PATHOGENESIS OF WEST NILE VIRUS INFECTION IN DOGS TREATED WITH GLUCOCORTICOIDS

RICHARD A. BOWEN,* MELISSA M. ROUGE, LEONARDO SIGER, JULES M. MINKE, ROBERT NORDGREN, KEMAL KARACA, AND JEREMY JOHNSON

Department of Biomedical Sciences and Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado; Merial Limited, Athens, Georgia; Merial SAS, Lyon, France.

Abstract. Control and glucocorticoid-treated dogs were infected with West Nile virus (WNV) through the bites of infected mosquitoes to study the effect of a commonly used immunomodulator on the magnitude and duration of viremia and on development of clinical disease. All dogs became viremic after challenge. The peak viremia and integrated magnitude of viremia were approximately 40 and 50 times higher, respectively, in the five dogs treated with methylprednisolone for 1 month compared with untreated dogs. None of the five control or treated dogs developed signs of clinical disease, nor was histopathologic evidence of neuroinvasion observed in any case. Neutralizing antibodies to WNV were produced in all dogs, with no apparent effect of glucocorticoid treatment. Considering the dramatic effect of glucocorticoid treatment on magnitude of viremia, it is likely that this therapy had suppressive effects on some aspect of innate immunity or T cell function.

INTRODUCTION

West Nile virus (WNV) has been shown to infect a broad range of mammalian, avian, and reptilian hosts, with substantial differences among species in both the magnitude of viremia and the clinical attack rate.1,2 In humans, a majority of WNV infections are asymptomatic or associated with a mild disease known as West Nile fever, and meningoencephalitis seems to develop in less than 1% of infected people.3,4 One factor that seems to enhance the development of severe disease after WNV infection in humans is immunosuppression, and several studies have reported West Nile encephalitis in immunocompromised patients undergoing organ transplants or cancer chemotherapy.5–12 Like humans, dogs are readily infected with WNV and develop a viremia of low magnitude.13–15 It also seems that most canine WNV infections are asymptomatic, based on serosurveys in endemic areas and experimental infections.13,16 However, a small number of naturally occurring clinical cases of canine WNV encephalitis have been described.17,18 Glucocorticoids are commonly used in both humans and dogs as anti-inflammatory and immuno-suppressive agents for a large array of conditions. In the study reported here, we evaluated the effect of glucocorticoid therapy on the course of WNV infection in dogs.

MATERIALS AND METHODS

Animals and mosquitoes. Ten female beagles, 5–6 months of age, were housed together, loose in a room within a BL3 biocontainment building, and examined at least twice daily for the duration of the study. They were fed commercial dog food and had free access to water at all times. None of the animals had detectable antibodies to WNV at the onset of the study as determined by plaque reduction neutralization.

Three-day-old, female Aedes albopictus mosquitoes were immobilized by chilling and inoculated intrathoracically with approximately 500 pfu of WNV (NY99-4132 isolate). Inoculated mosquitoes were given 10% sucrose for maintenance and incubated in an insectary for 8 days as described previously.14 On the afternoon before feeding on dogs, mosquitoes were transferred to cylindrical cartons with netting on both ends, denied sucrose solution, and held overnight with a pad moistened with water on their cages. Two or three mosquitoes that engorged on each dog were frozen, homogenized using a mixer mill (Retsch GmbH, Haan, Germany), and assayed for virus content by plaque assay.

Experimental design. Dogs were assigned to two groups based on a random number generator. Five were treated four times at weekly intervals with 10 mg/kg of methylprednisolone acetate (Depo-Medrol; Pharmacia-Upjohn Co., Kalama-zoo, MI) by intramuscular injection; the other five dogs were not treated. This dose of methylprednisolone was considered to be on the high end of what is used clinically in dogs. All 10 dogs were weighed weekly during the treatment period. One week after the last glucocorticoid treatment, all dogs were sedated with xylazine (5 mg intravenously) and challenged with WNV by allowing infected mosquitoes to feed on the medial aspect of their rear leg for 5–10 minutes. Blood was collected from all dogs into EDTA-containing tubes immediately before the first glucocorticoid treatment and at the time of WNV challenge, and cell counts were determined using a QBC hematology analyzer (Becton Dickinson Corp., Franklin Lakes, NJ). For determination of viremia and antibody response to infection, blood was collected twice daily from day 0 (day of challenge) through day 6, once daily from days 7 through 14, and on day 21. Body temperature was recorded at the same times as blood sampling. All dogs were killed 21 days after challenge. Brains were fixed in buffered formalin, and two areas of the brain stem were sectioned and examined for histopathological lesions by a board-certified veterinary pathologist.

