The Effect of Trypsin on the Growth of Rotavirus

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(Accepted 2 March 1978)

SUMMARY

It has been found that 1000-fold more bovine rotavirus is obtained when trypsin is incorporated in the maintenance medium and allowed to remain throughout the growth cycle. This holds true for primary calf kidney (CK) cells and also for several continuous and semi-continuous cell lines. In the presence of trypsin it has been possible to pass the virus serially on continuous cell lines seven times. Concentrations of 1 to 10 μg/ml of trypsin are found to be effective. Preliminary results suggest that the same technique will be effective for the in vitro propagation of human rotavirus.

INTRODUCTION

Studies by electron microscopy have revealed virus particles of characteristic morphology in diarrhoeal faeces of the young of many mammalian species, including humans (Flewett et al. 1974; Kapikian et al. 1974; Holmes et al. 1975; Totterdell et al. 1976). Based on their distinct morphology, these viruses are placed in a genus known provisionally as 'rotavirus' within the Reoviridae family. Calf rotavirus strains can be adapted to propagate in primary calf kidney but no distinct cytopathic effect occurs. It has not proved possible to carry out serial passage of calf rotavirus on established cell lines, although in continuous pig (IBRS-2) and monkey (BSC-1) cell lines calf rotavirus produces virus antigen but little infectious virus. The only consistent in vitro activity so far achieved with human rotavirus is the production of virus antigen after centrifugation of virus-containing faecal extracts on to the surface of monolayer cell cultures (Banatvala et al. 1975; Thouless et al. 1977).

It has been shown that trypsin enhances the infectivity of reoviruses in cell culture (Spendlove & Schaffer, 1965) and permits certain strains of influenza, which hitherto have been refractory, to form plaques (Appleyard & Maber, 1974). Furthermore, Matsuno et al. (1977), by incorporating trypsin in the overlay medium, produced plaques in monkey kidney cell cultures inoculated with bovine rotavirus. Theil et al. (1977) showed that, provided porcine rotavirus suspensions harvested from infected cell cultures were treated with pancreatin before inoculation into and during adsorption on to primary porcine kidney cultures, successful passage to high titres could be accomplished. Based on these observations, we incorporated trypsin into the culture medium during the whole period of virus/cell interaction, to increase the yield of bovine rotavirus by 3-0 log10 in different cell cultures. (Since preparing this manuscript we have discovered that Babiuk et al. (1977) have used the same approach to obtain similar results.)
METHODS

Stools containing bovine rotavirus were obtained from three field cases of neonatal calf diarrhoea. Ten % stool suspensions in PBS were prepared for tissue culture by clarification at 2000 rev/min for 10 min and passage of the resultant supernatant through a 0.22 μm Millex filter. Two isolates (WRV505 and L248) were used without passage. The third isolate (WRV310) was adapted to tissue culture by passage five times through primary CK cells. A second tissue-culture-adapted strain, TC27/31, was also obtained. Cell cultures used were primary CK, continuous monkey kidney (BSC-1 and LLC-MK2), semi-continuous human fibroblasts (MRC-5) and a continuous pig kidney (IBRS-2).

In all experiments, 1 ml of virus isolate was seeded on to Corning 150 cm² tissue culture flasks containing confluent cells. After 60 min adsorption at 37 °C, the volume of medium in each bottle was adjusted to 60 ml. Control flasks contained standard Eagle's basal medium (serum free) but for test flasks the medium additionally contained trypsin to give a final concentration of 1, 5 or 10 μg/ml. The enzyme used was prepared as a 1 % stock crystalline trypsin in 1 mM-HCl. Uninfected cells were maintained using either control or trypsin medium. As an additional control, monolayers that had been adsorbed in the presence of trypsin were changed to control medium. Incubation was continued at 37 °C and cultures maintained for 48 h.

Virus yields were estimated by immunofluorescence (IF) and by electronmicroscopy (EM). For IF, cell harvests, at 48 h, were sonicated for 30 s and 10-fold dilutions of the resultant homogenates centrifuged on to IBRS-2 coverslip cultures. After incubation at 37 °C for 24 h, these cells were examined for the presence of virus antigen (Totterdell et al. 1976). Five ml samples of cell harvests at 24 and 48 h were prepared for EM by centrifugation at 12000 g for 1 h and the supernatant drained from the tube. The pellet was resuspended in a small amount of distilled water and a drop of this mixed with an equal amount of 4% phosphotungstic acid adjusted to pH 6.0. A drop of this mixture was then placed on a 400 mesh Formvar-carbon coated grid, The excess fluid removed and the specimen examined immediately in a Philips 300 electron microscope.

