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Tick-Borne Langat/Mosquito-Borne Dengue Flavivirus Chimera, a Candidate Live Attenuated Vaccine for Protection against Disease Caused by Members of the Tick-Borne Encephalitis Virus Complex: Evaluation in Rhesus Monkeys and in Mosquitoes

ALEXANDER G. PLETNEV,[1]* MICHAEL BRAY,[2] KATHRYN A. HANLEY,[1] JIM SPEICHER,[1] AND RANDY ELKINS[1]

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892,[1] and Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702[2]

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Langat virus (LGT), strain TP21, a naturally avirulent tick-borne flavivirus, was used to construct a chimeric candidate virus vaccine which contained LGT genes for premembrane (preM) and envelope (E) glycoprotein and all other sequences derived from dengue type 4 virus (DEN4). The live virus vaccine was developed to provide resistance to the highly virulent, closely related tick-borne flaviviruses that share protective E epitopes among themselves and with LGT. Toward that end the chimera, initially recovered in mosquito cells, was adapted to grow to high titer in qualified simian Vero cells. When inoculated intraperitoneally (i.p.), the Vero cell-adapted LGT TP21/DEN4 chimera remained completely attenuated for SCID mice. Significantly, the chimera protected immunocompetent mice against the most virulent tick-borne encephalitis virus (TBEV). Subsequently, rhesus monkeys were immunized in groups of 4 with 10^5 or 10^7 PFU of LGT strain TP21, with 10^5 PFU of DEN4, or with 10^3, 10^5, or 10^7 PFU of the chimera. Each of the monkeys inoculated with DEN4 or LGT TP21 became viremic, and the duration of viremia ranged from 1 to 5 days. In contrast, viremia was detected in only 1 of 12 monkeys inoculated with the LGT TP21/DEN4 chimera; in this instance the level of viremia was at the limit of detection. All monkeys immunized with the chimera or LGT TP21 virus developed a moderate to high level of neutralizing antibodies against LGT TP21 as well as TBEV and were completely protected against subsequent LGT TP21 challenge, whereas monkeys previously immunized with DEN4 virus became viremic when challenged with LGT TP21. These observations suggest that the chimera is attenuated, immunogenic, and able to induce a protective immune response. Furthermore, passive transfer of serum from monkeys immunized with chimera conferred significant protection to mice subsequently challenged with 100 i.p. 50% lethal doses of the highly virulent TBEV. The issue of transmissibility of the chimera by mosquitoes was addressed by inoculating a nonhematophagous mosquito, *Toxorhynchites splendens*, intrathoracically with the chimera or its DEN4 or LGT parent. Neither the LGT TP21/DEN4 vaccine candidate nor the wild-type LGT TP21 virus was able to infect this mosquito species, which is highly permissive for dengue viruses. Certain properties of the chimera, notably its attenuation for monkeys, its immunogenicity, and its failure to infect a highly permissive mosquito host, make it a promising vaccine candidate for use in immunization against severe disease caused by many tick-borne flaviviruses.

The tick-borne flavivirus complex includes Kyasanur forest disease, Langat, Louping ill, Negishi, Omsk hemorrhagic fever, Powassan, and tick-borne encephalitis (formerly called Russian spring-summer encephalitis) viruses (TBEV) (5, 17). These viruses are endemic throughout most of the Northern Hemisphere and, except for Langat, cause disease of various severity that can have a mortality rate as high as 20 to 30%. The tick-borne flaviviruses share envelope glycoprotein epitopes that can induce cross-resistance among viruses of the group (26, 27). Approximately three decades ago, these properties of antigenic cross-reactivity and the existence of virulence polymorphism among tick-borne flaviviruses suggested that successful immunization might be achieved using a live, naturally attenuated tick-borne flavivirus. The impetus for this approach was the recovery of a virus from ticks in Malaysia, namely Langat virus (LGT), strain TP21, that did not appear to be associated with human disease under natural conditions (33). Shortly after LGT TP21 was isolated, an attenuated mutant of LGT, designated strain E5, was selected by 42 passages in embryonated chicken eggs (35). LGT E5 exhibited less virulence for monkeys inoculated intracranially and intraspinally than its TP21 parent. Also of note was the fact that E5 showed reduced neurovirulence and very little evidence of neuroinvasiveness in normal mice. Before evaluating the wild-type LGT strain TP21 or its more attenuated mutant E5 as a possible vaccine candidate for use in prophylaxis of severe human disease caused by certain members of the tick-borne flavivirus group, we sought to reduce or ablate the last vestiges of viru-

* Corresponding author. Mailing address: Building 7, Room 236, NIAID, NIH, 7 Center Dr., MSC 0740, Bethesda, MD 20892. Phone: (301) 402-7754. Fax: (301) 496-8312. E-mail: apletnev@niaid.nih.gov.

lence of these LGT strains for mice, specifically SCID mice that are 10^6 to 10^8 times more sensitive than immunocompetent mice for detection of neuroinvasiveness (24).

With the exception of the yellow fever virus 17D vaccine that is used extensively throughout the world, attempts to produce an effective live attenuated vaccine against other viruses of the *Flaviviridae* family have not yielded a licensed product. However, recent advances in recombinant DNA technology have made possible a novel approach for developing live attenuated flavivirus vaccines. This strategy includes recovery of infectious virus from RNA transcripts derived from a full-length cDNA clone of the viral genome. The availability of infectious cDNA clones of several flaviviruses has made it possible to construct viable viruses bearing attenuating mutations that had been introduced into the cDNA clone by site-directed mutagenesis (13, 14, 16, 18, 23, 39).

Using this technology it has also been possible to create new chimeric flaviviruses in which the structural protein genes of a full-length cDNA clone of a flavivirus are replaced by the corresponding viral genes of another flavivirus belonging to another antigenic group (1, 4, 7, 9, 22, 36). Substitution of genes is facilitated by the fact that the organization of the viral genome is highly conserved among all flaviviruses. The genome consists of a single 11-kb positive-strand RNA that contains a 5′ noncoding region followed by the genes for three structural proteins, namely, capsid (C), premembrane (preM), and envelope glycoprotein (E), followed by the genes for seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, and terminating in a 3′ noncoding region.

In some instances the two parents of a chimera can also differ in their insect vector specificity. We used both the antigenic and host range restriction approaches to develop a live attenuated vaccine for prevention of disease caused by the highly virulent tick-borne flaviviruses (22–25). The chimeras used initially for this purpose contained the genes for structural preM and E glycoproteins of TBEV and the remaining sequences from the mosquito-borne dengue type 4 virus (DEN4). Later this strategy was applied to tick-borne LGT strain TP21 or E5. The TBEV/DEN4 and LGT/DEN4 chimeras exhibited a modest reduction in neurovirulence for mice, as measured by intracerebral inoculation. However, considerably more impressive was the effect of chimerization on neuroinvasiveness, a property that reflects the capacity of virus to replicate at a peripheral site and then spread to the central nervous system, where it causes encephalitis. Chimerization of TBEV or LGT (TP21 or E5) with DEN4 completely ablated neuroinvasiveness when assayed by the most sensitive indicator system, the SCID mouse. For example, peripheral inoculation with 10^7 PFU of any of these chimeras failed to produce encephalitis in SCID mice (24). Also, in a previous study the TBEV/DEN4 chimera protected normal mice against challenge by homotypic, highly virulent TBEV (22). More recently it was observed that the preM and E proteins of LGT TP21 or LGT E5 in the LGT/DEN4 chimera provided significant protection when immunized mice were challenged intraperitoneally (i.p.) with the wild-type LGT strain TP21 (24) or with either the European strain or the highly virulent Far Eastern strain of TBEV (25).

