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Chimeric Tick-Borne Encephalitis/Dengue Virus Is Attenuated in *Ixodes scapularis* Ticks and *Aedes aegypti* Mosquitoes

Amber R. Engel,[1,*] Dana N. Mitzel,[2,*,†] Christopher T. Hanson,[1] James B. Wolfinbarger,[2] Marshall E. Bloom,[2] and Alexander G. Pletnev[1]

Abstract

In an effort to derive an efficacious live attenuated vaccine against tick-borne encephalitis, we generated a chimeric virus bearing the structural protein genes of a Far Eastern subtype of tick-borne encephalitis virus (TBEV) on the genetic background of recombinant dengue 4 (DEN4) virus. Introduction of attenuating mutations into the TBEV envelope protein gene, as well as the DEN4 NS5 protein gene and 3′ noncoding region in the chimeric genome, results in decreased neurovirulence and neuroinvasiveness in mice, and restricted replication in mouse brain. Since TBEV and DEN4 viruses are transmitted in nature by ticks and mosquitoes, respectively, it was of interest to investigate the infectivity of the chimeric virus for both arthropod vectors. Therefore, parental and chimeric viruses were tested for growth in mosquito and tick cells and for oral infection *in vivo*. Although all chimeric viruses demonstrated moderate levels of replication in C6/36 mosquito cells, they were unable to replicate in ISE6 tick cells. Further, the chimeric viruses were unable to infect or replicate in *Aedes aegypti* mosquitoes and *Ixodes scapularis* tick larvae. The poor infectivity for both potential vectors reinforces the safety of chimeric virus-based vaccine candidates for the environment and for use in humans.

Key Words: *Aedes aegypti*—Dengue—Flavivirus—*Ixodes scapularis*—Live attenuated vaccine—Tick-borne encephalitis virus.

Introduction

Many arthropod-borne viruses (arboviruses) of the Flaviviridae family, including tick-borne encephalitis (TBEV), yellow fever, dengue (DEN), Japanese encephalitis, and West Nile viruses, are important human pathogens and represent a significant public health threat in many regions of the world (Lindenbach et al. 2007). TBE is a severe neurologic disease in Europe and Asia and is caused by several antigenically related viruses within the TBEV serocomplex. Viruses in the TBEV complex include the European, Siberian, and Far Eastern subtypes of TBEV, as well as Langat (LGT), louping ill, Powassan, Kyasanur forest disease, and Omsk hemorrhagic fever. TBEV is maintained in nature through an enzootic cycle between small mammals and ticks. *Ixodes* ticks are the primary vectors for transmission of TBEV; however, *Dermacentor* and *Hyalomma* spp. ticks have also been associated with TBE epidemics (Gritsun et al. 2003). Humans may become infected with TBEV via not only the bite of an infected tick, but also by ingesting unpasteurized milk products obtained from infected goats or by aerosol exposure (Gritsun et al. 2003).

Although the majority of these viruses cause symptoms that range from a nonspecific febrile illness to meningoencephalitis, Omsk hemorrhagic fever and Kyasanur forest disease viruses are able to cause hemorrhagic fever (Gritsun et al. 2003). Despite the availability of formalin-inactivated vaccines, there are >10,000 hospitalized TBE cases reported annually in endemic areas of Europe and Russia. Further, there has been an increase in TBE incidence over the last 20 years in Europe and TBEV has emerged in new areas of the world, where it has not previously been endemic or associated

[1]Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.
[2]Laboratory of Virology, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana.
*These two authors contributed equally to this work.
†*Present address:* Lovelace Respiratory Research Institute, Albuquerque, New Mexico.

with human disease (Kunze 2006, Randolph 2008). In addition, although rare, several instances of incomplete protection and vaccine failure (>65 cases to date) have recently been demonstrated with use of the current inactivated TBEV vaccines (Bender et al. 2004, Kleiter et al. 2007, Lotric-Furlan et al. 2008, Plisek et al. 2008, Stiasny et al. 2009, Andersson et al. 2010, Grgic-Vitek et al. 2010). Thus, an effective vaccine that induces a durable immunity against TBEV is urgently needed to protect humans in endemic regions since the geographic range and magnitude of TBE continues to expand and increase.

Previously, we developed two live attenuated TBEV vaccine candidates using a strategy based on chimerization of tick-borne flaviviruses with mosquito-borne dengue type 4 (DEN4) virus (Pletnev and Men 1998, Pletnev et al. 2001, Rumyantsev et al. 2006). LGT/DEN4 virus was generated by replacing the premembrane (prM) and envelope (E) structural protein genes of DEN4 with the corresponding genes of the antigenically distant, but naturally attenuated tick-borne LGT (Pletnev and Men 1998, Pletnev et al. 2001). TBEV/DEN4Δ30 virus was generated by introducing the prM and E protein genes of a highly virulent Far Eastern TBEV strain into the corresponding region of DEN4 containing a genetically stable 30 nucleotide deletion in the 3′ noncoding region (Rumyantsev et al. 2006). Although LGT/DEN4 is safe in mice, monkeys, and humans (Pletnev and Men 1998, Pletnev et al. 2001, Wright et al. 2008), the level of induced cross-reactive antibody response to heterologous TBEV in humans is low (Wright et al. 2008). In contrast, TBEV/DEN4Δ30 demonstrates moderate levels of immunogenicity and protective efficacy in mice and monkeys compared to LGT/DEN4, but retains an unacceptably high level of neurovirulence in these animals (Rumyantsev et al. 2006, Maximova et al. 2008). Therefore, further attenuation of TBEV/DEN4Δ30 was achieved by introducing additional amino acid substitutions (K→D at residue 315 in the structural E protein of TBEV and/or DR→AA at residues 654, 655 in the NS5 protein of DEN4) into the genome. These mutations attenuate TBEV/DEN4Δ30 for neurovirulence and replication in the brains of highly sensitive suckling mice, ablate neuroinvasiveness in immunocompromised mice, and are thus considered to be promising TBEV vaccine candidates (Engel et al. 2010).

