Long-Term Mouse Cytomegalovirus Infection of Tracheal Organ Culture: Relation to Host Cell Replication

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SUMMARY

Tracheal organ cultures infected with mouse cytomegalovirus (MCMV) were shown to produce virus for up to 180 days with virus replication primarily in epithelial cells. Persistent virus infections were established in tracheal organ cultures from both MCMV-susceptible and MCMV-resistant strains of mice. In acutely infected tracheal organ cultures, cellular DNA synthesis appeared to precede the production of significant amounts of virus antigens and the release of virus into culture fluids. Since persistently infected tracheal organ cultures continued to synthesize cellular DNA, the results suggest that host cell turnover may continually renew the population of MCMV-susceptible cells. The results suggest a possible mechanism for virus persistence in tracheal organ culture based on continuing serial infection of newly susceptible cells combined with prolonged release of virus from infected cells.

INTRODUCTION

Cytomegaloviruses (CMV) establish latent and persistent infections in man and in experimental animals. Mouse CMV, which has many biological similarities to the human virus and some important differences as well (for review, see Osborne, 1982), has often been used as a model for human CMV. In man, congenital and neonatal CMV infections and reactivated virus infections in immunosuppressed adults often result in excretion of CMV that persists in the throat or in the urine for months or years (Hanshaw, 1971; Cheeseman et al., 1979). Although most infections are asymptomatic, CMV infection has been correlated with increased morbidity and mortality in recipients of organ transplants (Neiman et al., 1977; Light & Burke, 1979; Peterson et al., 1980) and with sequelae of motor and mental retardation in infected infants (Hanshaw, 1971; Reynolds et al., 1974). Although the salivary gland and the kidney appear to be major sites of chronic CMV production, the detailed pathobiology of persistent CMV infections, especially on the cellular level, is largely unexplored.

Two general mechanisms through which viruses might establish persistent infections are limited pathogenicity for some host-cell types and deficient or restricted immune response to the virus (Mims, 1974). If it is assumed that a restricted immune response to CMV might allow CMV-infected cells to persist, then limits to pathogenicity of the virus that are determined by cell type might in turn favour chronic CMV infections that are asymptomatic or benign.

Cytomegaloviruses replicate in vivo in many cell types including lymphocytes, macrophages, nerve tissue, many types of epithelial cells, and cells of the reproductive tract (Weller, 1971; Olding et al., 1975; Mims & Gould, 1978, 1979; Wu & Ho, 1979). While
various lymphoid cells have been implicated in latent CMV infections, in both pathological states and in chronic infections the epithelial cell seems to be a primary site of replication. Additionally, CMV does indeed appear to possess a restricted pathogenicity for epithelial cells; for many years cultured epithelial cells were considered incapable of supporting replication of CMV (Benyesh-Melnick, 1969; Weller, 1971). Moreover, recent reports of replication of CMV in vitro in epithelial cells have highlighted the relatively slow course of infection in this cell type (Michelson-Fiske et al., 1975; Vesterinen et al., 1975; Knowles, 1976; Vonka et al., 1976; Mäntyjärvi et al., 1977). We report here that in the epithelium of tracheal organ culture, mouse cytomegalovirus (MCMV) replicates in persistent, long-term infections which enable study of cell-determined parameters of chronic CMV infections.

METHODS

Mice. Balb/c, C57BL/6 and C3H/Hej were obtained from Jackson Laboratories (Bar Harbor, Me., U.S.A.). CD-1 outbred mice were obtained from Charles River Breeding Laboratories (Wilmington, Mass., U.S.A.).

Virus. Mouse cytomegalovirus (Smith strain) was obtained from the American Type Culture Collection (Rockville, Md., U.S.A.). The seed virus was passed twice in mouse embryo fibroblasts and three times through mice. Virus pools were prepared by intraperitoneal inoculation of 4- to 10-week-old CD-1 mice with 10⁴ to 10⁵ p.f.u. of virus. Two to three weeks after infection, salivary glands were removed aseptically, and a 10% (w/v) homogenate was prepared. The virus-containing extract was clarified by low-speed centrifugation, dimethyl sulphoxide (10%) was added, and the virus was then frozen at -70 °C in 1 ml vials.