Taken together, these observations suggest that chimeric viruses bearing the protective antigens of various highly virulent as well as attenuated flaviviruses may prove to be useful in immunization against flaviviruses of public health importance. Chimeric viruses should also be useful in studies that address the molecular basis of flavivirus pathogenesis and insect vector specificity.

The present study addresses preclinical issues, such as the effect of adapting the LGT TP21/DEN4 chimera to grow efficiently in certified simian Vero cells, an acceptable substrate for production of virus vaccines for humans. Also, the Vero cell-grown chimera was evaluated for attenuation, immunogenicity, and protective efficacy in rhesus monkeys to determine if adaptation to this approved cell substrate compromised any of the chimeras' desirable properties.

Another relevant issue was the possible effect of the chimera on the environment and flavivirus ecology. An initial assessment of the impact of the chimera on the environment was undertaken to determine if the virus replicated in the *Toxorhynchites splendens* mosquito, a laboratory surrogate that is highly permissive for replication of dengue viruses (29, 38).

MATERIALS AND METHODS

Cells and viruses. Simian $LLCMK_2$ cells and mosquito C6/36 cells were obtained from the American Type Culture Collection (Manassas, Va.). Qualified Vero cells (W.H.O. Seed, 143 passage; Novavax, Inc., Rockville, Md.) were used between passages 143 and 148.

The chimeric LGT TP21/DEN4 virus that contained preM and E genes of wild-type LGT strain TP21 with remaining sequences derived from DEN4 was originally recovered after transfection of mosquito C6/36 cells with full-length RNA transcripts of the full-length cDNA chimeric genome (24) and then adapted to grow in Vero cells as already described in detail (25). Novavax, Inc., using qualified Vero cells (passage 144) and serum-free VP-SFM medium (GIBCO BRL, Gaithersburg, Md.), prepared the final LGT TP21/DEN4(vac) virus suspension by ultrafiltration using a 500,000-MW hollow fiber cartridge. The stock virus had a titer of 10^8 PFU per ml in Vero cells. RNA was extracted from 1 ml of virus suspension, reverse transcribed to cDNA, and then sequenced as described previously (3, 25).

The wild-type LGT strain TP21 used in this study was kindly provided by R. Shope (University of Texas Medical Branch, Galveston) from the Rockefeller Foundation Collection and was originally isolated from ticks in Malaysia in 1956 (33). It had undergone nine passages in mouse brain and two in Vero cell culture when received by us. LGT TP21 was plaque purified three times in Vero cells before preparation of a virus stock, the titer of which was 2.4×10^8 PFU/ml in these cells. The Vero cell preparation of DEN4 Caribbean strain 814669 with a titer of 7.9×10^7 PFU/ml was kindly provided by S. Whitehead (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.). TBEV, strain Sofjin, which originally was isolated in 1937 from a TBE patient in far eastern USSR, was also prepared in Vero cells (31).

The procedures used for plaque assay and analysis of virus replication in cell culture were described earlier (22, 23). Also, the immunostaining focus-forming assay (12) was used in parallel with the plaque assay for determination of virus titer.

Immunization and challenge of rhesus monkeys. The studies involving monkeys were carried out at Bioqual, Inc. (Rockville, Md.), in accordance with procedures described in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, Md.). Twenty-four rhesus monkeys (*Macacca mulatta*), weighing 3 to 5 kg, were prebled and shown to be seronegative by neutralization assay for LGT and DEN4. Groups of 4 monkeys were inoculated subcutaneously (s.c.) with 10^3, 10^5, or 10^7 PFU of LGT TP21/DEN4(vac) chimera, 10^5 or 10^7 PFU of wild-type LGT strain TP21, or 10^5 PFU of wild-type DEN4 strain 814669. Inoculum (0.5 ml) was administered at two sites, one on each upper shoulder. The inoculum was then frozen for subsequent titration to confirm the quantity of virus administered. Blood was collected under ketamine anesthesia before immunization, and inoculated monkeys were bled daily for 12 days for detection of viremia. Blood samples for antibody response were collected on days 14, 21, 28, 35, and 42. On day 43 postimmunization each of the immunized monkeys was challenged by the s.c. route with 10^5 PFU of LGT TP21. These monkeys were bled daily for 12 days to test for viremia as well as on days 56, 70, and 84 for measurement of neutralizing antibody.

Viremia following virus inoculation. The quantity of virus in monkey serum was determined by direct titration on Vero and $LLCMK_2$ cells using the immunostaining focus-forming assay (12). Undiluted and serial 2- or 10-fold dilutions of serum in minimal essential medium (MEM) containing 2% heat-inactivated fetal bovine serum (FBS) were inoculated (0.2 ml) onto duplicate wells of 24-well tissue culture plates containing a monolayer of Vero or $LLCMK_2$ cells. After 1 h of adsorption at 37°C, the inoculum was removed and the cell monolayers were overlaid with MEM containing 2% FBS, 50 μg of gentamicin/ml, 0.25 μg of fungizone/ml, and 1% tragacanth gum (Sigma Chemical Co., St. Louis, Mo.) and were incubated for 4 days at 37°C and 5% CO_2. Medium was then removed, and the cell monolayers were fixed for 30 min with methyl alcohol and rinsed twice with phosphate-buffered saline (PBS). Cells in each well were treated sequentially with a 1:1 mixture of DEN4-specific and LGT TP21-specific mouse antibodies, each diluted 1:1,000, followed by peroxidase-labeled polymer conjugated to anti-mouse immunoglobulins (Dako Co., Carpinteria, Calif.) diluted 1:10 in PBS. Antibody-stained foci of infected cells were developed using 0.01% H_2O_2 and 0.04% 3,3′-diaminobenzidine (Sigma Chemical Co.) in PBS, and foci were counted; virus titer was expressed as focus-forming units per milliliter (FFU/ml).

FFU reduction neutralization assay. For determination of LGT or DEN4 virus-neutralizing antibody titer, fivefold-diluted serum, without the addition of complement, was heat inactivated for 30 min at 56°C. Serial twofold dilutions of serum (starting at a serum dilution of 1:10) were mixed with an equal volume of LGT TP21 or DEN4 virus suspension containing approximately 100 FFU. The mixture was incubated for 30 min at 37°C, and 0.1 ml of mixture was added to duplicate wells of $LLCMK_2$ or Vero cells. After 1 h of adsorption at 37°C, inoculum was removed and cells were overlaid and assayed for virus using the focus-forming assay as described. The antibody titer was the highest dilution of antibody that reduced the number of foci by 50% compared to the focus-forming titer of a mixture of virus with serum from the same monkey collected prior to immunization.

