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Murine Cytomegalovirus Infection of Mouse Testes

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With the aim of illustrating a mechanism of cytomegalovirus (CMV) venereal transmission, we induced murine CMV infection in the mouse testes of immunologically competent mice. Using in situ cytohybridization, we were able to show that murine CMV-specific DNA was associated with spermatocytes and mature sperm. Electron microscopy studies also supported sperm infection. The virus could be reisolated from infected epididymal sperm by cocultivation with mouse embryo fibroblasts. We found no difference in either the sexual performance or the fertilization efficiency of the sperm between infected and uninfected males.

Human cytomegalovirus (HCMV), a member of the herpesvirus group, is a ubiquitous agent that still poses a serious health problem. Endemic throughout the world, it is the most frequent cause of human congenital viral infections that result in fetal death, stillbirth, developmental abnormalities, and lesions of the central nervous system and liver (10, 24, 25, 26). The genital system is often among the affected areas. Recent evidence suggests that this virus can be sexually transmitted (7, 11, 15, 22). HCMV has been found in seminal fluid (5, 12, 14, 16, 17) and commonly found in the cervices of pregnant women and patients attending venereal disease clinics (11, 15, 27). Lang and his co-workers have also demonstrated that virions, in the form of infectious extracellular aggregates, were present in the semen of patients with HCMV mononucleosis (16, 17). Semen specimens examined by electron microscopy (EM) have been found to contain HCMV particles in both intracellular and extracellular locations (14, 16). Infections of females, including ascending genital tract infection, caused by sexual contact with infected males, could lead to the infection of the embryo and to subsequent deleterious effects during fetal development.

The genital organs of mice are also susceptible to murine cytomegalovirus (MCMV) infection (3, 6, 8, 20). Since MCMV can be found in the ejaculate of infected males, the virus may be sexually transmitted, thereby affecting the outcome of pregnancy (20; our unpublished data). As an initial step in the determination of the origin of testicular infection and the susceptible cell types in the testes, we examined the interaction of MCMV with the testicular interstitial Leydig cells of mice. Our results indicated that Leydig cells are susceptible to MCMV both in vivo and in vitro (3). In this paper, we are reporting evidence that MCMV can replicate in sperm cells of immunologically competent mice. This raises the possibility that infected sperm may serve as a vector in transmitting the MCMV genome into the egg cell.

Sendai virus-free, randomly bred, albino Swiss mice (strain CF-1) were used throughout this study. Random samples of salivary gland extracts from the colony were tested on mouse embryo fibroblasts (MEF) and found to be free of MCMV. These mice were also found to be seronegative when examined by an indirect immunofluorescence test against MCMV-specific antigens in virus-infected MEF.

In vivo infection of the testes was affected by inoculating both juveniles (age 4 to 6 weeks) and adults (10 weeks or older) intratesticularly (i.t.) with MCMV. The mice were anesthesized with an intraperitoneal (i.p.) injection of 250 mg of tribromoethanol (Avertin; Winthrop Laboratories, New York, N.Y.) per kg of body weight. We gently lifted each testis out of the scrotum through a small ventral incision and injected it with approximately 10 μl of an inoculant containing 10^4 PFU of MCMV. After slipping the testes back into the scrotum, the incision was sutured. Testes of control mice were similarly inoculated with 10 μl of heat-inactivated MCMV. All procedures were performed under aseptic conditions. Later, we periodically sacrificed the mice, removed the testes, and examined them for virus-tissue involvement. Inoculation with MCMV i.t. resulted in the infection of the sperm cells located in the lumen of the seminiferous tubules (Fig. 1). It also caused infection of the Leydig cells located in the testicular interstitial spaces, as shown in our previous report (3). The testes were examined by subjecting frozen testicular sections to in situ [^{3}H]cRNA-DNA cytohybridizations (6, 13). Discrete focal areas of the testicular sections contained distinctive silver grains indicative of positive hybridization of the probe with MCMV DNA. In these areas, grains were heavily concentrated over spermatids inside the lumen of the seminiferous tubules. The infected areas were found in testicular sections of testes obtained from juveniles between 3 and 20 days after i.t. MCMV injection and from adults between 4 and 16 days after MCMV inoculation. In contrast, only scattered background grains were seen in testicular sections taken from control mice that had received heat-inactivated MCMV. Testes sectioned 1 day after MCMV inoculation of both juvenile and adult mice were similarly negative in these hybridizations. The results of these experiments are summarized in Table 1.

