Vertical Transmission of Murine Cytomegalovirus

(Accepted 3 October 1978)

SUMMARY

Congenital abnormalities induced by murine cytomegalovirus have previously been suggested to be an indirect effect of maternal illness as no infectious virus has been isolated from the foetus. However, in this article we report that latent virus detectable by immunofluorescence and in situ hybridization, may frequently be present in foetal tissues.

Congenital infections with cytomegalovirus are believed to be one of the major causes of human birth defects including mental retardation, microcephaly, epilepsy, blindness and cerebral palsy (Stern et al. 1969; Hanshaw, 1970). As many as 1% of all infants may be born with CMV infection (Stern & Tucker, 1973) and although many of these infections are asymptomatic, they too may represent significant but subtle effects on the CNS preventing full development of a child's potential (Stern et al. 1969; Hanshaw, 1971).

Several investigators have examined the mouse cytomegalovirus as a model for the human infection. The cytomegaloviruses as a group are fairly host-specific viruses, characterized by their ability to initiate long-term persistent infections which are often accompanied by the shedding of virus in tissue secretions for considerable lengths of time. The mouse virus, like its human counterpart, is known to cause foetal injury which is manifested as decreased litter size, abortion or the delivery of stillborn macerated foetuses (Mannini & Medearis, 1961; Medearis, 1964) after inoculation of mice at the time of implantation (Neighbour, 1976) or during pregnancy (Mannini & Medearis, 1961; Johnson, 1969).

However, a major difference has been suggested in the course of congenital infections by the human and murine viruses. In the human case, the virus has frequently been isolated from the foetus and is believed to be transmitted across the placenta from the maternal blood system (Potter, 1957; Weller, 1971). In addition it has been suggested that reactivation of latent virus in the cervix or urinary tract may be important (Montgomery et al. 1971), or that venereal transmission in semen may occur (Lang & Kummer, 1972). However, in persistent murine infections no virus has been detected in the foetus. Medearis (1964) and Johnson (1969) were unable to demonstrate intra-uterine infection with MCMV either during acute infection of the mother, i.e. when a generalized viraemia was occurring, or during persistent infection when the virus is probably localized in salivary glands, kidneys and possibly the lymphocytes (Olding et al. 1975, 1976). They therefore suggested that the abnormalities were an indirect result on the foetus of maternal illness or a change in the placental environment. These results indicated a prime limitation in the use of the murine system as a model for the human infection. However, in this article we report that we have been able to detect the presence of latent MCMV in embryos from persistently-infected mice by immunofluorescence and in situ hybridization.

Several 2 to 3 week old female mice (SWR, Jackson Laboratories, Bar Harbor, Maine) were injected intraperitoneally with 10⁴ p.f.u. of tissue-culture passed MCMV (Smith strain; Hudson et al. 1976) and were observed periodically for signs of illness. No obvious symptoms were detected but high titres of virus were found in the salivary glands of selected mice up to 90 days after infection. Over the next few months, the offspring of these infected mice were occasionally observed to be runty with ruffled coats which had an oily appearance.
In addition, litter sizes were repeatedly small and the new-borns had diarrhoea and running eyes. These effects were observed sporadically. Some litters appeared to be normal although some of the offspring had ruffled coats and thus may not have been completely healthy. Stocks of uninfected animals did not exhibit these effects, except for small litter sizes which were observed occasionally.

Approximately one year after intraperitoneal inoculation, three pregnant mice were sacrificed and found to contain abnormal uteri. Two of these contained only two embryos as opposed to the normal complement of 7 to 14 while the third contained a single large dead foetus. Each pair of viable embryos was treated by standard procedures for preparing primary embryo cultures (Younger, 1954), and passaged for several generations. Extreme care was taken while removing the embryos from the mothers and in their subsequent cultivation to ensure that contamination with MCMV from either the laboratory or the maternal tissues did not occur. The two lines were kept separate, and designated EM-2 and EM-3. The single large foetus (EM-1) was discarded as it did not yield viable cells and had no infectious virus associated with it.

At passage 4 the EM-3 cells developed a characteristic MCMV-induced cytopathic effect (c.p.e.). On examination of the medium of these cultures under the electron microscope, herpes-like virions were detected with the multicapsid structure unique to MCMV (Hudson et al. 1976), indicating that activation of latent MCMV had occurred. In contrast, there was no infectious virus associated with EM-2 cells from passage 1 to passage 7, after which the line degenerated without any obvious production of c.p.e. Virus-like particles were not detected in the medium, nor in cell lysates of EM-2 cells and therefore the search was extended to include the possibility that latent MCMV was present in these cells. Indirect immunofluorescence and in situ hybridization tests were performed on EM-2 cells fixed on coverslips from early passages, while solution hybridization was conducted using cells from later passages when more material could be obtained.

The result of an indirect immunofluorescence test is shown in Fig. 1. The antisera for these experiments were prepared in rabbits against total infected cell lysates as has been described elsewhere (Chantler & Hudson, 1978). The sera were adsorbed thoroughly against mock-infected cells before use and gave minimal background fluorescence in uninfected cells (Fig. 1 a) but striking fluorescence with tertiary mouse embryo cells productively infected in vitro (Fig. 1 b). The result for EM-2 cells is shown in Fig. 1 (c) and (d). These cells have a characteristic 'stringy' appearance which readily distinguishes them from normal EM cells during early passage. Although not all the EM-2 cells exhibited fluorescence, a significant number showed distinct membrane fluorescence as well as a small amount of granular fluorescence within the cell. In addition to this, occasional foci of bright fluorescence were observed. These results indicated that MCMV was indeed present in these cells and that a low level of virus expression was occurring in discrete areas.