Virus and antibody assays. Virus titers in homogenized mosquitoes and dog serum were determined by plaque assay in six-well plates of Vero cells, as described previously.19 For virus titration, 0.1-mL samples of serum diluted in BA-1 medium (MEM salts, 1% bovine serum albumin, 250 mg/L sodium bicarbonate, 50 μg/mL gentamicin, 1 μg/mL amphotericin B in 50 mmol/L Tris, pH 7.6) were assayed in duplicate. The initial dilution used was 1:1 (serum:BA-1) because of toxicity observed initially with undiluted serum. Plaques were counted on days 3–5 after inoculation, and titers were expressed as plaque-forming units (pfu) per milliliter or per
mosquito. Neutralizing antibody titers were determined using a plaque reduction neutralization test (PRNT) previously described.\textsuperscript{19} Dilutions of serum in BA-1 medium were mixed with an equal volume of WNV (NY99 strain) such that 0.1 mL of the mixture contained 100–150 pfu. The serum-virus mixtures were incubated at 4°C overnight and inoculated onto Vero cells as described for the plaque assay. Plaque counts were obtained 3 days after inoculation, and titers were determined as the reciprocal of the highest dilution showing 80% neutralization. Titers greater than 10 were considered positive.

**Statistical analyses.** Differences between treated and control groups in magnitude of viremia over time and body temperature were analyzed by repeated-measures ANOVA. Differences in other parameters were evaluated using paired t tests with original (number of engorged mosquitoes, hematologic parameters, body weight) or log<sub>10</sub>-transformed data (peak viremia and viremia area under the curve). Probability values less than 0.05 were considered significant.

**RESULTS**

Each of the 10 dogs remained generally healthy during the pre-challenge period, although the dogs treated with methylprednisolone showed clear signs of hyperadrenocorticism during the period, manifested primarily as abdominal enlargement. The mean body weight of the five control dogs increased 22% during the month before challenge compared with a 16% weight gain for the treated dogs (P > 0.1). At the time of virus challenge, control and treated dogs had similar total white blood cell counts (P > 0.5; means 16,560 and 15,600, respectively). However, the dogs treated with methylprednisolone had a significantly lower (P < 0.001) absolute number and percentage of mononuclear cells (means 1,960 and 13.2%) than control dogs (means 5,300 and 33.0%). Each dog was exposed to between three and six mosquitoes for a period of 5–10 minutes (Table 1). It seemed that essentially all of the mosquitoes probed to some extent. The number of mosquitoes that visibly engorged on each dog ranged from two to five and was not different between treated and control animals (means of 3.4 and 2.8; P > 0.5). Similarly, there was no difference in the mean number of mosquitoes exposed to each of the control and treated dogs (4.4 versus 3.6, respectively; P > 0.1) Three engorged mosquitoes from each of nine dogs and two mosquitoes from one dog (PV) were frozen immediately after feeding, homogenized, and assayed for virus content. All of the mosquitoes were found to be infected, with titers that ranged from 4.0 × 10<sup>7</sup> to 2.2 × 10<sup>9</sup> pfu/mosquito (mean 6.6 × 10<sup>8</sup> pfu).

Each of the 10 dogs became viremic after challenge (Table 1). The mean peak titer for the dogs treated with methylprednisolone was approximately 40 times that observed for the control dogs (57,680 ± 89,818 versus 1421 ± 1356 pfu/mL; P < 0.01). Additionally, mean virus titers integrated over the period of viremia was greater in treated compared with control dogs (150,532 versus 2,829 pfu; P < 0.01). Finally, the mean duration of viremia for methylprednisolone-treated dogs was greater than for untreated dogs (4.1 versus 2.9 days; P < 0.05). Viremia was not detected in any of the dogs 6 days after mosquito feeding or thereafter.

None of the 10 dogs showed clinical signs of disease in the 3 weeks after challenge. Similarly, body temperature did not differ between treated and control groups, and there were no significant differences during the course of infection. Histopathologic examination of brains collected 21 days after WNV challenge failed to reveal evidence of encephalitis or other neurologic disorders in any of the 10 dogs.

Each of the 10 dogs produced neutralizing antibodies to WNV within 3 weeks after virus challenge. There were no apparent differences (P > 0.1) in humoral immune responses between dogs in the methylprednisolone-treated and control groups (Table 2).

**DISCUSSION**

There is great variability among mammals and birds in the magnitude of viremia and in the clinical attack rate observed after infection with WNV. For humans, it has been estimated that approximately 1 in 150 individuals infected with WNV develop severe disease.\textsuperscript{3,4} Based on experimental studies and field observations, dogs develop a viremia of low magnitude and seem to show clinical signs of WNV infection only rarely.\textsuperscript{13–16} Differences in innate immunity among individuals within a given species or exposure to factors that modulate immunity likely contributes to individual differences in outcome of any virus infection.