To see whether testicular infection could also occur after distant inoculation, we administered sublethal i.p. doses of MCMV to newborn mice (approximately 10^2 PFU) and to adult males (10^4 PFU). Similarly, control animals were injected i.p. with heat-inactivated MCMV. While many of the newborn mice succumbed in the 2 weeks after treatment, these live virus inoculations resulted in the infection of the testes in both the surviving newborns and the adults (Table 1), as determined by our [^{3}H]cRNA-DNA cytohybridization experiments. Again, sparse and distinct focal areas of testicular sections collected on 34 and 42 days (newborn) and between 4 and 58 days (adult) after i.p. inoculation of

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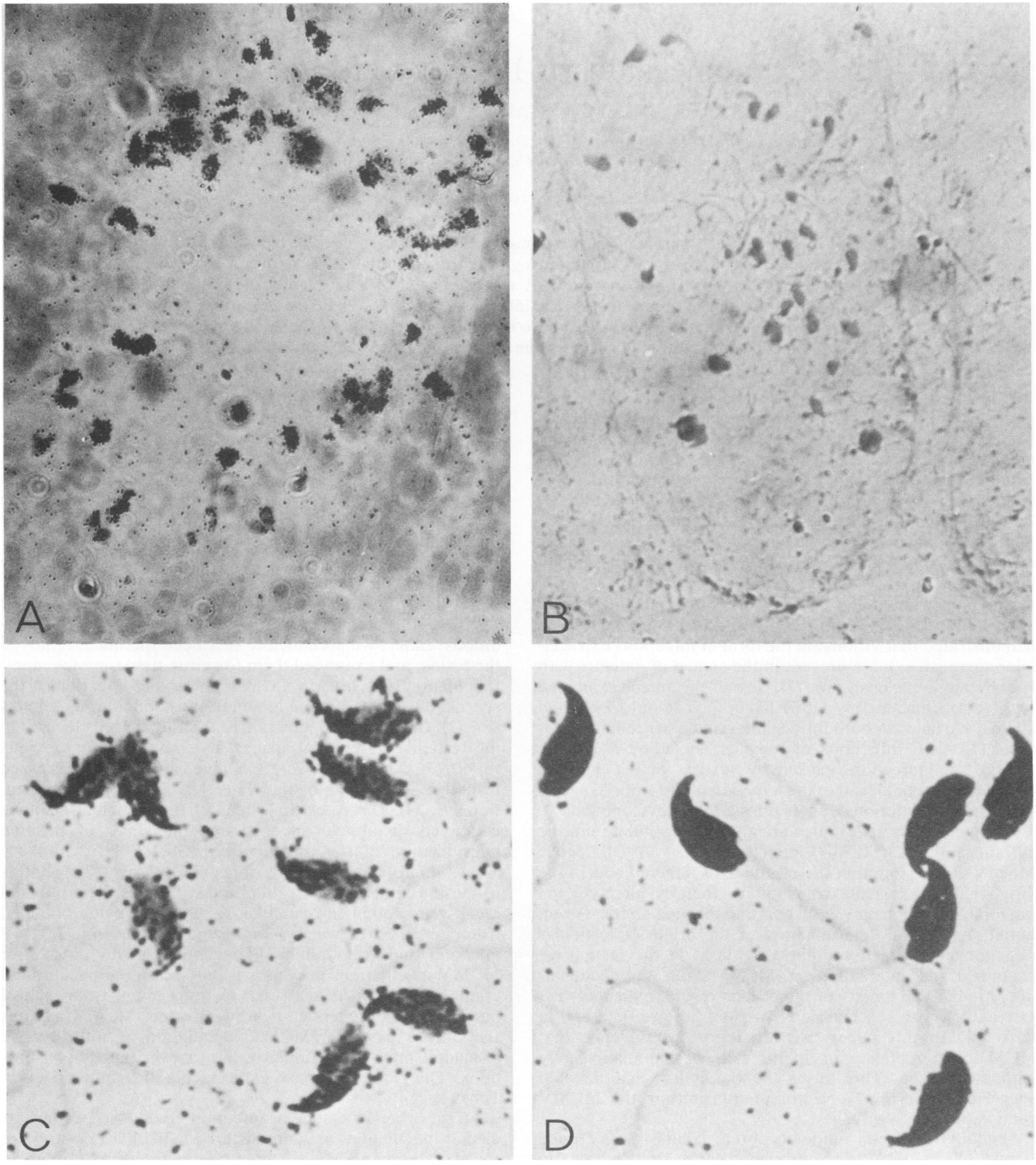

