Ultrastructural and Immunofluorescence Studies of Early Events in Adenovirus–HeLa Cell Interactions

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SUMMARY

Scanning electron microscopy and immunofluorescence techniques show that the attachment of adenovirus type 5 to HeLa cells is followed by a temperature-dependent redistribution of virus particles on the cell surface. Metabolic inhibitors and cytochalasin B, a drug that impairs microfilament function, blocked this redistribution. Transmission electron microscopy studies demonstrated that inhibition of redistribution was paralleled by an inhibition of virus internalization. In further experiments virus and cells were incubated at 4 °C and then treated with adenovirus ‘soluble’ antigens to block unoccupied virus receptors. On warming these preparations to 37 °C, the internalization of attached virions was found to be impaired. It is proposed that energy-dependent redistribution of attached adenovirus particles on the cell surface facilitates entry by bringing virions into contact with sufficient receptors to allow internalization.

INTRODUCTION

Viruses utilize a number of different mechanisms to enter the host cell (for review, see Dales, 1973). However, attachment of the virus particle to the appropriate receptor molecules in the host cell plasma membrane is considered to be the initial step in all virus infections. Recent results from several viruses suggest that, after attachment, virus particles may be redistributed over the cell surface (Gschwender & Traub, 1979; Helenius et al., 1980; Patterson & Macnaughton, 1981; Alstiel & Landsberger, 1981) by a mechanism that is temperature- and energy-dependent (Patterson & Macnaughton, 1981). Migration on the cell surface may or may not be an important prerequisite for virus internalization. After attachment, Semliki Forest virus (SFV) migrates to coated pits and is subsequently internalized into coated vesicles (Helenius et al., 1980). Uptake of SFV is inhibited by blocking oxidative phosphorylation (Marsh & Helenius, 1980). Entry of influenza virus, on the other hand, is not prevented by metabolic inhibitors (Patterson et al., 1979), although there is evidence of an energy-dependent redistribution of virus particles on the cell surface (S. Patterson & J. S. Oxford, unpublished data). The factors determining whether uptake is energy-dependent or -independent are not clear. It may be determined by a requirement for specific entry sites on the cell surface and/or the nature and density of the virus receptor molecules in the host cell plasma membrane. Influenza virus appears to bind to sialic acid residues and thus a large heterogeneous array of glycoproteins and glycolipids may be expected to serve as receptors. Other viruses such as Epstein–Barr virus (Yefenof et al., 1976), adenoviruses (Hughes & Mautner, 1973; Svensson et al., 1981) and picornaviruses (Crowell, 1976) appear to have more specific and probably less numerous receptors. Thus, it may be necessary for these viruses to migrate across the cell surface to contact sufficient receptor molecules to enable them to become internalized.

For adenovirus, where the protruding fibre capsomeres seem to play a key role in the initial adsorption step, it has been estimated that there are $10^5$ to $10^6$ fibre receptors on a single HeLa cell, allowing a maximum of about $10^4$ virus particles to bind (Philipson et al., 1968). The
receptor has also been isolated and shown to be a glycoprotein with a mol. wt. of approximately 42000 (Hughes & Mautner, 1973; Svensson et al., 1981). Thus the HeLa cell–adenovirus system provides a suitable model for investigating the early interactions between a virus with highly specific receptor requirements and the cell surface. Scanning (SEM), thin section transmission electron microscopy (TEM) and fluorescence microscopy have been employed to study this system. Furthermore, metabolic inhibitors and cytochalasin B, a drug that impairs microfilament function, have been used to examine the relationship between redistribution on the cell surface and internalization.

**METHODS**

**Virus and cells.** Adenovirus type 5 (ad 75) was propagated in KB cells growing in suspension in Joklik’s minimal essential medium (MEM). Virus was extracted from infected cells by homogenization in fluorocarbon and partially purified by velocity gradient centrifugation in CsCl as described previously (Winters & Russell, 1971). The virus band obtained from the gradient was dialysed against H21 medium (Gibco) containing 5% calf serum, and stored with 10% glycerol at -70°C. Titres of such virus seeds were in the range 2 × 10^9 to 5 × 10^9 p.f.u./ml with a particle to p.f.u. ratio between 50 and 500:1. HeLa cells were cultured in Eagle’s MEM containing 4% tryptose phosphate broth, 10% newborn calf serum, 100 units/ml penicillin and 100 µg/ml streptomycin. They were infected either when fully confluent or at 50% confluence with virus at approximately 100 p.f.u./cell.

**Anti-hexon serum.** This was obtained by immunization of rabbits with purified crystalline hexon (Pereira et al., 1968; Mautner & Wilcox, 1974) from adenovirus type 5.

