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VIROLOGY

Virology 315 (2003) 345–352

www.elsevier.com/locate/yviro

Needle-free Biojector injection of a dengue virus type 1 DNA vaccine with human immunostimulatory sequences and the GM-CSF gene increases immunogenicity and protection from virus challenge in *Aotus* monkeys

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Received 20 March 2003; returned to author for revision 19 June 2003; accepted 10 July 2003

Abstract

A dengue-1 DNA vaccine containing sequences encoding premembrane and envelope proteins (DIME) was previously shown to elicit virus neutralizing antibodies in rhesus and *Aotus* monkeys, and the primates were partially protected from viremia upon challenge. To increase the neutralizing antibody levels and subsequent protection from virus challenge, four strategies were evaluated: (a) coimmunization with a plasmid expressing *Aotus* GM-CSF gene; (b) coimmunization with a plasmid containing human immunostimulatory sequences (ISS); (c) coimmunization with both the GM-CSF gene and ISS; and (d) delivery of vaccine using the needle-free Biojector system. Vaccination with the mixed formulation containing DIME, GM-CSF gene, and ISS, by either needle injection or Biojector, led to neutralizing antibody titers that were stable for up to 6 months after vaccination. Furthermore, 6 of 7 monkeys (85%), and 7 of 8 monkeys (87%) receiving this formulation were completely protected from viremia when challenged 1 and 6 months after vaccination, respectively. This is a significant improvement compared to our previous study in which one of three monkeys (33%) receiving just the DIME vaccine was completely protected from viremia at 6 months after immunization.

Keywords: Dengue; GM-CSF; Immunostimulatory; Vaccine; Biojector

Introduction

Dengue is one of the most important arboviral diseases of humans. An estimated 100 million dengue infections are caused each year by four antigenically distinct serotypes of dengue virus, resulting in widespread dengue fever (DF) and at least 250,000 cases of dengue hemorrhagic fever (DHF) (Halstead, 1988). These viruses are transmitted primarily by the mosquito *Aedes aegypti* and are endemic throughout the tropical and subtropical regions of the world. A major expansion of dengue transmission has occurred throughout the tropical Americas accompanied by a large increase in the number of DHF cases. Despite the public health importance of DF and DHF, vector control, which has proven difficult and costly to sustain over time, is the only available control measure (Gubler, 1994). Vaccines are available for two related flaviviruses, yellow fever (YF) virus and Japanese encephalitis (JE) virus (Barrett, 1997a, 1997b). However, decades of effort have not produced a successful dengue virus vaccine. Live-attenuated dengue viruses, while immunogenic, have been associated with high reactogenicity and clinical complications (Edelman et al., 1994; McKee et al., 1987). Inactivated dengue viruses

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doi:10.1016/S0042-6822(03)00542-7

Table 1
Study design

Group	Vaccine, (0, 1, and 5 months)	*n*	No. challenged	
			Month 6	Month 11
1	PBS	8	4	4
2	D1ME (D)	4	4	—
3	D1ME + VR1721 (DG)	4	4	—
4	D1ME + pHis64 (DI)	4	4	—
5	D1ME + VR1721 + pHis64 (DGI)	8	4	4
6	D1ME + VR1721 + pHis64(BJ)[a] (DGI-BJ)	7	4	3
7	D1WP74[b]	7	3	4

[a] BJ denotes injection by Biojector.
[b] D1WP74 is dengue-1 virus Western Pacific 74 strain.

have been less immunogenic (Schlesinger, 1977), and recombinant dengue proteins have failed to consistently protect nonhuman primates from homologous dengue virus challenge (Deubel et al., 1988; Eckels et al., 1994).

