PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 1288–1292 (1974)

Erythrocyte Association and Interferon Production by Minute Virus of Mice[1] (37998)

ROBERT E. HARRIS,[2] PHILIP H. COLEMAN, AND PAGE S. MORAHAN

Department of Microbiology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

Minute virus of mice (MVM) is a member of the single-stranded DNA Parvovirus group. In infected animals, MVM, like Kilham's rat virus (KRV), Toolan's H-1 virus, and feline panleucopenia virus (other Parvoviruses), has the capacity of producing lethal teratogenic diseases (1–6). During the viremic phase of infections with KRV and H-1 viruses (2, 5), these viruses are found in association with the erythrocyte fraction of the blood; a factor which, as suggested by Mims (6), may be important in enhancing their teratogenic potential. It is not known whether MVM is also associated with erythrocytes in the infected animal.

Another aspect of the teratogenic potential of MVM is that this virus induces lethal teratogenic disease in hamsters, but only mild fetal infections in rats and mice (3, 4). The mechanism responsible for the differences in disease severity between these species is unknown. Induction of interferon in the pregnant mouse during MVM infection could be a possible mechanism limiting the severity of the disease. There are no published reports on the interaction of MVM and interferon. Indeed, the role of interferon in Parvovirus infections has not been studied extensively (7).

The present studies were undertaken to determine (a) if MVM is found in association with erythrocytes in infected mice, (b) if the virus induces interferon in mice, and (c) if the virus is sensitive to interferon.

Materials and Methods. Viruses. MVM (strain C. R. 10 × CP, Lot 7331/16, courtesy of Dr. John C. Parker, Microbiological Associates, Rockville, MD) was grown in secondary rat embryo cell culture monolayers. After 7 days incubation at 36°C, the virus was harvested, and a pool of seed virus prepared according to the methods of Schell (8) and Rowe (9). The $TCID_{50}$ (50% tissue culture infectivity dose) was $10^{6.7}$/ml in rat embryo cells and the hemagglutinin (HA) titer was 1:512.

For interferon assays the following viruses were used: The GD-7 strain of mouse polio was grown in BHK-21 cells, and the Indiana strain of vesicular stomatitis virus (VSV) was grown in L929 cells. These viruses titered 5×10^3 HA units and $10^{6.7}$ $TCID_{50}$/ml, respectively. As a reference for mouse interferon production, the Minnesota strain of encephalomycarditis virus (EMC) was used. EMC was grown in L929 cells and titered 2.8×10^8 plaque-forming units (PFU)/ml.

Animals. Male, nonpregnant and pregnant female mice (DUB/ICR), and pregnant rats (DUB/SDS) were purchased from Flow Laboratories, Dublin, VA.

Cell cultures. Rat embryo cells (REC) were prepared from 15- to 18-day, decapitated and eviscerated embryos and stored as a 10% suspension in liquid nitrogen. Primary REC monolayers were grown in 32-oz bottles in growth media consisting of Eagle's minimal essential medium (MEM), 10% fetal calf serum (FCS), 200 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml

[1] During this research REH was supported by the United States Air Force, A.F.I.T.

[2] Current address: Wilford Hall USAF Hospital, Lackland Air Force Base, San Antonio, TX 78238.

Fungizone. When primary cultures were confluent, secondary cultures were prepared in plastic 24-well trays (Linbro, FB 16-24-TC) seeded with 7×10^5 cells/ml and grown in 2% CO_2.

For interferon assays, L929 cells (Microbiological Associates, Rockville, MD) were grown in MEM with Hanks' balanced salt solution (HBSS), 10% FCS and antibiotics; and primary Swiss mouse embryo cells (Flow Laboratories Inc., Rockville, MD) were grown in MEM, with Earle's balanced salt solution (EMEM), 10% FCS, and antibiotics.

Animal inoculations and sample collections. For MVM viremia and interferon production studies, 10 adult male, 10 nonpregnant, and 10 pregnant female mice, known to be free of MVM antibodies, were inoculated intraperitoneally with $10^{5.5}$ $TCID_{50}$ of MVM. Uninoculated mice served as controls. Five mice in each group were bled daily via the retroorbital plexus using sterile heparinized capillary pipets. Their blood was pooled and centrifuged at 4°C for 10 min at 300*g*. The plasma was collected aseptically for viral assays and interferon studies and stored in liquid nitrogen. The buffy coat was discarded. The erythrocyte fraction was washed three times by centrifugation in 100% FCS. To further remove any virus which might be associated with white blood cells, after each wash the upper 10% of the erythrocyte fraction was discarded. After the third wash, the bottom fraction of erythrocytes was stored in liquid nitrogen for viral studies.

