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Characterization of Persistent Modoc Viral Infections in Syrian Hamsters

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Adult Syrian hamsters were readily infected by intranasal inoculation with Modoc virus. Viremias were detected 2 to 6 days after infection and peak viremia titers ($10^{6.2}$ plaque-forming units/ml of blood) occurred 4 days postinoculation. All infected animals developed neutralizing and hemagglutination-inhibiting antibodies by 7 days, and complement-fixing antibodies by 14 days postinoculation. High titers of antibodies persisted for at least 4 months. Modoc virus was recovered from throat swabs at 7 days postinoculation, but not at 14 days or later. Urine samples were positive for virus throughout a 12-week observation period. Isolation of virus from lungs and kidneys of one and three animals, respectively, at 151 to 221 days after inoculation confirmed chronic infection. Viral isolations were made only when organs were cultivated in vitro and were unsuccessful by tests on 10% homogenates of the organs. Horizontal viral transmission of virus by infected hamsters that were viruric was demonstrated in only 1 of 27 normal hamsters that were cocaged for 4 weeks under crowded conditions. General failure to obtain horizontal viral transmission may relate to rapid inactivation of virus in excreted urine. Vertical viral transmission was not demonstrated from five chronically infected female hamsters to their 34 offspring. However, if primary infection occurred during pregnancy, the progeny were either stillborn or died shortly after birth, and thus appeared to represent transplacental viral transmission.

Modoc virus is grouped taxonomically as a group B arbovirus because it is related antigenically to viruses that are known to be transmitted by arthropod vectors (2). However, no arthropod vector is known for this virus (10). Modoc virus has been isolated only from the organs of the deer mouse, *Peromyscus maniculatus*, collected in California, Oregon, and Colorado (15). Attempts to infect mosquitoes and ticks with this virus have been unsuccessful (10; J. L. Hardy, unpublished data).

Johnson (11) suggested that Modoc virus might survive in nature by vertical or horizontal transmission in persistently infected small mammals. He described a hamster infected with Modoc virus that excreted virus in its urine for at least 153 days after infection, and he isolated virus from in vitro cultures of kidney cells derived from the same hamster 424 days after infection. He could not recovery Modoc virus from a homogenate of kidneys from the same hamster.

This paper further characterizes the persistent infection by Modoc virus and its potential for horizontal and vertical transmission in hamsters.

(This paper is based on part of a paper submitted to the University of California in partial fulfillment of the requirement for the Ph.D. degree.)

MATERIALS AND METHODS

Virus. The prototype strain (M544) of Modoc virus (15) was obtained from H. N. Johnson. The virus had undergone only one intramuscular (i.m.) passage in hamsters and was received as viremic blood from these hamsters. After two further i.m. passages in hamsters, we prepared a stock suspension of virus for our studies. Clotted blood obtained from hamsters 72 h after infection was triturated in 4 volumes of Dulbecco phosphate-buffered saline (PBS) (pH 7.4), with 20% heat-inactivated (56 C for 30 min) fetal calf serum (FCS), and then was centrifuged at 400 × *g* for 10 min. The supernatant fluid was used as the stock virus and titered $10^{5.8}$ plaque-forming units (PFU) per ml of blood.

We prepared a second stock of virus for viral neutralization tests. It represented two intracranial (i.c.) passages and was a 10% homogenate of infected brains from suckling mice. The brain homogenate was prepared in 50% heat-inactivated FCS and 50% beef heart infusion broth. After centrifugation at 400 × *g*

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for 10 min, the supernatant fluid was ampouled and stored at −70 C.

The identity of Modoc virus in the viral stocks prepared in hamsters and mice was established by serum-dilution neutralization (SDN) tests in Vero cells using group B arboviral immune sera or ascitic fluids that were provided by R. W. Emmons, Infectious Disease Section, California State Department of Public Health. The immune sera or ascitic fluids included rabbit immune sera to Modoc and Powassan viruses, hamster immune sera to St. Louis encephalitis (SLE) virus, and mouse immune ascitic fluids to Rio Bravo virus. Antibody titers obtained by SDN with rabbit immune sera to Modoc virus were 1:800 and 1:2,560 for hamster- and mouse-passaged stock of Modoc virus, respectively. Neither Modoc viral stock was neutralized by immune sera or ascitic fluids to the other three group B arboviruses (SDN titers of $<1:4$).

