

Letter to the Editor

Neuropathogenesis and Neurovirulence of Live Flaviviral Vaccines in Monkeys

The stated goal of the work by Maximova et al. (12) was to evaluate the safety of a new live vaccine candidate for tick-borne encephalitis (TBE), the chimeric virus TBEV/DEN4Δ30, in a monkey neurovirulence test (MNVT) (7, 25). In the first part of the study, 22 monkeys were inoculated intrathalamically with the candidate virus or Langat TP21 virus. Clinical signs demonstrated higher neurovirulence for TBEV/DEN4Δ30 than for Langat TP21, and it was concluded that TBEV/DEN4Δ30 was unacceptable as a live vaccine. Pathomorphological examination was not performed.

The Langat TP21 virus should not have been used as a reference because some TP21 clones have shown “unsafe” levels of neurovirulence in monkeys (1, 2, 6, 8, 13) and people (3, 24). In the absence of a safe and approved TBE vaccine, yellow fever vaccine strain YFV17D has been used and recommended as the standard comparator for the MNVTs of new live flaviviral vaccines (4, 5, 14–16, 25, 26).

In the second part of this work, 40 more monkeys were used to study neuropathogenesis of TBEV/DEN4Δ30 and to compare neurovirulence of this vaccine candidate with those of Langat TP21 and YFV17D viruses. The MNVT of live flavivirus vaccines requires that the test be performed not earlier than the time of peak specific morphological lesions, while using an adequate number of animals for statistical reliability. Flavivirus-induced lesions in the central nervous system (CNS), particularly with attenuated strains, may develop and progress very slowly (13, 18–21, 23). Therefore, the minimum time for histopathological assessment of flavivirus neurovirulence has been established at approximately 30 days postinoculation (p.i.) (4, 5, 7, 9–11, 14–16). In this study, the majority of monkeys were euthanized earlier (days 3 to 21 p.i.). Also, great interanimal variability in severity of histological lesions following infection with flaviviral attenuated strains (6, 13, 21) deems two animals per group inadequate for reliable comparisons, conclusions, and recommendations.

Definitive works by Nathanson et al. with five flaviviruses (18–20) have provided the methodology for clear and reliable pathohistological assessment of the level of flavivirus neurovirulence in the MNVT. This methodology has been successfully applied for YFV17D (7–11, 25), as well as for new live flaviviral vaccines (4, 5, 14–16). However, Maximova et al. have used their own procedures for MNVT, which makes it difficult to compare the degree of neurovirulence for TBEV/DEN4Δ30 with those of preexisting vaccines and standards of practice.

Finally, it should be mentioned that the term “gliosis” (focal and diffuse) was used by Maximova et al. incorrectly (see the pathohistological description, determination of grading scales, and a microphotograph in Fig. 4 of their study). Those small cellular foci that are indicated as “focal gliosis” by arrowheads and white arrows in Fig. 4 do not represent the process of “gliosis.” The term “gliosis” refers to proliferation and hypertrophy of astrocytes and their processes (special staining is required) in the neuronal destruction areas, acute (i.e., infarction) or chronic (17, 22). In the cases of flaviviral infections, gliosis may develop after the cessation of acute inflammatory changes, in the course of a chronic process, mostly in the cerebellum, in the areas of necrosis and spongy degeneration of the Purkinje cells (27).

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Authors' Reply

Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV), and St. Louis encephalitis (SLEV) virus are in the *Flavivirus* genus of the family *Flaviviridae* and are very important human pathogens, causing a devastating and often fatal infection of the central nervous system (CNS). Since immunization with the yellow fever 17D (YF 17D) or JEV live virus vaccine can provide protective long-term immunity in humans, our major goal is to develop live attenuated flavivirus vaccine candidates effective against the neurotropic flaviviruses using modern recombinant DNA technology (11–13). Recently, we created a chimeric TBEV/DEN4Δ30 virus by replacing the membrane precursor and envelope glycoprotein genes of a mosquito-borne dengue type 4 virus (DEN4) with the corresponding genes from the highly virulent TBEV strain (13). However, the development of safe vaccines against neurotropic flaviviruses demands a detailed knowledge of virus-associated neuropathogenesis in permissive animal models, which include mice and nonhuman primates. Thus, one focus of our studies with a novel chimeric attenuated flavivirus is a comprehensive analysis of virus-associated neuropathogenesis and neurovirulence (5). This information will guide our decision making in determining whether we feel it is safe to proceed with evaluation of the attenuated vaccine candidate in humans. There are two tiers in the decision-making process prior to the initiation of clinical trials: first, we must

convince ourselves that a vaccine candidate is sufficiently safe, as indicated by highly reduced neuroinvasiveness and neurovirulence; and second, we need to provide sufficient information to the Food and Drug Administration (FDA), as well to the numerous regulatory committees that evaluate clinical protocols, that the virus should be safe for vaccinees and the community. Our recently published paper (5) largely addresses the first of these two decisions: should we as vaccine developers proceed to a clinical trial with this specific TBEV vaccine candidate? We decided not to submit an “Investigational New Drug” application to the FDA and not to initiate such a clinical trial based on the behavior of the vaccine candidate following intracerebral (i.c.) infection of nonhuman primates. The letter by Levenbook and Draper largely addresses issues regarding the second tier of the decision-making process, which was not needed in this specific case since this vaccine candidate was not submitted for approval by the various regulatory authorities.

