Aerosol Exposure to Western Equine Encephalitis Virus Causes Fever and Encephalitis in Cynomolgus Macaques

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Cynomolgus macaques were exposed by aerosol to a virulent strain of western equine encephalitis virus (WEEV). Between 4 and 6 days after exposure, macaques had a significantly elevated temperature that lasted for 3–4 days. Clinical signs of encephalitis began as the body temperature decreased, and then they rapidly increased in severity. Cynomolgus macaques with clinical signs of encephalitis had elevated white cell counts in the blood caused mostly by increased numbers of segmented neutrophils and monocytes. Elevated serum glucose levels also correlated with the severity of the clinical signs of encephalitis. Three cynomolgus macaques died; immunohistochemical evidence of viral antigen was present in the brain and central nervous system (CNS). Microscopic analysis also revealed a marked lymphocytic infiltrate in the CNS. Cynomolgus macaques will serve as a useful model of aerosol exposure to WEEV for the evaluation of potential vaccine candidates.

Western equine encephalitis virus (WEEV) is a positive-stranded RNA virus in the genus Alphavirus of the family Togaviridae. WEEV is endemic in the western portions of North America [1]. Natural transmission is by mosquito; outbreaks in human and equine populations occur but are uncommon. In natural outbreaks of WEEV, the clinical signs of illness in humans include fever, nausea, headache, and encephalitis, and the case-fatality rate is between 1% and 5%.

Like the related Venezuelan and eastern equine encephalitis viruses (VEEV and EEEV, respectively), WEEV is infectious by aerosol exposure. There are reports in the literature of 3 laboratory accidents in which personnel were exposed to WEEV, and 2 of these resulted in fatalities [2–4]. Two of the fatal illnesses were the probable result of aerosol exposure; for the third, the route of exposure was never identified. A report in 1967 put the total of fatalities at 2 in 5 exposed laboratory workers, for 40% mortality [5].

Wyckoff and Tesar [6] reported in 1939 that intranasal challenge with either WEEV or EEEV could cause severe, lethal encephalitis in rhesus macaques. Although there were differences in the onset and duration, the diseases caused by WEEV and EEEV were remarkably similar. For both, the clinical signs of encephalitis began after the fever had peaked, and they worsened until death. Intracranial injection of either virus was fatal. Results of other studies were similar [7, 8]. Virus could not always be detected in the blood of WEEV-infected rhesus macaques, but the difference in detection seemed to be due, in part, to differences in either the route of infection or the time the blood was drawn. Results of previous studies agreed that, after clinical signs of encephalitis were present, virus was not recovered from the blood but was found in tissues of the central nervous system (CNS).

A nonhuman primate model is needed to evaluate the efficacy of medical products to protect against al-
phaviruses are potential biological terrorism threats, an ideal multivalent vaccine would protect against VEEV, WEEV, and EEEV. Because there are problems with cross-reactivity among alphaviruses and possible interference between vaccines [13], evaluating vaccine candidates for efficacy would be easiest if the same animal model was used for all 3 viruses. The present study reports our evaluation of the cynomolgus macaque as a suitable animal model for human illness caused by aerosol exposure to WEEV.

MATERIALS AND METHODS

Macaques. Healthy, adult cynomolgus macaques (Macaca fascicularis) of both sexes from the nonhuman primate colony at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) [14] were screened by plaque-reduction neutralization testing (PRNT) and ELISA for previous exposure to VEEV, WEEV, and EEEV before assignment to the present study. Telemetry implants (Data Sciences International) were implanted subcutaneously on the dorsal surface, and the macaques were allowed to recover from surgery for at least 14 days before exposure to WEEV; this time frame allowed for the acquisition of sufficient telemetry data for the temperature data to be modeled. Research was conducted in compliance with the Animal Welfare Act, other federal statutes and regulations relating to animals and experiments involving animals, and the principles stated in the Guide for the Care and Use of Laboratory Animals [14]. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Virus. WEEV strain CBA-87 was isolated from the brain of a horse during an epizootic outbreak in Argentina [15]. For aerosol exposures, virus was diluted to an appropriate concentration in Hank’s balanced salt solution (HBSS) containing 1% fetal bovine serum.

