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Assurance of neuroattenuation of a live vaccine against West Nile virus: A comprehensive study of neuropathogenesis after infection with chimeric WN/DEN4 30 vaccine in comparison to two parental viruses and a surrogate flavivirus reference vaccine

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Abstract

The upsurge of West Nile virus (WNV) human infections in 2012 suggests that the US can expect periodic WNV outbreaks in the future. Availability of safe and effective vaccines against WNV in endemic areas, particularly for aging populations that are at high risk of West Nile neuroinvasive disease (WNND), could be beneficial. WN/DEN4 30 is a live, attenuated chimeric vaccine against WNV produced by replacement of the genes encoding the pre-membrane and envelope protein genes of the vaccine virus against dengue virus type 4 (DEN4 30) with corresponding sequences derived from a wild type WNV. Following intrathalamic inoculation of nonhuman primates (NHPs), a comprehensive neuropathogenesis study was performed and neurovirulence of WN/DEN4 30 vaccine candidate was compared to that of two parental viruses (i.e., WNV and DEN4 30), as well as to that of an attenuated flavivirus surrogate reference (i.e., yellow fever YF 17D). Clinical and virological data, as well as results of a semi-quantitative histopathological

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analysis, demonstrated that WN/DEN4-30 vaccine is highly attenuated for the central nervous system (CNS) of NHPs in comparison to a wild type WNV. Importantly, based on the virus replicative ability in the CNS of NHPs and the degree of induced histopathological changes, the level of neuroattenuation of WN/DEN4-30 vaccine was similar to that of YF 17D, and therefore within an acceptable range. In addition, we show that the DEN4-30 vaccine tested in this study also has a low neurovirulence profile. In summary, our results demonstrate a high level of neuroattenuation of two vaccine candidates, WN/DEN4-30 and DEN4-30. We also show here a remarkable sensitivity of our WNV-NY99 NHP model, as well as striking resemblance of the observed neuropathology to that seen in human WNND. These results support the use of this NHP model for translational studies of WNV neuropathogenesis and/or testing the effectiveness of vaccines and therapeutic approaches.

Keywords

West Nile Virus (WNV); live attenuated WNV vaccine; neuropathogenesis; neurovirulence; nonhuman primates

Introduction

West Nile virus (WNV) is a mosquito-borne neurotropic flavivirus that is emerging as a human pathogen of global scale (reviewed in [1–3]). WNV first appeared in North America in 1999 [4] and rapidly spread throughout the US producing large regional outbreaks of West Nile neuroinvasive disease (WNND) ([5, 6]. There had been a general decrease of WNND after 2004 with uncertainty whether WNV would remain a serious public health concern and whether universal vaccination would be cost-effective [6–10]. However, the resurgence of WNV in the US in 2012 resulted in 5,674 WNV human infections with 2,873 cases of WNND and 286 deaths [11]. This epidemiological pattern suggests that the US can expect periodic WNV outbreaks in the future [10]. WNV is now the major cause of arboviral neuroinvasive disease in the US [12]. It is estimated that over 3 million WNV infections and up to 1 million resulting illnesses have occurred so far with over 98% of the US population remaining at risk [13]. Thus, there is a need for effective and safe vaccines to prevent WNV disease in humans. Making a vaccine available particularly for people of advanced age, which is a population at high risk of development of severe WNND [5], could certainly be of benefit [8, 14].

Currently, there are no approved vaccines or treatments for WNV infection. Many technologies are being used to develop a WNV vaccine [reviewed in [8, 14, 15]. Four WNV vaccine candidates have been tested for safety and immunogenicity in clinical trials: (i) WNV/YFV 17D vaccine (live attenuated virus, based on the yellow fever 17D vaccine strain) [16–18]; (ii) WN/DEN4-30 vaccine (live attenuated virus, based on dengue type 4 vaccine strain) [19]; (iii) WNV prM-E DNA vaccine [20, 21]; and (iv) recombinant subunit E protein vaccine [22]. To date, only the WNV/YFV 17D vaccine candidate has undergone Phase II clinical trials in humans. Despite the results of clinical trials showing that the vaccine was immunogenic and well tolerated in all age groups studied, further development of this vaccine was stopped due to uncertainty in the market [9, 23].

