Dhori Virus *(Orthomyxoviridae: Thogotovirus)* Infection of Mice Produces a Disease and Cytokine Response Pattern Similar to That of Highly Virulent Influenza A (H5N1) Virus Infection in Humans

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Abstract. Mice infected with Dhori virus (DHOV) develop a fulminant, systemic, and uniformly fatal illness that has many of the clinical and pathologic findings seen in H5N1 influenza A virus infection. However, the role of host’s immune response in DHOV infection remains unclear. In this study, the concentrations of 23 inflammatory cytokines and chemokines were measured in the liver, lungs, and sera of mice during the course of DHOV infection. Liver function, level of viremia, and hematologic response were also monitored. Infected animals exhibited significant leukopenia and lymphopenia, which directly correlated with the disease progression. High yields of infectious virus along with strikingly elevated expression of various inflammatory mediators, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, IL-10, macrophage inflammatory protein (MIP)-1α, monocyte chemoattractant protein (MCP)-1, and interferon (IFN)-α, indicated that these responses play an important role in the observed disease and pathology. The overall clinical, pathologic, and immunologic responses of ICR mice to DHOV infection closely resemble those described for highly virulent influenza A virus infection in humans, thereby offering a realistic, safe, and alternative animal model for studying the pathogenesis and treatment of highly pathogenic avian influenza virus.

INTRODUCTION

The family *Orthomyxoviridae* currently consists of five genera: *Influenzavirus A*, *Influenzavirus B*, *Influenzavirus C*, *Thogotovirus*, and *Isavirus*.1 Previous studies2,3 have shown that Dhori virus (DHOV), a member of the genus *Thogotovirus*, is lethal to mice and causes systemic pathologic changes similar to those reported in humans with virulent influenza A (H5N1) virus infection. Histopathologic findings in DHOV-infected mice include inflammation, edema, and hemorrhage in lungs, necropsis and atrophy in liver, and marked lymphocytic apoptosis and karyorrhexis in lymph nodes and spleen, as seen in severe human cases of highly pathogenic H5N1 avian influenza A virus infection. Although the exact mechanism of DHOV pathogenesis in mice is unknown, the extensive pathology in the lungs, liver, and other tissues, along with the observation of prominent lymphopenia, strongly suggested that an exacerbated inflammatory response by the host, in response to DHOV infection, plays a major role in the observed disease.

One of the conclusions from our previous study3 was that mice experimentally infected with DHOV could serve as a surrogate model for severe H5N1 influenza A virus infection in humans. Although mouse, ferret, and monkey models for H5N1 influenza virus infection have been described,4,6 a major difficulty in working with these models is that the biosafety, security, and agricultural regulations governing use of virulent H5N1 virus effectively restrict experimental animal studies to a few high-security laboratory facilities. Safer and more accessible animal models are needed for further studies of the pathogenesis and therapy of virulent H5N1 virus infection.

The term “cytokine storm” refers to an inappropriate immune response caused by rapidly proliferating T cells or natural killer cells activated by infected macrophages and epithelial cells that are activated directly by certain viral infections. Acute viral infection of the respiratory system (i.e., H5N1 influenza) can result in a cytokine storm affecting the lungs, with subsequent damage to alveoli and lung tissue.7–9 If not treated and aggressively suppressed, death may result.10

To test the hypothesis that cytokine storm is responsible for the severe pathology observed in DHOV-infected mice, an experiment was carried out to determine the cellular immune responses to DHOV infection. In this study, the levels of 23 inflammatory cytokines and chemokines in the liver, lungs, and sera of mice infected with DHOV were determined during the course of infection. The results, combined with data from our previous study,3 indicate that the DHOV mouse model is a realistic and relatively safe11 alternative system to study the pathophysiology of virulent influenza A viruses, such as H5N1, and for the initial testing of viable treatments for severe influenza.

MATERIALS AND METHODS

Viruses. The prototype Dhori virus strain (IG 611313) was used to infect the animals. It was originally isolated from *Hyalomma dromedarii* ticks collected from camels in Gujarat State, India, in 1961,12 and had been passaged four times by intracerebral inoculation of sucking mice.

