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Effect of Murine Cytomegalovirus on Implantation Stage Mouse Embryos (41219)

JOHN F. BASKAR AND ENG-SHANG HUANG

Cancer Research Center and Department of Medicine, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Abstract. To investigate the effect of murine cytomegalovirus (MCMV) infection on implantation stage embryos, mouse blastocysts were exposed to MCMV *in vitro* and then surgically transferred to foster mice. When denuded blastocysts were exposed to MCMV, only 1 mother out of 14 gave birth to two live babies. In contrast when intact blastocysts were exposed to MCMV, the percentage of live births occurring in all mothers was not significantly different from that of controls. MCMV was also isolated from uterine washings of five out of seven mice which had received denuded MCMV-exposed blastocysts. Serological and DNA restriction enzyme analyses showed that the stock MCMV and the virus isolates were identical. MCMV microinjected into uterine lumen of pregnant mice at the time of embryo implantation resulted in a fetal loss with occasional inflammation of uteri. Tissue extracts obtained from implantation sites of the mice shed MCMV when cocultivated with mouse embryo fibroblasts (MEF). In contrast, little fetal loss occurred in pregnant mice whose uteri were microinjected with heat-inactivated MCMV. Tissue extracts derived from implantation sites of these mice did not yield virus when cocultivated with MEF. Thus, MCMV has a detrimental effect on mouse implantation stage embryos and the presence of MCMV at the time of embryo implantation may result in fetal loss.

Cytomegalovirus (CMV) infection is one of the most common congenital infections in man and can lead to a wide variety of birth defects including mental retardation, microcephaly, epilepsy, blindness, deafness, cerebral palsy, and muscular deficiency (1–3). There are at least two possible modes of CMV transmission to developing embryos: (i) Embryos could be infected during implantation by CMV associated with semen and/or the female genital tract (4–6). (ii) cytomegalovirus may cross the placenta and infect the embryo (7, 8).

Transplacental murine cytomegalovirus (MCMV) infections have not been established (9, 10) because MCMV does not infect the three-layered trophoblastic murine placenta (two of which are syncytial in nature) in a short 20-day gestation period. However, the mouse model is ideally suited to studies of CMV infections during implantation and consequences of such infections for several reasons. First, the mouse is easy to manipulate and it has a short gestation period so experimental results are obtained quickly. In addition, sophisticated technology exists for *in vitro* culture and transplantation of developing manipulated embryos into foster mothers (11, 12). Finally, the overall picture of MCMV infections in the mouse bears striking resemblance to human CMV infection and disease (13, 30). To investigate the possible effect of CMV infection on implantation stage embryos, we have exposed mouse blastocysts to MCMV *in vitro* and then surgically transferred the eggs to pseudopregnant surrogate mice. Alternatively, MCMV has been microinjected into the uterine horns of 3½-day-pregnant mice just prior to the blastocyst implantation and the outcome of the infection was studied. Our findings suggest that mouse early stage embryos are susceptible to MCMV.

Materials and Methods. *Cells and virus.* Mouse embryo fibroblasts (MEF) from CF-1 late pregnancy embryos were cultured in minimum essential medium (MEM), containing 100 units of penicillin and 100 μg/ml streptomycin, and supplemented with heat-inactivated 10% fetal calf serum (FCS). After infection with MCMV the cells were maintained in MEM–4% FCS. Stock salivary gland MCMV (Smith strain) was passaged three times in MEF before use.

Animals and collection of blastocysts. Germ-free random-bred albino Swiss mice, CF-1, from Charles River Breeding Labo-

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ratory, Wilmington, Massachusetts, were used. Prior to the experiments, salivary gland extracts from a few randomly selected mice from the colony were tested on MEF and found free of MCMV. Female mice, 6–8 weeks old, were superovulated (11) with 5 IU of pregnant mare serum gonadotropin (Sigma), followed 48 hr later with 5 IU of human chorionic gonadotropin (Sigma). The females were caged overnight with males and an insemination was verified the next morning by the presence of a vaginal plug (Day 0). Blastocysts were collected 3.5 days later. Blastocysts pooled from several pregnant mice were used in all experiments.