A live attenuated WN/DEN4 30 vaccine has been developed in our laboratory and preclinical studies with parenterally administered virus have demonstrated that it is highly attenuated, immunogenic in mice and monkeys, and safe for the environment [19, 24–26]. This vaccine was also well-tolerated and immunogenic in healthy adult volunteers (18–50 years of age) [27]. Based on these encouraging results, additional studies are being planned to evaluate vaccine safety and immunogenicity in subjects older than 50 years of age – a potential target population for vaccination. In order to further assess the safety of this vaccine before Phase II clinical trials, we performed a set of neuropathogenesis studies in nonhuman primates (NHPs) comparing the neurovirulence of WN/DEN4 30 vaccine to that of two parental viruses, WNV [4] and DEN4 30 [28], and also to the YF 17D vaccine following intrathalamic inoculation of virus. The licensed YF 17D has been used as a surrogate reference to help determine acceptability of live flavivirus vaccines in terms of their neurovirulence [29–32]. This report documents a very low neurovirulent potential of WN/DEN4 30 vaccine in NHPs. We also show a remarkable sensitivity of our WNV-NY99 NHP model, as well as striking resemblance of the observed neuropathology to human WNND.

2. Materials and methods

2.1. Viruses, animals and inoculations

The WNV wild-type strain was NY99-35262 (hereafter WNV) [24]. Vaccine viruses were: WN/DEN4 30 [27], DEN4 30 [28], and YF 17D [29]. 2–3 year old (4 kg) rhesus monkeys (*Macaca mulatta*) were seronegative for WNV, DEN4, and YF virus (PRNT₆₀). All animal experiments were approved by the NIAID/NIH Institutional Animal Care and Use Committee. In two experiments (Table 1), NHPs were inoculated bilaterally intrathalamically (i.t.) with the dose of 5.0 log₁₀ PFU of each virus as previously described [29], except that a smaller inoculum volume (0.25 ml) was used.

2.2. Clinical evaluation

Animals were observed twice daily for clinical signs and euthanized at scheduled time points or when moribund (Table 1). The following observations were recorded: neurological signs, coat condition, food consumption, body weight, rectal temperature, pulse, and respiration rate. Neurological illness was graded as follows: grade 0, no abnormalities; grade 1, rough coat and decreased food consumption; grade 2, clumsy and lethargic; grade 3, shaky movements, incoordination, limb weakness, tremors, or seizures; grade 4, inability to stand, moribund state, or multiple long-lasting seizures. The highest grade was assigned as the individual daily grade.

2.3. Sample collection and analyses

Following euthanasia and cardiac perfusion with sterile saline, brain, spinal cord, and selected peripheral tissues were collected, dissected, and processed for virology (virus titrations) and histopathology. The cerebrospinal fluid (CSF) was collected immediately before necropsy. Serum samples to measure viremia and neutralizing antibody titers were collected on days 0, 1, 3, 5, 7, 9, 10, 14, and 21 post infection (dpi). Brain dissection and sampling were slightly modified from that previously described [29]. Briefly, after a

parasagittal cut, the right brain hemisphere was fixed in 10% buffered formalin for 7 days and then Rhesus Monkey Brain Matrix (Ted Pella, Redding, CA) was used to make 4 mm coronal slices that were trimmed and routinely processed and embedded in paraffin. Two 5 µm sections (1st and 4th) from each paraffin block were mounted onto single slides and stained with hematoxylin and eosin (H&E). The left hemisphere was aseptically dissected at the same coronal levels. The analyzed regions of interest (ROI) of the central nervous system (CNS) were: cerebral cortex (frontal, temporal, parietal, and occipital), basal ganglia, thalamus, midbrain, pons, medulla oblongata, cerebellum, and spinal cord (cervical, thoracic, and lumbar). Core tissue samples (5 mm in diameter; 4 mm thick) were extracted using sterile Harris Uni-Cores (Ted Pella, Redding, CA) and 10% homogenates of at least three samples from each brain region were prepared using FastPrep-24 Instrument (MP Biomedicals, Solon, OH). Virus titers were determined by plaque-forming assay (PFA) and neutralizing antibody titers were measured by the 60% plaque reduction neutralization assay (PRNT₆₀) as previously described [24, 25, 29]. Spinal cord was dissected transversely and adjacent slices of the cervical, thoracic, and lumbar regions were used for histopathological or virological examination.

