Severe Encephalitis in Cynomolgus Macaques Exposed to Aerosolized Eastern Equine Encephalitis Virus

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Cynomolgus macaques exposed to an aerosol containing a virulent strain of eastern equine encephalitis (EEE) virus developed neurological signs indicating encephalitis that corresponded with the onset of fever and an elevated heart rate. Viremia was either transient or undetectable even in animals that succumbed to the illness. The onset of illness was dose dependent, but once a febrile response was observed, macaques were moribund within 36 h. Simultaneously, a prominent leukocytosis was seen; 1 day before being moribund, macaques had a white blood cell count $>20,000$ cells/$\mu$L. The leukocytes were predominantly granulocytes. Increases in serum levels of blood urea nitrogen, sodium, and alkaline phosphatase were also seen. The rapid onset and severity of neurological signs mirror what has been reported for human cases of disease caused by EEE.

Eastern equine encephalitis (EEE) is a zoonotic disease with high mortality caused by a group of arboviruses found in the eastern half of North America and portions of Central and South America [1]. EEE viruses are small, positive-strand RNA viruses of the family Togaviridae and genus Alphavirus. Like other alphaviruses, such as the related Venezuelan equine encephalitis (VEE) and western equine encephalitis (WEE) viruses, EEE viruses are mosquito transmitted and circulate through a natural reservoir; humans are not part of the natural host transmission cycle.

The disease was first recognized in equines and subsequently in humans in the 1930s. In humans, EEE viruses cause an acute febrile disease with encephalitis that has a high mortality rate ($\sim30\%$); a high percentage of survivors (as high as 70% in some outbreaks) have long-term neurological sequelae [1–4]. Fortunately, only a small number of cases ($\sim200$) have been reported since 1964. Clinical symptoms are not specific, and diagnosis relies on serological findings or isolation of virus from the central nervous system, although imaging of the brain through computed tomographic or magnetic resonance imaging scans have been shown to aid the diagnosis. Currently, however, there are no licensed antiviral drugs with efficacy against EEE infection, and treatment relies on symptom management [4]. A vaccine does exist; however, it is not licensed for human use and is currently given only to at-risk laboratory personnel as an investigational new drug.

The incidence of laboratory-acquired infections caused by VEE and WEE viruses led to the realization early on that alphaviruses are highly infectious by the aerosol route [5–10]. The most “desirable” trait of a biological weapon is the ability to infect by the aerosol route; in addition to being infectious by aerosol, VEE, WEE, and EEE possess a number of other properties considered desirable in biological weapons. VEE viruses...
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eastern equine encephalitis virus, EEE, aerosol infection, neurology, leukocytosis, animal model, laboratory animas, nonhuman primates
were studied as potential weapons by both the United States (before 1969) and the former Soviet Union. Because of these properties and prior history, there is concern that VEE, WEE, or EEE viruses could be relatively easily developed and deployed as a biological weapon.

In the past, both cynomolgus and rhesus macaques have been used after either subcutaneous or intranasal inoculation of alphaviruses [11–16]. We previously showed that cynomolgus macaques are susceptible to aerosol infection by epizootic and enzootic strains of VEE virus and WEE virus. The disease resembles what has been reported for humans [17–19]. We report here that cynomolgus macaques are also suitable as a model for aerosol exposure to EEE viruses.

MATERIALS AND METHODS

**Animals.** 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) were screened by plaque-reduction neutralization test and ELISA for previous exposure to VEE, WEE, and EEE viruses before assignment to these studies. This study was reviewed and approved by USAMRIID’s Laboratory Animal Care and Use Committee. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