The search for the virus genome was conducted initially by DNA-DNA hybridization in solution, using DNA extracted from EM-2 cells at passage 7, and 125I-labelled MCMV DNA (Misra et al. 1978). However, because only a small amount of EM-2 DNA was obtained, only 50 μg of unlabelled DNA could be used per assay and no virus sequences were detected. This may have been due to the lack of sensitivity of the experiment due to the low concentration of cellular DNA, or alternatively, to a selective reduction of cells carrying the virus genome during successive passage of the EM-2 line. To surmount these problems, in situ hybridization was performed on coverslips of EM-2 cells obtained from passages 2 and 3. The protocol for this technique is described elsewhere (Haase et al. 1977) and outlined in the legend to Fig. 2. Hybridization was carried out with 125I-labelled MCMV probe DNA.
Fig. 1. An indirect immunofluorescence test using rabbit antiserum to MCMV proteins and FITC-conjugated goat anti-rabbit IgG, examined on a Leitz Wetzlar fluorescence microscope. Original magnification ×40. (a) Mock-infected; (b) lytic infection (20 h); (c) and (d) EM-2 cells.

Fig. 2. In situ hybridization of 125I-labelled MCMV DNA to mouse embryo tissues: (a) mock-infected; (b) 20 h after infection in vitro; (c) and (d) EM-2 cells. Hybridization was performed on monolayers of cells fixed in 0.2 N-HCl on coverslips. After pre-treatment with ribonuclease, the DNA was denatured in 95% formamide 0.1 × SSC at 65 °C for 2 h. Hybridization was in 50% formamide 4 × SSC at 45 °C for 72 h using 5 μl of 125I-labelled MCMV DNA (0.225 μg/ml: 1.6 × 10^5 cpm/μg). The coverslips were then washed in acetate buffer (30 mm-sodium acetate, pH 4.5, 100 mm-NaCl, 2 mm-ZnCl₂) and treated with 10 units/ml of S1 nuclease at 50 °C. They were coated with Ilford K2 emulsion and after being exposed for 1 week, were developed and stained with Giemsa.
at 45 °C in 50 % formamide and autoradiography performed on the coverslips for 1 week. Embryo cells from uninfected mice, and cells infected with MCMV *in vitro* and harvested at 20 h p.i. were used as controls (Fig. 2a and b). The results show very low numbers of background grains in uninfected cells (a) while in contrast the infected cells at 20 h.p.i. contained many grains concentrated in the nucleus although also present in the surrounding cytoplasm (b). In comparison, the results for EM-2 cells are shown in Fig. 2(c) and (d). In (c), a single cell with cytoplasm barely visible can be seen to contain a high number of grains confined within the nuclear membrane. In (d) another EM-2 cell is shown with 4 or 5 grains in the nucleus and essentially none in the surrounding area. No foci of active replication could be identified by *in situ* hybridization as, during the procedures involved, a large number of the cells detach and only isolated cells remained on the coverslip. However, approximately the same proportion of cells as exhibited granular fluorescence were found to contain intra-nuclear grains by *in situ* hybridization.

These preliminary results in contrast to those of other workers (Medearis, 1964; Johnson, 1969) show that vertical transmission of MCMV from mother to foetus does occur. However, the virus appears to exist in the foetus in a latent form and has thus previously escaped detection. In the EM-3 cell line, the virus was reactivated by successive passage of the cells *in vitro*, but this may be an unusual occurrence. In the case of the EM-2 cells, infectious virus was not detected at any stage although significant virus gene expression was found by immunofluorescence and *in situ* hybridization. These results indicate that the asserted limitations of the murine system as a model for human cytomegalovirus infection may be unjustified.

Jordan *et al.* 1977 recently reported the reactivation of MCMV from latently infected mice using immunosuppression with anti-lymphocyte serum and corticosteroid. In this study, 97 % of mice inoculated subcutaneously with MCMV could be shown to harbour a latent infection some 4 to 8 months later. This finding, taken in conjunction with the possibility of impaired maternal responsiveness to foreign antigen during pregnancy (Hellstrom *et al.* 1969), indicates a possible mechanism for reactivation of the virus under natural conditions. In addition, the genome of MCMV has been detected recently in germ-line cells of testicular origin by *in situ* hybridization (F. J. Dutko & M. B. A. Oldstone, personal communication). This suggests two possible mechanisms for transmission of the virus to the foetus; transplacental passage of a reactivated virus, or direct vertical transmission through a germ-cell. We cannot distinguish between these possibilities at present.

These findings, together with our detection of MCMV in viable embryos from latently infected mothers, have considerable implications for the proposal to vaccinate humans with live attenuated virus in an attempt to prevent CMV-induced birth defects. Indeed in preliminary results from a current experiment, we have found that both virulent strains of MCMV (passaged in mouse sub-maxillary gland) and attenuated strains (passaged in cell-culture) have very similar effects on pregnancy wastage in the offspring.

We thank Dr A. T. Haase for supplying details of his protocol for *in situ* hybridization. This work was supported by grant MA-4762 from the Medical Research Council of Canada.

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(Received 25 July 1978)