Break-through neutralization tests were done in suckling mice to ascertain if either stock preparation of Modoc virus was contaminated with other viruses. Serial 10-fold dilutions of virus were mixed with 1:10 dilutions of rabbit immune sera to Modoc virus. After incubation for 1 h at 36 C, each virus-serum mixture was inoculated i.c. (0.01 ml per mouse) into a litter of 7 to 8 suckling mice. Virus obtained from brains of moribund mice at the neutralization break-through point were identified by neutralization tests. No virus other than Modoc virus was detected in the brains of these mice.

Cell cultures. Stock cultures of Vero cells that had been passed 120 to 150 times were grown in 16-oz. (0.473 liter) glass bottles in Eagle minimal essential medium (MEM) that was prepared in Earle balanced salt solution (BSS), supplemented with 5% FCS and sterilized by membrane filtration (Millipore Corp.). Cultures to be used for viral assay were grown in 60-mm plastic petri dishes in the same medium except that autoclavable MEM was used. All media contained 1.6 g of $NaHCO_3$ per liter, 100 units of penicillin per ml, and 100 mg of streptomycin per ml. Cultures were incubated at 36 C in a humidified atmosphere of 5% CO_2 in air.

Infection of hamsters. Syrian hamsters, 6 to 8 weeks old, were inoculated intranasally (i.n.) with 0.05 ml of viral suspension. The hamster blood passaged stock virus was always used for the experimental infection of hamsters. Each animal was caged individually unless otherwise indicated.

Collection of specimens. Hamsters were bled from either the heart or an orbital sinus to obtain samples for viremia and antibody determinations. When bled from the heart, 1 volume of blood was drawn into a syringe that contained 4 volumes of either a stabilizing medium for freezing if the sample was for viral tests, or into 4 volumes of saline if the sample was for antibody tests. The stabilizing medium consisted of 0.75% bovine albumin (fraction V) in a solution of 0.12 M NaCl to 0.05 M borate (pH 9.0). Blood was collected from the orbital sinus into Pasteur pipettes and 5 drops of blood (approximately 0.25 ml) was added to 1 ml of the stabilizing medium or saline. These samples were considered to be 1:5 dilutions of blood or 1:10 dilutions of serum. Clotted blood for viral tests was triturated in Ten Broeck grinders and clarified by centrifugation at 400 × *g* for 10 min.

Throat swabs were taken by gently rotating sterile cotton swabs on applicator sticks in the oral cavity of hamsters. Each swab was then immersed in 1 ml of stabilizing medium.

Individual urine samples were collected from the hamsters while they were housed in a 7 C room. Each animal was placed in a beaker on a platform of $\frac{1}{8}$-inch (about 0.3-cm) wire mesh screen. Excreted fluid was transferred to a tube and centrifuged at 1,500 rpm for 10 min. The supernatant fluid was mixed with an equal volume of the stabilizing medium.

Samples to be tested for virus were stored at −70 C and serum samples for antibody tests were stored at −20 C.

Viral isolation from organs. Attempts to demonstrate long-term persistence of Modoc virus in organs (lymph nodes, salivary-submaxillary glands, spleens, kidneys, and lungs) of infected hamsters were done both by direct and indirect isolation techniques. Dissected organs were minced with iris scissors into approximately 1-mm pieces and the fragments were rinsed with PBS until free of blood. A 10% homogenate was prepared in stabilizing medium from a portion of each organ by grinding in a mortar and pestle with Alundum. After centrifugation at 400 × *g* for 10 min, the supernatant fluid was tested directly for virus by plaquing on Vero cells.