Exposure of blastocysts in vitro and their transfer to the uteri of surrogate mothers. Blastocysts, with zona pellucida (intact) and without zona pellucida (denuded), were exposed to MCMV (10^4 PFU/100 blastocysts in 0.8 ml of MEM–4% FCS in organ culture dishes (Falcon No. 3010) for 1 hr at 37°, rinsed several times with MEM–10% FCS and cultured for 2 hr in the same medium at 37° in a humidified atmosphere of 5% CO_2 and 95% air. To remove the zona pellucida, intact blastocysts were incubated with 0.2% pronase in Dulbecco's phosphate-buffered saline (DPBS) at 37°. The lysis of the zona was periodically monitored using a dissecting microscope. When the zona began to dissolve, the eggs were transferred to fresh medium, washed, and cultured for 2 hr to allow the membrane to recover before exposing to MCMV. Control blastocysts were exposed to culture fluid, which was identical to the virus inoculum except it contained no virus. Additional controls were run with heat-inactivated preparations of MCMV. After culture, embryos were washed once with DPBS and surgically transferred to the uteri of pseudopregnant surrogate mothers as described (12) to allow further development *in vivo*. The pseudopregnant mice were prepared by mating females which were in proesterus cycle with vasectomized males.

Microinjection of MCMV into uterine lumen prior to blastocyst implantation. Approximately 2 μl of MCMV preparation containing 10–20 PFU was microinjected into the uterine lumen of naturally mated mice 3½ days postcoitum (prior to blastocyst implantation) as described.[1] As a control, 3½-day-pregnant mice were microinjected similarly with 2 μl of heat-inactivated MCMV. Experimental and control animals were caged in separate quarters. Six to ten days later the mice were sacrificed and the implantation sites observed.

Virus isolation. Pooled blastocysts were exposed to MCMV (10^4 PFU/100 blastocysts) for 90 min, washed four times with MEM–4% FCS for 4 hr at 37°, and then plaque assayed by cocultivating with MEF (20 blastocysts/35-mm petri dish) for 10–14 days. For control, blastocysts exposed to heat-inactivated MCMV were cocultivated with MEF. In addition, uterine washes and 10% extracts (w/v) of fetal and placental tissues were also plaque assayed on MEF.

Characterization of the viral isolates by restriction enzyme analysis. The analysis was performed as described (14). Mouse embryo fibroblast monolayers were infected with MCMV isolates (MOI:2). After 90 min adsorption at 37°, MEM–4% FCS was added, and the cells were incubated for 24 hr. The medium was then replaced with low-phosphate MEM containing 4% dialyzed FCS and 30 μCi/ml of carrier-free [^{32}P]orthophosphate (New England Nuclear Company). After culture for an additional 48–72 hr, the ^{32}P-labeled viral DNA was purified as described (15). Purified ^{32}P-labeled viral DNA (10 μl, 10^4 cpm) in Tris-buffered saline (TBS: 0.15 *M* NaCl–0.05 *M* Tris–hydrochloride, pH 7.4) was digested with 5 μl (10 units) of *Eco*RI (16), *Bam*HI (17), or *Xba*I (18) for 6 hr at 37° in the presence of 0.01 *M* $MgCl_2$ and 0.006 *M* β-mercaptoethanol. The samples were then subjected to electrophoresis in E buffer (30 m*M* Tris, 20 m*M* sodium acetate, and 1 m*M* EDTA, pH 7.2) on a 1% agarose slab-gel at a constant voltage of 4 V/cm (15). The gels were vacuum dried onto Whatman filter

[1] J. D. Biggers, J. F. Baskar, and D. F. Torchiana. Reduction of the fertility of mice by the intrauterine injection of prostaglandin antagonists. Submitted for publication.

paper and exposed to X-ray film (Kodak RP/R2).

