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Evaluation of the Langat/Dengue 4 Chimeric Virus as a Live Attenuated Tick-Borne Encephalitis Vaccine for Safety and Immunogenicity in Healthy Adult Volunteers

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Abstract

With the steady rise in tick-borne encephalitis virus (TBEV) infections in Europe, development of a live attenuated vaccine that will generate long-lasting immunity would be of considerable benefit. A chimeric flavivirus, designated LGT/DEN4, was previously constructed to have a genome containing the prM and E protein genes of Langat virus (LGT), a naturally attenuated member of the TBEV complex, and the remaining genetic sequences derived from dengue 4 virus (DEN4). LGT/DEN4 was highly attenuated in rodents and non-human primates, and clinical trials in humans were initiated. Twenty-eight healthy seronegative adult volunteers were randomly assigned in a 4:1 ratio to receive 10^3 PFU of LGT/DEN4 or placebo. Volunteers were closely monitored for clinical responses and for blood chemistry and hematological changes, and the level of viremia and the magnitude and duration of the neutralizing antibody response were determined. The LGT/DEN4 vaccine was safe and viremia was seen in only one vaccinee. Infection induced a neutralizing antibody response to wild-type LGT in 80% of volunteers with a geometric mean titer (GMT) of 1:63 present on day 42 post-immunization; however the antibody response against TBEV was both much less frequent (35%) and lower in magnitude (GMT = 1:9). To assess the response to a booster dose, 21 of the original 28 volunteers were re-randomized to receive a second dose of either 10^3 PFU of vaccine or placebo given 6 to 18 months after the first dose. The immunogenicity against either LGT or TBEV was not significantly enhanced after the second dose of vaccine. Thus, chimerization of LGT with DEN4 yielded a vaccine virus that was highly attenuated yet infectious in humans. The level of replication was sufficiently restricted to induce only a weak cross-reactive antibody response to TBEV. To provide a sufficient level of immunity to widely prevalent, highly neurovirulent strains of TBEV in humans, vaccine candidates will likely need to be based on the TBEV structural protein genes.

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Keywords

Tick-Borne Encephalitis; Live Virus Vaccine; Human

1. Introduction

Tick-borne encephalitis (TBE) is a severe disease caused by antigenically closely related RNA viruses belonging to the *Flaviviridae* family [1]. Members of the tick-borne encephalitis virus (TBEV) antigenic complex, which includes Kyasanur forest disease, Central European and Far Eastern tick-borne encephalitis, Langat, Louping ill, Omsk hemorrhagic fever, and Powassan viruses, are transmitted in nature in a cycle between ticks and various mammalian species with humans serving as incidental hosts. These viruses are endemic throughout most of the Northern Hemisphere, and, except for Langat virus (LGT), cause human disease of varying severity with up to 30% mortality [1]. Despite immunization of human populations living in endemic areas with an inactivated virus vaccine, TBE remains a serious public health problem in Europe and Asia, where up to 14,000 human cases are reported annually [2]. Licensed inactivated TBEV vaccines are available in Europe and Russia that induce effective short-term protection; however, three doses of vaccine are required for primary immunization. Since the titers of neutralizing antibodies induced by the inactivated TBEV vaccine decline with time after vaccination and with age, booster vaccinations every 3 years are needed to maintain protective immunity [3,4]. A TBEV vaccine that induces a more durable immunity would be an improvement over the existing vaccine. Use of a live attenuated virus vaccine is the most likely approach to achieve this goal since a single dose of the yellow fever (YF) 17D virus vaccine provides relatively long-term immunity in humans [1].

Immunity to members of the TBEV complex is mediated in large part by neutralizing antibodies directed to the structural envelope (E) glycoprotein of the virus. Since the amino acid identity among the E glycoproteins of the TBEV complex of flaviviruses is 78% or greater [5–7], these viruses share many protective E protein epitopes and hence form a single serogroup. Infection or immunization with one member of the TBEV complex induces a moderate to highly cross-reactive neutralizing antibody response to other members of the TBEV complex and confers cross-resistance [8,9]. The E protein of LGT virus is 88% percent related in amino acid sequence to that of strains of TBEV prevalent in Eurasia and is known to induce cross-protective immunity to TBEV viruses [8,10,11]. However, the antibody response of infected animals to LGT is higher than that to prevalent TBEV strains. It was hoped that the naturally attenuated LGT virus could serve as a live attenuated virus vaccine candidate and induce a protective and durable immune response to most members of the group [8,12–14]. Parenteral immunization with live LGT virus induced immunity against TBEV, but unfortunately retained residual virulence for the CNS of humans [13]. Clearly, LGT would require further attenuation before it could be used again as a vaccine candidate.