Aerosol exposures. Each macaque was anesthetized by intramuscular (im) injection of tiletamine/zolazepam (6 mg/kg), and a whole-body plethysmograph was performed to determine

<p>| Table 1. Fever response in cynomolgus macaques after aerosol exposure to western equine encephalitis virus. |
|----------------------------------|------------------|--|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Inhaled dose, log$_{10}$ pfu</th>
<th>Onset of fever, study day$^a$</th>
<th>$T_{max}$$^b$</th>
<th>Duration of fever, h</th>
<th>Duration of fever, days$^c$</th>
<th>Fever-hours$^d$</th>
<th>Average elevation in temperature$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3 ± 0.5</td>
<td>4.0</td>
<td>2.3</td>
<td>50.5</td>
<td>3.0</td>
<td>56.3</td>
<td>1.4</td>
</tr>
<tr>
<td>7.3 ± 0.4</td>
<td>5.0</td>
<td>3.5</td>
<td>92.5</td>
<td>4.5</td>
<td>165.9</td>
<td>1.7</td>
</tr>
</tbody>
</table>

$^a$ Defined as the first day with >8 h of significant elevation in temperature (as determined by autoregressive integrated moving average modeling).

$^b$ Defined as the maximum residual elevation in temperature in degrees Celsius.

$^c$ Calculated as the no. of days with >12 h of significant elevation in temperature.

$^d$ Calculated as the sum of the significant elevations in temperature; the result is expressed as degrees Celsius–hours.

$^e$ Calculated by dividing fever-hours by the duration of fever in hours; the result is expressed as degrees Celsius.
its respiratory capacity. Subsequently, each macaque was inserted into a class 3 biological safety cabinet located inside a biosafety level 3 suite and was exposed in a head-only aerosol chamber for 10 min to an aerosol created by a Collison nebulizer, as described elsewhere [10]. Samples were collected from the all-glass impinger attached to the aerosol chamber and were analyzed by use of a plaque assay to determine the inhaled dose of WEEV.

**Postexposure monitoring.** Macaques were observed daily for 3 days before and at least twice daily for 14 days after aerosol exposure to WEEV. Macaques were given scores for neurological signs of encephalitis in accordance with the following system: 5, normal; 4, loss of balance/muscle control; 3, occasional tremors/seizures; 2, frequent tremors/seizures; 1, comatose/moribund. Macaques were also given scores for changes in activity (5, normal; 4, active; 3, slow active; 2, sluggish; 1, inactive), behavior (5, normal; 4, antisocial; 3, depressed; 2, hunched with back to observer; 1, ignoring everything), and response to stimuli (5, normal; 4, when observer enters room; 3, when observer approaches cage; 2, when observer rattles cage; 1, on being pinched). Behavior was scored as antisocial if it was different from the macaque’s normal behavior. The clinical score for each macaque was recorded as the sum of scores for these 4 criteria. Macaques that were either comatose or had a clinical score <6 were euthanatized promptly by barbiturate overdose.

**Virological and clinical laboratory determinations.** Beginning 3 days before exposure and continuing until day 10 after exposure, macaques were anesthetized im with 50 mg/mL tiletamine hydrochloride and 50 mg/mL zolazepam hydrochloride, and blood samples were collected to assess antibody titers, complete blood counts, and viremia. Throat swabs for virus isolation were obtained during blood collection. Viremia in blood samples and throat swab samples was measured by standard plaque assay by use of Vero cells [9]. Blood cell counts were determined using a Coulter T-series instrument and a manual differential count. Serum chemistries were measured using a VITROS 250 (Ortho-Clinical Diagnostics).

**ELISA.** Standard ELISA procedures were used. Briefly, dilutions of serum were incubated for 1 h at room temperature on Microlite 2+ 96-well plates (Thermo Labsystems) coated with sucrose-gradient–purified, γ-irradiated WEEV. After washing with PBS-Tween, either secondary horseradish peroxidase (HRP)–conjugated goat anti–monkey IgG or HRP-conjugated goat anti–monkey IgM (both from Research Diagnostics) was added, and the plates were incubated for an additional 1 h at room temperature. After repeated washing with PBS-Tween, BM Chemiluminescence substrate (Roche Applied Sciences) was added for 5 min before analysis on an Lmax chemiluminescence plate reader (Molecular Devices).