RESULTS

Detection of rotavirus particles by electron microscopy

Both the adapted (WRV310) and unadapted (WRV505) strains of bovine rotavirus grew without difficulty in primary CK cells maintained on non-trypsin medium, and an average microscope plate of virus, harvested at 48 h, taken at a magnification of 57000 times, contained 3 to 6 virus particles (Fig. 1). However, the same seed virus, grown on the same cell batch but maintained on trypsin medium, showed greatly enhanced numbers of virus particles, even at the 1 μg/ml level of enzyme. At the 5 and 10 μg/ml levels of trypsin, the grids contained large rafts of virus particles that were frequently associated with membranous material (Fig. 2). Large inclusions, consisting of virus particles within a membranous sac, were also a feature of these preparations (Fig. 3). Because of the presence of these aggregates, it was difficult to estimate the average number of particles in random fields (but the number was certainly greater than 50). ‘Rough’ and ‘smooth’ forms of rotavirus particles were present in both trypsin-treated and control cell-culture harvests. However, it was our impression that trypsin-treated specimens contained a higher ratio of ‘smooth’ to ‘rough’ particles. Despite being grown in the presence of trypsin, the harvested particles did not show signs of degradation.
Fig. 1. A typical group of bovine rotavirus particles harvested from primary calf kidney (CK) cells without trypsin.

Fig. 2. An area from the same virus-cell batch but with 10 μg/ml trypsin in the medium. As here, the virus is frequently associated with tags of membrane. Only complete virus is present in this area.

Fig. 3. Much of the virus is contained within membranous sacs. The presence of these inclusions almost certainly influences the titres obtained by the immunofluorescence test.

Fig. 4. Another illustration of virus-containing inclusions, this time from the sixth serial pass in the BSC-1 cell line.

(All micrographs are at the same magnification.)
Examination of the virus propagated on BSC-1, LLCMK-2, MRC-5 and IBRS-2 cultures showed a similar picture to that found in CK. However, it was more difficult to detect virus in non-trypsin-treated preparations and the yield from continuous cell lines, although much better than from untreated CK, was not as great as that from trypsin-treated CK. These results are summarized in Table 1.

Examination by EM of the harvests from MRC5 and BSC-1 cell cultures was carried out to passage 7, and virus was readily detected at each passage level (Fig. 4). Examination by EM of culture harvests at 24 and 48 h showed a marked increase in numbers of virus particles by 48 h. The control experiments, carried out by adsorbing the virus in the presence of trypsin and then transferring to control medium, showed only a small increase over the non-trypsin-treated control.

Detection of rotavirus antigen by immunofluorescence

Titrations of the virus yield by IF from CK and MRC5 cell lines inoculated with WRV505 and both BSC-1 and IBRS-2 cell lines inoculated with L248 and TC27/31 are shown in Table 2 and in every case demonstrated an increase of virus in the presence of trypsin. Maximum titres were obtained following incorporation of 5 or 10 μg/ml of trypsin in the maintenance medium. However, since the EM studies above revealed the presence of virus aggregates when higher levels of trypsin were used, virus yields in this range estimated by IF may well have been an underestimate.

Effect of trypsin on cell viability

There was no apparent difference, by light microscopy, between cells grown in the presence of trypsin and control cells. Furthermore, trypan blue-stained IBRS-2 cells and BSC-1 cells, maintained on 10 μg/ml trypsin, showed 95% viability after 48 h.

DISCUSSION

EM observations show that greatly increased amounts of bovine rotavirus are produced when trypsin is present in the medium during the entire period of virus propagation. Control experiments, in which virus was adsorbed in the presence of trypsin but the cells were then maintained on trypsin-free medium, show only slight enhancement from control preparations.

With reovirus (Spendlove & Schaffer, 1965; Spendlove et al. 1970) and influenza (Klenk et al. 1975; Lazarowitz & Choppin, 1975) trypsin can increase the virus yield by cleaving surface components before the virus is introduced into cell culture. However, in this study with rotavirus, it appears that for full effect the trypsin is required to be present during the entire course of virus replication. This is similar to the findings of Matsuno et al. (1977), who demonstrated that incorporation of trypsin into the overlay medium was necessary for plaque production. Similarly Appleyard & Maber (1974) showed that certain strains of influenza virus would plaque only if trypsin were present throughout the growth cycle. However, Theil et al. (1977) have demonstrated passage of porcine rotavirus in primary porcine kidney cells which is dependent only on treatment of inocula with trypsin during adsorption, after which monolayers are washed and maintained in trypsin-free medium.