Indirect isolation of virus was done by first cultivating in vitro the enzymatically dispersed cells or tissue fragments from a portion of each organ and then testing the tissue culture fluids for virus after 14 days of cultivation at 36 C. For preparation of cultures from dispersed cells, tissue fragments were incubated for 1 to 2 h at room temperature in a solution of 0.5% trypsin and 0.2% ethylenediaminetetraacetic acid that was prepared in PBS without divalent cations. Dispersion of cells was augmented by aspiration with a pipette. Tissue fragments were allowed to settle and the supernatant fluid was removed and centrifuged at 400 × *g* for 10 min. Packed cells were resuspended in 3 ml of growth medium that consisted of MEM in Earle BSS containing 10% heat-inactivated FCS, 0.3 g of $NaHCO_3$ per liter, and antibiotics. One milliliter of cell suspension was dispensed into each of three screw-capped plastic tubes (16 by 125 mm) and incubated at 36 C. Growth medium changes of cultures were done when deemed necessary until cell growth was established. Thereafter, cultures were maintained in MEM in Earle BSS with 5% FCS and variable concentrations of $NaHCO_3$ dependent upon the metabolic rates of the cell cultures. At 14 days after initiation, cultures were frozen and thawed once to lyse cells. Fluids from three cultures prepared from the same organ were mixed with an equal volume of stabilizing medium and saved for viral tests.

The second type of in vitro culture was prepared by placing rinsed fragments of each organ into a 25-ml plastic tissue culture flask that contained 2 ml of Liebowitz medium supplemented with 30% heat-inactivated FCS and antibiotics. Culture fluids were changed with the same medium 3 days after initiation. Subsequent medium changes were done at 2- to

4-day intervals, and the maintenance medium described previously for cultures initiated from dispersed cells was used. At 14 days after initiation, a portion of fluid from each culture was mixed with an equal volume of stabilizing medium and was subsequently tested for virus.

Viral assay. Serial 10-fold dilutions of samples for viral assay were made in the stabilizing medium. Virus was titrated by plaquing each serial dilution on two replicate cultures of Vero cells. Cultures were rinsed twice with PBS, and virus in 0.2 volumes was adsorbed onto drained cultures for 1.5 h at 36 C. Inoculated cultures were rinsed once with PBS and overlaid with a medium that consisted of 1.5% methyl cellulose in autoclavable MEM supplemented with 2% heat-inactivated FCS, 1.6 g of $NaHCO_3$ per liter, and antibiotics. After incubation at 36 C for 4 days, cultures were overlaid again with the same medium, except neutral red (1:20,000) was added and methyl cellulose was replaced by 1.0% purified agar. Plaques were counted on days 7 to 9, and viral titers were expressed in PFU/ml.

Antibody tests. The following three serological methods were used to titrate antibodies: the microtest adaptation of the standardized diagnostic complement fixation (CF) method (3), the SDN test performed in microcultures of Vero cells as described by Earley et al. (7), and the hemagglutination inhibition (HI) test of Clarke and Casals (5) as adapted to the microtiter technique by Sever (14). For the HI test, sera were extracted with acetone and adsorbed with goose cells. Hemagglutinins were prepared by the sucrose-acetone technique (5) from brains of infected suckling mice. CF, SDN, and HI titers of 1:5, 1:10, and 1:20, respectively, were considered positive for antibody.

Fluorescent antibody technique. A pool of hamster hyperimmune sera to Modoc virus was prepared as previously described (6). This immune serum pool was negative (titer of $<1:4$) for antibodies of lymphocytic choriomeningitis virus, a *Peromyscus* herpesvirus, and a *Peromyscus* myxovirus by indirect fluorescent antibody tests (R. W. Emmons, California State Department of Public Health, Berkeley, Calif., personal communication).

The hyperimmune hamster sera were fractioned and conjugated with fluorescein isothiocyanate by the procedure of Cherry et al. (4), as modified by Lennette et al. (12). Monolayer cultures of Vero cells grown on glass cover slips were inoculated with virus, incubated for 3 to 4 days, fixed in acetone at −20 C, and stored at −70 C until stained with the conjugate. The staining procedure and fluorescence microscopy examination were done as related by Emmons and Lennette (8). All slides were counterstained with a 0.1% solution of Evans blue. A 1:20 dilution of the conjugate in a 10% suspension of bovine brain stained cells infected with Modoc virus but did not stain normal cells or cells infected with vesicular stomatitis virus.

Vertical transmission. One procedure used to study vertical transmission of Modoc virus in persistently infected hamsters is diagramed in Fig. 1. The organs selected to test for virus from weanling and adult hamsters were the lungs, spleens, salivary-submaxillary glands, kidneys, and lymph nodes (axial, inguinal, and popliteal). In vitro cultures were prepared from dispersed cells for all hamsters tested and were also grown in flasks from tissue fragments of organs removed from the mother hamsters. Viral isolates were identified by the fluorescent antibody technique.