DNA vaccines offer an alternative strategy for developing effective dengue virus vaccines. This approach, in which plasmid DNA expressing an antigen of interest is used as a vaccine, induces immune responses in animal models against a number of different viruses, including several flaviviruses (Colombage et al., 1998; Konishi et al., 1998; Phillpotts et al., 1996; Schmaljohn et al., 1997). We have previously shown that a dengue-2 DNA vaccine containing the genes for premembrane (prM) and 92% of envelope (E) protein induced neutralizing antibody response in mice (Kochel et al., 1997) and the vaccinated mice had a higher survival rate following dengue-2 virus challenge (Porter et al., 1998). When four different dengue-1 DNA vaccine candidates expressing truncated or full-length E with or without prM were compared, we observed that the candidate expressing prM and full-length E produced virus-like particles and elicited long-lasting neutralizing antibodies in mice (Raviprakash et al., 2000a). We further showed that this dengue-1 vaccine was immunogenic in nonhuman primates and afforded complete to partial protection from challenge (Kochel et al., 2000; Raviprakash et al., 2000b). These primate trials provided proof of principle that a dengue DNA vaccine could elicit protective immune responses in nonhuman primates. However, the studies also indicated that it was necessary to find ways to improve neutralizing antibody responses and protection from challenge. In this study, we have evaluated three different strategies to achieve this goal.

Results

Aotus monkeys were immunized as detailed in Table 1 and under Materials and methods. Three DNAs were used, as follows: DIME (D) is plasmid VR1012 containing dengue-1 prM and E genes; VR1721 (G) is a plasmid expressing the *Aotus* GM-CSF gene; and pHis64 (I) is a plasmid containing multiple copies of the human immunostimulatory sequences. The vaccines were delivered either by needle injection or by using the Biojector (BJ).

Antibody response

The antibody responses in each group of animals were measured by single dilution (1:100) indirect ELISA, and the results are shown in Fig 1. All of the vaccinated animals except one in the DI group had seroconverted after the first immunization (not shown). The antibody levels increased somewhat after the second dose of vaccine and remained stable until time of challenge (Fig. 1). All seven monkeys that received a single subcutaneous dose of dengue-1 virus (positive control) and none of the eight PBS injected (negative control) monkeys had dengue-specific antibody.

The ability of antidengue antibodies elicited by dengue-1 DNA vaccines to neutralize dengue-1 virus in vitro was measured by plaque reduction neutralization test (PRNT). Table 2 shows $PRNT_{50}$ titers for individual animals at months 3 (2 months after the first boost), 6 (1 month after the second boost), and 11 (6 months after the second boost). The third dose of vaccine given at month 5 had a strong boosting effect on neutralizing antibody response, as reflected by neutralization titers in sera obtained at month 6. For monkeys challenged at month 11, the neutralizing antibody titers remained stable or only declined slightly between month 6 and 11. When mean titers (3 months) for groups vaccinated with different formulations were compared, only the virus-infected positive control group was different from all other groups ($P < 0.0009$). However at 6 months, significant differences were indicated only between the positive control group compared to the PBS group ($P = 0.001$), the DI group ($P = 0.045$), and the DGI group ($P = 0.009$), indicating that the neutralization titers for groups vaccinated with D, DG, or DGI-BJ were not different from that of the virus-infected positive control group. At 11 months, the titers for the positive control group differed from the DGI group ($P = 0.026$) but not the DGI-BJ group ($P = 0.207$).

