Biological and Immunological Studies of a Dengue 2 Candidate Virus Vaccine

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OBJECTIVE : To investigate the immunological efficacy and mosquito infectivity of a candidate dengue 2 virus vaccine.

BACKGROUND : Dengue Hemorrhagic Fever (DHF) is a severe public health problem in Southeast Asia and represents a potential threat to military troops operating in areas where the dengue viruses are prevalent. For these reasons dengue virus strains are being sought for the preparation of vaccines. Over the past several years, a dengue 2 virus strain has been isolated under conditions suitable for vaccine development (1). The candidate vaccine virus (S1) is a small plaque variant isolated by plaque selection from dengue 2 PR-159, a strain obtained during the 1969 dengue 2 epidemic in Puerto Rico. Compared to the parent strain, (PR-159 PGMK-6), the small plaque variant (PR-159 S1-PGMK-19) was temperature sensitive and appeared to have decreased virulence in suckling mice. While the parent strain produced relatively consistent viremia in Indian Rhesus monkeys on days three to seven following inoculation, the S1 strain only occasionally produced viremia. When viremia did occur, it was as late as the tenth day following inoculation. Despite the inconsistency of viremia, the S1 strain appeared to be strongly immunogenic.

Following initial testing in the rhesus monkeys the S1 strain was further passed four times in fetal rhesus lung cells. A candidate vaccine (PR-159 (S1) Lot 1,PGMK-19 FRL-4) was prepared.

The purpose of this investigation was to study the clinical and immunological affect of this candidate vaccine strain on Indian Rhesus monkeys and to determine its infectivity for mosquitoes biting these monkeys.

METHODS : Two strains of dengue 2 virus were received from Dr. Walter E. Brandt, Department of Virus Diseases, Walter Reed Army Institute of Research. At the time of preparation the frozen parent strain (PR-159 PGMK-6) was reported to have a titer 1.5×10^5 PFU/ml at 35°C and the lyophilized candidate vaccine strain (PR-159 (S1) PGMK-19 FRL-4, Lot 1) was reported to have a titer of 1.9×10^5 PFU/ml at 35°C. The viruses were shipped on dry ice and held at -70°C. The virus preparations were rapidly thawed and reconstituted just prior to use. Indian Rhesus monkeys (Macaca mulatta) were screened for hemagglutination inhibition (HI) antibody against dengue 1-4, Japanese encephalitis, Wesselsbron and Langat antigen and for neutralizing (N) antibody against dengue 2. Fifteen

monkeys were selected which were negative for these antibodies. Monkeys received either the candidate vaccine or the parent strain. Each monkey was inoculated subcutaneously in the left arm with 0.5 ml of the appointed virus strain containing an estimated 0.5 - 1 x 10^5 PFU. The monkeys were examined before inoculation and daily throughout the course of the experiment. General condition, appetite, temperature organomegaly, lymphadenopathy and presence or absence of petechia were noted. Blood was obtained prior to inoculation and on days one through 10, 15, 30 and 60 following inoculation. Blood was submitted for hemogram and platelet count.

Heparinized blood was separated and plasma and leukocytes were submitted for virus assay. Methods for separation of leukocytes are described elsewhere in this annual report (The Isolation of Dengue Viruses from Leukocytes). Virus isolations were attempted by the direct and delayed plaque assay technique. Serum was submitted for serology by HI tests and plaque reduction neutralizations tests (PRNT) against prototype dengue 1-4 antigens.

On days one through ten following inoculation; 30, 3-5 day old female mosquitoes provided by the Department of Medical Entomology were allowed to feed on each monkey. Engorged mosquitoes were separated, incubated for 14 days at 32°C, sacrificed by quick freezing and stored at -70°C. Ten mosquitoes were pooled, (one pool/monkey/day) sonicated in 1.0 ml of Roswell Park Memorial Institute (RPMI) 1640 media supplemented with glutamine, sodium bicarbonate and 10% fetal calf serum, and centrifuged at 1000 x g for 30 minutes. The supernatant was assayed for virus by direct and delayed plaque techniques. Six mosquitoes/day/monkey were decapitated; head squashed preparations were examined for dengue antigen by a direct fluorescent antibody technique (FA).

Monkeys immunized with either the candidate vaccine or the parent dengue 2 strains were challenged with the candidate vaccine strain or with low passage Southeast Asian wild type strains of dengue 2 (BM-50-76 LLCMk$_2$-2) or dengue 3 (CH 1337-74 LLCMk$_2$-10). Dengue 2 (BM-50-76, LLC-Mk$_2$-2) was isolated from an *Aedes aegypti* mosquito collected in the home of a DHF patient; dengue 3 (CH 1337-74, LLC-Mk$_2$-10) was isolated from a DHF patient. The monkeys were handled and samples were collected for isolation and serology in a manner similar to that used in the initial infections.

