The Growth of Echovirus 1 in HEp-2 Cells Treated with Antibody to the Host Cells

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SUMMARY

Antibody prepared against HEp-2 cells inhibited the replication of echovirus 1. The number of infective centres was reduced and the cells which were infected produced less virus per cell than control cells. The attachment of virus to cells was not affected. The inhibition was reduced by increasing the multiplicity of infection. Application of antibody 5 min. after virus adsorption at 4° reduced the inhibition and there was little inhibition when it was applied 10 min. after adsorption. The antiserum had no inhibitory effect when infection was initiated by virus RNA; the antiserum may act by preventing uncoating of the virus.

INTRODUCTION

Antibody to the host cells inhibits the growth of certain enteroviruses in tissue culture (Habel et al. 1958; Quersin-Thiry, 1958). In primary human amnion cells and in HEp-2 cells treated with antiserum plaque counts by echoviruses are markedly reduced although those of polioviruses and Coxsackie viruses of group B are unaffected (Timbury, 1962, 1969). In human amnion cells adsorption takes place normally but a later stage in virus replication is blocked. This report describes further investigations into the effect of antibody to host cells on the growth cycle of echovirus 1 which suggest that the antibody acts by preventing uncoating of the virus.

METHODS

Virus. The prototype strain of echovirus type 1, FAROUK, was used after triple plaque purification and propagation in HEp-2 cells. The virus stock had a titre of $2.75 \times 10^8$ p.f.u./ml.

Cells. The HEp-2 cells were kindly supplied by Dr Yvonne Cossart, Virus Reference Laboratory, Central Public Health Laboratory, London, N.W. 9, and were grown and maintained in Eagle’s medium with 10% calf serum.

Antiserum. This was prepared in rabbits by immunization with suspensions of HEp-2 cells emulsified in Freund’s complete adjuvant. It was inactivated at 56° for 30 min. before use. It had no visible cytotoxic effect when applied undiluted to HEp-2 cells but had a titre of $1/1024$ in complement-fixation tests with HEp-2 cells and a haemagglutinin titre of $1/2048$ with human group O erythrocytes. At a dilution of $1/32$, 0.3 ml. applied to monolayers of HEp-2 cells reduced the plaque counts of echovirus 1 in the cells by an average of 84% (Timbury, 1969).

Virus assay. The inhibitory effect of the antiserum was estimated by comparing single-step growth curves or yields of virus 6 hr after infection in antiserum-treated cells with those in cells treated with normal rabbit serum. Monolayers of HEp-2 cells
were grown in 60 mm. diameter Petri dishes and treated for 1 hr at 37° with 0·3 ml. of antiserum diluted 1/8 or normal rabbit serum at the same dilution. Excess antiserum was removed and each culture was infected with 0·2 ml. of virus at m.o.i. of 1 for 30 min. at 37° and then washed 3 times with phosphate buffered saline (Dulbecco & Vogt, 1954); after addition of 5 ml. of fresh medium incubation was continued for 6 hr. Samples were obtained by scraping the cells into the medium with a rubber policeman and mixing 1 ml. of the suspension with 1 ml. of 0·4% sodium dodecyl sulphate in distilled water for 10 min. at room temperature. Samples were diluted 1/100 in PBS and plated on 4 replicate monolayer cultures of HEp-2 cells after further dilution as required. Infective centres were estimated by adding 0·5 ml. of 0·25% trypsin in tris saline to cultures which had been washed after the period of virus adsorption. When the cells were detached they were suspended in medium and plated after suitable dilution.

Estimation of rate of virus adsorption. Virus diluted to contain from 5000 to 10,000 p.f.u./0·1 ml. was inoculated in 0·1 ml. amounts on to a series of monolayers of HEp-2 cells in Petri dishes which had been treated for 1 hr at 37° with anti-HEp-2 cell serum diluted 1/32 or normal rabbit serum at the same dilution. At intervals after inoculation unadsorbed virus was sampled by adding 2·5 ml. from a container with 9·9 ml. phosphate buffered saline to the appropriate plate; diluent mixed with unadsorbed virus was then removed to the container and plated. This 100-fold dilution was to dilute any residual antiserum beyond the highest titre at which inhibitory activity could be demonstrated.