Organ cultures. Tracheal organ cultures were prepared and infected with MCMV, and the organ culture fluids were assayed for virus p.f.u. of MCMV as described by Nedrud et al. (1979).

Autoradiography and histology. MCMV-infected or uninfected tracheal rings were incubated for 16 to 18 h in organ culture medium containing 2 μCi/ml [3H]thymidine (sp. act. 1·9 Ci/mmol; Schwarz/Mann, Orangeburg, N.Y., U.S.A.). The organ cultures were then washed three times with sterile phosphate-buffered saline (PBS) and incubated for 30 min in fresh organ culture medium. The tracheal rings were washed again with PBS and frozen in blocks of OCT embedding compound (Lab-Tek Division, Miles Laboratories, Naperville, Ill., U.S.A.). Frozen sections (6 μm thick) were cut, fixed twice for 10 min in ice-cold methanol, and extensively washed in PBS. The slides were air-dried, stained for CMV antigens by immunofluorescence and/or dipped in Kodak NTB-2 emulsion. After 4 days exposure, the slides were developed with Kodak Microdol-X, and the number of cells per complete tracheal section with heavy accumulations of grains was counted and tabulated. Alternatively, after [3H]thymidine labelling and PBS washing, tracheal rings were fixed for 6 to 18 h in 2% paraformaldehyde, 2% glutaraldehyde, 0·1 m-phosphate buffer pH 7·2. The samples were then washed overnight in 0·1 m-phosphate, 0·2 m-sucrose buffer pH 7·2 and post-stained with 1% OsO₄, and then dehydrated in graded ethanols and embedded in plastic. The embedded specimens were cut into 0·5 μm sections which were processed for autoradiography (exposure time 2 weeks) and/or toluidine blue O-stained for light microscopy. Ultrathin (50 nm) sections were cut for examination in the electron microscope.

Immunofluorescence. Antiserum to MCMV was raised by injecting CD-1 mice with two sublethal doses of virus 30 days apart. Serum collected 10 days later was stored in aliquots at -20 °C. Frozen, fixed sections of tracheal rings were incubated under 20 μl of a 1:20 dilution of antiserum or normal serum for 45 min at 37 °C. The samples were washed three times for 10 min in PBS, rinsed for 15 s in distilled water, air-dried and then incubated with FITC goat anti-mouse IgG (Cappel Laboratories, Cochranville, Pa., U.S.A.) and washed as before.
Persistent MCMV infection of organ cultures

RESULTS

MCMV-infected tracheal organ cultures continued to excrete virus into the culture medium for at least 50 to 75 days post-infection when the cultures were usually terminated. Twenty-four sets of infected cultures from seven separate experiments were followed for longer times. Eighteen cultures continued to excrete virus for 90 days or longer, 15 longer than 100 days, six longer than 130 days, three longer than 150 days, and one culture was still producing MCMV after 180 days. Slightly fewer than half of the cultures were terminated for histological studies while still producing virus between 70 and 150 days after infection. Of the remaining cultures approx. one-third spontaneously ceased virus production between 70 and 100 days after infection, and the other two-thirds were terminated between 80 and 180 days after infection either because of fungal contamination or due to cessation of virus production.

MCMV-infected tracheal organ cultures from resistant strains of mice produce lower peak titres of virus (by 1 to 2 log_{10}) than identically infected organ cultures from susceptible strains of mice (Nedrud et al., 1979). Persistent MCMV infections could be established in tracheal organ cultures from both susceptible and resistant strains of mice. Data from representative experiments plotted in Fig. 1 show that the virus titres from an infected organ culture of a susceptible mouse strain remained higher than titres observed in a culture from a resistant mouse strain for well over 100 days. This difference (1 to 2 log_{10}) was duplicated in other experiments for at least 70 to 80 days after which the titres from susceptible cultures sometimes dropped below the titres from resistant cultures. However, the susceptible cultures exhibited acute destruction of large areas of epithelium after 70 to 80 days while cultures from resistant mice exhibited minimal cytopathic effect (c.p.e.) and retained areas of relatively undamaged epithelium after this time.