**PRNT.** To determine virus-neutralizing antibody titers, 2-fold serial dilutions of serum, starting at a concentration of 1:
Figure 4. Changes in leukocytes in cynomolgus macaques after aerosol exposure to western equine encephalitis virus. Blood samples were obtained from macaques daily, beginning 3 days before exposure through day 10 after exposure, for assessment of changes in leukocytes. Graphs show the change from baseline levels (in percentages) for each day after exposure for individual macaques in the low-dose ($\log_{10}$ pfu; left panels) and high-dose ($\log_{10}$ pfu; right panels) groups ($n = 6$ macaques/group). Graphs are shown for total white blood cells (WBCs; A), lymphocytes (B), neutrophils (C), and monocytes (D).
Table 2. Values for leukocytes in cynomolgus macaques after aerosol exposure to western equine encephalitis virus.

<table>
<thead>
<tr>
<th>Inhaled dose, log_{10} pfu</th>
<th>Average change, %&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lymphocytes</th>
<th>Neutrophils</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WBCs</td>
<td>Macrophages</td>
<td>Maximum, no.</td>
<td>Minimum, no.</td>
</tr>
<tr>
<td>6.3 ± 0.5</td>
<td>−0.4</td>
<td>30.3</td>
<td>5.9</td>
<td>2.6</td>
</tr>
<tr>
<td>7.3 ± 0.4</td>
<td>21.0</td>
<td>64.0</td>
<td>6.5</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**NOTE.** WBCs, white blood cells.

*Group mean of the average change in the percentage from baseline levels for the 10 days after exposure.

20, were mixed with equal volumes of medium containing 200 pfu of virus and were incubated overnight at 4°C. The next day, 6-well plates containing confluent monolayers of Vero cells were incubated with the virus-antibody mixtures for 1 h at 37°C and then were overlaid with 0.6% agarose in Eagle MEM. Two days later, 1 mL of a solution of 5%–6% neutral red in 1× HBSS (without phenol red) was added to each well, and the plaques were counted the next morning.

**Data analysis.** Body temperatures were recorded every 30 min by the DataQuest A.R.T. 2.1 system (Data Sciences). Monitoring began 10–14 days before exposure, to develop a training period of temperature data to fit an autoregressive integrated moving average model [10]. Forecasted values for temperatures during the postexposure time periods were based on those obtained during the training period and extrapolated forward in time. Residual temperature changes after exposure were determined by subtracting the predicted temperature from the actual temperature recorded for each time point. Residual temperature changes >3 SDs above those in the training period were used to compute the duration of fever (number of hours of significant elevation in temperature), the fever-hours (sum of the significant elevations in temperature and expressed as degrees Celsius–hours), and the average elevation in temperature (fever-hours divided by the duration of fever and expressed as degrees Celsius).

**Necropsy and histopathological assessment.** A complete necropsy was performed under biosafety level 3 conditions on all macaques that were euthanatized when moribund from either the staircase or the disease course experiments. Representative tissue samples were obtained from all major organ systems, and an expanded selection of samples was obtained from the CNS. Specific areas of the CNS from which tissue samples were obtained for histopathological assessment included the frontal cortex, corpus striatum, thalamus, mesencephalon, pons, cerebellum, medulla oblongata, and cervical and lumbar spinal cord at the level of the intumescences. Tissue samples were fixed in 10% neutral buffered formalin for 21 days, processed routinely, embedded in paraffin, cut in 5–6-μm slices, and stained with hematoxylin-eosin (HE).