RESULTS : Fifteen monkeys were inoculated with 0.5 ml of dengue 2 virus preparations. Ten of these received the candidate vaccine strain (PR-159 (S1) PGMK-19, FRL-4, Lot #1) and 5 received the parent strain (PR-159, PGMK-6) (Table 1). At the time of inoculation these virus preparations were titered at 37°C and found to contain 6.7 x 10^2 and 1.3 x 10^5 PFU/ml, respectively. Examination of the 15 monkeys throughout the course of the experiment showed no significant physical or hematological changes.

Using direct and delayed plaque assays at 37°C and 35°C no virus was recovered from the plasma of any of the ten monkeys who received the candidate vaccine virus. Antibodies against dengue 2 virus had developed by 30 days following inoculation in six of these monkeys and HI antibody was found in three.

Viremia was documented in all five of the monkeys inoculated with the parent strain. Virus was isolated from the plasma of one monkey by the second day following inoculation and was found in all monkeys on days six and seven. The viremia persisted in most monkeys through day eight but had cleared by day nine. Dengue 2 virus was isolated from leukocytes taken from monkeys on days eight and nine, late in the course of infection. On two occasions virus was isolated from leukocytes but could not be detected in plasma. Both HI and N antibody to dengue 2 developed by the 15th day after immunization in all of the monkeys inoculated with the parent strain. The antibody titers at the time of measurement did not appear to be related to the time or duration of viremia.

Dengue virus was not detected by either isolation or FA in mosquitoes that fed on monkeys inoculated with the candidate vaccine strain. Infection was detected by direct and delayed plaque isolation and by FA only in mosquitoes fed on monkeys inoculated with the parent virus. Virus was intermittently isolated from mosquitoes that fed on these monkeys on day three through six following inoculations. Isolations could be obtained from mosquitoes at times when no virus could be detected in the plasma, and virus was usually isolated from mosquitoes before or early in the stage of detectable viremia.

Challenge experiments were carried out 126 days following initial inoculation (Table 2). The monkeys which had received the candidate vaccine strain were divided into groups based upon the N antibody titer to dengue 2 antigen at 30 days. Three pairs were established each contained one monkey with an N antibody titer $\leq$ 1:20 and one with a titer of > 1:20. These pairs were inoculated with the candidate vaccine strain, and low passage Southeast Asian wild type strains of dengue 2 (BM-50-76, LLC-Mk_2-2) and dengue 3 (CH 1337-74, LLC-Mk_2-10) respectively. Of the remaining four monkeys who did not developed N antibody following inoculation with the candidate vaccine strain, one was inoculated with the candidate vaccine strain, two were challenged with the wild type dengue 2 strain and one received the wild type dengue 3 virus.

Plasma collected from all monkeys just prior to challenge were retested for HI and N antibodies. Of the monkeys which had received the candidate vaccine strain, only one monkey had N and/or HI antibody at this time, suggesting that the antibody detected at 30 days might have been of the IgM class. All the monkeys which had received the parent strain retained both N and HI antibodies.

The titers of inoculum at the time of injection, determined by plaque assay at 35°C were 5×10^3 PFU/ml for the candidate vaccine strain, 1.1×10^5 PFU/ml for the wild type dengue 2 strain (BM-50-76) and 4.6×10^5 PFU/ml for the wild type dengue 3 strain (CH 1337-74). In the monkeys reimmunized with the candidate vaccine strain, there was no detectable viremia; none the less all of these monkeys developed appreciable titers of dengue 2 HI and N antibody by day 141, 15 days after reimmunization. These antibodies persisted at essentially the same titer for at least one month after immunization.

Of the four vaccinated monkeys challenged with the dengue 2 strain (BM-50-76), three developed viremia and leukocyte infections on the fourth through the sixth day after challenge. The fourth which developed no demonstrable infection was the one monkey which had appreciable amounts of dengue 2 HI and N antibody

just prior to challenge. Challenge of two monkeys that received the parent strain of dengue 2 virus (PR-159, PGMK-6), with wild type dengue 2 (BM-50-76) resulted in HI titers rises but no demonstrable viremia. N antibody was not tested at a level necessary to detect a titer rise, it was already $\geq$ 1:640 at the time of challenge.

Four monkeys were challenged with the dengue 3 wild type virus (CH 1337-74) Two of these, having previously received candidate dengue 2 vaccine virus, developed dengue 3 viremia and cross reactive HI and N antibodies. The other two were initially inoculated with the dengue 2 parent strain (PR 159, PGMK-6). Of these, only one developed a viremia. This occurred at approximately the same time as the viremia occurred in the previous immunization with the dengue 2 parent strain. Both of these monkeys developed cross reactive HI and N antibodies.