**Surgical implantation of telemetry devices.** A radiotelemetry device (Data Sciences International) to monitor body temperature, heart rate, blood pressure, and activity was surgically implanted into macaques at least 24 days before exposure. To implant the device, a 10-cm paramedian incision was made over the left abdomen, exposing the abdominal musculature. A pocket was formed between the external and internal abdominal oblique muscle layers. A 4-cm incision was made in the left inguinal area exposing the femoral artery. Two fenestrations were created through the external abdominal oblique; the arterial catheter and the electrocardiogram (ECG) lead pair were routed through these openings, and the telemetry body was placed into the pocket between the muscle layers. With a trocar, the arterial catheter was routed through the subcutaneous space to the inguinal incision. With an 18-gauge needle, the arterial catheter was introduced into the femoral artery and secured with silk sutures. A 2-cm incision was made between the ninth and 10th ribs on the left side of the thorax. The ECG lead pair was routed through the subcutaneous space to the thoracic incision. A 2-cm incision was made over the right pectoral muscle just lateral to the right nipple. The silver ECG lead was routed to the pectoral incision. The ECG leads were trimmed to length and tied with a single cerclage suture to form terminal loops at the ends of the leads (2-0 Silk). The remaining slack in catheter and ECG leads was placed into the abdominal pocket and the external abdominal oblique was closed in a simple interrupted pattern (2-0 PDS). All skin incisions were closed in a subcuticular pattern (3-0 Vicryl). One macaque lost its implant 1 week before the study was initiated; the device was replaced with a device that monitored only temperature and activity (Data Sciences International).

**Virus.** EEE virus strain FL91-4679 was originally isolated from a mosquito in Florida [20]. The virus was passaged in cultured *Aedes albopictus* cells followed by 3 passages in Vero cells and 3 passages in baby hamster kidney cells. For aerosol exposures, virus was diluted to an appropriate concentration in Hank’s buffered saline solution (HBSS) containing 1% fetal bovine serum.

**Aerosol exposures.** Immediately before aerosol exposures,
macaques were anesthetized by intramuscular injection of tiletamine/zolazepam (6 mg/kg) and a whole-body plethysmograph was performed (Buxco Research Systems) to determine the animal’s respiratory capacity. The macaque was then inserted into a class III biological safety cabinet located inside a biosafety level 3 suite and exposed in a head-only aerosol chamber for 10 min to an aerosol created by a 3-jet Collison nebulizer (BGI). HBSS containing 1% fetal calf serum and 0.001% antifoam A was used as the collection medium in the all-glass impinger (AGI). Virus concentration in starting solutions and AGI was determined by plaque assay. Determination of presented dose was calculated using respiratory minute volume \((V_m)\) from the plethysmograph. Presented dose was calculated by multiplying the total volume \((V_t)\) of experimental atmosphere inhaled \((V_t = V_m \times \text{length of exposure})\) by the aerosol concentration \((C_e)\) (“presented dose” = \(C_e \times V_t\)).

**Postexposure monitoring.** Macaques were observed daily for 3 days preexposure and at least twice daily for 14 days after aerosol exposure to EEE virus. Animals were scored for neurological signs indicating infection according to the following score system: 1 = normal, 2 = loss of balance/muscle control, 3 = occasional tremors/seizures, 4 = frequent tremors/seizures,
and 5 = comatose/moribund. Animals were also scored for changes in activity (1 = normal, 2 = active, 3 = slow active, 4 = sluggish, and 5 = inactive), behavior (1 = normal, 2 = antisocial, 3 = depressed, 4 = hunched with back to observer, and 5 = ignoring everything), and response to stimuli (1 = normal, 2 = enter room, 3 = approach cage, 4 = rattle cage, and 5 = pinch). Observers were blinded and were not aware of which animals belonged in the 2 dose groups. The clinical score for each animal was recorded as the sum of those 4 criteria. Animals that were either comatose or moribund (defined as a combined score of ≥15) were euthanized promptly by a barbiturate overdose.

**Virologic and clinical laboratory determinations.** Beginning 3 days before exposure and continuing until day 10 after exposure, macaques were anesthetized with tiletamine/zolazepam (3 mg/kg; intramuscularly), and blood samples from the femoral vein were collected to assess complete blood counts (CBCs) and viremia. Throat swabs were taken during blood collection for virus isolation. Viremia in serum samples and throat swabs was measured using standard plaque assay methodologies in Vero cells [17]. Blood cell counts were determined using a Coulter ACT-Diff instrument and a manual differential count. Serum chemistries were measured using a VITROS 250 (Ortho-Clinical Diagnostics).