**"Soluble" antigens.** These were prepared from adenovirus-infected KB cells after fluorocarbon extraction and CsCl density gradient centrifugation (Russell et al., 1967). The material above the opalescent virus band containing the ‘soluble’ antigens was dialysed against phosphate-buffered saline (PBS) before use. Polypeptide analysis by SDS–polyacrylamide gel electrophoresis of this preparation indicated that about 80% of the total protein was derived from adenovirus capsid polypeptides; about 70% of this was hexon, 20% fibre and 10% penton base polypeptides.

**Experimental procedures**

**Thin section TEM.** For thin section electron microscopy, cells were grown on 3 cm Petri dishes. Prior to inoculation with virus, when appropriate, HeLa cells were incubated for 20 min at 37°C in 2 ml of L15 medium containing a combination of sodium azide (BDH) and 2-deoxy-D-glucose (Sigma) at 10^{-3} M and 5 × 10^{-2} M respectively or cytochalasin B (IC1) at 10 µg/ml. Control cells, not inhibitor-treated, were included in each set of experiments. The cells were cooled on ice after incubation, excess medium was removed and 50 µl of concentrated virus was added. After incubation for 1 h on ice, with occasional shaking, warm (37°C) medium containing the appropriate inhibitor was added and the cells incubated for 20 min at 37°C. They were subsequently fixed for 1 h at 20°C with 3% glutaraldehyde in 0-1 M-cacodylate buffer pH 7.2 containing 5% (w/v) sucrose. They were then processed for thin section TEM as previously described (Patterson & Bingham, 1976).

**"Soluble" antigens.** In experiments employing the adenovirus ‘soluble’ antigens, confluent cell monolayers were first inoculated with virus or ‘soluble’ antigen (100 µl) and kept for 1 h on ice. After washing three times with cold medium, cells that had been inoculated with virus were treated with 100 µl of ‘soluble’ antigens and the control, inoculated with antigen, was similarly inoculated with virus for 1 h on ice. They were then overlaid with warm medium and incubated at 37°C for 20 min before glutaraldehyde fixation. Control cells were inoculated with virus for 1 h on ice, washed three times with cold medium, and incubated for a further 1 h on ice before being warmed to 37°C.

**SEM.** Cells for SEM experiments were grown on 1 cm diam. coverslips and treated with inhibitors as described above. After incubation with virus for 1 h on ice, the cells were washed five times in cold medium (containing the appropriate inhibitor) in order to remove unattached virus and then incubated for 20 or 40 min at 37°C. Some cells which were not treated with inhibitor were incubated on ice after washing with ice-cold medium. The cells were then fixed for 24 h at 4°C in 3% glutaraldehyde buffered with 0-1 M-cacodylate buffer pH 7-2 containing 5% (w/v) sucrose. They were processed for SEM as previously described (Patterson & Macnaughton, 1982).

**Immunofluorescence.** For immunofluorescence experiments, virus was incubated for 1 h on ice with confluent cells grown on glass coverslips. The coverslips were then washed five times with cold medium and subsequently overlaid with warm medium and incubated for 20 or 40 min at 37°C. In some experiments, virus-treated cells were incubated for 20 min on ice after washing. Cells were fixed for 30 min at 20°C with 1% paraformaldehyde in 0-1 M-cacodylate buffer pH 7-2. After three washes, each of 5 min, in 0-1 M-cacodylate buffer (pH 7-2, including 0.1 M-lysine to block any free aldehyde groups in the second wash), the fixed cells were incubated for 20 min at 20°C with rabbit anti-hexon serum diluted 1:40 in PBS. They were subsequently washed four times with PBS and incubated
for 20 min at 20 °C with fluorescein-conjugated goat anti-rabbit immunoglobulin (Miles Laboratories, Stoke Poges, U.K.) diluted 1:10 in PBS. Labelled preparations were washed four times before examination.

**Quantification.** In the TEM inhibitor experiments, the number of attached and internalized virus particles were counted and the results are expressed as the percentage of internalized particles. The proportion of these lying free in the cytoplasm or in vacuoles was also noted. Between 400 and 500 particles were counted in each sample. In experiments employing 'soluble' antigen, sections through approximately 100 cells were examined and the number of attached and internalized particles noted. Approximately 50 cells from each sample were closely examined by TEM. For a quantitative assessment of possible changes in the surface distribution of virus particles, a series of SEM micrographs were taken. Each series started at a lamella edge and extended 12 μm across the cell surface in the direction of the nucleus. Transects, 4 μm wide, were drawn across each series of micrographs and divided into 12 × 1 μm steps. The number of particles in each 4 μm² area was counted and the results from five randomly selected cells from each experiment were pooled.

**Nomenclature.** The terminology proposed by Ginsberg *et al.* (1966) for the major capsid components (hexon, penton, fibre) is used.