Modern recombinant DNA technology has made possible novel approaches for developing live attenuated flavivirus vaccines (reviewed in [11] and [15]), and this technology has been applied to further attenuate LGT for humans. The human trial reported here is part of the development plan for a live attenuated TBEV vaccine [10,16–20] that is based on chimerization of a non-neuroinvasive, mosquito-borne dengue type 4 virus (DEN4) with a neurotropic, tick-borne LGT virus. The chimeric virus tested here is referred to as LGT/DEN4. Specifically, chimeric LGT/DEN4 virus was created by replacing the membrane precursor (prM) and envelope (E) structural protein genes of DEN4 with the corresponding genes from LGT strain TP21 [18,20]. The resulting LGT/DEN4 virus exhibited greatly reduced neuroinvasiveness and neurovirulence in mice compared to the LGT parent and was immunogenic, providing complete protection against lethal challenge with TBEV or LGT [18–20]. LGT/DEN4 was also

found to be attenuated, immunogenic, and efficacious in monkeys [10,20]. In addition, the LGT/DEN4 virus failed to infect mosquitoes after intrathoracic inoculation [10], indicating that the chimeric virus has a limited potential for transmission by mosquitoes. Based on these preclinical studies, LGT/DEN4 was selected as the initial TBEV vaccine candidate for evaluation in a clinical trial in humans at a dose of 10^3 PFU, since this dose of vaccine candidate was able to induce protective immunity against LGT or TBEV in mice and monkeys [10,18–20]. Similarly attenuated vaccine constructs have been generated using a Far Eastern TBEV strain as the tick-borne virus parent [16,20].

Two major questions were addressed in the present study of LGT/DEN4 in adult volunteers. First, did chimerization between the genetically unmodified LGT and DEN4 viruses lead to attenuation in humans as it did in mice and monkeys? Second, did the level of antibody induced by the attenuated infection with LGT/DEN4 in humans stimulate a sufficient level of cross-reactive antibodies to pathogenic Eurasian TBEV strains to warrant continued development? The findings indicate that chimerization indeed led to attenuation of the LGT/DEN4 vaccine candidate for humans as it did for monkeys. However, the reduced level of LGT/DEN4 replication resulted in the induction of a low titer of antibodies against TBEV.

2. Materials and methods

2.1. Study population

The clinical phase 1 study was conducted in the General Clinical Research Center at Vanderbilt University Medical Center (supported by GCRC grant M01RR00095). Laboratory assays were performed at Johns Hopkins School of Public Health and the Laboratory of Infectious Diseases at NIH. The clinical protocol was reviewed and approved by the Vanderbilt University Institutional Review Board, the Vanderbilt University Biosafety Committee, the Committee on Human Research of the Bloomberg School of Public Health, and the Johns Hopkins University Institutional Biosafety Committee. A medical monitor, Dr. Robin McKenzie and the NIAID Data Safety and Monitoring Board reviewed the study at predetermined intervals.

Twenty-eight healthy adult volunteers were recruited from the Nashville area. Informed consent was obtained from each volunteer in accordance with the Code of Federal Regulations Title 21, Part 50 – Protection of Human Subjects. The individuals who were enrolled met the following eligibility criteria: age 18–50 years; no history of chronic illness; normal findings during physical examination; negative (<1:10) for serum neutralizing antibodies to LGT, TBEV, West Nile virus, dengue virus types 1–4, and yellow fever virus; negative for antibodies to Saint Louis encephalitis virus and Japanese encephalitis virus by hemagglutination-inhibition (titer <1:10); negative human immunodeficiency virus antibody test and hepatitis C virus antibody test; negative for hepatitis B surface antigen; normal hematologic and hepatic values; and normal urinalysis. Female volunteers had a negative result on a urine pregnancy test prior to vaccinations and on the days of vaccination and agreed to use contraception or abstain from sexual intercourse for the duration of the study.

2.2. Study design

The initial phase of the study was a double-blind placebo-controlled trial in which volunteers were randomly assigned in a 4:1 ratio to receive the LGT/DEN4 vaccine or placebo. Twenty-eight volunteers were enrolled in the study; 20 received 10^3 plaque forming units (PFU) of the LGT/DEN4 vaccine and eight received placebo consisting of vaccine diluent (L-15 Medium without phenol red; Cambrex Bio Science Walkersville, Inc., Rockville, MD). Volunteers were enrolled in a step-wise manner: four volunteers were initially enrolled; after study day 21, all safety data was reviewed by the medical monitor before proceeding with enrollment of the next 10 volunteers; and the set of safety data collected for these volunteers was then reviewed

by the medical monitor before enrollment of the remaining 14 volunteers. Randomization was done such that no more than 1 volunteer in the first group of 4 volunteers and no more than 4 volunteers in the first group of 10 volunteers received placebo.

