EFFECTS OF IMMUNOSUPPRESSION ON WEST NILE VIRUS INFECTION IN HAMSTERS

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Abstract. A research study, comparing the pathogenesis of experimental West Nile virus (WNV) infection in immunocompetent and immunosuppressed golden hamsters, is described. Cyclophosphamide was used to immunosuppress the animals. The immunosuppressed hamsters had a prolonged period of viremia, depressed humoral immune response, more extensive and severe pathology, and higher fatality rate than the untreated immunocompetent animals. Histopathological and immunohistochemical studies of tissues from the two groups showed that pathologic changes in the untreated infected animals were confined to the brain and spinal cord, whereas the histopathological changes and WNV antigen distribution in the immunosuppressed animals were much more extensive and diffuse, involving the adrenal, kidney, heart and lung, and brain and spinal cord. Results of this study in the hamster model provide insight into the increased severity of WNV infection observed in immunosuppressed people.

INTRODUCTION

West Nile virus (WNV), a member of the Japanese encephalitis serogroup of the genus Flavivirus, family Flaviviridae, first appeared in the Western Hemisphere in 1999, during a meningoencephalitis epidemic in New York City. Since then, WNV has spread rapidly in North America, with a corresponding increase in the number of reported human cases. Based on retrospective serologic studies done in New York during 1999–2000, it was estimated that ~80% of human WNV infections were asymptomatic and that about 1 of every 150 human infections resulted in neuroinvasive disease. Among patients hospitalized with WNV neuroinvasive disease, the fatality/case ratio was 12%. Advanced age was identified as the most common risk factor for neuroinvasive disease and death; but more recent reports have indicated that immunosuppressed persons are also at high risk of developing severe neuroinvasive disease if they are infected with WNV.

To better understand the pathophysiology of WNV infection in immunosuppressed persons, a hamster model of WNV encephalitis was used. Adult golden hamsters were pretreated with and maintained on cyclophosphamide, an alkylating agent with broad immunosuppressive activity, and were infected with WNV. Their response was compared with that of immunocompetent (untreated) hamsters experimentally infected with the virus. Our findings show that immunosuppressed hamsters develop more severe disease and much more extensive pathology than untreated immunocompetent hamsters after challenge with WNV. We believe that this model could be used to study the pathogenesis and management of WNV infection in immunosuppressed people.

MATERIALS AND METHODS

Virus. WNV strain NY385-99, originally isolated from a dead bird at the Bronx Zoo in 1999 and passaged three times in Vero cells, was used to infect the animals. The virus dose was 10⁴ tissue culture infectious dose₅₀ units (TCID₅₀), given intraperitoneally.

Animals. Adult female golden hamsters (Mesocricetus auratus), 8–12 weeks of age, obtained from Harlan Sprague-Dawley, Indianapolis, IN, were used. The animals were cared for in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council) under an animal use protocol approved by the University of Texas Medical Branch. All work with the infected animals was carried out in ABSL-3 facilities.

Immunosuppression of animals. Cyclophosphamide (Cytoxan; Baxter Healthcare Corp., Princeton, NJ) was used as the regimen for immunosuppression. Forty-nine hamsters received three doses of cyclophosphamide (100 mg/kg, IP) over a 9-day interval to maintain immunosuppression. The first dose was given 5 days before infection; the second was given 1 day before infection; and the third dose was given 4 days after infection. Five additional hamsters were given cyclophosphamide on the same schedule, but they did not receive WNV. These five animals comprised Group 1 and served as a cyclophosphamide control; they were bled just before the first injection of cyclophosphamide (pretreatment) and again 3 and 6 days later to monitor the level of immunosuppression in hamsters receiving the drug. The level of immunosuppression was monitored by total white blood cell (WBC), neutrophil, and lymphocyte counts done on the serial blood samples, using a Hemavet HV950FS multispecies hematology analyzer (Drew Scientific, Oxford, CT), following the manufacturer’s instructions.

