An Animal Model for Studying the Pathogenesis of Chikungunya Virus Infection

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Abstract. Newborn and 14-day-old mice inoculated subcutaneously with chikungunya virus (CHIKV) developed lethargy, difficulty walking, dragging of hind limbs, and reduced weight gain within 7–10 days after infection (PI). During the initial 6–7 days PI, the animals had viremia; high levels (10^6–10^8 PFU) of CHIKV were also present in leg muscle. The virus persisted in muscle for several days after viremia disappeared. The major histopathologic changes were in skeletal muscle, which were focal necrosis and inflammation, followed by fibrosis and dystrophic calcification. Some mice also showed dystrophic calcification in the joint cartilage, but there were few deaths, and most of the animals eventually recovered. CHIKV antigen was shown by immunohistochemistry in the muscle for several weeks after infection. Based on the clinical and pathologic similarities with CHIKV infection in humans, young ICR and CD-1 mice offer a useful and realistic model for further study of the pathogenesis and treatment of CHIKV infection.

INTRODUCTION

Chikungunya virus (CHIKV), the etiologic agent of chikungunya fever (CHIKF), is a mosquito-transmitted alphavirus belonging to the family Togaviridae. It is included within the Semliki Forest complex of alphviruses, based on its antigenic and phylogenetic relationships.1 CHIKV was first isolated during an epidemic of dengue-like disease in Tanganyika (Tanzania) in 1952.2 The word “chikungunya” is Swahili and means “that which bends up”; it was the name used by indigenous people of the region to describe the characteristic posture assumed by patients afflicted with the severe muscle and joint pains associated with this illness.3,4 The current known geographic distribution of CHIKV includes sub-Saharan Africa, India, islands in the Indian Ocean, Southeast Asia, and the Philippines.3–5 Carey6 presented historic evidence suggesting that CHIKF probably has occurred sporadically in India and Southeast Asia for at least 200 years, although the clinical and epidemiologic similarities of CHIKF and dengue fever make precise differentiation of the two diseases difficult without laboratory confirmation.

During the past several years, there has been renewed interest in CHIKV because of large outbreaks of the disease in India, Sri Lanka, and a number of smaller islands in the Indian Ocean4,5 and by the appearance of cases of CHIKF in Europe and the Americas among tourists and other travelers returning from the affected regions.4,7 During the summer of 2007, a small outbreak occurred in Italy, apparently the result of an introduction from a traveler visiting from India.8 These recent outbreaks illustrate the ability of certain vector-borne viral diseases to be introduced into non-epidemic regions, when the appropriate ecologic conditions for their transmission exist.9

A number of alphaviruses within the Semliki Forest complex produce an illness in humans or equines characterized by fever, arthralgia, and rash. This group includes chikungunya, o’nyong-nyong, Getah (horses), Ross River, Mayaro, and Barmah Forest viruses.3,10 Each of these viruses has a unique geographic distribution and mosquito vector(s), but the illnesses associated with them are clinically very similar. The myalgia and arthralgia associated with these alphavirus infections tends to be transient (5–7 days), but in some patients, it persists longer or is recurrent.4,10

The pathogenesis of CHIKV in humans and the mechanism by which it causes arthritic disease is poorly understood. Ross River virus (RRV) is the best studied of the arthritogenic alphaviruses; and a mouse model of RRV-induced arthritis/arthralgia has been used to study the pathology and immunology of the disease.11–15 In the RRV mouse model, infection results in severe inflammation and necrosis of skeletal muscle. To determine whether the pathogenesis of CHIKV is similar and to gain insight into the cause of the severe muscle and joint pain observed in humans with the infection, a series of experiments was carried out in mice experimentally infected with a recent epidemic strain of CHIKV. This report describes our results.

MATERIALS AND METHODS

Animals. Two outbred mouse lines were used in this study; the Institute for Cancer Research (ICR) strain obtained from Harlan Sprague-Dawley (Indianapolis, IN) and the CD-1 strain obtained from Charles River Laboratories (Wilmington, MA). Mice were cared for in accordance with guidelines of the Committee on Care and Use of Laboratory Animals (Institute for Laboratory Animal Resources National Research Council, Washington, DC). All experiments were conducted in an animal biosafety level 3 (ABSL-3) facility under a protocol approved by the University of Texas Medical Branch (UTMB) Institutional Animal Care and Use Committee (IACUC).

Virus. CHIKV strain LR 2006-OPY1 was used to infect the mice. The virus isolate was obtained from a CHIKF patient during an outbreak on La Reunion Island in 2006.7 The virus was kindly provided to us by Dr. Remi Charrel, Emerging Virus Unit, Faculty of Medicine, University of the Mediterranean, Marseilles, France; it had been passed five times in Vero cell cultures.