2.4. Digital pathology and scoring

Sections were scanned at 20x magnification using ScanScope XT (Aperio, Vista, CA); Spectrum Plus and ImageScope software were used for digital slide organization, viewing, and analysis complemented by neuroanatomical maps [33]. Semi-quantitative scores (1, no lesions; 2, minimal; 3, mild; 4, moderate; 5, severe) were assigned to each CNS ROI to assess the degree of: (i) cellular infiltration (CI); (ii) microgliosis (MG) and neuronal degeneration (ND) (see Table S1).

2.5. Statistical analysis

For all groups of animals inoculated with each virus, virus titers or histopathological scores were compared using multivariable analysis and linear mixed models computed with the Fit Model platform in JMP 9.0.0 (www.jmp.com). Post hoc tests for each main effect and interaction were computed using Tukey Honest Significant Differences (HSD). Graphs were generated using Least Squares Means from the Tukey HSD Connected Letters Report, while significant differences were identified by sorting and filtering results from the Tukey HSD Ordered Differences Report (see Supplemental Materials). *P* values <0.05 were considered significant.

3. Results

3.1. Clinical observations

The clinical course of viral infection in NHPs inoculated with WNV, WN/DEN4 30, DEN4 30, or YF 17D virus is summarized in Table 2. WNV-infected monkeys developed fever soon after the inoculation that lasted until animals became moribund (maximum temperature 40.8°C). Twenty two percent of monkeys infected with WNV developed neurological signs of Grade 2 on 4 dpi. Grade 3 signs began to appear on 6 dpi and, by 9 dpi, all remaining monkeys developed Grade 4 signs. None of the monkeys inoculated with WN/DEN4 30, DEN4 30, or YF 17D developed neurological signs, but febrile reaction

(>39.3°C) was present in many animals indicating subclinical infection. None of mock-inoculated monkeys developed neurological signs or febrile reaction.

3.2. Viremia and neutralizing antibody response

The majority (92%) of WNV-inoculated monkeys had a viremia (mean titer $2.5 \log_{10}$ PFU/ml) on the day following inoculation (Figure 1). WNV viremia decreased thereafter both in terms of the level and frequency ($1.7 \log_{10}$ PFU/ml in 83% of monkeys on 3 dpi; $0.8 \log_{10}$ PFU/ml in 22% of monkeys on 5 dpi). In contrast, monkeys inoculated with YF 17D or DEN4-30 had viremia only on 3 dpi, albeit the level and frequency of viremia were higher in the former ($1.8 \log_{10}$ PFU/ml in 75% of monkeys and $0.9 \log_{10}$ PFU/ml in 42% of monkeys, respectively). Viremia was not detected in any monkeys inoculated with WN/DEN4-30 (limit of detection: $0.7 \log_{10}$ PFU/ml). Although WNV and YF 17D induced serum neutralizing antibody responses somewhat more rapidly, the antibody levels against each homologous virus were similar by 21 dpi (Figure 1).

3.3. Virus replication in the CNS and peripheral tissues

WNV was detected at 3 dpi in the inoculation site (thalamus), adjacent cortical regions (frontal, parietal, and temporal), and in the basal ganglia and midbrain (Figure 2). At this time, WNV was also detected in the CSF of one of three monkeys ($1.9 \log_{10}$ PFU/ml). It took WNV only four days to spread throughout the entire CNS. The regions having the highest virus loads (up to $8 \log_{10}$ PFU/g) were: pons, medulla oblongata, cerebellum, and spinal cord. It appears that as soon as WNV spread to these preferred structures, it continued to replicate there (increase or plateau in virus loads), whereas virus loads in the sites of its initial replication sharply decreased. By 9/10 dpi, all WNV-infected animals invariably had neurological signs of Grade 4, signifying development of fulminant encephalitis.

YF 17D was detected in the CSF of two of three monkeys at 3 dpi (titers 1.3 and $4.0 \log_{10}$ PFU/ml). Initial anatomical sites of YF 17D replication resembled those of WNV - moderate virus loads (up to $5 \log_{10}$ PFU/g) in the cerebral cortex, basal ganglia, thalamus, and midbrain at 7 dpi with a sharp decline by 10 dpi (Figure 2). However, by 10 dpi, striking differences between the replication of these two viruses had become evident in the pons, medulla oblongata, cerebellum, and spinal cord. In contrast to WNV, YF 17D titers at 10 dpi were low in these regions and virus was rapidly cleared from the entire CNS.

DEN4-30 virus was detected only in the frontal cortex, basal ganglia, and spinal cord at 7 and 10 dpi in very low titers (up to $1.9 \log_{10}$ PFU/g) and virus was cleared by 14 dpi (Figure 2).