MCMV replicates primarily in epithelial cells in tracheal organ culture (Mäntyjärvi et al., 1977). Characteristic c.p.e. of the MCMV infections are rounded swollen epithelial cells projecting from the tracheal rings beginning 7 to 10 days after infection. The epithelium of both infected and uninfected cultures gradually lose their cilia and become squamous beginning after 1 to 2 weeks.

This flattening of the epithelium continued in long-term cultures although sporadic areas of epithelium with active cilia were sometimes observed in both control and infected cultures for
Fig. 2. Tracheal organ culture persistently infected with MCMV (light micrograph). CD-1 mice were infected with MCMV and 82 days after infection tracheal rings were prepared for light microscopy as described in Methods. Bar marker represents 20 μm.

Fig. 3. Tracheal organ culture persistently infected with MCMV (electron micrograph). Preparation of specimen was the same as in Fig. 2 except that ultrathin sections were cut and examined by electron microscopy. 'A' marks a cell with lacy nucleus as in Fig. 2; C, cartilage. Bar marker represents 5 μm. Inset is a higher magnification showing a portion of cell 'A' with desmosomes (D) and virus particles. Bar marker represents 1 μm.
Fig. 4. DNA synthesis in uninfected, freshly explanted or precultured tracheal rings as determined by autoradiography. Tracheal organ cultures prepared from Balb/c mice were incubated overnight in 1 μCi/0.5 ml [3H]thymidine. Frozen sections were prepared and processed for autoradiography as described in the text. (a) Tracheal ring labelled beginning 2 h after explantation in organ culture. (b) Tracheal ring labelled after culturing for 8 days. Bar marker represents 250 μm for (a) and (b). Phase-contrast microscopy of unstained frozen sections is shown. Insets show a higher magnification to bring out details. Bar marker represents 50 μm for (a) and (b).

up to 120 days. In persistently infected cultures the enlarged rounded areas of epithelium representing MCMV c.p.e. began to gradually degenerate and slough after 30 to 40 days, but focal areas of c.p.e. persisted for as long as virus could be detected in culture fluids. When
Table 1. Preculturing of tracheal rings increases the number of cells incorporating tritiated thymidine and increases virus titres after infection with mouse cytomegalovirus (MCMV)*

<table>
<thead>
<tr>
<th>No. of days preincubation before [3H]thymidine labelling or MCMV infection</th>
<th>0</th>
<th>1</th>
<th>4</th>
<th>8-9</th>
<th>96-120</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells per tracheal ring with heavy accumulations of grains</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(log_{10} p.f.u./ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.90</td>
<td>4.15</td>
<td>4.65</td>
<td>5.15</td>
<td>ND†</td>
</tr>
<tr>
<td>1</td>
<td>3.90</td>
<td>4.15</td>
<td>4.65</td>
<td>5.15</td>
<td>ND†</td>
</tr>
<tr>
<td>4</td>
<td>3.90</td>
<td>4.15</td>
<td>4.65</td>
<td>5.15</td>
<td>ND†</td>
</tr>
<tr>
<td>8-9</td>
<td>3.90</td>
<td>4.15</td>
<td>4.65</td>
<td>5.15</td>
<td>ND†</td>
</tr>
<tr>
<td>96-120</td>
<td>3.90</td>
<td>4.15</td>
<td>4.65</td>
<td>5.15</td>
<td>ND†</td>
</tr>
</tbody>
</table>

* Uninfected tracheal rings were cultured for the indicated number of days before labelling overnight with [3H]thymidine as described in Methods. Frozen sections for autoradiography were prepared and developed 4 days later. The number of cells per complete tracheal section with heavy accumulations of grains was then counted. Numbers are averages ± standard deviation; numbers in parentheses are the number of rings counted at each time point. Additional groups of tracheal rings were precultured for the number of days shown, infected with MCMV and cultured further as described in Methods. Virus titres in the culture fluids harvested 10 days after infection were determined.

† ND, Not done.

examined by light microscopy (Fig. 2), the enlarged cells of the epithelium in persistently infected cultures exhibited the lacy-looking nucleus characteristic of infected cells in this system. Fig. 2 also shows an apparently uninfected area of epithelium that has retained cilia and does not possess the typical virus c.p.e. seen in the adjacent area. Electron microscopy of persistently infected tracheal cultures showed that cells with lacy nuclei (such as the cell marked ‘A’ in Fig. 3) contained virus particles in various stages of maturation both in the nucleus and in the cytoplasm. Desmosomes, indicative of the epithelial nature of these cells, were also seen.