Animals. Adult female mice (ICR strain), 8–11 weeks of age, were obtained from Harlan Sprague-Dawley (Indianapolis, IN). 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 Resource Council) under an animal use protocol approved by the University of Texas Medical Branch.

Infection of animals and samples collection. A total of 38 mice were used. Twenty-eight animals were randomly divided into seven groups of four each. One group of four animals served as controls and were not inoculated with DHOV. The...
other six groups plus 10 additional mice were given ~10^6.9 plaque-forming units (PFU) of DHOV, administered by the intranasal (IN) route. Animals were first anesthetized with Halothane (Halocarbon Laboratories, River Edge, NJ), and two drops (~100 μL) of a virus solution were instilled into the nares. For 6 consecutive days, one group (four animals) was euthanized daily to collect samples (liver, lungs, and blood) for study of viral levels, as well as cytokine, chemokine, hematologic, and liver function assays. Similar samples were also collected from the four uninfected (control) mice. The 10 remaining infected mice were not sampled; they were simply observed daily to determine the time of death and to generate a survival curve.

**Virus titrations.** Titrations of daily blood samples from the infected mice were done in 24-well tissue culture plates seeded with Vero cells, as previously described.3 Serial 10-fold dilutions from 10^{-1} to 10^{-6} were made of each blood specimen, using phosphate-buffered saline (pH 7.4) with 10% fetal bovine serum as the diluent. Four wells of the tissue culture plate were inoculated with 100 μL of each dilution. After absorption at 36°C for 2 hours, 1.0 mL of agar overlay medium was added to each well, and the plates were incubated at 37°C in a 5% CO₂ atmosphere for 72 hours. A second overlay, containing 1% neutral red, was added on Day 4, and plaques were counted on the sixth day. Virus titers were calculated as the number of PFU per milliliter of blood.

**Hematologic studies.** White blood cell (WBC), neutrophil (NE), lymphocyte (LY), monocyte (MO), eosinophil (EO), basophil (BA), red blood cell (RBC), hemoglobin (Hb), hematocrit (HCT), and platelet (PLT) counts were determined using a Hemaver 950 analyzer (Drew Scientific, Oxford, CT). For this period, NE and EO counts increased gradually on Days 4–6 p.i., but by the third day, they showed signs of illness (ruffled fur, emaciation, lethargy, and labored breathing). On the fifth day, two mice died. The remaining animals died on Day 6 p.i. The survival curve is shown in Figure 1A.

**Liver function studies.** Determinations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were done on fresh serum using a Prochem-V clinical chemistry analyzer (Drew Scientific, Oxford, CT), according to the manufacturer’s instructions.

**Measurement of inflammatory cytokines and chemokines.** Samples of liver and lung were weighed and homogenized in diluent, using a Qiagen TissueLyser (Retsch, Haan, Germany), to prepare 10% (wt/vol) tissue suspensions. After clarification, the tissue suspensions and sera were subsequently inactivated by gamma (cobalt-60) irradiation at a total dosage of 2 million rads, using a Model 109 laboratory irradiator (Shepherd & Associates, San Fernando, CA). The inactivated samples were used to define cytokine and chemokine profiles using the Bio-Plex Cytometric Bead Array (Bio-Rad, Hercules, CA) analysis, according to the manufacturer’s recommendations. This technology was used to simultaneously quantify the 23 inflammatory mediators described in the results.

**Statistical analysis.** The levels of inflammatory cytokines and chemokines were compared between each group of infected mice and the controls; each pair was tested for significance in differences by analysis of variance (ANOVA) Student’s t-test with StatView software; P < 0.05 was considered statistically significant.