Experimental design. A total of 101 hamsters were used in this study. The animals were randomly assigned into three groups as follows: Group 1 (N = 5) received only cyclophosphamide; Group 2 (N = 49) received cyclophosphamide plus WNV; and Group 3 (N = 47) received only WNV. After inoculation of WNV, eight hamsters from Group 2 and seven animals from Group 3 were bled daily (200 µL from the retro-orbital vein) for 8 consecutive days to determine the level and duration of viremia and the antibody response. To compare the pathology in the immunosuppressed and immunocompetent hamsters, six animals (three each in Groups 2 and 3) were killed on the sixth and eighth days after infection. A random sample of 15 other moribund hamsters in the two groups were...
killed 8–11 days after infection. At the time of death, these animals were immediately perfused with 10% buffered formalin, as described before.\textsuperscript{13} After refrigeration overnight at 5°C, the entire brain and samples of spinal cord, liver, spleen, kidney, heart, lungs, and adrenals were collected from each animal and stored for an additional 24 hours in 10% formalin, before being transferred to 70% ethanol for storage until processing.

All hamsters were evaluated daily for clinical manifestations of disease; any deaths were recorded.

**Histologic evaluation of tissues.** After fixation, tissues samples were processed for routine paraffin embedding and sectioning. Tissues sections of 4 to 5 μm thickness were made and stained with hematoxylin and eosin (H&E) and evaluated microscopically; additional sections were prepared for immunoperoxidase staining by direct primary antibody labeling, as described previously.\textsuperscript{13} The primary antibody was a mouse hyperimmune ascitic fluid (MIAF), prepared against WNV, with a working dilution of 1:200. For assessment of relative staining intensity of various areas of the brain and spinal cord, a semi-quantitative immunohistochemical scale (IHC score) was used as follows: 0, no specific staining; 1, one or two foci of staining involving single or a few cells; 2, focal staining but involving more cells; 3, diffuse staining but still with discrete foci; and 4, diffuse staining involving the entire region examined (e.g., Figure 3E).

**Virus assay.** Blood from the infected hamsters was titrated by plaque assay in monolayer cultures of Vero cells.\textsuperscript{15} Serial 10-fold dilutions from $10^{-1}$ to $10^{-8}$ of blood were prepared in phosphate-buffered saline (pH 7.4), containing 10% fetal bovine serum. Duplicate wells of 24-well microplate cultures of Vero cells were inoculated with each dilution. After virus absorption for 1 hour and addition of an overlay, the cultures were incubated at 37°C; plaques were counted 4 days later. Virus titers were defined as the number of plaque-forming units (PFU) per milliliter of blood.

**Immune response.** The humoral immune response of the hamsters to WNV infection was measured by hemagglutination-inhibition (HI) test.\textsuperscript{13} A standard HI technique was used. Antigens for the HI test were prepared by the sucrose-acetone extraction method from brains of newborn mice infected with WNV and treated with β-propiolactone.\textsuperscript{16} Hamster sera were tested by HI at serial 2-fold dilutions from 1:20 to 1:5,120 at pH 6.6, using four units of antigen and a 1:200 dilution of goose erythrocytes.

**Statistical analysis.** The primary endpoint of the study was mortality at 3 weeks after infection. Secondary endpoints included evaluation of patterns of viremia, immune response, and central nervous system (CNS) pathology. To estimate a sample size, we assumed an 85% mortality rate in the immunosuppressed hamsters and 50% mortality in the immunocompetent group; this assumption was based on prior animal studies of WNV and other *Flavivirus* infections in immunocompetent and immunosuppressed hosts.\textsuperscript{13,17–19} A minimum of 33 hamsters per group provided 80% power analysis and α = 0.05 two-sided significance level to detect a 35% difference in mortality. The Kaplan-Meier method and Logrank test were used to analyze survival data; differences between viral titers and antibody response were analyzed using the Wilcoxon rank-sum test. Differences were considered to be significant at a $P < 0.05$.