WN/DEN4-30 virus was unable to replicate in the CNS of NHPs - virus titers were below the limit of detection ($1.7 \log_{10}$ PFU/g).

Statistical analysis has confirmed that the above differences in the magnitude and spatiotemporal patterns of replication between WNV and three vaccine viruses were statistically significant (Table 3). At 3 dpi, WNV mean titer was significantly higher in the thalamus compared to that of WN/DEN4-30, suggesting more efficient replication of WNV in the inoculation site. At 7 dpi, WNV titers were significantly higher than those of both

DEN4 30 and WN/DEN4 30 in many CNS regions (Table 3). However, when compared to YF17D, WNV titers were significantly higher only in the pons, medulla oblongata, cerebellum, and the spinal cord. YF 17D titers were also higher than those of both DEN4 30 and WN/DEN4 30 in the frontal cortex, basal ganglia, thalamus, midbrain, pons, and medulla oblongata. At 9/10 dpi, WNV titers significantly exceeded those of all three vaccine viruses in the thalamus, pons, medulla oblongata, cerebellum, and spinal cord, suggesting that these regions were most permissive for WNV replication.

WNV was detected in the adrenal glands or mesenteric lymph nodes (LNs) of monkeys at 7 dpi and in the mandibular, deep cervical, or inguinal LNs, as well as in the adrenal glands at 9/10 dpi (Table 4). YF 17D virus was only detected in one monkey in the axillary and mesenteric LNs, spleen, and bone marrow at 7 dpi. Neither DEN4 30, nor WN/DEN4 30 was found in peripheral tissues at any time point.

3.4. Histopathological analysis of the CNS

Digital slides containing multiple sections from twelve CNS ROI of each monkey (i.e., frontal, temporal, parietal, and occipital cortex, basal ganglia, thalamus, midbrain, pons/medulla oblongata, cerebellum, and cervical, thoracic, and lumbar regions of the spinal cord) were analyzed for presence of (i) cellular infiltration (CI) by peripheral inflammatory cells; (ii) microgliosis (MG), as evident by presence of microglial nodules (MGNs), glial hypercellularity, neuronophagia; and (iii) neuronal degeneration (ND) as evident by the central chromatolysis, swelling and/or shrinkage and/or eosinophilia of neuronal cytoplasm and eccentric pyknotic neuronal nuclei.

The severity of these changes was then assessed semi-quantitatively (Table S1). A comparison of high (WNV) and low (WN/DEN4 30) scores for the cerebellum of infected monkeys is shown in Figure 3. An apparent gradient in the scores from the highest (WNV) to intermediate (YF 17D) to low (DEN4 30) and to the lowest (WN/DEN4 30) can be seen in the spinal cord (Figure 4). Mock-inoculated monkeys had no histopathological changes (CI and MG/ND scores: 1- no changes; see Supplemental Table 1).

Spatiotemporal distributions of mean scores in each CNS ROI of infected monkeys are shown in Figure 5. Statistically significant differences in scores are listed in Table 5. Judging by this data, it is evident that WNV induced the most severe histopathological changes in the cerebellum and spinal cord compared to all vaccine viruses. However, despite the impression that the YF 17D scores were somewhat higher than those of DEN4 30 and WN/DEN4 30 (Figure 4 and 5), a comprehensive multivariable statistical analysis employed in this study showed that there were no significant differences between these three vaccine viruses. These results argue that all three vaccine viruses behaved similar in terms of induced histopathology.

4. Discussion

The purpose of this study was to assess the neuroattenuation of the WN/DEN4 30 vaccine before advancing to clinical evaluation in subjects older than 50 years of age. As recommended by the World Health Organization, live attenuated vaccines against

neurotropic viruses (e.g. polio, Japanese encephalitis), potentially neurotropic viruses (e.g., measles, mumps, rubella, and dengue) viruses, as well as chimeric virus vaccines (e.g., based on YF 17D or DEN4 backbones), should be tested for neurovirulence in NHPs to assure their safety for humans. NHPs have a unique value in translational research and have served as models for human diseases for over a century. Because of the natural susceptibility of NHPs to a wide range of human pathogens and the high degree of genetic similarity to humans, NHPs have provided invaluable insights into pathogenesis, immunity, and vaccine development for many infectious diseases [34–36]. However, WNV infection of NHPs, either natural or experimental by peripheral routes of inoculation, induces only viremia and humoral immune response and does not result in neurological disease [22, 25, 37–43]. This is similar to the situation in humans - less than 1% of infections result in WNND [3, 5]. Major risk factors for development of WNND are immunosuppression and advanced age [3, 5]. However, immunodeficient or aged macaques do not develop clinical signs even with very high peripheral virus doses [43]. Therefore, although mimicking the natural route of infection and being useful for monitoring viremia and immunological correlates of protection by vaccines, the peripheral model of WNV infection in NHP is unsuitable for evaluation of therapeutic interventions once the virus has reached the CNS.

