Comparative Pathogenesis of Epidemic and Enzootic Chikungunya Viruses in a Pregnant Rhesus Macaque Model

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Abstract. Since 2004, an East African genotype of Chikungunya virus (CHIKV) has emerged, causing significant epidemics of an arthralgic syndrome. In addition, this virus has been associated for the first time with neonatal transmission and neurological complications. In the current study, pregnant Rhesus macaques were inoculated with an enzootic or epidemic strain of CHIKV to compare pathogenesis and transplacental transmission potential. Viremias were similar for both strains and peaked at 2–3 days post-inoculation (dpi). Viral RNA was detected at necropsy at 21 dpi in maternal lymphoid, joint-associated, and spinal cord tissues. The absence of detectable viral RNA and the lack of germinal center development in fetuses indicated that transplacental transmission did not occur. Neutralizing antibodies were detected in all dams and fetuses. Our study establishes a non-human primate model for evaluating vaccines and antiviral therapies and indicates that Rhesus macaques could serve as a competent enzootic reservoir.

INTRODUCTION

Chikungunya virus (*Togaviridae*, *Alphavirus*; CHIKV) is transmitted in nature among humans or non-human primates and mosquitoes and typically causes debilitating arthritogenic syndromes that can last for weeks or months.^{1–3} CHIKV was first isolated from the serum of a febrile human in Tanganyika (Tanzania) in 1952 during an epidemic of dengue-like illness.^{4,5} Over the past 50 years, CHIKV has expanded its geographic range into eastern Africa and central and southeastern Asia, where it has been associated with an increasing frequency and intensity of outbreaks.^{4–16} Sequence analysis of the E1 gene of CHIKV isolates has indicated the presence of three distinct CHIKV clades including Asian, West African, and East/Central/South African genotypes (ECSA).^{17–21} In West Africa, CHIKV is transmitted mainly among several *Aedes* spp. mosquitoes and non-human primates, but occasionally it infects other wild animals and spills over to cause small sporadic outbreaks in humans.²² The recent 2004–2010 outbreak has involved a monophyletic lineage virus that diverged from the ECSA clade.^{17–21} This novel East African strain was first observed in Kenya and later dispersed to a number of Indian Ocean islands, the Indian subcontinent, and Southeast Asia. The emergence of this strain sparked significant epidemics, most notably in India, where more than 2 million human cases have been reported.²³ The virus also established a focal autochthonous outbreak in Italy, the first report of CHIKV activity in Europe.²⁴

In addition to the magnitude of the epidemic, previously unreported neurological manifestations in adults and fetal encephalopathy have been reported.^{25–29} CHIKV infection of the human central nervous system was first described in the 1960s.^{30,31} However, novel neurological syndromes, such as seizures, meningoencephalopathy, myelitis, and choroiditis, have only been reported during the recent outbreaks.^{25–29} In addition to neurological manifestations, the first observations of

pre-partum neonatal transmission were associated with infection with this novel CHIKV strain; however, it remains to be determined whether these novel disease syndromes observed during the 2004–2010 outbreak reflect a change in tissue tropism or an increase in pathogenesis, or if they are simply indicative of the magnitude of the recent outbreaks and improved case reporting.^{32–35} Additionally, the association of CHIKV disease in neonates with mothers infected during gestation largely has been epidemiological, without a case-controlled study and without assessment of the potential for transplacental viral passage under experimental conditions.^{32–35} Although CHIKV mouse models exhibit many of the disease symptoms observed during human infection, the reproductive, neurological, and immune systems of non-human primates are more similar to humans than murine systems. Therefore, pregnant macaques may serve as an ideal model for overcoming limitations of rodent models.^{36–41}

In the current study, an epidemic East African strain isolated from an infected traveler from India in 2006²¹ and an enzootic strain isolated in West Africa were selected for inoculation. Rhesus macaques in the third trimester of pregnancy were infected subcutaneously with a biologically relevant dose of either the epidemic or the enzootic CHIKV strains to replicate natural transmission of the virus by mosquito bite. The macaques were held for 21 days post-inoculation (dpi) to allow adequate time for observation of disease presentations, viremia kinetics, tissue tropisms, and humoral immune responses in both dams and fetuses. Fetal condition was monitored throughout infection, and transplacental transmission was assessed at 21 dpi by examining whether viral RNA was present in the placenta and fetal tissues as well as by assessing follicle development in fetal lymph nodes. Viral infection outcomes and host responses were examined to determine pathophysiological differences between the epidemic and enzootic CHIKV strains. In addition to comparing the pathogenicity of the recently emergent ECSA strain of CHIKV with a historical enzootic isolate and assessing the potential for Rhesus macaques to serve as amplifying hosts of this virus in Asia, this study serves to develop an animal model of CHIKV disease for evaluating the applications of rationally engineered vaccines and antivirals in pregnant women.