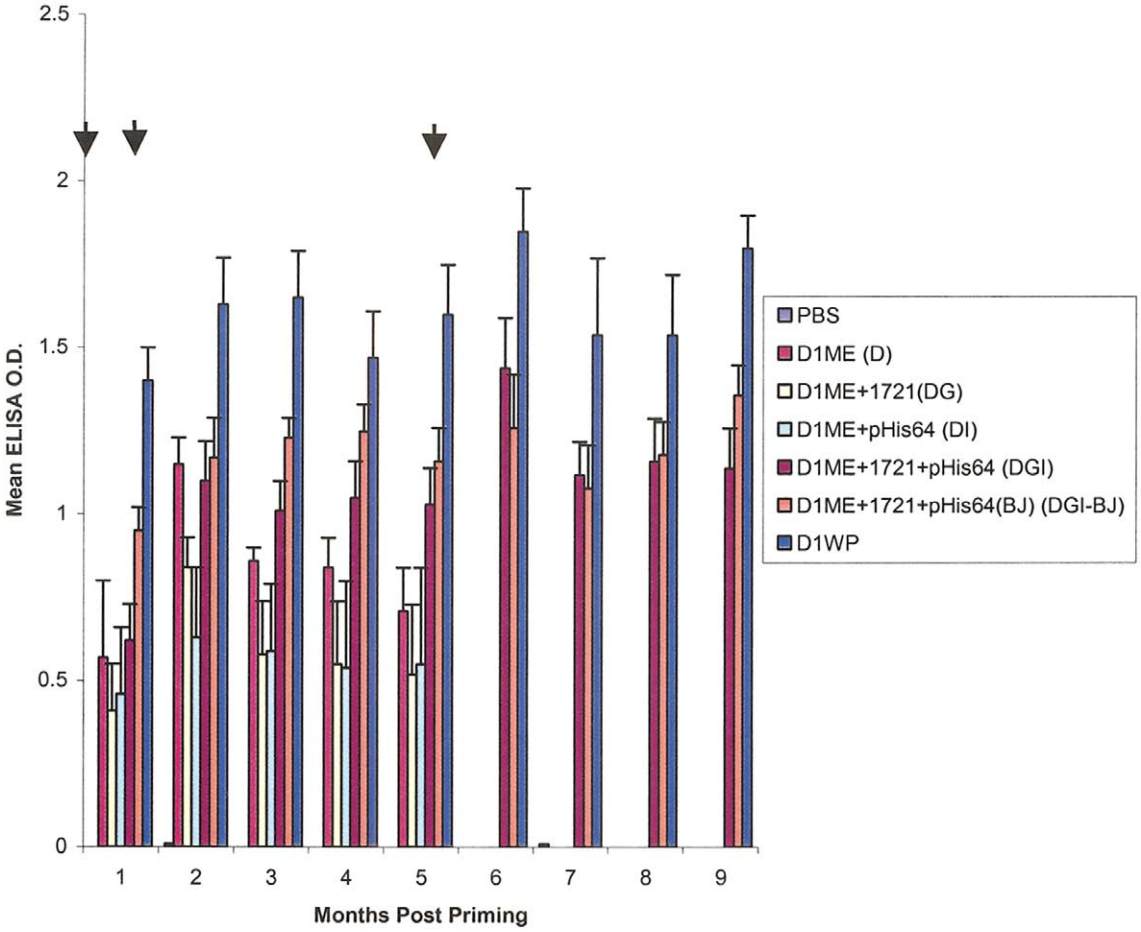

Fig. 1. Animals were vaccinated at months 0, 1, and 5 (↓). Sera from monthly bleeds were diluted 1:100 and assayed for dengue-specific antibody by ELISA as described under Materials and methods. The mean ELISA OD and the standard deviation for each group are shown.

Protection from live virus challenge

Animals were challenged with live dengue-1 virus at month 6 (1 month after the third vaccine dose) and at month 11 (6 months after the third vaccine dose) to assess the protective efficacy of vaccine-induced immune responses. Four monkeys from groups vaccinated with D, DG, DI, DGI, and three monkeys from group DGI-BJ were challenged at month 6 by subcutaneous injection of live dengue-1 virus, and serum samples from daily bleeds were analyzed for the presence of infectious virus (viremia). Four monkeys each from the PBS and D1WP74 groups were included as controls. The PBS control animals developed 2–4 days of viremia (mean = 3.25 days), whereas animals previously infected with dengue-1 virus (D1WP74 group) did not develop detectable viremia (Table 3). Compared to PBS control animals, the duration of viremia was significantly reduced in animals vaccinated with D ($P = 0.021$), DGI ($P = 0.039$), DGI-BJ ($P = 0.011$), and the previously infected positive control group ($P = 0.006$). Some breakthrough viremia was observed in the presence of neutralizing antibody, for example, in animals 0835, WR323, and WR339. Most significantly, six of the seven animals (85.7%) vaccinated with DGI or DGI-BJ were completely protected from viremia. The only viremic animal in this group (WR399) failed to develop neutralizing antibody. Another group of eight animals vaccinated with the same formulation (four with DGI; four with DGI-BJ) were challenged 6 months after the last dose of vaccine. Seven of the eight vaccinated animals (87.5%) were completely protected from viremia and one animal had a single day of viremia (Table 4). The mean viremia days for the vaccinated group ($n = 7$) was 0.14 compared to 3.75 days for PBS control group ($n = 4$) ($P < 0.0009$).