Since arboviruses are transmitted in nature by their specific arthropod vector, the environmental safety of live attenuated vaccines is a significant concern. Due to the potential of these vaccines to be transmitted back into nature from vaccinated individuals via the bite of the given arthropod, they must not only be safe and immunogenic in the vertebrate host, but should also be unable to infect, replicate, or disseminate from its vector host. Thus, before testing in humans, the ability of the TBEV/DEN4 mutant viruses to infect and disseminate in their respective arthropod vectors was investigated in this study. Because the TBEV/DEN4 viruses are comprised of sequences derived from both mosquito- and tick-borne viruses, we assessed the ability of the chimeric viruses to infect mosquito or tick cell culture, and infect, replicate, and/or disseminate in mosquitoes or ticks.

Materials and Methods

Cell culture and viruses

Simian Vero cells (ECACC and World Health Organization seed) were maintained as described previously (Best et al. 2005, Engel et al. 2010). ISE6 tick cells, derived from *Ixodes scapularis* embryonated eggs, were kindly provided by Drs. Munderloh and Kurtti (University of Minnesota) and maintained at 34°C (Munderloh et al. 1994), whereas *Aedes albopictus*–derived C6/36 cells (ATCC) were cultured in Eagle's minimal essential medium (Invitrogen) at 32°C (Hanley et al. 2003).

A total of seven viruses were used in these studies (Supplementary Fig. S1; Supplementary data are available online at www.liebertonline.com/vbz). Chimeric LGT/DEN4 virus contains the prM and E protein genes of LGT strain TP21 and the remaining sequence derived from recombinant DEN4 (Pletnev and Men 1998). Chimeric TBEV/DEN4Δ30 virus (GenBank accession no. FJ828987) consists of the prM and E protein genes of Far Eastern TBEV strain Sofjin with the remaining sequence derived from DEN4Δ30 (GenBank accession no. AF326827) (Rumyantsev et al. 2006). Three additional TBEV/DEN4Δ30 mutant viruses that were used in these studies contained engineered mutation(s) within the E protein at residue 315 (E_{315}; Lys→Asp) and/or the NS5 protein at residues 654 and 655 ($NS5_{654,655}$; AspArg→AlaAla), as described previously (Engel et al. 2010). The TBEV/DEN4Δ30 viruses containing the engineered E_{315}, $NS5_{654,655}$, or both $E_{315}/NS5_{654,655}$ mutations are designated as vΔ30/E_{315}, vΔ30/$NS5_{654,655}$, or vΔ30/E_{315}/$NS5_{654,655}$, respectively. Wild-type DEN4 (GenBank accession no. AF375822), derived from a cDNA clone of DEN4 Caribbean strain 814669, and tick-borne LGT strain TP21 (GenBank accession no. AF253419) viruses were used as parental controls in these studies. The parental Far-Eastern TBEV strain Sofjin was not used because it is a BSL-4 agent.

Virus infection in cell culture

To investigate the kinetics of virus replication in cell culture, Vero or C6/36 cells were infected in triplicate with virus at a multiplicity of infection of 1 for 1 h at 37°C or 32°C, respectively. The cell culture medium was harvested daily. The virus titer in each cell culture preparation was determined on Vero cells using a plaque-forming assay in which virus foci were detected by immunostaining (Engel et al. 2010). Mean viral titers from each time point were determined from three replicates. The significance of replication at each time point was defined as a difference of ≥15-fold in mean virus titer.

To investigate the ability of the viruses to infect and replicate in tick cells, infection of Vero and ISE6 cells was undertaken as previously described (Mitzel et al. 2007). Both cell lines were initially infected with virus at a multiplicity of infection of 1, incubated at 34°C or 37°C, respectively, for 1 h, followed by addition of fresh maintenance medium. For ISE6 cells, all viruses were serially passaged two times after an initial infection of cells. The virus in the ISE6 culture medium was harvested on day 7 postinoculation and was used in subsequent infections of new ISE6 cells. The infected cells were stained for viral antigen by immunofluorescence assay (IFA) or used to extract viral RNA.

Detection of viral antigen in cell culture

After 4 (Vero cells) or 7 (ISE6 cells) days, infected cells were tested for the presence of virus antigen by IFA (Best et al. 2005). Briefly, slides were prepared by cytocentrifugation of 5×10^4 cells, followed by fixation in 100% acetone for 30 min.

Cells were then stained with a 1:1 mixture of TBEV- and DEN4-specific antibodies from hyperimmune mouse ascetic fluid at a final concentration of 1:1000, followed by staining with goat anti-mouse immunoglobulin G conjugated with Alexa Fluor 488 (Invitrogen) at a 1:500 dilution. The slides were mounted using ProLong Gold antifade with 4′6-diamidino-2-phenylindole (Invitrogen). Images were captured using an Olympus BX51 microscope and an Olympus DP70 camera.

Virus infection in mosquitoes and ticks

Aedes aegypti mosquitoes were reared at 27°C in 70% relative humidity with a 16-h daylight cycle. For oral infection of mosquitoes, 10^6 PFU of each virus was mixed separately in a 1:2 dilution with defibrinated rabbit blood (Spring Valley Laboratories) containing 2.5% sucrose to yield a final virus concentration of $10^{5.5}$ PFU/mL. Five-day-old female mosquitoes that had been deprived of a sugar source for 24 h were exposed to the virus blood meal for 25 min. The blood meal was prepared immediately before feeding and was offered to mosquitoes in a 37°C preheated water-jacketed feeder covered in stretched parafilm. Virus in the blood meal was quantitated after oral infection of mosquitoes and found to be stable. Fully engorged mosquitoes were transferred to a new container and allowed continuous access to a cotton pad soaked in a solution of 10% sucrose. Mosquitoes were maintained as described above for either 14 or 21 days postinfection (dpi), after which they were harvested and stored at −80°C until dissection.

To assess virus infection and dissemination in mosquitoes, the legs were removed from the mosquitoes and the bodies and heads were separately triturated in 250 μL Hanks balanced salt solution (Invitrogen) supplemented with 10% fetal bovine serum, 0.0025 mg/mL amphotericin, 0.05 mg/mL ciprofloxacin, and 0.07 mg/mL clindamycin. Virus titers of head and body suspensions were determined on Vero cells by plaque-forming assay. The Fisher's exact test ($p < 0.001$; GraphPad Prism 5 Software) was used to determine statistical significance for the rates of infection and dissemination, whereas one-way analysis of variance followed by the Tukey *post hoc* test ($p < 0.001$) was used to test for significant differences between the virus titers in the bodies and heads of mosquitoes.