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MATERIALS AND METHODS

Animals. Six pregnant colony-bred female Rhesus macaques (*Macaca mulatta*) housed at the animal facility at the California National Primate Research Center, aged 7–15 years and at gestational days 121–132, were used for CHIKV inoculation. Animals were housed and maintained according to regulations and guidelines set forth by the University of California, Davis, Institutional Animal Care and Use Committee (IACUC) under an approved IACUC protocol. All animals were screened for viral infection status before inoculation. All monkeys were negative for Simian T-cell lymphotropic/leukemia virus type 1 (STLV-1), Simian retrovirus (SRV), and Simian retrovirus (SIV). Two animals in each group were positive for Simian foamy virus (SFV), Cytomegalovirus (CMV), and Herpes B virus; one animal in each group was positive for only SFV and CMV. All animals were bled 2 days before viral inoculation to confirm the absence of CHIKV neutralizing antibodies.

Cell lines and viruses. African green monkey kidney (Vero), baby hamster kidney (BHK-21), and *Ae. albopictus* (C6/36) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Vero and C6/36) and minimal essential medium (MEM) (BHK-21) supplemented with 5% fetal bovine sera (FBS) and antibiotics, and then were incubated in a humidified environment with 5% CO₂ at 37°C and 28°C, respectively. Rhesus macaques were inoculated with a West African strain of CHIKV (37997) isolated from a mosquito pool during enzootic transmission in Senegal in 1983 (obtained from the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention reference collection) and a human CHIKV strain (DHS-4263) isolated by the California Department of Public Health from a traveler infected in India during the epidemic in 2006.⁴² The CHIKV epidemic strain was originally isolated in rabbit keratinocyte cells (RK), passed one time in BHK-21, and passed one time in Vero cells before experimentation. The CHIKV enzootic strain was isolated in mosquito cells (*Ae. pseudoscutellaris*; AP), passed one time in Vero cells, and passed one time in BHK-21 cells. Both virus stocks were prepared by inoculating C6/36 cells at a multiplicity of infection (MOI) of 0.1 and were harvested at 2 days. Groups of three pregnant macaques each were inoculated subcutaneously on the right upper arm with a 100- μ L inoculum comprising 1,000–10,000 plaque forming units (PFU) of either the epidemic East African or the enzootic West African strain. Animals were fasted 3–4 hours before sedation with ketamine (5–20 mg/kg body weight) by intramuscular injection for viral inoculation, physical examination, and blood collection.

Clinical signs and fetal viability. Macaques were monitored daily for clinical signs of CHIKV disease, including fever, pain as evidenced by reduced mobility, joint swelling, nose/gum bleeding, rash, and peripheral lymphadenopathy. These examinations included monitoring body weight, taking rectal temperature, and measuring the circumference and local temperature of joints (wrists and ankles). The joint circumference was assessed daily across wrists and ankles. To ensure consistency, the sites for each measurement were marked in indelible ink. Local joint temperatures were detected using a DermaTemp DT-1001RS Remote Sensor Model (Exergen, Watertown, MA). An Ultrasonic Doppler Flow Detector (Parks Medical Electronics, Inc., Aloha, OR) was used to monitor fetal heart rate as a measure of fetal viability.

Tissue collection and processing. Peripheral blood samples were collected 2 days before virus inoculation and daily up to 21 dpi by venipuncture. Blood samples were collected with ethylenediaminetetraacetic acid (EDTA) and heparin-impregnated tubes and centrifuged at 1,000 $\times$ g for 10 minutes to isolate plasma and buffy coats. Plasma was frozen at –80°C until assayed for virus titer (quantified by Vero cell plaque assay), viral RNA, anti-CHIKV antibody levels, plasma chemistry, and cytokine profiles. Whole-blood samples were collected with EDTA-coated tubes for blood cell counts. Dams and fetuses were euthanized at 21 dpi by deep ketamine anesthesia followed by intravenous (IV) barbiturate overdose using sodium pentobarbital at 60 mg/kg. Maternal and fetal tissues were collected and assayed to determine the viral load and extent of histopathology, including blood, joint-associated skeletal muscle/synovium/epiphysis/connective tissues, brain, spinal cord, heart, lung, kidney, spleen, liver, bone marrow, joints, and lymph nodes (axillary and inguinal lymph nodes). In addition, mammary glands, placental tissue, and skin and brachial lymph nodes from arms proximal and distal to the inoculation site were collected from dams. Maternal and fetal tissues were collected in 10% buffered formalin for histopathology, were collected in 4% paraformaldehyde for electron microscopy, and were snap-frozen in liquid nitrogen for viral RNA detection and infectious virus isolation.

Histopathology. Maternal and fetal tissues were fixed in 10% neutral buffered formalin. Selected dam and fetal tissues were collected, including skin from arms (inoculation side and opposite arm), joints, joint-associated skeletal muscle and connective tissue, synovium, epiphysis, bone marrow, brain, spinal cord, heart, lung, kidney, spleen, liver, mammary tissue, vagina, brachial lymph nodes close to the inoculation site and from opposite arm, axillary and inguinal lymph nodes, and placenta. These tissues were routinely processed and embedded in paraffin, sectioned at 7 μ m, and mounted on positive-charged glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). Tissue sections were stained with hematoxylin and eosin (HE).

