of time. Geometric mean titers in three different serological tests were lower for the S-1 vaccine group than for monkeys that received S-1 virus at the 14th or 18th PGMK cell passage level. These results point to an apparent decrease in immunogenicity of the S-1 clone after passage in

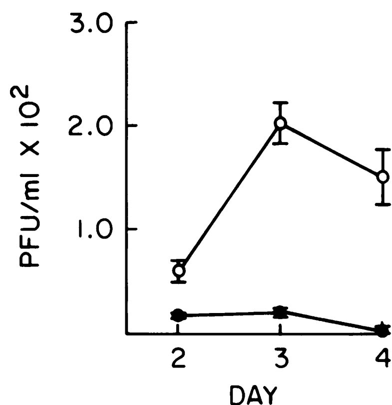

FIG. 2. *Replication of S-1 vaccine (○) and S-1 PGMK (●) viruses in monkey PBL cultures. Bars indicate the standard error for triplicate cultures.*

TABLE 4. *Relative neurovirulence for S-1 and parent viruses inoculated intracerebrally in suckling mice*

Virus	$\log_{10}$ LD_{50}/ml [a]	PFU/ml	LD_{50}/PFU
S-1 PGMK	2.9	1.1×10^{5}	0.006
S-1 vaccine	3.8	1.4×10^{6}	0.004
Parent (PGMK-6)	5.6	3.0×10^{6}	0.13

[a] LD_{50}, 50% lethal dose.

TABLE 5. *Summary of serum antibody titers in monkeys receiving the S-1 clone*

Virus	No. of monkeys	Inoculum	Reciprocal geometric mean antibody titers [a]		
			CF	HI	N
S-1 p-14	2	1.5×10^{5}	90	113	560
S-1 p-18	3	5.0×10^{4}	101	137	760
S-1 vaccine	5	3.8×10^{4}	18	15	60

[a] CF, Complement-fixing titer on day 30; HI, hemagglutination inhibition titer on day 30; N, neutralization titer on day 30 (for monkey receiving S-1 p-14) or day 45.

DBS-FRhL-2 cells. Virulence of these viruses in monkeys can only be measured by production of viremia. Since viremia occurs only occasionally if at all in monkeys inoculated with S-1 vaccine and S-1 PGMK viruses, we can only conclude that there has been no change in this marker upon passage. Details of monkey experiments with the vaccine virus are given elsewhere (14).

DISCUSSION

In vitro markers including replication and plaque formation at superoptimal temperatures in LLC-MK_2 monolayers, as well as replication in monkey PBL cell suspensions, indicate a greater capacity for the S-1 vaccine virus to overcome temperature or host restriction than the S-1 PGMK virus. Similarly, superior replication of the S-1 vaccine virus in comparative growth curves with S-1 PGMK virus using human monocytes has recently been demonstrated (1). In addition, it appears that the S-1 vaccine can generate leaky virus at 37°C, whereas this temperature-dependent phenomenon was not seen for S-1 PGMK virus. The only marker examined where no difference could be detected between the two viruses was mouse neurovirulence.

Previously, loss of attenuation for the S-1 clone was correlated with the emergence of a large-plaque, temperature-resistant population of virus which appeared to be revertant (1a). The concomitant increase in mouse and monkey virulence made it appear that these properties of the S-1 clone were covariant. In the present study, the S-1 clone passaged in DBS-FRhL-2 cells appeared to gain some degree of temperature resistance without emergence of large-plaque, revertant virus or an increase in animal virulence.

Another change in the virus that could be measured was a significant decrease in immunogenicity of the virus for monkeys. Reduced immunogenicity for monkeys may be associated with a lower capacity for the S-1 vaccine to replicate in the host animal. It is not possible to demonstrate this directly at the present time, and in vitro markers indicate no decreased replicative capacity for the S-1 vaccine.

Selection for a variant of the S-1 clone may have occurred during passage in DBS-FRhL-2 cells. However, there appears to be no detectable virus population with an altered temperature-sensitive phenotype or increased plaque size. Host modification of viral infectivity and possibly also immunogenicity may have played some part in the observed changes in the clone upon passage.

Although the S-1 vaccine virus demonstrates leakiness when it is grown at 37°C, there is no evidence of reversion. There is also no indication that the S-1 clone reverts in rhesus monkeys or chimps (6). In addition to the relative stability of the clone, there were minimal changes in the temperature-sensitive phenotype and lack of any measurable neurovirulence in monkeys. Examination of these and other characteristics of the S-1 vaccine prepared in DBS-FRhL-2 cells indicates that this vaccine is a reasonably safe and potentially useful immunogen for humans.