The TBEV neutralizing antibody titer of pooled sera from the 4 monkeys in each group was determined by plaque reduction assay on Vero cells under BL-4 biosafety conditions as described previously (31).

Passive protection of mice. The studies involving TBEV challenge were carried out in a biosafety level 4 laboratory at the U.S. Army Medical Research Institute of Infectious Diseases (Fort Detrick, Md.). For evaluation of passive protection, 4-week-old female BALB/c mice were injected s.c. with single doses (100 μl) of pooled monkey sera 16 h before the s.c. inoculation with 100 i.p. 50% lethal doses (LD_{50}s) of TBEV (strain Sofjin). Another group of mice received pooled sera twice, at 16 h before TBEV challenge and 6 days after challenge. Mice were observed daily for 28 days for signs of illness or death.

Mosquito inoculation. One- to 10-day-old adult *T. splendens* mosquitoes of both sexes were immobilized by immersion in an ice water bath and inoculated intrathoracically with 0.22 μl of virus suspension or PBS using the technique of Rosen and Gubler (28). A Harvard Apparatus microinjector (Medical Systems Corp., Greenvale, N.Y.) was used for inoculation of mosquitoes. Inoculated mosquitoes were held at 24°C and 75% relative humidity with a 12-h daylight cycle for 14 days and then stored frozen at −20°C. To assay for the presence of virus, head squashes were prepared for each mosquito as described by Sumanochitraporn et al. (34). Slides were fixed in acetone for 20 min and processed for immunofluorescence assay (IFA) by adding a 1:1 mixture of LGT- and DEN4-specific antibodies present in hyperimmune mouse ascitic fluid diluted 1:100 in PBS with 0.05% Tween 20. Afterward, the secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (KPL, Gaithersburg, Md.), was added and the slides were viewed with an Olympus BX60 microscope.

RESULTS

Derivation and characterization of Vero cell-passaged LGT TP21/DEN4(vac) chimera. Initially, the LGT/DEN4 chimeras were recovered following transfection of mosquito C6/36 cell culture with full-length RNA transcripts of full-length cDNA, as described previously (24). Subsequently, these chimeras were adapted to replicate efficiently in qualified simian Vero cells, an approved cell substrate for human vaccines (25). The increased replicative efficiency and cytopathic effect of the Vero cell-adapted chimeras in Vero cells was the result of host range mutations in the virus genome that were selected during adaptation and propagation of these viruses in simian cells. Complete sequence analysis of the mosquito cell-derived LGT TP21/DEN4 chimera and its Vero cell-adapted progeny identified three amino acid changes (Lys_{296}→Gln, Thr_{310}→Ala, and Cys_{480}→Phe) in the E protein of the Vero cell-passaged chimera (25). One or more of these changes played a role in altering cell tropism.

During a previous study (25), it was observed that the LGT TP21/DEN4(vac) chimera appeared to be more immunogenic and protective than LGT E5/DEN4(vac), because mice inoculated with two doses of the former chimera but not the latter chimera were fully protected against subsequent challenge with TBEV European subtype (strain Absettarov) or TBEV Far Eastern subtype (strain Sofjin). For this reason we selected the LGT TP21/DEN4(vac) chimera as our lead vaccine candidate for evaluation in nonhuman primates.

To prepare high-titer suspensions of chimeric virus for use in immunization of monkeys, LGT TP21/DEN4(vac) was passaged twice more in the qualified Vero cells using serum-free medium. The virus suspension was concentrated by ultrafiltration to achieve a titer of 10^8 PFU/ml. At this point the nucleotide sequence of the complete chimeric genome was again determined. An amino acid difference (Thr→Ser) at position 335 in the E protein sequence of the LGT TP21/DEN4(vac) chimera was identified following the two additional passages in Vero cells. The same amino acid change in E protein had been observed previously in the Vero cell-adapted LGT E5/DEN4(vac) chimera (25).

Vero cell-adapted LGT TP21/DEN4(vac) chimera (10^6 PFU) inoculated i.p. failed to cause fatal disease in immunocompetent 3-week-old Swiss mice or immunodeficient SCID mice (C.B.-17 ICR/scid/scid; Taconic Farms, Germantown, N.Y.). This indicated that the Vero cell-passaged virus, like its mosquito cell-recovered and propagated virus parent, was completely attenuated for both Swiss and SCID mice with respect to neuroinvasiveness.

Immunization of monkeys with chimeric LGT TP21/DEN4(vac) virus and parental DEN4 or LGT TP21 virus. (i) Viremia and neutralizing antibody response following immunization. Twenty-four rhesus monkeys (*Macacca mulatta*) in groups of 4 were inoculated s.c. with 10^3, 10^5, or 10^7 PFU of LGT TP21/DEN4(vac) chimera, 10^5 or 10^7 PFU of wild-type LGT TP21, or 10^5 PFU of DEN4. All 8 monkeys inoculated with LGT TP21 developed viremia that lasted 1 to 5 days (Table 1). The mean duration of viremia was at least 2 days longer, and peak virus titer in serum was higher for monkeys inoculated with 10^5 PFU of LGT TP21 than for monkeys inoculated with 10^7 PFU of LGT TP21. Each of the monkeys that received DEN4 had 1 to 3 days of viremia. In contrast, viremia was not detected in monkeys that received 10^3 or 10^5 PFU of the chimera, and only 1 of 4 monkeys inoculated with 10^7 PFU of the chimera exhibited a low-level, 1-day viremia; only one plaque was detected on cell monolayers inoculated with 200 μl of the undiluted serum collected from monkey number 10 on the day after inoculation. Most likely this represented residual inoculum. These observations indicate that the chimera replicates in monkeys less well than either the LGT TP21 or DEN4 parent.