As a positive control in the mouse interferon production studies, and in order to insure that the assay system employed was sufficiently sensitive to detect interferon, a known interferon-producing virus (EMC) was studied. Thirty male mice were inoculated intraperitoneally with 56 PFU of EMC virus. Groups of five mice were bled daily by cardiac puncture; the serum was collected and frozen at −70°C for subsequent interferon assays.

Viral assays. For viral assays, growth medium was removed from 75–85% confluent monolayers of secondary REC, 10-fold dilutions of MVM were made in growth medium. Cell cultures were inoculated in quadruplicate with 0.1 ml each of the appropriate dilution. After adsorption for 2 hr at 36°C, the cells were washed twice with 1 ml of HBSS. Finally, 1 ml of Wistar EMEM (10) containing 2% FCS and antibiotics was added. Cells were observed microscopically for cytopathic effects (CPE) daily for 12 days. Cultures showing 2 + CPE or greater were used to calculate the $TCID_{50}$ by the method of Reed and Muench (11).

Interferon assays. Blood samples from MVM- or EMC-infected mice were assayed for interferon. Because plasmas from MVM-infected mice were free of detectable virus, these samples were not treated prior to assay. Sera from EMC-infected mice were acidified at pH 2.0 for 2 days in order to inactivate the virus. The interferon assay method was essentially as described by Oie *et al.* (2) measuring the yield reduction of GD-7 virus HA in L929 cells. Duplicate tube cultures were each incubated with 0.5 or 1 ml of the sample interferon dilution. The titer of interferon was determined graphically as the reciprocal of the interferon dilution producing 0.5 $\log_{10}$ reduction in HA yield. A laboratory standard murine interferon was titered simultaneously in each assay.

In other studies the sensitivity of MVM to the laboratory standard murine interferon was determined. Dilutions of interferon were added to quadruplicate cultures of rapidly growing secondary mouse embryo cells (MEC). After incubation overnight at 36°C, the cultures were washed twice with HBSS and challenged with 10^4 $TCID_{50}$ of MVM. Because MVM did not produce characteristic CPE in the MEC cultures, infected MEC cultures were incubated for 7 days and then frozen and thawed three times. The yield of MVM in pools of each interferon dilution was determined by titration of the virus in secondary REC. The titer of interferon against MVM was calculated as the reciprocal of the interferon dilution producing 0.5 $\log_{10}$ reduction of the MVM yield from the noninterferon-treated virus-infected cells. As a control for the sensitivity of the assay, a duplicate

set of interferon-treated cultures were inoculated with 10^2 $TCID_{50}$ of VSV. After 2 hr of viral adsorption, cultures were washed twice with HBSS and then fed with Wistar EMEM maintenance media. After 3 days, the titer of interferon against VSV was calculated as the reciprocal of the interferon dilution which resulted in a 50% reduction in CPE from that in noninterferon-treated controls.

Results. In the viremic mouse, MVM was closely associated with the erythrocyte fraction of the blood (Fig. 1). The peak erythrocyte viremia in infected animals was noted on day 5 or 6 after MVM inoculation. There was no detectable virus ($\leq 10^{1.5}$ $TCID_{50}$/ml) found in the plasma fraction of the blood. The peak erythrocyte MVM viremia titer ($10^{7.0}$/ml) for male mice was statistically higher ($P < 0.01$) than those corresponding titers obtained with either pregnant or nonpregnant female mice.

MVM did induce interferon in the plasma of infected mice; however, the levels of interferon induced were relatively low

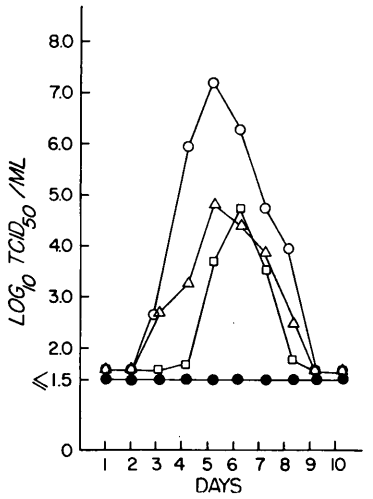

FIG. 1. Viremia levels of MVM after intraperitoneal inoculation of mice with $10^{6.5}$ $TCID_{50}$ of virus. Each point represents titers in pooled samples of five mice: ○—○—○, Erythrocyte fraction of male mice; □—□—□ erythrocyte fraction of nonpregnant female mice; △—△—△ erythrocyte fraction of pregnant female mice; ●—●—●, plasma fraction of male and nonpregnant and pregnant female mice.