Electron microscopy. For transmission electron microscopy, 4% paraformaldehyde-fixed tissues were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 90 minutes at room temperature, rinsed, and dehydrated in ascending concentrations of acetone from 30% to 100% for 10 minutes for each step, with three changes of acetone at 95% and two changes at 100%. Infiltration was carried out in a microwave (Pelco 34700 BioWave; Ted Pella Inc., Redding, CA) for 3 minutes for each step under vacuum and between 250 and 450 W.⁴³ Tissues were placed into capsules and polymerized at 70°C overnight. Blocks were trimmed and ultrathin-sectioned using a Diatome diamond knife (Hatfield, PA) at 60–90 nm on a Leica UTC ultramicrotome (Leica Ultracut UCT, Vienna, Austria). The sections were post-stained by standard protocol for transmission electron microscopy using uranyl acetate in 70% lead citrate. Images were taken on a Philips CM120 (FEI Company, Hillsboro, OR) with a GATAN MegaScan digital camera (Pleasanton, CA) at the Diagnostic and Research Electron Microscopy Laboratory, University of California, Davis (Davis, CA).

Quantification of viral RNA. Maternal and fetal tissue samples from CHIKV-inoculated dams were weighed individually and then homogenized in 1 mL of DMEM with 5% FBS on ice before RNA extraction and inoculation of cell cultures for viral isolation. CHIKV RNA from homogenized maternal/fetal tissues, plasma, and buffy coats was extracted

by MagMAX RNA isolation kits and a MagMAX Express magnetic particle processor (Ambion, Foster City, CA) according to the manufacturer's instructions. Viral RNA was detected by TaqMan One-Step reverse transcription polymerase chain reaction (RT-PCR) Master Mix Reagents Kits with an ABI 7500 instrument (Applied Biosystems, Foster City, CA) using previously described CHIKV-specific primers and probe (forward primer 5'-AAGCTYCGCGTCCTTTACCAAG-3', reverse primer 5'-CCAAATTGTCCYGGTCTTCCT-3', and probe 5'-FAM-CCAATGTCYTCMGCCTGGACACCTTT-TAMRA-3') based on the manufacturer's recommendations.⁴⁴ RNA was extracted from serially diluted samples of enzootic and epidemic CHIKV stocks (range = 0–6 log₁₀ PFU/mL) and assayed by the method described above to establish a standard curve for quantification. CHIKV real-time RT-PCR positive tissues were blind-passaged two times in C6/36 cells (7 days per passage). RNA was extracted from the supernatants of blind passages using MagMAX viral RNA isolation kits, examined for the presence of CHIKV RNA as described above, and titrated for infectious virus by plaque assay on Vero cells.

Enzyme-linked immunosorbent assay. Ninety-six-well plates were seeded with Vero cells and infected with the CHIKV epidemic strain at an MOI of 0.1. Negative control plates were seeded with uninfected Vero cells. Plates were fixed 20 hours post-infection (hpi) by treatment with fixative solution (0.2% w/v of bovine serum albumin and 20% v/v of acetone in 1× phosphate-buffered saline; PBS) for 1 hour at 4°C. After fixation, plates were air-dried overnight and stored at –20°C. Enzyme-linked immunosorbent assay (ELISA) plates were blocked with TENTC buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 0.15 M NaCl, 0.05% v/v Tween 20, and 0.2% casein) at 28°C for 1 hour and then treated with 2-fold serial dilutions (1:50 to 1:10,240) of macaque plasma (diluted in blocking buffer) for 1 hour at 28°C. Unbound antibody was removed by washing plates three times in PBST washing buffer (1× phosphate buffered saline (PBS) with 0.05% v/v Tween 20, pH 7.2). Horse radish peroxidase (HRP)-conjugated goat anti-human immunoglobulin G (IgG)/IgM/IgA Ab (KPL, Gaithersburg, MD) was diluted 1:6,000 in blocking buffer and added to the plates before incubation for 1 hour at 28°C. The plates were again washed, and bound antibody was detected by the addition of substrate buffer (1 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 3 mM H₂O₂ in a buffer prepared by mixing 0.1 M citric acid with 0.2 M Na₂HPO₄ to give a pH of 4.2). Reactions were incubated for 30 minutes in the dark and read at 405 nM. All samples were run in duplicate. Anti-CHIKV Ab titers were determined by taking the reciprocal of the highest plasma dilution to have at least a 2-fold higher optical density (OD) on CHIKV-infected plates than on uninfected control plates. CHIKV-positive human sera served as positive control (provided by the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO), and uninfected monkey plasma served as negative control.