Preparation of antiserum. Antiserum to MCMV (Smith stain) was prepared as described (31) by infecting 10-week-old male CF-1 mice with two sublethal doses of MCMV 3 weeks apart. Mice were bled 2 weeks after the second inoculation. The antiserum has an immunofluorescence titer of 1:256. We have used 1:32 dilution (in DPBS) in our tests.

Virus neutralization. Serial twofold dilutions of anti-MCMV serum were prepared. Equal volumes of serum dilutions and virus calculated to contain approximately 100 PFU were mixed, incubated at 37° for 1 hr, and immediately titrated on MEF monolayers.

Anti-complement (C3) immunofluorescence (ACIF). The test was done as described (19). Briefly, virus-infected and uninfected MEF cells, and denuded blastocysts exposed to live and heat-inactivated MCMV, cultured for 24 hr and air dried on cover slips, were fixed with ice-cold acetone for 10 min. Anti-MCMV mouse serum mixed with human complement was applied to the coverslips. The coverslips were then incubated at 37° in a humidified atmosphere for 1 hr. After extensively washing with phosphate buffer saline (PBS), FITC-conjugated anti-human C3 globulin (Hyland) along with rhodamine counterstain (BBL) were applied to the samples and incubated for another 45 min. After incubation, the coverslips were drained and washed in PBS as before. The coverslips were washed in distilled water and mounted in phosphate-buffered glycerol. Samples were viewed on a Leitz Ortholux microscope, using a uv light source.

Results. *Transfer of MCMV-exposed blastocysts to surrogate mothers.* To determine our ability to successfully transfer fertilized eggs, we obtained blastocysts on four different occasions from C57BL females (black) which had been mated with their C57 littermates. These embryos, intact and denuded, were surgically transferred to the uteri of Swiss surrogate mothers (white) which had been induced to pseudopregnancy by mating with vasectomized Swiss males (white). All the surrogate mothers gave birth to black mice. At present we achieve 50–59% live births from this surgical egg transfer technique, as good or better than previously reported results (20).

In seven experiments MCMV-exposed blastocysts were transferred to the uteri of pseudopregnant mice, 160 denuded blastocysts to 14 mice (Group A), and 89 intact blastocysts to 8 mice (Group B). As controls, blastocysts bathed in culture fluid were similarly transferred to the uteri of pseudopregnant mice, 116 denuded blastocysts to 10 mice (Group C), and 76 intact blastocysts to 7 mice (Group D). In addition, 40 denuded blastocysts exposed to heat-inactivated MCMV were transferred to 4 pseudopregnant mice (Group E). The results of the embryo transfer experiments are shown in Table I. One out of 14 surrogate mothers in Group A gave rise to two live babies (<2%). However, the babies were subsequently eaten by their surrogate mother and we were unable to observe the progress of any congenital defects. In contrast, all the surrogate mothers in the other four groups gave live births at term: 47 in Group B (53%) 65 in Group C (56%), 40 in Group D (53%), and 19 in Group E (48%). All the offspring looked healthy and there was no cannibalism.

Among the experimental foster mothers in Group A, three showed a massive swelling in the external genitals 10–14 days after the blastocyst transfer. Inclusion bodies similar to that of CMV were seen in some areas of the swollen tissues in 6-μm-thick sections stained with May–Grünwald. Focal to diffuse infiltrations of lymphocytes and other white blood cells were also seen in these tissues. The uterine washings of these mice, when incubated with MEF, produced a cytopathic effect (CPE) characteristic of MCMV. In addition, uterine washings from two other mice (out of four) in the same group also gave rise to CPE typical of MCMV infection when cocultivated with MEF. In contrast, none of the uterine washings tested from other groups gave rise to CPE when cocultivated with MEF.