Each of the 24 inoculated monkeys developed serum neutralizing antibodies to the virus inoculated. Antibody levels increased rapidly in all animals during the second and third week after inoculation; the titer attained by day 21 remained at

TABLE 1. Viremia and serum neutralizing antibody response of rhesus monkeys infected with LGT TP21 or DEN4 or their TP21/DEN4(vac) chimera[a]

Group	Immunizing virus (dose in PFU)	Monkey no.	Viremia (log_{10} FFU/ml) on day postinoculation							Serum neutralizing antibody titer on postimmunization day 42 for:		
			0	1	2	3	4	5	6	TP21	DEN4	TBEV
1	TP21/DEN4(vac) (10^3)	1	—[b]	—	—	—	—	—	—	876	<20	
		2	—	—	—	—	—	—	—	797	<20	
		3	—	—	—	—	—	—	—	840	<20	
		4	—	—	—	—	—	—	—	1,004	<20	
	GMT[d]									**871**	**<20**	**320**
2	TP21/DEN4(vac) (10^5)	5	—	—	—	—	—	—	—	375	<20	
		6	—	—	—	—	—	—	—	232	<20	
		7	—	—	—	—	—	—	—	303	<20	
		8	—	—	—	—	—	—	—	732	<20	
	GMT									**372**	**<20**	**320**
3	TP21/DEN4(vac) (10^7)	9	—	—	—	—	—	—	—	1,777	<20	
		10	—	0.7[c]	—	—	—	—	—	3,597	<20	
		11	—	—	—	—	—	—	—	2,969	<20	
		12	—	—	—	—	—	—	—	1,530	<20	
	GMT									**2,344**	**<20**	**640**
4	TP21 (10^5)	13	—	—	2.5	2.8	2.3	0.7	—	2,089	<20	
		14	—	—	2.9	2.8	2.5	1.3	—	4,181	<20	
		15	—	1.5	1.9	2.2	1.7	1.0	—	7,378	<20	
		16	—	—	1.2	1.6	1.3	—	—	1,867	<20	
	GMT									**3,311**	**<20**	**1,280**
5	TP21 (10^7)	17	—	—	1.7	1.3	0.7	—	—	1,189	<20	
		18	—	—	1.2	—	—	—	—	349	<20	
		19	—	—	1.0	—	—	—	—	339	<20	
		20	—	—	0.7	—	—	—	—	940	<20	
	GMT									**603**	**<20**	**160**
6	DEN4 (10^5)	21	—	1.7	2.2	1.0	—	—	—	<10	1,170	
		22	—	—	1.2	—	—	—	—	<10	2,328	
		23	—	1.0	1.7	1.2	—	—	—	<10	1,118	
		24	—	1.2	1.6	—	—	—	—	<10	2,248	
	GMT									**<10**	**1,622**	**<20**

[a] Serum TP21 or DEN4 neutralizing antibody titer was determined for individual serum samples using wild-type LGT TP21 or DEN4 strain 814669, respectively. A serum TBEV neutralizing antibody titer was determined for pooled sera from 4 monkeys in each group using TBEV strain Sofjin.
[b] Plaques were not observed when simian Vero or $LLCMK_2$ cells in 24-well plates were inoculated with 200 μl of undiluted monkey serum and stained 5 days later using a focus-forming assay.
[c] A single plaque was detected when $LLCMK_2$ cells were inoculated with 200 μl of undiluted monkey sera.
[d] GMT, geometric mean titer.

this level until 42 days postimmunization, the day before the monkeys were challenged with LGT TP21 virus (data not shown). In addition, the pooled 42-day convalescent-phase sera of each of the five groups of monkeys immunized with LGT TP21 or its DEN4 chimera each contained a moderate to high level of neutralizing antibodies for the antigenically related TBEV.

Monkeys inoculated with 10^5 PFU of LGT TP21 developed high levels of LGT and TBEV neutralizing antibodies. Paradoxically, a higher dose of the TP21 strain (10^7 PFU) induced a lower titer of neutralizing antibodies. Reduced viremia and reduced immune response of monkeys that received the high dose of LGT TP21 might have been due to accumulation of defective interfering particles in the LGT TP21 virus suspensions with higher titers. It should be noted that evidence for interference in a high-titer suspension of dengue virus was reported more than 48 years ago by Walter Schlesinger (reviewed in reference 30). In addition, a weak antibody response

TABLE 2. Response of rhesus monkeys immunized with LGT TP21 or DEN4 or their TP21/DEN4(vac) chimera to challenge with LGT TP21 (10^5 PFU) on day 43 following immunization

Group	Immunizing virus (dose in PFU)	Monkey no.	Viremia ($\log_{10}$ FFU/ml) on indicated day postchallenge with TP21							Serum TP21 neutralizing antibody titer	
			0	1	2	3	4	5	6	42 days after immunization	41 days after challenge
1	TP21/DEN4(vac) (10^3)	1	—	—	—	—	—	—	—	876	2,010
		2	—	—	—	—	—	—	—	797	3,989
		3	—	—	—	—	—	—	—	840	7,164
		4	—	—	—	—	—	—	—	1,004	2,045
	GMT[a]									**871**	**3,311**
2	TP21/DEN4(vac) (10^5)	5	—	—	—	—	—	—	—	375	425
		6	—	—	—	—	—	—	—	232	5,805
		7	—	—	—	—	—	—	—	303	548
		8	—	—	—	—	—	—	—	732	4,974
	GMT									**372**	**1,622**
3	TP21/DEN4(vac) (10^7)	9	—	—	—	—	—	—	—	1,777	4,338
		10	—	—	—	—	—	—	—	3,597	5,873
		11	—	—	—	—	—	—	—	2,969	1,675
		12	—	—	—	—	—	—	—	1,530	2,681
	GMT									**2,344**	**3,311**
4	TP21 (10^5)	13	—	—	—	—	—	—	—	2,089	2,285
		14	—	—	—	—	—	—	—	4,181	6,331
		15	—	—	—	—	—	—	—	7,378	5,341
		16	—	—	—	—	—	—	—	1,867	1,204
	GMT									**3,311**	**3,090**
5	TP21 (10^7)	17	—	—	—	—	—	—	—	1,189	2,557
		18	—	—	—	—	—	—	—	349	4,222
		19	—	—	—	—	—	—	—	339	949
		20	—	—	—	—	—	—	—	940	4,147
	GMT									**603**	**2,570**
6	DEN4 (10^5)	21	—	—	2.6	2.4	2.1	1.3	—	<10	821
		22	—	—	2.5	2.3	2.1	0.7	—	<10	786
		23	—	—	1.7	1.3	—	—	—	<10	341
		24	—	—	2.4	2.0	1.2	—	—	<10	500
	GMT									**<10**	**576**

[a] GMT, geometric mean titer.

was observed in mice that had been immunized with a high dose of yellow fever 17D virus compared to the high level of antibody response induced by a low dose of virus (6).

Each of the monkeys that received DEN4 developed a high level of DEN4 neutralizing antibodies but failed to produce a detectable immune response to LGT or TBEV. Monkeys inoculated with 10^3 or 10^5 PFU of chimera developed a moderate to high titer of LGT TP21 and TBEV neutralizing antibodies, but an even higher immune response developed when monkeys were inoculated with 10^7 PFU of the chimera (Table 1).

(ii) Response to challenge. On day 43 postimmunization, each of the immunized monkeys was challenged by the s.c. route with 10^5 PFU of LGT TP21. This dose of challenge virus was chosen because it induced a greater viremia and a higher antibody response than the larger 10^7 PFU dose (Table 1). During the 12 days postchallenge, viremia was not detected in monkeys previously immunized with the chimeric virus or its LGT TP21 parent, whereas each of the monkeys previously inoculated with DEN4 developed viremia lasting 2 to 4 days (Table 2). This indicated that even at the lowest dose (10^3 PFU), the chimera induced an immune response to LGT preM and E structural proteins that provided complete protection from LGT TP21 virus challenge.