**RESULTS**

**Clinical manifestations and mortality.** The 10 DHOV-infected mice used for the survival curve remained well during the first 2 days post-infection (p.i.), but by the third day,
Hematologic values in ICR mice after infection with DHOV

<table>
<thead>
<tr>
<th>Test</th>
<th>Baseline</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (cells/mm³)</td>
<td>6,454 ± 0.01</td>
<td>6,764 ± 0.01</td>
<td>3,468 ± 0.01</td>
<td>4,652 ± 0.01</td>
<td>1,956 ± 0.01</td>
<td>2,303 ± 0.01</td>
<td>2,030 ± 0.01</td>
</tr>
<tr>
<td>NE (cells/mm³)</td>
<td>1,014 ± 0.01</td>
<td>1,542 ± 0.01</td>
<td>1,776 ± 0.01</td>
<td>2,130 ± 0.01</td>
<td>564 ± 0.01</td>
<td>538 ± 0.01</td>
<td>1,203 ± 0.01</td>
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<tr>
<td>LY (cells/mm³)</td>
<td>4,922 ± 0.01</td>
<td>4,754 ± 0.01</td>
<td>1,326 ± 0.01</td>
<td>1,848 ± 0.01</td>
<td>90 ± 0.01</td>
<td>976 ± 0.01</td>
<td>976 ± 0.01</td>
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<tr>
<td>MO (cells/mm³)</td>
<td>361 ± 0.01</td>
<td>304 ± 0.01</td>
<td>150 ± 0.01</td>
<td>222 ± 0.01</td>
<td>88 ± 0.01</td>
<td>90 ± 0.01</td>
<td>88 ± 0.01</td>
</tr>
<tr>
<td>EO (cells/mm³)</td>
<td>124 ± 0.01</td>
<td>136 ± 0.01</td>
<td>166 ± 0.01</td>
<td>252 ± 0.01</td>
<td>84 ± 0.01</td>
<td>60 ± 0.01</td>
<td>53 ± 0.01</td>
</tr>
<tr>
<td>BA (cells/mm³)</td>
<td>30 ± 0.01</td>
<td>26 ± 0.01</td>
<td>44 ± 0.01</td>
<td>90 ± 0.01</td>
<td>22 ± 0.01</td>
<td>10 ± 0.01</td>
<td>15 ± 0.01</td>
</tr>
<tr>
<td>RBC (cells/mm³)</td>
<td>8,822 ± 0.01</td>
<td>9,346 ± 0.01</td>
<td>9,484 ± 0.01</td>
<td>9,544 ± 0.01</td>
<td>9,824 ± 0.01</td>
<td>9,874 ± 0.01</td>
<td>9,558 ± 0.01</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>12.5 ± 0.01</td>
<td>13.1 ± 0.01</td>
<td>13.2 ± 0.01</td>
<td>13.6 ± 0.01</td>
<td>14.1 ± 0.01</td>
<td>13.6 ± 0.01</td>
<td>14.0 ± 0.01</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>49.8 ± 0.01</td>
<td>50.5 ± 0.01</td>
<td>52.7 ± 0.01</td>
<td>51.9 ± 0.01</td>
<td>53.6 ± 0.01</td>
<td>54.1 ± 0.01</td>
<td>55.2 ± 0.01</td>
</tr>
<tr>
<td>PLT (10³/mm³)</td>
<td>1,107.1 ± 0.01</td>
<td>1,187.0 ± 0.01</td>
<td>970.6 ± 0.01</td>
<td>599.0 ± 0.01</td>
<td>418.8 ± 0.01</td>
<td>361.6 ± 0.01</td>
<td>285.0 ± 0.01</td>
</tr>
</tbody>
</table>

Baseline (mean value of the four non-infected control mice).

WBC, white blood counts; NE, neutrophils; LY, lymphocytes; MO, monocytes; EO, eosinophils; BA, basophils; RBC, red blood cells; Hb, hemoglobin; HCT, hematocrit; PLT, platelets.

tor [TNF]-α in lungs, liver, and sera of the DHOV-infected mice were determined using a cytometric bead array assay. In general, the infected animals mounted a robust immune response compared with the control (uninfected) mice. It was characterized by a cascade of inflammatory mediators.

