Infection of Mouse Ectoplacental Cone Cells with Murine Cytomegalovirus

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SUMMARY

In order to understand the mechanism of congenital cytomegalovirus (CMV) infection, we studied the effect of murine cytomegalovirus (MCMV) on murine ectoplacental cone (EPC) cells in vitro. Cytopathic effects (c.p.e.) were seen in many MCMV-infected EPC cell cultures 5 to 7 days after exposure to MCMV. The infected cells showed intranuclear inclusions characteristic of CMV infection when stained with May-Grunwald-Giemsa. Culture fluids collected from MCMV-infected EPC cells after 4 or more days of culture caused c.p.e. when co-cultivated with mouse embryo fibroblasts (MEF). Employment of the anti-complement immunofluorescence (ACIF) test detected MCMV-specific antigens, in situ hybridization localized MCMV DNA and electron microscopy detected the presence of the viral particles in the MCMV-infected EPC cells. Thus, exposure of EPC cells to MCMV in vitro resulted in a productive infection.

Cytomegalovirus (CMV) infections are 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 (Weller & Hanshaw, 1962; Davis, 1969; Huang & Pagano, 1977; Kumar & Nankervis, 1979). There are at least two possible modes of CMV transmission to developing embryos. (i) Embryos could potentially be infected during implantation by CMV associated with semen and/or the female genital tract (Lang & Kummer, 1972; Neighbour & Fraser, 1978; Dutko & Oldstone, 1979). (ii) Cytomegalovirus may cross the placenta and infect the embryo (Cochard et al., 1963; Benirschke et al., 1964; Eachempati & Woods, 1976).

Transplacental mouse cytomegalovirus (MCMV) infections have not been established (Medearis, 1964; Johnson, 1969) because of the difficulty of MCMV-infecting the three-layered trophoblastic murine placenta (two of which are syncytial in nature) in a short gestation period. However, the mouse model is ideally suited to studies of CMV infections during implantation and the consequences of such infections, for several reasons. First, the mouse is easy to manipulate and it has a short gestation period so that experimental results are obtained quickly. In addition, sophisticated technology exists for transplantation of developing manipulated embryos into foster mothers (Kirby, 1971; Mintz, 1971). Finally, the overall picture of MCMV infections in the mouse bears striking resemblance to human CMV and disease (Weller, 1971).

A possible mode of CMV infection during embryo implantation and early pregnancy is for CMV to pass through the infected precursor placental cells (by replication) from the endometrium. These precursor placental cells are called ectoplacental cone (EPC) cells in mouse and the embryological origin is as follows. The early 3 day-old embryo (blastocyst) is partitioned into an inner cell mass from which the embryo proper will develop and an outer ring of cells called the primary trophoblast whose chief function is to anchor the blastocyst to the endometrium. Subsequently, the cells of the primary trophoblast degenerate but are replaced by a cone-shaped mass of cells originating from the inner cell mass. This mass of cells is mitotically active and is called the ectoplacental cone (Kirby, 1971). Cells of the EPC later come in contact with the chorion, the allantois and the endometrium; this association
results in the development of a placenta (Gardner et al., 1973). Although EPCs per se have not been identified in human embryos, the latest evidence suggests that similar precursor placental cells probably exist (Luckett, 1978). If EPC cells are susceptible to MCMV, it is possible that the virus may infect the embryo via these cells at an early stage of embryonic development. In view of this, we have studied the effect of MCMV on EPC cells in vitro and have found that EPC cells are susceptible to MCMV infection.