Measurement of neutralizing antibody using a plaque reduction neutralization test. Six-well plates were seeded with Vero cells in DMEM containing 5% FBS and incubated until confluent. Plasma was heat-inactivated at 56°C for 30 minutes. Serial 2-fold dilutions of plasma were prepared in DMEM with 5% FBS (1:40–1:10,240). An equivalent volume of the homologous or heterologous CHIKV suspension comprising 100 PFU was added to the diluted plasma and incubated

for 60 minutes at 37°C. After pre-incubation, the plasma-virus mixtures were added to cells in the six-well plate and incubated for 60 minutes. The inoculum was then removed, the monolayers were overlaid with 2 mL per well of Ye-Lah medium, 3% sodium bicarbonate, and 1% agarose, and they were incubated for 1 day at 37°C. A second overlay was added to each well containing 3% neutral red. Plaques were visualized after incubation for 1 day. Plaque reduction neutralization test (PRNT₈₀) values were calculated by taking the reciprocal of the lowest dilution to reduce ≥ 80% of plaques. All plasma samples were tested in duplicate.

Cytokine profiles. EDTA-treated maternal plasma samples collected before CHIKV inoculation (baseline is day –2) and at 3, 6, and 9 dpi were examined for cytokine profiles using a Milliplex Map non-human primate cytokine kit (Millipore, Danvers, MA) and a BioPlex instrument (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Analyte panels of the Milliplex Map kit include CD40 Ligand, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12/23 (p40), IL-13, IL-15, IL-17, IL-18, IL-1ra, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, MCP-1, MIP-1α, MIP-1β, TGF-α, TNF-α, and VEGF.

Statistics. Data after viral inoculation were compared using an analysis of variance (ANOVA), with viral strain and time as main effects and means compared by Fisher's least significant difference multiple comparison test.⁴⁵ Repeated measures ANOVA was not used because of the relatively large variation among macaques during the time course and limit of detection, which precluded reliable statistical analyses. PRNT data were transformed by natural logarithm (*y*) before analysis, and the results are expressed as the inverse of the geometric mean titer.

RESULTS

Disease presentation and fetal viability. All CHIKV-inoculated macaques showed intermittent fever of variable duration with mean body temperatures significantly elevated over time ($F = 1.69$; $df = 22, 92$; $P = 0.04$). Significant differences were observed in the timing of fever between strains (time by strain interaction: $F = 1.82$; $df = 22, 92$; $P = 0.03$), peaking at 39.4°C on 2 and 17 dpi for the epidemic strain and at 39.7°C on 19 dpi for the enzootic strain. Joint (wrist and/or ankle) swelling was observed in two macaques inoculated with the enzootic strain and one macaque inoculated with the epidemic strain. An increase in local joint temperature (1.1–9.4°C increase in the epidemic group and 5–6.1°C increase in the enzootic group) and joint circumference (5- to 10-mm increase in the epidemic group and 9- to 11-mm increase in the enzootic group) were used as indicators of local joint swelling. One enzootic CHIKV-infected macaque exhibited an erythematous rash and severe leg swelling at 7 dpi (Figure 1).

Fetal viability was assessed daily from 2 days before CHIKV inoculation by monitoring heart rate. A normal range for fetal heart rate was determined using both the heart rate data obtained before virus inoculation and evaluation of archival data for similar aged Rhesus macaque fetuses collected at the California National Primate Research Center. Average fetal heart rates in each cohort were within normal ranges after CHIKV inoculation. However, lower heart rates were recorded for individual fetuses within 1 day of peak viremia and then again at approximately 14 and 18 dpi. There were no significant differences in fetal heart rates ($P > 0.05$) between the viral strains (Figure 2).

FIGURE 1. Skin. Erythematous rash and muscle/joint swelling in Chikungunya virus enzootic-infected macaque. This figure appears in color at www.ajtmh.org.

Systemic viremia. All macaques in both groups developed serum viremias during days 1–5 after CHIKV inoculation, with peak viral titers of 5–6 $\log_{10}$ PFU/mL recorded at 2–3 dpi (Figure 3). When tested by two-way ANOVA, mean viral titers on 1–4 dpi for the endemic strain (4.4 $\log_{10}$ PFU/mL) were significantly greater ($F = 4.7$; $df = 1, 16$; $P = 0.04$) than the epidemic strain (3.7 $\log_{10}$ PFU/mL); however, the viremia temporal pattern was consistent, because the viral $\times$ time interaction term was not significant ($P > 0.05$). Notably, the macaque infected with the enzootic strain with the highest peak viral titer (6.4 $\log_{10}$ PFU/mL) also exhibited the most pronounced rash and joint swelling (shown in Figure 1).

