Dengue-2 Vaccine: Viremia and Immune Responses in Rhesus Monkeys

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Studies were undertaken in Indian rhesus monkeys (*Macaca mulatta*) to determine the safety, potency, immunogenicity, and mosquito infectivity of a small-plaque, temperature-sensitive variant of dengue type 2 (DEN-2) virus, a vaccine candidate. Fifteen monkeys were inoculated subcutaneously with the vaccine virus, ten receiving $10^{3.1}$ plaque-forming units (PFU) and five receiving $10^{4.5}$ PFU. After primary immunization, viremia was detected in only one monkey, a recipient of the higher dose of vaccine. The recovered virus had the same growth characteristics as the vaccine strain. *Aedes aegypti* mosquitoes did not become infected when they were allowed to feed on monkeys that received the lower dose of vaccine. As expected, the immunization produced no evidence of illness in any of the animals. A dose response to vaccine was detected; all five of the high-dose recipients developed neutralizing antibodies, whereas only five of ten low-dose recipients did so. In both groups, neutralizing antibody was often transient. Its presence at 30 days did not always correlate with protection from viremia in those animals challenged 4 to 6 months after vaccination with wild-type DEN-2 virus. However, immunized animals developed anamnestic antibody responses after challenge, and none demonstrated adverse effects to infection. Reimmunization of monkeys 4 months after primary immunization led to the production of low-titered but persistent neutralizing antibody which protected the animals from a wild-type virus challenge.

A candidate dengue type 2 (DEN-2) virus vaccine has been prepared from the previously described small-plaque (S-1) strain of PR-159 by passage in fetal rhesus lung cells, DBS-FRHL-2. Characterization of the vaccine virus and comparison with precursor virus passed in primary green monkey kidney cells (PGMK) is reported elsewhere (4). This report describes the viremia and immune response produced by the vaccine strain in rhesus monkeys (*Macaca mulatta*), the failure of the dengue vector mosquito *Aedes aegypti* to acquire infection from these monkeys after immunization, and the ability of the immunized monkeys to resist infection after challenge with wild strains of DEN-2 virus.

MATERIALS AND METHODS

Viruses. The candidate vaccine, DEN-2, PR-159, S-1, lot no. 1, in the form of a freeze-dried preparation, was rehydrated in sterile water for injection just before

inoculation into rhesus monkeys. Since immunization studies were performed at two different laboratories, vaccine virus was assayed at both locations. The vaccine averaged 1.2×10^5 plaque-forming units (PFU) per 0.5-ml dose at the Armed Forces Research Institute of Medical Sciences (AFRIMS) and 3.8×10^4 PFU per 0.5-ml dose at the Walter Reed Army Institute of Research (WRAIR). Vaccine doses will be referred to as the low dose and the high dose, respectively. To challenge the vaccinated monkeys, three wild strains of DEN-2 virus were used. Two were isolated from patients. The vaccine parent strain PR-159 (PGMK-6) was derived from a patient with uncomplicated dengue fever seen during the 1969 dengue epidemic in Puerto Rico, and the Asian strain 21868 was isolated in 1967 from a Thai child with hemorrhagic fever. Strain BM50-76 was isolated from a pool of *A. aegypti* collected in 1976 from the home of a dengue hemorrhagic fever patient in Thailand.

Plaque assay. Methods to assay the infectivity of the vaccine inoculum, determine the levels of viremia, identify the characteristics of plaque morphology, and isolate the virus from mosquitoes used modifications of plaque assays previously described (3, 13). Preformed monolayers of LLC-MK₂ cells in 25-cm² plastic flasks (Costar, Bellco Glass Inc., Vineland, N.J.) were inoculated and incubated at 35°C.

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Serology. Hemagglutination inhibition (HI) and complement fixation (CF) tests used microtiter modifications of standard methods (2, 8, 11). Sucrose-acetone-extracted antigens were prepared from prototype strains of DEN-1, -2, -3, and -4 viruses (7) and were used in HI and CF tests at 8 and 2 U, respectively. Plaque reduction neutralization tests were used to assay neutralizing (N) antibodies (9).

Primate inoculation. Wild-caught Indian rhesus monkeys (*M. mulatta*) free of HI and CF antibodies against all four dengue serotypes and N antibodies against DEN-2 virus were housed in individual cages in mosquito-proof rooms. Immunizations and challenges were performed at two different laboratories; the monkeys used at AFRIMS are designated series A, and those at WRAIR are designated series W. Each animal received 0.5 ml of vaccine subcutaneously and was examined for signs of disease before inoculation and daily throughout each experiment. Blood for serology was obtained on days 0 through 10 inclusive and on days 15, 30, and either 45 or 60 after inoculation. For the first 10 days after inoculation, additional heparinized plasma was collected for virus isolation.

