Serial Propagation of Astrovirus in Tissue Culture with the Aid of Trypsin

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SUMMARY

Astrovirus could be serially passed at least 13 times in primary human embryo kidney (HEK) cells when 10 μg/ml of crystalline trypsin was incorporated in a serum-free maintenance medium. In the presence of trypsin the virus was also passed and adapted to a continuous line of rhesus monkey kidney cells (LLCMK₂) and primary baboon kidney (PBK) cells in which it was passed 25 and 16 times respectively, without evidence of diminishing infectivity. Attempts to adapt the virus to other cell lines (Vero, Hep II, MRC-5, BHK and HRT-18) were unsuccessful. After 11 passages in HEK cells, a titration of virus grown in different concentrations of trypsin showed that virus propagation was still trypsin-dependent.

Astrovirus, as described by Madeley & Cosgrove (1975), has been incriminated in outbreaks of diarrhoea and vomiting amongst children and staff of paediatric wards (Kurtz et al., 1977; Ashley et al., 1978). Cultivation of astroviruses and other gastroenteritis-causing viruses by conventional methods in cell monolayers has not been successful. A limited growth cycle without virus production, however, was demonstrated for rotaviruses (Banatvala et al., 1975) and astroviruses (Lee & Kurtz, 1977) when virus antigen was detected in infected cells using a fluorescence antibody technique.

The use of trypsin was shown to enhance the growth of reoviruses in cell culture (Spendlove & Schaeffer, 1965; Spendlove et al., 1970), and Almeida et al. (1978) reported that yields of bovine rotavirus could be increased by up to 3·0 log₁₀ in cell cultures when trypsin was incorporated in the medium for the whole of the virus replication cycle. More recently, Graham & Estes (1980) showed the same pattern of enhanced infectivity when growing simian and porcine, as well as bovine, rotaviruses in the presence of the proteolytic enzymes, trypsin or elastase. We report here the effect of incorporating trypsin in the cell medium on the replication of astroviruses.

Crystalline beef pancreas trypsin (BDH) was prepared as a stock 2% solution in 1 mM-HCl and stored in 0·1 ml amounts at −40 °C. Working strengths were prepared in serum-free 199 medium containing 100 units/ml penicillin, 100 units/ml kanamycin and 0·088% sodium bicarbonate. Trypsin inhibitor (soybean; Sigma) was also prepared as a stock 2% solution and stored at −40 °C. For use, it was diluted appropriately to effect neutralization of trypsic activity.

The virus used was obtained from an infected volunteer, DM (Kurtz et al., 1979). A 10% faecal suspension in phosphate-buffered saline (PBS) was clarified by centrifugation at 2300 g for 30 min. A 0·1 ml amount of the supernatant was used as the initial inoculum. HEK, PBK, LLCMK₂, Vero, Hep II, MRC-5, BHK and HRT-18 cells were grown to confluence in Eagle's minimum essential medium (MEM) or 199 medium containing antibiotics, sodium bicarbonate and 10% newborn calf serum in 50 ml plastic tissue culture flasks (Nunclon). Before inoculation, the monolayers were washed twice in 199 medium to remove any residual serum present in the growth medium. The faecal supernatant was then added, together with 5 ml serum-free 199 medium containing 10 μg/ml trypsin and the cell cultures were incubated.
Table 1. Effect of trypsin concentration and host cell on the multiplication of astrovirus*

<table>
<thead>
<tr>
<th>(a) Trypsin (μg/ml)</th>
<th>24 h</th>
<th>48 h</th>
<th>96 h</th>
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<tbody>
<tr>
<td>50</td>
<td>3.3</td>
<td>3.7</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>2.6</td>
<td>3.6</td>
<td>3.3</td>
</tr>
<tr>
<td>1</td>
<td>1.6</td>
<td>2.8</td>
<td>3.1</td>
</tr>
<tr>
<td>0</td>
<td>1.0</td>
<td>&lt;1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) Virus grown in</th>
<th>HEK (passage 8 virus)</th>
<th>LLCMK₂ (passage 16 virus)</th>
<th>PBK (passage 14 virus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK</td>
<td>5.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>LLCMK₂</td>
<td>7.0</td>
<td>6.0</td>
<td>3.0</td>
</tr>
<tr>
<td>PBK</td>
<td>6.0</td>
<td>1.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

*Titres of astrovirus (log_{10} infective units virus/ml inoculum) were determined when virus was grown (a) in HEK cells in the presence of different concentrations of trypsin and (b) in HEK, LLCMK₂ and PBK cells and titrated in homologous and heterologous cell systems in the presence of 10 μg/ml trypsin.

at 35 °C. The presence of the trypsin caused the cells to become detached from the flask walls and remain in suspension. After 3 days, passage was effected by adding 1 ml of the infected cell suspension to another cell monolayer to which, after a 2 h adsorption period, 5 ml of the trypsin-containing maintenance medium were added.

At each passage cell deposits were prepared for immunofluorescence staining by centrifuging 0.3 ml of a 1:10 dilution of cell suspension at 90 g for 5 min on to glass slides in a Shandon Cytospin cytocentrifuge. After staining by the indirect fluorescent antibody technique, preparations were examined for specific cell fluorescence by incident light illumination using a Leitz Dialux microscope.