Blood cell count and plasma chemistry. Complete blood cell counts and serum chemistry were performed at –2, 0, 3, 6, 9, 12, 15, and 21 dpi. Leukocyte counts obtained before CHIKV infection were used to establish normal ranges for the macaques (Figure 4). Leucopenia was observed during peak viremia, with a significant decrease on 3 and 6 dpi compared with days –2 and 15 ($P < 0.05$, least significant difference test). Compared with baseline levels, neutrophil counts decreased significantly after CHIKV infection, with counts on 3–9 dpi lower than on –2 dpi ($P < 0.05$, least significant difference test), remaining low until 4 days after viral clearance from the circulation, and recovering late in infection. Lymphocyte counts in all macaques decreased significantly ($P < 0.05$, least significant difference test) at peak viremia followed by a rapid increase to above baseline values. Overall, mean counts were significantly elevated for the enzootic strain (1,777 per mm^3) compared with the epidemic strain (1,340 per mm^3). Eosinophil

FIGURE 2. Mean ($\pm$ STD) of fetal heart rate after CHIKV inoculation. (A) CHIKV epidemic strain. (B) CHIKV enzootic strain. Normal fetal heart rate range is shown in the dashed box area. There were no significant differences in mean fetal heart rates ($P > 0.05$) between the viral strains (ANOVA).

FIGURE 3. Mean ($\pm$ STD) systemic viremia in $\log_{10}$ plaque forming units (PFU) per milliliter determined by Vero cell plaque assay (detection limit: 1.7 $\log_{10}$ PFU/mL). The viremia temporal pattern was consistent between the epidemic and enzootic strains ($P > 0.05$), but the overall mean viremia titer for the enzootic strain was significantly greater than for the epidemic strain ($P < 0.05$, ANOVA).

counts varied significantly over time ($P < 0.05$, least significant difference test) but were similar for both groups. The number of eosinophils decreased on days 3–6, increased on 12 dpi to baseline levels, and then decreased again at 21 dpi. Analysis of blood chemistry revealed a small increase in fibrinogen (300–400 mg/dL) and a minor reduction in hemoglobin and hematocrit across infection times.

Histopathology. Complete necropsy and histopathological examinations were performed on maternal and fetal tissues at 21 dpi. By necropsy, clinical signs (fever, joint swelling, and rash) were abated in all animals. Among the tissues examined, histological findings were limited to the liver (all infected animals), spinal cord (one of six animals, infected with enzootic strain), and muscle (two of six animals, infected with enzootic strain). When compared between age-matched uninfected animals, both CHIKV epidemic and enzootic strain-infected maternal livers exhibited sinusoidal lining endothelial cells that were diffusely prominent, often protruding into the hepatic sinusoidal space (Figure 5A). Although this histological finding was non-specific and was seen rarely and multifocally in the age-matched controls the endothelium was diffusely affected in six of six livers examined from infected animals. Few randomly scattered foci of neutrophils, lymphocytes, and karyorrhectic debris were observed in all CHIKV-infected maternal livers (Figure 5A and B). This hepatic microabscessation change may have been caused by fever. Individual hepatocytes or scattered clusters of hepatocytes in two of three CHIKV epidemic-infected animals contained cytoplasmic eosinophilic protein bodies (presumed proteinaceous inclusions) (Figure 5B). In macaques infected with the epidemic strain, scattered regions of dissecting sinusoidal fibrosis (Figure 5D) corresponded by electron microscopy to subendothelial deposits of collagen (space of Disse) (Figure 5E). In one section examined by electron microscopy, Kupffer cells contained degenerate cells (Figure 5F), indicating recent necrosis. Among the central nervous system (CNS) tissues examined, one CHIKV enzootic strain-inoculated macaque had an accumulation of lymphocytes and plasma cells, with fewer histiocytes present within the meninges adjacent to a ventral root ganglion of the lumbar spinal cord (Figure 5G). Two CHIKV enzootic strain-infected macaques showed mild scattered individual muscle fiber necrosis (Figure 5H), with occasional mononuclear infiltrates presumed to be satellite cells in combination

FIGURE 4. Mean (±STD) peripheral leukocyte population after CHIKV inoculation. Normal ranges of each leukocyte population are shown in the dashed box areas. Total leukocyte, lymphocyte, neutrophil, and eosinophil counts significantly decreased after CHIKV infection (* $P < 0.05$, least significant difference test). Lymphocyte counts in all macaques decreased significantly at peak viremia, followed by a rapid increase to above baseline values. Mean lymphocyte count was significantly elevated for the enzootic strain compared with the epidemic strain (** $P < 0.05$, least significant difference test).

with mononuclear infiltrates. The majority of maternal lymph nodes (brachial, axillary, and inguinal lymph nodes) had follicular hyperplasia, including primary and secondary follicle formation with para-follicular expansion, subcapsular lymphocytosis, and histiocytosis. These features of the lymph nodes were considered non-specific, because age-matched control macaques showed similar changes. No histological findings were present within any fetal tissues examined. Most notably, all fetal lymph nodes were histologically unremarkable (Figure 5I).