**Immunohistochemical analysis.** Unstained 5–6-μm sections of the tonsil, submandibular lymph node, lung, pancreas, and CNS were mounted on positive-charged slides (Superfrost Plus; Fisher Scientific) and were evaluated for WEEV antigen by use of an immunoperoxidase-based system (DAKO Envision+; DAKO). The tissues were pretreated with citrate buffer (pH 6.0) for 30 min at 97°C and were blocked with serum-free protein block and 5% normal goat serum. A rabbit polyclonal antibody (gift from C. Rossi, USAMRIID) made against an alphavirus cocktail consisting of WEEV, VEEV, EEEV, and Sindbis virus was applied to the sections for 30 min at room temperature. The Envision+ HRP-labeled dextran polymer conjugated to a rabbit secondary antibody was applied in accordance with the manufacturer’s instructions. Bound antibody was detected by an incubation with 3,3′-diaminobenzidine substrate–chromagen for 5–8 min, and the sections were counterstained with HE.

**RESULTS**

Preliminary studies were conducted to determine the aerosol ID<sub>50</sub> of WEEV strain CBA-87 for macaques. Changes in body temperature and clinical signs of encephalitis were used to assess infection and disease; macaques were not anesthetized during the preliminary studies, because the anesthesia could affect the temperature data and, therefore, the subsequent determi-
Figure 6. Changes in serum glucose levels in cynomolgus macaques after aerosol exposure to western equine encephalitis virus. Blood samples were obtained from macaques daily, beginning 3 days before exposure through day 10 after exposure, for assessment of changes in serum chemistry. Graphs show the serum glucose levels (A–B) or the change in serum glucose levels from baseline levels (in percentages; C–D) for individual macaques in the low-dose (log 10 pfu; A, C) and high-dose (7.3 ± 0.4 log10 pfu; B, D) groups (n = 6 macaques/group).

nation of the ID50 [16]. According to the staircase method, the aerosol ID50 was 6.25 log10 pfu [17]. One macaque died of the infection during the determination of the ID50.

Twelve macaques were assigned to 1 of 2 groups of 6 macaques each and were exposed to WEEV strain CBA-87 by aerosol for 10 min. One group received an inhaled dose of 6.3 ± 0.5 log10 pfu, and the other group received an inhaled dose of 7.3 ± 0.4 log10 pfu. These doses correspond to 1 and 10 ID50, respectively. The doses were chosen to provide information concerning the relationship between physiological changes seen after exposure and the development of encephalitis.

Between day 4 and day 5 after exposure, a notable elevation in body temperature was evident in 3 macaques in the low-dose (log10 pfu) group and 5 macaques in the high-dose (7.3 ± 0.4 log10 pfu) group (figure 1). In macaques that developed a fever, body temperature continued to increase for 2 or 3 days until it reached its maximum on day 6 or 7. For several macaques, particularly those in the high-dose group, body temperature decreased significantly below baseline levels for an extended period before returning to normal. Table 1 shows a summary of the fever response. Both the duration and the severity (as measured by fever-hours and the average elevation in temperature) of the fever response were greater in the high-dose group than in the low-dose group. The average elevation in temperature during the fever response was well correlated with the clinical score (r = −0.87 [Pearson correlation coefficient]; P < .001) (figure 2).

Macques were also monitored for clinical signs of encephalitis. Figure 3 shows the clinical scores of macaques monitored for neurological signs of encephalitis and changes in activity, behavior, and response to stimuli. Beginning on day 6, the first visible signs of illness were observed. In macaques in the low-dose group, the observed signs of illness were mild, and the macaques recovered rapidly (figure 3A). The most commonly noted signs of illness in the low-dose group were antisocial behavior and a reduction in appetite and overall activity (data not shown). One macaque in the low-dose group developed occasional tremors on day 10, but these disappeared by day 11, and it subsequently recovered. Another macaque in the low-dose group also began showing signs of illness on day 6 and occasional tremors on day 7; by day 8, the frequency of the tremors had increased, and it was found prostrate, although it sat up when stimulated, and it ate. On day 9, the macaque was found comatose, convulsing, and unresponsive to stimulation; it was subsequently euthanatized. The macaque was necropsied, and tissue samples were obtained for histological examination and viral isolation.