To verify whether the virus used to infect

TABLE I. SUMMARY OF EGG TRANSFER EXPERIMENTS

Group	Total number			Percentage of live births at term
	Blastocysts transferred	Foster recipient mothers	Live births at term	
A. Denuded blastocysts[a] exposed to MCMV	160	14	2	1.3
B. Intact blastocysts exposed to MCMV	89	8	47	52.8
C. Denuded blastocysts[a] exposed to MEF culture fluid[b]	116	10	65	56.0
D. Intact blastocysts exposed to MEF culture fluid[b]	76	7	40	52.6
E. Denuded blastocysts[a] exposed to heat-inactivated MCMV	40	4	19	47.5

Note. Denuded or intact blastocysts were transferred to the uteri of pseudopregnant foster mothers for development to term after exposure to MCMV.

[a] Blastocysts were treated with 0.2% pronase to remove the zona pellucida.

[b] Fluid identical to MCMV stock except it did not contain virus.

the blastocysts was the same as that isolated from uterine washings, we subjected these two viruses to serological and restriction enzyme analyses. Anti-MCMV mouse serum gave a positive reaction in ACIF tests with MEF infected with either the stock MCMV or the virus isolated from uterine washings. The antiserum also neutralized both the stock MCMV and the viral isolate. However, the antiserum did not react with WI-38 cells infected with HCMV (Towne strain) nor did it neutralize HCMV. Three restriction endonucleases, *Eco*RI (16), *Bam*HI (17), and *Xba*I (18), which recognize and cleave specific DNA base sequences were used for analysis of the viral DNAs. We obtained identical fragment patterns for the two viral DNAs. From this, we concluded that MCMV isolated from uterine washings is a reisolation of the virus employed to infect blastocysts.

Susceptibility of blastocysts to MCMV in vitro. Blastocysts were exposed to MCMV *in vitro*, and after washing extensively in culture medium, 83 denuded blastocysts (Group 1) and 81 intact blastocysts (Group 2) were cocultivated with MEF (20 blastocysts/dish). As controls, blastocysts obtained from the same pool were exposed to heat-inactivated MCMV and 81 denuded blastocysts (Group 3) and 80 intact blastocysts (Group 4) were cocultivated with MEF. The results of these experiments are summarized in Table II. In three out of four experiments, distinct plaques were observed in MEF cultures exposed to denuded blastocysts infected with MCMV (Group 1) 7–12 days after cocultivation. Supernatants of these cultures showed low levels of infectious MCMV when assayed on MEF. In addition, the cells involved in the formation of plaques were positive for MCMV-specific antigens when tested with anti-MCMV serum in the ACIF test. In contrast, no plaques nor infectious virus were detected in any other cultures after cocultivation with MEF.

To detect MCMV-specific antigens in blastocysts, we performed two additional experiments in which 31 denuded blasto-

TABLE II. COCULTIVATION OF MCMV-EXPOSED BLASTOCYSTS WITH MOUSE EMBRYO FIBROBLASTS

Experiment[a]	No. of blastocysts employed	After cocultivation of blastocysts with MEF		
		No. of plaques observed	MCMV titer[b]	MCMV antigens[c]
1	21	15	2×10^2	Positive
2	20	0	0	Negative
3	21	25	1.1×10^3	Positive
4	21	11	1.7×10^2	Positive

[a] Denuded blastocysts exposed to MCMV. No infectious virus nor MCMV antigens were detected when (i) denuded blastocysts were exposed to heat-inactivated MCMV; (ii) intact blastocysts were exposed to MCMV; and (iii) intact blastocysts were exposed to heat-inactivated MCMV.

[b] Infectious virus in culture fluid on Day 12 after the cocultivation as determined by a plaque assay on MEF.

[c] Determined by an anticomplementary immunofluorescence test.

cysts were exposed to MCMV and cultured for 24 hr. No fluorescence was observed in any of the blastocysts when reacted with anti-MCMV sera in the ACIF test.