Viral distribution among tissues. Maternal and fetal tissues were assayed for CHIKV RNA using a real-time RT-PCR assay. CHIKV RNA was undetectable in peripheral leukocytes after the clearance of circulating infectious virus from the plasma. Viral RNA was detected in all maternal spleens and lymph nodes, including brachial, axillary, and inguinal lymph nodes, except for the left brachial lymph node from macaque MMU33907 (enzootic group) (Table 1). RNA was also detected in the joint-associated connective tissue of MMU29320 (enzootic group), the spinal cord of MMU28764 (enzootic group), and the joint-associated connective tissue and skeletal muscle of MMU33347 (epidemic group) (Table 1). RNA detection at 21 dpi provided evidence of CHIKV persistence more than 14 days after the clearance of the systemic viremia. However, CHIKV RNA was not detected in fetal tissues and placentas. All RT-PCR positive tissues were blind-passaged two times in a C6/36 cell line for 7 days per passage and then assessed by Vero cell plaque assay. No infectious virus or viral RNA was detected after blind passage of homogenized tissues.

Detection of antiviral antibodies. Anti-CHIKV total antibodies and neutralizing antibodies were detected in monkeys

inoculated with both strains of CHIKV by 7 dpi. Antibody responses were generally similar between monkeys challenged with both strains. Exposure to the epidemic strain elicited a 3-fold higher mean antibody titer at day 14 than exposure to the enzootic strain (Figure 6A). It is plausible that the use of antigen derived from the epidemic CHIKV strain for the ELISA contributed to minor variation in the detection sensitivity of our assay to antibody generated by these viruses. However, there was no significant difference in the levels of anti-CHIKV antibody elicited to either virus at days 7 or 21 ($P > 0.05$). In all six fetuses, anti-CHIKV antibodies were detected at statistically similar levels ($P > 0.05$) at 21 dpi (Figure 6B).

Exposure to the epidemic CHIKV strain elicited a significantly higher ($F = 17.5$; $df = 1, 20$; $P < 0.001$) PRNT titer on both 14 and 21 dpi (inverse of the geometric mean titer [GMT] = 1,016 at 14 dpi, GMT = 2,032 at 21 dpi) than exposure to the enzootic strain (GMT = 403 at 14 dpi, GMT = 640 at 21 dpi). PRNT titers measured at 14 and 21 dpi using viruses homologous (Figure 6C) and heterologous (Figure 6D) to the infecting viral strain produced similar GMT titers (less than 4-fold different), indicating that neutralizing antibody responses to these strains were indistinguishable.

Cytokine profile. Profiles of 23 cytokines were examined at -2, 3, 6, and 9 dpi using a Milliplex Map non-human primate cytokine kit with a BioPlex instrument following the manufacturer's protocols (Bio-Rad) for macaques inoculated with either the CHIKV epidemic or enzootic strain. Macaque 33347 was treated for intermittent idiopathic diarrhea and exhibited elevated baseline cytokine levels, and therefore, it was excluded from cytokine analyses. Cytokines IL-2, IL-6, IL-15, IL-1ra, MCP-1, and VEGF did not differ significantly between virus strains ($P > 0.05$), but increased significantly

FIGURE 5. Histopathology of Chikungunya virus-infected liver, spinal cord, and skeletal muscle. (A) Endothelium lining hepatic sinusoids is prominent and protrudes into the sinus spaces (black arrow). There are randomly scattered hepatic microabscessations with neutrophils, lymphocytes, and karyorrhectic debris (open arrow; hematoxylin and eosin [HE] stain). (Scale bar, 20 μ m.) (B) Hepatocytes contain cytoplasmic eosinophilic protein bodies (arrow). Epidemic strain infected animal (HE stain). (Scale bar, 10 μ m.) (C) Sinusoidal lining endothelium is flat and non-reactive in uninfected liver (arrow; HE stain). (Scale bar, 20 μ m.) (D) Dissecting fibrosis within hepatic sinusoids (arrow; HE stain). (Scale bar, 20 μ m.) (E) Segmental, subendothelial regions contain collagen fibers (arrow; transmission electron microscopy [EM]). (Scale bar, 2 μ m.) (F) A Kupffer cell contains presumed degenerate necrotic cells (arrow; EM). (Scale bar, 2 μ m.) (G) There is a focal accumulation of lymphocytes, plasma cells, and histiocytes within the meninges adjacent to a ventral root ganglion of lumbar spinal cord (arrow; HE stain). (Scale bar, 100 μ m.) (H) Scattered individual muscle fiber necrosis with occasional perimysial accumulation of mononuclear cells (arrow; HE stain). (Scale bar, 50 μ m.) (I) Fetal lymph nodes are histologically unremarkable (HE stain). (Scale bar, 500 μ m.) This figure appears in color at www.ajtmh.org.

($P < 0.05$) at 3 dpi in parallel with peak systemic viremia and then decreased rapidly to baseline levels (Figure 7). Cytokine IL-13 was significantly increased ($P < 0.05$) for the epidemic strain on day 3 compared with the enzootic strain, which was indicated by the significant virus strain $\times$ time interaction term in the ANOVA. The remaining cytokines (such as IFN γ , MIP-1 α , TNF α , GM-CSF, IL-8, and IL-10) showed minimal increase post-inoculation or were highly variable among replicates precluding statistical inference.