Four to six months after immunization, monkeys were challenged with 0.5 ml of wild-type dengue virus subcutaneously or were reimmunized with 0.5 ml of vaccine. The reimmunized monkeys were challenged with 0.5 ml of wild-type dengue virus subcutaneously 10 months after reimmunization.

Mosquito infections. On days 1 through 10 after immunization, laboratory-reared female *A. aegypti* were allowed 0.5 h to feed on monkeys that had received wild-type DEN-2 virus and the low-dose vaccine. Engorged mosquitoes were incubated for 14 days at 32°C, killed by quick freezing, and stored at -70°C until tested. For individual animals on each day, 10 engorged mosquitoes were pooled and sonicated in 1.0 ml of RPMI 1640 medium (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal calf serum, glutamine, and antibiotics. The resulting mosquito suspensions were centrifuged at $100 \times g$ for 30 min, and the supernatants were tested for virus by plaque assay. The heads of an additional six *A. aegypti* were examined for dengue antigen by a direct fluorescent-antibody technique (8). The antibody used in fluorescent-antibody studies was a fluorescein isothiocyanate-labeled pool of human convalescent serum, which was obtained from dengue hemorrhagic fever patients in Bangkok. After labeling, the HI titer of the conjugate was $\geq 1:1,280$ against each of the dengue serotypes.

RESULTS

Wild-type DEN-2 infections of rhesus monkeys. The three low-passage wild-type DEN-2 strains, PR-159 (PGMK-6), Asian strain 21868 (BSC-5, LLC-MK₂-1), and BM50-76 (LLC-MK₂-3), regularly led to infection when inoculated into unimmunized rhesus monkeys (Table 1). Low-level viremias began as early as day 2 and continued for as long as 6 days. Virus was isolated by a direct plaque assay from plasma obtained from all of the monkeys. Titers ranged up to 6.5×10^1 PFU/ml. All isolates

contained virus which produced plaques of mixed sizes, characteristic of wild-type virus strains. Viremia was invariably followed by the development of CF, HI, and N antibodies. As expected, there was no clinical illness in any of the infected monkeys.

Pools of *A. aegypti* fed on the five series A monkeys which received 1.3×10^5 PFU of PR-159 (PGMK-6); infection was detected in mosquitoes fed on each monkey. Mosquitoes were infected by blood meals taken from 3 to 8 days after inoculation. The days on which virus could be detected in mosquitoes were usually but not always the same as those on which viremia was detected.

Primary immunization of rhesus monkeys with candidate DEN-2 vaccine. The vaccine strain was inoculated without dilution into 15 monkeys; 10 series A monkeys received vaccine which titered 2.1×10^3 PFU per dose (low dose), and 5 series W animals received portions of the same vaccine which titered 3.8×10^4 PFU per dose (high dose) (Table 2). Viremia was detected only in the plasma of one monkey which received the high dose of vaccine; small-plaque virus in low titer was isolated from blood taken on the 7th day after vaccination.

Antibody responses varied according to the dose of vaccine virus administered. All five monkeys which received the high dose of vaccine developed CF, HI, and N antibodies by the 45th day after immunization, with geometric mean titers at 1:24, 1:19, and 1:62, respectively. Three monkeys developed N titers of 1:100 or greater. Among the 10 low-dose recipients, only 8 had detectable antibody. The highest geometric mean titers for CF, HI, and N antibodies, which were found on day 30, were 1:9 (eight monkeys), 1:40 (three monkeys), and 1:41 (five monkeys), respectively.

In Bangkok, *A. aegypti* pools were allowed to feed on the series A monkeys which received the low dose of vaccine. None of these monkeys were viremic at any time after vaccination. In no case, even in those monkeys that showed an antibody response, was transmission of virus to mosquitoes detected by either virus isolation or fluorescent-antibody technique.

Wild-type DEN-2 challenges of immunized monkeys. During the 3- to 6-month period after immunization, antibody levels in the 15 vaccinated monkeys declined markedly. Nine immunized monkeys were challenged with wild-type DEN-2 virus (Table 3). At the time of challenge, only four of the nine monkeys had detectable N antibodies.