Cell DNA synthesis and MCMV replication in acutely infected tracheal organ cultures

Muller & Hudson (1977) reported that only dividing cell cultures would optimally replicate MCMV. We had previously noted that at least a few cells in both infected and uninfected tracheal organ cultures would incorporate [3H]thymidine in detectable amounts for up to 100 days (J. G. Nedrud, unpublished observations). We therefore investigated what role cellular DNA synthesis might play in MCMV replication and persistence in tracheal organ culture. When freshly explanted uninfected tracheal rings were cultured overnight in [3H]thymidine, only a few cells (<1%) incorporated label as determined by autoradiography. However, culturing the uninfected tracheal rings for 1 to 8 days before [3H]thymidine labelling increased the number of epithelial cells per section incorporating label to the order of 10% or more (see Fig. 4). Grains were concentrated over cell nuclei. Culturing of tracheal rings for 8 days before infection by MCMV also resulted in a 10-fold increase in virus yield measured at 10 days after infection. These results are summarized in Table 1.

Although preculture of tracheal rings resulted in both active cell DNA synthesis and increased yields of virus at 10 days after infection, the peak virus titres obtained were nearly identical in both instances, around 10^5 p.f.u./ml of culture fluid. The increased yield of virus at 10 days was due to accelerated kinetics of virus production by precultured tracheal rings. Thus, as shown in Fig. 5, in freshly explanted cultures, virus titres above 10^4 p.f.u./ml were reached 10 days or more after infection, and peak titres were not reached until 15 to 20 days.
Persistent MCMV infection of organ cultures

In contrast, with tracheal rings precultured for 7 to 9 days before MCMV infection, titres greater than $10^4$ p.f.u./ml were found in culture fluids from the first change of medium, and peak virus titres were reached about 10 days after infection.

To examine further the temporal relation between cellular DNA synthesis and virus replication, additional experiments were designed. Freshly explanted tracheal organ cultures were infected with MCMV. At predetermined intervals cultures were labelled overnight with $[^3]$H]thymidine, and then harvested and scored for both cellular DNA synthesis by autoradiography and for virus antigen production by MCMV-specific immunofluorescence. The labelling conditions and autoradiographic exposure times chosen for these experiments were sufficient to detect cellular, but not virus DNA synthesis (DeMarchi & Kaplan, 1976). Virus antigens were assayed because they are an earlier marker for replication of MCMV than the appearance of infectious virus in culture fluids. In these experiments maximum rates of cellular DNA synthesis (i.e. maximum number of cells with grains in autoradiography per ring

Fig. 5. Accelerated kinetics of MCMV production in precultured tracheal rings. Tracheal organ cultures from MCMV-susceptible mice were prepared and either infected immediately (▼) or after preculturing the rings for 7 to 9 days (▼). Points plotted are geometric mean titres of virus from a total of six experiments. Error bars represent the range of values observed in individual experiments.

Fig. 6. Cumulative cellular DNA synthesis, antigen expression and virus production by MCMV-infected tracheal organ cultures. Freshly explanted tracheal organ cultures were infected with MCMV and labelled for 18 h on days 1, 3, 7, 12 and 15. Three to six individual tracheal rings at each time point were then prepared for antigen determination by MCMV-specific immunofluorescence and autoradiography. Fluorescence (+) was scored on a scale of +1 (definite positive fluorescence involving small foci and small numbers of cells) to +4 (very bright, widespread fluorescence) with ± representing marginally positive specific fluorescence. Sections reacted with normal mouse serum plus conjugate served as a control and were completely negative. The integer fluorescence scores for individual rings were averaged to give the plotted values. Cumulative numbers of cells per section exhibiting a heavy accumulation of grains (△) was determined by assuming that cells labelled overnight for 18 h represented the number of cells which would be labelled in a 24 h period. Cell numbers with grains for days between labellings were interpolated from the values obtained on days where labelling occurred. Cumulative numbers of cells with grains were then calculated by adding the numbers obtained for individual days. Cumulative p.f.u.s of MCMV released into the culture medium (●) were calculated using the data plotted in Fig. 5 as the additive number of p.f.u.s observed at days 4, 7, 10, 14, 18, 22 and 26 for the respective days.
Table 2. Infected tracheal organ cultures continue to synthesize cellular DNA in areas associated with foci of MCMV cytopathic effects