I. scapularis adult females with egg sacs (Oklahoma State University, Stillwater, OK) were housed in a relative humidity of 98% with a 16-h daylight cycle for oviposition and larvae emergence. The larvae were used within 6 months of emergence.

Infection of ticks by the immersion method has been previously described (Mitzel et al. 2007). Briefly, 60 larvae per group were collected in a sterile 1.5-mL screw cap centrifuge tube and pretreated by exposure to a reduced relative humidity. The tick larvae were then immersed in 0.5 mL of complete Dulbecco's modified Eagle's medium (Invitrogen) containing 10^6 PFU/mL of virus and incubated at 34°C for 45 min. Larvae were removed from the medium and maintained at a relative humidity of 98% for 21 and 45 days postimmersion (dpim).

Detection of viral RNA from cell culture or whole ticks

Total RNA was isolated from virus-infected Vero or ISE6 cells using the RNeasy mini kit and QIAshredder spin columns (Qiagen) (Mitzel et al. 2007).

To isolate total RNA from ticks, a pooled group of 25 ticks were homogenized using one of two methods. In the first method, ticks were frozen in liquid nitrogen and triturated with mortar and pestle, and the tick suspension was passed through a QIAshredder spin column (Mitzel et al. 2007). For the second method, ticks were frozen in liquid nitrogen and transferred to Lysing Matrix D tubes (MPBio) containing 800 μL of RLT buffer (Qiagen RNeasy kit). The ticks were homogenized using the Fastprep 24 (MPBio) set at speed level 6 m/s for 40 s and the homogenate was clarified by centrifugation at 21,000 *g* for 3 min. For both methods, RNA from the homogenates was purified using an RNeasy mini kit. RNA tested with both methods demonstrated similar results.

Viral RNA (250 ng) from cell culture and ticks was detected using SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen) and primers that were specific for positive- or negative-sense strand RNA of TBEV (Sofjin), LGT, or DEN4 viruses (Table 1) (Schwaiger and Cassinotti 2003, Mitzel et al. 2007). Amplification of this amount of RNA resulted in the possible detection of at least one positively infected ticks per group.

Results

Replication of chimeric viruses in C6/36 mosquito cells

Since the chimeric viruses are comprised mainly of mosquito-borne DEN4, we first investigated the ability of the chimeric and parental viruses to infect and replicate in mosquito C6/36 cells and compared their pattern of replication with that in Vero cells. LGT and DEN4 viruses were used as parental controls. In Vero cells, all viruses demonstrated similar growth kinetics over 8 days and replicated efficiently to high titers (mean peak titers between $10^{7.2}$ and $10^{7.8}$ PFU/mL) (Fig. 1A). As expected, DEN4 virus replicated well in C6/36 cells, attaining a mean titer of $10^{8.2}$ PFU/mL by 3 dpi, whereas the growth of tick-borne LGT virus was completely restricted in this cell line and resulted in no productive infection (Fig. 1B). In contrast, chimerization of LGT with DEN4 or TBEV with DEN4Δ30 resulted in viruses that replicated well in C6/36 cells and attained a mean titer of $10^{7.3}$ or $10^{8.2}$ PFU/mL, respectively, by day 6. However, although the TBEV/DEN4Δ30 mutants (vΔ30/E_{315}, vΔ30/$NS5_{654,655}$, and vΔ30/E_{315}/$NS5_{654,655}$) reached mean peak titers between $10^{5.8}$ and $10^{7.4}$ PFU/mL in C6/36 cells, their replication was significantly reduced in mosquito cells on multiple days compared to DEN4 or TBEV/DEN4Δ30 viruses. Further, introduction of both mutations into TBEV/DEN4Δ30 decreased the efficiency of virus replication to a greater level than each mutation individually.

Replication and dissemination of chimeric viruses in Ae. aegypti *mosquitoes*

Since they are the principal mosquito vector that transmits DEN virus (Gubler 1998), *Ae. aegypti* were next orally infected with a high titer ($10^{5.5}$ PFU/mL) of DEN4, LGT, or the chimeric viruses to assess their ability to infect, replicate, and disseminate in mosquitoes. To measure infectivity and dissemination, the presence of virus in the bodies and heads of mosquitoes, respectively, was assayed on days 14 and 21 after feeding mosquitoes on a blood meal (Table 2). DEN4 virus infected a significant ($p < 0.001$) percentage of mosquitoes on

Table 1. Primers Used for RT-PCR

	Primer	Primer position	Amplicon size (bp)
Positive-sense strand			
DEN4[a]	5′-CCAGAGTCCCCAGCGAGACTAG-3′	2529	1942
	5′-GCCAAGGGGTAGAGACCTGAC-3′	4471	
TBEV[b]	5′-GCCACAGTGCGGAAGGAAAGAG-3′	478	1866
	5′-GGATCTTGGGCAAGAACCCCACTC-3′	2344	
LGT[c]	5′-CAGCGACTGTGATTGTGGATATTC-3′	44	2355
	5′-AAGGTTGGGTTCCTCATGTTCAAGC-3′	2397	
Negative-sense strand			
DEN4[a]	5′-CTCCATGACGCCACACAACCCATGTC-3′	2449	1627
	5′-CTCAGAAACCCAGGATTCGCGCTCTTGG-3′	822	
TBEV[b]	5′-CACCGCCAAGAACTGTGTGCA-3′	2299	1399
	5′-GACCGTGGAAAGTGTGGTGAC-3′	900	
LGT[c,d]	5′-ACTGGCCGGTAGAAACAGCTT-3′	3199	1748
	5′-AAGTCAGCCTCGTTCACAGTGT-3′	1451	
LGT/DEN[c,e]	5′-CGCTCCTCCCAGGACGGTGTGC-3′	2302	1334
	5′-GCGTCGAGATGCACCCACCTGGA-3′	968	

[a]Sequence of DEN4 vector p4 (GenBank accession no. AY648301).
[b]Sequence of TBEV Sofjin strain (GenBank accession no. X07755).
[c]Sequence of LGT virus TP21 strain (GenBank accession no. AF253419).
[d]Negative-sense primer used to detect LGT virus infection.
[e]Negative-sense primer used to detect LGT/DEN4 virus infection.
DEN4, dengue 4; LGT, Langat; TBEV, tick-borne encephalitis virus.

day 14 and reached a mean virus titer of $10^{3.5}$ PFU/mosquito body. In addition, DEN4 was able to disseminate to the head in the majority ($p < 0.001$) of mosquitoes, but reached a five-fold lower titer. In contrast, LGT, LGT/DEN4, and all TBEV/DEN4Δ30 mutants were unable to infect or disseminate in mosquitoes by 14 dpi.