Extraction and assay of virus RNA. Virus RNA was extracted by a modification of the method described by Alexander et al. (1958). Virus was propagated in monolayers of HEp-2 cells grown in rotating 80 oz bottles, and after low-speed centrifugation to remove coarse cell debris was concentrated by centrifugation at 30,000 rev./min. for 4 hr. The pellet was resuspended in 2 to 3 ml. phosphate buffered saline. In some experiments the virus was purified by isopycnic centrifugation in caesium chloride but usually was used as a crude concentrate. RNA was extracted by shaking the virus for 5 min. with an equal volume of water-saturated phenol at room temperature. After centrifugation at 3000 rev./min. for 5 min. the aqueous phase was removed and shaken with fresh phenol while the lower phenol phase was re-extracted with 1 ml. of solution A of Dulbecco & Vogt (1954). The aqueous phases were pooled and the phenol was removed by shaking for 1 to 2 min. with an equal volume of ether; this was repeated 4 times and then excess ether was sucked off and the remainder blown off by bubbling with 5% (v/v) carbon dioxide in nitrogen. The RNA preparations were plated on HEp-2 cells in solution A containing 300 µg./ml. DEAE dextran (Pharmacia, Sweden). To ensure that the extract did not contain intact virus 0·1 ml. of each RNA suspension was incubated with 0·1 ml. of pancreatic ribonuclease (20 µg./ml.) for 1 hr at 37°, and the mixture was plated after being diluted 1/10 in solution A containing 300 µg./ml. DEAE dextran.

RESULTS

Single-step growth cycle of echovirus 1 in antiserum-treated cells

Virus replication was inhibited in antibody-treated cells (Fig. 1). Virus was attached to the cells normally since the amount of virus associated with the antibody-treated cells was the same as that associated with untreated cells during the first 2 hr of the
growth cycle. In both treated and untreated cultures the amount of virus recoverable 2 hr after infection was less than that recoverable immediately after adsorption. After 2 hr, however, there was a striking difference in the two curves as the formation of new virus was both delayed and diminished in antiserum-treated cells. At the end of

![Graph showing single-step growth cycle of echovirus 1 in HEp-2 cells.](image)

**Fig. 1.** Single-step growth cycle of echovirus 1 in HEp-2 cells treated with antiserum to HEp-2 cells or normal rabbit serum. ○ --- ○, Cell-associated virus and □, infective centres in antiserum-treated cells; ● --- ●, cell-associated virus and ■, infective centres in untreated cultures.

**Table 1.** Effect of increasing multiplicity of infection on inhibition of yield of echovirus 1 by antiserum to HEp-2 cells

<table>
<thead>
<tr>
<th>Multiplicity of infection</th>
<th>Log. reduction in 6 hr yield of virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>0.5*</td>
</tr>
<tr>
<td>20</td>
<td>0.8*</td>
</tr>
<tr>
<td>10</td>
<td>1.4*</td>
</tr>
<tr>
<td>5</td>
<td>1.5*</td>
</tr>
<tr>
<td>1</td>
<td>1.4†</td>
</tr>
</tbody>
</table>