Each volunteer received a single 0.5 ml dose of vaccine or placebo as a subcutaneous injection in the deltoid region. Volunteers were observed 30 minutes after vaccination for any immediate reaction to the vaccine. They were given a diary card and a digital oral thermometer to record their temperature 3 times daily for 19 days. Volunteers returned to the clinic every other day for 16 days after vaccination and on study days 19, 21, 28, 42, and 180. A clinical assessment was done at each visit and blood was obtained for hematological and clinical chemistry testing and for virologic or immunologic analysis. Serum from study days 0, 2, 4, 6, 8, 10, 12, 14, and 16 was titered for vaccine virus as described below; serum from study days 0, 28, 42, and 180 was analyzed for neutralizing antibody.

After completion of initial vaccination, the study was modified to include a second dose to assess the response of vaccinees to a booster dose. Twenty-one of the original 28 volunteers agreed to participate. Six to 18 months after initial vaccination they were re-randomized to receive either 10^3 PFU of vaccine or the placebo control (vaccine diluent). Sixteen volunteers received vaccine as the primary and secondary dose, 3 placebo recipients from the primary phase received vaccine in the booster phase, while 2 volunteers received placebo in both study phases.

2.3. Adverse Event Monitoring

Local reactogenicity was defined as injection site erythema, tenderness or swelling. Erythema and swelling at the injection site were graded as mild (1 - ≤ 20 mm diameter), moderate (>20mm - <50 mm diameter), or severe (≥50 mm diameter). Clinical signs and symptoms such as fever, headache, photophobia, nuchal rigidity, mental status change, nausea, malaise, myalgia, arthralgia, and rash were assessed at each follow-up visit during the acute 19-day post-vaccination period. Injection site pain and other solicited adverse events were graded as mild (no effect on activities of daily living, no treatment given); moderate (partial limitation in activities of daily living or treatment given); or severe (activities of daily living limited to <50% of baseline or medical evaluation required). A serious adverse event was defined as any event resulting in a life-threatening condition, hospitalization, congenital anomaly or birth-defect, persistent or significant disability, or death. All adverse events were determined to be either definitely, probably, possibly, remotely, or not related to vaccine. A vaccine-related meningoencephalitic-like syndrome was defined as infection associated with 2 or more of the following symptoms: grade 2 headache lasting ≥ 12 hours, grade 2 photophobia lasting ≥ 12 hours, nuchal rigidity (if present is grade 3, by definition), or generalized myalgia, grade 2, lasting ≥ 12 hours. LGT/DEN4 virus infection was defined as recovery of vaccine virus from the serum of a volunteer or a ≥4-fold rise in titer of serum neutralizing antibodies against LGT.

2.4. Laboratory testing

Clinical laboratory studies done for safety assessment included the following: a complete blood count including a white cell differential was tested at every visit for the first 19 days of the trial; serum alanine amino transferase (ALT) was tested at every follow-up visit from day 4 through day 19 and again on study day 28; prothrombin time, partial thromboplastin time, serum creatinine, and creatine phosphokinase were tested at every other visit through study day 16 and again on study day 28. The above studies were performed in the Vanderbilt Medical Center Clinical Pathology Laboratories. Serum from each visit through study day 19 was titered for vaccine virus and, if positive on study day 19, serum from study day 21 was also titered. Virus titer was determined at the Center for Immunization Research Laboratory, Bloomberg School of Public Health (Baltimore, MD).

2.5. Vaccine virus

A full-length cDNA copy of LGT/DEN4 in which the prM and E structural protein genes of DEN4 were replaced with those of LGT strain TP21 was constructed as previously described [18,20]. Infectious RNA transcripts derived from the chimeric LGT/DEN4 cDNA were used to transfect qualified mosquito C6/36 cells. Recovered virus was passaged once in qualified Vero cells, terminally diluted twice and then amplified in Vero cells. The final amplification of vaccine seed virus was made in serum-free medium. The clinical vaccine lot was produced at Novavax (Rockville, MD) in compliance with Good Manufacturing Practices and passed all safety tests confirming microbial sterility and animal safety. The LGT/DEN4 vaccine lot (designated LGT TP21/DEN4#2) had a titer of 4×10^6 PFU/ml. Vaccine virus in OptiPRO serum-free medium (Invitrogen, Carlsbad, CA) containing SPG buffer (final concentration: 218 mM sucrose, 5.4 mM monosodium glutamate, 3.8 mM potassium phosphate, monobasic and 7.2 mM potassium phosphate, dibasic, pH 7.2) was diluted with sterile L-15 medium to yield 10^3 PFU/0.5 ml just prior to vaccination and was kept on wet ice until administration. The titer of the LGT/DEN4 vaccine virus at the time of administration was confirmed by titration on Vero cell monolayer cultures as described previously [10]. Titration of the vaccine virus was done immediately after preparation and then 4 hours after preparation to assure stability throughout the vaccination period. Back titrations of the LGT/DEN4 inocula indicate that vaccine virus was stable during the time of administration, with its titers ranging from $10^{2.6}$ to $10^{2.9}$ PFU/dose.