DISCUSSION

The rapid recent expansion of CHIKV and the appearance of atypical neurological syndromes and pre-partum neonatal transmission highlight the need for development of an appropriate animal model for studying this disease. All

Rhesus macaques in this study survived infection and exhibited viremias of sufficient magnitude to infect blood-feeding *Aedes* spp. mosquitoes. Given the urban/periurban distribution of Rhesus macaques and their overlapping distribution with *Ae. aegypti* and *Ae. albopictus* mosquitoes, these results as well as the introgression of the ECSA genotype into much of Asia highlight the importance of these non-human primates as potential amplifying hosts for CHIKV in Asia.

Strikingly, pronounced joint swelling, similar to that observed in human Chikungunya cases, was evident in three macaques. Because arthralgia is difficult to assess in animal models, the identification of inflammation in ankle and wrist joints serves as a valuable marker of CHIKV arthritogenic disease. With the exception of one macaque infected with the enzootic strain of CHIKV that developed the highest and most prolonged viremia and exhibited skin rash and

TABLE 1
Chikungunya viral RNA persistence at 21 dpi presented as the Taqman RT-PCR cycle threshold (Ct) values

	Animal Identification Number					
	30375	28160	33347	33907	28764	29320
Virus strain	Epidemic	Epidemic	Epidemic	Enzootic	Enzootic	Enzootic
Spleen	37.1	41.6	36.7	36.2	38.3	35.3
Axillary LN	34.6	38.0	38.6	39.4	37.7	36.1
Inguinal LN	34.0	36.8	36.6	35.2	32.9	42.8
Brachial LN—inoculation arm	33	41.4	32.9	36.8	39.0	34.1
Brachial LN—opposite arm	37.8	36.3	38.5	UD	39	NA
Joint-associated connective tissue	UD	UD	43.1	UD	UD	37.5
Joint-associated skeletal muscle	UD	UD	38.6	UD	UD	UD
Cervical spinal cord	UD	UD	UD	UD	37.9	UD
Thoracic spinal cord	UD	UD	UD	UD	33.1	UD
Brain/bone/skin/liver/heart/vagina/ bone marrow/kidney/lung	UD	UD	UD	UD	UD	UD

LN = lymph node; UD = undetermined, > 45 Ct; NA = not available.

severe muscle/joint swelling, joint swelling (presumed inflammation) was short-lived (3–15 days) and resolved by the time of necropsy in all animals affected. This observation is similar to CHIKV infection in humans, where protracted muscle/joint lesions are limited to more severe cases.^{38,46} Muscle biopsies were collected at necropsy (21 dpi) proximal to the joints of two macaques that exhibited marked inflammation. Individual muscle fiber necrosis and occasional perivascular infiltration of leukocytes were observed. Viral RNA was also detected 21 dpi in these biopsy samples, possibly indicating persistent infection at these sites. Corresponding evidence of muscle fiber necrosis was observed in biopsies taken from two patients exhibiting myalgia during CHIKV infection.⁴⁷ At the time of necropsy, no evidence of inflammation or necrosis was evident in synovial tissue collected from the same sites.

Sites of viral persistence also were evaluated. Viral RNA was detected in all maternal lymphoid tissues, some joint-associated tissues, spleen, and spinal cord at 21 dpi, providing

evidence for CHIKV persistence more than 14 days after the clearance of the systemic viremia. In agreement with our findings, viral RNA has also been detected in lymphoid tissues of Cynomolgous macaques infected with a La Réunion isolate of CHIKV as late as 55 dpi.³⁸ In that study, macrophages seemed to be a major reservoir of virus, both in the lymph nodes and in other tissues; however, we did not detect CHIKV in synovial tissues, CSF, the CNS, or the spleen late in infection in Rhesus macaques. Although persistent focal hepatocellular apoptosis/necrosis was observed in both Rhesus and Cynomolgous macaques, viral RNA was only evident in the latter species after 21 dpi. Ultimately, the variation in the sites of viral RNA persistence observed between these two studies may reflect differences in the virus strain, dose, route of inoculation, pregnancy status, macaque species, or age of the animals selected. The relationship between viral persistence and disease severity in the Cynomolgous macaque model is unclear, because evidence of arthritogenic signs was not reported in that study.

FIGURE 6. CHIKV-specific antibody levels in dams and fetuses after CHIKV inoculation. (A) Anti-CHIKV Ab levels measured by ELISA in inoculated dams. *Anti-CHIKV Ab levels from macaques inoculated with the epidemic strain were 3-fold higher than those with the enzootic strain at 14 dpi. No differences were observed between groups by 21 dpi. (B) Anti-CHIKV Abs were detected by ELISA in all six fetuses with similar levels. Neutralizing anti-CHIKV antibody titers (PRNT₈₀) in CHIKV epidemic/enzootic strain-infected dams accessed using both the homologous (C) and heterologous (D) CHIKV strains. Exposure to the epidemic CHIKV strain elicited a significantly higher PRNT₈₀ titer on days 14 and 21 than animals infected with the enzootic strain (** $P < 0.001$, ANOVA).

