Pathogenesis of Modoc Virus (*Flaviviridae*; *Flavivirus*) in Persistently Infected Hamsters

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Abstract. The long-term persistence of Modoc virus (MODV) infection was investigated in a hamster model. Golden hamsters (*Mesocricetus auratus*) were infected by subcutaneous inoculation with MODV, in which fatal encephalitis developed in 12.5% (2 of 16). Surviving hamsters shed infectious MODV in their urine during the first five months after infection, and infectious MODV was recovered by co-cultivation of kidney tissue up to eight months after infection. There were no histopathologic changes observed in the kidneys despite detection of viral antigen for 250 days after infection. Mild inflammation and neuronal degeneration in the central nervous system were the primary lesions observed during early infection. These findings confirm previous reports of persistent flavivirus infection in animals and suggest a mechanism for the maintenance of MODV in nature.

INTRODUCTION

Modoc virus (MODV) is a rodent-associated flavivirus that has no known arthropod vector. Taxonomically, it is included in the MODV group of the family *Flaviviridae* and genus *Flavivirus*. The virus was first isolated from the mammary gland tissue of a white-footed deer mouse (*Peromyscus maniculatus*) in Modoc County, California, in 1958,¹ and subsequently, it was found in other western regions of the United States and Canada.^{1–3} Previous studies suggest that MODV is maintained in nature by horizontal transmission among rodents,⁴ and a small number of experimental studies have demonstrated that deer mice⁵ and hamsters⁶ show development of persistent MODV infection and chronic viremia.

Davis and Hardy⁶ showed that MODV was chronically shed in the urine of experimentally infected hamsters for at least three months after intranasal inoculation, but virus could not be directly isolated from organ homogenates of the persistently infected hamsters. However, by co-cultivation of the tissues *in vitro*, it was possible to recover MODV from the kidneys and lungs of chronically infected hamsters. These findings are analogous to those previously reported in a hamster model of West Nile virus (WNV) infection⁷; however, unlike MODV, WNV is a mosquito-borne flavivirus.

Hamsters experimentally infected with WNV show development of a chronic renal infection and can persistently shed the virus in urine for up to eight months.⁷ Despite the presence of high levels of neutralizing antibodies in the rodents, infectious WNV was recovered by direct culture of the urine and by co-cultivation of kidney tissue for up to 247 days after intraperitoneal inoculation. West Nile virus antigen was also readily detected by immunohistochemical analysis within the tubular epithelial cells, interstitial cells, and macrophages of the distal renal tubules, and moderate histopathologic changes were observed in the kidneys and brains of the chronically infected hamsters.⁷ Persistent renal infection and chronic viremia were also demonstrated in hamsters experimentally infected with St. Louis encephalitis virus (SLEV), another mosquito-borne flavivirus.⁸ To date, there have been no comprehensive studies that match viral antigen expression with the histopathologic changes that have been associated with chronic MODV infection.

Recent reports of chronic infection with a number of different flaviviruses^{7–11} has renewed interest in the subject of persistent flavivirus infection and the role it might play in virus maintenance and pathogenesis in the vertebrate host. In the present study, we extend findings of earlier studies by investigating virus localization in tissues and histopathologic changes associated with long-term MODV infection in hamsters. In an attempt to elucidate the mechanism of persistent viral infection, we compared the findings of the present study with those of previous studies of chronic flavivirus infections.

MATERIALS AND METHODS

Virus. The MODV strain M544 was used for these studies¹ and obtained from the World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch in Galveston, Texas. H. N. Johnson originally isolated the virus from the mammary gland tissue of a white-footed deer mouse (*Peromyscus maniculatus*) in Modoc County, California, in 1958; it had received nine intracerebral passages in suckling mice and one passage in Vero cells before use.

Animals. Adult female Syrian golden hamsters (*Mesocricetus auratus*), 5–6 weeks of age, were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). The animals were cared for in accordance with the guidelines of the University of Texas Medical Branch Committee on Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, DC). All work with infected animals was carried out in animal biosafety level-2 facilities under an approved Institutional Animal Care and Use Committee protocol.

