The effect of maternal cytomegalovirus infection on preimplantation development in the mouse

P. A. Neighbour

Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, U.K.

Summary. Mice were inoculated with murine cytomegalovirus at 14 and 7 days before and 1 and 4 days after mating. The effects of maternal infection on early pregnancy were investigated. Inoculation 7 days before and 1 day after mating, i.e. around ovulation and implantation, significantly reduced pregnancy rate. Embryos in these females were developmentally retarded, perhaps because of the inflammatory effect of the infection on the genital tract. Retarded embryos developed normally when cultured.

Introduction

The materno-fetal interactions of virus infections during human pregnancy have been frequently reviewed (Blattner, Williamson & Heys, 1973; Fucillo & Sever, 1973; Hardy, 1973), and have been studied in laboratory animals (Ferm & Kilham, 1964; Hassan & Cochran, 1969; Selzer, 1969; Coid & Wardman, 1971). Certain viruses have been shown to cause fetal wastage, congenital infection, morphological anomaly and other forms of reproductive failure following maternal infection in the postimplantation stages of pregnancy. However, little attention has been given to the possible effects of viral infection occurring before conception or during the preimplantation period. Mims (1966) has demonstrated by immunofluorescence that transmission of lymphocytic choriomeningitis virus may occur from parent to offspring through the infected ovum, and many leukoviruses are also transmitted by this vertical route (Bentvelzen, Daams, Hageman & Calafat, 1970; Calarco & Szollosi, 1973; Kalter *et al.*, 1975). The preimplantation embryo is a favourable environment for the replication of certain viruses: Mengo encephalitis virus (Gwatkin & Auerbach, 1966); human adenovirus type 5 (Chase, Winters & Piko, 1972); simian virus 40, Moloney sarcoma virus and polyoma virus (Baranska, Sawicki & Koprowski, 1971; Biczysko, Solter, Pienkowski & Koprowski, 1973) have been shown *in vitro* to infect and replicate within preimplantation mouse embryos, usually resulting in the death of the embryo.

Thus, maternal infection occurring at the times of ovulation and implantation might jeopardize the success of pregnancy, particularly if the infection produces an acute viraemia with an associated dissemination of infection in the genital tract.

Cytomegalovirus (CMV) has been isolated from the human genital tract during chronic infection (Medearis, Montgomery & Youngblood, 1970), and Lang & Kummer (1972) have isolated infectious CMV in the semen of a patient with a CMV mononucleosis, suggesting that venereal transmission might result in an ascending genital tract infection. In the mouse, the virus causes an acute, generalized infection which reaches its maximum 3–4 days after inoculation and persists for a further 7–9 days. Medearis (1964) and Johnson (1969) have attempted to produce intrauterine infections with the murine cytomegalovirus (MCMV) during pregnancy and found that infection caused fetal wastage, which was almost certainly due to the indirect effects of maternal ill health. The authors were unable to isolate virus from the surviving fetuses and concluded that the virus does not cross the placenta. However, the MCMV was inoculated either after implantation, when the pregnancy was well established, or several weeks before mating, by which time the maternal viraemia had subsided.

In the experiments described in the present paper, viraemia was timed to coincide with ovulation and implantation. Thus, the effects of maternal infection on the development *in vivo* of preimplantation mouse embryos were studied.

Materials and Methods

Mice

Specific pathogen-free mice of the BALB/c strain were obtained from the Animal Division of the C.R.C., and were conventionally housed. Females were virgin and approximately 6–8 weeks old at the time of mating. Males were 10–12 weeks old and of proven fertility. In most experiments, one male was placed overnight with three females. The females were examined on the following morning for the presence of a vaginal plug which was taken as evidence of successful mating. The day of finding such a plug was considered to be Day 1 of pregnancy. In some experiments (as indicated in the Tables), ovulation was induced by i.p. injections of 2 i.u. PMSG (Gestyl: Organon) and 2 i.u. HCG (Pregnyl: Organon) 48 hr later. Mice were paired after the second injection and overnight mating was again detected by the presence of a vaginal plug.