The titer of serum neutralizing antibodies against LGT TP21

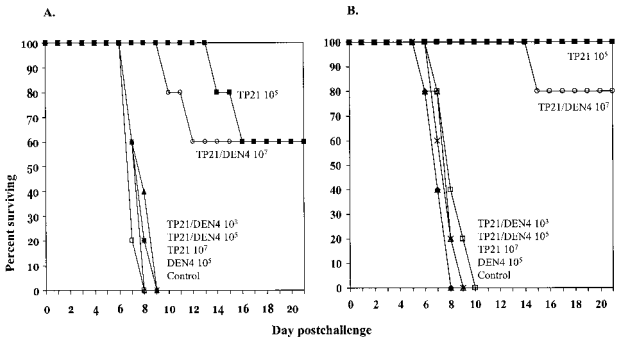

FIG. 1. Passive transfer of pooled sera from rhesus monkeys immunized with DEN4 or LGT TP21 or their TP21/DEN4(vac) chimera protects mice from s.c. challenge with 100 i.p. LD_{50}s of TBEV. Groups of 5 mice were injected s.c. once (A) or twice (B) with 100 μl of pooled sera from monkeys inoculated with 10^3 (Δ), 10^5 (□), or 10^7 (o) PFU of TP21/DEN4 chimera, with 10^5 (♦) PFU of DEN4, with 10^5 (■) or 10^7 (▲) PFU of LGT TP21, or with culture medium (×, control). Sixteen hours after serum transfer, the mice were challenged by intraperitoneal injection of 100 i.p. LD_{50}s of TBEV, strain Sofjin. Mice shown in panel B were inoculated 6 days after TBEV challenge with the second dose of indicated monkey pooled sera. Mice were observed daily for morbidity, and the day of death was recorded.

was measured 41 days following challenge with this virus. Six of the 20 monkeys immunized with the chimera or its LGT TP21 parent developed a significant (≥4-fold) increase in serum neutralizing antibody titer after LGT TP21 challenge, indicating that LGT TP21 had stimulated an anamnestic immune response (Table 2). This type of response was not observed in monkeys immunized with 10^5 PFU of LGT TP21 or 10^7 PFU of its chimera. These groups had responded to primary immunization by developing the highest antibody level.

(iii) Passive transfer of serum from immunized monkeys protects mice against TBEV challenge. We evaluated the ability of serum collected from vaccinated monkeys on day 42 postimmunization to passively protect mice from TBEV challenge. Four-week-old female BALB/c mice were injected s.c. with 100 μl of pooled sera from a group of 4 monkeys that had been immunized with parental or chimeric virus, as shown in Fig. 1. Sixteen hours after serum transfer the mice were challenged s.c. with 100 i.p. LD_{50}s of the Far Eastern subtype of TBEV, strain Sofjin. Sixty percent protection from the TBEV challenge was provided by pooled sera from the monkeys that were immunized with 10^7 PFU of the chimera or 10^5 PFU of LGT TP21 and had a serum TBEV neutralizing antibody titer of 1:640 or 1:1,280, respectively (Table 1 and Fig. 1A). Also, mice that died in these two groups showed delayed time of death compared with those of the control group, namely, mice that did not receive pooled sera. None of the mice inoculated with pooled sera from the other groups of monkeys survived the TBEV challenge. These results indicate that the TBEV neutralizing antibodies measured in vitro were also functional in vivo, providing protection when mice were challenged peripherally with TBEV.

In a second experiment, an effort was made to maintain a protective level of LGT TP21 antibodies in passively immunized mice: groups of 5 mice received 100 μl of the pooled sera from monkeys twice, initially 16 h before TBEV challenge and later 6 days after TBEV challenge (Fig. 1B). All of the mice that received pooled sera from monkeys inoculated with 10^5 PFU of TP21 and 80% of mice that received pooled sera from monkeys inoculated with 10^7 PFU of chimera remained healthy after TBEV challenge. None of the mice in the other groups survived TBEV challenge. These data are encouraging and suggest that the chimeric vaccine might induce protective immunity in primates against a closely related, highly virulent heterologous tick-borne flavivirus.

Does the chimera or parental LGT TP21 or DEN4 replicate in mosquitoes following intrathoracic inoculation? Because DEN4 is transmitted to humans by infected mosquitoes, it would be desirable if the LGT/DEN4 vaccine candidate could not be transmitted by mosquitoes in order to prevent introduction of the chimeric virus into the environment during clinical trials and subsequent routine use. Previously, the Vero cell-adapted chimeras LGT TP21/DEN4(vac) and LGT E5/DEN4(vac) were compared to their parental DEN4 virus with respect to plaque morphology and maximum yield in simian Vero cells and mosquito C6/36 cells (25). The peak titers of the two chimeric viruses were not significantly different in either the Vero or the C6/36 cells. Also, both Vero cell-adapted chimeras and parental DEN4 were able to replicate efficiently in mosquito cell culture and produce 5- to 7-mm-size plaques. In contrast, the growth of the original LGT TP21 or E5 virus in mosquito cells was totally restricted (24). In the present study we addressed the question of replication of chimeric viruses or parental viruses in mosquitoes. Assay for infectivity of mosquito-borne dengue viruses by parenteral inoculation of susceptible mosquitoes is considered a very sensitive system for recovery and titration of these flaviviruses (28, 29). *T. splendens* mosquitoes were injected intrathoracically with wild-type DEN4 or LGT TP21 or LGT E5 or their chimeric viruses. This route of inoculation permitted experimental bypass of the midgut escape barrier. These large mosquitoes are considered to be unusually permissive for the dengue viruses (29, 38). Viral infection was determined by scoring the presence of viral antigen in head tissues by IFA. The wild-type DEN4, which grew to high titer in C6/36 cells, also replicated efficiently in these mosquitoes (Table 3). The calculated 50% mosquito infectious dose was $10^{2.8}$ PFU. In contrast, both strains of LGT virus, i.e.,

TABLE 3. Replication of parental DEN4 or LGT virus or their LGT/DEN4 chimera in *T. splendens* mosquitoes following intrathoracic inoculation as determined by IFA

Virus	Dose of virus inoculation (log_{10} PFU)	No. mosquitoes inoculated	No. (%) mosquitoes infected[b]
DEN4 (814669)[a]	4.2	14	11 (79)
	3.2	9	6 (67)
	2.2	10	3 (30)
	1.2	6	1 (17)
	0.2	9	1 (11)
	0.02	8	0
TP21	4.6	22	0
E5	4.3	6	0
TP21/DEN4(vac)	4.3	20	0
E5/DEN4(vac)	1.6	12	0

[a] The mosquito 50% infectious dose for DEN4 was calculated as $10^{2.8}$ PFU.
[b] Number (percentage) of mosquitoes with detectable antigen in head tissue; mosquitoes were assayed 14 days postinoculation by IFA using DEN4- and LGT-specific hyperimmune mouse ascitic fluid.

