DHORI VIRUS (ORTHOMYXOVIRIDAE: THOGOTOVIRUS) INFECTION IN MICE: A MODEL OF THE PATHOGENESIS OF SEVERE ORTHOMYXOVIRUS INFECTION

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Abstract. After intranasal, subcutaneous, or intraperitoneal infection with Dhori virus (DHOV), adult mice developed a fulminating and uniformly fatal illness with many of the clinical and pathologic findings seen in mice infected with H5N1 highly pathogenic avian influenza A virus. Histopathologic findings in lungs of DHOV-infected mice consisted of hemorrhage, inflammation, and thickening of the interstitium and the alveolar septa and alveolar edema. Extrapulmonary findings included hepatocellular necrosis and steatosis, widespread severe fibrinoid necrosis in lymphoid organs, marked lymphocyte loss and karyorrhexis, and neuronal degeneration in brain. Similar systemic histopathologic findings have been reported in the few fatal human H5N1 cases examined at autopsy. Because of the relationship of DHOV to the influenza viruses, its biosafety level 2 status, and its similar pathology in mice, the DHOV-mouse model may offer a low-cost, relatively safe, and realistic animal model for studies on the pathogenesis and management of H5N1 virus infection.

INTRODUCTION

Since the appearance of human disease caused by avian influenza A H5N1 viruses in Hong Kong in 1997,1 there has been renewed interest in the pathogenesis of highly pathogenic avian influenza (HPAI) viruses.2–13 One of the major deterrents to research with H5N1 and other HPAI viruses is that the biosafety, security, and agricultural regulations governing their use effectively restrict experimental animal work to a very few high security laboratory facilities. Safer and more accessible animal models are needed.

The family Orthomyxoviridae currently consists of five genera: *Influenzavirus A, Influenzavirus B, Influenzavirus C, Thogotovirus,* and *Isavirus.*14 Felipe and others15,16 previously reported that two thogotoviruses, Dhori and Thogoto, were lethal to mice inoculated intraperitoneally and that they produced pathologic lesions in the animals similar to those described with mouse-adapted strains of influenza virus. To further study this observation, experiments in which ICR mice were inoculated intraperitoneally, subcutaneously, or intranasally with Dhori virus were carried out, and the clinical outcome, level of viremia, and histopathology after infection by the three routes were determined. This report describes our findings that indicate that Dhori virus, a biosafety level 2 (BSL-2) non-select agent,17 could serve as a safe alternative model in mice for studies on the pathogenesis and therapy of HPAI virus infection.

MATERIALS AND METHODS

Virus. The prototype strain (IG 611313) of Dhori virus (DHOV) was used in this study; it was originally isolated from *Hyalomma dromedarii* ticks collected from camels in Gujarat State, India, in 1961.18 The virus used to infect the mice had been passaged four times previously by intracerebral inoculation of suckling mice.

Animal. The mice used in this study were 8- to 11-week-old females (ICR strain), obtained from Harlan Sprague-Dawley (Indianapolis, IN). The animals were cared for in accordance with 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.

Virus assay. Blood samples from the infected mice were titrated by plaque assay in monolayer cultures of Vero cells, as described previously.19 Serial 10-fold dilutions of the blood from 10⁻¹ to 10⁻⁶ were prepared in phosphate-buffered saline, pH 7.4, containing 10% fetal bovine serum (PBS). Duplicate wells of 24-well microplate cultures of Vero cells were inoculated with 100 μL of each dilution. After virus absorption for 1 hour and addition of an agar overlay, the cultures were incubated at 37°C; a second overlay containing 1% neutral red was added 72 hours later, and plaques were counted 6 days after inoculation. Virus titers were calculated as the number of plaque forming units (PFU) per milliliter of blood.