* Mean of results from three separate experiments.
† Mean of results from seven separate experiments run as controls in parallel with the experiments above.
the growth cycle the titre of virus formed in cultures treated with antiserum was 1.5 log. lower than that in untreated cultures. This was a true reduction in virus yield and not merely a delay in virus replication because estimates of virus yields 24 hr after infection showed the same degree of inhibition. The number of infective centres in antibody-treated cells was also reduced although to a lesser extent—in this instance by 1.2 log.—than the titre of virus produced by the treated cells. This shows that fewer cells were producing virus in antiserum-treated cultures and that those that became infected produced less virus than untreated cells. Because the formation of new virus reached a peak about 5 to 6 hr after infection, the reduction in yield of virus from cells 6 hr after virus adsorption was used as a measure of the effect of the antiserum on a single-step virus growth cycle. In 16 separate experiments with echovirus 1 the reduction in titre of virus at 6 hr from cells treated with antibody ranged from 0.9 log. to 2.2 log. with a mean reduction of 1.6 log.

![Graph](image)

**Fig. 2.** Rate of adsorption of echovirus 1 to HEp-2 cells treated with antiserum to HEp-2 cells or normal rabbit serum. O--O, % virus adsorbed to antiserum-treated cells; •--•, % virus adsorbed to untreated cells.

**The effect of increasing dose of virus on the inhibition**

The degree of inhibition was next investigated in cells infected at multiplicities of infection which ranged from 1 to 300. Although the inhibition of virus yield was unaffected in cells infected at input multiplicities of 5 p.f.u./cell and 10 p.f.u./cell, there was a reduction in the degree of inhibition when cells were infected with virus at a multiplicity of 20. Increase in the dose of virus to 300 p.f.u./cell caused a further reduction in the degree of inhibition although this was proportionately less than the considerable increase in the virus dose employed (Table 1).

**Application of antiserum at different times during the virus growth cycle**

In human amnion cells antibody to the host cells does not prevent adsorption of virus (Timbury, 1963), and in HEp-2 cells also the rates of virus adsorption were the same in antibody-treated as in untreated cells (Fig. 2). The single-step growth curve
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confirms this since the amount of virus recoverable from antibody-treated cells immediately after the adsorption period was the same as in untreated cultures (Fig. 1).

Further experiments were made in which antiserum was added to cells at different times after infection to determine the stage in the growth cycle which was blocked by the antibody. The antiserum was applied for 1 hr at 37° to cells which had previously been infected with virus at a multiplicity of 5 for 30 min. at 4°. Adsorption was permitted at 4° rather than 37° because the penetration and uncoating of enteroviruses do not take place at the lower temperature (Holland, 1962; Mandel, 1967). After virus adsorption the cells were washed three times with phosphate buffered saline and incubated in fresh medium before application of antiserum. The reductions in the yield of virus 6 hr after infection were estimated in replicate cultures which had been treated with antiserum at different times after infection (Table 2). The antiserum had little effect when it was added 10 min. after virus adsorption. Application of antiserum as soon as 5 min. after adsorption considerably reduced the inhibitory effect although application immediately after adsorption resulted in undiminished inhibition. The inhibition was thus due to an action of the antibody on an early stage in the virus growth cycle. Similar experiments in which the virus was adsorbed at 37° instead of 4° showed that the antiserum had much less effect when applied immediately after adsorption at this temperature—perhaps because some virus penetration or uncoating had already taken place during the period of adsorption at 37°.

Table 2. Inhibition of virus yield from cells treated with antiserum to HEp-2 cells applied at different times after virus adsorption

<table>
<thead>
<tr>
<th>Time of application of antiserum</th>
<th>Log. reduction in 6 hr yield of virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>After virus adsorption*</td>
<td></td>
</tr>
<tr>
<td>0 min.</td>
<td>2.2</td>
</tr>
<tr>
<td>5 min.</td>
<td>0.8</td>
</tr>
<tr>
<td>10 min.</td>
<td>0.3</td>
</tr>
<tr>
<td>15 min.</td>
<td>0.2</td>
</tr>
<tr>
<td>20 min.</td>
<td>0.2</td>
</tr>
<tr>
<td>30 min.</td>
<td>0.1</td>
</tr>
<tr>
<td>1 hr</td>
<td>0</td>
</tr>
<tr>
<td>Before viral adsorption*</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Virus was adsorbed at a multiplicity of 5 p.f.u./cell for 30 min. at 4°.