Four monkeys from the low-dose group were challenged by subcutaneous inoculation of 1.1

TABLE 1. *Viremia and antibody responses of unimmunized rhesus monkeys after infection with wild strains of DEN-2 virus*

Inoculum	Amt (PFU)	Monkeys		Duration of viremia (days)		Maximum titer of viremia (PFU/ml)	Reciprocal geometric mean antibody titer on day 30		
		No.	Series	Mean	Range		CF	HI	N
PR-159, PGMK-6	1.3×10^5	5	A	4.2	3-6	5.5×10^1	48	211	560
BM50-76, LLC-MK ₂ -3	2.0×10^6	2	A	4.5	4-5	6.5×10^1	128	452	640
Asian strain 21868; BSC-5, LLC-MK ₂ -2	3.4×10^5	3	W	4.5	4-5	2.0×10^1	181	113	Not done

TABLE 2. *Viremia and antibody responses of rhesus monkeys to a first dose of DEN-2 vaccines*

Monkey series and no.	Inoculum (PFU)	Viremia	Reciprocal antibody titer								
			CF on day:			HI on day:			N on day:		
			15	30	45/60 ^a	15	30	45/60 ^a	15	30	45/60 ^a
A 228	1.2×10^{3b}	None	4	<4	<4	<10	20	10	<10	20	ND ^c
A 231		None	<4	<4	<4	<10	<10	<10	<10	<10	ND
A 290		None	8	8	<4	<10	<10	<10	<10	<10	ND
A 293		None	4	4	<4	<10	<10	<10	<10	<10	ND
A 294		None	8	8	4	<10	<10	<10	<10	<10	ND
A 297		None	8	8	8	<10	<10	<10	70	80	ND
A 298		None	<4	4	<4	<10	<10	<10	<10	<10	ND
A 299		None	4	32	16	<10	80	10	25	140	ND
A 301		None	32	16	8	<10	40	40	20	50	ND
A 315		None	4	8	4	<10	<10	<10	10	10	ND
W 179	3.8×10^{4d}	None	16	16	32	20	20	20	ND	ND	20
W 792		None	8	8	16	10	10	10	ND	ND	10
W 810		None	32	32	32	20	20	40	ND	ND	200
W 853		1 day ^e	16	32	32	20	20	40	ND	ND	220
W 867		None	16	16	16	10	10	10	ND	ND	100

^a Samples were collected on day 60 and on day 45 for monkeys receiving the low and high doses of vaccine, respectively.

^b Titer is the mean of three determinations.

^c ND, Not done.

^d Titer is the mean of 11 determinations.

^e Small-plaque virus isolated on day 7.

TABLE 3. *Viremia and antibody responses of immunized rhesus monkeys to challenge^a with wild strains of DEN-2 virus*

Monkey series and no.	Original inoculum of vaccine (PFU)	Wild-type challenge inoculum (PFU)	Postchallenge viremia (days)	Reciprocal antibody titer					
				HI on day:		CF on day:		N on day:	
				0	30	0	30	0	30/45 ^b
A 231	1.2×10^{3c}	BM50-76, 1.1×10^5	3	<4	128	<10	≥640	<10	≥640
A 290			2	<4	128	10	640	<10	≥640
A 293			1	<4	128	<10	80	<10	170
A 299			0	8	32	80	≥160	100	≥640
W 810	3.8×10^{4d}	21868, 6×10^5	3	<4	256	10	≥640	<10	≥640
W 853			0	8	64	40	160	250	≥640
W 867			0	<4	512	<10	≥640	<10	≥640
W 179		PR-159, 3×10^5	3	<4	256	10	≥640	60	≥640
W 729			0	<4	64	<10	160	40	≥640

^a Challenged 4 and 6 months after immunization for series A and W monkeys, respectively.

^b Samples collected on day 30 and day 45 for series A and W monkeys, respectively.

^c Titer is the mean of three determinations.

^d Titer is the mean of 11 determinations.

$\times 10^5$ PFU of BM50-76 4 months after the initial immunization. Viremia occurred in three of four monkeys, which had an N titer of $<1:10$ at the time of challenge. Viremia began on the 3rd day after inoculation and was short-lived, lasting only 1 to 3 days. The mean titer was 18 PFU/ml, with a range of 3 to 69 PFU/ml. Each monkey showed significant rises in homologous N antibody and both homologous and heterologous CF and HI antibodies (data not shown). One monkey with an N titer of 1:100 before challenge did not have detectable viremia, although the antibody response was similar to that of monkeys that were viremic.