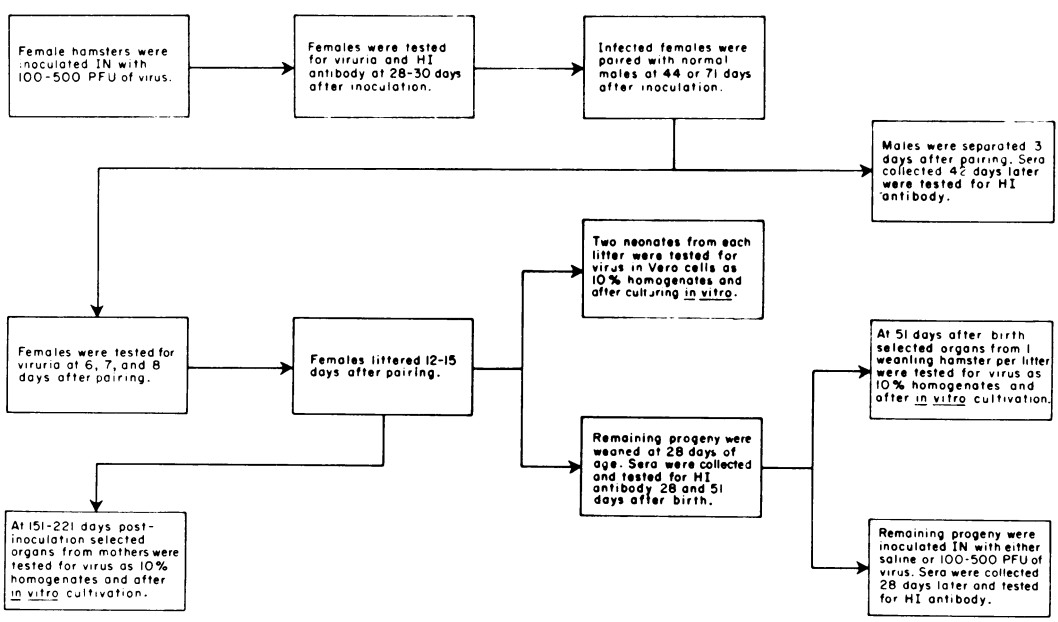

FIG. 1. *Procedure used to study vertical transmission of Modoc virus in hamsters.*

In a second experiment, vertical transmission was studied in four hamsters inoculated i.n. during pregnancy 7 to 9 days before they littered.

Horizontal transmission. In experiments to demonstrate horizontal transmission, hamsters were inoculated i.n. with 100 to 500 PFU of Modoc virus. At 14 days postinoculation, each infected hamster was housed with three normal hamsters for a period of 28 days in cages (20 by 30 cm) which contained autoclaved sand that was changed once a week. Horizontal transmission in the contact hamsters was measured by testing for antibody development at the day of separation from the infected hamsters and again 28 days later. Throat swab and urine samples were taken from the inoculated hamsters at selected intervals during the exposure period. One or more positive samples collected at each time were identified as Modoc virus by the fluorescent antibody technique. Undiluted urine samples collected from nine hamsters at 28, 56, 84, and 85 days after infection were tested for infectious virus by inoculating each sample i.n. (0.05 ml) into one normal hamster. Antibody samples were taken from these animals 28 days after inoculation with urine.

RESULTS

Infection of hamsters. Adult hamsters were susceptible to infection with Modoc virus by i.n. inoculation and the mean infectious dose (ID_{50}) was 10 to 20 PFU (Table 1).

The viremia profile in hamsters inoculated i.n. is presented in Fig. 2. Separate groups of four or five hamsters were bled on alternate days beginning on day 1 or day 2 after injection. Maximal viremia titers of $10^{5.2}$ to $10^{6.4}$ PFU per ml of blood developed 4 days after inoculation. Viremia was not detected after day 5 with the exception of a single plaque in one blood sample taken 8 days postinoculation.