In both human and veterinary medicine, glucocorticoids are among the most widely used drugs used to suppress autoimmune and inflammatory responses. A study of adults in Britain indicated that, at any given time, approximately 1% of all adult patients under care of a general practitioner were

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**Table 1**

Viremia in dogs after WNV challenge (log<sub>10</sub> pfu/mL serum)

<table>
<thead>
<tr>
<th>Treatment group</th>
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<th>3.5</th>
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<td>2.7</td>
<td>1.8</td>
<td>&lt;1.0</td>
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</table>

<sup>a</sup> Number of mosquitoes engorged/total number of mosquitoes exposed.
in both dogs and humans and widely used pharmacologically for that purpose. As expected, the dogs in this study that were treated with methylprednisolone had significantly lower absolute and relative mononuclear cell counts. Previous studies have indicated that glucocorticoids do not have major effects on humoral immune responses (i.e., to vaccines), and the antibody response to WNV infection in this study was not different between treated and control dogs. Considering the dramatic effect of glucocorticoid treatment on magnitude of viremia, it is likely that this therapy had suppressive effects on some aspect of innate immunity (i.e., synthesis of interferon-γ or other cytokines) or T cell function.

The magnitude of viremia observed in methylprednisolone-treated dogs was in some cases above the threshold expected to allow infection of feeding mosquitoes. It may be that dogs and other species such as horses, which do not normally serve as amplifying hosts for WNV, may play a role in the natural transmission of WNV if immunosuppressed or stressed, either naturally or pharmacologically. This type of situation would be of minimal epidemiologic significance in areas where other competent hosts are abundant (i.e., birds), but may be of potential importance for introduction of the virus into new locations.

The dogs used in this study were young, in excellent general health, and treated with glucocorticoids for a relatively short period of time, which may partially explain the lack of clinical disease observed in the face of a relatively high WNV viremia. In contrast, human and veterinary patients undergoing glucocorticoid therapy inevitably have one or more concurrent diseases, and often are chronically ill and are treated with glucocorticoids and other, more potent immunosuppressive drugs for prolonged periods of time. In such real-world patients, an effect of glucocorticoids on enhancement of viremia may contribute to significantly elevated risk of clinical disease.

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Authors’ addresses: Richard Bowen, Department of Biomedical Sciences, Colorado State University, Fort Collins, CO, E-mail: rbowen@colostate.edu. Melissa Rouge, Department of Biomedical Sciences, Colorado State University, Fort Collins, CO, E-mail: melissarouge@yahoo.com. Leonardo Siger, Merial Limited, Athens, GA, E-mail: Leonardo.Siger@Merial.com. Jules Minke, Merial SAS, Lyon, France, E-mail: Julius.Minke@Merial.com. Robert Nordgren, Merial Limited, Athens GA, E-mail: Bob.Nordgren@Merial.com. Kemal Karaca, Merial Limited, Athens GA, E-mail: Kemal.Karaca@Merial.com. Jeremy Johnson, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins CO, E-mail: jsjohnson9@yahoo.com.

Reprint requests: Richard Bowen, Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523. E-mail: rbowen@colostate.edu.

REFERENCES


### NOTE

#### Table 2

**Development of neutralizing antibodies to WNV after challenge**

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<th>Treatment group</th>
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being treated with oral glucocorticoids, and the highest frequency of use was in patients 70–79 years old. In addition to pharmaceutical use, many types of stress are associated with elevated secretion of endogenous glucocorticoids from the adrenal glands. Glucocorticoids in both natural or synthetic forms have diverse effects on immune responses, including influences on dendritic cell maturation and suppression of secretion of cytokines such as interleukin-1, interleukin-2, and tumor necrosis factor. Treatment with glucocorticoids would thus be expected to influence the course of viral infections, including that of WNV. Indeed, mice treated with corticosterone, the major glucocorticoid in rodents, and infected with WNV developed significantly higher viremia and mortality than controls.

Our results showed that a 1-month treatment of dogs with a glucocorticoid resulted in substantially higher viremia after infection with WNV in comparison with control dogs. Specifically, although each of the dogs in this study developed viremia after mosquito-mediated challenge, the mean peak viremia and integrated magnitude of viremia in glucocorticoid-treated dogs was roughly 40 times greater than in untreated dogs. In fact, ranges in peak viremia in control dogs versus treated dogs did not overlap. For unknown reasons, but possibly because of their younger age, the magnitude of viremia in the control dogs in this study was higher than seen in two previous studies from this laboratory.

Despite the significantly higher levels of viremia seen in glucocorticoid-treated dogs, none of these animals developed any sign of clinical disease, including fever. We cannot rule out the possibility that neuroinvasion took place in some of these animals, but histopathologic evaluation of two regions of the brain stem failed to detect any indication of encephalitis. Because of the lack of clinical disease and microscopic lesions in the brain stem, we did not conduct more extensive evaluations to detect virus or viral RNA in tissues. It may be that substantially higher levels of circulating WNV are necessary for any neuroinvasion. Although higher magnitude viremia per se is not known as a risk factor for neuroinvasion, these results support numerous clinical observations in humans that implicate immunosuppression as a risk factor for West Nile disease.

Dogs and humans are considered to represent glucocorticoid-resistant species, and their lymphocytes are more resistant to the lytic effects of high concentrations of glucocorticoids than those of species such as rodents and rabbits. Nonetheless, glucocorticoids are clearly immunosuppressive...