To assess whether the viruses replicated slower in mosquitoes and therefore have a longer extrinsic incubation period, infected mosquitoes were also harvested on day 21. Similar to studies by others (Hanley et al. 2003, Blaney et al. 2007), DEN4 virus still infected a significant ($p < 0.001$) percentage of mosquitoes by 21 dpi, and disseminated to the heads in all mosquitoes infected (Table 2). Comparable mean virus titers were observed in the bodies and heads on day 21 as on day 14 in DEN4-infected mosquitoes. In contrast, despite undergoing a longer incubation, none of the mosquitoes that fed on a blood meal containing LGT or any of the chimeric viruses became infected. Notably, pilot studies using the unmodified TBEV/DEN4 virus demonstrated that it could infect and replicate in a low number (1/18; 5.6%) of *Ae. aegypti* after oral infection (data not shown), indicating that introduction of Δ30 was necessary to completely ablate TBEV/DEN4 infectivity and dissemination in these mosquitoes. Thus, these results demonstrate that LGT- or TBEV-derived viruses were significantly attenuated in *Ae. aegypti*, an arthropod vector that is highly susceptible to DEN4 virus infection and dissemination.

Replication of chimeric viruses in ISE6 tick cells

Since the Δ30 mutation was necessary to completely attenuate TBEV/DEN4 for infectivity and dissemination in mosquitoes, TBEV/DEN4Δ30, its derivatives, and the

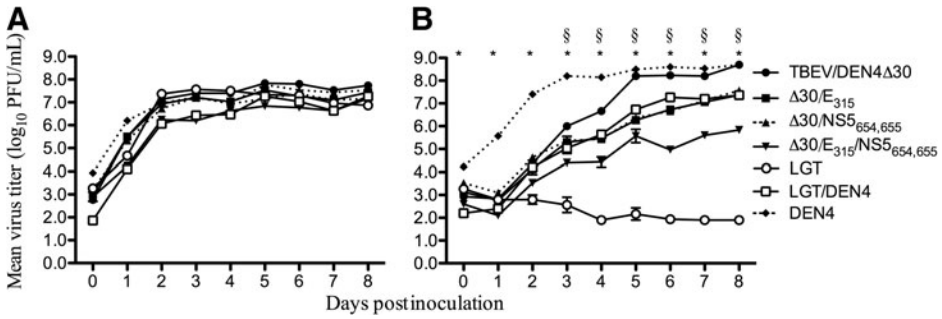

FIG. 1. Replication kinetics of TBEV/DEN4Δ30, its mutants, LGT, DEN4, or LGT/DEN4 in **(A)** simian Vero or **(B)** mosquito C6/36 cells infected at a multiplicity of infection of 1. Each time point represents the mean viral titer (±standard error) from three replicates. The significance of viral replication was defined as a difference of ≥15-fold in mean virus titer at the indicated time point. Asterisks indicate that the replication of DEN4 virus is significantly different from that of the TBEV/DEN4Δ30 mutants, LGT, or LGT/DEN4 viruses, whereas § indicates that LGT virus is significantly different in replication from all remaining viruses on the indicated days. The limit of detection was 2.0 $\log_{10}$ PFU/mL. DEN4, dengue 4; LGT, Langat; TBEV, tick-borne encephalitis virus.

Table 2. Infectivity of the Dengue 4, Langat, and Chimeric Viruses in *Aedes aegypti* Mosquitoes Fed on a Blood Meal Comprised of $10^{5.5}$ PFU/mL of Virus

	No. infected/no. tested (percentage infected)[b]			
	Day 14		*Day 21*	
Virus[a]	*Body*	*Head*	*Body*	*Head*
DEN4	16/18 (89)[c] 3.5 ± 0.38[d]	13/16 (81)[c] 2.8 ± 0.28[d]	15/18 (83)[c] 3.7 ± 0.40[d]	15/15 (100)[c] 3.0 ± 0.32[d]
TBEV/DEN4Δ30	0/18 (0)	0/18 (0)	0/18 (0)	0/18 (0)
vΔ30/E_{315}	0/18 (0)	0/18 (0)	0/18 (0)	0/18 (0)
vΔ30/$NS5_{654,655}$	0/18 (0)	0/18 (0)	0/18 (0)	0/18 (0)
vΔ30/E_{315}/$NS5_{654,655}$	0/18 (0)	0/18 (0)	0/18 (0)	0/18 (0)
LGT	0/18 (0)	0/18 (0)	0/18 (0)	0/18 (0)
LGT/DEN4	0/18 (0)	0/18 (0)	0/18 (0)	0/18 (0)

[a] *Aedes aegypti* mosquitoes were orally infected with the indicated viruses and incubated for 14 or 21 dpi. Mosquitoes ingested ~2.8 $\log_{10}$ PFU of virus, assuming that each mosquito ingested 2 μL of a 5.5 $\log_{10}$ PFU/mL blood meal.

[b] On the indicated day, mosquito bodies and heads were harvested, triturated, and virus titer was determined on Vero cells.

[c] Indicates that percent infected is statistically significant from remaining groups ($p < 0.001$; Fisher's exact test).

[d] Mean virus titer ± standard error ($\log_{10}$ PFU/mosquito) of DEN4-infected mosquito bodies and heads are shown. These titers are significantly different from the remaining virus titers ($p < 0.001$; one-way analysis of variance, followed by Tukey *post hoc* test).

LGT-derived viruses were next tested in tick cells and ticks. To determine the susceptibility of tick cells to infection with the chimeric viruses, the presence of virus antigen in infected ISE6 cells was first examined by IFA. After infection of ISE6 cells with tick-borne LGT virus, antigen was found to be distributed in the cytoplasm (Fig. 2), which is the primary intracellular site for flavivirus replication (Lindenbach et al. 2007). Despite the detection of viral antigen in simian Vero cells, no antigen could be detected in ISE6 cells infected with DEN4 or any of the chimeric viruses (Fig. 2). To ascertain whether the lack of antigen could be due to low infection rates of DEN4 and the chimeric viruses, they were serially passaged two additional times in ISE6 cells. Again, virus antigen was detected in the cytoplasm of cells infected with LGT virus, but not in cells infected with DEN4 or the chimeric viruses (data not shown). Thus, infection of ISE6 cells with any of the chimeric viruses tested did not result in detectable levels of virus antigen. These data suggest that tick cells are not susceptible to infection with mosquito-borne DEN4-based viruses.