2.6. Virus quantitation and serologic assessment

The amount of virus in blood was determined by plaque-forming assay after inoculation of serial 10-fold dilutions of serum onto Vero cell monolayer cultures as described previously [10]. LGT- or TBEV-specific neutralizing antibody titer of each serum sample was determined by a plaque-reduction neutralizing assay using LGT TP21 or TBEV/DEN4Δ30 virus, respectively [20]. A chimeric TBEV/DEN4Δ30 virus contained the prM and E protein genes of the highly virulent Sofjin strain of Far Eastern TBEV. The E protein of Sofjin strain shares 94–96% of amino acid identity with TBEV strains endemic in Europe and Asia [21]. Neutralizing antibody titer was defined as the dilution of serum that neutralized 60% of virus used in the assay. Serum of rhesus monkeys previously immunized with LGT/DEN4 or TBEV/DEN4Δ30 virus and then challenged with wild-type LGT virus [20] was used for positive control.

The serological responses were quantified as neutralizing antibody titers and classified in two ways: subjects with detectable antibody (≥1:5) and subjects with a fourfold rise in antibody titer (≥1:20). The former category was enumerated separately since the day 0 serum specimens of all subjects and all post-immunization specimens in placebo recipients had titers of <1:5, suggesting that vaccinees with detectable antibody had a response to vaccine. Such serum specimens are referred to as seropositive. Vaccinees with titers of ≥1:20 were considered unequivocally infected with vaccine, and vaccinees with this level of antibody were considered to have seroconverted.

3. Results

3.1. Volunteers

Twenty-eight volunteers ranging in age from 18 to 50 years were enrolled in the initial phase of the study and followed for the next 6 months. Seventeen volunteers were male (100% white) and 11 were female (64% white, 27% black and 9% Asian). Twenty received vaccine and eight received placebo.

Of the 21 volunteers in the booster dose phase of the study, 14 were male and 7 were female (6 white and 1 Asian). Nineteen volunteers (12 male and 7 female) received vaccine while 2 volunteers, both male, received placebo.

3.2. Clinical Safety

3.2.1. Initial dose—Vaccine was well tolerated by volunteers with minimal local reactogenicity (2/20 vaccinees had mild tenderness at the injection site, 2 additional vaccinees had bruising at the injection site, and 1/10 placebo recipients had mild arm stiffness related to the injections). None of the volunteers experienced any signs or symptoms of systemic or meningoencephalitic-like illness and there were no severe reactions (Table 1). Of the 20 LGT/DEN4 vaccinees, 5 reported headache, a frequency similar to that reported by 3 of 8 placebo recipients. One vaccinee and one placebo recipient developed neutropenia, and both cases resolved uneventfully. One placebo recipient reported a fever of 101.5°F; no temperature elevations were reported by vaccinees.

3.2.2. Booster dose—All 19 volunteers in the booster phase of the study tolerated the LGT/DEN4 vaccine well (Table 1). Two vaccinees had mild local tenderness at the injection site. Three vaccinees reported having headaches that were judged possibly related to the vaccine. All the headaches were classified as not serious and the longest duration was 42 hours. Rash was not reported or observed in any of the volunteers during this phase of the study.

3.3. Evidence of viremia

One vaccinee (subject #25 in Table 3) had vaccine virus detected in the blood following vaccination. This viremia occurred during the booster dose phase of the trial in a vaccinee who had received placebo at first vaccination. Vaccine virus was recovered on study day 8 at the lowest detectable level (titer = 0.7 $\log_{10}$ PFU/ml). The low level of viremia in infected vaccinees indicated that LGT/DEN4 virus was highly restricted in replication in humans.

3.4. Serologic responses to LGT/DEN4

Ninety-five percent (19/20) of vaccinees were seropositive to LGT as tested on study days 28, 42, or 180 (Table 2), but only 80% were considered to have seroconverted. Geometric mean titers (GMT) of neutralizing antibody to LGT were highest on day 28 and 42 (66 and 63 reciprocal GMT, respectively) with about a three-fold drop in GMT by study day 180. By using an attenuated TBEV/DEN4Δ30 virus as the test virus in the neutralization assay, TBEV-specific cross-reactive antibodies in the serum of volunteers could be measured following vaccination with LGT/DEN4. Of the 20 volunteers, 13 (65%) had detectable TBEV-specific neutralizing antibody titers (≥1:5) on study days 28, 42, or 180, but antibody titers to heterologous TBEV were much less than to the homologous LGT virus (Table 2). Overall, only 35% of vaccinees seroconverted to TBEV. Antibody was not detected against either LGT or TBEV in any placebo recipient.