**Telemetry data analysis.** Body temperature, heart rate, and blood pressure were recorded every 15 min by the DataQuest A.R.T. 2.3 system (Data Sciences International). Monitoring began 14 days before exposure to develop a baseline period to fit an autoregressive integrated moving average model [18]. Forecasted values for the postexposure period were based on the baseline extrapolated forward in time using NCSS software (version 2004; Number Cruncher Statistical Systems). Residual changes were determined by subtracting the predicted value from the actual value recorded for each point. For body temperature, residual changes >3 SDs were used to compute fever duration (number of hours of significant temperature elevation), fever hours (sum of the significant temperature elevations), and average fever elevation (fever hours divided by the fever duration in hours).

**Statistical analysis.** Before analysis, log10 transformations were applied to inhaled dose values. For the CBC and telemetry data, the following observations for each animal were used in the analysis: last recorded observation before euthanasia or the end of the study, maximum recorded observation, and minimum recorded observation. CBC and telemetry values as well as log10 inhaled dose values met assumptions of normality and homogeneity of variance. Mixed-model analysis of variance (ANOVA) was used to compare titers between survival outcome groups with log10 inhaled dose as a covariate. Analyses were conducted using SAS (version 9.1.3; SAS Institute; SAS Online-Doc [version 9]).

**RESULTS**

**Lethality of aerosolized EEE in cynomolgus macaques.** A study was conducted to examine the disease course of aerosolized EEE in cynomolgus macaques at 2 different doses (3.65 × 10^6 pfu for the lower-dose group and 1.27 × 10^7 pfu for the higher-dose group). Based on preliminary studies that demonstrated the lethality of EEE in cynomolgus macaques (data not shown), we expected half the animals to succumb to the disease in the lower-dose group, whereas the higher-dose group represented a suitable challenge dose for vaccine studies. Four days after exposure the first clinical signs were noted; 4 of the macaques in the high-dose group began developing clinical signs of illness as well as slight tremors (figure 1). One day later (day 5) those 4 macaques were unresponsive to stimulation and had frequent tremors; 1 had a body temperature <32°C. All 4 macaques were promptly euthanized. Both remaining macaques in the high-dose group began developing signs of encephalitis on day 5, were found comatose on day 6, and were promptly euthanized. Two macaques in the low challenge dose group developed signs of encephalitis and illness, one on day 6 and the other on day 8; in both cases, the macaques were found comatose the following day (days 7 and 9, respectively) and were promptly euthanized. Necropsy findings will be reported in a separate manuscript (J.L.R., unpublished data). The remaining 4 of 6 macaques in the lower-dose group survived.

### Table 1. Summary of fever data in cynomolgus macaques.

<table>
<thead>
<tr>
<th>Inhaled dose</th>
<th>No. of survivors/total no. of macaques</th>
<th>Onset</th>
<th>ΔTmax, °C</th>
<th>Duration, days</th>
<th>Fever hours</th>
<th>Average elevation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.65 × 10^6 pfu</td>
<td>4/6</td>
<td>7.5</td>
<td>1.7</td>
<td>23.7</td>
<td>0.5</td>
<td>106.2</td>
</tr>
<tr>
<td>1.27 × 10^7 pfu</td>
<td>0/6</td>
<td>3.5</td>
<td>3.8</td>
<td>48.8</td>
<td>2.2</td>
<td>105.9</td>
</tr>
</tbody>
</table>

**NOTE.** ΔTmax, maximum change in temperature.

- a Defined as the first day with 8 or more hours of significant temperature elevation (as determined by autoregressive integrated moving average modeling).
- b Calculated as the no. of days with 12 or more hours of significant temperature elevation.
- c Calculated as the sum of the significant temperature elevations.
- d Calculated by dividing fever hours by the fever duration in hours.
challenge with little or no external signs of disease, although 1 survivor did have slight tremors on day 5.