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Time after infection at harvest (days)</th>
<th>No. of cells with heavy accumulation of autoradiographic grains*</th>
<th>No. of cells of c.p.e. characteristic of MCMV†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>With or near cells having grains</td>
<td>Without cells having grains</td>
</tr>
<tr>
<td>IF2-20A</td>
<td>84</td>
<td>53</td>
<td>2</td>
</tr>
<tr>
<td>IF2-20B</td>
<td>109</td>
<td>45</td>
<td>3</td>
</tr>
<tr>
<td>1372-1</td>
<td>100</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>1372-5</td>
<td>100</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
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<td>9</td>
<td>2</td>
</tr>
<tr>
<td>1372-8</td>
<td>96</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>1574</td>
<td>82</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1575</td>
<td>82</td>
<td>36</td>
<td>4</td>
</tr>
<tr>
<td>1578</td>
<td>107</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>1580</td>
<td>84</td>
<td>126</td>
<td>2</td>
</tr>
<tr>
<td>1581</td>
<td>109</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1582</td>
<td>88</td>
<td>25</td>
<td>2</td>
</tr>
</tbody>
</table>

* Average number of cells per ring with heavy accumulation of grains was 33.3.
† Total of nine rings containing 24 foci with autoradiographic grains, and seven rings with 12 foci without autoradiographic grains.

per labelling period) always preceded the appearance of virus antigens. When plotted against cumulative numbers of cells synthesizing cellular DNA, virus antigen expression appeared to lag behind yet parallel cellular DNA synthesis (Fig. 6). In addition, Fig. 6 shows that cumulative virus released into the culture fluids also paralleled cell DNA synthesis and virus antigen production, but with a further lag of 5 to 10 days. These results indicate that cell DNA synthesis precedes virus synthesis in a manner which suggests but does not prove that the two events may be causally related.

In this acute stage of virus infection MCMV antigens were concentrated in areas of tracheal epithelium showing gross virus c.p.e. and were also diffusely distributed over areas which appeared to be normal. In experiments where simultaneous immunofluorescence and autoradiography were done between 10 and 20 days after infection, slightly more than 50% of the autoradiographically labelled cells were either antigen-positive or very closely associated with intensely fluorescent (antigen-positive) areas. In these experiments tracheal organ cultures were pulse-labelled for 16 to 18 h, and then refed with non-radioactive medium; 0 to 3 days later MCMV antigens were determined. There was no apparent difference in the results obtained with cultures labelled 3 days before antigen determination and those with cultures labelled immediately before antigen determination.

Cell DNA synthesis and MCMV replication in persistently infected tracheal organ cultures

Since we had already observed continued cellular DNA synthesis in long-term uninfected tracheal organ cultures (Table 1) the idea that non-permissive cells might become permissive for virus replication during or after cellular DNA synthesis emerged as a possible mechanism for persistent MCMV infection in organ culture.

The first test of this hypothesis was to determine whether or not long-term infected organ cultures continued to synthesize cellular DNA. Table 2 shows that in 10 out of the 12 rings examined between 82 and 109 days after infection cellular DNA synthesis was detectable by autoradiography. Furthermore, 75% of the rings contained foci of c.p.e. which had a direct or close association with heavily labelled cells, and two-thirds of all the foci observed in these
Fig. 7. Light and electron microscopic examination of cells with heavy accumulation of grains in persistently infected tracheal organ cultures. Persistently infected cultures were labelled overnight with \(^{3}H\)thymidine and then processed and embedded in Epon plastic for light microscopy/autoradiography and electron microscopy. Serial 0.5 \(\mu\)m tissue sections for light microscopy and autoradiography were cut, followed immediately by cutting 50 nm sections from the same tissue blocks which were examined under the electron microscope. Using photographs of the autoradiograms as a guide it was then possible to identify and photograph specific, individual areas and cells under the electron microscope. (a) Light micrograph of focal c.p.e. on CD-1 mouse tracheal ring infected 82 days previously with MCMV. (b) Autoradiogram from serial section of specimen in (a). Bar marker represents 20 \(\mu\)m in (a) and (b). (c) Electron micrograph from serial section of specimen in (a) and (b). The cell marked with an arrow in (c) is the cell with a heavy accumulation of grains marked with an arrow in (b) and also marked with an arrow in (a). Bar marker represents 4 \(\mu\)m.
rings were associated with heavily labelled cells. These numbers are conservative estimates since the tracheal rings and the bleb-like areas of c.p.e. are three-dimensional structures, whereas autoradiographic analysis encompasses only a single flat plane of these structures. All of the tracheal rings described in Table 2 were from cultures which demonstrated the production of MCMV in culture fluids at the time of harvest or shortly before harvest.

