Correlates to Increased Lethality of Attenuated Venezuelan Encephalitis Virus Vaccine for Immunosuppressed Hamsters

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Splenectomy or pretreatment of adult hamsters with cyclophosphamide (Cytoxan) increased the lethality of the TC-83 vaccine strain of Venezuelan encephalitis virus (VEE), inoculated subcutaneously, from 12% for normal hamsters to 75% and 76%, respectively. Neither splenectomy nor cyclophosphamide treatment significantly increased the lethality of Pixuna virus. Cytoxan-treated (Cy) hamsters developed and maintained levels of TC-83 virus higher than normal infected controls in blood, brain, spleen, and femoral bone marrow; splenectomy had a similar but less intense effect. A severe myeloid necrosis of femoral bone marrow developed 4 to 9 days after TC-83 virus inoculation in 78% of the Cy hamsters and in 48% of the splenectomized (Sx) hamsters. In contrast, only 13% of normal TC-83-infected hamsters developed this lesion. Extensive hemorrhagic lesions in the olfactory lobes and adjacent areas of the brain also developed more frequently in Cy or Sx hamsters than in normal infected controls. Lethally infected hamsters developed and maintained a severe thrombocytopenia, which may be related to the bone marrow lesion and to the hemorrhagic manifestations of lethal VEE infections.

In previously reported studies on the pathogenesis of Venezuelan encephalitis virus (VEE) infections of adult golden Syrian hamsters, several virulent and attenuated VEE virus strains have been compared with respect to viral growth (1, 5) and development of histopathological lesions in target organs (4). It was suggested (4, 5) that the determinants of VEE virulence for hamsters were not only invasiveness or tropism of virus for hematopoietic and brain tissues, but also the interaction of virus with host defense mechanisms. Immunosuppressive procedures might, therefore, significantly increase the lethality of low-virulence VEE strains for hamsters. Immunosuppression has been shown to increase significantly the lethality for rodents of a variety of neurotropic arboviruses (7, 13); this potentiating can often be correlated with increased replication of virus in the brain and with histological destruction in that organ. However, some strains of VEE are not truly neurotropic for hamsters since they destroy extraneural, lymphoid tissues so rapidly and completely that the animals die before the encephalitic phase can develop (1, 4). Other VEE strains, such as VEE subtype III, kill hamsters more slowly, and an encephalitic syndrome does develop (5). The following experiments were designed to test whether immunosuppressive procedures would increase the lethality of two low-virulence strains of VEE and, if so, to correlate this increased lethality with the growth of virus and the development of lesions in target organs.

MATERIALS AND METHODS

Viruses and virus assays. The live attenuated VEE vaccine, strain TC-83, was obtained in the lyophilized state from Merrell-National Drug Co. (lot 3, run 9); it was reconstituted with 1.2 ml of sterile water for injection and was diluted to 500 ml in diluent containing 1% bovine albumin in Hanks balanced salts solution (BA/H) adjusted to pH 8.0. Multiple samples were stored at ~60 °C in vials; the samples contained 1.3 x 10^3 plaque-forming units (PFU) per 0.2 ml. The Trinidad donkey strain of VEE virus (9) was obtained from the American Type Culture Collection and was passed one additional time in primary chicken embryonic cell cultures (CEC) prepared and used essentially as described previously (10). Pixuna virus, strain BeAr 35645 (11), was in the fourth mouse passage from a pool of Anoploites nimbus and was passed one additional time in CEC. Viruses were titrated by counting PFU in CEC grown in 8-cm² wells of plastic plates, maintained under a medium containing 1% agarose, basal medium (Eagle) with Earle salts (Gibco G-11) and N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES) buffer (25 mM). After the cells had been incubated for 48 h at 37°C in a 5% CO,

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atmosphere, 1 ml of a 1:7,500 dilution of neutral red (Gibco-533) was added, and plaques were counted after 4 h.

Inoculation of hamsters and harvest of tissues. Both normal and Sx male hamsters were obtained from Lakeview Hamster Colony, Newfield, N.J., and were used when they weighed 70 to 80 g (7 weeks old). Hamsters were inoculated subcutaneously (sc) over the back with 1,000 PFU of virus in 0.2 ml of BA/H. Heparinized plasma and organ suspensions were obtained from inoculated hamsters as described previously (5). For platelet counts, heparinized blood was diluted 1:100 with Unopettes (no. 5855) and was counted in a hemocytometer with the use of a phase-contrast optical system.

