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Penetration of Host Cell Membranes by Adenovirus 2

DENNIS T. BROWN[1] AND BYRON T. BURLINGHAM[2]

Department of Cell Biology and Pharmacology, University of Maryland School of Medicine, Baltimore, Maryland 21201

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Highly purified human adenovirus type 2 directly penetrated the plasma membranes of KB cells. The process of membrane penetration resulted in the appearance of large numbers of adenovirions free in the cytoplasm of the infected cells. The virions underwent a morphological change as they penetrated the cell surface. Penetration of the plasma membranes and the accompanying alteration in virion morphology was dependent on a function associated with the intact cells, because neither event occurred when purified virions were added to isolated cell membranes. Inactivation of the adenovirions with heat or antibodies before inoculation of the cells reduced the infectivity of the virus population and prevented the appearance of virions free in the cytoplasm. The inactivation of the virions did not significantly reduce the number of virus particles which were found in cell vacuoles and pinocytotic vesicles.

The events which occur during penetration of host cells by adenoviruses have been studied by physical and morphological techniques (8, 9, 10, 14, 17). Early ultrastructural studies using thin section techniques suggested that adenovirions enter the host cell by phagocytosis (10). However, a fraction of the virions was detected free in the cytoplasm, suggesting that some of the adenovirions may have penetrated directly through the plasma membrane. A more recent study of ultrathin sections by Morgan et al. (17) has provided additional evidence that adenovirions directly penetrate the host cell membrane. Morgan et al. concluded that penetration of the plasma membrane is initiated by the attachment of the virion to the cell membrane by the penton fiber. The attached virion was then drawn closer to the surface until in some instances the capsid appeared to touch the cell. Virions could be seen free in the cytoplasm of the cell within 10 min after infection. Morgan et al. (17) suggested that the virions which penetrated the plasma membrane lost their distinctive icosahedral structure and appeared rounded. These investigators suggested that this configurational change may have resulted from a loss of the pentons from the vertexes of the virion. Dunnebecke et al. (12) have presented morphological data which suggests that polioviriions also directly penetrate the host cell membranes. Lonberg-Holm and Philipson (14) have studied the early events in adenovirus penetration by using physical techniques. Their results show that adenovirions are rapidly converted to a DNase-sensitive structure within the cell. The virions recovered from the cell extract had an increased buoyant density, which could be explained if the virions had lost a small portion of capsid protein.

In the ultrathin section studies of Dunnebecke et al. (12) and Morgan et al. (17), the penetrating virions were seen in positions close to the cell membrane outside the cell and free in the cytoplasm. However, these investigators did not show virions halfway through the cell membrane, a position essential to the hypothesis of direct membrane penetration by viruses. The inability to detect the penetrating virions may be a sampling problem inherent to the thin section technique. After the adsorption of the viral capsid to the plasma membrane, the process of membrane penetration may occur very rapidly; therefore, only an extremely small portion of the virions would be in a state of partial penetration, and the probability of detecting this fraction in ultrathin sections is very low. In addition, the interruption in the continuity of the plasma membrane by the penetrating virion may weaken the membrane so that it does not withstand the stresses imparted to it

[1] Present address: The Institut für Genetik der Universität zu Köln, 5-Köln 41, West Germany.

[2] Present address: Division of Biology, Kansas State University, Manhattan, Kan. 66506.

by dehydration and embedding. Areas of the plasma membrane where direct penetration is taking place may therefore be poorly preserved in thin sections.

The freeze-etching technique provides a method for examining large areas of cell surfaces. Furthermore, it has been demonstrated that membranes of frozen cells are cleaved between the inner and outer leaflets so that replicas reveal the interior morphology of the membrane (18). The preparative procedures utilized in freeze-etching do not subject the specimen to chemical dehydration or to the stresses of polymerization during embedding. We have used freeze-etching in addition to ultrathin sectioning to study the penetration of cell membranes by human adenoviruses.

MATERIALS AND METHODS

Materials. Thymidine-6-H^3 (>9.0 Ci/mmol) was purchased from New England Nuclear Corp.

Phosphate-buffered saline (PBS) was prepared as described by Dulbecco and Vogt (11).

Reticulocyte standard buffer is 0.01 M Tris, pH 7.4, 0.01 M KCl, and 0.0015 M $MgCl_2$.