Virus assay. Samples of mouse blood, brain, and skeletal muscle were titrated by plaque assay in monolayer cultures of Vero cells.17 Tissues were first triturated in individual sterile 2-mL glass TenBroeck tissue grinders (Kimble/Kontes, Vineland, NJ) in 1.0 mL of diluent (phosphate-buffered saline with 10% fetal bovine serum) to prepare ~20% (wt/vol) suspensions. After centrifugation, serial 10-fold dilutions from 10^{-1} to 10^{-6} of the tissue supernatants and of the blood samples
RESULTS

Clinical manifestations and mortality. Newborn mice inoculated subcutaneously with CHIKV showed signs of illness (lethargy, loss of balance and difficulty walking, dragging of the hind limbs, and hair loss around the inoculation site on the back) 7–10 days after infection. In CD-1 mice, the alopecia on the back was more severe and sometimes included skin vesicles. In the ICR mice, alopecia was observed, but it was less diffuse and without blistering of the skin. The clinical signs in the CD-1 mice were also more severe, although the range of clinical manifestations were similar for both groups. Mortality from CHIKV infection was low, and the surviving mice eventually recovered hair growth and use of their hind limbs within 6 weeks of infection. Figure 1 shows the mortality in newborn ICR and CD-1 mice infected with CHIKV. The fatality rate in the newborn CD-1 mice was 17%, whereas the ICR mice had a slightly lower rate of 8%.

Decreased weight gain was observed in the newborn mice infected with CHIKV as shown in Figure 2. The failure to gain weight was greater in the CD-1 mice than in the ICR mice. In CD-1 mice, there was a significant difference in weight gain from Day 5 until Day 13 between the control and infected mice (Figure 2B). On Day 13 after infection (PI), the mean difference between the infected and control groups was 1.3 g, a 30% difference. In the ICR mice, failure to gain weight occurred earlier, from Days 2 to 6, and was transient; the infected and control ICR mice were gaining weight at a similar rate by Day 14 PI (Figure 2A).

Virus titrations. The mean daily CHIKV titers (PFU/mL) obtained from titrations of blood and 10% homogenates of brain and leg muscle are shown in Figure 3. All of the mice developed viremia that lasted 5–8 days, depending on their age at the time of infection. CHIKV titers were higher and persisted longer in the newborn mice (Figures 3A and B), but the levels of viremia were not significantly different between the newborn CD-1 and ICR mice (P = 0.18). The 14-day-old mice had a shorter period of detectable viremia (Figures 3C and D) than the newborn mice. Viremia ended by Day 5 PI in the 14-day-old ICR mice and by Day 7 PI in CD-1 mice of the same age.

The levels of infectious CHIKV in the brain were markedly different in the newborn and the 14-day-old mice. Mean levels of virus in brains of the newborn mice (Figures 3A and B) were lower than titers in blood for the first 3 days PI; however, afterward they closely paralleled each other. CHIKV was detected in brains of all of the newborn mice sampled...
during the first 9 or 10 days, but it was absent from brains of newborn mice sampled after Day 10. In contrast, CHIKV was only detected in the brains of three of the 14-day-old mice for brief periods of 1–2 days PI (Figures 3C and D). These data indicate that virus was present in the brains of the newborn mice for 9 or 10 days after infection but that it was subsequently cleared, and most of the newborn mice (83% of CD-1 and 92% of ICR) survived.

Overall, CHIKV titers were highest and persisted longest in leg muscle (Figures 3A–D). Mean CHIKV titers in leg muscle were not significantly different among the newborn CD-1 and ICR mice ($P = 0.16$), although the mean titers in
TABLE 1

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>ICR mice</th>
<th>CD-1 mice</th>
<th>HI antibody positive* / Total mice tested</th>
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<tr>
<td>7</td>
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* Positive = HI titer ≥ 1:20.

the 14-day-old CD-1 mice were slightly higher than those in ICR mice of the same age. The higher levels of virus in leg muscle of the 14-day old mice compared with those in the blood and brain of the same mice indicate a tropism of CHIKV for skeletal muscle.