Fever response. Radiotelemetry devices implanted in the macaques allowed monitoring of body temperature, heart rate, and blood pressure after exposure. Telemetry data comparing actual and predicted body temperature and heart rate from 1 macaque in the high-challenge dose exposure group are shown in figure 2A and 2B. The first signs of fever were noticeable toward the end of the day 2 after exposure, while elevations in heart rate and blood pressure first become apparent a day later. The heart rate in this animal peaked at around 240 beats per minute. Body temperature began to drop precipitously between days 4 and 5, reaching a low of 27°C by the morning of the fifth day. Remarkably, heart rate remained elevated, while body temperature dropped, only dropping back to near normal on the fifth day. No significant changes in blood pressure were seen until late in the infection (data not shown).

The averaged daily residual body temperatures and heart rates after exposure for each of the macaques are shown in figure 2C–2F and table 1. In the high-dose group, 5 of the 6 macaques had a 1°C–2°C elevation in temperature on day 3 after exposure (figure 2C). On day 4, all the high-dose macaques had elevated temperatures between 1.5°C and 3°C above normal. Body temperatures began to decline by day 5, when 4 of the high-dose macaques were found moribund and were euthanized. Body temperatures in the remaining 2 macaques in the high-dose group had begun to drop on day 6, when both animals were found comatose and were euthanized. Elevations in heart rate were seen in all 6 macaques but lagged the fever response by 1 day (figure 2D). In some of the high-dose macaques, heart rate had declined to normal by the time the animals were euthanized, whereas in others, the heart rate remained elevated.

In the low-dose group, only 2 of the 6 macaques showed significant temperature changes; both animals were moribund within 36 h of fever onset, although it was delayed by 3–4 days, compared with that of the high-dose group (figure 2E). There were few changes in heart rate after exposure in the low-dose group, even in the 2 macaques that developed fever and were ultimately euthanized (figure 2F). The differences in fever response and heart rate changes between the low- and high-dose groups suggests that, at a lower dose, the pathogenesis of EEE virus infection may be different, although the number of animals was too small to be certain.

Blood samples and throat swabs found evidence of viremia in both groups after exposure (figure 3). Although in 1 animal, serum virus titers reached as high as 10⁵ pfu/mL, the viremia in most of the macaques was transient or undetectable, lasting
Figure 4. Changes in peripheral blood leukocyte populations after exposure to eastern equine encephalitis (EEE) virus. Macaques were bled daily beginning 3 days before exposure through day 10 after exposure to assess changes in peripheral blood leukocytes. Graphs show the no. of cells per microliter ($\times 10^3$) on the y axis for individual macaques on each day after exposure for the low (left panels) and high (right panels) EEE virus challenge dose groups. Graphs are shown for total white blood cells (A), lymphocytes (B), monocytes (C), and granulocytes (D).

an average of 1.5 days in the high-dose group and 1.3 days in the low-dose group. Levels of virus found in the throat and blood were quite variable, were not related to the inhaled dose of virus, and did not predict outcome. Five of the 8 animals that succumbed to the exposure did not have detectable virus in their throats, whereas 3 of the macaques that survived did. Two of the macaques in the low-dose group that were viremic did develop a fever after exposure but survived; at 28 days after exposure, both animals were seropositive for antibody to EEE virus. The 2 macaques in the low-dose group that did not become viremic also did not develop fevers and, 28 days after exposure, were negative for antibody to EEE virus, suggesting
Table 2. Statistically significant differences in clinical parameters between survivors and nonsurvivors.