FIG. 1. Detection of MCMV DNA on sperm by [^{3}H]cRNA-DNA in situ hybridization. (A) Testicular section from a 34-day-old mouse showing heavy silver grains on spermatids in the testicular lumen. This mouse was inoculated i.p. at birth with MCMV (10^2 PFU). (B) Testicular section from a 34-day-old control mouse showing no silver grains on the spermatids. This mouse was inoculated i.p. at birth with heat-inactivated MCMV. (C) Sperm from epididymis of a 12-week-old mouse 9 days after MCMV (10^4 PFU) i.p. inoculation. Note the heavy cluster of silver grains on the sperm heads. (D) Sperm from epididymis of a 12-week-old control mouse 9 days after heat-inactivated MCMV i.p. inoculation. The testicular sections and the sperm penetrations were fixed on slides with methanol and acetic acid (3:1) and dehydrated with ethanol. After treatment with proteinase K (10 μg/ ml) for 10 min at 37°C, washing, and dehydration, the cellular DNA was denatured with 0.07 N NaOH for 2 min. After dehydration with ethanol, hybridizations were carried out on all of the above specimens with MCMV [^{3}H]cRNA probe at 66°C for 20 h. The observation of silver grains on the cells indicates the presence of MCMV DNA.

TABLE 1. Detection of MCMV DNA in testes and on sperm heads of mice after MCMV inoculation

Mice (MCMV inoculation)	Inoculation route	Day tested	Presence (+) or absence (−) of MCMV DNA[a]	
			Testes	Epididymal sperm
Newborns (before 24 h after birth)	i.p. (10^2 PFU/animal)	34	+ + +	ND[b]
		42	+ +	ND
		60	−	ND
Juveniles (4–6 wk)	i.t. (10^4 PFU/testis)	1	−	ND
		3	+ +	ND
		6	+	ND
		9	+	ND
		20	+	ND
Adults (10 wk or older)	i.t. (10^4 PFU/testis)	1	−	NT[c]
		4	+	+ +
		5	+	+ +
		6	NT	+ + + +
		7	+ +	+ + + +
		9	+ +	+ + + +
		16	+	+
		33	NT	−
	i.p. (10^4 PFU/animal)	2	−	−
		3	−	−
		4	+	+
		6	+	+ + + +
		8	+ +	+ + +
		9	+	+ + +
		11	+	+
		16	+	+
		22	+	+
		36	−	−
		58	+	−

[a] Detected by [^{3}H]cRNA-DNA cytohybridization. +, About 25% positive for MCMV DNA; + +, 50% positive for MCMV DNA; + + +, 75% positive for MCMV DNA; + + + +, 100% positive for MCMV DNA.
[b] ND, Epididymal sperm are not developed at this stage of development.
[c] NT, Not tested.

MCMV contained heavy silver grains inside the lumen of the seminiferous tubules, notably over spermatids. None or few background grains were found in testicular sections obtained from several test animals and from all control animals. The results of these experiments are summarized in Table 1.

Mature sperm was also examined for infection by periodically removing the epididymides from adult males after either i.t. or i.p. inoculations of MCMV. After squeezing the sperm out of the tubules into tubes containing minimal essential medium supplemented with 4% fetal calf serum and washing the sperm several times in the same medium, we used them for several different assays and observations: (i) recovery of virus in tissue culture, (ii) localization of MCMV DNA by in situ cRNA-DNA cytohybridization, (iii) detection of MCMV-specific antigens, and (iv) EM examination for the presence of virus particles.

Low levels of MCMV were recovered on three occasions (2 and 6 days after i.t. and 5 days after i.p. infection) when the sperm samples were cocultivated and passaged with MEF for 14 or more days. In contrast, no virus was recovered from any of the control specimens tested. Virus thus recovered was examined by restriction enzyme analysis, using the endonucleases *Eco*RI and *Xba*I (2, 4). Coelectrophoresis of these enzyme digests gave identical DNA fragment patterns for both the reisolated virus and the original virus inoculum. In addition, MEF infected by both the viruses produced MCMV-specific antigens as detected by an indirect immunofluorescence test.