Tissues harvested for histopathological examination were fixed in neutral buffered Formalin and were stained with hematoxylin and eosin as described previously (4).

Pretreatment of hamsters with cyclophosphamide. Cyclophosphamide (Cytoxan) was obtained from Mead-Johnson, Evansville, Ind., and was diluted in sterile saline for injection to contain 7.5 mg/ml. Hamsters weighing 70 to 80 g were inoculated intraperitoneally with 7.5 mg in 1 ml, 16 h prior to, and simultaneously with, sc inoculation of virus.

RESULTS

Increased lethality of TC-83 vaccine for immunosuppressed hamsters. Seventy-five of 100 adult Sx hamsters died after sc inoculation of 1,000 PFU of TC-83 vaccine, as did 76 of 100 Cy hamsters (Fig. 1). In contrast, only 12 of 100 normal hamsters died after this virus challenge. The majority of Sx hamsters died 5 to 6 days after inoculation, whereas most Cy hamsters died 6 to 10 days after inoculation. In contrast, all of 100 normal hamsters inoculated sc with 1,000 PFU of the parental, virulent Trinidad strain of VEE died within 5 days.

Effect of immunosuppression on the lethality of Pixuna virus (VEE subtype IV) for hamsters. Eleven of 50 Cy hamsters died 4 to 7 days after sc inoculation of 1,000 PFU of Pixuna virus. Only 1 of 50 normal inoculated hamsters and none of 25 Sx hamsters died after challenge with Pixuna virus. Treatment with Cytoxan thus appeared to be the more vigorous immunosuppressive procedure. However, since Cytoxan did not increase the lethality of Pixuna virus to the high level observed for TC-83, further studies comparing the pathogenesis of Pixuna virus in Cy and normal hamsters were not initiated.

Growth of TC-83 vaccine in target tissues of normal versus immunosuppressed hamsters. Infectious TC-83 virus levels in tissues harvested from Sx and Cy hamsters were generally higher than in tissues harvested at the same time from normal TC-83-infected hamsters (Fig. 2).

By day 4, viremias in Cy hamsters had progressed to levels similar to those attained earlier in Trinidad strain-infected hamsters; the high-level viremia was maintained at least until day 9, which was the last time at which randomly sacrificed Cy hamsters were tested. Virus levels in blood harvested from individual Cy hamsters dying relatively late on days 11, 14, and 15 were 4.5, 5.1, and 3.6 log10 PFU/ml, respectively. Viremias in Sx hamsters were also elevated and prolonged relative to viremias in controls, but not to the extent observed for Cy animals.

Similar results were obtained when TC-83 viral growth curves in brain, spleen, and bone marrow were compared. The virus levels in brain tissues of Cy and Sx hamsters were significantly higher than in controls from day 4 until the animals died, although TC-83 virus did not reach the 8 to 9 log10 PFU/g level observed for the Trinidad strain in brain tissues. The virus concentrations detected in brain tissues did not exceed viremia levels until day 4.
for TC-83 in normal and Sx hamsters and until day 6 for Cy hamsters, suggesting that most of the virus detected in brain at earlier times may have represented virus in the contained blood. High levels of virus were maintained in the brains of Cy hamsters; in contrast, viral titers in the brains of Sx hamsters decreased slowly, and virus was more rapidly cleared from brains of normal TC-83-infected hamsters.

The high mean titer of TC-83 virus in spleens of Cy hamsters on day 1 was significantly higher ($P < 0.01$) than for TC-83 in normal hamsters.
and even slightly higher than for Trinidad strain virus (all day 1 titers were based on 25 animals per point). Likewise, Sx and Cy hamsters developed significantly higher titers of virus than controls in bone marrow and maintained high titers in bone marrow until they died.

**Lethality of virus isolated from sick Cy hamsters.** It was previously reported that TC-83 vaccine strain can revert to virulence after serial passage in hamsters or after a single sc inoculation of a large dose (3). To test the possibility that Cytoxan may potentiate a selective in vivo amplification of viral particles more virulent that the TC-83 inoculum population as a whole, virus was isolated from the brains of five sick Cy hamsters killed on day 6. Each isolate was tested for virulence by inoculating 10 normal hamsters sc with 1,000 PFU. The fractions (dead/inoculated) for the five isolates tested were 0/10, 2/10, 1/10, 3/10, and 0/10, demonstrating an overall lethality of 6/50 (12%), the same as for the original TC-83 inoculum.