It seems unlikely that trypsin effects this enhancement of virus growth by cleavage of cell components, as ‘rough’ particles have been demonstrated to be non-infectious (Bridger & Woode, 1976). Furthermore, it is known that, with rotavirus, the multiplicity of infection is high and in the presence of trypsin the course of the replicative cycle appears to be
Effect of trypsin on rotavirus replication

Table 1. Enhancement of growth by trypsin of four bovine rotavirus strains in various cell cultures

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>CK</th>
<th>LLCMK-2</th>
<th>BSC-1</th>
<th>MRC-5</th>
<th>IBRS-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRV505</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>L248</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>WRV310</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>TC27/31</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
</tbody>
</table>

* +, ++, Growth of virus enhanced.

Table 2. Effect of trypsin on yield (logs) of three bovine rotavirus strains in CK, BSC-1 and IBRS-2 cell cultures

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inoculum</th>
<th>0</th>
<th>1μg</th>
<th>5μg</th>
<th>10μg</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>WRV505</td>
<td>2.5</td>
<td>5.0</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>BSC-1</td>
<td>L248</td>
<td>&lt;0.5</td>
<td>ND</td>
<td>ND</td>
<td>3.5</td>
</tr>
<tr>
<td>IBRS-2</td>
<td>TC27/31</td>
<td>2.5</td>
<td>ND</td>
<td>ND</td>
<td>5.5</td>
</tr>
<tr>
<td>MRC-5</td>
<td>L248</td>
<td>&lt;0.5</td>
<td>ND</td>
<td>ND</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>TC27/31</td>
<td>1.5</td>
<td>ND</td>
<td>ND</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>WRV505</td>
<td>1.0</td>
<td>ND</td>
<td>ND</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Considerably shorter (48 h) than in non-trypsin-treated primary CK cultures in which peak yields occur at five days.

Alternatively, trypsin may produce an effect on the cell surface, thereby enhancing virus penetration. This would be in accord with the ‘stick and grip’ theory put forward by Rees et al. (1977). Additional weight to this theory is provided by the fact that rotavirus infectivity is enhanced by centrifuging the virus on to the cell’s surface. It seems improbable that the virus is actually impacted on to the cell surface, since centrifugation may be carried out at speeds as low as 1200 g. Centrifugation is more likely to alter the properties of the cell surface, permitting viral uptake perhaps in a mechanism similar to that produced by the trypsin.

Yet another theory is offered by the fact that trypsin enhances cell growth (Carney & Cunningham, 1977). Studies on primary chick fibroblasts have shown that cell metabolism is enhanced and the time of the mitotic cycle is reduced in the presence of trypsin. In the present context, this is of interest because of the greatly reduced harvest time in the presence of trypsin.

Trypsin also destroys interferon (Isaacs et al. 1957) and it could be that rotavirus is highly interferon-sensitive. In addition, we have found that foetal calf serum contains antibody to rotavirus, and the trypsin could negate the effect of this. However, it could well be argued that all the above points may be relevant in the production of enhanced amounts of rotavirus and that the enzyme is acting in different ways at different points on the growth cycle.

The technique described is an unusual one as the cells detach from their plastic base after approximately 12 h. However, this does not appear to interfere with cell metabolism, as there is a considerable increase in the amount of virus produced between 24 and 48 h. It should also be pointed out that, although the virus-infected cells contained large virus inclusions that could be visualized by electron microscopy, there was no obvious difference
between virus-infected cells grown in trypsin and control cells maintained in the same medium when they were examined by light microscopy as unstained viable preparations.

Many of the results discussed in this paper could have been presented more accurately if a convenient titration method for bovine rotavirus and its antibodies were available. Now that it appears possible to produce large amounts of the virus on readily obtainable cell substrates, this should become a more practical proposition.

Although the present paper deals exclusively with the effect of trypsin on bovine rotavirus, it might be found that low levels of this or other enzymes could enhance the growth of other virus types. It is particularly encouraging that preliminary experiments (J. E. Banatvala & J. D. Almeida, unpublished data), in which faecal extracts containing rotaviruses from children with acute diarrhoea were inoculated into trypsin cell cultures, also suggest that replication of rotaviruses occurs with the production of infectious complete forms of virus particles.

We wish to thank Mrs A. Wilson for expert assistance and Mr Gerald Woode, Institute for Research into Animal Diseases, Compton, Berks, for supplying the L248 and TC27/3I strains of bovine rotavirus.

REFERENCES


(Received 27 February 1978)