However, we will address some of the issues raised in the letter to the editor in which Levenbook and Draper raised concerns with our studies. They indicated the following.

(i) According to Levenbook and Draper, “The stated goal of the work by Maximova et al. (12) was to evaluate the safety of a new live vaccine candidate for tick-borne encephalitis (TBE), the chimeric virus TBEV/DEN4Δ30, in a monkey neurovirulence test (MNVT).”

This statement is only partially correct, and indeed one of the reasons for doing this study was to determine the safety of the indicated vaccine candidate. However, there were other scientific questions that we wanted to address, such as (i) replication kinetics of the TBEV/DEN4Δ30 vaccine candidate in the CNS of rhesus monkeys, (ii) tropism of the virus for different CNS regions and type of infected cells, (iii) spatio-temporal patterns of virus-associated histopathology, and (iv) phenotype of cells involved in the inflammatory response. We specifically indicated this in the article: “Comprehensive analysis of the neuropathogenesis in nonhuman primates is needed to further our understanding of the neurovirulence potential of new live attenuated chimeric TBEV vaccine candidates. ... Thus, our major objective was to study neuropathogenesis in rhesus monkeys following i.c. inoculation with TBEV/DEN4Δ30 virus and to compare the level of neurovirulence of the chimeric vaccine candidate with that of LGTV or YF 17D vaccine” (5). The standard MNVT referred to by Levenbook and Draper evaluates only a single time point (day 30 postinoculation) when the virus replication has ceased and is insufficient to meet our stated objectives and does not provide the detailed neuropathogenesis data that we needed to make our decision.

(ii) Levenbook and Draper state, “The Langat TP21 virus should not have been used as a reference because some TP21 clones have shown ‘unsafe’ levels of neurovirulence in monkeys (1, 2, 6, 8, 13) and people (3, 24). In the absence of a safe and approved TBE vaccine, yellow fever vaccine strain YFV17D has been used and recommended as the standard comparator for the MNVTs of new live flaviviral vaccines (4, 5, 14–16, 25, 26).”

In our article, we have clearly stated the rationale for using LGTV virus, a TBEV variant, as a comparator for chimeric TBEV/DEN4Δ30 virus: “The naturally attenuated LGTV that retains an unacceptable level of residual neurovirulence for humans (2, 14) acts as the surrogate for the [neurovirulent] TBEV parent, which is a BSL-4 [biosafety level 4] agent” (5). The comment by Levenbook and Draper that “in the absence of a safe and approved TBE vaccine, yellow fever vaccine strain YFV17D has been used and recommended as the stan-

standard comparator for the MNVTs of new live flaviviral vaccines" is misleading the reader on several important points. First, Levenbook and Draper refer to extensive studies of chimeric vaccine candidates against dengue and Japanese encephalitis created on the YF 17D background, in which they participated (3, 7–9). None of these vaccine candidates was against a tick-borne encephalitis virus (TBEV). Second, while it seems prudent to use YF 17D as a comparator for new chimeric viruses created on the YF 17D background, evidence is lacking to justify the "recommendations" of using YF 17D as "the standard comparator" when antigenically distant chimeric flaviviruses based on dengue virus type 4 need to be tested. Information justifying such a recommendation has yet to be generated. Third, we clearly stated that we chose to use the YF 17D as a "surrogate" comparator since this vaccine virus, although antigenically distant from the TBEV/DEN4Δ30 virus, has long been used for human vaccination with a remarkable record of safety and efficacy (6).

(iii) Levenbook and Draper state, "The MNVT of live flavivirus vaccines requires that the test be performed not earlier than the time of peak specific morphological lesions, while using an adequate number of animals for statistical reliability." Levenbook and Draper also imply, "The minimum time for histopathological assessment of flavivirus neurovirulence has been established at approximately 30 days postinoculation (p.i.) (4, 5, 7, 9–11, 14–16)" and indicate that, in our studies, "the majority of monkeys were euthanized earlier (days 3 to 21 p.i.)."