While heavily labelled cells were often associated with foci of c.p.e. in persistently infected tracheal organ culture, the heavily labelled cells themselves did not exhibit the hallmarks of MCMV infection. As Fig. 7 (a, b) shows, the heavily labelled cells were typically located in a basal position (underlying the areas of virus c.p.e. and adjacent to an often intact basement membrane) and did not possess a CMV-induced lacy nucleus or inclusions.

The ultrastructure of these heavily labelled cells was disclosed by carefully cutting ultrathin (50 nm) sections serial to the thicker (0.5 μm) sections used for autoradiography. Fig. 7 (c) is an electron micrograph which shows again that the heavily labelled cell marked with an arrow in Fig. 7 (a, b) does not possess the morphology of a CMV-infected cell. Immediately above this cell, however, is an infected cell containing incomplete virus particles.

Fluorescent antibody/autoradiographic analysis confirmed these results. MCMV antigens were generally localized to the cytopathic foci of persistently infected cultures. While cells in DNA synthesis were often associated with these antigen-positive foci (see Table 2 and Fig. 7) less than 5% of the autoradiographically labelled cells were antigen-positive themselves.

**DISCUSSION**

The epithelium of murine tracheal organ culture supports the long-term replication and excretion of cytomegalovirus. The results presented here also confirm the positive correlation first reported by Muller & Hudson (1977) between cellular DNA synthesis and permissive MCMV replication and suggest that this correlation may at least partially provide a mechanism for the observed persistent infection.

Tissue culture models of persistent (human) CMV infections have recently been established in fibroblasts and lymphoblastoid cell lines (Mocarski & Stinski, 1979; Furakawa, 1979), but the model described here is the first to make use of the organized tissue inherent in organ culture. This model is also unique in demonstrating persistent infection of epithelial cells, an important target cell type *in vivo* (Henson et al., 1967; Weller, 1971; Mims & Gould, 1979).

Organ culture models more closely approximate *in vivo* tissue complexity and organization than do other culture systems. Even the lack of interactions with an intact host immune system may approximate the situation for CMV infection *in vivo* in as much as immunological effector mechanisms may have restricted access to CMV-infected cells facing ductal or tubular lumina in persistently infected salivary gland or kidney (Henson et al., 1967; Henson & Neapolitan, 1979; Mims, 1974).

The tracheal organ culture system described here should be viewed only as a model system for the persistent CMV infection of epithelial cells and not as a model for respiratory tract infection. This culture model has been used previously to postulate a cellular basis for the genetics of MCMV susceptibility and resistance. Tracheal organ cultures from resistant strains of mice were shown to be resistant to CMV infections *in vitro* (Nedrud et al., 1979) while fibroblast and macrophage cultures from resistant mice were not resistant *in vitro* [Nedrud et al., 1979; Brautigam et al., 1979; Grundy (Chalmer) et al., 1981].

Although CMV does have the capacity to infect respiratory tissues in the lung of both man and experimental animals (Neiman et al., 1977; Jordan, 1978) the virus has not been shown to infect the trachea *in vivo* (Mims & Gould, 1979). One reason that CMV may not establish infections in tracheal epithelium *in vivo* is the relatively low turnover rate of this tissue *in vivo* (Boren & Paradise, 1978). There is abundant evidence linking the metabolic state of target cells and their capacity to replicate CMV. Muller & Hudson (1977) showed that infected,
serum-starved cultures of mouse fibroblasts would not replicate MCMV DNA, but that these arrested cells could be induced into supporting virus replication using fresh media and serum. Fluorouracil-arrested cultures of human embryonic lung cells were shown by DeMarchi & Kaplan (1977) to produce up to 700-fold less human CMV than actively growing cells. Mocarski & Stinski (1979) have also suggested that reduced host cell metabolic state may be responsible for blockage of human CMV replication in the non-productive fraction of persistently infected human fibroblasts.