TP21 and E5, which were totally restricted in mosquito cell culture, also exhibited complete growth restriction in mosquitoes following intrathoracic inoculation. Significantly, neither chimera replicated in the mosquitoes, although both chimeric (LGT TP21/DEN4 and LGT E5/DEN4) viruses replicated efficiently in mosquito C6/36 cell culture. The preceding observations indicate that the chimeras retain the mosquito negative phenotype of their LGT parent, and hence they are unlikely to be transmitted by mosquitoes. The question of infectivity of the chimeras for ticks is presently under investigation.

DISCUSSION

The pattern of viremia of LGT TP21 and DEN4 in rhesus monkeys observed in this study is similar to that reported earlier (2, 16, 19). Each of 8 monkeys inoculated with LGT TP21 virus and each of 4 monkeys inoculated with DEN4 virus became viremic, and the duration of viremia ranged from 1 to 5 days. In contrast, viremia was not detected in 11 of the 12 monkeys inoculated with the LGT TP21/DEN4(vac) chimera; the single PFU was detected in the serum of the 12th monkey on the day after inoculation. These observations indicate that the LGT TP21/DEN4(vac) chimera is less efficient for replication in monkeys than either the LGT TP21 or DEN4 parent.

Each of the gene products of LGT TP21 or DEN4 has been selected over its evolutionary history to interact efficiently with other products of its viral genome in the complex program of virus replication. It is likely that substitution in the chimera of two LGT TP21 genes for the corresponding genes of DEN4 created LGT TP21-DEN4 protein incompatibilities that compromised virus replication in vivo. However, chimerization does not always lead to attenuation, as indicated by the robust DEN1/DEN4 and DEN2/DEN4 chimeras, which do not appear to be attenuated in monkeys (2). This can explained by the fact that DEN4 and DEN1 or DEN4 and DEN2 are more closely related than LGT TP21 (a tick-borne flavivirus) and DEN4 (a mosquito-borne flavivirus).

In humans, dengue virus viremia generally begins on the second to sixth day after infection and usually lasts 3 to 5 days (8). In contrast, in an earlier study in Russia, experimental inoculation of LGT TP21 induced viremia in human recipients that began on the sixth to eighth day after inoculation, providing additional evidence that this virus was partially attenuated for humans (10). It appears that restriction of replication of dengue virus mutants or LGT virus in monkeys, as measured by days of viremia or the peak serum titer, may be a marker of attenuation of these viruses for humans (10, 11, 26, 27, 32). In addition, a recombinant DEN4 vaccine candidate that exhibited a restricted pattern of viremia in rhesus monkeys due to a deletion in the 3′ noncoding region of the viral genome (16) also exhibited a satisfactory level of attenuation and immunogenicity in adult human volunteers (A. P. Durbin, R. A. Karron, W. Sun, D. T. Vaughn, M. J. Reynolds, J. R. Perreault, R. Men, C.-J. Lai, W. R. Elkins, R. M. Chanock, B. Murphy, and S. S. Whitehead, unpublished data). For these reasons the attenuation phenotype of the LGT TP21/DEN4(vac) chimera in monkeys also may be considered a predictor of attenuation of this chimera for humans.

Although attenuated, the chimera stimulated a moderate to high level of serum neutralizing antibodies against LGT TP21 as well as the highly virulent TBEV. The strongest immune response was observed in the group of monkeys inoculated with 10^7 PFU of the chimera or 10^5 PFU of LGT TP21 (Table 1). The level of TBEV neutralizing antibodies in monkey convalescent-phase serum was lower by a factor of 2 to 4 compared to that of LGT TP21 neutralizing antibody titer, and this was consistent with observations made previously for antibodies to LGT and TBEV in sera of volunteers immunized with the further attenuated E5 mutant of LGT (26). Finally, none of the 8 monkeys immunized with LGT TP21 nor any of the 12 monkeys immunized with its chimera became viremic following challenge with 10^5 PFU of TP21, whereas each of the 4 monkeys previously inoculated with DEN4 developed viremia lasting 2 to 4 days. This indicated that, even at the lowest dose tested (10^3 PFU), the chimera induced an immune response to LGT TP21 preM and E structural proteins that completely protected animals from LGT TP21 virus challenge.

Consistent with the close antigenic relationship of LGT and TBEV, our previous studies with LGT TP21/DEN4(vac) in mice have shown a high degree of cross-protection between LGT and the TBEV European subtype (strain Absettarov) or the TBEV Far Eastern subtype (strain Sofjin) (25). In the present study, we observed that TBEV antibodies in the serum of monkeys immunized with TP21 or its DEN4 chimera measured in vitro were functional in vivo; these antibodies were able to passively protect mice against challenge with 100 i.p. LD_{50}s of TBEV strain Sofjin. All of the mice that received pooled convalescent-phase sera twice from monkeys inoculated with 10^5 PFU of TP21 and 4 of 5 mice that received convalescent-phase sera from monkeys inoculated with 10^7 PFU of chimera remained healthy after a fatal TBEV challenge. These data suggest that the chimeric vaccine may induce protective immunity to TBEV in nonhuman primates. Studies in monkeys are under way to determine optimal dose and number of inoculations of LGT TP21/DEN4(vac) chimera required to elicit durable protective immunity against peripheral challenge with highly virulent TBEV strains.

Because the candidate LGT TP21/DEN4(vac) vaccine virus contains sequences derived from a mosquito-borne flavivirus (DEN4) as well as a tick-borne flavivirus (LGT), we were concerned about (i) its potential impact on the environment, (ii) the creation of an abnormal virus life cycle, or (iii) the development of a new reservoir in nature. To address these issues, it is necessary to identify the insect vector tropism of the vaccine virus, i.e., mosquitoes, ticks, or both. Initially, the large, nonhematophagous mosquitoes of the species *T. splendens*, which is a sensitive host for determining the infectivity of mosquito-borne dengue viruses (29, 38), were inoculated intrathoracically with various doses of DEN4, LGT (TP21 or E5), or their chimeras and then later were analyzed by IFA for evidence of infection. DEN4 virus, which replicates efficiently in mosquito C6/36 cell culture, was highly infectious for mosquitoes. In contrast, the LGT/DEN4 chimeras were not able to infect these large mosquitoes despite efficient growth of chimeric virus in the mosquito C6/36 cell line. This observation indicates that the chimeras have a limited potential for transmission by mosquitoes. In future studies the transmissibility of the LGT/DEN4 chimeras by other species of mosquitoes, such as *Aedes aegypti* and *Aedes albopictus*, which are primary vectors of dengue virus, will be studied. Also, transmission by ticks

will be examined by allowing insects to feed on infected animals during the time of peak viremia.