To date, it is known that only intracerebral WNV inoculation produces clinical signs and neuropathology in NHPs. However, the number of studies of WNV encephalitis in NHPs, in comparison to mouse models, is limited. Since the first study in 1940 that described neurovirulent properties of WNV in monkeys [37], there were several reports dealing with the histopathological aspects of the CNS infection in monkeys inoculated intracerebrally with various virus isolates from Africa [38, 39, 44, 45], Europe, and Asia [39]. However, very little is known about the neuropathogenesis associated with the North American WNV strains (i.e., lineage 1, WNV NY99 strain) in NHPs. In one study, two rhesus macaques (without any prior vaccination) were challenged intracerebrally with WNV NY99 and developed fever and neurological signs with a lethal outcome, but no virological and/or histopathological analyses of the CNS were reported [46]. In published neurovirulence studies with different WNV isolates [37–39, 44, 45], CNS disease in rhesus monkeys had common clinical and pathological features that can be summarized as follows: (i) neurovirulence of different WNV strains vary; (ii) majority of monkeys develop viremia, febrile reaction, and encephalitis (neurological signs include tremors, spasticity, incoordination, weakness, convulsions, ataxia, and paralysis); (iii) encephalitis can be fatal, but asymptomatic infection can also be present; (iv) clinical course of fatal encephalitis is acute, leading to death or moribund state that require euthanasia at 9–15 days, whereas non-fatal encephalitis is characterized by subacute course often followed by virus persistence; (v) pathology is confined to the CNS with perivascular and subpial inflammatory cell infiltration, microglial nodules, and neuronal cell degeneration; (vi) the most severely affected CNS regions are cerebellum, pons, medulla oblongata, spinal cord; the basal ganglia and thalamic nuclei can be affected to various degrees, but involvement of the cerebral cortex is generally mild.

All things considered, the pathological features in our model of WNV infection in NHPs are in line with previous studies that used different WNV strains. However, our model allows monitoring the kinetics of virus replication within the CNS, and demonstrated a devastating

neuropathology leading to clinical signs that remarkably recapitulate human WNND in the US, manifestations of which include meningitis, movement disorders, pons/medulla oblongata encephalitis, cerebellar ataxia, and/or acute flaccid paralysis syndrome [2, 3, 47–49]. Thus, the results of this study provide valuable data on WNV NY99 neuropathogenesis in NHPs and can be used as a baseline in evaluations of vaccines and treatments for humans. Having this baseline, we next analyzed the neurovirulence of our vaccine candidate against WNV (WN/DEN4 30), DEN4 30 vaccine (backbone of our vaccine), and YF 17D vaccine (a surrogate reference with an acceptable level of neurovirulence). As anticipated based on the previous studies [19, 24, 25], current results show that WN/DEN4 30 vaccine is highly neuroattenuated for NHPs, when compared to a wild type WNV. However, in the absence of reference vaccine against WNV, it is difficult to judge what level of neuroattenuation is safe. Here, we show: (i) the ability of WN/DEN4 30 to replicate in the CNS of NHPs was lower than that of YF 17D and similar to that of DEN4 30; (ii) the degree of virus-induced neuropathology after i.t. inoculation was not significantly different between WN/DEN4 30, DEN4 30 and YF 17D. These are strong indications of a high level of neuroattenuation of WN/DEN4 30. Therefore, this vaccine against WNV is suitable for further evaluation in target population older than 50 years of age. Our results also support the use of an improved NHP model for translational studies of WNV neuropathogenesis and/or testing the effectiveness of vaccines and therapeutic approaches. We strongly support the idea that a safe and effective vaccine against WNV is needed to prevent future outbreaks and that it would be beneficial for aged population [5, 8, 14]. Future studies will be focused on further clinical evaluation of WN/DEN4 30 vaccine.