All volunteers who received two doses of LGT/DEN4 administered with an interval of at least 6 months after primary inoculation became seropositive (≥1:5) against LGT (Table 3). Geometric mean LGT-specific neutralizing antibody titers in the serum of vaccinees were similar on day 28, 42, or 180 between groups that received one or two doses of vaccine. In both groups, antibody titers peaked at day 28 or 42 and tended to decrease about two-fold by day 180. Two subjects (#17 and 28) in the booster group who did not seroconvert following primary inoculation seroconverted 28 days after receiving the booster inoculation, however, the antibody response of one of them (#28) became undetectable again by day 180. The second dose of LGT/DEN4 failed to significantly increase the number of vaccinees with antibody to TBEV (6/16 before the second dose to 8/16 after second dose) or to increase the mean titer of TBEV antibody.

4. Discussion

Experience with an extensive vaccination campaign against TBEV in Austria during the last 20 years has shown that active immunization of human populations living or traveling in endemic areas can result in a decline of illness caused by TBEV [22]. The currently available TBEV vaccines licensed in Europe (FSME-IMMUN® vaccine in Austria and Encepur® vaccine in Germany) are derived from inactivated whole virus (Central European TBEV strain Neudoerfl and strain K23, respectively). Although each vaccine is administered on a slightly different schedule, three immunizations are recommended: on day 0 (1st dose), from days 21 to 72 (2nd dose), and after 9–12 months (3rd dose) [23]. These licensed vaccines are safe, well tolerated by both adults and children, and immunogenic with seroconversion rates of 90–99% following the primary immunization course [22,23]. For these vaccines, seroconversion is defined as a change from an undetectable immunological response (titer <1:2) to a detectable level (titer ≥1:2) in the neutralization assay. However, titers of neutralizing antibodies wane in a log-linear fashion within 2–4 years after vaccination, falling below protective levels (neutralizing antibody titer of <1:10) in 5–30% of the recipients [3]. The decrease in post-vaccination antibody levels is observed with increased frequency in the elderly, such that approximately 30% of persons over 60 years of age do not have protective immunity. Currently, booster vaccinations are recommended every 3 years after completion of primary immunization.

Despite the effectiveness of TBE vaccination in Austria, the inactivated vaccines have several limitations, including the long schedule of primary immunization, the need for repeated booster vaccinations due to the short duration of immunity, the rare occurrence of severe TBE disease in vaccinees in TBEV endemic areas [4,24], and the high cost of manufacture. These disadvantages and difficulties associated with the current inactivated TBEV vaccines have encouraged research to develop new improved vaccines. Based on the success of live attenuated vaccines for other flaviviruses, such as the yellow fever 17D vaccine [25] and Japanese encephalitis (JE) SA 14-14-2 vaccine [26], live attenuated vaccines offer the possibility of inducing the desired protective and durable immune responses. Early attempts to develop an effective live attenuated TBEV vaccine using the naturally attenuated LGT strains were unsatisfactory due to a low frequency of post-vaccination encephalitis [11,13,27]. Nevertheless, these vaccine candidates induced a broad cross-protective immune response against TBEV strains that lasted 3 or more years following a single immunization [8,12,13, 27]. The LGT virus vaccine candidate tested in Russia was effective in preventing TBE in endemic regions, reducing the incidence of TBE by more than 10-fold compared to the previously used inactivated TBEV vaccine (reviewed in [11] and [13]).

The pre-clinical studies with LGT/DEN4 indicated that chimerization of LGT with DEN4 strongly attenuated the resulting chimeric virus for rodents and non-human primates. Attenuation was manifest as reduced replication in human cells of neural origin, decreased neurovirulence in newborn mice as measured by intracerebral inoculation, and decreased neuroinvasiveness in immunocompetent or immunodeficient mice, a property that reflects the capacity of virus to spread from a peripheral site of the body into the CNS where it causes encephalitis [20]. In addition, LGT/DEN4 was found to be highly restricted in replication in rhesus monkeys, replicating to levels significantly less than either parent virus [10], indicating that chimerization itself led to an increased level of attenuation. It is likely that the presence of the LGT prM and E genes in the chimeric virus creates LGT-DEN4 protein incompatibilities that compromise virus replication *in vivo*. Although chimerization between LGT and DEN4 indeed resulted in an acceptable level of attenuation for monkeys, chimerization between two flaviviruses does not always lead to a satisfactory level of attenuation. YF/JE-N [28,29], St. Louis encephalitis/DEN4 (A.G. Pletnev; unpublished data), intertypic DEN1/DEN4 [30], or intertypic JE virus chimeras [31] do not appear to be satisfactorily attenuated in mice or

monkeys. All chimeric viruses evaluated to date involved a parent virus that contained one or more attenuating mutations [32,33]. Since the current human study was the first to evaluate a chimeric flavivirus generated from two unmodified parent viruses, this permitted an evaluation of the independent effect of chimerization on virus attenuation for humans.