In contrast to the viral infectivity assay, we were able to detect MCMV DNA associated with epididymal sperm heads of all the samples tested between days 4 and 16 after i.t. inoculation and days 4 and 22 after i.p. inoculation by in situ [^{3}H]cRNA-DNA cytohybridization (Table 1 and Fig. 1). MCMV DNA was not detectable in the sperm heads when tested on day 33 after i.t. inoculation and on days 2, 3, 36, and 58 after i.p. inoculation. None of the sperm from control animals was found to contain MCMV DNA by this method.

Indirect immunofluorescence tests with anti-MCMV mouse serum (1) failed to detect MCMV-specific antigens in any of the sperm obtained after either i.t. or i.p. inoculations. On several occasions, we have noticed MCMV-specific fluorescence on unidentified cells in sperm preparations derived from infected mice. None of the sperm preparations from control animals were positive in this test.

Sperm preparations were also examined by using EM to detect the presence of virus particles. Since the mature sperm is completely electron dense, we were unable to determine the presence or absence of virus particles in it. However, infected animals often had sperm with indeterminate particles adhering to the outer membrane near the acrosomal region that were not present in uninfected animals (Fig. 2C and D). Immature sperm contained in preparations from infected mice were more easily examined, and we were able to locate complete, enveloped virions in the cytoplasm of some of these sperm (Fig. 2A and B). Incomplete and defective virus particles were also observable in specimens from infected mice. None of the sperm obtained from normal adults and examined under the electron microscope contained any viruslike particles.

To evaluate the sexual performance of infected males, we mated them with uninfected receptive females 8 days after i.p. MCMV inoculation. The results of these experiments are summarized in Table 2. Uninfected males of comparable age were also mated with uninfected receptive females. There were no significant differences between these two groups of males in their sexual preformance: 14 of 21 and 16 of 21 of the females were successfully mated by the infected and uninfected males, respectively (Table 2). Epididymal sperm cells collected from all MCMV-infected mice in this experiment were positive for MCMV DNA by in situ [^{3}H]cRNA-DNA cytohybridization. None of the sperm collected from uninfected males was positive in this test. The efficiency of fertilization was the same for both infected and uninfected males. There was no significant difference in the number of two-celled eggs collected 24 h after mating from the two groups of females (i.e., those mated to infected males versus those mated to uninfected males) (Table 2).

It has been speculated that the venereal acquisition and transmission of cytomegalovirus (CMV) may occur both in humans and in mice (6, 7, 15–17, 20, 27). Lang et al. (16, 17) have found a high CMV titer in the semen of some patients. Coupled with recent reports that MCMV is harbored in the reproductive tissues of male mice (6, 8, 20), our observations of the infection of both sperm and testicular interstitial Leydig cells (3) of immunologically competent mice raise a strong possibility that MCMV infection can be transmitted venereally.

In one study on sexual transmission, male mice were inoculated i.p. with MCMV, producing an acute, general infection (20). Then epididymal sperm samples were col-