Experimental design. Sixteen hamsters were inoculated subcutaneously with 5.2 log₁₀ plaque-forming units (PFU) of the virus stock, and blood samples (100 µL) were obtained from the hamsters on day 10 after infection to determine the antibody response (as described below). Beginning on day 10 after infection and at monthly intervals for eight months, one or two hamsters were euthanized, and blood and organ samples were collected for virus isolation, serologic, histopathologic, and/or immunohistochemical analysis.

Viruria titration. To assay for viremia, fresh urine was collected at monthly intervals from the hamsters for eight months. Urine was diluted 1:10 with phosphate-buffered saline, pH 7.4, that contained 30% fetal bovine serum. The diluted urine (200 µL) was inoculated into flasks with confluent Vero cell cultures, as described.^{7,8} If cytopathic effect (CPE) was observed in the cultures, the Vero cells were

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subsequently examined for the presence of MODV antigen by indirect fluorescent antibody (IFA) assay, using a MODV-specific mouse immune ascitic fluid and a commercially prepared fluorescein-conjugated goat antimouse immunoglobulin (Sigma, St. Louis, MO). To quantify the amount of virus present, the MODV-positive urine samples were titrated by plaque assay as described.⁷

Serologic analysis. The antibody responses of hamsters to MODV infection were measured by hemagglutination-inhibition (HI) and IgM-capture enzyme-linked immunosorbent assays (ELISAs), as described.¹² Antigens for the HI test were prepared from brains of MODV-infected newborn mice by the sucrose-acetone extraction method. Hamster serum samples were tested by HI at serial two-fold dilutions from 1:20 to 1:5,120 at pH 6.0 with 4 units of antigen and a 1:200 dilution of goose erythrocytes. The results were expressed as the inverse of the last dilution of sera that resulted in HI. For the IgM ELISA, serum samples were screened at a single 1:40 dilution, and results were recorded using an optical density at 405 nm (OD₄₀₅). Absorbance values ≥ 0.20 OD₄₀₅ were considered positive.

Virus detection by co-cultivation. Tissue samples of kidney and lung from the experimentally infected hamsters were assayed for MODV by co-cultivation as described.⁷ In brief, tissue fragments were incubated at 37°C for 45–60 minutes in 50-mL conical tubes with a trypsin-EDTA solution to dissociate cells. After several washes with phosphate-buffered saline, the cells were resuspended in 3–5 mL of Medium 199 with Earle's salts (Gibco, Grand Island, NY), 10% fetal bovine serum, and penicillin-streptomycin. Equal volumes of the suspended cells from each tissue sample were inoculated into three culture flasks, each containing a monolayer of Vero cells. Co-cultures were maintained at 37°C for 15 days and examined for evidence of CPE. If CPE was observed, the presence of MODV in the culture medium was confirmed by sub-culturing the culture medium on another monolayer of Vero cells. These Vero cells were subsequently examined for the presence of MODV antigen by IFA.

Histologic and immunohistochemical examinations. To collect tissues for histopathologic and immunohistochemical analysis, hamsters were exsanguinated by cardiac puncture while under halothane anesthesia (Halocarbon Laboratories, River Edge, NJ). For two animals (hamsters 88 and 93), the chest cavity was opened quickly, and 20–30 mL of 10% buffered formalin was injected directly into the left ventricle of the heart to perfuse the body. After refrigeration overnight

at 4°C, each body was dissected, and samples of lung, liver, spleen, kidney, and spinal cord, as well as the entire brain, were removed and placed in 10% buffered formalin solution for an additional 24 hr to enable proper fixation. The next day tissue samples were transferred to 70% ethanol for storage. For all other animals, samples of lung, liver, spleen, kidney, and spinal cord, as well as the entire brain, were collected immediately after killing and placed in 10% buffered formalin solution for 24 hours, and then transferred to 70% ethanol for storage. Tissue specimens were subsequently processed, and histologic slides were prepared. Tissues from age-matched uninfected hamsters were processed as described above and used as negative controls.

Immunohistochemical staining for MODV antigen was performed as described.¹² A MODV mouse hyperimmune ascitic fluid obtained from the World Reference Center for Emerging Viruses and Arboviruses was used as the primary antibody at a dilution of 1:100, and bound primary antibody was directly labeled and detected by use of a commercially available ISO-IHC AEC Kit (Inno-Genex, San Ramon, CA).