Cells and virus. Human adenovirus type 2 (Ad 2) was obtained from the American Type Culture Collection and propagated in KB cells maintained in suspension culture as described by Burlingham and Doerfler (4, 6). All adenovirus preparations used in the described experiments were purified by chromatography on a 4% agarose column followed by three cycles of equilibrium centrifugation in CsCl density gradients and dialysis against PBS as described by Burlingham and Doefler (4, 6). An electron micrograph of virus purified by this procedure is shown in Fig. 1. Some virus preparations were isotopically labeled with thymidine-6-H^3 as previously described (4). The infectivity of the adenovirus preparations was determined by plaque assay on primary human embryonic kidney cells (5).

Preparation of cells infected with adenovirus. Monolayers of KB cells were grown in petri plates (60 mm diameter) with Eagle medium (13) supplemented with 5% calf serum. The growth medium was aspirated from the plates and the cells were washed twice with PBS warmed to 37 C. The cells were inoculated with a multiplicity of 5,000 to 10,000 plaque-forming adenovirions per cell. After incuba-

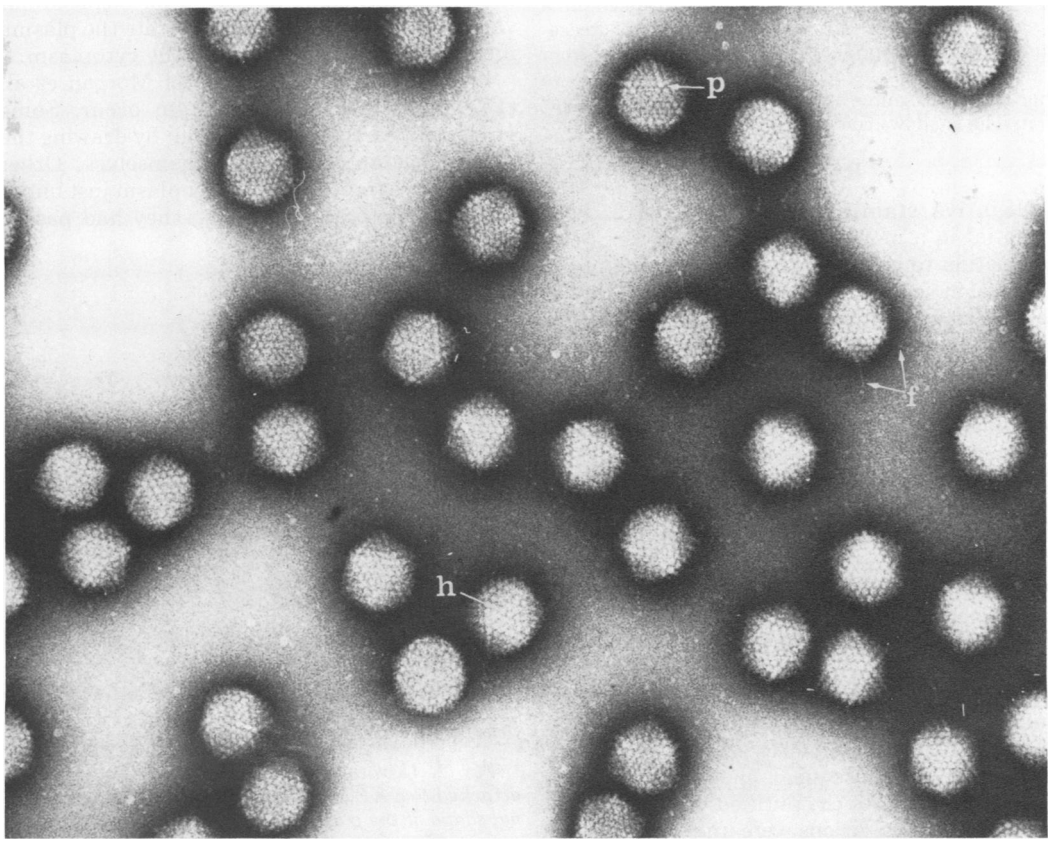

FIG. 1. *Purified virions of Ad 2 negatively stained with phosphotungstic acid. H, hexon; p, penton; f, fiber. Magnification ×120,000.*

tion (generally at 37 C), the petri plates containing the cells were transferred to an ice bath and 2.0 ml of ice-cold 2% glutaraldehyde in PBS was added to each dish. After fixation on ice for 60 min the cells were scraped from the plates and stored at 4 C until prepared for electron microscopy.

Preparation of KB cell membranes. Large quantities of KB cell membranes were prepared as described by Caliguiri and Tamm (7), with 4×10^6 KB cells per preparation. The membrane fractions were examined by electron microscopy.