The LGT/DEN4 vaccine candidate was highly attenuated for humans following either the first or second dose without significant local or systemic reactogenicity. The clinical reactogenicity observed in recipients of the LGT/DEN4 vaccine was less than that observed in recipients of a similar dose of a previously reported live attenuated dengue DEN4 vaccine [32]. Rash and neutropenia were observed in some recipients of the previously tested DEN4 vaccine candidate, but not in LGT/DEN4 vaccinees. Additional evidence of the further attenuation of this vaccine came from the observation that only one of 17 vaccinees infected with LGT/DEN4 (those with at least a 4-fold rise in antibody titer) had detectable viremia. Since dengue viruses replicate in humans up to $10^{6–8}$ infectious units per ml blood [34] and since previous studies in humans with LGT TP21 virus revealed that over 70% of volunteers became viremic for a duration ranging from 3 to 10 days [27], it is clear that chimerization of LGT with DEN4 greatly decreased the replication of the chimeric virus in humans compared to that of either parent. The level of replication of LGT/DEN4 virus in humans was similar to that observed in rhesus monkeys [20], indicating that chimerization resulted in attenuation in both species. This interpretation is offered with the caveat that the replication of the wild-type DEN4 parent has not been evaluated directly in humans. However, LGT/DEN4 induced viremia much less frequently in healthy adult volunteers than that observed for live attenuated DEN4Δ30 vaccine candidate derived from the same DEN4 parent and administered at the same dose [32].

A single 10^3 PFU dose of LGT/DEN4 vaccine candidate induced a moderate neutralizing antibody response against LGT: 95% of volunteers became seropositive post-vaccination (titer ≥1:5) and 80% seroconverted as defined by a ≥4-fold increase in serum neutralizing antibody titer (titer ≥1:20). However, the antibody response against heterologous TBEV was unacceptably low in terms of both the frequency and the magnitude of the response. The levels of antibody to TBEV achieved in LGT/DEN4-immunized volunteers appeared less than that observed in recipients of the licensed inactivated TBEV vaccines described above. Two independent factors may have contributed to this weak heterologous TBEV antibody response: (1) the 12% divergence in amino acid sequence of the structural E proteins of LGT and the Far Eastern strain of TBEV [35] and (2) the low level of replication of the LGT/DEN4 virus in humans. The observation that almost all vaccinees were without detectable viremia and did not develop a strong neutralizing antibody response to the homologous LGT virus suggests that LGT/DEN4 was highly attenuated for humans. Clearly the additional restriction of replication resulting from the chimerization changed the LGT virus from an immunogenic vaccine candidate to one that is over-attenuated.

Interestingly, the neutralizing antibody response against either LGT or TBEV was not boosted after the second dose of LGT/DEN4 vaccine, suggesting that the relatively low level of immunity associated with a single dose of LGT/DEN4 immunization was able to restrict the virus replication to levels insufficient to stimulate an anamnestic response. In contrast, booster antibody responses are frequently observed in monkeys after second doses of dengue virus [36]. Perhaps the secondary immune response to the encephalitic flaviviruses is fundamentally different from that of the dengue viruses, and resolution of this interesting question will require additional clinical trials.

The failure to induce a vigorous immune response against TBEV in humans using the LGT/DEN4 chimeric virus confirms that the required balance between attenuation and immunogenicity of live virus vaccines is difficult to achieve. It may be possible to overcome the low level of immunogenicity against TBEV by increasing the dose. However, previous

studies with other flavivirus vaccine candidates in rhesus monkeys and humans have shown that the magnitude of the immune response is not necessarily a function of the vaccine dose [10,32]. Therefore, we believe that achieving >95% seroconversion to TBEV while maintaining a satisfactory safety profile will be difficult to accomplish by increasing the dose of immunization. As an alternative strategy, our current live attenuated TBEV vaccine candidate is the chimeric TBEV/DEN4Δ30 virus that is highly attenuated in mice and rhesus monkeys and induces a higher level of neutralizing antibodies in the monkeys against TBEV than did LGT/DEN4. The structural proteins of the TBEV/DEN4Δ30 chimera are antigenically closely related (94–96% of amino acid identity) to the TBEV strains that are responsible for causing most TBEV illness in Eurasia. We anticipate that the TBEV/DEN4Δ30 chimera will induce a more satisfactory immune response against TBEV in humans than that observed in the present study using divergent E protein of the LGT/DEN4 chimera. However, its safety in primates will require additional studies.