**Antibody response.** Blood was collected from the newborn mice before death on Days 1–12 and on Days 15, 17, and 22 PI. The sera from these samples were subsequently tested for HI antibodies to CHIKV antigen, as described before. Table 1 shows the antibody prevalence (number of animals with HI antibody titers ≥ 1:20/total animals tested) of the ICR and CD-1 mice from Day 7 onward. The HI antibody response of the newborn mice to CHIKV infection was delayed; in comparison, older ICR mice (≥ 4 weeks old) usually developed HI antibodies within 5–7 days PI (unpublished data). CHIKV HI antibody was first detected on Day 9 PI in the ICR mice, but only some of the animals seroconverted. The HI antibody response of the CD-1 mice was even slower; the first seroconversion was not detected until 12 days PI. When tested 22 days PI, only ~62% of the newborn mice had developed specific HI antibodies (Table 1).

**Histopathology and immunohistochemical analysis.** A full histopathologic examination of the various organ systems was only done on tissue samples from the newborn ICR mice. For the CD-1 mice and the 14-day-old ICR mice, just skin and hind legs were examined. H&E-stained sections of small intestine, colon, kidneys, and adrenals from the infected newborn ICR mice appeared normal. There were no significant abnormalities observed in the brain of the newborn ICR mice. Occasional neurons showed degenerative changes in some animals, but the pattern of injury was more consistent with a postmortem artifact rather than true pathologic changes. In the heart, there were no abnormalities involving the endocardium or the pericardium, and for the most part, myocardium. One animal on Day 7 and another on Day 8 PI showed focal dystrophic calcification in the myocardium. The significance of this could not be determined, because it occurred in only two mice, and there were no active injuries, such as muscle necrosis or inflammation, observed in these or the other mice. Starting from Day 3 PI, rare foci of spotty hepatocytic necrosis were noted scattered in the parenchyma, without a specific zonal distribution or changes in severity. Between Days 2 and 7 PI, there was a mild increase in macrophages containing cellular nuclear debris (tingible body macrophages) in the spleen, as well as a mild increase in lymphocytic necrosis. These changes appeared to be transient, because no histologic abnormalities were identified in spleens of the newborn ICR mice examined afterward.

The most significant and consistent pathologic changes in the newborn ICR mice were in the skeletal muscle and adjacent connective tissues (Figure 4). From Days 2 to 4 PI, there were non-specific changes, including mild shrinkage/atrophy of focal muscle bundles, characterized by a slight increase in nuclear density, but with an absence of inflammatory cellular infiltration (Figure 4A). Starting on Day 5 PI, there was increasing muscle necrosis, as foci appeared in which the individual muscle bundles became "wavy" and contained aggregates of small dark granules (nuclear dust) because of destruction of nuclei. There was also minimal mononuclear inflammatory cellular infiltration. The peri- and intramuscular brown fat became more abundant, with focal necrosis and dystrophic calcification. These changes became most severe on Day 7 PI and beyond (Figures 4B and C). In some specimens, a prominent lymph node was also present, which showed brisk lymphocytic necrosis. Throughout the course of the infection, these findings were always focal, involving only portions of the muscle tissue, usually adjacent to the joints. There was no preference in distribution regarding front or rear leg or left or right side. Two newborn ICR mice were examined 17 days PI; their legs showed residual foci of prominent muscle necrosis, with calcification, and prominent fibrosis (scar formation; Figure 4D). Immunohistochemically, CHIKV antigens were not shown in the brain of the newborn ICR mice. Antigen staining started to appear focally in leg skeletal muscle on Day 3 of infection (Figure 4E). Subsequently, the antigen positivity in muscle increased in intensity and distribution, and it was usually associated with muscle degeneration and necrosis (Figure 4F). In some mice, the skin on the back also showed inflammation of the dermis, with necrosis and focal calcification of the subcutaneous muscle (Figures 4G). This was associated with positive viral antigen staining (Figure 4H).

The histopathologic changes in the hind limbs were examined in newborn and 14-day-old CD-1 mice and in the 14-day-old ICR mice. In general, the pathology seen in the skeletal muscle was more severe in newborn CD-1 mice compared with newborn ICR mice. In the newborn CD-1 mice, each limb specimen showed extensive multifocal myositis with necrosis. In addition, the skin exhibited extensive necrosis in the subcutaneous muscle, with calcification. Similar dystrophic calcifications were also present in the joint cartilage.