Four hamsters inoculated i.n. with virus all developed SDN, HI, and CF antibodies (Fig. 3). Neutralizing and HI antibodies were detected in the four animals 1 week after inoculation and there was a parallel increase in titers of both types of antibodies for the following 3 weeks. Only one hamster had detectable CF antibodies 7 days after inoculation, but CF antibodies were

TABLE 1. *Susceptibility of hamsters to infection with Modoc virus by intranasal inoculation*

No. of PFU inoculated per hamster	No. of hamsters infected[a] per no. inoculated
38	3/4
3.8	1/4
0.4	1/4
0.04	0/4

[a] Development of an HI antibody titer of 1:20 or greater by 28 days postinoculation was considered positive for infection.

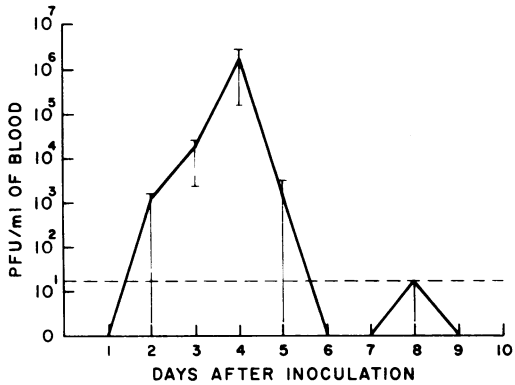

FIG. 2. *Viremia profile in hamsters inoculated intranasally with Modoc virus. Solid line indicates mean of titers; vertical bar indicates range of titers; and broken line indicates minimum titer detectable.*

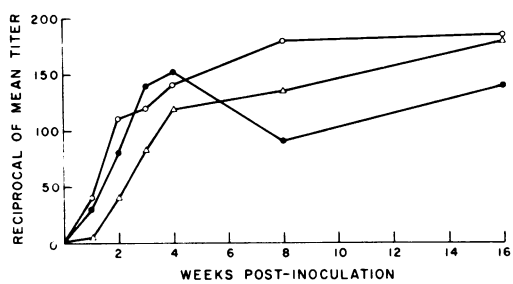

FIG. 3. *Development of hemagglutination-inhibition, complement fixation, and neutralizing antibodies in four hamsters inoculated intranasally with Modoc virus. Symbols: O, mean of serum-dilution neutralization titers; ●, mean of HI titers; Δ, mean of CF titers.*

detectable in all four hamsters at 14 days after inoculation and the titers increased steadily for the following 2 weeks. Neutralizing, HI, and CF antibodies persisted for at least 4 months postinoculation in all animals.

The viruria titers in hamsters inoculated i.n. with Modoc virus are given in Table 2. Virus was detected in the urine of all nine animals one or more times during each of the 3 months tested. Maximal viruria titers were detected 7 days after infection.

Modoc virus was isolated from the throat swabs of all nine hamsters on day 7 after infection. The titers in these samples ranged from 3.5×10^1 to 9.0×10^2 PFU/ml with a mean titer of 2.3×10^2 PFU/ml. Virus was not detected in throat swabs taken 14, 21, and 28 days postinoculation.

Vertical transmission. All six female hamsters used in the vertical transmission study had virurias at 28 to 30 days postinoculation. These females were bred 7 to 11 weeks after inocula-

TABLE 2. *Virurias in hamsters inoculated intranasally with Modoc virus*

Days after inoculation	No. positive/ no. tested[a]	Log_{10} titers of virus per ml in positive urine samples	
		Mean	Range
7	8/8	4.6	2.8-5.4
14	5/9	2.3	0.7-2.8
21	5/9	2.6	1.7-3.0
28	4/9	2.7	1.7-2.9
35	6/9	2.1	2.3-3.7
42	3/9	2.3	2.0-2.5
49	6/9	2.9	1.5-3.4
56 and 57	10/17	2.5	1.3-2.9
63	5/9	2.7	1.3-3.2
70	6/9	3.5	1.3-4.3
77	5/9	2.5	0.7-2.8
84 and 85	7/16	2.6	1.0-3.0

[a] Nine animals were included in the study but urine samples were not always obtained from each animal at each collection period.

tion. The breeding males did not develop antibodies to Modoc virus after three days of contact with these females. Five of the females littered.