5. Conclusions

We report here valuable clinical, virological, and histopathological data on the pathogenesis of the CNS infection with the WNV NY99 in an improved NHP model. This model should be useful for preclinical studies of treatments for the WNND, as well as for in-depth safety testing of WNV vaccines, and may facilitate vaccine approval for human use within the concept of “The Animal Rule”. Based on the comparison to two parental viruses (WNV and DEN4 30) and a surrogate flavivirus reference vaccine (YF 17D) our WN/DEN4 30 vaccine shows an acceptable safety profile as evidenced by its very low neurovirulence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Availability of safe and effective WNV vaccines for high risk aging populations could be beneficial
- We assessed the neurovirulence of a chimeric WN/DEN4-30 vaccine in nonhuman primates (NHPs)
- WN/DEN4-30 was compared to a wild-type WNV and two other vaccines (DEN4-30 and YF 17D)
- Comprehensive data demonstrate that WN/DEN4-30 vaccine is highly neuroattenuated for NHPs
- The results support further clinical evaluation of WN/DEN4-30 vaccine in older adults

Figure 1.

Viremia (columns) and serum neutralizing antibody response to homologous virus (lines) in NHPs. Viremia is expressed as mean virus titer $\pm$ SE. The limit of virus detection in serum was 0.7 log₁₀ PFU/ml. Virus titers in sera of monkeys inoculated with WN/DEN4 Δ 30 were below the limit of detection (not shown). Neutralizing antibody response is shown by the mean reciprocal PRNT₆₀ values. The lowest serum dilution tested by PRNT₆₀ was 1:5.

Figure 2.

Virus titers in the CNS of NHPs inoculated with WNV, WN/DEN4 Δ30, DEN4 Δ30, or YF 17D virus. Mean virus titer $\pm$ SE in each CNS region are shown for each virus on indicated dpi (3 dpi was assessed only for WN NY99 and WN/DEN4 Δ30 viruses). The limit of virus detection was 1.7 log₁₀ PFU/g of tissue.

Figure 3. Representative areas of the cerebellum (spinocerebellum) demonstrating different combinations of histopathological scores (H&E). Inoculated virus and the CI and MG/ND score combinations are shown for each panel (A and B). Insets show the corresponding circled areas in overview images at higher magnification. *Abbreviations:* M, molecular layer; P, Purkinje cell layer (also outlined); G, granular layer. Note: leptomeningeal inflammatory cell infiltration (*arrows*); degeneration (*arrowheads*) and loss of Purkinje cells; prominent rarefaction of the granular layer (white asterisks). Bars: 1000 μ m (overviews in A and B); 100 μ m (insets).

Figure 4.

Representative sections of the spinal cord (lumbar region) demonstrating different combinations of histopathological scores (H&E). Inoculated virus and the CI and MG/ND score combinations are shown for each panel (A – D). Insets show the corresponding circled areas in overview images at higher magnification. Vh, ventral horn. Gray matter is outlined. Note: perivascular inflammatory cell infiltration in A, B, and C (*white arrows*); focal glial proliferation in C (*white asterisk*); microglial nodule in A (*black arrowhead*); degenerative neuronal changes - central chromatolysis, swelling, and eccentric pyknotic nuclei (A and B, *black arrows*). Bars: 1000 μ m (overviews in A-D); 100 μ m (insets).

Figure 5.

Spatiotemporal distributions of mean CI and MG/ND scores in the CNS of monkeys infected with WNV, WN/DEN4 Δ30, DEN4 Δ30, or YF 17D. Mean histopathological scores and SEs are shown for each CNS ROIs on the indicated dpi. *3 dpi was assessed only for WNV and WN/DEN4 Δ30. No changes other than injection-related lesions were found in mock-inoculated NHPs (Score 1 – normal; not shown). N/A – not applicable (all WNV-infected animals developed fulminant encephalitis and were euthanized moribund at 9/10 dpi).

Table 1

Study experimental design and end-points

Experiment ^a	Inoculated virus	No. of NHPs	Number of NHPs euthanized on indicated dpi				
			3 dpi ^b	7 dpi	9/10 dpi ^c	14 dpi	21 dpi
1	Mock	4	ND	1	1	1	1
1	WNV	12	3	3	6	N/A	N/A
1	WN/DEN4 30	15	3	3	3	3	3
2	DEN4 30	12	ND	3	3	3	3
2	YF 17D	12	ND	3	3	3	3

^aDue to a large number of animals and safety concerns the study was done in two separate experiments which were executed in the same manner.