This work is now completed and will prepared for publication.

REFERENCE

1. Eckels, K.H., Brandt, W.E., Harrison, V.R. McCown, J.M., and Russell, P.K., 1976. Isolation of Temperature-Sensitive Dengue-2 Virus under Conditions Suitable for Vaccine Development. Inf. & Imm., 14:1221-1227.

Table 1. Viremia and Antibody Responses Following Primary Inoculation of Indian Rhesus Monkeys with Dengue Viruses.

Monkey Number	Primary Inoculum	Viremia Day										Dengue 2 Antibody Day			
		1	2	3	4	5	6	7	8	9	10	0	10	15	30
E228	Vaccine (D2)[a]											0/0[c]	0/	0/10	20/20
E231	Vaccine (D2)											0/0	0/	0/5	0/5
E290	Vaccine (D2)											0/0	0/	0/0	0/0
E293	Vaccine (D2)											0/0	0/	0/0	0/0
E294	Vaccine (D2)											0/0	0/	0/0	0/0
E297	Vaccine (D2)											0/0	0/	0/65	0/80
E298	Vaccine (D2)											0/0	0/	0/0	0/0
E299	Vaccine (D2)						-					0/0	0/	0/0	80/140
E301	Vaccine (D2)						-					0/0	0/	0/20	40/53
F15	Vaccine (D2)							-				0/0	0/	0/10	0/10
F17	Parent (D2)[b]	-	-	+	+	⊞	⊞	+	⊞	⊞	-	0/0	0/	80/130	160/220
F66	Parent (D2)	-	-	-	-	+	⊞	+	⊞	-	-	0/0	0/	160/220	160/680
F67	Parent (D2)	-	-	-	-	-	⊞	+	⊞	-	-	0/0	40/	320/1000	320/1000
F70	Parent (D2)	-	-	-	-	⊟	⊞	+	⊞	-	-	0/0	20/	320/220	160/400
F74	Parent (D2)	-	+	⊟	⊞	-	⊞	+	-	-	-	0/0	10/	320/340	320/1000

[a] Dengue 2 Candidate Vaccine (PR-159, (S1) Lot 1, June 76)
[b] Dengue 2 Parent Strain (PR-159, PGMK-6)
[c] Reciprocal Hemagglutination Inhibition/Neutralization Titers, 0 = < 1:10
+ Isolation of Dengue 2 from Plasma
- No isolation
⊞ Isolation of Dengue 2 from Mosquitoes
⊞ Isolation of Dengue 2 from Leukocytes

Table 2. Viremia and Antibody Responses Following Secondary Inoculation of Indian Rhesus Monkeys with Dengue Viruses

Monkey Number	Secondary Inoculum	Viremia Day										Dengue 2 Antibody Day		
		1	2	3	4	5	6	7	8	9	10	126	141	156
E228	Vaccine (D2)[a]							-				0/0[d]	160/200	80/250
E231	BM50-76 (D2)[b]				+	▲	▲					0/0	1280/≥640	640/≥640
E290	BM50-76 (D2)				▲	▲						0/0	5120/≥640	640/≥640
E293	BM50-76 (D2)				+	▲						0/0	80/90	80/170
E297	CH1337-74 (D3)[c]				+	+						0/0	640/250	320/320
E298	Vaccine (D2)											0/0	160/300	80/160
E299	BM50-76 (D2)					-	-	-		-		80/100	320/≥640	160/≥640
E301	Vaccine (D2)											0/0	80/180	40/160
F15	CH1337-74 (D3)		-		▲	+						0/0	320/320	160/550
F17	CH1337-74 (D3)				+	+	+					160/300	1280/340	1280/190
F66	CH1337-74 (D3)											160/≥640	5120/≥640	2560/≥640
F67	BM50-76 (D2)											320/≥640	1280/≥640	1280/≥640
F70	BM50-76 (D2)							-				640/≥640	5120/≥640	256/≥640

[a] Dengue 2 Candidate Vaccine (PR-159, (S1) Lot 1, June 76)
[b] BM-50-76, LLC-Mk_2, Southeast Asian Dengue 2
[c] CH 1337-74, LLC-Mk_2, Southeast Asian Dengue 3
[d] Reciprocal Hemagglutination Inhibition/Neutralization Titers 0 = < 1:10
+ Isolation of Dengue 2 from Plasma
No isolation
□ Isolation of Dengue 2 from Mosquitoes
▲ Isolation of Dengue 2 from Leukocytes