RESULTS

Clinical outcome and antibody response. Two of the 16 experimentally infected hamsters (12.5%) showed development of signs of severe encephalitis. On day 6 after infection, hamster no. 93 showed signs of bilateral hindlimb paralysis and was euthanized. On day 7 after infection, hamster no. 88 showed signs of complete paralysis with an intact corneal reflex and was euthanized. Tissues were collected for histopathologic and immunohistochemical examination.

The remaining hamsters appeared healthy, remained active, and gained weight throughout the eight-month duration of the experiment. For all surviving hamsters, IgM and HI antibodies to MODV were present in the blood on day 10 after subcutaneous infection. Absorbance values of IgM ranged from 0.386 to 0.655 OD₄₀₅ (mean $\pm$ SD = 0.533 ± 0.079), and HI titers ranged from 320 to 1,280 (mean $\pm$ SD = 594 ± 394).

Persistent MODV infection in hamsters. Beginning on day 10 after infection, urine was collected at approximately monthly intervals from the 14 surviving hamsters and cultured for MODV (Table 1). During the first four months after infection, most (5 of 6) hamsters had infectious MODV in their urine (as determined by IFA assay), and by month 5 after infection, only 1 of 5 hamsters had viruria. Over the next

TABLE 1
Isolation of Modoc virus (MODV) from urine (top) and selected tissues (bottom) of chronically infected hamsters

Day of urine collection after infection	Animal number*													
	85	86	89	94	95	91	92	87	97	96	98	90	99	100
10	+	NA	NA	+	+	+	+	NA	+	NA	+	NA	+	+
28	+†	+†	+	NA	+	NA	NA	NA	+	+	+	NA	0	+
51			+†	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
79				+†	0†	+	0	+	NA	+	NA	+	0	+
111						+†	+†	+	+	NA	NA	0	NA	+
147								0†	0†	0	NA	0	NA	+
182										0†	0†	0	0	0
219												0†	0†	0
250														0†
Lung co-cultivation	0	0	0	+	0	0	0	0	0	0	0	0	0	0
Kidney co-cultivation	0	+	+	+	+	0	+	+	+	0	0	+	+	+

* As indicated, results of urine or tissue co-culture. + = MODV isolated; NA = urine specimen not available; 0 = no MODV isolated.

† Day of euthanasia and lung/kidney co-cultivation experiments; results are indicated in bottom portion of table.

three months, MODV could no longer be detected in the urine of the hamsters. Despite the presence of viral antibodies in the blood, virus titers in urine on day 10 after infection ranged between 1.3 and 4.0 log₁₀ PFU/mL (mean $\pm$ SD = 3.3 $\pm$ 0.9 log₁₀ PFU/mL); for later time points, virus titers were not determined in the urine samples.

Results of lung and kidney co-cultures performed on the 14 persistently infected hamsters at the time of euthanasia are shown in Table 1. Overall, MODV was detected in the kidneys of 10 (71.4%) of 14 hamsters and throughout the eight-month period of the experiment. In contrast, MODV was only detected in the lung of one hamster (no. 94) on day 79 after infection.

Histopathologic findings in hamsters with MODV infection.

The liver, spleen, lung, and heart of all infected hamsters showed no substantial microscopic lesions, except for rare focal portal inflammation of the liver and mild reactive lymphoid hyperplasia of the spleen. On day 6 after infection, the adrenal gland of hamster no. 93 showed cortical necrosis

with infiltration of mononuclear cells and occasional neutrophils. However, there were no other microscopic changes observed in the adrenal glands of the remaining infected hamsters. With the exception of rare tubular dilation in the renal cortex and focal-to-multifocal microcalcification, there were no substantial pathologic changes observed in the kidneys.

During early infection, considerable pathologic changes were observed in the brain and spinal cord of the infected hamsters. On days 6–7 after infection, the spinal cord (at the thoracic region) showed a subacute-to-acute non-suppurative myelitis with moderate astrocytosis/microgliosis and focal neurophagia (Figure 1A). In the ventral horn of the spinal cord, individual neuronal cell degeneration and apoptosis was prominent, and a marked perivascular infiltration of mononuclear cells was observed with small foci of hemorrhage. After days 6–7 of infection, the lesions in the spinal cord of the remaining infected hamsters consisted of occasional, mild infiltrations of inflammatory cells, including astrocytes, and individual neuronal cell degeneration.