In summary, LGT/DEN4 vaccine virus administered as a single 10^3 PFU dose was highly infectious and highly attenuated in human volunteers and induced a neutralizing antibody response to LGT in at least 80% of recipients. However, seroconversion against heterologous TBEV was infrequent and the level of TBEV antibodies was significantly lower in magnitude than that observed against homologous LGT virus. The neutralizing antibody titers against either LGT or TBEV were not significantly increased after a second dose of LGT/DEN4 vaccine indicating that an immunization with a single dose of vaccine was sufficient to restrict virus replication and blunt an anamnestic response. Nevertheless, the second dose was safe with no evidence of enhanced replication or potentiation of disease.

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Table 1

Clinical summary of volunteers inoculated with a primary or booster dose of LGT/DEN4, a live attenuated vaccine candidate for TBE.

Volunteers given	Dose cohort (log_{10}PFU)	No. of volunteers	No. of viremic	Number of volunteers with indicated sign:					
				Systemic illness[a]	Headache	Rash	Fever	Neutropenia[c]	Elevated ALT[d]
Initial LGT/DEN4	3.0	20	0	0	5	0	0	1	0
Initial placebo		8	0	0	3	0	1[b]	1	0
Boost LGT/DEN4	3.0	19	1	0	3	0	0	0	0
Boost placebo		2	0	0	0	0	0	0	0

[a] Systemic illness is defined as ≥2 of the following symptoms lasting ≥2 days: headache, malaise, vomiting, arthralgia/malaise, nausea, or photophobia. There was no significant difference between vaccines and placebo recipients in the occurrence of any of the individual solicited symptoms used to define systemic illness. All solicited adverse events were mild or moderate in severity.

[b] Fever occurred on study day 40; maximum temperature was 101.5°F.

[c] Neutropenia is defined as an absolute neutrophil count of <1500 cells/mm^3.

[d] Elevated ALT level is defined as any value above the upper limit of normal (for males, >72U/L; for females, >52 U/L).

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Table 2

Immunologic response of volunteers immunized with a single 10^3 PFU dose of the chimeric LGT/DEN4 vaccine candidate.

Volunteer ID#	Code	Serum neutralizing antibody titer on indicated day against[a]:							
		TBEV/DEN4Δ30				LGT			
		0	*28*	*42*	*180*	*0*	*28*	*42*	*180*
02	Placebo	< 5	< 5	< 5	NT[b]	< 5	< 5	< 5	< 5
06	Placebo	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
12	Placebo	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
14	Placebo	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
16	Placebo	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
19	Placebo	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
25	Placebo	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
26	Placebo	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
01	Vaccine	< 5	**14**	**9**	< 5	< 5	**300**	**404**	**101**
03	Vaccine	< 5	< 5	< 5	**12**	< 5	**10**	**20**	< 5
04	Vaccine	< 5	**23**	**8**	**33**	< 5	**210**	**97**	**175**
05	Vaccine	< 5	**318**	**164**	**186**	< 5	**3005**	**1702**	**283**
08	Vaccine	< 5	**9**	**6**	**18**	< 5	**43**	**27**	**41**
09	Vaccine	< 5	< 5	**7**	**10**	< 5	**35**	**25**	**11**
10	Vaccine	< 5	< 5	< 5	< 5	< 5	**18**	**11**	**5**
11	Vaccine	< 5	**11**	**8**	**25**	< 5	**250**	**119**	**25**
13	Vaccine	< 5	< 5	< 5	< 5	< 5	**41**	**26**	**30**
15	Vaccine	< 5	< 5	< 5	**27**	< 5	**54**	**64**	**65**
17	Vaccine	< 5	< 5	< 5	< 5	< 5	< 5	< 5	**15**
18	Vaccine	< 5	< 5	< 5	< 5	< 5	**90**	**75**	< 5
20	Vaccine	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
21	Vaccine	< 5	**5**	**5**	NT	< 5	**88**	**119**	NT
22	Vaccine	< 5	< 5	< 5	< 5	< 5	**67**	**47**	< 5
23	Vaccine	< 5	**21**	**304**	**276**	< 5	**145**	**833**	**217**
24	Vaccine	< 5	**30**	**48**	**175**	< 5	**264**	**516**	**45**
27	Vaccine	< 5	**8**	**5**	**9**	< 5	**55**	**33**	**8**
28	Vaccine	< 5	< 5	< 5	**6**	< 5	< 5	**6**	< 5
29	Vaccine	< 5	**16**	**25**	**131**	< 5	**1252**	**850**	**191**
	GMT[c]:	**9**	**9**	**16**		**66**	**63**	**24**	
	Seropositive:	**10/20 50%**	**11/20 55%**	**12/19 63%**		**17/20 85%**	**18/20 90%**	**14/19 74%**	
	Seroconversion:	**4/20 20%**	**4/20 20% 7/20 = 35%**	**7/19 37%**		**15/20 75%**	**16/20 80% 16/20 = 80%**	**10/19 53%**	

[a] Plaque reduction (60%) neutralizing antibody titers (reciprocal) were determined against wild-type LGT TP21 strain or chimeric TBEV/DEN4Δ30 virus as indicated.