Microinjection of MCMV into uterine lumen of 3½-day-pregnant mice. In order to study the effect of MCMV on blastocysts at the time of implantation we surgically microinjected 2 μl of MCMV (10–20 PFU) into the uterine lumen of ten 3½-day-pregnant mice. The mice were sacrificed 6 to 10 days postinoculation and the implantation sites were examined. In total, 25 implantations occurred in these 10 mice. However, the implantation sites, in many cases, contained neither fetuses (14/25) nor placentas (8/25) (Fig. 1). In addition, the tissue extracts (4/11 embryos and 7/14 placentas) yielded MCMV 10–16 days after cocultivation with MEF. In contrast, 67 implantations occurred in 9 mice whose uteri were microinjected with heat-inactivated MCMV. Most of these implantation sites contained both fetuses (64/67) and placentas (65/67). In addition, none of the tissue extracts (18 fetuses and 18 placentas) yielded MCMV when cocultivated with MEF for 16 days.

Discussion. In order to explore the effect of MCMV infection on implantation stage embryos we exposed mouse blastocysts to MCMV *in vitro* and, after implanting them in surrogate mothers, observed the consequences for both mother and offspring. The results obtained in our studies showed that MCMV exerts harmful influence on the implantation stage embryo, the blastocyst. The exposure of denuded blastocysts to

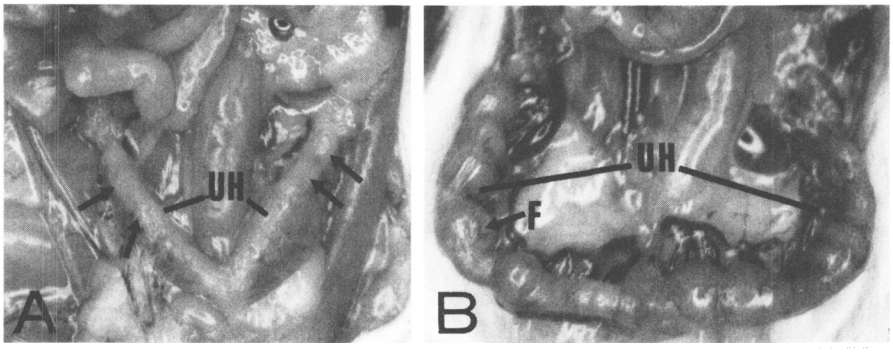

FIG. 1. Viscera of 3½-day-pregnant Swiss mice showing uterine horns (UH). (A). Each mouse, at the time of embryo implantation (3½ days postcoitum), was microinjected with 2 μl of MCMV (10–20 PFU) into both uterine horns. Ten days later the mouse was sacrificed. Note the uterine horns are devoid of implants except in places where there are signs of fetal absorption (arrows). (×3). (B). Control mouse sacrificed 10 days after microinjection of heat-inactivated MCMV into its uterine horns (2 μl/horn) at 3½ days postcoitum. Unlike the mouse in (A), the uterine horns of this mouse bear numerous viable embryos (F). (×3).

MCMV resulted in fetal loss (Table I). However, it is not known in this study whether the fetal loss was due to direct MCMV infection of the blastocysts or indirect consequences of endometrial secondary infection.

We were able to isolate MCMV from uteri of mice (5 out of 14) which had received MCMV-exposed denuded blastocysts. The presence of MCMV in the uterine lumen of these mice indicates that the virus may have been released by the infected blastocysts. It is also possible that a portion of the virus recovered in the uterine washes came from secondarily infected endometrium. Observation of CMV-bearing cells in the genital tissues of the mice which received MCMV-exposed denuded blastocysts indicates MCMV infection. This observation is in agreement with studies reported previously by Neighbour (21). Isolation of MCMV on a few occasions by cocultivation of MCMV-exposed denuded blastocysts with MEF suggests MCMV infection of blastocysts. In our earlier studies, we have observed viral particles with a typical herpesvirus morphology in a few blastomeres of MCMV-exposed denuded early blastocysts cultured for 48 hr *in vitro* (unpublished data). However, in the present study, we failed to detect MCMV-specific antigens in blastomeres 24 hr postinfection. The reasons for not detecting these antigens are not known at the present time. It is very unlikely that the replicative cycle of MCMV in blastomeres is considerably delayed and further studies need to be done to investigate this problem.