The optimal time for performance of a histopathological assessment of flavivirus neurovirulence has not been established as 30 days p.i. for all flaviviruses and certainly not for new, untested chimeric viruses. For this to be established, one must first confirm "the time of peak specific morphological lesions" for each flavivirus strain or chimeric virus which contains genes from different flaviviruses. Thus, to determine the optimal time for histopathological examination of the CNS of monkeys infected with our specific virus, we analyzed the evolution of the CNS infection at multiple time points over a 30-day period (including 30 days p.i.) using an integral set of clinical, virological, histopathological, and immunohistochemical data. This approach provided insight into the neuropathogenesis beyond the traditional 30-day-p.i. time point and allowed us to demonstrate that the three attenuated flaviviruses under study were better discriminated by their histopathological profile (as well as by kinetics of their replication in the CNS) at earlier time points.

It is important to indicate that there are a number of concerns regarding the standardized MNVT testing. A scientific workshop arranged by the World Health Organization (WHO; Geneva, Switzerland, 2005) was entirely dedicated to addressing the ethical and methodological issues regarding neurovirulence tests for live virus vaccines in nonhuman primates (19). As stated in the WHO report, there is often no clear definition of criteria for assessment of the test results or the absence of the use of appropriate reference vaccines in the tests. The WHO also concluded that requirements of test procedures for yellow fever vaccine (18) could be clarified (19). In the United States, requirements for the monkey neurovirulence test specified by the *Code of Federal Regulations* (CFR) have been revoked, and vaccines are currently assessed on a case-by-case basis, relying in part on the revoked CFR for guidance.

(iv) According to Levenbook and Draper, "The term 'gliosis' (focal and diffuse) was used by the authors incorrectly." Regarding the concern about the misuse of the term "gliosis," the current view on gliosis implies reaction of all elements of the

neuroglia including not only astrocytes (in which case it should be called astrogliosis), but also activation and proliferation of microglia (microgliosis). This new understanding has come from rapid advances in the field of microglial and astrocytic neurobiology over the past two decades. These advances have led to the recognition that glia, particularly microglia, respond to tissue insult in a complex manner including both the secretion of inflammatory cytokines and modification of cellular functions that transcend the historical vision of phagocytosis and structural support that has long been enshrined in the term "reactive gliosis" (16). The histopathological basis of encephalitis is derived from the triad of damage to the CNS parenchyma (nerve cell damage or loss), reactive gliosis, and inflammatory cellular infiltration (1, 15). This classical response is represented by (multi)nodular encephalitis, as in the majority of viral encephalitides, and consists of damage to neurons, followed by neuronal cell death and neuronophagia, focal/nodular proliferation of microglia and astroglia (i.e., gliosis), and focal/nodular infiltration by lymphocytes and macrophages. The classical encephalitic nodules are composed of a mixture of microglia, astrocytes, and lymphocytes usually around affected neuron(s) (1). "Those small cellular foci [our Fig. 4]" which "do not represent the process of gliosis" were consistently described by us as a combination of mononuclear inflammatory cell infiltration, focal gliosis, and neuronal degeneration/neuronophagia. Furthermore, using immunohistochemistry we demonstrated that these inflammatory foci were largely composed of CD68⁺ activated microglia/macrophages around degenerating neurons and parenchymal infiltrating lymphocytes. It should be noted that in a recently published paper by Johnson et al. (4), the authors, including Dr. Draper, also used the term "gliosis" to describe similar virus-associated histopathology in the CNS of nonhuman primates following i.c. inoculation with recombinant vesicular stomatitis virus vectors (Fig. 3; hematoxylin and eosin staining). Additionally, focal gliosis (glial shrunkenness or Bergmann gliosis) in the molecular layer of the cerebellar cortex accompanying degenerating Purkinje cells and shown in our Fig. 4 is considered typical in fatal cases of human TBE and West Nile virus encephalitis, and was also described in dogs with TBE (10, 17). Glial fibrillary acidic protein staining might be performed in future studies to address the extent of astrogliosis in flavivirus encephalitis, but this was beyond the scope of our paper.

In summary, our studies in nonhuman primates identified the chimeric TBEV/DEN4Δ30 virus as insufficiently attenuated for further consideration as a vaccine candidate. This was based on a set of clinical, virological, histopathological, and immunohistochemical data that examined the time course of CNS infection following i.c. inoculation in nonhuman primates. We have proceeded to construct new, further attenuated vaccine candidates against TBEV. Furthermore, based on our data, we are developing a high-throughput computerized morphometric analysis that will allow objective quantitative analysis of the cellular inflammatory responses to neurotropic viruses in the CNS of the primate host. This methodology might help to define the unique phenotypic virus signatures and guide the development of safe and effective live virus vaccines.

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