Discussion

This study has evaluated early and sustained antibody responses to a dengue-1 DNA vaccine in *Aotus* monkeys, administered with or without immune enhancing agents, by needle injection and Biojector delivery. Significantly higher neutralizing antibody titers were achieved by delivering D1ME (D), D1ME plus VR1721 (DG), or a mixture of D1ME, VR1721, and pHis64 via the Biojector (DGI-BJ). In DGI-BJ-vaccinated animals which were followed for 6

Table 2
50% virus neutralization titers in vaccinated *Aotus* monkeys

Vaccine	Animal I.D.	3 months		6 months		11 months	
		$PRNT_{50}$	Grp. Mean ± SE	$PRNT_{50}$	Grp. Mean ± SE	$PRNT_{50}$	Grp. Mean ± SE
PBS	1251	0	0.0 ± 0	0	0.0 ± 0		0.0 ± 0
	2209	0		0			
	WR406	0		0			
	WR437	0		0			
	ICD2	0		0		0	
	1960	0		0		0	
	2164	0		0		0	
	WR423	0		0		0	
D1ME (D)	WR419	0	2.5 ± 2	480	365.0 ± 167		
	WR420	0		770			
	WR413	10		0			
	WR371	0		210			
D1ME + 1721 (DG)	0835	0	33.7 ± 19	95	357.5 ± 282		
	0610	65		1200			
	0999	0		0			
	WR409	70		135			
D1ME + pHis64 (DI)	WR323	0	3.7 ± 3	180	156.2 ± 74		
	WR454	0		0			
	WR339	0		95			
	WR417	15		350			
D1ME + 1721 + pHis64 (DGI)	1985	24	21.1 ± 5	415	161.8 ± 42		78.7 ± 24
	0664	35		225			
	WR441	35		160			
	WR399	0		0			
	2339	20		150		150	
	2358	0		130		55	
	WR449	40		135		50	
	WR432	15		80		60	
D1ME + 1721 + pHis64 (BJ) (DGI-BJ)	WR445	0	60.0 ± 18	80	307.8 ± 71		332.5 ± 117
	T218	70		510			
	WR411	60		270			
	WR424	40		270		300	
	WR455	120		510		640	
	WR374	10		65		70	
	WR396	120		450		320	
D1WP74	1958	90	402.8 ± 89	240	859.2 ± 253		753.3 ± 309
	WR416	580		1920			
	WR370	320		1535			
	T211	830		640			
	T261	320		1090		1280	
	0990	300		270		210	
	WR384	380		320		770	

months after immunization, the antibody levels did not decline as observed in earlier trials of rhesus and *Aotus* monkeys that were needle injected with vaccine DNA alone. The antibody levels were completely protective with the exception of one monkey that developed a single day of detectable viremia. This is a significant improvement compared to an earlier study in which only 33% protection was achieved in *Aotus* monkeys vaccinated with D1ME alone and challenged 6 months after third dose (Kochel et al., 2000).

Most naked DNA vaccines have suffered from lower than desired antibody responses. It is therefore important to identify means by which to enhance antibody responses. We have considered four such strategies for improving dengue-1 DNA vaccine. First, we chose to evaluate coimmunization with pHis64 plasmid. This plasmid contains multiple copies of human-specific ISS. In a previous study, we had observed that a dengue-2 DNA vaccine elicited better antibody responses in mice when coinjected with pUC-19 plasmid DNA containing mouse-specific ISS (Porter et al., 1998). Immunostimulatory sequences have been used as adjuvants with both protein- and DNA-based vaccines. In the case of gp120-depleted, whole killed HIV-1 vaccine, it was shown that simultaneous injection of HIV-1 and oligonucleotides containing ISS led to higher p24-specific antibody response, stronger lymphocyte proliferation and β-chemokine production (Moss et al., 2000, 2001). An HIV-1 gp160 subunit vaccine induced a typical Th2 domi-

Table 3
Viremia in *Aotus* monkeys vaccinated (0, 1, and 5 months) and challenged (6 months) with live dengue-1 virus