Since the lack of DEN4 or chimeric virus antigen detected in tick cells by IFA may be due to low levels of virus infectivity and replication, confirmation of ISE6 cell infection was performed by RT-PCR, a more sensitive method of virus detection, using primers specific for the positive- and negative-sense strands of virus RNA. Flavivirus negative-sense strand RNA, or complementary RNA, is not a component of the virion and its presence is an obligatory marker for virus replication (Lindenbach et al. 2007). After initial infection, positive-sense RNA was identified in LGT-, DEN4-, TBEV/DEN4Δ30-, vΔ30/E_{315}-, and vΔ30/$NS5_{654,655}$-infected cells, but not in cells infected with vΔ30/E_{315}/$NS5_{654,655}$ or LGT/DEN4 virus (Fig. 3A). Although negative-sense RNA was detected in TBEV/DEN4Δ30-, vΔ30/E_{315}-, and vΔ30/$NS5_{654,655}$-infected ISE6 cells, it was not detected in cells infected with vΔ30/E_{315}/$NS5_{654,655}$, LGT/DEN4, or DEN4 viruses (Fig. 3B), further suggesting that ISE6 cells are not permissive to these viruses. The presence of DEN4 positive- but not negative-sense RNA suggests that the detected RNA may have been derived from the input virus and was not a direct result of virus replication.

Since negative-sense RNA of LGT, TBEV/DEN4Δ30, vΔ30/E_{315}, and vΔ30/$NS5_{654,655}$ was detected in ISE6 cells, further evaluation of all viruses was performed in tick cells. The presence of viral RNA in ISE6 cells was examined after two serial passages of the supernatant. Both positive- and negative-sense RNA from LGT virus were detected after the second and third passage in ISE6 cells. In contrast, viral RNA was not detected for any of the chimeras or DEN4 virus at passage 2 or 3 (Table 3). The presence of TBEV/DEN4Δ30, vΔ30/E_{315}, and vΔ30/$NS5_{654,655}$ positive- and negative-sense RNA in the initial infection but not in subsequent passages suggests that early replication of these chimeras did not result in a productive infection in ISE6 cells.

Replication of chimeric viruses in tick larvae

To determine the potential of chimeric LGT/DEN4 and TBEV/DEN4Δ30 viruses to infect the vector important for transmission of tick-borne flaviviruses, the susceptibility of *I. scapularis* to virus infection was examined utilizing a method recently developed for infecting tick larvae with tick-borne LGT virus (Mitzel et al. 2007). After immersion of tick larvae in the medium containing a high virus concentration (10^6 PFU/mL), infection and replication of the chimeric viruses in the larvae were evaluated and compared to that of LGT virus using RT-PCR. Since previous experience with LGT virus has shown that ~75% of *I. scapularis* larvae become infected after immersion with 10^6 PFU/mL (Mitzel et al. 2007), it was used in the current study as a positive control. At 21 dpim, positive-sense viral RNA was detected by ticks infected with LGT virus, but not in ticks immersed in the medium containing LGT/DEN4, TBEV/DEN4Δ30, vΔ30/E_{315}, vΔ30/$NS5_{654,655}$, or vΔ30/E_{315}/$NS5_{654,655}$ (Fig. 4A). Although a very faint PCR product was obtained for positive-sense DEN4 RNA at 21 dpim, no product was detected for the negative-sense RNA in ticks infected with the chimeric or DEN4 viruses (Fig. 4B). The complementary strand of LGT RNA was detected at 21 dpim, confirming replication of LGT virus in the tick larvae.

Since the presence of positive-sense but not negative-sense strand DEN4 RNA may represent input virus or inefficient