**RESULTS**

**Scanning electron microscopy**

Adenoviruses were readily recognized as particles approximately 100 nm in diameter on the cell surface (Fig. 1b). Similar particles were not observed on the surface of non-infected cells (Fig. 1a). There was considerable variation in the total number of virus particles adsorbed to different cells. However, the distribution of these particles across the surface of different cells subjected to the same experimental treatment was consistent.

The distribution of virus particles that attached in the cold was not random. Thus, the density of particles at the lamella edge was greater than over the rest of the cell surface (Fig. 1b and 2a). On warming the cells in the absence of inhibitors, there was a redistribution of virus particles. The density of particles up to 2 μm from the edge of lamellae of cells incubated for 20 min at 37 °C was less than over the rest of the cell surface (Fig. 1c and 2b). Extending the warm-incubation period up to 40 min resulted in a marked loss of particles up to 5 μm from the edge of lamellae (Fig. 2c). The extent of redistribution was similar on confluent and semi-confluent cell cultures. In some experiments the increased concentration of virus at the lamella edge after cold incubation was less than in the experiment illustrated in Fig. 2(a); however, warming to 37 °C always resulted in a similar striking redistribution of virus particles. Treatment of cells with a combination of sodium azide and 2-deoxy-D-glucose inhibited virus redistribution at 37 °C (Fig. 2d). As has been shown in other SEM studies (Miranda *et al.*, 1974), cytochalasin B was found to cause HeLa cells to contract, lose microvilli and develop blunt, knobby projections. Such cells were unable to mediate redistribution of virus particles across their surface (Fig. 2e). There was an apparent loss of particles on cells treated with cytochalasin B; the reason for this is not clear.

**Immunofluorescent staining**

Cells that were inoculated with virus in the cold, fixed and labelled with a rabbit anti-hexon antibody followed by a fluorescein-conjugated anti-rabbit immunoglobulin reagent showed particulate staining over their entire surface. The intensity of staining over the cell surface was relatively even, except at the cell boundaries where more intense staining was observed (Fig. 3a). On incubating preparations for 20 min at 37 °C, the staining pattern changed such that there was reduced staining over the peripheral areas of the cell (Fig. 3b). The effect of warming for 40 min was more pronounced: the cells showed discrete central caps of immunofluorescence overlying their nuclei, whilst the peripheral cellular regions had become devoid of stain (Fig. 3c).

**Thin section electron microscopy**

Thin sections of cells incubated with virus in the cold showed attached, but not internalized, virus particles on examination by electron microscopy. After they had been warmed, virus particles in cytoplasmic vacuoles and free in the cytoplasm were observed (Fig. 4d, e, f). Uptake was observed at coated pits and at uncoated sites on the plasma membrane (Fig. 4a, b, c).
Between 5 and 15% of vacuoles containing virus were coated vesicles (Fig. 4d) and particles lying free in the cytoplasm accounted for 30 to 60% of the internalized particles in all experiments. Similar results were obtained with confluent and semi-confluent cells. Although both inhibitor treatments reduced the percentage of internalized virions, the most marked inhibition of uptake occurred in cells treated with sodium azide and 2-deoxy-D-glucose. In these cells the percentage of internalized particles was reduced to 1 or 2% of the total number of cell-associated particles, compared with 39 to 64% in controls and 10% in cytochalasin B-treated cells.
Fig. 2. Histograms showing the distribution of adenovirus particles on the cell surface after: (a) inoculation with virus on ice, washing and then incubating for 20 min on ice; (b) inoculation with virus on ice, then washing followed by incubation at 37 °C for 20 min; (c) inoculation with virus on ice, then washing followed by incubation for 40 min at 37 °C; (d) pretreating cells with sodium azide and 2-deoxy-D-glucose, inoculation with virus on ice, washing and then incubating for 20 min at 37 °C in the presence of the inhibitors; (e) pretreating cells with cytochalasin B, inoculation with virus on ice, washing and then incubating for 20 min at 37 °C in the presence of the inhibitor.

Table 1. Analysis of TEM observations on the effect of 'soluble' antigen on adenovirus binding and uptake*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell-associated virions per cell profile/no. of cells examined</th>
<th>Internalization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt. 1</td>
<td>Expt. 2</td>
</tr>
<tr>
<td>Control</td>
<td>3.23</td>
<td>7.51</td>
</tr>
<tr>
<td>Virus followed by 'soluble' antigen</td>
<td>0.84</td>
<td>2.65</td>
</tr>
<tr>
<td>'Soluble' antigen followed by virus</td>
<td>0.87</td>
<td>0.63</td>
</tr>
</tbody>
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* The results of two series of experiments employing different virus preparations are shown. For each experiment, data were obtained from thin sections through approximately 100 cell profiles.
The results of experiments employing 'soluble' antigens containing fibre protein to block plasma membrane virus receptors are given in Table 1. Quantitative data were derived by examining approximately 100 cells and, as expected from the results of other similar investigations (Philipson et al., 1968), showed that treatment of cells with fibre protein reduced the subsequent binding of virions. Obviously, this kind of analysis can only give an indication of binding inhibition since the length of membrane profile and the area of cell cytoplasm examined will not be exactly the same for each experiment. Analysis of uptake by counting the ratio of attached to internalized particles is not subject to this criticism. Such analyses showed that blocking unoccupied receptor sites with 'soluble' antigens, after adsorption of virus in the cold, impaired the temperature-dependent internalization process.