<table>
<thead>
<tr>
<th>Clinical parameter, outcome*</th>
<th>Last recorded</th>
<th>Maximum</th>
<th>Minimum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>P</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>ALKP, IU/L</td>
<td>.0133b</td>
<td>.0238b</td>
<td>.5832</td>
</tr>
<tr>
<td>Euthanized (n = 8)</td>
<td>177.38 (49.35)</td>
<td>71.63 (29.71)</td>
<td></td>
</tr>
<tr>
<td>Alive (n = 4)</td>
<td>72.75 (12.34)</td>
<td>68.25 (15.78)</td>
<td></td>
</tr>
<tr>
<td>BUN, mg/dL</td>
<td>.9091</td>
<td>.0437b</td>
<td>.0685</td>
</tr>
<tr>
<td>Euthanized (n = 8)</td>
<td>37.63 (11.89)</td>
<td>11.50 (1.60)</td>
<td></td>
</tr>
<tr>
<td>Alive (n = 4)</td>
<td>13.00 (1.41)</td>
<td>10.75 (0.90)</td>
<td></td>
</tr>
<tr>
<td>Granulocytes, cells/μL</td>
<td>.0137b</td>
<td>.2882</td>
<td></td>
</tr>
<tr>
<td>Euthanized (n = 8)</td>
<td>29.06 (9.65)</td>
<td>3.39 (0.88)</td>
<td></td>
</tr>
<tr>
<td>Alive (n = 4)</td>
<td>3.88 (1.80)</td>
<td>3.15 (1.31)</td>
<td></td>
</tr>
<tr>
<td>WBC, cells/μL</td>
<td>.0158b</td>
<td>.1350</td>
<td></td>
</tr>
<tr>
<td>Euthanized (n = 8)</td>
<td>32.31 (10.30)</td>
<td>5.45 (0.95)</td>
<td></td>
</tr>
<tr>
<td>Alive (n = 4)</td>
<td>7.68 (2.17)</td>
<td>5.98 (2.12)</td>
<td></td>
</tr>
<tr>
<td>Heart rate change, bpm</td>
<td>.8383</td>
<td>.3122</td>
<td>.6705</td>
</tr>
<tr>
<td>Euthanized (n = 8)</td>
<td>50.44 (48.13)</td>
<td>5.72 (2.07)</td>
<td></td>
</tr>
<tr>
<td>Alive (n = 4)</td>
<td>8.80 (8.63)</td>
<td>4.47 (2.27)</td>
<td></td>
</tr>
<tr>
<td>Temperature elevation, °C</td>
<td>.9502</td>
<td>.0016b</td>
<td>.6048</td>
</tr>
<tr>
<td>Euthanized (n = 8)</td>
<td>0.34 (2.21)</td>
<td>1.06 (1.63)</td>
<td></td>
</tr>
<tr>
<td>Alive (n = 4)</td>
<td>0.15 (0.19)</td>
<td>0.44 (0.10)</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** ALKP, alkaline phosphatase; bpm, beats per minute; BUN, blood urea nitrogen; WBC, white blood cell.

* Moribund animals were euthanized.
  b Statistically significant differences between survivors and nonsurvivors (P<.05).

that a productive infection was not established in those 2 animals.

**Hematological changes after exposure.** Hematological changes were also assessed daily after exposure and compared with preexposure values for each animal. Unlike the prominent lymphopenia typically seen early after infection with VEE viruses, there was very little change in WBC count the first few days after exposure (figure 4). Beginning on day 3, however, an elevation in WBC counts was seen in all the high-dose animals. Similar increases were seen 1–2 days later in the 2 animals in the lower dose group that succumbed to EEE. Increases in WBC counts corresponded with increases in body temperature. Further breakdown of the WBC into granulocytes, lymphocytes, and monocytes indicated that the increase in WBC counts was almost entirely due to increases in granulocytes. A WBC count >20 cells/μL and granulocyte count >15 cells/μL was always associated with a poor outcome (table 2). Monocyte counts also increased in sick animals; however, the maximum number of monocytes was <10% of that of granulocytes.

Serum chemistry was also assessed for any changes after exposure. In the high-dose group, changes in lactate dehydrogenase, aspartate aminotransferase, alkaline phosphatase (ALKP), blood urea nitrogen (BUN), and sodium levels were first noted around 4 days after exposure, the same day that animals in the high-dose group became febrile and elevated WBC counts were seen (figure 5). The 2 low-dose animals that succumbed to EEE did not show the same degree of elevation of these markers, except for an increase in ALKP levels. Other markers analyzed (γ-glutamyl transpeptidase, creatinine, total bilirubin, glucose, and albumin) did not change appreciably over the course of the infection (data not shown).