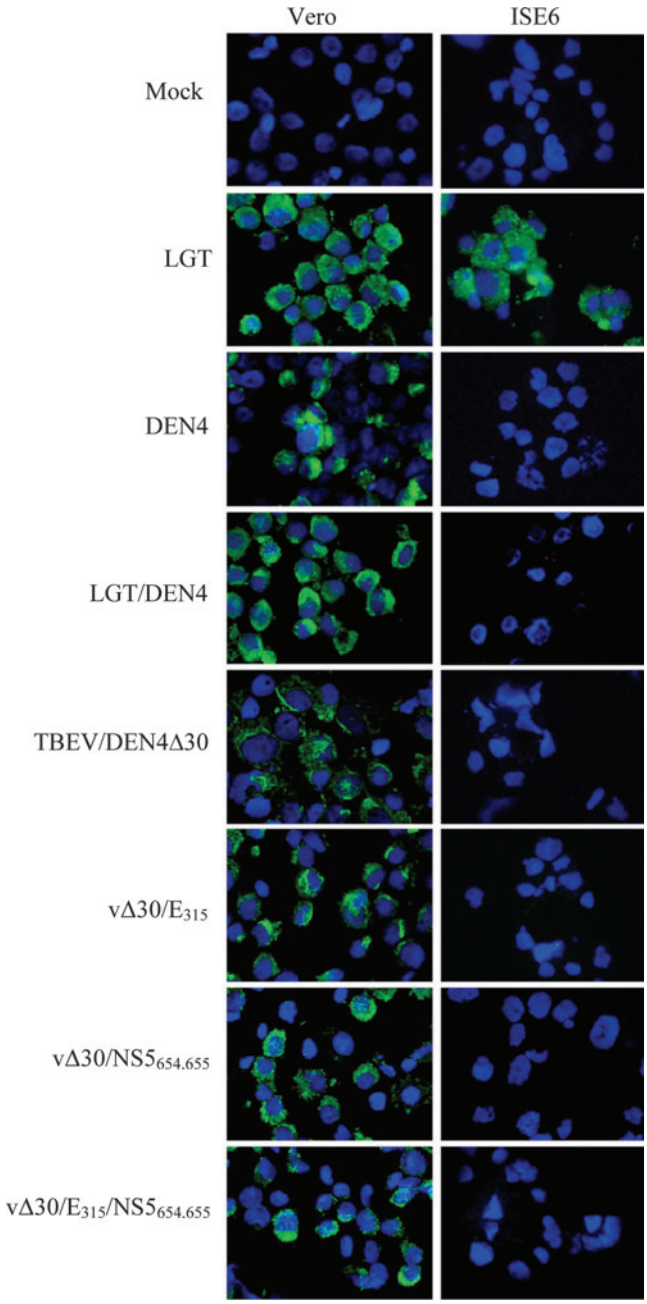

FIG. 2. Viral antigen expression in Vero and ISE6 cells. Vero (left panel) or ISE6 (right panel) cells were infected with DEN4, LGT, LGT/DEN4, TBEV/DENΔ30, or TBEV/DENΔ30 mutant viruses at a multiplicity of infection of 1 or with medium (mock) and stained on day 4 (Vero) or 7 (ISE6) postinfection for the presence of virus antigen (green). 4′6-Diamidino-2-phenylindole (blue) was used to counterstain the nuclei.

replication, it was of interest to determine whether DEN4 RNA could be detected at a later time point. Therefore, total RNA was isolated from ticks infected with LGT or DEN4 virus at 45 dpim. Although viral RNA was detected for LGT virus, DEN4 RNA could not be detected (data not shown). These data suggest that naïve *I. scapularis* larvae take up DEN4 when exposed to high concentrations of virus, but that the larvae are not permissive for productive virus replication by DEN4 or the chimeric viruses.

Discussion

Experience with yellow fever 17D and Japanese encephalitis SA-14-14-2 viruses has demonstrated that live attenuated vaccines are highly efficacious and protective against wild-type viruses that cause disease (Monath 2005, Halstead and Jacobson 2008). Although live attenuated vaccines are advantageous in many ways over other vaccine platforms, safety concerns have arisen with this vaccine strategy. Principal among these are the potential reversion to wild-type virus phenotype and the risk of introducing the vaccine virus into the environment. These concerns are of particular importance if the viruses are normally transmitted by arthropod vectors. To reduce the capacity of the vaccine virus to revert to virulent phenotype or to be transmitted back to humans by the given vector in nature, two safety barriers should be in place. The vaccine should demonstrate (1) a low and short-duration of viremia in the vertebrate host and (2) poor infectivity and replication in the arthropod vector. A short duration of viremia in the vaccinated individual would lower the likelihood for introduction of mutations that lead to reversion and also lower the likelihood of feeding mosquitoes or ticks to become infected. Further, poor infectivity and dissemination in the vector would reduce the chance of transmission back into the environment if the vaccine is taken up by a feeding mosquito or tick.

We have previously demonstrated that our chimeric vaccine candidates exhibit low levels of viremia in monkeys and/or in humans, indicating that the first safety barrier is in place (Pletnev et al. 2001, Rumyantsev et al. 2006, Wright et al. 2008). Further, we have demonstrated that the TBEV/DEN4Δ30 vaccine candidates are greatly restricted for replication in mice (Engel et al. 2010) and in monkeys (unpublished data), indicating that the likelihood of reversion to virulence, their potential recombination with wild-type virus, and/or uptake from a vaccine by the feeding vector would be extremely low. However, the ability of these vaccine candidates to be transmitted by mosquitoes and ticks remained undetermined. Although the attenuated TBEV/DEN4Δ30-based vaccine candidates have not yet been assessed in humans, in the present study, we measured their ability to infect and replicate in mosquitoes and ticks and compared them with LGT and LGT/DEN4, two viruses that have previously been tested in human clinical trials (Il'enko et al. 1968, Gritsun et al. 2003, Wright et al. 2008).

Similar to previous observations (Pletnev and Men 1998, Kuno 2007), tick-borne LGT virus replicated poorly in mosquito C6/36 cells, suggesting that these cells are refractory to LGT infection since no increase in titer was observed over time. In contrast, LGT/DEN4 and TBEV/DEN4Δ30 mutant viruses, containing ~80% of the DEN4 genome, replicated well in mosquito cells. However, consistent with observations by Lawrie et al. (2004) and Kuno (2007), tick-borne LGT virus infected and replicated efficiently in ISE6 tick cells. Although three chimeric viruses (TBEV/DEN4Δ30, vΔ30/E_{315}, and vΔ30/$NS5_{654,655}$) were able to infect tick cells, they, along with LGT/DEN4 and vΔ30/E_{315}/$NS5_{654,655}$, were unable to establish a productive infection in tick cells. Both DEN4 and the chimeric viruses failed to replicate in the tick-borne virus permissive cells (ISE6), despite replicating efficiently in cells (C6/36) permissive for mosquito-borne virus. This indicates that some molecular determinants of virus tropism may be found outside of the prM-E structural region, such as in the