The effect of the antiserum on cells infected with virus RNA

Experiments were next made to investigate the effect of the antiserum on virus uncoating by testing for inhibition in monolayers of cells infected with virus RNA. If the antiserum were to inhibit the replication of uncoated virus or virus RNA this would be evidence that the antibody blocked a stage in the growth cycle after the uncoating had taken place. With intact echovirus 1 the average reduction in plaque counts on antiserum-treated monolayers was 84% (Timbury, 1969). Antiserum inhibited plaque formation by virus RNA much less than it inhibited plaque formation by intact virus; the reduction was only 36% (Table 3). Since a plaque assay is a multi-step growth experiment, further studies were undertaken to investigate the action of the antiserum on single-step growth cycles initiated by RNA. The RNA was prepared by extracting concentrated suspensions of virus with cold phenol. It was inoculated
Table 3. *Inhibition of plaque counts by antiserum to HEp-2 cells in cells infected with echovirus 1 RNA*

<table>
<thead>
<tr>
<th>Mean plaque count in cultures with normal rabbit serum diluted 1/32</th>
<th>Mean plaque count in cultures with antiserum to HEp-2 cells diluted 1/32</th>
<th>Reduction in plaque counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>72.25</td>
<td>46.25</td>
<td>36%*</td>
</tr>
</tbody>
</table>

* P = 0.05.

![Graph showing cell-associated virus in HEp-2 cells treated with antiserum to HEp-2 cells or normal rabbit serum after infection with echovirus 1 RNA.](image)

Table 4. *Effect of antiserum to HEp-2 cells on virus yield from cells infected with echovirus 1 RNA or intact virus*

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Log. reduction in 6 hr yield of virus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>0.1</td>
</tr>
<tr>
<td>Intact virus</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Mean of results from three separate experiments.

to HEp-2 cell monolayers in solution A containing 300 μg./ml. of DEAE dextran; dilution of intact virus in DEAE dextran at this concentration did not affect the inhibitory action of the antiserum. The monolayers were treated with antiserum in the
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usual way but were washed once with phosphate-buffered saline before inoculation of the RNA. The titres of the various suspensions of RNA varied from $2 \times 10^4$ p.f.u./ml to $4.25 \times 10^6$ p.f.u./ml, with a mean titre of $8.1 \times 10^5$ p.f.u./ml; these represented approximately 0.1% of the infectivity of the original virus preparations. The infectivity of a sample of each RNA preparation was destroyed by ribonuclease.

Judged by the 6 hr yields of virus in cells infected with RNA the antiserum had no inhibitory action on the course of infection when this was initiated with virus RNA, whereas there was marked inhibition in parallel cell cultures inoculated with intact virus (Table 4). Single-step growth experiments were made with cells which had been treated with antiserum to HEp-2 cells or normal rabbit serum and then infected with RNA (Fig. 3). No cell-associated infectivity could be detected until 3 hr after infection in both sets of cultures, but from 3 hr onwards the titres of virus recovered from the cells treated with antibody were the same as the titres of virus from untreated cells. The number of infective centres was the same in both antiserum-treated and untreated cultures. Thus the antiserum did not affect the course of virus replication in cells infected with RNA or uncoated virus; which suggests that the antiserum acts by preventing the normal virus uncoating process.