Implantation

Females were killed by CO₂ asphyxiation on Day 9 of pregnancy and the total number of living and dead implantations was counted. Examination of the ovaries revealed whether ovulation had occurred at the time of mating, and the number of oocytes ovulated was determined by counting the corpora lutea. Females which had failed to ovulate were excluded from analysis. Pregnancy rates, preimplantation losses and implantation rates (pregnant females only) were calculated for each experimental group.

Development in vivo

Mice were killed on Days 2, 3 or 4 of pregnancy. Embryos were collected by flushing the oviducts or uteri with phosphate-buffered saline. All embryos were examined microscopically to determine their developmental stage and whether they were morphologically normal.

Development in vitro

The embryos collected on Days 2 and 3 of pregnancy were pooled within each experimental group. All degenerate and apparently arrested embryos showing any degree of necrosis were discarded. The remaining viable embryos were rinsed three times in Whittingham's medium based on Tyrode's solution (Whittingham, 1971), and transferred to droplets of this medium under paraffin oil in sterile plastic dishes (Falcon Plastics) for culture. They were then incubated at 37°C in a water-saturated atmosphere of 5% CO₂ in air for 72 hr (Day 2 embryos) or 48 hr (Day 3 embryos). After culture, the stage of development reached was determined by microscopic examination.

Virus

The Smith strain of murine cytomegalovirus (Smith, 1954) was obtained from Dr R. D. Smith of Cincinnati, Ohio. It had been passed repeatedly *in vivo* in the salivary glands of Swiss Webster mice. It was passed three times in this laboratory in 6-week-old female BALB/c mice by i.p. inoculation. Viral stocks were a clarified suspension of homogenized salivary glands from infected mice in Hanks B.S.S. supplemented with 5% fetal calf serum and antibiotics. The stock virus was titrated by the i.p. inoculation of serial 10-fold dilutions into 6-week-old female BALB/c mice. Histological examination of salivary glands was used to detect infection. The titre of this stock virus was 1.1×10^7 ID₅₀/ml.

Inoculation

Female mice were inoculated 14 and 7 days before (*ante coitum*) and 1 and 4 days after (*post coitum*) mating with uninoculated males. Infected mice received an i.p. injection of 0.3 ml of a 1×10^{-2}

dilution of stock virus in Eagles MEM containing 5% fetal calf serum and antibiotics. Control mice were inoculated with a similar dilution of stock virus which had been heated to 56°C for 30 min and found to be free of infectious virus on mouse embryo fibroblast cultures.

The salivary glands of all animals were removed and fixed in formol-acetic-ethanol before embedding in paraffin wax. Sections, stained with haematoxylin and eosin, were examined microscopically for the presence of cytomegaly, monocytic infiltration and intranuclear inclusions, indicative of chronic cytomegalovirus infection.

Statistical significance was determined by application of χ^2 tests or analysis of variance (Snedecor & Cochran, 1967) and significance was accepted at the 5% level.

Results

Histological examination of the salivary glands showed that animals which had received infectious virus were chronically infected, while control mice were uninfected.

Implantation

Infection 14 days before, or 4 days after mating did not affect the pregnancy rate or the preimplantation loss (Table 1), but when mice were infected either 7 days before or 1 day after mating, there was a significant reduction in pregnancy rate and increase in preimplantation loss. There was no significant effect of infection on the implantation rate or on the mean numbers of implantations in those mice which became pregnant, irrespective of the timing of inoculation.

These results show that the time of inoculation is critical and that infection exerts an all-or-none effect on implantation, leading to normal pregnancy or complete failure.

Table 1. Implantation in mice infected with cytomegalovirus before or after mating

Time of inoculation and treatment	No. of females mated	No. of females with implantations	Pregnancy rate (%)	Pre-implantation loss† (%)	Pregnant females		
					Mean no. $\pm$ S.E.M./female		Implantation rate (%)
					Corpora lutea	Implantations	
14 days <i>a.c.</i>							
Uninfected	19	13	68.4	47.7	8.2 $\pm$ 0.6	6.2 $\pm$ 0.8	75.7
Infected	21	15	71.4	47.2	10.1 $\pm$ 0.4*	7.0 $\pm$ 0.7	69.5
7 days <i>a.c.</i>							
Uninfected	16‡	15	93.8	44.6	12.5 $\pm$ 0.7	7.5 $\pm$ 0.5	60.1
Infected	31‡	19	61.3*	65.3***	12.3 $\pm$ 1.1	6.3 $\pm$ 0.7	50.9
1 day <i>p.c.</i>							
Uninfected	57	41	71.9	46.0	8.4 $\pm$ 0.3	6.1 $\pm$ 0.5	72.2
Infected	63	28	44.4***	62.1***	9.5 $\pm$ 0.3	7.3 $\pm$ 0.4	76.4
4 days <i>p.c.</i>							
Uninfected	34	27	79.4	31.2	9.1 $\pm$ 0.3	7.2 $\pm$ 0.6	79.2
Infected	32	23	71.9	36.5	8.6 $\pm$ 0.4	6.7 $\pm$ 0.4	78.3