Vaccine		Monkey I.D.	Nt. ab. titer[a]	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Group mean	Group median
PBS	1	1251	0	−	−	+	+	−	−	−	−	3.25	3.50
	2	2209	0	−	+	+	+	+	−	−	−		
	3	WR406	0	−	+	+	+	−	−	−	−		
	4	WR437	0	−	−	+	−	+	+	+	−		
D1ME (D)	5	WR419	480	−	−	−	−	−	−	−	−	0.50	0.00
	6	WR420	770	−	−	−	−	−	−	−	−		
	7	WR413	0	−	−	+	−	+	−	−	−		
	8	WR371	210	−	−	−	−	−	−	−	−		
D1ME +	9	0835	95	+	−	+	+	+	−	−	−	1.00	0.00
1721	10	610	1200	−	−	−	−	−	−	−	−		
(DG)	11	0999	0	−	−	−	−	−	−	−	−		
	12	WR409	135	−	−	−	−	−	−	−	−		
D1ME +	13	WR323	180	−	−	−	−	+	−	−	−	1.25	1.00
pHis64	14	WR454	0	−	−	+	+	+	−	−	−		
(DI)	15	WR339	95	−	−	−	+	−	−	−	−		
	16	WR417	350	−	−	−	−	−	−	−	−		
D1ME +	17	1985	415	−	−	−	−	−	−	−	−	0.75	0.00
1721 +	18	0664	225	−	−	−	−	−	−	−	−		
pHis64	19	WR441	160	−	−	−	−	−	−	−	−		
(DGI)	20	WR399	0	−	−	+	+	+	−	−	−		
D1ME +	21	WR445	80	−	−	−	−	−	−	−	−	0.00	0.00
1721 + pHis64 (BJ) (DGI-BJ)	22	T218	510	−	−	−	−	−	−	−	−		
	23	WR411	270	−	−	−	−	−	−	−	−		
D1WP74	24	1958	240	−	−	−	−	−	−	−	−	0.00	0.00
	25	WR416	1920	−	−	−	−	−	−	−	−		
	26	WR370	1535	−	−	−	−	−	−	−	−		
	27	T211	640	−	−	−	−	−	−	−	−		

Note. Animals were challenged on day 0 by subcutaneous inoculation of live dengue-1 virus. Animals were bled daily thereafter and viremia was determined as described under Materials and methods. + and − indicate presence or absence of virus, respectively.

[a] $PRNT_{50}$ at the time of challenge.

Table 4
Viremia in *Aotus* monkeys vaccinated (0, 1, and 5 months) and challenged (11 months) with live dengue-1 virus

Vaccine		Monkey I.D.	Nt. ab. titer[a]	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Group mean	Group median
PBS	1	ICD2	0	−	−	+	+	+	−	−	−	3.75	3.5
	2	1960	0	+	−	+	+	+	−	−	−		
	3	2164	0	−	−	+	+	+	−	−	−		
	4	WR423	0	−	−	+	+	+	+	+	−		
D1ME +	5	2339	150	−	−	−	−	−	−	−	−	0	0
1721 +	6	2356	55	−	−	−	−	−	−	−	−		
pHis64	7	WR449	50	−	−	−	−	−	−	−	−		
(DGI)	8	WR432	60	−	−	−	−	−	−	−	−		
D1ME +	9	WR424	300	−	−	−	−	−	−	−	−	0.25	0
1721 + pHis64 (BJ) (DGI-BJ)	10	WR455	640	−	−	−	−	−	−	−	−		
	11	WR374	70	−	−	−	+	−	−	−	−		
	12	WR396	320	−	−	−	−	−	−	−	−		
D1WP74	13	T261	1280	−	−	−	−	−	−	−	−	0	0
	14	0990	210	−	−	−	−	−	−	−	−		
	15	WR384	770	−	−	−	−	−	−	−	−		

Note. Animals were challenged on day 0 by subcutaneous inoculation of live dengue-1 virus. Animals were bled daily thereafter and viremia was determined as described under Materials and methods. + and − indicate presence or absence of virus, respectively.