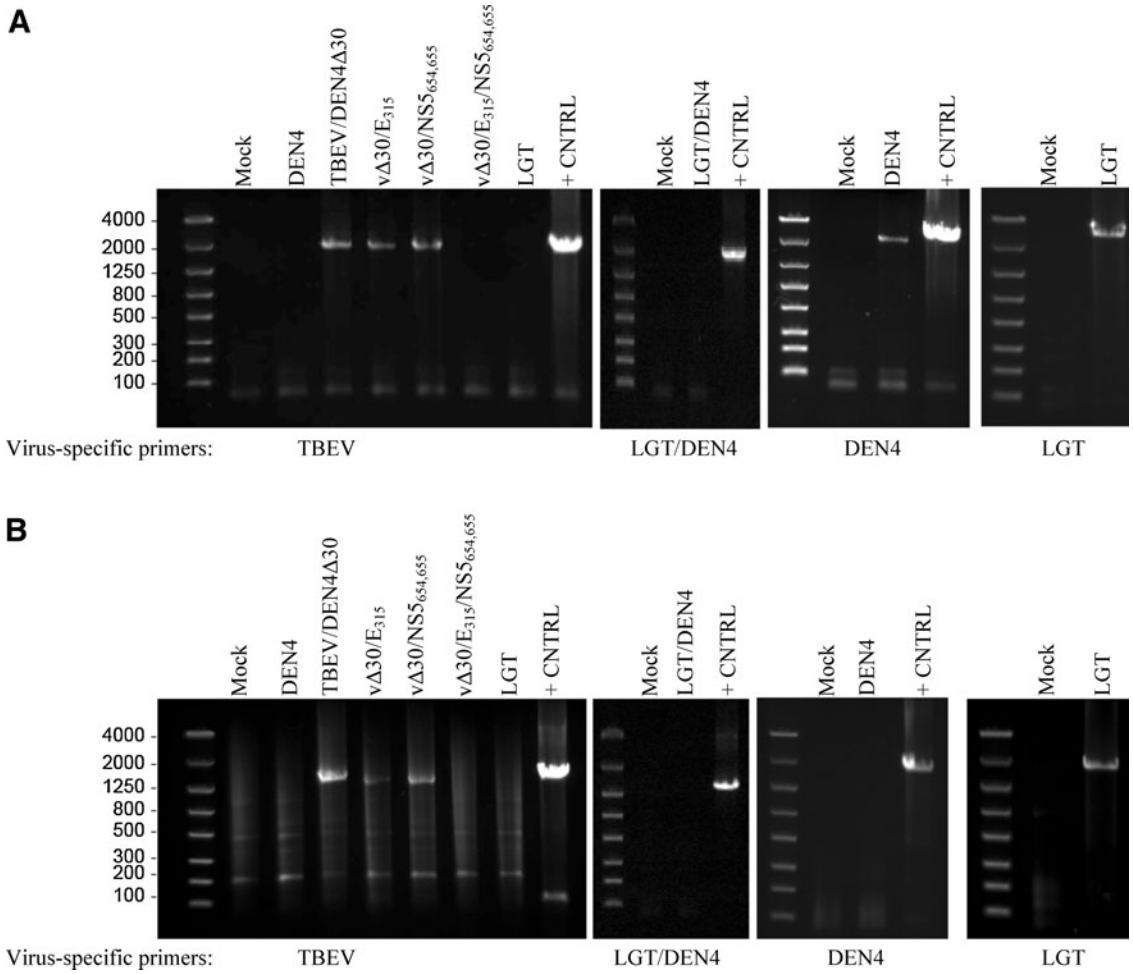

FIG. 3. Detection of viral RNA in ISE6 tick cells by RT-PCR. Total cellular RNA was isolated from cells infected with the indicated virus or from mock-inoculated cells. Viral RNA was detected using RT-PCR and primers specific for **(A)** positive- and **(B)** negative-sense strands of TBEV, LGT, or DEN4 virus RNA. Primers specific for TBEV were used to detect TBEV/DEN4Δ30 and its derivatives (vΔ30/E_{315}, vΔ30/$NS5_{654,655}$, and vΔ30/E_{315}/$NS5_{654,655}$). Primers specific to LGT or DEN4 virus genome were used to detect LGT, LGT/DEN4, or DEN4 RNA. Total RNA isolated from Vero cells infected with the corresponding virus was used as a positive control (+CNTRL) for detection of TBEV/DEN4Δ30, DEN4, LGT, or LGT/DEN4 RNA.

capsid, NS2A, or NS4B proteins, regions that have previously been demonstrated to affect tick cell or mosquito tropism (McElroy et al. 2006, Schrauf et al. 2009).

DEN4 virus demonstrated a high level of vector competence in *Ae. aegypti*, as >80% of mosquitoes demonstrated infectivity and dissemination on 14 and 21 dpi. However, neither LGT nor LGT/DEN4 virus were able to infect, replicate, or disseminate in *Ae. aegypti* mosquitoes, despite the ability of LGT/DEN4 virus to replicate in C6/36 cells. These results are in agreement with the previous observation that

Table 3. Detection of Viral RNA in ISE6 Tick Cells During Three Serial Passages

	Passage 1[a]		*Passage 2*		*Passage 3*	
Virus	*Positive*[b]	*Negative*[b]	*Positive*	*Negative*	*Positive*	*Negative*
LGT	+	+	+	+	+	+
DEN4	+	−	−	−	−	−
LGT/DEN4	−	−	−	−	−	−
TBEV/DEN4Δ30	+	+	−	−	−	−
vΔ30/E_{315}	+	+	−	−	−	−
vΔ30/$NS5_{654,655}$	+	+	−	−	−	−
vΔ30/E_{315}/$NS5_{654,655}$	−	−	−	−	−	−

[a]Presence or absence of viral RNA is denoted by + or −, respectively.
[b]Positive- or negative-sense RNA strand detected by RT-PCR.