(Table I). In the nonpregnant female mice, plasma interferon titers ranged from less than 20 units on all days except for titers of 38 and 45 units on days 2 and 3 after infection. In pregnant mice, plasma interferon titers ranged from less than 20 units on all days except for titers of 42, 56, and 42 units on days 7–9 after infection. In male mice, all plasma samples were free of detectable interferon in the lowest dilution tested (1:40). The low levels of interferon seen with this single-stranded DNA virus are in contrast to the results obtained with the single-stranded RNA virus, EMC. Infection of male mice with EMC led to the production of 1500 units of interferon on day 2 after inoculation, when the virus titer was 3.7×10^4 PFU/ml (Table I).

Although MVM was a poor inducer of interferon, the virus was very sensitive to the inhibiting action of interferon (Fig. 2). The titer of the laboratory standard murine interferon against VSV was 4000 units as assayed by the CPE method. Interferon assayed simultaneously against MVM resulted in an interferon titer of 20,000 units as determined by the yield reduction method.

Discussion. Four members of the single-stranded DNA Parvovirus group (Toolan's H-1 virus, Kilham's rat virus, feline panleucopenia virus, and minute virus of mice) have the propensity to attack animal fetuses *in utero* (1–6). The teratogenic action of these viruses is probably based upon their affinity for replicating cells and for the proliferating vascular bed of the developing fetus (2, 3, 5).

Mims (6) has stated that viruses circulating free in the plasma are rapidly cleared from the blood stream, but that whenever viruses are associated with erythrocytes, the viremia may persist for longer periods of time. The form in which a virus arrives at placental villi, whether free in the plasma or closely bound to erythrocytes, may determine whether a high or low level or viremia is requisite for transplacental infection (6). Viruses that have been found to be associated with or localized intracellularly within erythrocytes include Toolan's H-1 virus, KRV virus, Colorado tick fever virus, Friend

TABLE I. Blood Interferon Titers Obtained from Mice After Inoculation with Minute Virus of Mice (MVM) or Encephalomycarditis Virus (EMC).

	Interferon titers[a]			
	MVM			EMC
Day[b]	Nonpregnant	Pregnant	Male	Male
1	<20	<20	<40	<20
2	38	<20	<40	1500
3	45	<20	<40	60
4	<20	<20	<40	<20
5	<20	<20	<40	ND[c]
6	<20	<20	<40	ND
7	<20	42	<40	ND
8	<20	56	<40	ND
9	<20	42	<40	ND
10	<20	<20	<40	ND
Laboratory standard mouse interferon	45,000	40,000	35,000	75,000

[a] Interferon titer = reciprocal of greatest dilution resulting in 0.5 $\log_{10}$ reduction of GD-7 virus hemagglutinin yield.

[b] Days after inoculation of mice with $10^{5.5}$ $TCID_{50}$ of MVM or 56 PFU of EMC virus.

[c] ND indicates not done since there were no survivors.

leukemia virus, Rauscher leukemia virus, mammary tumor virus, and various viruses of lower vertebrates (2, 5, 13–18). Colorado tick fever virus, reovirus, and bluetongue virus, members of the Diplornavirus group, have all been shown to be teratogenic in animals (Harris and Coleman, Abstr. Ann. Meeting A.S.M.—1973, p. 226, 19, 20). Toolan's H-1 virus and KRV are teratogenic viruses which are members of the Parvovirus group. The present studies clearly show that MVM, another teratogenic Parvovirus, is also closely associated with the erythrocyte fraction of the blood.

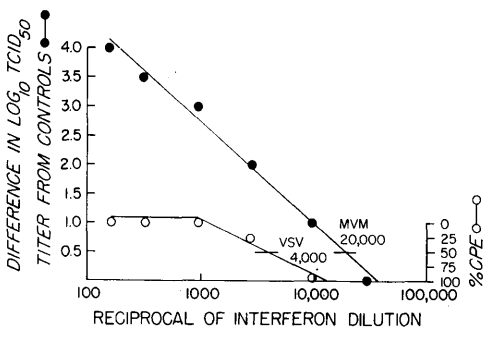

FIG. 2. Sensitivity of MVM and VSV to murine interferon. Mouse embryo cells were incubated with dilutions of murine interferon, washed, and either MVM or VSV adsorbed. The titer of interferon against VSV was calculated as the reciprocal of the dilution of interferon resulting in 50% reduction of cytopathic effect (CPE) as compared to virus control cultures. The titer of interferon against MVM was calculated as the reciprocal of the interferon dilution that reduced the yield of MVM ($TCID_{50}$/ml) by 0.5 $\log_{10}$ from the yield in noninterferon-treated, virus-infected cells.