Persistent infection with Modoc virus was demonstrated in each of the five mother hamsters after mating. Virurias were detected in four females during pregnancy and virus was recovered from the cultured organs of two of these animals (one from cultured lungs and one from cultured kidneys) when they were sacrificed. Modoc virus was isolated from the cultured kidneys of the fifth mother that had no detectable viruria during pregnancy and from the sixth female that failed to become pregnant after pairing. These viral isolations were made 151 to 221 days after inoculation. Whenever virus was isolated, it was recovered from in vitro cell cultures. No isolations were made when homogenates of the same organs were assayed similarly for virus. The isolates were identified by the fluorescent antibody technique. All animals from which virus was recovered had SDN antibody titers of 1:160 or greater to Modoc virus at the time of sacrifice.

None of the 34 offspring from the five chronically infected mothers showed evidence of infection. Virus was not isolated from tissue homogenates or in vitro cultures that were prepared from 10 newborn hamsters and from five progeny that were tested when 51 days of age. The sera that were collected from 24 of the hamsters at 28 and 51 days after birth were all negative when tested for HI antibody to Modoc virus. Fourteen hamsters born of the five females were challenged with Modoc virus at 51 days of age and all developed HI antibody. Five hamsters from the same mothers were injected with saline and none had detectable antibody.

Modoc virus was transmitted transplacentally to offspring born to hamsters that experienced a primary viral infection during pregnancy. Four hamsters were infected i.n. 7 to 9 days before they littered. All their progeny were either stillborn or developed symptoms of encephalitis and died by 7 days after birth. Since the incubation period of this strain of Modoc virus in suckling hamsters inoculated i.c. was 10 to 14 days, it was assumed that these progeny were infected in utero and that they died from the Modoc viral infection. Unfortunately, no attempt was made to confirm the latter by recovery of virus from the progeny.

Horizontal transmission. To study horizontal transmission, infected hamsters were caged in close contact with groups of three normal hamsters. Only one of 27 contact hamsters caged for 4 weeks with nine infected hamsters became infected as demonstrated by antibody development. Modoc virus was excreted in the urine of all of the infected hamsters during this period. The means and ranges of the viruria titers of samples collected from the infected hamsters were previously presented in Table 2. The excreted virus was infectious for normal hamsters as shown in Table 3. The contact hamsters that did not become infected were separated from the inoculated animals for 35 days and then were challenged by i.n. inoculation of 100 to 500 PFU of Modoc virus. All were negative before challenge and developed antibody by 28 days after challenge.

The possibility was tested that the low transmissibility of Modoc virus was due to a rapid

TABLE 3. *Infectivity of Modoc virus isolated from the urine of persistently infected hamsters for normal hamsters*[a]

No. of hamsters shedding virus	Viral titers in urine of shedding hamsters[b]	Fraction of hamsters inoculated with urine that developed antibody to Modoc virus[c]
17	≤5	5/17
8	5-50	7/8
5	51-500	5/5
6	501-5,000	5/6

[a] Each urine sample was inoculated i.n. (0.05 ml) into one normal hamster.

[b] The minimum titer that was detectable in Vero cells was 5 PFU/ml.

[c] An HI antibody titer of 1:20 or greater at 28 days postinoculation was considered as evidence of infection.

inactivation of virus in urine. The rate of decline of infectious virus at 7, 22, and 37 C in urine collected from male hamsters is shown in Fig. 4. The half-life of the virus decreased rapidly with increasing temperature and ranged from hours at 7 C to minutes at 37 C. A similar phenomenon was observed in a more limited study with urine from female hamsters.

DISCUSSION

Adult Syrian hamsters were found to be quite susceptible to i.n. infection with Modoc virus. Infected animals developed high-level viremias that peaked on day 4 and usually were undetectable by day 6. Disappearance of viremia coincided with the development of detectable neutralizing and HI antibodies by 7 days after infection. Antibodies persisted at high titers for at least 4 months after the primary infection. Virus was recovered from throat swabs of all animals 7 days after infection, but not later. This virus must have represented active infection in or near the respiratory tract, since viremias were negligible at this time. Virurias were readily detected in infected animals over a 12-week observation period after inoculation.