[a] Fifty percent virus neutralization titers ($PRNT_{50}$) at the time of challenge.

nated immune response in mice; however, when the mice were boosted with vaccine plus ISS oligonucleotides, a 7-fold increase in the production of IgG2a antibodies and a 10-fold increase in the interferon-γ secreted by restimulated splenic cells (Deml et al., 1999). In a study contrasting immunostimulatory sequences (ISS) contained in plasmids or oligonucleotides, it was shown that human monocyte-derived dendritic cells are activated by ISS contained in plasmid DNA but not by ISS in oligonucleotides (Schattenberg et al., 2000). All of these studies have utilized either cell-culture models or mouse model systems and no data on in vivo efficacy of ISS in higher animal systems are available. Although the differences were not statistically significant, this study suggests that coinjection of pHis64 plasmid resulted in decreased neutralizing antibody levels. Groups DI and DGI [($PRNT_{50}$) = 156 and 161, respectively], both had decreased antibody titers compared to vaccine DNA alone ($PRNT_{50}$ = 365).

Second, we evaluated coimmunization with VR1721, a plasmid expressing *Aotus* GM-CSF gene. We have previously observed in mice that neutralizing antibody produced by a dengue-2 DNA vaccine increased significantly when the vaccine DNA was coinjected with plasmid expressing murine GM-CSF gene. Several cytokine genes have been used as genetic adjuvants for DNA vaccines. Among the nine different cytokine genes tested with a rabies virus DNA vaccine, GM-CSF consistently enhanced the immune responses (Xiang et al., 1997). Infiltration of CD11c+ immature dendritic cells at the site of GM-CSF DNA injection has been postulated as a mechanism by which immune responses are enhanced (Haddad et al., 2000). There has been some concern regarding use of plasmids expressing highly potent molecules such as GM-CSF. In a recent study in mice and rabbits, repeated ID and IM injections of a multivalent malaria DNA vaccine which included GM-CSF plasmid was found to be safe and well tolerated without any evidence of autoimmune pathology (Parker et al., 2001). In this study, use of *Aotus*-specific GM-CSF gene did not significantly affect the neutralizing antibody levels. In groups vaccinated with vaccine DNA alone (D) or in combination with GM-CSF plasmid (DG), three of four animals seroconverted with mean $PRNT_{50}$ titers of 365 and 476, respectively.

Although the DGI formulation which contained both ISS and GM-CSF gene in addition to vaccine DNA, did not significantly increasing neutralizing antibody titers, it did afford 100% protection to animals challenged 6 months after vaccination. This is in contrast to 33% protection observed in an earlier study in which animals were vaccinated with D1ME DNA alone and challenged 6 months after (Kochel et al., 2000). When animals were vaccinated with vaccine DNA alone, only three of the four animals were protected when challenged 1 month after vaccination. It is therefore possible that the immune enhancing agents are enhancing protective immune responses other than neutralizing antibody.

Our third strategy to improve dengue-1 DNA vaccine utilized an alternate method of delivering the vaccine. Delivery of agents via the needle-free Biojector results in a better tissue distribution of the material. Use of Biojector greatly increased the uptake of plasmid DNA expressing human papilloma virus E7 gene (Manam et al., 2000). Ten- to 50-fold higher antibody titers were reported for a malaria DNA vaccine delivered by Biojector compared to that obtained by needle injection (Aguiar et al., 2001). In a recent clinical trial comparing hepatitis A virus vaccine delivered by needle and Biojector, a higher proportion of people vaccinated by Biojector seroconverted (Williams et al., 2000). We immunized 15 Aotus monkeys with the mixed formulation DGI, 8 by needle injection, and 7 by Biojector. The needle-injected group had a mean $PRNT_{50}$ titer of 161 at 6 months compared to 307 for the group injected by the Biojector. In contrast to needle injection, the titers among animals injected by the Biojector were more uniform and consistent and did not decline from month 6 to month 11. Although the titers in needle-injected animals were sufficient to protect animals from challenge, we speculate that the higher titers in Biojector-injected animals may afford longer term protection from virus challenge. It will be interesting to study some of these aspects in a human trial.

Materials and methods

Animals

Aotus monkeys *(Aotus nancymae)*, of either sex, age 5 to 15 years, weighing between 0.85 and 1.1 kg, were housed at the Naval Medical Research Center/Walter Reed Army Institute of Research animal facility in Silver Spring, MD. The monkeys were prescreened for the presence of dengue-specific antibody by enzyme-linked immunosorbant assay (ELISA) and PRNT. Only those animals that did not show evidence of previous dengue exposure were included in the study. Animals were excluded if the ELISA OD exceeded two or more standard deviations at a 1:100 dilution of their sera when tested against dengue-1 antigen compared to the OD obtained with a negative control antigen, or if the sera exhibited greater than 30% dengue-1 virus neutralization at 1:10 dilution in a standard PRNT.