**RESULTS**

**Clinical manifestations and mortality.** The hamsters in Group 1 (cyclophosphamide control) remained well throughout the 21-day observation period, although a marked decrease in total WBC occurred after treatment with cyclophosphamide (Table 1), indicating that the animals were immunosuppressed.

During the first 5 days post-infection (p.i.), hamsters in both Groups 2 and 3 appeared normal, except for one animal in Group 2 that was found dead on day 3 p.i. By the seventh day, most of the animals in Groups 2 and 3 appeared lethargic and remained huddled together in the corners of their cages. Between days 7 and 10 p.i., all of the animals in Group 2 and about one half of the hamsters in Group 3 developed neurologic signs, including hind limb paralysis, tremors, difficulty walking, loss of balance, somnolence, and coma.

Figure 1 compares the survival in the two WNV-infected groups. By day 16 p.i., all of the hamsters in Group 2 were dead; in contrast, only 54% of the animals in Group 3 were dead on day 21. Treatment with cyclophosphamide reduced survival significantly ($P < 0.05$).

**Level and duration of viremia.** Figure 2 shows the pattern of viremia in 15 hamsters from Group 2 ($N = 8$) and Group 3 ($N = 7$). Viremia was detected in both groups within 1 day p.i. During the first 3 days p.i., there was little difference in the mean levels of viremia between the two groups. As observed in earlier studies,\textsuperscript{13,17} the viremia in the immunocompetent animals (Group 3) peaked at day 3 (mean titer, $10^{5.4}$ PFU mL) and then decreased. WNV was not detected in the blood of the immunocompetent hamsters after day 6.

In contrast, mean virus titers in the blood of the eight immunosuppressed hamsters (Group 2) continued to increase for 8 days after infection. At this point, no further bleedings

<table>
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<td>Effect of cyclophosphamide (three intraperitoneal injections of 100 mg/kg given over a 9-day period) on total white blood cell, neutrophil, and lymphocyte counts in five uninfected hamsters (Group 1)</td>
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<th>Hamster no.</th>
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<th>Start NE</th>
<th>Start LYM</th>
<th>Day 3 WBC</th>
<th>Day 3 NE</th>
<th>Day 3 LYM</th>
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\[\text{WBC, total white blood cells}/\mu\text{L}; \text{NE, neutrophils}/\mu\text{L}; \text{LYM, lymphocytes}/\mu\text{L}.\]
were done, because several of the immunosuppressed animals were dead or moribund, and the remainder were seriously ill. From days 3 through 8, there was a significant difference ($P < 0.05$) in the mean levels of viremia between hamsters in Group 2 and Group 3.

**Antibody response.** Table 2 compares the HI antibody response of hamsters in Groups 2 and 3 p.i. The immunocompetent animals (Group 3) developed detectable HI antibodies between days 5 and 6 p.i. and the antibody titers continued to increase through day 8. In contrast, none of the eight immunosuppressed animals had developed a detectable HI antibody response by day 8 p.i. The lack of detectable antibodies in this latter group coincided with the prolonged and increasing levels of viremia (Figure 2).

**Histopathology and immunohistochemistry.** No histologic abnormalities were observed in the uninfected, cyclophosphamide-treated hamsters (Group 1). In the untreated WNV-infected hamsters (Group 3), no histologic changes were seen in the visceral organs; but the brain exhibited multifocal changes, including neuronal degeneration within 8 days p.i. In contrast, the changes were much more severe in the cyclophosphamide-treated, infected animals (Group 2); the latter animals had marked neuronal degeneration in the brain and spinal cord (Figure 3B). In addition, there was focal necrosis involving the adrenal cortex. Immunohistochemically, there was intensive and diffuse antigen distribution in many tissues of the immunosuppressed, infected hamsters. For example, on day 6 p.i., no significant antigen was observed in the liver or the spleen of animals in either group. However, intense antigen staining was seen in the adrenal cortex and kidneys of the cyclophosphamide-treated, infected animals. Hamsters in the latter group also exhibited antigens in the lungs (interstitial cells of the alveolar wall) and the heart (myocardium). This became evident on the eighth day of infection or later, as shown in Figure 4A–C.

