Aerosol Infection of Cynomolgus Macaques with Enzootic Strains of Venezuelan Equine Encephalitis Viruses

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Because Venezuelan equine encephalitis viruses (VEEVs) are infectious by aerosol, they are considered to be a biological-weapons threat. Nonhuman-primate models are needed to evaluate the efficacy of candidate vaccines. In the present study, cynomolgus macaques, after aerosol exposure to either VEEV-IE or VEEV-IIIa, developed fever, viremia, and lymphopenia; the severity of the fever response, viremia, and lymphopenia correlated with the inhaled dose of VEEV. Of the 10 macaques in our study, 7 developed clinical signs indicative of encephalitis, including loss of balance and hypothermia. In the macaque, the enzootic strains used are infectious by aerosol and lead to disease, including clinical encephalitis.

Venezuelan equine encephalitis viruses (VEEVs) are a group of family, Togaviridae; genus, Alphavirus) of small, related, positive-stranded RNA viruses endemic to Central and South America. Six subtypes containing 9 varieties of VEEVs have been identified by serology; most of them are enzootic and are thought to circulate through rodent hosts by a mosquito vector [1]. Widespread equine and human outbreaks have occasionally emerged, most of which have been caused by the IA/B and IC strains [2]. Isolated cases of human and equine disease caused by infection with the enzootic strains of other varieties and subtypes have also been reported [3, 4]. Typical human symptoms or signs of VEEV infection include a rapid onset of fever, headache, lymphopenia, muscle pain, malaise, and neck stiffness [5]. Although the disease caused by infection with VEEV is incapacitating, such infections are rarely fatal in humans.

VEEVs have also been found to be highly infectious by aerosol and are considered to be potential biological weapons [6]. In the case of the epizootic strains of VEEV, the illness that results from aerosol exposure is similar to that caused by transmission by mosquito [5]. After a mosquito bite, VEEVs circulate through the blood and eventually infect the olfactory bulbs, leading to infection of the brain. In animal models, surgical removal of the olfactory bulbs prevents or delays infection of the brain [7]. In the case of aerosol exposure to VEEV, direct infection of an animal’s olfactory region bypasses the requirement for viremia [8].

Laboratory accidents and animal studies have shown that the enzootic strains of VEEV can cause disease after aerosol exposure [3, 9, 10]. The severity of illness and symptoms as a result of aerosol exposure to the enzootic strains of VEEV appears to be comparable to the illness and symptoms caused by infection with the epizootic strains. Cases have also occurred in individuals vaccinated against the epizootic VEEV-IA/B strains, highlighting the need for vaccine candidates that could protect against both the epizootic and the enzootic strains of VEEV [3, 9].

The illness caused by the enzootic strains of VEEV has not been extensively studied in animal models, especially in nonhuman primates. Monath et al. [11] compared the illness caused by 3 epizootic and 2 enzootic strains injected subcutaneously in nonhuman primates. Virus was detected in the blood of rhesus macaques experimentally infected with the enzootic ID and IE strains; however, these macaques did not mount a febrile response, as detected by rectal thermometer. Also, although the macaques infected with the enzootic ID and IE strains were leukopenic, the duration of leukopenia was shorter than that seen with the epizootic strains. In another study, macaques injected subcutaneously with VEEV-IIIa failed to develop a fever, whereas a single macaque infected by intracranial inoculation developed fever and signs of encephalitis [12]. The conclusion from these studies was that the enzootic strains are less pathogenic than the epizootic strains, at least by subcutaneous inoculation that mimicked a mosquito bite; whether this would hold true for aerosol exposure was not examined. To evaluate the efficacy of candidate vaccines against aerosol exposure to the enzootic strains of VEEV, a nonhuman primate model was needed. Viruses from 2 varieties were chosen, on
the basis of both genetic divergence from the epizootic IA/B strains and prior evidence that they cause illness in humans. Within the subtype I VEEVs, the IE strains are the most genetically divergent from the IA/B strains [13]. VEEV-IIIA strains are also genetically divergent from VEEV-IA/B strains and, as with IE strains, there have been isolated reports of accidental laboratory exposure, even in individuals vaccinated against the epizootic IA/B strains [9, 10].

**Materials and methods.** Healthy, adult cynomolgus macaques (Macaca fascicularis) of both sexes were obtained from the US Army Medical Research Institute of Infectious Diseases primate colony. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and to experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council) [14]. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Before assigning the macaques to study groups, we obtained blood samples and, using plaque-reduction neutralization and ELISA, screened them for previous exposure to VEEV-IA/B, VEEV-IE, VEEV-IIIA, western equine encephalitis viruses, and eastern equine encephalitis viruses. To monitor temperature, telemetry implants (Data Sciences International) were implanted subcutaneously on the dorsal surface, and the macaques were allowed 30 days to recover from surgery and to acclimate; this time was also used to acquire, prior to exposing the macaques to VEEV, sufficient telemetry data for baseline modeling of temperature data.