FIGURE 7. Cytokine profiles before infection and at various time points post-inoculation with CHIKV strains. Cytokine changes of CHIKV epidemic (●) and enzootic (□) strain-inoculated macaques compared between baselines and 3, 6, and 9 dpi show that the proinflammatory cytokines and chemotactic/leukocytotropic factors exhibit a significant time series increase (* $P < 0.05$, ANOVA).

Because CHIKV infection is principally denoted by fever and joint inflammation, we accordingly sought to identify proinflammatory cytokines and chemotactic factors that may contribute to disease and could be potential targets for therapeutics. Cytokines IL-2, IL-6, IL-13, IL-15, IL-1ra, MCP-1, and VEGF were up-regulated during the peak viremic phase and rapidly returned to baseline levels shortly after virus clearance from the blood. Notably, the leukocytotropic factor IL-6 was significantly activated at 3 dpi in all infected Rhesus macaques. Retrospective analyses of CHIKV infections in Singapore positively associated IL-6 expression with severe disease.⁴⁸ Several other immune factors, such as IL-2 and VEGF, have also been observed to be overexpressed in humans with CHIKV disease.⁴⁸ The observation that expression of these cytokines was also elevated during infection in Rhesus macaques highlights the congruence between disease in humans and non-human primates. Interestingly, an increase in MCP-1 was also observed during CHIKV infection in all of the Rhesus macaques. This is consistent with findings of elevated MCP-1 and IL-6 levels observed in Cynomolgus macaques during early CHIKV infection.³⁸ Moreover, Rulli and others⁴⁹ recently showed that administration of inhibitors of MCP-1 to

mice challenged with the closely related arthritogenic alphavirus, Ross River virus, ameliorated disease. Whether inhibition of the proinflammatory cytokines identified in this study may have therapeutic potential for CHIKV disease remains to be determined.

Intrauterine CHIKV infections were first reported during the recent outbreak in the Indian Ocean. Retrospective studies of the La Réunion epidemic of CHIKV in 2006 indicated that two modes of intrauterine infection occurred. Most commonly, fetal infection was observed intrapartum. During this outbreak, approximately 50% of neonates birthed to viremic mothers acquired infection.³³ Couderc and Lecuit³⁷ proposed that infection might result from disruption of the placental barrier to infection during delivery. In less than 1% of pregnancies, fetal infection occurred pre-partum. Although viral antigen was evident in the placenta in two cases of pre-partum infection, the underlying factors that promote fetal infection in these rare circumstances remain elusive. In our study fetal infection was not detected, despite establishment of robust viremias in monkeys challenged with both epidemic and enzootic strains of CHIKV and evidence of viral persistence in tissues as late as 21 dpi. Moreover, maternal infection did not

seem to have a significant effect on fetal condition as assessed by monitoring fetal heart rate. This result reinforced the observation that pre-partum intrauterine infection is a rare event. It is possible that factors such as maternal immune status and pre-term placental abruption may contribute to pre-partum infection; however, in light of the small number of reported cases and the difficulty of reconstructing such rare events in animal models, the mechanism of infection remains undefined.

Our report extends previous studies on CHIKV infection in macaques and provides new data comparing viremia and clinical signs in non-human primates inoculated with enzootic West African and ECSA viral strains. Interestingly, although infection with the enzootic strain produced significantly higher viremias than the epidemic strain, pathophysiological differences were not identified. We observed sites of viral persistence that may contribute to the protracted arthralgic and neurological syndromes observed in humans. Although the selection of pregnant dams for our study limited our ability to directly compare our results on disease severity and progression with results from non-pregnant Cynomolgus macaque models, the congruent viral persistence and host humoral immune responses observed in both species showed that these species can both serve as valuable models to assess efficacy of vaccines and antiviral therapies. Moreover, the similarity in presentation of CHIKV disease between Rhesus macaques and humans and the reconstitution of cellular and immunological hallmarks of human disease in this model make it an ideal system to evaluate the mechanisms of CHIKV pathology.

Received May 21, 2010. Accepted for publication September 28, 2010.

Acknowledgments: We would like to thank Dr. Christopher Barker (Center for Vectorborne Diseases, University of California, Davis, CA) and Dr. Brad J. Biggerstaff (Centers for Disease Control and Prevention [CDC], Fort Collins, CO) for statistical analysis assistance. We would also like to thank Drs. Robert Lanciotti and Barbara W. Johnson (CDC, Fort Collins, CO) for providing CHIKV-positive human sera for ELISA controls. We appreciate the input of Linda Hirst and the support of Dr. Sona Santos, Wilhelm von Morgenland, and the animal care staff of California National Primate Research Center (University of California, Davis, CA) for their assistance with the animal work. Finally, we are thankful for technical support from JoAnn Yee (California National Primate Research Center, Davis, CA) for performing cytokine assays and Dr. Michael Glanzberg for reviewing the manuscript. This project was supported by the California National Primate Research Center (NCRR RR000169-48) and Pacific Southwest Research Center of Excellence for Biodefense and Emerging Infectious Diseases (U54 AI065359).

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