In this study, we have used pronase to remove the zona pellucida from the blastocyst. Data are now available which suggest that *in vivo* enzyme-like pronase may also be involved in the release of the embryo (blastocyst) from the zona pellucida before implantation (22).

Small amounts of MCMV were introduced into uterine lumen of mated mice just prior to embryo implantation, during the time the embryo is exposed to its environment. During implantation the blastocyst hatches out of its protective coat, the zona pellucida, and implants onto the endometrium. After implantation, the embryo is once again separated from the uterine environment by the development of a placenta. This short implantation period may be the only time the embryos are susceptible to foreign agents including viruses. The results obtained from the microinjection studies show that MCMV in the genital tract at the time of implantation has a detrimental direct effect on embryonic development. We concluded that implantation stage embryos (blastocysts) and the endometrium were susceptible to MCMV infection because MCMV was isolated from embryonic and placental tissues after MCMV microinjection.

Other laboratories have also attempted to infect mouse embryos with MCMV. Medearis (9) and Johnson (10) induced intrauterine infection in midgestation with MCMV. Consistent with our findings, they observed that MCMV infection caused fetal loss. However, the authors were unable to isolate virus from the surviving fetuses. This failure was attributed to the inability of MCMV to cross the placenta and to infect the fetus, and the fetal loss was attributed to the influence of maternal infection on the fetus.

In a recent investigation Neighbour (21) studied the effects of indirect maternal MCMV infection on the *in vivo* development of mouse preimplantation embryos by depositing MCMV into the peritoneal cavity. The intraperitoneal inoculation produced an acute maternal infection, retarding embryonic development *in vivo*. The embryos themselves were not productively infected and they developed into apparently normal fetuses when transferred to uninfected mice. Most recently Young *et al.* (23) attempted infection and fertilization of mice after artificial insemination with a mixture of sperm and MCMV. In such studies, fertilization occurred and embryogenesis appeared normal on gross examination. The experiments described in this communication demonstrate that direct exposure of mouse blastocysts to MCMV during implantation results in fetal loss.

Results from our previous study (24) and those of others (21, 25) indicate that mouse preimplantation embryos are not susceptible to MCMV infection. However, in an *in vitro* study Heggie and Gaddis (26) have re-

ported a slight reduction of mouse blastocysts developing from two-cell embyros exposed to MCMV compared to the blastocysts developing from two-cell embryos exposed to control fluids. In contrast, mouse postimplantation embryos beyond egg cylinder stage are susceptible to MCMV infection (21, 24). MCMV infection of the egg cylinders could be aided by ectoplacental cone cells which are susceptible to MCMV infection *in vitro* (32). The data presented in this communication suggest that mouse implantation stage embryos (blastocysts) may also be susceptible to MCMV. However, more direct and conclusive evidence is required to prove the susceptibility of the blastocyst to MCMV. Further experiments on the direct susceptibility of the blastocyst to MCMV are now being designed and executed in our laboratory.

Transplacental CMV can be studied with the guinea pig (GP) model without *in vitro* manipulations. However, with the GP system it is difficult to study the effect of CMV infection and the virus–host interactions during implantation. Similar to the work of others (27–29) we have succeeded in establishing transplacental GP CMV infections. The two experimental animal model systems, the mouse system for genital route CMV infection and the GP system for transplacental CMV infection, will complement each other in our further study of CMV congenital infections leading to birth defects.

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