**DISCUSSION**

Lectin- and antibody-mediated redistribution of plasma membrane receptors into patches and caps is well documented (for reviews, see Nicolson, 1974; Singer, 1974). The phenomenon was shown to require a ligand that was at least divalent, to be temperature- and energy-dependent, and to be inhibited by drugs which impaired the functioning of the cytoskeletal system. From
Fig. 4. Micrographs from TEM experiments showing cells which had been inoculated with virus and then incubated for 20 min at 37 °C. (a, b) Virus uptake at uncoated sites on the plasma membrane. (c) Virus uptake at a coated pit. (d) Virus in a coated vesicle. (e) Virus in a cytoplasmic vacuole. (f) Virus lying free in the cytoplasm adjacent to a nuclear pore. All bar markers represent 100 nm.
the present experiments, which provide evidence for a temperature- and energy-dependent redistribution of adenovirus particles on the cell surface, it seems reasonable to postulate that a virus particle may be regarded as a multivalent ligand able to cross-link membrane receptors and activate the cytoskeletal system to mediate redistribution. The immunofluorescence studies indicated that redistribution results in an accumulation of virus particles on the plasma membrane overlying the nucleus. A biological advantage may thus be conferred on these particles, since their route through the cytoplasm to the nucleus would be shortened.

SEM and immunofluorescence studies of adenovirus particles attached to HeLa cells in the cold showed an increased concentration of virions at the edge of cellular lamellae. The reason for this distribution is not clear. It may indicate an increased number of binding sites; however, further evidence is required before such an interpretation can be confidently proposed.

Philipson et al. (1976) have proposed that, after attachment, a temperature-dependent lateral diffusion of receptors is required for internalization. The need for contact with further receptors is supported by our experiments with 'soluble' antigen. However, the inhibitor studies, which demonstrated a correlation between cell surface redistribution and uptake of virus suggest that a temperature-dependent diffusion of receptors is insufficient to enable internalization to proceed efficiently and that an active process is required. Differences in the energetics of influenza virus and adenovirus internalization may reflect the number of available receptor molecules in the plasma membrane.

Two modes of entry into cells have been described for adenovirus: uptake into cytoplasmic vacuoles followed by penetration of the vacuolar membrane (Chardonnet & Dales, 1970) and direct penetration of the plasma membrane (Morgan et al., 1969; Brown & Burlingham, 1973). Our own data and those of Chardonnet & Dales (1970) suggest that entry by the former route may occur at coated or uncoated sites on the plasma membrane. By TEM, the majority (85 to 95%) of virus-containing vacuoles appear to be uncoated. However, this may underestimate the proportion of particles entering via coated pits since the clathrin coat may dissociate from the vacuolar membrane soon after internalization (Pearse & Bretcher, 1981). Inhibition of virus redistribution on the cell surface was paralleled by a marked reduction in the number of cytoplasmic and vacuolar particles; this suggests that movement on the cell surface may play a role in both entry routes.

Simultaneous inhibition of oxidative phosphorylation and glycolysis reduces the uptake of adenovirus and SFV (Marsh & Helenius, 1980) more than does treatment with cytochalasin B. This may reflect the blocking of cytoskeleton-independent movement of virus particles, or alternatively the movement of virus receptor molecules. In this context it is interesting to note that Braun et al. (1978) have reported that the capping of Thy.1 and H2 antigens on mouse lymphocytes is independent of microfilaments.

Electron microscopy has been employed by many investigators to trace the route of virus entry into the cell. Although it is not unreasonable to extrapolate from electron microscopical findings and propose an infectious route into the cell, it should be remembered that in most studies, including the present, the majority of particles observed are non-infectious. Bearing this in mind, our electron microscopical findings have provided evidence for an energy-dependent redistribution, probably mediated by the cytoskeletal system, of adenovirus particles on the surface of HeLa cells. Blocking of redistribution was paralleled by an inhibition of virus uptake, suggesting that movement of adenovirus particles on the cell surface is an important step in the internalization process.

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REFERENCES


Adenovirus–HeLa cell interactions


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