Significantly different compared with uninfected mice of the same group: * $P < 0.05$; *** $P < 0.001$.

† $[(\text{Total no. of CL} - \text{total no. of implantations})/\text{Total no. of CL}] \times 100$.

‡ Ovulation was induced in these mice.

Development in vivo

To investigate the reasons for the failure of implantation, mice infected 7 days before and 1 day after mating were killed on Days 2, 3 and 4 of pregnancy. Examination *post mortem* revealed that the uteri and oviducts of infected mice were inflamed, there being increased vascularization and enlargement of the mesenteric vessels. The lumbar and renal lymph nodes, which drain the uterus, were greatly enlarged. The developmental stages of the embryos collected are shown in Table 2.

Table 2. Development *in vivo* of preimplantation embryos in mice induced to ovulate and infected with cytomegalovirus before or after mating

Time of inoculation and treatment	Day (p.c.) of embryo collection	No. of females	Total no. of embryos collected	Embryonic stage at collection (%)							
				1-cell	2-cell	3-cell	4-cell	8-cell	Morula	Blastocyst	Degenerate
7 days a.c.											
Uninfected	2	17	174	3.4	74.7	5.2	7.5	—	—	—	8.6
Infected		16	161	4.3	85.1*	0.6*	5.0	—	—	—	5.0
Uninfected	3	16	122	—	0.8	—	5.7	67.2	13.9	—	12.3
Infected		27	141	—	2.1	—	14.2*	60.2	2.8**	—	20.6
Uninfected	4	24	220	—	2.3	—	1.8	2.3	29.5	53.6	10.5
Infected		22	160	—	3.1	—	6.9*	3.8	51.9***	15.6***	18.8*
1 day p.c.											
Uninfected	2	15	150	5.3	75.3	1.3	1.3	—	—	—	16.7
Infected		17	174	29.3***	56.3***	0	0	—	—	—	14.4
Uninfected	3	12	114	7.9	0	0	7.0	74.6	—	—	10.5
Infected		16	154	11.7	0.6	0.6	4.5	54.5***	—	—	27.9***
Uninfected	4	15	141	—	—	—	0	—	37.6	53.2	9.2
Infected		16	169	—	—	—	0.6	—	53.3**	27.2***	18.9*

Significantly different compared with uninfected mice of the same group: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

On Day 4 of pregnancy there were significantly fewer blastocysts, with correspondingly more morulae and degenerate embryos in the uteri of infected mice than in those of uninfected controls. This retardation in development was also observed earlier in the preimplantation period. Most of the embryos collected on Days 2 and 3 of pregnancy from infected mice were one developmental stage behind those from uninfected donors (Table 2).

These results show that preimplantation development is retarded when maternal infection is timed appropriately. Further analysis of these data also suggests the all-or-none nature of the response to infection described earlier. On Day 4 of pregnancy, 32.5% of 40 infected mice had a number of blastocysts equal to or greater than the mean found in a group of 45 uninfected mice, while 42.5% had no blastocysts at all. Therefore, while some mice would fail to become pregnant due to an absence of blastocysts from the uterus at implantation, others, albeit a minority, would exhibit normal pregnancy.

Development in vitro

There was no significant difference in the development *in vitro* of embryos collected on Days 2 and 3 of pregnancy from mice inoculated 7 days before or 1 day after mating with infectious or inactivated virus (Table 3).