LITERATURE CITED

1. **Brandt, W. E., J. M. McCown, F. H. Top, Jr., W. H. Bancroft, and P. K. Russell.** 1979. Effect of passage history on dengue-2 virus replication in subpopulations of human leukocytes. Infect. Immun. **26**:534-541.

1a. **Eckels, K. H., W. E. Brandt, V. R. Harrison, J. M. McCown, and P. K. Russell.** 1976. Isolation of a temperature-sensitive dengue-2 virus under conditions suitable for vaccine development. Infect. Immun. **14**: 1221-1227.

2. **Ehrenkranz, N. J., A. K. Ventura, R. R. Cuadrado, W. L. Pond, and J. E. Porter.** 1971. Pandemic dengue in Caribbean countries and the southern United States—past, present, and potential problems. N. Engl. J. Med. **285**:1460-1469.

3. **Halle, S., and E. Zebovitz.** 1977. A spontaneous temperature sensitive mutant of Japanese encephalitis virus: preliminary characterization. Arch. Virol. **54**:165-176.

4. **Halstead, S. B., and E. J. O'Rourke.** 1977. Dengue viruses and mononuclear phagocytes. I. Infection enhancement by nonneutralizing antibody. J. Exp. Med. **146**:201-217.

5. **Hammon, W. McD., S. Rohitaydhin, and J. S. Rhim.** 1963. Studies on Japanese B encephalitis virus vaccines from tissue culture. IV. Preparation and characterization of a pool of attenuated Oct-541 line for human vaccine trial. J. Immunol. **91**:245-305.

6. **Harrison, V. R., K. H. Eckels, J. W. Sagartz, and P. K. Russell.** 1977. Virulence and immunogenicity of a temperature-sensitive dengue-2 virus in lower primates. Infect. Immun. **18**:151-156.

7. **Hotta, S.** 1952. Experimental studies on dengue. I. Isolation, identification and modification of the virus. J. Infect. Dis. **90**:1-9.

8. **Mayer, V.** 1963. Two variants of tick-borne encephalitis showing different plaque morphology. Virology **20**:372-390.

9. **Mayer, V.** 1973. The highly attenuated E4"14" plaque-cloned derivative from the Langat TP21 E5 strain. Isolation and properties. Acta Virol. **17**:263.

10. **McCown, J. M., and W. H. Bancroft.** 1977. Identification of dengue viruses from the Caribbean and the Bahamas. W.H.O. Pan Am. Health Org. Newslett. **6**: 11-12.

11. **Paul, S. D.** 1966. Some biological properties of two variants of Kyasanur Forest disease virus. Indian J. Med. Res. **54**:419-424.

12. **Sabin, A. B., and R. W. Schlesinger.** 1945. Production of immunity to dengue with virus modified by propagation in mice. Science **101**:640-642.

13. **Schlesinger, R. W., I. Gordon, J. W. Frankel, J. W. Winter, P. R. Patterson, and W. R. Dorrance.** 1956. Clinical and serologic response of man to immunization with attenuated dengue and yellow fever viruses. J. Immunol. **77**:352-364.

14. **Scott, R. McN., A. Nisalak, K. H. Eckels, M. Tingpa-**

lapong, V. R. Harrison, D. J. Gould, F. E. Chapple, and P. K. Russell. 1980. Dengue-2 vaccine: viremia and immune responses in rhesus monkeys. Infect. Immun. **27**:181-186.

15. **Tarr, G. C., and A. S. Lubiniecki.** 1975. Chemically-induced temperature-sensitive mutants of dengue virus type 2. I. Isolation and partial characterization. Arch. Virol. **48**:279-287.
16. **Tarr, G. C., and A. S. Lubiniecki.** 1976. Chemically-induced temperature-sensitive mutants of dengue virus type 2: comparison of temperature sensitivity in vitro with infectivity in suckling mice, hamsters, and rhesus monkeys. Infect. Immun. **13**:688-695.
17. **Wallace, R. E., P. J. Vasington, J. C. Petriciani, H. E. Hopps, and D. E. Lorenz.** 1973. Development of a diploid cell line from fetal rhesus monkey lung for virus vaccine production. In Vitro **8**:323-332.
18. **Wisseman, C. L., and B. H. Sweet.** 1961. The ecology of dengue, p. 15-40. *In* J. M. May (ed.), Second series of studies in medical geography. Hafner Publishing Co., New York.
19. **Wisseman, C. L., B. H. Sweet, E. C. Rosenzweig, and O. R. Eylar.** 1963. Attenuated living type 1 dengue vaccines. Am. J. Trop. Med. Hyg. **12**:620-623.