^bThe 3 dpi time point was included in the first experiment. However, this time point was omitted in the second experiment due to relatively little informational value obtained.

^cSix remaining NHPs infected with WNV became uniformly severely ill by 9 dpi. However, five monkeys were euthanized on 9 dpi (during late afternoon) and one monkey on the following morning (10 dpi). Since the difference in the time of euthanasia between WNV-infected monkeys was less than 24 hours, and because the corresponding time of euthanasia/necropsy for all vaccine viruses was 10 dpi, this time point is referred as 9/10 dpi hereafter. N/A – not applicable (all WNV-infected monkeys were euthanized at 9/10 dpi). ND – not done.

Table 2

Clinical course of infection in NHPs inoculated with WNV, WN/DEN4 30, DEN4 30, or YF 17D

Inoculum	Clinical signs ^a	Clinical course on indicated dpi									
		0-3	4	5	6	7	8	9/10	14	21	
WNV	Grade ^b	0 100%	2 22%	2 22%	3 33%	3 56%	3 83%	4 100%	N/A	N/A	
	Temperature ^c	39.9°C 25%		40.8°C 56%		40.7°C 78%		40.8°C 50%	N/A	N/A	
WN/DEN4 30	Grade	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	
	Temperature	39.4°C 27%						39.7°C 33%	39.7°C 22%		
DEN4 30	Grade	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	
	Temperature	39.6°C 8%						40.4°C 78%			
YF 17D	Grade	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	0 100%	
	Temperature	39.8°C 25%		39.6°C 11%		39.8°C 22%		40.2°C 67%			

^a Clinical signs were monitored and recorded as described in the Materials and Methods.

^b Neurological grades and percent of animals having the corresponding grade are shown.

^c If temperature was elevated (>39.3°C), the maximum recorded temperature and percent of animals with febrile reaction are shown. N/A – not applicable (all WNV-infected monkeys were euthanized at 9/10 dpi). Mock-inoculated animals (not shown) remained normal during the entire experiment (neurological grade 0; normal temperature).

Table 3

Statistical comparison of virus titers in the CNS of NHPs inoculated with WNV, WN/DEN4 30, DEN4 30, or YF 17D virus

Dpi	CNS region	Virus 1: WNV	Virus 2: YF 17D	Virus 3: WN/DEN4 30	Virus 4: DEN4 30	Difference in mean titers* (log ₁₀ PFU/g)	P value
3	Thalamus	A	ND	A	ND	(A) = 1.3	0.013
7	Frontal cortex	A	B	A B		(A) = 1.7; (B) = 1.7	<0.0001
		A			A B	(A) = 1.7; (B) = 1.7	<0.0001
	Temporal cortex	A		A		(A) = 1.8	<0.0001
		A			A	(A) = 1.8	<0.0001
	Basal ganglia	A	B	A B		(A) = 2.3; (B) = 2.4	<0.0001
		A			A B	(A) = 2.3; (B) = 2.4	<0.0001
	Thalamus	A	B	A B		(A) = 3.5; (B) = 2.8	<0.0001
		A			A B	(A) = 3.5; (B) = 2.8	<0.0001
	Midbrain	A	B	A B		(A) = 2.9; (B) = 2.9	<0.0001
		A			A B	(A) = 2.9; (B) = 2.9	<0.0001
	Pons/medulla oblongata	A	A B			(A) = 1.5	0.004
		A		A B		(A) = 3.5; (B) = 2.0	<0.0001
		A			A B	(A) = 3.5; (B) = 2.0	<0.0001
	Cerebellum	A	A			(A) = 4.0	<0.0001
		A		A		(A) = 4.8	<0.0001
		A			A	(A) = 4.8	<0.0001
	Cervical spinal cord	A	A			(A) = 2.8	<0.0001
		A		A		(A) = 4.1	<0.0001
		A			A	(A) = 4.0	<0.0001
	Thoracic spinal cord	A	A			(A) = 2.9	<0.0001
		A		A		(A) = 4.0	<0.0001
		A			A	(A) = 3.9	<0.0001