Germ-free CF-1 mice, from Charles River Breeding Laboratory, Wilmington, Mass., U.S.A., were used in our studies. Before the experiments, salivary gland extracts from a few randomly selected mice from the colony were tested on mouse embryo fibroblasts (MEF) and found free of MCMV. Ectoplacental cones were surgically removed from 7.5-day-old embryos as described by Kirby (1971). Briefly, the pear-shaped decidual swellings containing embryos were removed aseptically from uteri of sacrificed pregnant mice and placed into a 60 mm Petri dish containing minimum essential medium with 100 units penicillin and 100 μg/ml streptomycin (MEM), supplemented with heat-inactivated 4% foetal calf serum (FCS). The entire embryo was removed from the surrounding decidual tissue with watchmakers forceps and washed free of maternal blood and decidual cells with MEM–4% FCS. The ectoplacental cone was then dissected from the egg cylinder under a dissecting microscope using a fine pair of scissors. The EPCs (pooled from two to four pregnant mice per experiment) were washed several times by repeated transfer to Petri dishes containing fresh medium. The repeated washings yielded pure populations of EPCs. One half of the EPCs in the pool were exposed to MCMV (Smith strain, m.o.i. 2, for 90 min). As a control, the remaining EPCs from the pool were treated identically, except that the heat-inactivated MCMV was used. After thorough rinsing with MEM–4% FCS, EPCs were cultured individually in flat-bottom wells of microtest II tissue culture plates (Falcon No. 3040) in 0.2 ml MEM–10% FCS. To obtain conclusive evidence that EPCs which were exposed to MCMV were supporting MCMV replication we employed anti-complement immunofluorescence (ACIF) for detection of MCMV-specific antigens, in situ hybridization for localization of MCMV DNA (Huang & Pagano, 1977), electron microscopy for observation of viral particles and MCMV plaque assay of culture fluids on embryo fibroblasts.

The results from three experiments are summarized in Table 1. In two other experiments, culture fluids were assayed for MCMV and in one other experiment cells were immunofluorescence stained for MCMV antigen. Data from these three experiments (not shown) were similar to the data in Table 1. Cytopathic effects (c.p.e.) were seen in many MCMV-infected EPC cultures 5 to 7 days after exposure to MCMV (Fig. 1). The cells involved in the c.p.e. revealed large intranuclear inclusions characteristic of CMV infection when stained with May-Grunwald-Giemsa. Culture fluids collected from MCMV-infected EPC cells after 4 or more days of culture caused c.p.e. when co-cultivated with MEF. This indicated a productive MCMV infection. Neither c.p.e. nor intracellular inclusions were detected in the uninfected EPC cell cultures. In addition, the supernatants from EPCs from five control experiments which had been treated with heat-inactivated MCMV did not cause c.p.e. when co-cultivated with MEF.

The ACIF tests which were used for the detection of CMV-related antigens have already been described by Huang & Pagano (1977). Five to 10% of EPC cells in two out of four experiments (only one shown in Table 1) were positive for MCMV antigens 3 days after infection with MCMV. The cells tested were from a pool of two mice in one experiment and three mice in the other experiment. Subsequently, the number of cells showing specific immunofluorescence increased to 20 to 30% when tested 4 or more days after MCMV infection (Table 1). However, the antiserum did not stain either the uninfected cells or the EPC cells exposed to heat-inactivated MCMV. A pool of non-immune mouse serum obtained from CF-1 mice did not stain in the fluorescent antibody test with EPC cells infected
Table 1. **Summary of results of MCMV-infected ectoplacental cone cells**

<table>
<thead>
<tr>
<th>Day tested</th>
<th>Expt. 1</th>
<th>Expt. 2</th>
<th>Expt. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Virus (p.f.u.)* in supernatant from a MCMV-treated EPC</td>
<td>Presence of MCMV</td>
<td>Virus (p.f.u.)* in supernatant from a MCMV-treated EPC</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>0.5 × 10¹</td>
<td>±</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>5.0 × 10¹</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>2.3 × 10²</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Assayed in mouse embryo fibroblasts; each cone contained approx. 10³ cells.
† Determined by indirect immunofluorescence.
‡ Determined by in situ DNA hybridization.
§ Determined by electron microscopy.
\[ ND, Not done. \]
with MCMV. The ACIF studies detected the presence of MCMV-induced proteins in the MCMV-infected EPC cells.