The concentrations of IL-3, IL-5, IL-6, IL-9, IL-13, IL-17, and TNF-α in the liver of infected mice showed no change compared with the controls (data not shown), but IL-1α, IL-2, IL-10, IL-12, KC, G-CSF, and RANTES showed significant increases (Figure 3). IL-1α and IL-12 (P70) production displayed a gradual increase as the infection progressed; on Days 5 and 6, these values were significantly higher than controls (P < 0.01). Cytokines IL-12 (P40), IL-10, KC, G-CSF, and RANTES and chemokines MIP-1α, Eotaxin, and MCP-1 increased markedly (P < 0.05; Figure 4). MIP-1β and MCP-1 increased markedly (P < 0.05), but MCP-1β did not change.

In lungs, the levels of IL-2, IL-3, IL-5, IL-6, IL-9, and IL-13 did not change compared with the controls, but IL-1α, IL-1β, IL-10, IL-12, and IL-17 increased significantly (P < 0.05; Figure 4). IL-1α, IL-1β, KC, G-CSF, and RANTES showed a gradual increase as the infection progressed. IL-10, IL-12, IL-17, Eotaxin, and MCP-1 reached peak levels on Day 4 of infection. TNF-2 was only slightly elevated. The chemokines MIP-1α, Eotaxin, and MCP-1 increased markedly (P < 0.05), but there was little change in the concentration of MIP-1β (Figure 4).

The concentration of inflammatory factors in sera of the infected mice was higher than in the liver and lungs (Figures 3–5). Compared with normal controls, the serum levels of IL-1β, IL-2, IL-3, IL-5, IL-10, IL-12 (P70), IL-17, GM-CSF, and TNF-α showed little change (data not shown). However, IL-1α, IL-6, IL-9, IL-12 (P40), and IL13 changed significantly compared with controls (P < 0.05; Figure 5). The chemokines MIP-1α, Eotaxin, and MCP-1 were elevated markedly (P < 0.05), but MCP-1β did not change.

High virus titers were detected in sera of infected mice. To observe if DHOV resists type I IFN-mediated antiviral activity, IFN-α and IFN-γ also were measured in liver, lungs, and sera. IFN-α in liver was elevated on Day 2 but decreased rapidly on Day 3 and subsequent days (Figure 6). It also increased on Days 2 and 3 in the lung and sera but decreased to lower levels on Days 4–6. IFN-γ was elevated, but it did not increase significantly (P > 0.05) in the liver or lungs compared with normal controls. However, it was elevated in sera on Days 5 and 6 (P < 0.05; data not shown).

**DISCUSSION**

Results of this study confirm our earlier findings that experimental infection of ICR outbred mice with DHOV produces an illness comparable to that observed in BALB/c and B6/129 wild-type mice infected with virulent influenza A (H5N1) virus.4,14,15 DHOV-infected mice exhibited significant leucopenia, lymphopenia, and thrombocytopenia; the degree of WBC, lymphocyte, and platelet depletion became greater with progression of the disease. The decrease in peripheral WBC and PLT counts coincided with hemorrhages and more inflammatory cells observed in histologic sections of DHOV-infected mouse lungs on the same days.3

In addition, the levels of aminotransferase (ALT and AST) were also elevated as the infection progressed, with hepatocyte-associated damage. Similar clinical laboratory findings have been reported in humans with H5N1 virus infection, namely leucopenia and lymphopenia, mild-to-moderate thrombocytopenia, and elevated aminotransferase levels.8 A report from Thailand16 indicated that an increased risk of death was associated with decreased leukocyte, platelet, and particularly lymphocyte counts at the time of admission.
The innate immune responses to virulent influenza A H5N1 virus infection also probably contribute to disease pathogenesis. In the early (1997) human outbreaks, elevated blood levels of IL-6, IFN-γ, TNF-α, and soluble IL-2 receptor were observed in individual fatal cases.17 In a subsequent outbreak in 2003, elevated levels of interferon-inducible protein 10, MCP-1, and monokine induced by IFN-γ were found in H5N1 influenza patients 3–5 days after the onset of illness.18 Plasma levels of inflammatory mediators (IL-6, IL-8, IL-1b, and MCP-1) also were reported to be higher among H5N1 patients who died than among persons who survived the infection. The average levels of plasma IFN-α were also about three times higher in the fatal cases than in healthy controls. These changes may be responsible in part for the sepsis, acute respiratory distress syndrome, and multiorgan failure observed in severe cases of H5N1 virus infection. Patients with H5N1 influenza A virus infection have a primary viral pneumonia complicated by acute respiratory distress, multiple organ dysfunction, and hemophagocytosis,17,19 all of which are compatible with cytokine dysregulation.20,21