The viruses that we used were 68U201 (VEEV-IE), first isolated in 1968, from the brain of a sentinel hamster in Guatemala [15]; and Mucambo (VEEV-IIIA), first isolated in 1957, from a nonhuman primate in Brazil [9]. Both were recovered from suckling mouse–brain suspensions and were passaged twice in BHK cells before use. For aerosol exposures, viruses were diluted to an appropriate concentration in Hanks’ buffered saline solution containing 1% fetal bovine serum.

For aerosol exposure, macaques were anesthetized by intramuscular injection of Telazol (6 mg/kg). Immediately before aerosol exposure, whole-body plethysmography for 3 min was performed on each macaque, to determine its respiratory capacity. Subsequently, the macaque was inserted into a class III biological-safety cabinet located inside a biosafety level-3 suite and, in a head-only aerosol chamber, was exposed for 10 min to a VEEV aerosol created by a collison nebulizer, as described elsewhere [16]. After exposure, the macaque remained in the chamber for an additional 5 min, with clean air. Samples were collected from the all-glass impinger attached to the aerosol chamber and were analyzed, by a plaque assay, to determine the inhaled dose of VEEV. The macaques were exposed in a staircase-type fashion (at the doses shown in the Results section below), to determine the median effective dose that causes illness.

Beginning 3 days before exposure and continuing for as long as 10 days after exposure, the macaques were anesthetized by use of Telazol (3 mg/kg), and blood samples were collected to assess lymphopenia and viremia. Viremia was measured by a plaque assay, by standard methods, in Vero cells [16]. Blood-cell counts were determined by use of a Coulter T-series machine and a manual differential count.

Body temperatures were recorded every 30 min by use of the DataQuest A.R.T. 2.1 System (Data Sciences International). Monitoring of temperature began 10–14 days before exposure, to develop a baseline period of temperature data to fit an autoregressive integrated moving-average model [16]. Forecasted values for the time periods after exposure were based on the training model extrapolated forward in time. Residual temperature changes after exposure were determined by subtracting the predicted temperature from the actual temperature recorded for each point. Residual temperature changes >3 SD above the temperature during the training period were used to compute fever duration (i.e., no. of hours of significant temperature elevation) and fever-hours (i.e., sum of significant temperature elevations).

**Results.** To develop a nonhuman-primate model of aerosol exposure to the enzootic strains of VEEV, cynomolgus macaques were implanted with telemetry devices to monitor temperature response after such exposure. At least 30 days after implantation of the telemetry devices, the macaques were exposed by aerosol to either VEEV-IE or VEEV-IIIA. Macaques exposed by aerosol to VEEV-IE developed a biphasic fever within the first day of exposure, with a second, smaller peak occurring some 1–2 days after the initial fever response (figure 1A). A different pattern of response was seen after aerosol exposure to VEEV-IIIA; even at the highest dose tested, onset of fever was not seen until >3 days after exposure, and the fever was not biphasic (figure 1B); furthermore, the peak of the fever did not occur until 3–4 days after onset, or ∼7 days after exposure.

Several of the macaques exposed to either VEEV-IE (3 of 5) or VEEV-IIIA (4 of 5) developed clinical signs of encephalitis during the postfebrile period. These signs included depression and loss of appetite (7 of 10) and problems with balance and muscle control (7 of 10). Of the 5 macaques exposed to VEEV-IIIA, 3 developed hypothermia and continued to display signs of depression and loss of appetite. All signs of encephalitis were relatively mild and tended to resolve at approximately the time when body temperatures returned to normal.

In addition, lymphopenia and viremia were assessed in the macaques during the course of the disease caused by VEEV-IE or VEEV-IIIA. Regardless of the strain of VEEV, viremia peaked
Figure 1. Development of fever, viremia, and lymphopenia after aerosol exposure of macaques to enzootic strains of Venezuelan equine encephalitis virus (VEEV). The graphs show average body-temperature elevation, percent change in peripheral blood lymphocytes (PBL), and viremia in macaques that developed significant fevers after aerosol exposure to either $7 \times 10^3$ pfu of VEEV-IE (A) or $3.3 \times 10^6$ pfu of VEEV-III A (B). Temperature data are the average residual temperature elevation for each day after exposure. Changes in lymphocyte counts after exposure were calculated as the percent change from the baseline, which was considered to be the average count of samples taken on each of the 3 days before challenge.
at 2 days after exposure (figure 1). Both the duration and the peak of the viremia were comparable between the group of macaques exposed to VEEV-IE and the group exposed to VEEV-IIIA, when the groups were compared at similar doses (table 1); however, in macaques exposed to VEEV-IE, peak viremia corresponded with the time at the onset of fever (figure 1A), whereas, in macaques exposed to VEEV-IIIA, virus was no longer detectable in the blood at the time at the onset of fever (figure 1B).