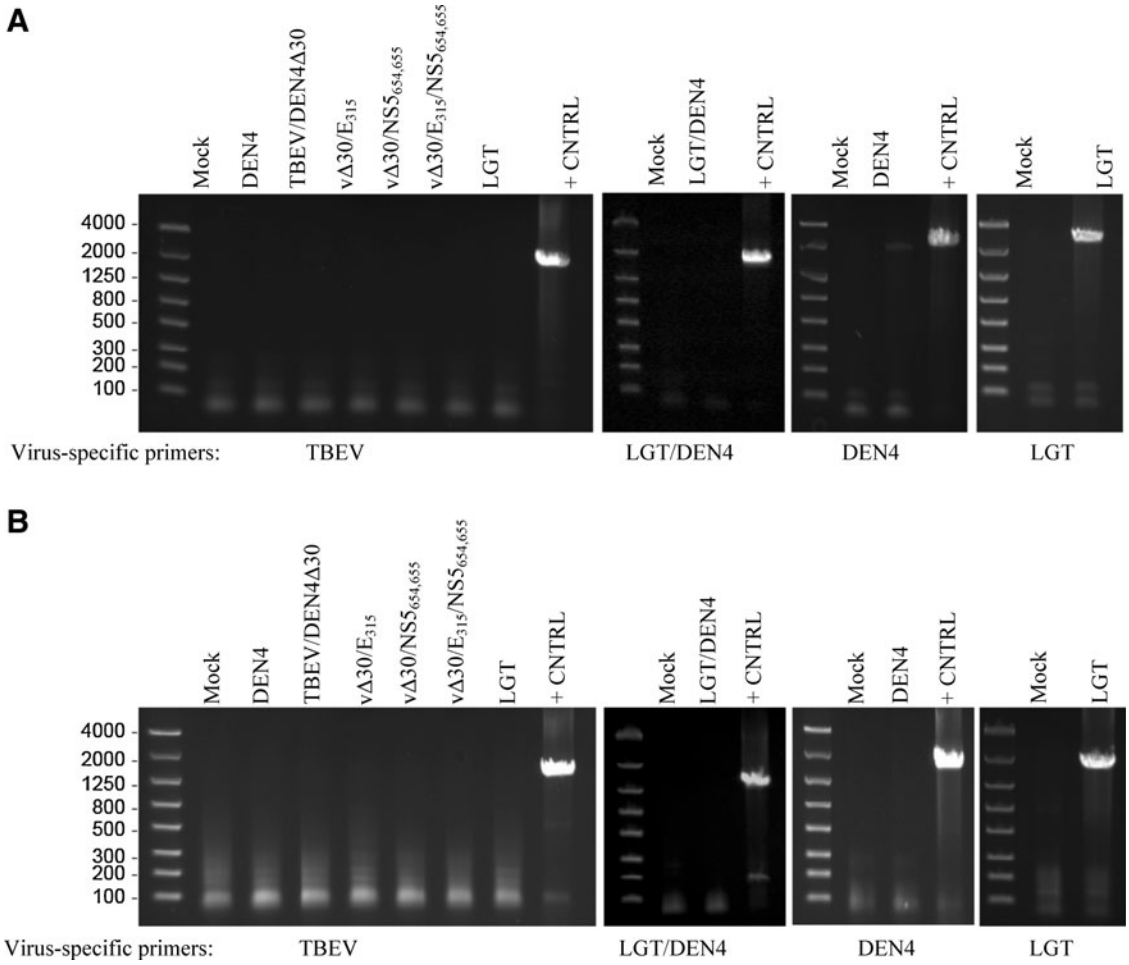

FIG. 4. Presence of viral RNA in ticks after infection. Total RNA was isolated from a group of 25 *Ixodes scapularis* larvae at 21 days postimmersion with medium only (mock) or the various viruses. Viral RNA was detected using primers specific for the **(A)** positive- or **(B)** negative-sense RNA. To detect TBEV/DEN4Δ30 and its derivatives (vΔ30/E_{315}, vΔ30/$NS5_{654,655}$, and vΔ30/E_{315}/$NS5_{654,655}$), TBEV-specific primers were used. LGT- or DEN4-specific primers were used to detect LGT/DEN4, LGT, or DEN4. Total RNA isolated from virus infections in Vero cells were used as positive controls (+CNTRL).

neither LGT nor LGT/DEN4 virus could infect or disseminate in *Toxorhinchites splendens* mosquitoes after intrathoracic inoculation (Pletnev et al. 2001), a highly sensitive model for detection of mosquito-borne viruses in which the route of inoculation bypasses the midgut escape barrier. Further, despite the ability to replicate well in C6/36 cells, all chimeric viruses (containing a majority of the DEN4 genome) were unable to infect, replicate, or disseminate in *Ae. aegypti* after oral infection. These data were consistent with studies of other chimeric flaviviruses that are able to replicate in C6/36 cells, but unable to replicate or disseminate in *Ae. aegypti* (Bhatt et al. 2000, Johnson et al. 2003, 2004, Blaney et al. 2004, 2007, Charlier et al. 2010).

In the present study, we demonstrated that attenuation of the TBEV/DEN4Δ30-derived viruses for mosquitoes results from (1) chimerization between two heterologous viruses that exhibit very different host and vector specificities, and (2) introduction of the Δ30 mutation. Our lab has previously shown that chimerization of different flaviviruses with DEN4 (i.e., LGT/DEN4, West Nile/DEN4, DEN2/DEN4, and DEN3/DEN4 viruses) can directly yield viruses that exhibit both reduced virulence in mammals and restricted replication in mosquitoes (Pletnev et al. 2001, Whitehead et al. 2003, Blaney et al. 2004, Hanley et al. 2005). However, although chimerization between TBEV and DEN4 virus reduced mosquito infectivity by ~16-fold compared to DEN4 (5.6% vs. 89%), introduction of Δ30 was necessary to completely ablate mosquito infectivity and dissemination. These data correlate with those by Hanley et al. (2003) and Troyer et al. (2001), who have shown that introduction of the Δ30 mutation restricts the ability of DEN4 virus to infect and disseminate in *Ae. aegypti* mosquitoes.

I. scapularis ticks were highly susceptible to tick-borne LGT virus infection, but were not susceptible to infection by any viruses containing the DEN4 genome. After infection by immersion, LGT virus was able to infect and replicate in tick larvae on 21 and 45 dpi, as detected by RT-PCR. However, LGT/DEN4 and TBEV/DEN4Δ30 mutants lost the ability to infect and replicate in ticks, suggesting that the DEN4 genetic background was a major contributor responsible for the attenuated phenotype of these viruses in ticks.

In the present studies, we have used a cell line and larvae from *I. scapularis* ticks, which is not the natural vector for European, Siberian, or Far Eastern subtypes of TBEV. However, the ability of Ixodid ticks to be competent for TBEV appears to be a general feature of these vectors; it has been well documented that TBEV can naturally and experimentally infect different members of the *Ixodes ricinus* complex and ticks from other genera (Varma and Smith 1972, Nosek and Kozuch 1985, Lawrie et al. 2004, Kuno 2007, Mitzel et al. 2007, Ruzek et al. 2008). For instance, Ruzek et al. (2008) and Lawrie et al. (2004) both demonstrated that different viruses in the TBEV complex were able to infect a variety of tick cell lines, indicating that it is likely that the results demonstrated in these studies would be similar in other tick cell lines and ticks. However, it is unclear whether the TBEV/DEN4Δ30 chimeric viruses could replicate in the natural vectors of European, Siberian, and Far Eastern subtypes of TBEV, *I. ricinus* and *Ixodes persulcatus*. Since the TBEV/DEN4Δ30 chimeric viruses do not replicate in *I. scapularis*, it would be of interest to test the susceptibility of *I. ricinus* and *I. persulcatus* to these viruses in future studies.

In summary, the combination of chimerization and introduction of the Δ30 mutation dramatically restricted the ability of the viruses to replicate and disseminate in mosquito and tick vectors. This suggests that the chimeric viruses have a limited potential for transmission and will not represent a threat for the environment.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Alexander G. Pletnev
Laboratory of Infectious Diseases
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Building 33, Room 3W10A
33 North Drive, MSC 3203
Bethesda, MD 20892

E-mail: apletnev@niaid.nih.gov