DISCUSSION

Antibody prepared against the host cells inhibits the growth of echovirus 1 in HEp-2 cells at an early stage in the virus growth cycle. Adsorption of the virus to monolayers of HEp-2 cells is not inhibited. Axler & Crowell (1968) have reported that antiserum to HeLa cells inhibited the attachment of various enteroviruses including echovirus 6 to HeLa cells. These conflicting results are probably due to the use by Axler and Crowell of suspended cells rather than cell monolayers for the estimation of virus attachment to the cells. The inhibition of attachment which they found may have been due to the intense clumping or agglutination which results when cells in suspension are treated with antibody prepared in rabbits (Goldberg & Green, 1959; Dumonde et al. 1961; M. C. Timbury, unpublished results) and not, as they concluded, to the covering by antibody of virus receptors on the cell surface. The strong inhibition observed in the single-step growth experiments in HEp-2 cells when antiserum was applied immediately after virus adsorption is further evidence that antibody to host cells inhibits a stage in the growth cycle after adsorption has taken place.

The lack of inhibition when infection is initiated by virus RNA instead of intact virus suggests that the antiserum acts by preventing virus uncoating. The alternative explanation is that penetration and not uncoating is affected by the antiserum. Virus RNA can infect cells which lack receptors for intact virus (Holland, McLaren & Syverton, 1959) and it is therefore possible that RNA not only adsorbs to but also penetrates the cell by a mechanism different from that of the intact virus. In human amnion cells the amount of virus recoverable from antiserum-treated cells decreased in the first hour after infection and this was interpreted as evidence that the virus underwent uncoating or eclipse in antibody-treated cells (Timbury, 1963). The same reduction in recoverable virus during the first 2 hr after adsorption is also seen in HEp-2 cells treated with antibody, but since either penetration or uncoating is inhibited in these cells, the reduction in infectivity cannot be due to viral uncoating. The loss of infectivity in antibody-treated cells shortly after adsorption could, however, be explained if it
were due to virus engulfment by the cell rather than uncoating—in which case penetration would take place normally with the antiserum blocking the subsequent uncoating process. Virus penetration *per se* is not usually regarded as being associated with loss of infectivity but it is possible that the engulfment of the virus by cellular membranes hinders its subsequent release as an infectious unit.

It is not in fact certain that enteroviruses uncoat intracellularly rather than at the cell surface. Although it has been suggested that the uncoating of poliovirus might take place at the cell membrane (Cooper, 1962; Fenwick & Cooper, 1962; Holland, 1962), Mandel (1967) has recently presented convincing evidence that this is an intracellular process. Philipson & Lind (1964) have shown that echovirus 7 may uncoat as a result of combination with cell surface receptors but intracellular mechanisms for virus uncoating clearly exist since poliovirus can replicate in cells which lack virus receptors if entry is accomplished by cell fusion (Enders, Holloway & Grogan, 1967). The balance of opinion at present is probably that virus enters cells by engulfment after attachment to the cell membrane and is uncoated within the cell.

The inhibitory effect of the antiserum is almost certainly a result of a reaction with antigens on the cell surface. In the absence of complement, antacellular antibody does not penetrate into the cells but appears to cause agglutination of adjacent portions of the cell membrane (Easton, Goldberg & Green, 1962; Carey, Kuhn & Harford, 1965). Perhaps, in tissue culture, this effect prevents the uncoating of engulfed virus particles. However, although antibody has a marked effect on the cell membrane, its effects are not restricted to the cell surface but involve cytoplasmic structures such as the cell lysosomes also (Dumonde et al. 1961; Dorling & Loewi, 1965). Antibody in the absence of complement causes a marked activation of lysosomal acid phosphatase but does not impair the viability of the cells (Dumonde et al. 1965). It has been suggested that lysosomes play a part in uncoating virus (Allison & Sandelin, 1963; Mallucci & Allison, 1965; Mallucci, 1966) and the effect of antiserum to host cells on virus replication may be a result of its action on cell lysosomes.

Finally, the difference in susceptibility to the effects of antacellular serum of echoviruses and Coxsackie virus A9 on the one hand, and polioviruses and group B Coxsackie viruses on the other, suggests that there may be some fundamental difference in the process of uncoating between enteroviruses which are not affected by the antiserum and those which are strongly inhibited by it.

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REFERENCES


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