In this study, we measured the concentrations of proinflammatory cytokines IL-1α, IL-1β, IL-2, IL-3, IFN-α, TNF-α, IL-6, IL-12 (P40), IL-12 (P70), G-CSF, GM-CSF, KC, RANTES, and IL-17; the Th2 cytokines IL-4, IL-5, IL-10, and IL-13; the Th1 cytokine IFN-γ; and chemokines MCP-1, MIP-1α, MIP-1β, and Eotaxin in the liver, lungs, and sera of DHOV-infected mice. The cytokine and chemokine patterns detected in the DHOV-infected mice were similar to those previously observed in BALB/c mice with H5N1 virus infection15,22; the latter animals also developed high concentr-
tions of IL-1β, IFN-γ, KC, IL-6, and MIP-1α in their liver, lungs, and sera.10,23 The possible roles of these responding cytokines are multiple and include stimulating immune cell proliferation, differentiation, and stimulating hematopoiesis.24,25 IL-12, IFN-γ, TNF-α, and transforming growth factor (TGF)-β were all elevated in the lungs and liver of the DHOV-infected mice, indicating that DHOV infection enhances type I immune responses and potentiates inflammation in the liver and lungs. Plasma IL-12 concentrations were also significantly elevated in the infected mice, which may trigger eosinophils, basophils, and mast cells to release cytokines for differentiation of Th cells. The Th2 cytokines may play roles in local infiltration and activation of eosinophils26–28 and in inducing airway hyperreactivity and allergic inflammation.29 In addition, the production of RANTES by bronchial epithelial cells contributes to the infiltration of the epithelium by inflammatory cells in viral infection30 and may be associated with the observed alveolar injury.

Although the cytokine response in the lungs may be more relevant to pathogenesis than that in the blood,31 intrinsic viral properties probably contribute to the increased cytokine and chemokine levels and to pathogenesis in the lungs. De Jong and others32 reported that plasma levels of MCP-1, IL-8, IL-6, and IL-10 correlated with H5N1 virus load in fatal human cases, similar to the findings in our study. In this study, most of the cytokines and chemokines reached peak levels in tissues on Day 4 after infection, when there were increased viremia and distribution of DHOV in the tissues. Recently, it was shown that increased production of cytokines and chemokines during infection with 1918 influenza virus can be attributed to the presence of neutrophils and alveolar macrophages in the lungs of infected mice.22,33 In our study, DHOV infection also resulted in increased neutrophil and macrophage recruitment, in association with a relevant to significant increase in cytokine and chemokine expression.

Because analysis of the relative importance of individual cytokines in disease production and immune response is limited by the redundant functions of many of these mediators and by their complex interactions, only the general patterns of some important cytokines and chemokines in response to DHOV infection could be characterized in this study. Overall, our results support two main concepts. First, DHOV infection triggered an intense host immune response in the mice, and the observed hypercytokinemia and hyperchemokinemia were associated with hepatic and pulmonary injuries. Second, the detailed immunologic response reported here, together with our previous pathologic study,7 shows that the DHOV mouse model has many similarities in terms of virus replication, tissue distribution, clinical signs, pathology, and immune response to H5N1 influenza A virus infection in humans. Thus, the DHOV-mouse model may be useful for further dissection of the molecular mechanisms and for studies on the prevention and therapy of virulent influenza A (H5N1) virus infection.

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FIGURE 6. IFN-α concentrations in sera, lungs, and livers of mice with DHOV infection.