Dpi	CNS region	Virus 1: WNV	Virus 2: YF 17D	Virus 3: WN/DEN4 30	Virus 4: DEN4 30	Difference in mean titers* (log ₁₀ PFU/g)	P value
	Lumbar spinal cord	A	A			(A) = 3.0	<0.0001
		A		A		(A) = 4.0	<0.0001
		A			A	(A) = 4.0	<0.0001
9/10	Thalamus	A	A			(A) = 1.7	<0.0001
		A		A		(A) = 1.9	<0.0001
		A			A	(A) = 1.9	<0.0001
	Pons/medulla oblongata	A	A			(A) = 2.4	<0.0001
		A		A		(A) = 2.9	<0.0001
		A			A	(A) = 2.9	<0.0001
	Cerebellum	A	A			(A) = 4.9	<0.0001
		A		A		(A) = 5.3	<0.0001
		A			A	(A) = 5.3	<0.0001
	Cervical spinal cord	A	A			(A) = 3.1	<0.0001
		A		A		(A) = 3.5	<0.0001
		A			A	(A) = 3.4	<0.0001
	Thoracic spinal cord	A	A			(A) = 3.5	<0.0001
		A		A		(A) = 3.9	<0.0001
		A			A	(A) = 3.8	<0.0001
	Lumbar spinal cord	A	A			(A) = 3.1	<0.0001
		A		A		(A) = 4.2	<0.0001
		A			A	(A) = 4.2	<0.0001

All four viruses were compared simultaneously using multivariable statistical analysis as described in the Material and Methods. Only statistically significant differences are shown and the *p* values are provided. If the viruses are connected by the letter A, then the mean titer of Virus 1 (WNV) was significantly higher than that of Virus 2 or Virus 3 or Virus 4. If the viruses are connected by the letter B, then the mean titer of Virus 2 (YF 17D) was significantly higher than that of Virus 3 or Virus 4.

* Difference in mean titers between the viruses connected by the same letter (i.e., (A) indicates: Virus 1 – Virus 2; or Virus 1 – Virus 3; or Virus 1 – Virus 4). ND – not done.

Table 4
Detection of virus in the peripheral tissues of NHPs inoculated with WNV or YF 17D virus

Tissue	WNV				YF 17D	
	7 dpi (n = 3)		9/10 dpi (n = 6)		7 dpi (n = 3)	
	# Positive (ID)	log ₁₀ PFU/g	# Positive (ID)	log ₁₀ PFU/g	# Positive (ID)	log ₁₀ PFU/g
Lymph nodes	0		0		0	
Mandibular	0		1 (C88)	1.8	0	
Deep cervical	0		1 (HXO)	3.8	0	
Axillary	0		0		1 (A8E061)	2.4
Bronchial	0		0		0	
Mesenteric	1 (DCL8)	2.3	0		1 (A8E061)	2.3
Inguinal	0		1 (DC5P)	2.5	0	
Spleen	0		0		1 (A8E061)	2.1
Bone marrow	0		0		1 (A8E061)	3.0
Heart	0		0		0	
Lungs	0		0		0	
Liver	0		0		0	
Pancreas	0		0		0	
Adrenal glands	1 (DSC3Z)	3.4	1 (DC5P)	3.1	0	
Kidneys	0		0		0	

The number of positive monkeys (#), their unique identifier (ID), and corresponding virus titers are shown for indicated dpi.

Table 5

Statistical comparison of histopathological scores for the CNS of NHPs inoculated with WNV, WN/DEN4 30, DEN4 30, or YF 17D virus

Dpi	CNS region	Virus 1	Virus 2	MG/ND		CI	
				Difference in mean score *	P value	Difference in mean score *	P value
7	Spinal cord	WNV	WN/DEN4 30	1.6	0.032		
7	Spinal cord	WNV	DEN4 30	1.6	0.032		
7	Spinal cord	WNV	YF 17D	1.6	0.032		
9/10	Cerebellum	WNV	WN/DEN4 30	3.5	<0.0001	3.6	<0.0001
9/10	Cerebellum	WNV	DEN4 30	2.5	0.0004	2.5	0.011
9/10	Cerebellum	WNV	YF 17D	3.0	0.0001	3.0	0.0002
9/10	Spinal cord	WNV	WN/DEN4 30	2.9	<0.0001		
9/10	Spinal cord	WNV	DEN4 30	2.2	<0.0001		
9/10	Spinal cord	WNV	YF 17D	2.2	<0.0001		

* Positive number indicates that the mean score for the group of animals inoculated with Virus 1 was significantly higher than the mean score for the groups of animals inoculated with Virus 2. Only statistically significant differences are shown.