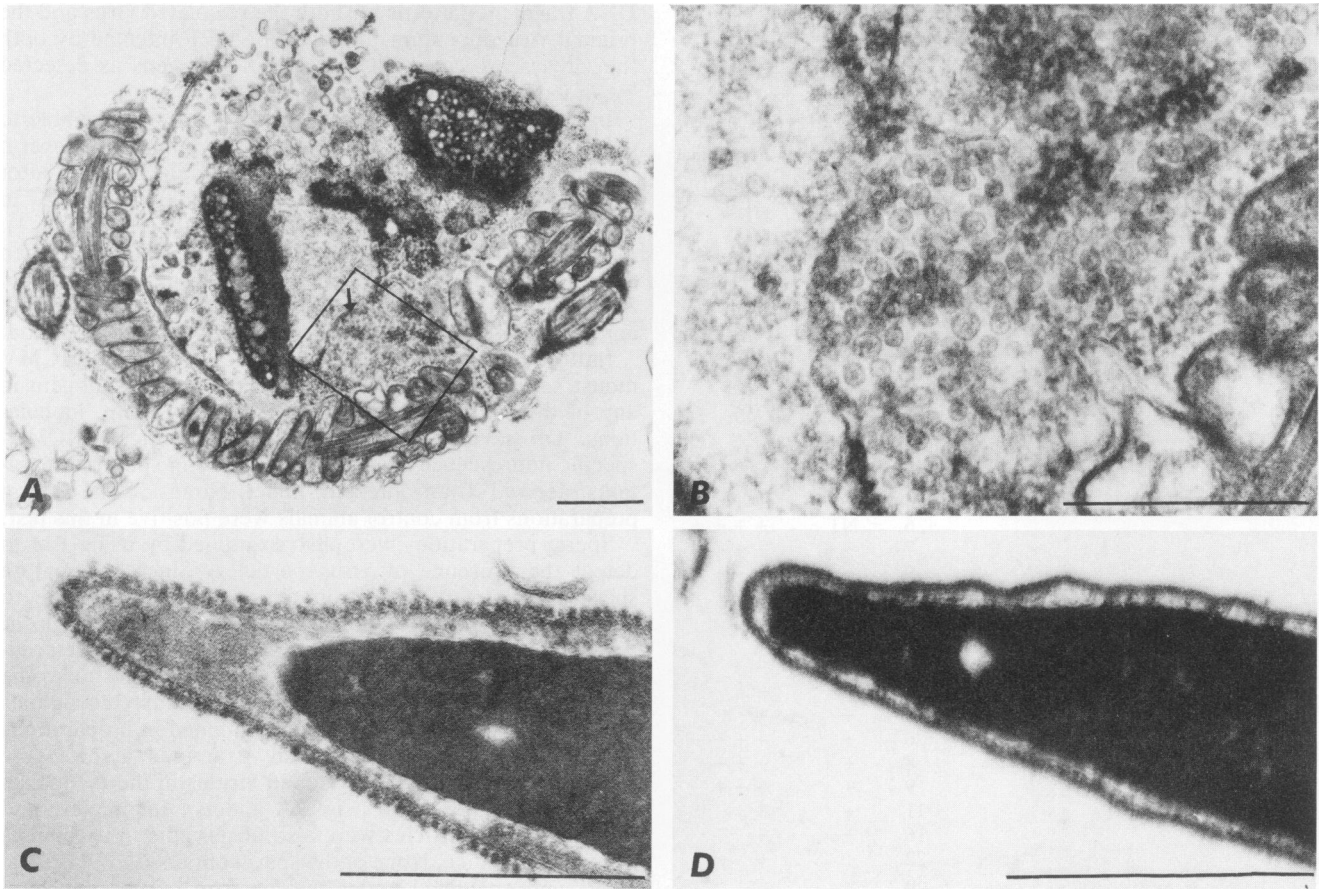

FIG. 2. EM of epididymal sperm derived from MCMV-inoculated mice. (A) An undeveloped sperm containing numerous complete, and a few incomplete, CMV-like viral particles (arrow) near the acrosome region. This sperm was derived from an 8-week-old mouse, 9 days after MCMV (10^4 PFU) i.t. inoculation. (B) A magnified view of the viral particles shown in panel A. (C) Observation of indeterminate particles on a sperm head on the apical and midportion surfaces. This sperm was obtained from the epididymis of a 10-week-old mouse 6 days after i.t. MCMV (10^4 PFU) inoculation. (D) An epididymal sperm head from a 10-week-old control mouse that received heat-inactivated MCMV i.t. Note the absence of the particles as seen on the surface of the sperm head shown in panel C above. Bars represent 1 μm.

lected and found to contain virus in nearly all cases between 2 and 14 days after infection. Virus was also consistently recovered from seminal vesicles between 6 and 16 days postinfection. To demonstrate further the presence of virus in the ejaculate of acutely infected mice, semen was recovered from the uteri of four out of six uninfected females that mated with infected males. Not shown in those studies were the actual infection site of MCMV in the male genital tract, the infectivity of the sperm themselves, and the question of whether the virions were extracellular or associated with other cells in the semen (20). Another study involving the fertilization of murine ova after artificial insemination of mice with a mixture of sperm and MCMV also does not demonstrate whether the sperm themselves were infected (28).