Experimental design. A total of 30 mice was used; the animals were divided into three groups of 10 animals each. The three groups were inoculated with a DHOV stock containing 10⁻⁸ PFU/mL, administered by the intraperitoneal (IP), subcutaneous (SC), or intranasal (IN) routes. Mice infected by the IP and SC routes received 100 μL of a 1:2 dilution of the virus stock (≈10⁻⁶ PFU). Animals infected by the IN route were first anesthetized with Halothane (Halocarbon Laboratories, River Edge, NJ), and two drops (≈100 μL) of the stock virus solution were instilled into the nares.

After infection, each mouse was examined daily for signs of illness, and 100 μL of blood was taken from the retro-orbital sinus for virus assay. Whole blood was diluted 1:10 in PBS in marked vials and frozen at −80°C for subsequent titration. If an animal died or appeared moribund, a necropsy was performed, and samples of the heart, lung, liver, spleen, kidney, adrenal glands, thymus, and cervical lymph nodes, as well as the brain, were collected and fixed in 10% buffered formalin for 48 hours, before being transferred to 70% ethanol for storage until processing.

Histopathologic and immunohistochemical methods. After fixation, the tissues were processed for routine paraffin embedding and sectioning. Histologic sections of 4–5 μm thickness were made and stained with hematoxylin and eosin (H&E) and evaluated microscopically.20 Selected tissue sections were also studied immunohistochemically, using a...
DHOV hyperimmune mouse ascitic fluid (1:100 dilution) as the primary antibody. A mouse-on-mouse ISO-IHC labeling kit (InnoGenex, San Ramos, CA) was used, according to the manufacturer’s instructions and a published protocol. The primary antibody was incubated with the sections at room temperature for 2 hours. Tissue sections from uninfected mice were used as negative controls. To further validate the specificity of the immunohistochemical staining, the primary antibody was replaced by an irrelevant mouse antibody (anti-West Nile virus), which yielded no staining.

RESULTS

Clinical manifestations and mortality. Within 24 hours after infection, the 10 mice in the IP group showed signs of illness (ruffled fur, emaciation, lethargy, and labored breathing). All of these animals were dead or moribund by the fourth day. Mice in the SC and IN groups appeared well during the first 2 days after infection (p.i.); by the third day, they had developed signs of illness similar to the IP group, and all were dead by Day 6 p.i. Figure 1 shows survival of mice in the three groups. The onset of illness and time of death were earlier in the IP group, but the clinical manifestations and ultimate outcome (death) were similar in the three groups.

Viremia. The mean daily virus titers (log_{10} PFU/mL) obtained on blood samples from mice in the three groups are shown in Figure 2. Except for a transient low-level viremia in one mouse in the IN group on the first day, the animals did not develop a detectable viremia until the second or third day p.i. In general, the highest levels of viremia were in the IP group; however, these animals were all dead or moribund by Day 4 p.i. Mice in the SC and IN groups had a slightly longer viremia until their death on the sixth day p.i.

Histopathologic and immunohistochemical studies. The brains of some animals in the IN group showed scattered mild neuronal degeneration, focally involving the cortex, hippocampus, cerebellum (Purkinje cells), and brainstem. This was rather inconsistent. The central white matter near the hippocampus, subcortical region of the cerebellum, and brainstem contained prominent vacuolation (Figure 3), caused by condensation and shrinkage of neurons.

The lungs of the IN-infected animals consistently showed multifocal hemorrhagic pneumonitis, with alveolar edema in some (Figure 4A). Many of the lungs showed thickening of the interstitium and the alveolar septa, with increased cellularity accompanied by scattered degenerating cells with fragmented nuclei (apoptosis; Figure 4B). These pulmonary changes may have been responsible for clinical signs of respiratory distress in the mice.