In our studies evaluating the viremia of MVM in mice, it was interesting to note that the virus was a poor inducer of interferon. Interferon levels ranging only from 38–56 units were detected. These results are consistent with the study of Kilham *et al.* (7) in which KRV induced only 5–70 units of interferon in serum specimens from infected rats. The fact that little interferon was produced is consistent with the knowledge that MVM and KRV possess single-stranded DNA of small molecular size, and thus, would not be expected to be potent interferon inducers (21). Further evidence that these results are valid is shown by the fact that EMC virus, a RNA virus, was a potent interferon inducer using similar conditions

of production and assay in our laboratory.

Although MVM appears to be a poor inducer of interferon in mice, the virus was sensitive to the action of interferon when compared to VSV, which is known to be sensitive to interferon (22). These results correlate well with the study of Kilham *et al.* (7) in which KRV was also shown to be very sensitive to interferon when compared to VSV. Thus, two members of the Parvovirus group have been shown to have similar characteristics in relationship to interferon induction and sensitivity to interferon.

Summary. Minute virus of mice was found to be closely associated with the erythrocyte fraction of the blood of infected mice, with peak viremia occurring on day 5 or 6 after intraperitoneal inoculation. The association with erythrocytes suggests a common mechanism of transplacental transport for the teratogenic Parvoviruses. In addition, MVM was found to be a relatively poor inducer of interferon and to be very sensitive to the inhibiting action of interferon.

The opinions expressed in this paper are those of the authors and not those of the Department of Defense nor the U. S. Air Force.

The technical assistance of Georgia Schuller, Rose Ragland, and Charles Fleming is gratefully acknowledged.

1. Kilham, L., Margolis, G., and Colby, E. D., Lab. Invest. **17**, 465 (1965).

2. Kilham, L., and Margolis, G., Teratology **2**, 111 (1969).

3. Kilham, L., and Margolis, G., Proc. Soc. Exp. Biol. Med. **133**, 1447 (1970).

4. Kilham, L., and Margolis, G., Teratology **4**, 43 (1971).

5. Margolis, G., and Kilham, L., Lab. Invest. **22**, 478 (1970).

6. Mims, C. A., Bacteriol. Rev. **28**, 30 (1964).

7. Kilham, L., Buckler, C. E., Ferm, V. H., and Baron, S., Proc. Soc. Exp. Biol. Med. **129**, 274 (1968).

8. Schell, K., Lane, W. T., and Casey, M. J., Proc. Nat. Acad. Sci. USA **55**, 81 (1966).

9. Rowe, W. P., Hartley, J. W., and Estes, J. D., Nat. Cancer Inst. Monogr. **4**, 189 (1960).

10. Sokal, F., Kuwert, E., Wiktor, T. J., Hummeler, K., and Koprowski, H., J. Virol. **2**, 836 (1968).

11. Reed, L. J., and Muench, H. A., Amer. J. Hyg. **27**, 493 (1938).

12. Oie, H. K., Buckler, C. F., Uhlendorf, C. P., Hill, D. A., and Baron, S., Proc. Soc. Exp. Biol. Med. **140**, 1178 (1972).

13. Portella, O. B., Arch. J. Virusforsch. **14**, 277 (1964).

14. Toolan, H. W., Proc. Soc. Exp. Biol. Med. **124**, 144 (1967).

15. Bernard, G. W., Cooper, E. L., and Mandell, M. L., J. Ultrastruct. Res. **26**, 8 (1968).

16. Emmons, R. W., Oshiro, L. S., Johnson, H. N., and Lennette, E. H., J. Gen. Virol. **17**, 185 (1972).

17. Nandi, S., and Haslam, S., Cancer Res. **31**, 479 (1971).

18. Reilly, C. A., Jr., and Schloss, G. T., Cancer Res. **31**, 841 (1971).

19. Hashimi, A., Carruthers, M. M., Wolf, P., and Lerner, A. M., J. Exp. Med. **124**, 33 (1966).

20. Schultz, G., and Delay, P. D., J. Amer. Vet. Ass. **127**, 224 (1955).

21. Grossberg, S. E., N. Engl. J. Med. **287**, 13, 79 and 122 (1972).

22. Wagner, R. R., and Huang, A. S., Virology **28**, 1 (1966).

Received Sept. 4, 1973. P.S.E.B.M., 1974, Vol. 145.