The five series W monkeys which received the high dose of vaccine were divided into two groups and challenged 6 months after primary immunization. Two of them were given the parent strain, PR-159 (3×10^5 PFU/dose), and the remaining three were given Asian strain 21868 (6×10^5 PFU/dose). Viremia occurred in one monkey from each group. One nonviremic monkey had an N titer of $<1:10$ before challenge, whereas another monkey, despite a prechallenge N titer of 1:60, produced a viremia. The duration of viremia for both monkeys was 3 days. All five of the monkeys exhibited a fourfold or greater titer rise in HI, CF, and N antibodies. The degree of titer rise in any of the challenged monkeys

did not appear to be related to the presence or absence of viremia.

Reimmunization and challenge. Three series A monkeys, initially immunized with the lower dose of vaccine, received booster immunizations at 4 months with 2.5×10^3 PFU of the vaccine virus (Table 4). There was little or no detectable HI or CF antibody in the sera of these monkeys before the booster immunization, although one of them had low titers of both N and HI antibodies at 30 days after the initial immunization. After the booster dose, viremia was not detected, and all three monkeys developed CF, HI, and N antibodies to DEN-2. HI antibody titers after reimmunization were declining by 6 months and undetectable at 10 months in two of the three animals. All three monkeys retained CF and N antibodies throughout the 10-month period. Monkeys which received the booster immunization were challenged approximately 10 months later with 2×10^6 PFU of wild-type DEN-2 virus (BM50-76). After immunization, none of the challenged monkeys developed viremia (Table 5), and the monkeys remained clinically well. Despite the absence of viremia, all monkeys demonstrated anamnestic responses in CF, HI, and N tests.

DISCUSSION

The disparity, by more than 10-fold, in the titers of the rehydrated vaccine at AFRIMS and at WRAIR was unexpected. The titers shown in this paper resulted from means of 3 determinations at AFRIMS and 11 determinations at WRAIR. The dissimilarity might have resulted either from rehydration of the freeze-dried vaccine or from its transportation in solid CO_2 in rubber-stoppered bottles. We do not feel that the disparity was due to differences in the sensitivity of the LLC-MK₂ cells for DEN-2 virus. The cells used in both laboratories were derived from the same tissue culture stock. Repeated titrations of wet frozen DEN-2 virus, shipped in the same container but sealed in glass ampoules,

TABLE 4. Viremia and antibody responses of rhesus monkeys to a booster dose of attenuated DEN-2 vaccine^a

Monkey series and no.	Viremia	Reciprocal antibody titer								
		CF on day:			HI on day:			N on day:		
		0	15	30	0	15	30	0	15	30
A 294	None	4	16	8	10	80	80	<10	90	160
A 298	None	<4	4	4	<10	160	80	<10	300	160
A 301	None	<4	32	16	<10	80	40	<10	160	160

^a Booster immunization with 1.2×10^3 PFU of DEN-2 vaccine given 4 months after primary immunization.

TABLE 5. Viremia and antibody responses of reimmunized rhesus monkeys to challenge^a with wild strains of DEN-2 virus

Monkey series and no.	Viremia	Reciprocal antibody titer								
		CF on day:			HI on day:			N on day:		
		0	15	30	0	15	30	0	15	30
A 294	None	4	256	512	<10	$\geq 10,240$	1,280	80	≥ 640	≥ 640
A 298	None	8	256	256	40	$\geq 10,240$	2,560	80	≥ 640	≥ 640
A 301	None	16	256	256	<10	1,280	1,280	80	≥ 640	≥ 640

^a Challenged with 2×10^6 PFU of BM50-76 10 months after booster immunization.

were similar at both laboratories. Furthermore, the immune response also differed between the two groups of monkeys immunized. A dose response was indicated by N antibody conversions in all high-dose recipients, whereas only 5 of 10 animals receiving the lower dose converted. As judged by N antibody, the 50% immunizing dose was approximately $10^{3.1}$ PFU.

The CF, HI, or N antibody did not always persist after immunization. For example, of the six monkeys that developed N antibody and were later challenged, only four retained the antibody at the time of challenge. The transient nature of the antibodies in some monkeys may have been due to the administration of a suboptimal dose of antigen, leading to the production of short-lived antibody (12), or to the subcutaneous route of immunization. The intracutaneous route was found to be more effective in early studies in humans (10). The presence or absence of detectable N antibody at the time of challenge, however, did not always correlate with protection from viremia. In one case, despite the loss of N antibody, no viremia occurred, whereas in another case viremia developed in the presence of a prechallenged antibody titer of 1:60. These findings indicate that protection from viremia induced by this vaccine may depend upon factors in addition to the presence of humoral antibody.