FIGURE 1. Histologic changes and immunohistochemical detection of Modoc virus antigen in spinal cord and brain of experimentally infected hamsters. **A**, Spinal cord with diffuse mononuclear cell infiltration and early neuronal degeneration (**arrow**), day 7 after infection. **B**, Brainstem with scattered neuronal degeneration (**arrow**), day 7 after infection. **C**, Strong cytoplasmic staining (red) of neurons in spinal cord, day 7 after infection. **D**, Strong cytoplasmic staining (red) of large neurons in brainstem, day 6 after infection. **E**, Negative antigen staining of neurons in spinal cord, uninfected hamster. **F**, Negative antigen staining of neurons in brainstem, uninfected hamster.

In the cerebrum during early infection, mild non-suppurative (mononuclear) encephalitis was consistently observed that included neuronal degeneration and necrosis. Neuronal degeneration was seen in many areas of the brain, demonstrated by contraction of the perikaryon with eosinophilia of the cytoplasm, central chromatolysis, and condensation of the nucleus (Figure 1B). On days 6–7 after infection, neuronal degeneration was scattered and mild, and there was a mononuclear cell infiltration in the basal nuclei and brainstem, psammoma bodies in the basal nuclei, and mild lymphocytic meningitis that included a rare number of neutrophils. For both hamsters that were examined on day 28 after infection, there was prominent degeneration and loss of neurons in the cerebellum (Purkinje cells) with mild, scattered degeneration of large neurons in the superficial and deep cortex, olfactory bulb, and hippocampus. Focal mild inflammatory cell (mononuclear) infiltration was evident, and rare perivascular microglial nodules were located near the olfactory bulb. For hamsters that were examined on days 51, 79, 111, 147, 182, 219, and 250 after infection, microscopic changes observed in the brain were consistent and included mild, focal degeneration of neurons in the superficial and deep cortex, hippocampus, brainstem, and cerebellum (Purkinje cells). Psammoma bodies were also observed in the basal nuclei, and focal accumulations of mononuclear cells and astrocytes were noted occasionally in the basal ganglion, meninges, and choroid plexus.

Immunohistochemical detection of MODV antigen.

Immunohistochemically, the heart, lung, liver, spleen, and adrenal gland of all MODV-infected hamsters were negative for viral antigen. However, large neurons in the spinal cords of the hamsters that showed development of neurologic disease were positive for antigen on days 6–7 after infection (Figure 1C).

Seven or eight large neurons from each side of the ventral horn were positive for MODV antigen. In contrast, there was no viral antigen detected in the spinal cords of MODV-infected hamsters between days 28 and 250 after infection.

In the brainstem of hamsters on days 6–7 after infection, MODV antigen was detected in a cluster of large neurons (Figure 1D), and other regions of the brain were negative for antigen. For hamsters that were examined between days 28 and 111 after infection, no viral antigen was detected in the brain. However, on days 147 and 182 after infection, single neurons in the brainstem of all examined hamsters were positive for antigen. During this timeframe, the choroid plexus was also consistently positive for antigen (Figure 2B). For hamsters that were examined on days 219 and 250 after infection, viral antigen was no longer detected in the brain.

On days 6–7 after infection, MODV antigen was localized to the tubular epithelial cells of the renal cortex and medulla. Between days 28 and 250 after infection, viral antigen continued to be detected in the kidney and was primarily localized to the tubular epithelial cells of the medulla with occasional involvement of the cortex (Figure 2D). For all days, the renal papillae were negative for viral antigen.

DISCUSSION

Previous work has reported a mortality rate as high as 50% in hamsters infected with MODV by either the intraperitoneal or intranasal route.¹³ However, in our study only a small percentage of hamsters showed development of severe neurologic disease after subcutaneous inoculation of MODV, and most (87.5%) survived infection and showed development of a robust antibody response by day 10 after infection.