There was widespread, severe fibrinoid necrosis involving the lymphoid organs, namely, the thymus (Figure 5A), spleen (Figure 5B and C), and in a few cases when available, thoracic and abdominal lymph nodes. It was characterized by marked karyorrhexis of the lymphocytes (with cellular and nuclear condensation and fragmentation), resulting in a diffuse loss of lymphocytes, which was replaced by pink, amorphous fibrinoid material. Sometimes scattered tingible body macrophages in a “blue” background were observed in the spleen (Figure 5C), representing increased phagocytosis of apoptotic lymphocytes. In addition, areas containing activated lymphocytes were noted, characterized by marked enlargement of cells and nuclei, with prominent nucleoli. Some of the cells at the center of the lymphoid follicles and periarteriolar lymphatic sheath manifested necrosis, with preservation of the overall follicular architecture (follicular fibrinoid necrosis; Figure 5B).

The liver initially showed mild foamy change, which quickly progressed to multifocal necroapoptosis of hepatocytes with no inflammation (Figure 5D) and to severe coagulative zonal necrosis (Figure 5E).

The adrenal glands of the IN-infected animals showed only minimal changes, from a few scattered degenerating cells in the cortical layer to small necrotic foci (no more than three necrotic cells), to large areas of cortical necrosis (Figure 5F). No significant abnormalities were observed in the heart, pancreas, or kidney.

The histologic findings in the SC and IP groups were similar to those of the IN-infected mice. There was increased mononuclear infiltration, particularly in the brainstem. In the liver, multifocal hepatic necrosis was consistently seen; early microvesicular steatosis was seen in about one half of the animals. As noted above, there were no abnormalities seen in the heart, pancreas (only examined from a few animals), or kidney. Focal cortical cell necrosis was seen in adrenals in about one half of the SC- and IP-infected animals.
Immunohistochemically, specific Dhori viral antigen was detected in many organs, with almost all of the antigen in the nuclei of the cells (Figure 6). Although a few scattered neurons were found to be positive (Figure 6A), positive staining was more readily seen in the lungs, thymus, spleen, liver, adrenals (Figure 6B–F), and lymph nodes (data not shown). Of these latter organs, staining in the liver was the most intense (Figure 6E).

DISCUSSION

DHOV has a wide distribution in the Old World; it has been isolated in southern and eastern Europe, Central Asia, and Africa from ticks, mainly species of the genus *Hyalomma*. 18,21,22 Antibodies against DHOV have been detected in large domestic animals (camels, horses, and goats), as well as in humans living in endemic regions. 18,23 Five human cases of accidental aerosol infection with DHOV were reported among a group of laboratory workers in the former USSR. 24 These individuals developed an acute febrile illness characterized by headache, retro-orbital pain, myalgia, general weakness, and a prolonged period of convalescence; two of these people also developed mild symptoms of cranial nerve involvement with radicular syndrome. 24

The genus Thogotovirus currently is made up of four viruses: Thogoto, Dhori, Batken, and Araguari. 22,25 The first three viruses are thought to be tick-transmitted, less is known about the natural transmission of Araguari virus. 25 The thogotoviruses contain six or seven segments of linear, negative sense ssRNA, 14 and they share many morphologic, genetic, and biochemical similarities with the influenza viruses, including the ability to block the expression of mammalian interferon genes. 4,25–28 Results of this study indicate that the pathology of DHOV infection in mice is also similar to that produced by influenza viruses in mice. 7,29–34

Results of our study confirm the earlier findings of Felipe and others, 15 namely that DHOV infection in mice produces a systemic disease with involvement of multiple organs. Histopathologic changes were observed in the lungs, brain, thymus, thoracic and abdominal lymph nodes, liver, spleen, and to a lesser degree, in adrenals of the DHOV-infected mice. One of the most striking findings was the lymphotropism of the virus; DHOV-infected mice had extensive lymphocytic loss. The process manifested initially as numerous lymphocytes with nuclear fragmentation and tingible body macrophages (Figure 5C), and later as large areas of effacement of lymphoid areas with fibrinoid changes in the spleen and thymus (Figure 5A and B). It seems that apoptosis was involved in the earlier phase of the lymphocytic loss; as the infection progresses, more cells and multiple factors become involved, incorporating the fibrinoid necrosis as part of the pathogenesis. Furthermore, the virus seemed to either stimulate or activate lymphocytes, resulting in the appearance of large numbers of immunoblast-like cells in the spleen and abdominal lymph nodes (Figure 5C). In this regard, it resembled the pathology of Epstein-Barr virus infection. 35 Other authors 9,11–13 have reported similar findings in fatal human cases of H5N1 virus infection. Focal necrosis of lymph nodes and atrophy of the splenic white pulp have been observed in these patients, along with marked hemophagocytosis.