All macaques in the high-dose group began to show signs of illness on day 7 or 8. Most macaques in the high-dose group (5 of 6) had occasional or frequent tremors on days 8, 9, and
10 after exposure. Three macaques had an increased frequency of tremors on days 8 and 9. Two of those 3 recovered, with the tremors disappearing by day 11 or 12. The third macaque, however, had nearly continuous tremors on day 9, and telemetry recorded a body temperature $<30^\circ\text{C}$. The macaque was euthanatized, and samples were obtained at necropsy for histological examination and virus isolation.

Blood samples for hematological, serum chemistry, and viremia assessments were obtained daily from all macaques during the pre- and postexposure periods. No virus was detected by plaque assay in any blood or throat swab sample obtained after exposure (data not shown). Analysis of the postexposure hematological data revealed evidence of a profound leukocytosis in individual macaques beginning on day 6 or 7 (figure 4A). Increased leukocyte counts were most pronounced in the high-dose group (table 2). This response was primarily composed of segmented neutrophils, although monocyte and lymphocyte counts were also elevated in some macaques (figure 4B–D). The maximum neutrophil counts were correlated with the clinical score ($r = -0.66$ [Pearson correlation coefficient]; $P < .001$) (figure 5). Leukocyte counts began to return to baseline levels by day 10. Table 2 summarizes the changes in leukocyte subsets by group.

Postexposure blood samples were assayed for serum chemistries, and the results were compared with those of preexposure blood samples. Figure 6 shows the serum glucose levels in individual macaques in both the low- and high-dose groups. In general, serum glucose levels were highly variable during the 10 days after exposure. In several macaques, however, elevations in serum glucose levels were 50%–100% above baseline levels at various points (figure 6B). In particular, the 2 macaques that were euthanatized had high serum glucose levels at the time of death. In all macaques, the maximum serum glucose levels were correlated with the clinical score ($r = -0.77$ [Pearson correlation coefficient]; $P < .001$) (figure 7). The other analytes measured showed no remarkable changes after exposure (data not shown).

The antibody response to WEEV infection was measured by both ELISA and PRNT. In both the low- and high-dose groups, anti-WEEV IgM was first detected on day 7, and the levels increased through day 10 (figure 8). Anti-WEEV IgG was first detected on day 9. Neutralizing antibody to WEEV began to be detectable at the same time as anti-WEEV IgG.

At necropsy, macroscopic changes were unremarkable, with the exception of marked congested meninges in 2 macaques and mild congestion in 1 macaque. The only significant microscopic findings were confined to the CNS, and, in general, these consisted of a nonsuppurative meningoencephalitis and demyelination. Multifocal expansion of Virchow-Robin’s space by moderate to marked numbers of lymphocytes and monocytes was noted in the brains of all macaques and in some
spinal cord sections (figure 9A). There were multifocal inflammatory and necrotic foci composed primarily of lymphocytes and microglia and lesser numbers of neutrophils, occasional well-formed glial nodules, necrotic and apoptotic cells, and low numbers of extravasated red blood cells within the gray matter. These inflammatory foci were prominent in the brain and, to a lesser degree, in the spinal cord, with the cervical region demonstrating more lesions than the lumbar region. Multifocal
areas of demyelination were present in the white matter of the brain and spinal cord; occasionally, inflammation was associated with these areas of demyelination.

Immunohistochemical staining was restricted to the CNS, and the strongest staining was localized to the inflammatory areas (figure 9B). Specifically, neurons, microglia, low numbers of cerebellar Purkinje cells, and α-motor neurons in the spinal cord gray matter were immunopositive. Occasionally, immunostaining was present in white matter areas characterized by demyelination and myelitis.

**DISCUSSION**

As was described elsewhere for aerosol exposure to VEEV [9, 10], we found that fever was the first and most obvious clinical sign of illness after exposure to WEEV. The duration and severity of fever after exposure to WEEV were also similar to what was previously reported for exposure to VEEV. The onset of fever after exposure to WEEV was delayed 2–3 days relative to that seen with epizootic VEEV but was similar to that seen with an enzootic strain of VEEV-IIIA.