Table 3. Development *in vitro* of preimplantation embryos collected from mice induced to ovulate and infected with cytomegalovirus before or after mating

Time of inoculation and treatment	Day (<i>p.c.</i>) of embryo collection	Duration of culture (hr)	Total no. of embryos cultured	Embryonic stage after culture (%)			
				2- to 8-cell	Morula	Blastocyst	Degenerate
7 days <i>a.c.</i>							
Uninfected	2	72	147	8.2	11.6	57.1	23.1
Infected			149	9.4	12.1	46.3	32.2
Uninfected	3	48	104	4.8	7.7	72.1	15.4
Infected			109	0.9	11.0	70.6	17.4
1 day <i>p.c.</i>							
Uninfected	2	72	118	3.3	13.6	67.8	15.3
Infected			113	0	10.6	74.3	15.0
Uninfected	3	48	92	0	14.1	80.4	5.4
Infected			90	1.1	6.7	84.4	7.8

These results show that despite developmental retardation *in vivo* preimplantation embryos collected from infected mice were not affected irreversibly and were able to develop normally *in vitro*.

Discussion

These experiments show that there is a failure in pregnancy when maternal infection with MCMV is timed appropriately. There appears to be a critical period when infection will jeopardize the onset of pregnancy. When the infection is timed to coincide with ovulation and preimplantation development, implantation frequently fails to occur. Implantation is not affected when the infection ends before ovulation and the apparent infertility is therefore only temporary. Similarly, when inoculation occurs late in the preimplantation period, generalized infection begins after implantation and the onset of pregnancy is unaffected.

These results also show clearly that infection during this critical period caused a retardation in early embryonic development. In a normal mouse, the uterus is only receptive to the implanting embryos for a few hours (Finn, 1966). The developmental retardation of the embryos from infected mice and the consequent asynchrony of uterine receptivity are probably responsible therefore for the implantation failure.

This pregnancy block occurred as an all-or-none response to maternal infection during this critical period. Despite normal ovulation and mating, mice either exhibited a total implantation failure, or became pregnant with a normal implantation rate. Therefore, infection acted primarily at a maternal rather than an embryonic level. Embryos which were removed from their maternal environment and cultured developed normally. From this evidence, and that of the inflammation of the reproductive tract of infected mice, it would appear that the infection affects development by altering the environment of the developing embryo. It is unlikely that the embryos themselves are infected, but this is being investigated.

Other viruses have been found to affect the onset of pregnancy in mice. Hassan & Cochran (1959) also reported that reovirus type 1 almost completely prevented implantation when inoculated on Days 1, 2 and 3 of pregnancy. Similarly, human influenza reduced the implantation rate in mice inoculated before mating (Adams, Heath, Imagawa, Jones & Shear, 1956).

Therefore, it can be concluded that maternal infection can affect the course of preimplantation development in the mouse. The outcome of CMV infection during human pregnancy, however, differs considerably from the murine situation. Placental transmission of CMV is known to occur in women and this can result in abnormal fetal development (Potter, 1957; Weller, 1971; Weller & Hanshaw, 1962). Cytomegalovirus is host-specific, and there is wide variation in the course of infection depending upon the host species. However, despite these differences, one may speculate that a similar situation to that described in this paper may arise in man. Infection with CMV, or other viruses, at the time of conception may directly or indirectly impose a temporary infertility, preventing the onset of an otherwise successful pregnancy.

I thank Dr D. A. J. Tyrrell and Dr R. D. Barnes for their advice and help with the preparation of this manuscript; and Mr Bruce Howard for his technical assistance. The work was carried out during the tenure of a Medical Research Council Studentship.