It appears that the live LGT TP21/DEN4(vac) chimeric virus vaccine candidate has a favorable safety profile. Chimerization of LGT with DEN4 completely ablated neuroinvasiveness of the LGT parent when assayed in both immunocompetent mice and highly permissive immunodeficient (SCID) mice (24, 25). Also, there was evidence for restriction of viral replication of the chimera in monkeys, as determined by reduction of viremia, and in mosquitoes, as demonstrated by failure to infect *T. splendens* after intrathoracic inoculation. These two characteristics of the chimera suggest that it is unlikely that the LGT TP21/DEN4(vac) vaccine candidate can be transmitted by mosquitoes. A high level of attenuation of the chimeric LGT TP21/DEN4(vac) virus for mice and monkeys as well as its satisfactory immunogenicity and protective efficacy makes this chimera a promising vaccine candidate that should be evaluated in humans. But before initiating clinical trials with the LGT TP21/DEN4(vac) candidate live virus vaccine, the safety of the chimera must be evaluated further. Histopathological studies will soon be initiated in monkeys to determine if the chimera exhibits neurovirulence when inoculated by the intracerebral or intraspinal route. Comparison will be made with the neurovirulence of the 17D vaccine strain of yellow fever virus, the attenuated E5 vaccine strain of LGT, and the attenuated poliovirus type 3 vaccine strain (20).

Concerns have been raised regarding the possibility that the LGT TP21/DEN4(vac) chimera might set the stage for a subsequent potentiation of dengue infection. This is a theoretical concern, but unfortunately there is no experimental animal model of dengue hemorrhagic fever (DHF) to test this concern, despite many attempts to develop such a model. Therefore, we must rely on epidemiological and clinical studies of human dengue virus infection and disease to guide us. The initial hypothesis for the etiology of DHF proposed that dengue virus surface glycoprotein cross-reactive antibodies induced by the dengue virus of a primary infection act to enhance the replication of a dengue virus of a different serotype responsible for a secondary infection. This is thought to occur by antibody-enhanced entry of virus into an expanded number of cells of the host. Recently (15, 21), the explanation for the pathogenesis of DHF has been enlarged to include the induction of dengue virus nonstructural protein cross-reactive cytotoxic T lymphocytes (CTLs) during primary infection that act to exacerbate disease during a subsequent secondary infection with a heterologous dengue virus serotype. Evidence to support the initial hypothesis is provided by the observation that in Asia the first peak of DHF occurs in infants (i.e., less than 12 months of age) who possess diminishing amounts of maternally derived dengue virus antibodies. In this situation, maternal cross-reactive dengue virus antibodies might play a role in pathogenesis of DHF. However, cross-reactive dengue virus CTLs could not play a role, because this form of immunity is not transferred from mother to infant. This strongly suggests that cross-reactive dengue virus nonstructural protein CTLs are not an absolute requirement for development of DHF. Thus, it is not clear whether the proposed exaggerated CTL response is an additional cause of DHF or the manifestation of a severe dengue virus infection. Without an animal model of DHF, it is unlikely that this question can be answered soon. We plan to approach these questions by immunizing monkeys with the chimera and subsequently challenging these animals with wild-type dengue virus or candidate attenuated dengue vaccine virus after an interval of 6 months. The readout for potentiation will be an increase in the level of viremia following the dengue virus challenge. The basis for using this approach is the tight correlation between the level of viremia and the extent and severity of dengue virus disease in humans (37).

Also, to reduce the hypothetical risk factor for vaccine recipients to develop DHF, it might be possible to combine a candidate LGT TP21/DEN4 vaccine virus with a dengue tetravalent vaccine that contains each of the four dengue virus serotypes.

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REFERENCES

1. **Bray, M., and C.-J. Lai.** 1991. Construction of intertypic chimeric dengue viruses by substitution of structural protein genes. Proc. Natl. Acad. Sci. USA **88:**10342–10346.
2. **Bray, M., R. Men, and C.-J. Lai.** 1996. Monkeys immunized with intertypic chimeric dengue viruses are protected against wild-type virus challenge. J. Virol. **70:**4162–4166.
3. **Campbell, M. S., and A. G. Pletnev.** 2000. Infectious cDNA clones of Langat tick-borne flavivirus that differ from their parent in peripheral neurovirulence. Virology **269:**225–237.
4. **Chambers, T. J., A. Nestorowicz, P. W. Mason, and C. M. Rice.** 1999. Yellow fever/Japanese encephalitis chimeric viruses: construction and biological properties. J. Virol. **73:**3095–3101.
5. **Clarke, D. H.** 1964. Further studies on antigenic relationships among the viruses of the group B tick-borne complex. Bull. W. H. O. **31:**45–56.
6. **Guirakhoo, F., Z.-X. Zhang, T. J. Chambers, S. Delagrave, J. Arroyo, A. D. T. Barrett, and T. P. Monath.** 1999. Immunogenicity, genetic stability, and protective efficacy of a recombinant, chimeric yellow fever-Japanese encephalitis virus (ChimeriVax-JE) as a live, attenuated vaccine candidate against Japanese encephalitis. Virology **257:**363–372.
7. **Guirakhoo, F., R. Weltzin, T. J. Chambers, Z.-X. Zhang, K. Soike, M. Ratterree, J. Arroyo, K. Georgakopoulos, J. Catalan, and T. P. Monath.** 2000. Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. J. Virol. **74:**5477–5485.
8. **Halstead, S. B., S. Udomsakdi, P. Singharaj, and A. Nisalak.** 1969. Dengue and Chikungunya virus infection in man in Thailand, 1962–1964. III. Clinical, epidemiologic and virologic observations on disease in non-indigenous white persons. Am. J. Trop. Med. Hyg. **18:**984–996.
9. **Huang, C. Y., S. Butrapet, D. J. Pierro, G. J. Chang, A. R. Hunt, N. Bhamarapravati, D. J. Gubler, and R. M. Kinney.** 2000. Chimeric dengue type 2 (vaccine strain PDK-53)/dengue type 1 virus as a potential candidate dengue type 1 virus vaccine. J. Virol. **74:**3020–3028.
10. **Il'enko, V. I., A. A. Smorodincev, I. N. Prozorova, and V. G. Platonov.** 1968. Experience in the study of a live vaccine made from the TP21 strain of Malayan Langat virus. Bull. W. H. O. **39:**425–431.
11. **Innis, B. L., K. H. Eckels, E. Kraiselburd, D. R. Dubois, G. F. Meadors, D. J. Gubler, D. S. Burke, and W. H. Bancroft.** 1988. Virulence of a live dengue virus vaccine candidate: a possible new marker of dengue virus attenuation. J. Infect. Dis. **158:**876–880.
12. **Ishimine, T., M. Tadano, T. Fukunaga, and Y. Okuno.** 1987. An improved micromethod for infectivity assays and neutralization test of dengue viruses. Biken J. **30:**39–44.
13. **Mandl, C. W., H. Holzmann, T. Meixner, S. Rauscher, P. F. Stadler, S. L. Allison, and F. X. Heinz.** 1998. Spontaneous and engineered deletions in the 3′ noncoding region of tick-borne encephalitis virus: construction of highly attenuated mutants of a flavivirus. J. Virol. **72:**2132–2140.
14. **Mandl, C. W., S. L. Allinson, H. Holzmann, T. Meixner, and F. X. Heinz.** 2000. Attenuation of tick-borne encephalitis virus by structure-based site-specific mutagenesis of a putative flavivirus receptor binding site. J. Virol. **74:**9601–9609.