The long-term shedding of virus in the urine of all animals tested indicated that Modoc virus readily established a persistent infection in hamsters. Evidence for persistent infection was obtained also by the isolation of virus from in vitro cultures of lungs from one hamster and the kidneys from three other hamsters when they were sacrificed 151 to 221 days after infection. Sera from all persistently infected animals contained high levels of neutralizing antibody. These results confirm the observations previously reported by Johnson (11).

The mechanism which allows Modoc virus to persist in hamsters that are circulating high levels of neutralizing antibody is unknown.

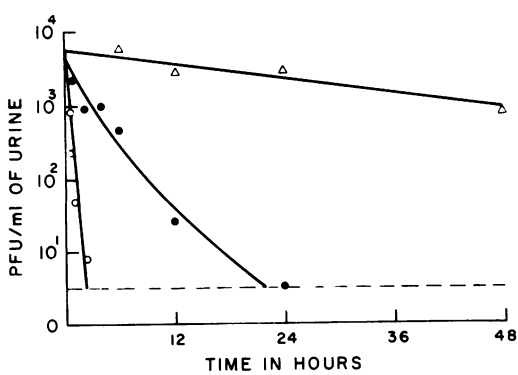

FIG. 4. *Survival of Modoc virus in hamster urine at 7 C, 22 C, and 37 C. △, 7 C; ●, 22 C; ○, 37 C; -----, minimum titer detectable.*

Since Modoc virus appeared to be quite dissociable from antibody prepared in hamsters (6), it is conceivable that infectious virus-antibody complexes circulate in the blood and are filtered out by the kidneys as is known to occur in several other persistent virus infections (9). However, further studies on the pathogenesis of this virus in hamsters are needed before the exact nature of the persistent infection can be determined.

The excretion of infectious virus in urine offered a potential source for horizontal transmission, but a very low level of transmission was found even under conditions of crowding. This apparent paradox is explained partially by the toxicity of urine for Modoc virus. The levels of excreted virus that were detected in urine would have been inactivated within 18 h at room temperature. Inactivation of virus probably was not due entirely to thermal inactivation because earlier studies had demonstrated that Modoc virus was inactivated slowly at room temperature in PBS (pH 7.4) (6). The reduction of infectious virus in urine may have been caused also by fecal matter introduced during the collection of urine. Further loss of virus could have occurred during evaporation of urine, through adsorption of urine by the bedding and from toxic components in the bedding and environment.

Chronically infected female hamsters that were mated 2 to 3 months after their primary infection did not transmit virus to their offspring. Neutralizing antibodies in the blood of mother hamsters may have prevented infectious virus from crossing the placenta or infecting the embryos. This conclusion seems warranted since female hamsters with primary infections during their second trimester of gestation appeared to transmit infection transplacentally. In this case, progeny were either stillborn, or died after birth, within 10 to 14 days after infection of the mothers. Suckling hamsters inoculated i.c. with the non-neuroadapted strain of Modoc virus used in this study normally had an incubation period of 10 to 14 days (unpublished data). Similar results have been reported for mice infected with SLE virus 7 or more days of gestation (1).

The lack of horizontal or vertical transmission of Modoc virus by persistently infected hamsters in the present study does not mean that such transmission cannot occur in the natural wildlife hosts of Modoc virus if they are persistently infected. Wildlife hosts might develop higher titered virurias, or may be more susceptible to infection with Modoc virus than are hamsters. Also, the behavioral patterns of wildlife hosts could bring them into more direct

contact with virus. Conversely, natural strains of Modoc virus might have different pathogenic and immunogenic properties than those of our laboratory adapted strain. Further studies in which wildlife hosts are infected with several strains of Modoc virus could determine if persistent infection in such animals allows survival of this virus in nature. Results of a study on Modoc viral infection in deer mice (*P. maniculatus*) will be reported at a later date.

It should be emphasized that Modoc virus was never isolated from homogenates of organs from persistently infected hamsters. Virus was only recovered after in vitro cultivation of cells from the lungs of one animal and the kidneys of three other animals. No virus was isolated by either method from organs of other hamsters that were known to have a persistent infection. Viral isolations from persistently infected animals were undoubtedly hampered by our inability to effectively eliminate antibody from tissue homogenates and possibly from some in vitro cultures.

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