We found that the clinical signs of encephalitis increased after the fever had peaked, which is consistent with what was reported previously for VEEV [6, 7]. We found that aerosol exposure to WEEV was far more likely than exposure to VEEV to induce neurological signs of encephalitis. In particular, after exposure to WEEV, the development of tremors was more frequent, and these tremors were more severe and prominent. The results of our study, however, also indicated that the development of tremors did not always predict a fatal outcome in macaques. There are several possible reasons for this discrepancy. In prior studies, an unknown quantity of virus was instilled in the nose, whereas, in the present study, a defined dose was delivered to the respiratory tract via a small-particle aerosol. Differences in the strain of WEEV used may also have altered the outcome. In the present study, we used CBA-87, a virulent strain that was isolated from the brain of a horse during an epizootic outbreak in Argentina; previous studies of WEEV in macaques lacked a description of the strain used.

It is striking how specific WEEV infection is to the CNS. No virus was detected in either the blood or the throat swab samples obtained after exposure. No virus or pathological changes could be detected in any of the tissue samples examined, except for those from the CNS. The failure to find virus in the blood or in any peripheral tissues of macaques that died in both the present study and in previous studies suggest that WEEV entering through the respiratory tract may indeed travel up the olfactory nerves directly to the brain, as has been proposed in aerosol exposure to VEEV [18–20]. The pathological changes in the CNS consisted of abundant viral antigen and a pronounced leukocyte infiltrate. An in-depth analysis of the findings of the pathological examination is under way (T.L., unpublished data). It is possible that no virus was found in peripheral organs or tissues, because the samples from these areas were collected during a late stage of the infection, when serum antibody may have controlled or eliminated WEEV in peripheral sites; however, it does not explain the failure to detect virus in the serum during an early stage of the infection.

Although no virus was found except for in the CNS, specific antibody to the virus could be detected on day 7. As has been postulated for other alphavirus infections, clearance of the virus with minimal immunopathological changes may depend on the production of neutralizing antibody. Three macaques died of infection; why they died, and why other macaques that developed clinical signs of encephalitis survived, cannot be explained at this time.

The level of leukocytosis, the serum glucose level, and the fever response were all correlated with the severity of encephalitis seen in the macaques. The maximum serum glucose levels were correlated with the disease outcome, but only on the day that moribund macaques were euthanatized; otherwise, changes in serum glucose levels were too variable to be useful for predicting the disease outcome. Leukocytosis was more consistent, but, like serum glucose levels, maximum levels were attained on the same day that moribund macaques were euthanatized. Of the 3 indicators, elevation in temperature appeared to be the most able to predict the disease outcome.

Given the restricted specificity of the infection, it is not clear what is driving the leukocyte and serum glucose changes. Leukocytosis has been reported in both EEEV and WEEV infections in humans [1, 4, 21, 22]. Increases in the number of neutrophils have only rarely been reported in other viral infections, so the importance of this finding is not clear. Neutrophils are not thought to play a role in protecting the host against viral infections. Because the level of leukocytosis correlated with the disease severity in the present study, it suggests that this response is inappropriate and could be contributing to the disease outcome. Similarly, it is difficult to assess the importance of elevation in serum glucose levels, because this has not been commonly reported for viral infections. Further investigation is needed to understand the mechanisms responsible and their importance to the disease outcome.

The utility of an animal model in the evaluation of medical products intended for human use depends greatly on the relevance of the model to the human disease. The natural transmission of WEEV is by mosquito bite; the only records of aerosol exposure in humans are from laboratory accidents. In humans, mortality appears to be higher for illness caused by aerosol exposure than for that caused by mosquito bite (40% vs. ≤5%), but those data may be misleading, because the number of reported laboratory accidents with WEEV is very low.
[5]. What we can infer from human disease caused by mosquito bite is that the most commonly observed clinical signs include fever, leukocytosis, and neurological signs of encephalitis [1]. The disease course, clinical signs, and disease outcome in cynomolgus macaques exposed to WEEV by aerosol are consistent with what has been reported in humans. We believe, therefore, that the cynomolgus macaque is a useful model for efficacy studies to evaluate vaccines and therapeutics for human use.

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References