15. **Mathew, A., I. Kurane, S. Green, H. A. F. Stephens, D. W. Vaughn, S. Kalayanarooj, S. Suntayakorn, D. Chandanayingyoong, F. A. Ennis, and A. L. Rothman.** 1998. Predominance of HLA-restricted cytotoxic T-lymphocyte responses to serotype-cross-reactive epitopes on nonstructural proteins following natural secondary dengue virus infection. J. Virol. **72:**3999–4004.
16. **Men, R., M. Bray, R. M. Chanock, and C.-J. Lai.** 1996. Dengue type 4 virus mutants containing deletion in the 3′ noncoding region of the RNA genome: analysis of growth restriction in cell culture and altered viremia pattern and immunogenicity in rhesus monkeys. J. Virol. **70:**3930–3937.
17. **Monath, T. P., and F. X. Heinz.** 1996. Flaviviruses, p. 961–1035. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
18. **Muylaert, I. R., T. J. Chambers, R. Galler, and C. M. Rice.** 1996. Mutagenesis of the N-linked glycosylation sites of the yellow fever virus NS1 protein: effects on virus replication and mouse neurovirulence. Virology **222:**159–168.
19. **Nathanson, N., and B. Harrington.** 1966. Experimental infection of monkeys with Langat virus. I. Comparison of viremia following peripheral inoculation of Langat and Japanese encephalitis viruses. Am. J. Epidemiol. **84:**541–556.
20. **Nathanson, N., I. S. Thind, W. O'Leary, and W. H. Price.** 1968. Histological studies of the monkey neurovirulence of group B arboviruses. IV. Evaluation of an attenuated strain (E5) of Langat virus. Am. J. Epidemiol. **88:**103–112.
21. **Okamoto, Y., I. Kurane, A. M. Leporati, and F. A. Ennis.** 1998. Definition of the region on NS3 which contains multiple epitopes recognized by dengue virus serotype-cross-reactive and flavivirus-cross-reactive, HLA-DPw2-restricted CD4 T cell clones. J. Gen. Virol. **79:**697–704.
22. **Pletnev, A. G., M. Bray, J. Huggins, and C.-J. Lai.** 1992. Construction and characterization of tick-borne encephalitis/dengue type 4 viruses. Proc. Natl. Acad. Sci. USA **89:**10532–10536.
23. **Pletnev, A. G., M. Bray, and C.-J. Lai.** 1993. Chimeric tick-borne encephalitis and dengue type 4 viruses: effects of mutations on neurovirulence in mice. J. Virol. **67:**4956–4963.
24. **Pletnev, A. G., and R. Men.** 1998. Attenuation of the Langat tick-borne flavivirus by chimerization with mosquito-borne flavivirus dengue type 4. Proc. Natl. Acad. Sci. USA **95:**1746–1751.
25. **Pletnev, A. G., G. G. Karganova, T. I. Dzhivanyan, V. A. Lashkevich, and M. Bray.** 2000. Chimeric Langat/dengue viruses protect mice from heterologous challenge with the highly virulent strains of tick-borne encephalitis virus. Virology **274:**26–31.
26. **Price, W. H., I. S. Thind, R. D. Teasdall, and W. O'Leary.** 1970. Vaccination of human volunteers against Russian spring-summer (RSS) virus complex with attenuated Langat E5 virus. Bull. W. H. O. **42:**89–94.
27. **Price, W. H., and I. S. Thind.** 1973. Immunization of mice against Russian spring-summer virus complex and monkeys against Powassan virus with attenuated Langat E5 virus. Am. J. Trop. Med. Hyg. **22:**100–108.
28. **Rosen, L., and D. Gubler.** 1974. The use of mosquitoes to detect and propagate dengue viruses. Am. J. Trop. Med. Hyg. **23:**1153–1160.
29. **Rosen, L.** 1981. The use of *Toxorhynchites* mosquitoes to detect and propagate dengue and other arboviruses. Am. J. Trop. Med. Hyg. **30:**177–183.
30. **Schlesinger, R. W.** 1977. Dengue viruses. Virol. Monogr. **16:**1–132.
31. **Schmaljohn, C., L. Vanderzanden, M. Bray, D. Custer, B. Meyer, D. Li, C. Rossi, D. Fuller, J. Fuller, J. Haynes, and J. Huggins.** 1997. Naked DNA vaccines expressing the prM and E genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. J. Virol. **71:**9563–9569.
32. **Scott, R. M., A. Nisalak, K. H. Eckels, M. Tingpalapong, V. R. Harrison, D. J. Gould, F. E. Chapple, and P. K. Russell.** 1980. Dengue-2 vaccine: viremia and immunoresponses in rhesus monkeys. Infect. Immun. **27:**181–186.
33. **Smith, G. C. E.** 1956. A virus resembling Russian spring-summer encephalitis virus from an *Ixodid* in Malaya. Nature **178:**581–582.
34. **Sumanochitraporn, W., D. Strickman, R. Sithiprasasna, P. Kittayapong, and B. L. Innis.** 1998. Effect of size and geographic origin of *Aedes aegypti* on oral infection with dengue-2 virus. Am. J. Trop. Med. Hyg. **58:**283–286.
35. **Thind, I. S., and W. H. Price.** 1966. A chick embryo attenuated strain (TP21 E5) of Langat virus. I. Virulence of the virus for mice and monkeys. Am. J. Epidemiol. **84:**193–213.
36. **Van Der Most, R. G., K. Murali-Krishna, R. Ahmed, and J. H. Strauss.** 2000. Chimeric yellow fever/dengue virus as a candidate dengue vaccine: quantitation of the dengue virus-specific CD8 T-cell response. J. Virol. **74:**8094–8101.
37. **Vaughn, D. W., S. Green, S. Kalayanarooj, B. L. Innis, S. Nimmannitya, S. Suntayakorn, T. P. Endy, B. Raengsakulrach, A. L. Rothman, F. A. Ennis, and A. Nisalak.** 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J. Infect. Dis. **181:**2–9.
38. **Watts, D. M., B. A. Harrison, A. Nisalak, R. M. Scott, and D. S. Burke.** 1982. Evaluation of *Toxorhynchites splendens* (Diptera: Culicidae) as a bioassay host for dengue viruses. J. Med. Entomol. **19:**54–59.
39. **Zeng, L., B. Falgout, and L. Markoff.** 1998. Identification of specific nucleotide sequences within the conserved 3′-SL in the dengue type 2 virus genome required for replication. J. Virol. **72:**7510–7522.