Although primary immunization did not always provide protection from viremia, immunological memory was stimulated in all monkeys challenged with wild-type DEN-2 virus, as demonstrated by the resulting secondary antibody responses. However, despite the evidence of immune priming, enhancement of viremia, as described in sequential heterotypic dengue infections of monkeys ending with DEN-2 virus, did not occur (5). The viremias seen in the immunized monkeys challenged with DEN-2 virus were shorter than those seen in primary wild-type infections. Also, there was no enhancement of viremia in monkeys challenged with DEN-3 virus 4 months after DEN-2 immunization. The titer and duration of viremia in these monkeys were not substantially different from that seen in unimmunized monkeys receiving DEN-3 virus (unpublished data). The observation of waning antibody after primary immunization suggested that a booster immunization might be used to increase immunity. Secondary immunization led to brisk N antibody responses which persisted at the same titers until the time of challenge by DEN-2 wild-type virus 10 months later. Monkeys which were boosted appeared to be protected against viremia, which might have resulted from the challenge.

Viruses could be isolated from female *A. aegypti* fed on monkeys inoculated with wild-type DEN-2 virus, whereas no virus was detected in mosquitoes biting monkeys receiving the low-dose vaccine. *A. aegypti* were capable of being infected with the vaccine strain of virus after intrathoracic inoculation (unpublished data). We cannot exclude the possibility that vector mosquitoes may be infected with vaccine virus by feeding, but a short or absent viremia should make infection of mosquitoes unlikely.

When used in rhesus monkeys, the candidate DEN-2 vaccine caused no unexpected physical effects and appeared to be immunogenic. Although it was less potent than precursor PGMK-passaged vaccine viruses when administered to monkeys (6), the candidate FRhL vaccine might prove to be acceptable in humans, who are more susceptible to dengue infections. The absence of detectable side effects, the lack of evidence for reversion, the low probability of infecting a vector mosquito, and the enhancement of immunity promoted by a second immunization are biological properties which are compatible with stable attenuation, suggesting that the candidate DEN-2 vaccine PR-159, FRhL-2 is suitable for testing in human volunteers.

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LITERATURE CITED

1. Berge, T. O. 1975. International catalogue of arboviruses, 2nd ed. Department of Health, Education, and Welfare publication no. (CDC) 75-8301. U.S. Government Printing Office, Washington, D.C.
2. Clark, D. H., and J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am. J. Trop. Med. Hyg.* 7: 561-573.
3. 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.
4. Eckels, K. H., V. R. Harrison, P. L. Summers, and P. K. Russell. 1980. Dengue-2 vaccine: preparation from a small-plaque virus clone. *Infect. Immun.* 27:175-180.
5. Halstead, S. B., H. Shortwell, and J. Casals. 1973. Studies on the pathogenesis of dengue infection in monkeys. II. Clinical laboratory responses to heterologous infection. *J. Infect. Dis.* 128:15-22.
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. Kent, J. F., and E. H. Fife, Jr. 1963. Precise standardization of reagents for complement fixation. *Am. J.*

- Trop. Med. Hyg. 12:103-116.
8. **Kuberski, T. T., and L. Rosen.** 1977. A simple technique for the detection of dengue antigen in mosquitoes by immunofluorescence. *Am. J. Trop. Med. Hyg.* 26:533-537.
 9. **Russell, P. K., A. Nisalak, P. Suckanavachana, and S. Vivona.** 1967. A plaque reduction test for dengue virus neutralizing antibodies. *J. Immunol.* 99:285-290.
 10. **Sabin, A. B.** 1952. Research on dengue during World War II. *Am. J. Trop. Med. Hyg.* 1:30-50.
 11. **Sever, J. L.** 1962. Application of microtiter technique to viral serological investigations. *J. Immunol.* 88:320-329.
 12. **Uhr, J. W., and M. S. Finkelstein.** 1967. The kinetics of antibody formation. *Prog. Allergy* 10:37-83.
 13. **Yuill, T. M., P. Sukhavachana, A. Nisalak, and P. K. Russell.** 1968. Dengue virus recovery by direct and delayed plaques in LLC-MK₂ cells. *Am. J. Trop. Med. Hyg.* 17:441-448.