**DISCUSSION**

CHIKV has been responsible for a number of recent outbreaks involving an estimated 266,000 people on the island of La Reunion in 2005–2006, 1.4 million people in India in 2006–2007, and > 200 people in Italy in the summer of 2007. As noted earlier, CHIKV infection generally causes an acute self-limited illness with clinical symptoms of fever, rash, and incapacitating joint and muscle pain. Direct mortality from CHIKV infection is uncommon, although death has been reported, particularly among older patients with other medical conditions. During the recent La Reunion outbreak, there were ~250 deaths (0.17%), mostly in elderly people. Hemorrhagic manifestations and seizures have also been reported occasionally, usually among children. During the recent...
FIGURE 4. Histopathologic changes in skeletal muscle and skin of newborn ICR mice after infection with CHIKV. A, Skeletal muscle taken 4 days PI shows focal individual muscle fiber wavy changes and necrosis (arrowhead). B, Skeletal muscle taken 7 days PI, showing focal, but severe muscle necrosis and inflammation (purple-colored areas). C, Skeletal muscle taken 9 days PI, with prominent muscle necrosis, atrophy, and infiltration by mononuclear inflammatory cells and neutrophils. D, Skeletal muscle taken 17 days PI shows focal muscle necrosis, with prominent dystrophic calcification (arrowheads). E, CHIKV antigen in focal degenerating muscle fibers (arrowheads) in skeletal muscle taken 3 days PI. F, More intensive viral antigen staining of skeletal muscle was seen at a later stage, associated with inflammation and degeneration (red-colored area). G, Skin tissue taken 17 days PI, showing marked inflammatory cellular infiltration of the upper dermis and necrosis of the subcutaneous muscle bundles (bottom), with calcification (arrowheads). H, Viral antigen staining in the subcutaneous muscle bundles of skin. A–D and G, H&E stain; E, F, and H, immunohistochemical stain.
CHIKF outbreak in La Reunion, mother-to-child transmission was reported among neonates. With the increased geographic distribution of this virus and its potential for more spread to new regions, it is important to understand the pathogenesis of CHIKV and to develop more targeted and effective treatment strategies.

Apart from the clinical manifestations of CHIKV infection, little is known about the pathogenesis of the virus in humans. A recent clinical study of CHIKF patients reported the occurrence of rhabdomyolysis with elevated creatine phosphokinase levels. During the La Reunion outbreak, muscle biopsies were done on two CHIKF patients. The first was done on the quadriceps muscle of a patient during the acute phase of his illness; histologic studies showed atrophy and necrosis of scattered muscle fibers with a limited number of infiltrating inflammatory cells. IHC examination of the tissue showed CHIKV antigen at the periphery of muscle fibers, either as single cells or groups of immunoreactive cells. Muscle fibers were not found positive for CHIKV antigen in this biopsy. The second patient was biopsied 3 months after her initial infection, during a recurrent episode of fever, myalgia, and arthralgia. The quadriceps muscle of this patient showed extensive interstitial mixed acute and chronic inflammation with large areas of necrosis and collagenosis. CHIKV antigen was also visualized by IHC in immunoreactive CD68 and CD3 cells of the second patient but at a lower magnitude than in the first patient. The histopathologic and IHC findings in these two human cases are similar to our findings in the CHIKV-infected mice, except that there was less inflammation in the mouse muscle, and CHIKV antigen was seen within degenerating muscle cells in the mice.

Our results with CHIKV infection of newborn and young mice are also comparable to previous reports of the pathogenesis of RRV infection in young mice. Young (14–21 day old) mice subcutaneously infected with RRV develop hind limb weakness, muscle wasting, weight loss, and mononuclear cell infiltrates into skeletal muscle. The primary sites of RRV replication in these infected mice occur within bone- and joint-associated connective tissue and skeletal muscle. In other laboratory studies, we also infected newborn and 14-day-old ICR mice subcutaneously with Mayaro virus; the resulting skeletal muscle and connective tissue changes were similar to those described above for CHIKV and RRV (unpublished data).

The behavior of CHIKV in the brain of the newborn mice (Figures 3A and B) is noteworthy. Our data indicate that the virus entered the brain and initially replicated but that replication was subsequently restricted. By the 10th day, no virus could be detected in the brain by culture. Likewise, no significant histopathology or evidence of viral antigen (by immunohistochemistry) was seen in serial brain specimens taken from the infected newborn mice during the 12-day sampling period. The phenomenon of age-dependent susceptibility to central nervous system invasion and to fatal encephalitis has been described before with Semliki Forest virus and RRV, and with Sindbis virus, a member of the western equine encephalitis antigenic complex of alphaviruses. In newborn mice, these viruses replicate to high titer in the brain, sometimes causing death within 3–5 days. However, in older mice, virus replication in the brain is restricted, and mice often recover. There are also strain differences in neurovirulence among these three alphaviruses.

In conclusion, the clinical similarities between CHIKV and RRV infection in humans and their apparent histopathologic similarities in mice suggest that the mouse model described in this publication represents a useful and realistic system for further studies of the pathogenesis and treatment of CHIKV infection in humans.

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