References

- ADAMS, J.M., HEATH, H.D., IMAGAWA, D.T., JONES, M.H. & SHEAR, H.H. (1956) Viral infections in the embryo. *Am. J. Dis. Child.* **92**, 109–114.
- BARANSKA, W., SAWICKI, W. & KOPROWSKI, H. (1971) Infection of mammalian unfertilized and fertilized ova with oncogenic viruses. *Nature, Lond.* **230**, 591–592.
- BENTVELZEN, P., DAAMS, J.H., HAGEMAN, P. & CALAFAT, J. (1970) Genetic transmission of viruses that incite mammary tumor in mice. *Proc. natn. Acad. Sci. U.S.A.* **67**, 377–384.
- BICZYNSKO, W., SOLTER, D., PIENKOWSKI, M. & KOPROWSKI, H. (1973) Interactions of early mouse embryos with oncogenic viruses—simian virus 40 and polyoma. 1. Ultrastructural studies. *J. natn. Cancer Inst.* **51**, 1945–1954.
- BLATTNER, R.J., WILLIAMSON, A.P. & HEYS, F.M. (1973) Role of viruses in the etiology of congenital malformations. *Prog. med. Virol.* **15**, 1–14.
- CALARCO, P.G. & SZOLLOSI, D. (1973) Intracisternal A particles in ova and preimplantation stages of the mouse. *Nature, New Biol.* **243**, 91–93.
- CHASE, D.G., WINTERS, W. & PIKO, L. (1972) Replication of human adenovirus in cultured mouse embryos. *J. Cell Biol.* **55**, 39a, 77.
- COID, C.R. & WARDMAN, G. (1971) The effect of para-influenza type 1 (Sendai) virus infection on early pregnancy in the rat. *J. Reprod. Fert.* **24**, 39–43.
- FERM, V.H. & KILHAM, L. (1964) Congenital anomalies induced in hamster embryos with H-1 virus. *Science, N.Y.* **145**, 510–511.
- FINN, C.A. (1966) Endocrine control of endometrial sensitivity during the induction of the decidual cell reaction in the mouse. *J. Endocr.* **36**, 239–248.
- FUCILLO, D.A. & SEVER, J.L. (1973) Viral teratology. *Bact. Rev.* **37**, 19–31.
- GWATKIN, R.B.L. & AUERBACH, S. (1966) Synthesis of a ribonucleic acid virus by the mammalian ovum. *Nature, Lond.* **209**, 993–994.
- HARDY, J.B. (1973) Fetal consequences of maternal viral infections in pregnancy. *Archs Otolar.* **98**, 218–227.
- HASSAN, S.A. & COCHRAN, K.W. (1969) Effects of reovirus type 1 on the developing mouse embryo. *Am. J. Path.* **55**, 147–161.
- JOHNSON, K.P. (1969) Mouse cytomegalovirus: placental infection. *J. infect. Dis.* **120**, 445–450.
- KALTER, S.S., HEBERLING, R.L., SMITH, G.C., PANIGEL, M., KRAEMER, D.C., HELMKE, R.J. & HELLMAN, A. (1975) Vertical transmission of C-type viruses: their presence in Baboon follicular oocytes and tubal ova. *J. natn. Cancer Inst.* **54**, 1173–1176.
- LANG, D.J. & KUMMER, J.F. (1972) Demonstration of cytomegalovirus in semen. *New Engl. J. Med.* **287**, 756–758.
- MEDEARIS, D.N., JR (1964) Mouse cytomegalovirus infection. III. Attempts to produce intrauterine infections. *Am. J. Hyg.* **80**, 113–120.
- MEDEARIS, D.N., JR, MONTGOMERY, R. & YOUNGBLOOD,

- L. (1970) Cytomegalovirus infection of the female genital tract. *Pediatr. Res.* **4**, 461.
- MIMS, C.A. (1966) Immunofluorescence study of the carrier state and mechanism of vertical transmission of lymphocytic choriomeningitis virus infection in mice. *J. Path. Bact.* **91**, 395–402.
- POTTER, E.L. (1957) Placental transmission of viruses. *Am. J. Obst. Gynec.* **74**, 505–513.
- SELZER, G. (1969) Transplacental infection of the mouse fetus by Coxsackie viruses. *Israel J. med. Sci.* **5**, 125–127.
- SMITH, M.G. (1954) Propagation of salivary gland virus of the mouse in tissue cultures. *Proc. Soc. exp. Biol. Med.* **86**, 435–440.
- SNEDECOR, G.W. & COCHRAN, W.G. (1967) *Statistical Methods*, 6th edn. Iowa State University Press.
- WELLER, T.H. (1971) The cytomegaloviruses: ubiquitous agents with protean clinical manifestations. *New Engl. J. Med.* **285**, 203–214.
- WELLER, T.H. & HANSHAW, J.B. (1962) Virologic and clinical observations on cytomegalic inclusion disease. *New Engl. J. Med.* **266**, 1233–1244.
- WHITTINGHAM, D.G. (1971) Culture of mouse ova. *J. Reprod. Fert., Suppl.* **14**, 7–21.

Received 24 January 1976