**Correlation of changes in clinical parameters with outcome.** Analysis of the CBC and clinical chemistry results by ANOVA in SAS found significant differences in the maximum recorded observations for WBC counts (P = .0098), granulocytes (P = .0125), ALKP (P = .0238), and BUN (P = .0437) levels between macaques that survived exposure to EEE and those that did not (table 2). Significant differences were also found in the maximum temperature elevation (P = .0016) between survivors and nonsurvivors. Changes in heart rate, however, were not significantly different between survivors and nonsurvivors (P = .3122). Monitoring these clinical signs should aid in predicting the outcome of EEE infection in macaques.

**DISCUSSION**

We report here that aerosol exposure to EEE virus causes lethal encephalitis in cynomolgus macaques. Given that the mortality rate of confirmed symptomatic cases in humans is ~30% and the considerable number of survivors reporting long-term neurological sequelae, the severity and outcome of the encephalitis
Figure 5. Elevated liver enzymes and sodium levels in the serum samples of macaques exposed to aerosolized eastern equine encephalitis virus. Macaques were bled daily beginning 3 days before exposure through 10 days after exposure to assess changes in serum chemistry. Graphs show the levels of lactate dehydrogenase (LDH) (A), aspartate aminotransferase (AST) (B), sodium (C), blood urea nitrogen (BUN) (D), and alkaline phosphatase (ALKP) (E) in serum samples from macaques in the low-dose (left panels) and high-dose (right panels) groups for 10 days after exposure.
caused by EEE virus in macaques was not surprising. Two of the animals that survived did develop viremia and a mild febrile response, one with mild neurological signs. This corresponds with what has been reported in human outbreaks—that some individuals may only develop mild illness, while the remainder develop severe encephalitis with potentially life-threatening consequences. This study also demonstrated that indeed EEE virus is infectious by aerosol and that the clinical signs and outcome are similar to mosquito transmission.

Rodent models are also available for studying EEE infection. Although adult mice are resistant to subcutaneous infection with EEE, they are susceptible to aerosolized EEE virus [21]. Hamsters have also been established as a model for subcutaneous infection with EEE, and it has been proposed that they are a better model of the human disease than mice because they are more prone to development of vasculitis than mice [22]; results from aerosol exposure have not been reported. Results from the pathological examination of the macaques that succumbed to EEE infection will be reported in a separate manuscript (J.L.R., unpublished data).

Similar to what we previously reported with WEE virus, a prominent leukocytosis was observed in animals that developed severe disease. Although, in the previous study, we were not able to definitively associate leukocytosis with a poor prognosis, in this study, a WBC count >20,000 cells/μL was seen in all the macaques that progressed to a comatose state and/or other signs of severe encephalitis. The cause of this increase in granulocytes and the role that this increase plays in the outcome of EEE virus infection is not clear at this time.

In this study, a prominent rise was seen in liver enzymes and other serum markers associated with liver damage, an unexpected finding. Moderate levels of virus were isolated from the livers of necropsied macaques, confirming infection in the liver. Changes in ALKP and BUN levels could be used as a predictor of outcome in macaques, but it is not clear whether this would hold true for humans, because there are no reports of changes (or the lack thereof) in human patients. Sodium levels were elevated in macaques that succumbed to the infection; this stands at odds with the one report of a human case of EEE in which sodium levels were examined and were found to be significantly lower than normal [3]. As with the increase in granulocytes, the cause or effect of these changes in EEE infection is not clear at this time. They may serve as helpful diagnostic indicators given the observation that viremia may be very low or not present in the serum at the onset of clinical signs.

Compared with our previous results with WEE virus, the disease course from time of exposure to illness and then to final outcome was very rapid. This agrees with the previous reports from the 1930s looking at intranasal infection of macaques with EEE and WEE viruses [16]. There are no reported cases of human exposure to aerosolized EEE virus for comparison; however, in both rhesus and cynomolgus macaques, the disease course and severity of clinical signs for the encephalitic alphaviruses (VEE virus, WEE virus, and EEE virus) has in general resembled that of the human infection quite closely [11, 13, 17–19]. In addition to demonstrating that cynomolgus macaques are a suitable model for aerosol exposure to EEE virus and vaccine efficacy studies, this study has provided criteria that would be suitable for earlier end points in nonhuman primate studies with EEE and potential areas for future research in understanding the lethality of EEE infection.

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