To observe the effects of different strengths of trypsin on astrovirus growth in HEK cells, passage 11 infected HEK cell suspension was inoculated into flasks with maintenance medium containing 50, 10, 1 or 0 μg/ml trypsin. The amount of free virus present after 24, 48 and 96 h was then determined by removing 1 ml amounts at these times and centrifuging at 250 g for 10 min to deposit cellular material. Trypsin inhibitor was added to the supernatant to neutralize residual trypsin. Serial 10-fold dilutions of the supernatant were then made in trypsin-free maintenance medium and 1 ml amounts inoculated on to HEK cells grown in 10 x 1 cm tissue culture tubes and incubated. No cytopathic effect was observed. After 2 days the cells were removed from the glass by treatment with a trypsin/versene mixture and suspended in 1 ml of 199 medium. A 0.3 ml amount of this suspension was used to prepare cytocentrifuge deposits which were stained as above, and the fluorescing cells counted.

In another experiment, virus which had been adapted to grow in HEK, LLCMK₂, and PBK cells, was titrated in homologous and heterologous systems in the presence of 10 μg/ml trypsin. Cells were examined for the presence of astrovirus antigen as previously described.

For electron microscopy, 4 ml of infected cell suspension were sonicated at a wavelength of 18 μm for 30 s and then centrifuged at 3500 g for 30 min. The supernatant was recentrifuged at 116 000 g for 1 h. The resultant pellet was suspended in a few drops of distilled water and grids prepared and examined in a Philips 300 microscope. For immune electron microscopy, the pellet was suspended in 0.1 ml PBS. To this was added 0.1 ml of a 1:20 dilution of the anti-astrovirus serum. The mixture was held for 1 h at room temperature followed by overnight at 4 °C. Four ml PBS were then added and the tubes centrifuged at 116 000 g for 1 h. Grids were prepared from the pellet and examined.

Astrovirus was passed serially 13 times in HEK cells, without evidence of diminishing titre, when serum-free 199 medium containing 10 μg/ml trypsin was used as maintenance medium.
Fig. 1. (a) Passage 24 astrovirus antigen in LLCMK₂ cells demonstrated by indirect fluorescent antibody technique. Acetone-fixed cells were incubated with a 1:20 dilution of a human anti-astrovirus serum for 30 min, washed and stained with a 1:40 dilution of fluorescein-conjugated sheep anti-human IgG serum (Wellcome). (b) Electron micrograph of astrovirus from passage 11 HEK cell suspension negatively stained with 2% phosphotungstic acid. Bar marker represents 100 nm.

The maximum yield of virus in the cell-free supernatant fluid was reached after 48 h (Table 1a). A more rapid yield of virus occurred when 50 μg/ml trypsin was incorporated in the medium, but this did not result in a higher titre of virus. Conversely, if the medium contained only 1 μg/ml trypsin, the maximum yield of virus was delayed. In the absence of trypsin very little virus was released from the cells as indicated by the titration, although subsequent fluorescence staining of these cells showed that many contained virus antigen.

After six passages, astrovirus-infected HEK cell suspensions were passed to other cell lines, again in the presence of 10 μg/ml trypsin. Vero, BHK, Hep II, MRC-5, MDCK and HRT-18 cells were all non-permissive. Very occasional fluorescing cells were observed after the primary inoculations and at first passage, but it is likely that this was due to 'carry-over' from the original inoculum. Further passes were all negative. Serial virus growth could not be established when faecal extract was inoculated directly into LLCMK₂ or PBK cells maintained in trypsin medium. When these cell lines were infected with sixth passage astrovirus-infected HEK cells, however, serial replication occurred, and the virus has been passed 25 times in LLCMK₂ cells (Fig. 1a) and 16 times in PBK cells, without signs of diminishing infectivity.

Titrations of virus grown in different cells are shown in Table 1(b). The titres in HEK and LLCMK₂ cells compare favourably with the virus titre (10⁹/ml) of the original inoculum of 10% faecal extract grown in HEK cells in the presence of trypsin. HEK cells appeared to be more sensitive than LLCMK₂ and PBK cells, even when virus was titrated in the same cell line as that in which it was grown.
Short communications

After 10 passages in LLCMK₂, an attempt was made to pass the virus in reducing concentrations of trypsin (10, 5, 2, 1 and 0 μg/ml). This yielded progressively fewer fluorescing cells despite prolongation of the incubation period, and no positive cells could be found when trypsin-free medium was used.

Electron microscopy of the cell suspension from passages 11 and 13 in HEK cells and passages 7, 19 and 24 in LLCMK₂ cells showed abundant virus particles (Fig. 1b) which morphologically appeared identical to those found in faeces. Surface antigenic similarity was also demonstrated as virus was clumped by immune serum.

The incorporation of trypsin in the cell medium permitted proliferative replication of astrovirus in HEK, LLCMK₂ and PBK cells; 13, 25 and 16 passages respectively have been achieved without loss of infectivity. Babiuk et al. (1977), found that the infectivity of bovine rotavirus was gradually lost in the absence of trypsin, but was restored again in the resumed presence of it. Attempts to adapt astrovirus to cells in a trypsin-free environment were unsuccessful. We found that virus, grown in cells in the presence of trypsin, could further infect cells in medium containing trypsin inhibitor, but only for one cycle as demonstrated by fluorescence of virus antigen. Release of astrovirus from cells appeared only to occur if trypsin was present throughout the whole of the incubation period.

Spendlove et al. (1970) found that 20 μg/ml chymotrypsin removed the virus capsid from reovirus. However, no obvious morphological changes were seen when rotavirus was propagated in the presence of 1 to 10 μg/ml trypsin (Almeida et al., 1978). In the case of astrovirus, examination of cell fluids by electron microscopy did not reveal any obvious defects in virus structure, and antigenicity, as demonstrated by immune electron microscopy, remained unchanged.

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REFERENCES

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