FIGURE 2. Immunohistochemical detection of Modoc virus antigen in tissues of chronically infected hamsters. **A**, Negative antigen staining of choroid plexus, uninfected hamster. **B**, Positive antigen staining (red) of choroid plexus, day 182 after infection. **C**, Negative antigen staining of tubular epithelium of kidney, uninfected hamster. **D**, Positive antigen staining (red) of tubular epithelium of kidney, day 250 after infection.

Despite the presence of circulating antibodies against MODV, infected hamsters continued to shed virus in the urine for up to four months (and in one case five months) after infection. Infectious MODV was also isolated from the kidneys for at least eight months after infection, but MODV was rarely isolated from the lungs. These results corroborate findings from a previous study⁶ in which hamsters infected intranasally with MODV were able to shed virus in urine for at least three months after infection, and the virus was isolated from lungs and kidneys of infected hamsters at seven months after infection.

Overall, the lesions in the spinal cord during early infection were more severe than those observed in the brain. Mild inflammation and neuronal degeneration in the brain and spinal cord were the primary lesions observed during early infection. Interestingly, the lesions in the central nervous system (CNS) were similar in pattern to, but to a lesser degree than, those described for WNV infection of hamsters during early infection.¹²

Modoc virus antigens were expressed in the neurons of the brainstem and spinal cord during acute infection, which coincided with inflammatory changes. The staining pattern of the neurons in the CNS was similar to that described for hamsters infected with WNV.¹² However, the large number of positive-staining neurons in the affected regions of the brain during WNV infection suggests a more extensive neurologic involvement than MODV infection. Co-cultures of the CNS tissues were not undertaken in this study, but it would be interesting to do so in future investigations.

There were no substantial histopathologic lesions observed in the kidney during acute and chronic MODV infection, which differed to some degree from those described after WNV infection.⁷ During WNV infection in hamsters, there appeared to be some changes after the first few months, including amorphous deposits in the interstitium of the renal papillae and progressive dilatation and atrophy of the renal tubules. These changes were also described in hamsters after experimental SLEV infection.⁸ However, as discussed,^{7,8} the significance of these findings are difficult to interpret because similar changes have been reported to occur spontaneously in older hamsters, especially in females.

Consistent with the results of co-cultivation, MODV antigen was only detected in the epithelium of the renal tubules throughout infection. Interestingly, antigen expression in the kidney after WNV infection differed from that of MODV infection. West Nile virus antigen was detected in the tubular epithelium as well as the interstitium, primarily involving macrophages and, rarely, vascular endothelial cells.⁷ In SLEV-infected hamsters, antigen expression in the kidneys was similar in pattern and distribution to that for WNV.⁸ It is not clear how these differences in viral antigen expression in the kidney contribute to chronic shedding of flaviviruses in nature.

Modoc virus is one of many flaviviruses that persist in vertebrate animals.¹⁴ Interestingly, several of these viruses are also shed in urine for an extended period. This property also appears to be independent of whether the flavivirus is mosquito-borne, tick-borne, or non-vector-borne, which may play a role in the maintenance of these viruses in nature. Of the mosquito-borne flaviviruses, Japanese encephalitis virus, WNV, SLEV, and more recently, Rocio virus are examples of viruses that have been shown to persist in multiple vertebrate

animals, including the development of viruria.^{7,8,15,16} Of the tick-associated flaviviruses, tick-borne encephalitis virus, Kyasanur forest disease virus, and Omsk hemorrhagic fever virus are some examples of viruses that persist in tissues after infection.^{17–19} However, only Omsk hemorrhagic fever virus has been shown to cause chronic renal infection and viruria in muskrats. As for non-vector-borne flaviviruses, Rio Bravo virus and MODV are two examples that have been shown to cause persistent infection.^{6,20} Rather than the development of chronic viruria, Rio Bravo virus localizes in the salivary glands of bats and is chronically shed in the saliva.

In summary, the findings of this study support and extend previous work on the effect of MODV infection in hamsters. We were able to demonstrate that MODV, when inoculated subcutaneously, is shed in urine and present in the kidneys for a longer duration than previously documented. Based on MODV antigen expression in the kidneys (and potentially histopathologic changes), its pathogenesis appears to differ from that of WNV and SLEV. Because MODV infection in hamsters has been proposed as a model for evaluating antiviral drugs,²¹ this finding could have an impact in determining whether these drugs are equally effective for treating other flavivirus infections.

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