DNA

The vaccine construct DIME containing dengue-1 prM and full-length E genes cloned into vector pVR1012 (Vical Inc., San Diego, CA) has been described previously (Raviprakash et al., 2000a). VR1721 is a plasmid expressing the *Aotus* GM-CSF gene. Plasmid pHis64 which contains multiple copies of human ISS was kindly provided by Dr. Heather Davis, University of Ottawa, Canada. All DNA were purified by alkaline lysis (Maniatis T, 1984) followed by chromatography on a Sepharose S-1000 (Pharmacia)

column (Horn et al., 1995). Endotoxin levels (30–50 EU/mg DNA) were determined by standard limulus amoebocyte lysate assay.

Immunization

One microgram of vaccine DNA (D) was administered either with or without 0.1 mg VR1721 (G) and/or 0.5 mg pHis64 plasmid (I), as shown in the Study Design (Table 1), and 0.4 ml of vaccine formulation (DNA in PBS) was administered intradermally (id) at 4–6 sites in the chest area. When Biojector was used to deliver vaccine, 4×100 μl aliquots were delivered id in the thigh area. The vaccinations were repeated at months 1 and 5 after the initial inoculation. The PBS control animals were injected id with 0.4 ml of PBS using a needle. The positive control (D1WP74) group was inoculated (at time zero) subcutaneously in the upper left arm with 0.5 ml of DMEM containing 2×10^4 PFU of dengue-1 virus.

Antibody analyses

The monkeys were bled at monthly intervals, and their sera were tested for dengue-1-specific antibody by ELISA and/or PRNT. IgG antibody was detected by ELISA as previously described (Ansari et al., 1993) except that polyethylene glycol (PEG) precipitated dengue-1 virions were used as the antigen, and a peroxidase-labeled antihuman immunoglobulin was used as the conjugate. PRNT was performed with vero cells as described (Russell et al., 1967), using twofold serial dilutions of serum samples. A pool of the sera of animals collected before they were primed was used as the negative control for PRNT. $PRNT_{50}$ titers were determined by probit analysis. $PRNT_{50}$ titers were considered zero if a 1:10 serum dilution caused less than 50% reduction.

Virus challenge and viremia

Monkeys were challenged at either month 6 or month 11 (1 and 6 months after third dose, respectively; Table 1). Each monkey was bled prior to challenge and then inoculated subcutaneously in the upper left arm with 0.5 ml DMEM containing 2×10^4 PFU of dengue-1 virus. The virus used for challenge (dengue-1 Western Pacific 74) was prepared from infected fetal Rhesus lung cells and was kindly provided by Dr. K. Eckels, Walter Reed Army Institute of Research (Silver Spring, MD). Monkeys were bled daily for 10 days and again on days 21 and 28 after challenge. Sera were used to measure viremia and antibody responses. Viremia was measured by inoculating 25-cm^2 flasks of confluent Vero cells in duplicate with 0.2 ml of a 1:10 diluted serum sample. The inoculated cells were incubated for 10 days at 37°C in a CO_2 incubator. The cells were then scraped off the flasks, washed with PBS, and spotted in duplicate onto immunofluorescence slides. The slides were processed for indirect immunofluorescent staining using mouse monoclonal antibody 7E11 (specific for NS-1 protein) and FITC-conjugated anti-mouse immunoglobulin. They were examined under a fluorescent microscope with appropriate positive and negative controls.

Statistical analyses

Virus neutralization titers (mean titers for groups) and the mean viremia days for each group were analyzed using one-way ANOVA with Dunnett's post-hoc test or Tukey HSD test to determine whether there were significant differences among groups in the virus neutralization titer and viremia.

Acknowledgments

We thank Heather Kelly for technical assistance. This work was supported by the NMRC work unit number 63002A.810.SA0085. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animals Resources, National Research Council, DHHS Publication No. (NIH) 86–23 (1985). The opinions and assertions contained herein are not to be considered official or to reflect the views of the U.S. Navy or the Naval Service at large.

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