The intensity of the antigen staining (IHC score) in the Group 2 animals was markedly increased in the brain and spinal cord (Figure 5). Examples of the intensity of antigen staining in the brain and spinal cord of several representative cyclophosphamide-treated hamsters are shown in Figure 5.
4D–H and in Figure 3D, respectively. Figure 3 contrasts the histologic changes and antigen intensity in spinal cord sections from an infected, immunocompetent hamster from Group 3 at 10 days p.i. (Figure 3A and C) and from an infected immunosuppressed animal from Group 2 at 8 days p.i. (Figure 3B and D). Although the Group 3 animal was examined 2 days later than the cyclophosphamide-treated hamster, there were only limited foci of antigen positive neurons in the spinal cord of the former animal compared with diffuse antigen positivity in the latter, indicating increased virus spread associated with immunosuppression. Likewise, the meninges and ependymal cells were negative in the Group 3 animals, but focal antigen positivity was noted in regions immediately beneath the ependymal lining cells in some of the immunosuppressed hamsters (Group 2).

**DISCUSSION**

The results of this study clearly show that cyclophosphamide-induced immunosuppression enhances WNV infection. Cyclophosphamide affects both the humoral and cell-mediated immune responses, and both components of the immune response are important in controlling the infection. The treated hamsters in Group 2 had a prolonged viremia, depressed humoral immune response, more extensive and severe pathology, and a higher fatality rate than the untreated infected animals in Group 3. These results concur with more limited studies done > 30 years ago, which showed that cyclophosphamide treatment potentiated WNV in rats and mice and that it transformed an essentially subclinical infection into a progressive fatal encephalitis. The immunosuppressed rodents in these earlier experiments also had increased viremia and little or no antibody response. The same phenomenon has been observed with other arboviruses in experimentally infected animals immunosuppressed with alkylating agents, radiation, corticosteroids, and thymectomy.

Our results obtained after WNV infection of immunosup-
Figure 4. WNV antigen distribution in cyclophosphamide-treated hamsters (9 days p.i.). A, Lung. B, Heart (myocardial fibers). C, Kidney (renal tubules). D, Cerebral cortex. E and F, Hippocampus (note the diffuse staining involving the neuronal bodies and neural processes). G and H, Cerebellum, showing antigen positivity in nearly all of the Purkinje cells and many small neurons of the granular layer (note staining of the axons in H; immunohistochemical staining, using anti-WNV antibody).
pressed hamsters were similar to a recent description of a case of persistent WNV infection in an immunosuppressed cancer patient. The patient, a 57-year-old man receiving chemotherapy (cyclophosphamide, mitoxantrone, vincristine, and prednisone) for lymphoma, was admitted to the hospital with fever, muscle weakness, tremors, and a total white blood cell count of 1,600 cells/μL. After admission, the patient rapidly deteriorated, became comatose, and was maintained for 3 months on life support systems until he died. On several occasions during his hospitalization, WNV RNA was detected...
in serum and cerebrospinal fluid (CSF) samples; at autopsy, WNV RNA and WNV antigens were detected in brain tissue by reverse transcriptase-polymerase chain reaction and immunohistochemistry, respectively. Antibodies to WNV were not detected by ELISA in serum or CSF at any time during his illness. As with the immunosuppressed hamsters, the patient failed to develop a serologic response and to clear his WNV infection.

Another important finding of our study was that the pathology of WNV infection in immunocompetent hamsters was largely confined to the brain and spinal cord, whereas the infected immunosuppressed animals had much more widespread organ involvement. Diffuse organ involvement also has been described in some cases of fatal WNV encephalitis in immunosuppressed persons. The diffuse nature of WNV infection in immunosuppressed animals and humans is probably a result of the depressed immune response and persistent viremia. Thus, we believe that the immunosuppressed hamster model offers some insight into the pathogenesis of WNV infection in immunosuppressed humans and why these people are at higher risk of severe disease and death, if they become infected with WNV.

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