Furthermore, the results presented in this paper show that in acutely infected tracheal organ cultures there is a correlation between cellular DNA synthesis as measured by autoradiography and the capacity of these cultures to replicate MCMV. Freshly prepared cultures neither incorporated [3H]thymidine nor efficiently produced MCMV. Such cultures are not deficient in taking up MCMV, however, since after a lag period of about 15 days they did produce $10^4$ to $10^5$ p.f.u./ml of virus. Additionally, the speed with which MCMV-infected organ cultures began to produce virus could be increased simply by culturing for a few days prior to infection (Fig. 5). Such preculturing also increased the level of cellular DNA synthesis in these cultures approx. 10-fold (Table 1). Finally, apparently uninfected but heavily labelled epithelial cells were observed in persistently infected tracheal organ cultures in a position immediately adjacent to cells containing virus particles (Fig. 7).

Our immunofluorescent/autoradiographic results differ somewhat from a previous report. MCMV, as reported here and by Muller & Hudson (1977), replicates best in actively dividing cultures. Similarly, DeMarchi & Kaplan (1977) reported that cultures with active cellular DNA synthesis yield up to 700-fold more human CMV than non-replicating cultures of infected human embryonic lung cells. DeMarchi & Kaplan (1976) also reported, however, that CMV antigens were not present in cells undergoing autoradiographically detectable DNA synthesis. Although this result is similar to what we observed in persistently infected organ cultures it differs from the results we observed in acutely infected cultures where 50% of the cells undergoing DNA synthesis also displayed CMV antigens. The reason for the differences between DeMarchi & Kaplan's results and ours could be related to the culture systems employed, to inherent differences between human CMV and MCMV, or to other undefined factors.

If the assumption that cells undergoing cellular DNA synthesis subsequently become permissive for MCMV replication is true, then presumably the heavily labelled but uninfected cells shown in Fig. 7 and other similar cells would soon become infected by MCMV from adjacent cells. Alternatively, the heavily labelled cells could already be infected and virus replication could be triggered after cellular DNA synthesis. Such a scheme might contribute to the persistent nature of tracheal organ culture MCMV infections by providing a continued supply of newly susceptible cells. This hypothesis assumes that non-differentiated basal cells do not become permissive for virus infection prior to cell division and differentiation. Recent studies by J. F. Baskar (personal communication) and by Dutko & Oldstone (1981) with teratocarcinoma cells suggest that while undifferentiated teratocarcinoma cells will not produce infectious MCMV, differentiated forms of these same cells will produce MCMV.

Ultimately, the persistent infection observed here must be ascribed to a limited cytopathology of the virus with productive (lytic) infection not occurring in all available cells. Already infected cells might move into the virus lytic cycle upon release from some type of metabolic block, perhaps an event associated with cellular DNA synthesis. An infection such as this could conceivably go on in vitro for as long as cells were available and continued to move from a virus non-permissive state into a virus permissive state. The limited capacity of the tracheal epithelium to repair itself in vitro may eventually lead to arrest of the persistent infection. It is also likely that virus-infected cells are not immediately destroyed, but continue to excrete virus for some time, leading to the piling up of infected cells into the characteristic
blebs observed in MCMV-infected tracheal organ cultures. Although we do not have any quantitative data on this point, the general impression emerged during these studies that blebs of c.p.e. appeared slowly, enlarged, and then persisted relatively unchanged (except for gradual sloughing) for weeks or even months.

In vivo a similar infection could be self-perpetuating as long as infected cells were in a site unavailable for elimination by the immune system. A few naturally cycling cells in vivo might be lytically infected initially and destroyed by the virus. This could trigger a lesion-healing response by neighbouring stem cells, which might proliferate and migrate into the damaged area. These proliferating cells would now presumably be susceptible to a lytic virus infection which would destroy them and induce even more cells to proliferate and become infected, thus perpetuating the infection.

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