Experimental studies with influenza viruses in animal mod-
els have also reported multiple organ involvement. Intranasal inoculation of some avian and equine influenza viruses resulted in systemic disease involving the lungs, brain, liver, and kidney. Two recent studies in ferrets inoculated with H5N1 influenza virus found a similar infectivity pattern. In addition to the expected pulmonary pathology (acute bronchiolitis, bronchopneumonia and interstitial pneumonia with suppurative exudates, epithelial necrosis, and intra-alveolar edema), the infected ferrets also showed neuronal degeneration with inflammatory infiltration of the brain and hepatocellular necrosis. Using an avian influenza virus that had been selected for hepatotropism, Haller described the progressive histologic changes occurring in livers of the mice after IP inoculation of the virus. Infected mice developed scattered hepatic lesions ranging from focal to widespread liver cell necrosis, with almost no inflammatory cell infiltration. The hepatocellular necrosis was characterized by acidophilic changes, nuclear pyknotic and karyorrhectic changes, and sometimes Councilman bodies. Similar cytologic changes were observed in our DHOV-infected mice. Autopsies on fatal human cases of H5N1 virus infection have also revealed extensive liver involvement with central lobular necrosis.
necrosis, fatty changes, extra-medullary hematopoiesis, and activated Kupffer cells with hemophagocytic activity.\textsuperscript{9,11–13} Severe human cases of H5N1 virus infection also have biochemical evidence of liver dysfunction.\textsuperscript{1,36}

Based on the above comparisons, we believe that the DHOV mouse model has many similarities in terms of tissue distribution and pathology to H5N1 influenza virus infection in the mouse and ferret models. It also has many similarities to the pathology observed in the few human cases that have been examined histopathologically. Despite worldwide concern about a possible H5N1 influenza pandemic, little is yet known about the pathogenesis of the H5N1 virus or the mechanism of the severe human disease it causes. Although >120 human deaths caused by the H5N1 virus have been reported, the pathogenesis of the disease in people is still poorly understood, in part because it has been difficult to get tissues from fatal cases. In many of the Asian countries where most of the human deaths have occurred, cultural issues prevent autopsies of victims. In addition, the biosafety, security, and agricultural restrictions regulating use of the H5N1 virus in experimental animal studies have effectively limited such work to a few high security containment laboratories. Be-

\textbf{FIGURE 6.} Immunohistochemical detection for Dhori viral antigens in tissues of infected mice. \textbf{A}, Brain, a few scattered small neurons in the granule layer in the cerebellar cortex are positive. \textbf{B}, Lung, many interstitial cells are stained. \textbf{C}, Thymus, residual antigen positive cells in the largely necrotic area. \textbf{D}, Spleen, a few splenic macrophages and lymphocytes are positive. \textbf{E}, Liver, many antigen-positive hepatocytes. \textbf{F}, Adrenal, a focus of positive antigen stain in cortical cells. Note that the positive antigen staining (red color) is mostly intranuclear.
cause of the taxonomic relationship of DHOV to the influenza viruses, its BSL-2 status, and its similar pathology in mice, the DHOV mouse model may offer a lower-cost, safer, and realistic animal model for studies on the pathogenesis and management of H5N1 influenza virus infection.

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