**Clinical signs and histopathological lesions.** Cy and Sx hamsters infected with TC-83 virus usually developed a moderate to severe petechial rash over most of the body about 4 days after infection, which persisted until death (Fig. 3). Sick hamsters did not develop central nervous system signs (paralysis, tremors). Sx hamsters usually died suddenly, within several hours of first appearing sick, whereas Cy hamsters often appeared "rough" for several days before dying.

Groups of Cy, Sx, or normal TC-83-infected hamsters were killed at random at daily intervals in two experiments, from 1 to 9 days after inoculation, for histopathological examination of tissues. In addition, two uninfected hamsters treated with Cytoxan alone were killed on days 2, 4, and 6. No lesions were detected in any tissues harvested from hamsters treated only with Cytoxan (Fig. 4a). No severe lesions were seen in the tissues harvested from any infected animal before day 4, and thus the frequencies of severe lesions were compared among groups for times selected after day 4 (Table 1). Multifocal areas of severe hemorrhage developed in the olfactory lobes of the brain in 18 of 22 Cy hamsters and in all of 27 Sx hamsters. In approximately half of these brains, the hemorrhagic lesions were more generalized, extending into the pyriform lobes and adjacent areas of the brain (Fig. 4b). Although 11 of 24 normal hamsters infected with TC-83 also developed hemorrhagic lesions in the olfactory lobes, these lesions were not so severe or so extensive in normal infected hamsters as in the infected immunosuppressed groups. The majority (14 of 18) of Cy hamsters developed severe myeloid necrosis of femoral bone marrow (Fig. 4c), as did half of the Sx hamsters. In contrast, only 3 of 24 normal hamsters developed myeloid necrosis in bone marrow. The frequency of hemorrhagic necrosis of the intestinal wall was higher for Cy hamsters than for the other two groups, but was seen in only 7 of the 20 specimens examined. Likewise, postnecrotic changes in the spleen, characterized by macrophages heavily engorged with necrotic cell debris (Fig. 4d), occurred more frequently for the Cy group, but still with a relatively low frequency.

**Development of thrombocytopenia.** One consequence of the bone marrow destruction, which might correlate with the hemorrhagic manifestations of lethal VEE infections, was the development of a severe thrombocytopenia. Platelet counts decreased to almost undetectable levels in Trinidad-infected hamsters (Fig. 5). However, severe but transient thrombocytopenia also developed in normal TC-83-infected hamsters, in the presumed absence of severe, irreversible bone marrow necrosis. Platelet counts in Cy hamsters decreased at the same rate as for normal TC-83-infected hamsters, but failed to recover. Sx hamsters were not tested, because platelet counts in uninfected Sx hamsters were very high.

![Fig. 3. Petechial rash on the skin of a Cytoxan-treated hamster. 5 days after infection with TC-83.](http://iai.asm.org/)
DISCUSSION

Hamsters that had been splenectomized or treated with Cytoxan were more susceptible than normal hamsters to the lethality of the TC-83 vaccine strain of VEE. This is consistent with the observation reported previously that...
young (2- to 3-week-old) hamsters are more susceptible to TC-83 lethality (3), presumably because they have not yet attained full immunological competence.

TC-83 virus replicated and was maintained at higher titers for longer periods in plasmas, brains, spleens, and femoral bone marrows of immunosuppressed hamsters, relative to normal infected controls. Since the population from which the Sx hamsters were randomly selected for the determination of mean virus titers after day 5 was composed primarily of animals that would have survived, the growth curves for Sx hamsters may not reflect the true virus levels in sick Sx hamsters on days 6 and 7.