Regardless of whether the macaques were exposed to VEEV-IE or to VEEV-IIIA, lymphocyte counts dropped >30% within the first 24 h after the exposure (figure 1A and 1B). The duration of lymphopenia was fairly comparable between the group of macaques exposed to VEEV-IE and the group exposed to VEEV-IIIA, when the groups were compared at similar doses (table 1); however, the degree of lymphopenia appeared to be more severe for macaques exposed to VEEV-IE than for those exposed to VEEV-IIIA (table 1).

Discussion. We have reported here the development of nonhuman-primate models of aerosol exposure to 2 enzootic strains of VEEV. Depending on the inhaled dose, primates developed signs of an active infection—including fever, viremia, lymphopenia, and clinical encephalitis—that are consistent with what have been reported in human cases of VEEV [5].

In a single earlier report of exposure of macaques to VEEV-IE, rhesus macaques developed significant viremia after subcutaneous injection of a different enzootic VEEV-IE strain (Mena II) but did not develop fever [11]. These results contrast with the data reported in the present study, which showed that, after aerosol exposure to VEEV-IE, macaques were only slightly viremic but did develop a fever. The fever at higher aerosol doses of VEEV-IE is similar to that reported in macaques exposed by aerosol to the epizootic VEEV-IA/B strains [16].

After aerosol exposure to VEEV-IIIA, the febrile response and signs of encephalitis in the macaques that we studied resembled those which a previous study had observed in a macaque after intracerebral injection of VEEV-IIIA [12]. For VEEV-IIIA, there appeared to be a significant delay in the onset of fever, which occurred at the time when the virus disappeared from the blood and the lymphopenia started to resolve. However, because of the small number of macaques used in the present study, it is not clear whether this delay was significantly longer than that seen for other strains of VEEV.

Disease in humans after exposure to the enzootic strains of VEEV is rare and has been confined to small outbreaks and laboratory accidents. What data exist suggest that, regardless of route, human illness after exposure to the enzootic strains can be as severe as that reported for the epizootic strains. The data reported here do indicate that the enzootic strains of VEEV can be infectious by aerosol in the macaque and that they can lead to clinical encephalitis. The disease that follows aerosol exposure of the macaque to either of the 2 enzootic strains

### Table 1. Summary of clinical responses in macaques after aerosol exposure to enzootic strains of Venezuelan equine encephalitis virus (VEEV).

<table>
<thead>
<tr>
<th>Virus, inhaled dose, pfu</th>
<th>Fever</th>
<th>Viremia</th>
<th>Lymphopenia</th>
</tr>
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<tr>
<td></td>
<td>$T_{max}$, ºC</td>
<td>Duration, h</td>
<td>Fever-hours</td>
</tr>
<tr>
<td>VEEV-IE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$6.7 \times 10^4$</td>
<td>3.8</td>
<td>18</td>
<td>35.6</td>
</tr>
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<td>$1.0 \times 10^5$</td>
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<td>38.5</td>
<td>55.6</td>
</tr>
<tr>
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<td>3.0</td>
<td>17.5</td>
<td>28.9</td>
</tr>
<tr>
<td>$7.0 \times 10^7$</td>
<td>4.5</td>
<td>111</td>
<td>238.4</td>
</tr>
<tr>
<td>VEEV-IIIA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1.00 \times 10^4$</td>
<td>1.3</td>
<td>3</td>
<td>3.2</td>
</tr>
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<td>4.1</td>
<td>118</td>
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</tr>
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</table>

**NOTE.** $T_{max}$, maximum residual elevated temperature.

$^a$ Inhaled dose was determined by plethysmograph of the macaque and by use of plaque assays of collison-nebulizer and all-glass-impinger contents.

$^b$ Fever-hours was calculated as the sum of the significant temperature elevations.

$^c$ Percent change in lymphocyte counts was calculated by subtracting the baseline no. of lymphocytes per milliliter of blood (determined in bleedings before exposure) from the no. of lymphocytes per milliliter of blood each day after exposure, then dividing by the baseline number and multiplying by 100. Values shown are the average for all 6 days after exposure.
used in the present study resembles that reported for human exposures to VEEV, making the cynomolgus macaque a useful model for vaccine-efficacy studies.

Acknowledgments

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