In a study similar to ours, Brautigam and Oldstone (6), attempted to establish an infection of testicular germ line cells, inoculating newborn mice i.p. with MCMV and, 1 week later, performing in situ [^{3}H]cRNA-DNA cytohybridizations on a small number of testes of these mice. Although the cytohybridization showed foci of infection in squamous epithelial cells external to the tunica albuginea, an examination of germ line cells was not possible because of the immaturity of the mice used in their experiments. Subsequently, Dutko and Oldstone (8) showed that MCMV replicates in the testes of adult, athymic, nude mice. However, it could not be conclusively demonstrated that the infection of sperm cells occurs in normal mice since the nude mouse is immunologically compromised.

The emphasis of this study is on the demonstration of the presence of MCMV DNA associated with the sperm heads of immunologicaly competent mice after infection by MCMV, as detected by in situ cytohybridization (Fig. 1), and the detection by using EM of virus particles in the cytoplasm of immature sperm (Fig. 2). Because the sensitivity of detection of the in situ hybridization technique is 20 gene copies per cell, the infected sperm that tests positive must contain at least 20 gene copies (8).

The MCMV used in this study was plaque purified and passaged twice in MEF before use. This process causes either partial or complete attenuation of the virus (21). The attenuated virus used in this study also caused infection of the murine testes similar to that caused by a virulent virus (salivary gland, in vivo-passaged virus) used in the study of Neighbour and Fraser (20). Brautigam and Oldstone (6) and Dutko and Oldstone (8) have also used tissue culture-passaged MCMV in their studies with results comparable to those reported in this investigation.

TABLE 2. Effect of murine sperm carrying MCMV DNA on in vivo fertilization

Treatment of adult males	No. of adult males used	Expt	Females[a] with vaginal plug/ total mated	Total no. of embryos[b] collected
MCMV[c]	4	1	3/5	82
	5	2	4/5	89
	5	3	3/5	79
	6	4	4/6	84
None	4	1	4/5	71
	5	2	4/5	95
	5	3	4/5	98
	6	4	4/6	93

[a] Normal adult receptive females were left with males overnight and in the morning checked for a vaginal plug for proof of mating.

[b] Females, 24 h after mating, were sacrificed, and the two-cell-stage embryos were collected from the oviducts.

[c] Six to nine days after intraperitoneal MCMV inoculation (about 10^4PFU per animal), the males were mated with normal females. Epididymal sperm collected from these mice right after mating contained MCMV DNA on the sperm head in a [^{3}H]cRNA-DNA cytohybridization.

Sperm contain an array of protease enzymes in the acrosome necessary to digest a path through the corona radiata and zona pellucida around the ovum during fertilization (19). Therefore, it may be speculated that the presence of the indeterminate particles on the cytoplasmic membrane near the acrosomal region of the infected sperm seen in the EM may be caused by the enzymatic removal of the viral envelope by the protease enzymes of the sperm. This speculation, and the fact that the mature sperm cell is metabolically inactive, may help to explain why we could not detect MCMV-specific antigen on the infected sperm by using our indirect immunofluorescence test; it is possible that MCMV-specific antigens may have been lost due to the enzymatic digestion of sperm-related proteases.

In the present study, epididymal sperm collected from all of the MCMV-infected mice immediately after they mated contained MCMV DNA associated with the sperm head in [^{3}H]cRNA-DNA cytohybridizations, suggesting MCMV infection of the sperm and a possible transfer of MCMV genome by the sperm into the ovum at fertilization.

Our in vivo fertilization results are consistent with those of Neighbour and Fraser (20), who showed earlier that murine embryos fertilized in vitro by sperm preincubated with MCMV were able to develop to the blastocyst stage. In addition, those embryos did not reveal evidence of any productive viral infection. Whether productive infection would occur after the initiation of differentiation remains to be seen.

Our previous studies (unpublished data), as well as others (9), with murine undifferentiated embryonal carcinoma cells, which are biologically, morphologically, and biochemically similar to undifferentiaed normal embryonic cells (18, 23), indicate that MCMV cannot productively infect undifferentiated cells. Recently, Dutko and Oldstone (9), confirming these results, have shown that latently infected undifferentiated teratocarcinoma cells can produce infectious virus and viral antigens after being induced to differentiate. From these results, one can infer that early embryos infected with CMV can support a latent infection and that viral gene expression can later occur as the embryonic cells proceed to differentiate. Work is now under way in our laboratory to confirm this speculation by transferring MCMV-infected murine eggs to surrogate mice and tracing the viral gene expression during the course of fetal development.

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