To localize the viral DNA in the intracellular EPC cells we performed DNA–DNA cytohybridization in situ. This technique is particularly valuable for decisive identification of viral genomes and it allows localization of the genetic material to specific cell type. To detect the specific genome in whole or in part an appropriate viral probe is required. In this study, the MCMV DNA used as the probe was purified and labelled with $^{32}$P-dATP in vitro by repair synthesis with DNA polymerase I (Huang & Pagano, 1977). Labelling with $^{32}$P resulted in a nucleic acid probe with high degree of purity and specific activity ($10^7$ ct/min/µg MCMV DNA). Cultured MCMV-infected and control (exposed to heat-inactivated MCMV) EPC cells were exposed to hypotonic 0-1 × balanced salt solution and then fixed in ice-cold methanol:acetic acid (3:1) for 10 min. After fixation, the coverslips were dipped in 90% alcohol and absolute alcohol to remove the residual acetic acid, and then dipped into 0.4% agarose at 60 °C and air dried to form a thin agarose layer on the coverslip to prevent detachment of cells during alkalinization and hybridization. The denaturation of DNA was carried out by mild alkalinization of the cells in 0.07 M-NaOH for 3 min. The coverslips were again washed extensively with 70% alcohol and absolute alcohol and air dried. Using the $^{32}$P-MCMV DNA probe ($10^5$ ct/min/sample) hybridization was carried out on the cells in a moist chamber for 22 h at 66 °C. After extensive washings with 2 × SSC and sequential dehydration with 70%, 95% and absolute alcohol coverslips were dipped in Kodak Nuclear Track NTB-2 emulsion and autoradiographed for 1 week. The coverslips were then developed, rapid fixed and stained with Giemsa. Distinct and clustered silver grains (more than 30 grains/cell) were observed over EPC cells infected with MCMV (Table 1). In contrast, none or less than five scattered grains/cell were seen in non-infected cells. The number of positive cells in the experimental groups varied from 18 to 43% compared with less than 1% positive cells in the control groups.

For direct visualization of MCMV infection we also carried out electron microscopy on samples of the EPC cells (Baskar, 1975; Montplaisir et al., 1972). Numerous viral particles...
with a typical herpesvirus morphology were observed both in the nucleus and the cytoplasm of cells examined on days 4 to 6 after MCMV infection (Table 1). No viral particles were seen either in the non-infected cells or in cells examined on days 1 and 3 after MCMV infection.

Employing restriction endonuclease analyses (Kilpatrick et al., 1976) we performed an experiment to verify whether the virus used to infect the EPCs was the same as that recovered from the infected cells. Two restriction endonucleases, EcoRI (Greene et al., 1974) and Xba (Zain & Roberts, 1977), which recognize and cleave specific base sequences were used for the study. Co-electrophoresis of EcoRI and Xba digests showed identical DNA fragment patterns for the original and the isolate. The similarity in the fragment patterns demonstrated that the virus isolated from the infected EPC cells is probably a re-isolation of the virus employed to infect the cells.

Contrary to our findings, Neighbour (1978) has reported that mouse EPC cells do not support MCMV replication. The EPCs employed in his study (P. A. Neighbour, personal communication) came from Balb/c mice. After isolation, EPCs were minced into 2 or 3 pieces, cultured in Falcon plastic plates in 2 ml RPMI 1640 medium containing 10% FCS for 72 h and then exposed to MCMV (m.o.i. 10) for 60 min. The cultures were monitored for infectious virus only up to 96 h post-infection. In contrast, EPCs in our study were derived from CF-1 mice. First, we exposed EPCs to MCMV (m.o.i. 2) for 90 min and then cultured individually in flat-bottom wells of microtest II tissue culture plates in 0.2 ml MEM–4% FCS. Unlike Neighbour (1978) we monitored our cultures for infection beyond 96 h after infection. We always observed signs of infection starting at 96 h post-infection, whereas Neighbour did not detect infectious MCMV at any time after inoculation except in one culture from which virus was recovered at low titres at 72 and 96 h post-infection. Perhaps our varying culture conditions might explain difference between our results and his. In any case, application of many different techniques in our study establishes the susceptibility of mouse EPC cells to MCMV infection in vitro.

We have shown in the present communication that at 7.5 days post-coitum, mouse precursor placental cells (EPC cells) are susceptible to MCMV in vitro. It is reasonable to assume that, if such infections occur in vivo, the virus may infect the embryo via EPC cells. Embryonic sacs (egg cylinders) devoid of EPCs and extra-embryonic membranes obtained on day 7.5 of pregnancy from Balb/c mice (Neighbour, 1978) and CF-1 mice (J. F. Baskar & E-S. Huang, unpublished data) are susceptible to MCMV in vitro.

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REFERENCES


Short communications


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