[b] NT, not tested.

[c] GMT, geometric mean titers (reciprocal) are calculated for all 20 vaccine recipients. For calculation of GMT, a negative titer (<1:5) was assigned a value of 4. Seroconversion (indicated by shading) is defined as a serum neutralizing antibody titer of ≥1:20 on study day 28, 42, or 180, compared with the pre-vaccination titer, which was <1:5 for each volunteer.

Table 3

Immunologic response of volunteers following a second 10^3 PFU dose of the chimeric LGT/DEN4 vaccine candidate.

Volunteer ID#	Code		Serum neutralizing antibody titer on indicated day against[a]:							
			TBEV/DEN4Δ30				LGT			
	1-st dose	2-nd dose	*0*	*28*	*42*	*180*	*0*	*28*	*42*	*180*
19	Placebo	Placebo	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
26	Placebo	Placebo	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
12	Placebo	Vaccine	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
16	Placebo	Vaccine	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
25	Placebo	Vaccine	< 5	**298**	**568**	**68**	< 5	**1008**	**649**	**147**
01	Vaccine	Vaccine	< 5	**6**	**5**	< 5	**19**	**39**	**22**	**28**
03	Vaccine	Vaccine	< 5	< 5	< 5	< 5	< 5	**17**	**11**	**5**
04	Vaccine	Vaccine	**38**	**36**	**37**	**36**	**157**	**187**	**164**	**83**
05	Vaccine	Vaccine	**39**	**51**	**31**	**28**	**289**	**446**	**381**	**86**
08	Vaccine	Vaccine	**14**	**39**	**33**	**28**	**28**	**100**	**88**	**26**
10	Vaccine	Vaccine	< 5	< 5	< 5	< 5	< 5	**6**	**5**	**7**
11	Vaccine	Vaccine	**8**	**9**	**5**	**37**	**34**	**48**	**52**	**56**
13	Vaccine	Vaccine	< 5	< 5	< 5	< 5	**14**	**29**	**31**	**20**
17	Vaccine	Vaccine	< 5	< 5	< 5	< 5	< 5	**58**	**92**	**30**
18	Vaccine	Vaccine	< 5	< 5	< 5	< 5	**11**	**21**	**14**	**13**
20	Vaccine	Vaccine	< 5	< 5	< 5	< 5	< 5	**16**	**14**	< 5
22	Vaccine	Vaccine	< 5	< 5	< 5	< 5	**10**	**36**	**44**	**11**
23	Vaccine	Vaccine	**35**	**30**	**76**	**10**	**55**	**85**	**67**	**49**
27	Vaccine	Vaccine	< 5	< 5	**5**	< 5	**5**	**11**	**12**	**7**
28	Vaccine	Vaccine	< 5	**7**	< 5	< 5	< 5	**50**	**19**	< 5
29	Vaccine	Vaccine	**17**	**11**	**9**	**14**	**127**	**163**	**164**	**80**
		GMT[b]:	**8**	**9**	**8**	**8**	**17**	**45**	**38**	**19**
		Seropositive:	**6/16 38%**	**8/16 50%**	**8/16 50%**	**6/16 38%**	**11/16 69%**	**16/16 100%**	**16/16 100%**	**14/16 88%**
		Seroconversion:		**0/16 0%**	**0/16 0% 1/16 = 6%**	**1/16 6%**		**2/16 13%**	**2/16 13% 3/16 = 19%**	**1/16 6%**

[a] Plaque reduction (60%) neutralizing antibody titers (reciprocal) were determined against wild-type LGT TP21 strain or chimeric TBEV/DEN4Δ30 virus as indicated.

[b] GMT, geometric mean titers (reciprocal) are calculated for 16 vaccine recipients and does not include 3 volunteers who received placebo in primary inoculation and vaccine in second inoculation. Seroconversion (indicated by shading) is defined as a ≥4-fold rise in serum neutralizing antibody titer on day 28, 42, or 180 after booster vaccination, compared with a titer observed in serum of volunteers prior to receive a booster dose of LGT/DEN4 vaccine.