A combination of several factors was probably responsible for the enhanced replication and maintenance of high virus levels in Cy and Sx hamsters. Preliminary (unpublished) data suggest that interferon levels in plasma and in bone marrow suspensions from Cy and Sx hamsters are lower than in normal TC-83 controls. Cytoxan may also directly increase the sensitivity of target (bone marrow) cells to destruction by TC-83 virus. Both Cytoxan and splenectomy may directly decrease the rate at which TC-83 virus is cleared from plasma and other tissues. This latter hypothesis will be tested directly by comparing clearance rates for TC-83 after intracardiac inoculation of Sx and normal hamsters. It is also possible that engorged macrophages (Fig. 4d) cannot clear virus efficiently. Clearance of virus may be an important determinant of viral virulence, since artificial blockade of the reticuloendothelial system with silica beads increased the lethality of the 17-D vaccine strain of yellow fever virus (12) and of coxsackievirus B3 for mice (8). Thorotrast has also been shown to decrease the rate at which Semliki forest virus is cleared from mouse plasma (6), and preliminary evidence has been reported (5) which indicates that hamsters pretreated with Thorotrast are less resistant than normal hamsters to the lethality of TC-83 virus infection.

The high, sustained levels of virus in the bone marrow of immunosuppressed hamsters correlated with the severe histological destruction of these tissues, and with the prolonged, severe thrombocytopenia. The hemorrhagic manifestations of VEE, including the petechial rash and the multifocal, noninflammatory hemorrhagic lesions in the brain, may be secondary to the thrombocytopenia. The failure of Cy hamsters to recover may be related to a decreased capacity of bone marrow to regenerate in the presence of Cytoxan. The severe hemorrhagic diathesis involving the intestines of hamsters inoculated with virulent VEE strains (1) may also be secondary to virus-induced destruction of bone marrow.

It is difficult to evaluate the importance of the hemorrhagic brain lesions which were usually more severe and occurred more frequently in the brains of Cy and Sx hamsters than in normal infected controls. Four of 24 normal TC-83 infected hamsters had equally severe and

### Table 1. Frequency* of severe lesions in tissues of immunosuppressed or normal hamsters 4 to 9 days after sc inoculation of 1,000 PFU of TC-83 vaccine virus

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Brain hemorrhages*</th>
<th>Femoral bone marrow*</th>
<th>Intestine*</th>
<th>Spleen*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Olfactory lobes</td>
<td>Generalized</td>
<td>PP</td>
<td>Wall</td>
</tr>
<tr>
<td>Cytoxan</td>
<td>18/22 (82%)</td>
<td>11/22 (50%)</td>
<td>14/18 (78%)</td>
<td>2/20 (10%)</td>
</tr>
<tr>
<td>Splenectomy</td>
<td>27/27 (100%)</td>
<td>13/27 (48%)</td>
<td>13/27 (48%)</td>
<td>2/17 (12%)</td>
</tr>
<tr>
<td>None</td>
<td>11/24 (46%)</td>
<td>4/24 (17%)</td>
<td>3/24 (13%)</td>
<td>0/22 (0%)</td>
</tr>
</tbody>
</table>

*Frequency: number of animals in which severe lesions were observed/total number of animals tested.

*Myeloid depletion and necrosis affecting >50% of the cells.

*Multifocal areas of hemorrhage with or without inflammatory cells plus resultant neuronal and glial cell necrosis.

*Severe necrosis of Peyer's patches (PP) or hemorrhage with ulceration of the mucosa.

*Necrosis of white pulp with or without engorged macrophages in red and white pulp.

![Platelet counts in blood from normal hamsters infected with Trinidad or TC-83 strains of Venezuelan encephalitis virus, or from Cytoxantreated hamsters infected with TC-83. Each point is an arithmetic mean ± 1 standard deviation, based on 10 hamsters.](http://iai.asm.org/Downloaded from http://iai.asm.org/ on August 29, 2017 by guest)
extensive lesions (Table 1), but were apparently healthy. The occurrence of this lesion in apparently healthy, TC-83-infected hamsters has been reported by other investigators (2). Although it is conceivable that hemorrhage may have been an important factor in the “sudden death” observed for SX hamsters, Cy hamsters, which exhibited similar hemorrhagic lesions, did not usually die as rapidly.

It is our working hypothesis, however, that these brain lesions occur secondarily to the interaction of TC-83 virus with bone marrow, and that this bone marrow interaction is more destructive when hamsters are pretreated with Cytoxan or splenectomy. Further experiments, in which host defense mechanisms are suppressed more selectively, and in which the interaction of virus with bone marrow cells and function is defined more precisely, may lead to a clearer understanding of the determinants for virulence and attenuation of VEE viruses.

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LITERATURE CITED