Electron microscopy. Negative staining was carried out with the procedures described by Anderson (1). Phosphotungstic acid was prepared as a 2% solution and was adjusted to pH 7.2 with NaOH.

Cells fixed in glutaraldehyde as described above were resuspended in the phosphate buffer described by Millonig (16). Postfixation was carried out for 1 h with 1% solution of osmium tetroxide in Millonig phosphate buffer. The fixed cells were embedded in Epon 812 by the procedure described by Luft (15). Ultrathin sections were cut on a Reichart OM U-2 ultra microtome by using a diamond knife (Dupont).

Freeze-etching of virus-infected cells was carried out by the procedure described by Brown et al. (3). Specimens for deep-etching were washed in distilled water before freezing (21).

Specimens were studied and photographed in a Siemens Elmiscope 101. The micrographs of freeze-etched specimens presented here were produced from contact copies of the original photographic plates so that the metal source appears as a source of illumination. The shadows containing no metal are dark.

RESULTS

Negative staining of purified Ad 2. It is essential in a morphological study of virus-cell interaction to establish that the infecting virus suspension is homogeneous and pure. This is particularly important in studies involving morphologically complex virions such as the adenoviruses. The presence of dissociated viral subunits and partially disrupted particles in the inoculum could distort studies of the early events in infection. Free pentons and fibers in the inoculum may compete with intact particles for virus receptor sites on the cell surface. Partially disrupted particles with no organelle of attachment could be expected to appear primarily in pinocytotic vesicles and vacuoles. Such contaminations or degradations of the infecting virions could not be detected in ultrathin sections or in freeze-etched preparations. Therefore, we used the technique of negative staining to establish the morphological integrity and purity of our adenovirus suspensions before using them in subsequent experiments. Electron micrographs of the purified virions (Fig. 1) revealed that the virions were uniformly intact, with easily distinguishable subunit morphology of hexons, pentons, and fibers as described by Valentine and Pereira (22).

Penetration of cell membranes by adenovirions. The penetration of KB cells by Ad 2 was observed in samples of cells fixed at 10 and 20 min after infection. Specimens fixed at both times revealed virions attached to the cell surface and free in the cell cytoplasm. Many more cytoplasmic virions were detected in the preparations fixed at 20 min after infection.

The adenovirions adsorbed to the KB cell membrane were generally separated from the plasma membrane by a uniform distance(Fig. 2). The thin-sectioned virion shows a distinctly hexagonal shape (Fig. 2, large arrows). Capsid subunits are seen at the outer edge, which surround an electron-dense core approximately 50 nm in diameter. The distance between the edge of the virion and the outer leaflet of the membrane bilayer is roughly equal to the length of the penton-associated fiber (Fig. 1). Therefore, it is presumed that the virion is attached to the membrane by this structure.

After the initial attachment to the cell surface, the virion appears to have two routes of entry into the cell (17). It may be engulfed by the cell and thereby appear in an intracellular vacuole, or it may directly penetrate the plasma membrane and appear free in the cytoplasm.

In our studies, as in those of Morgan et al. (17), both processes seemed to occur. Some virions appeared to enter the cell by drawing the plasma membrane around themselves. Other virions were free in the cell cytoplasm just under the cell membrane as though they had passed

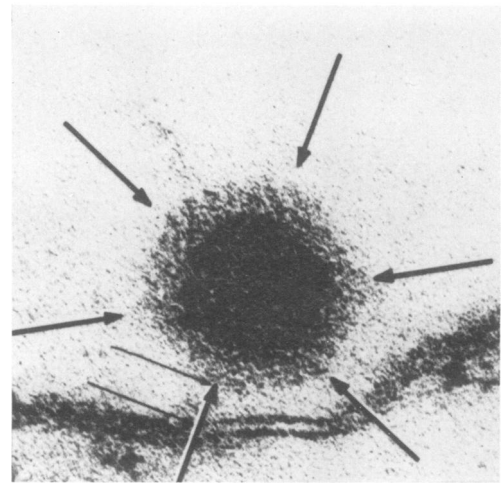

FIG. 2. *Ultrathin section of a type 2 adenovirion attached to a KB cell plasma membrane. The hexagonal shape of the particle is outlined by the large black arrows. The distance between the vertex of the virion and the cell membrane is delineated by small black arrows. The edges of this virion show a subunit structure. Magnification* ×400,000.

directly through it (Fig. 6). Attempts to unequivocally visualize virions in the process of directly penetrating cell membranes (partly inside and partly outside) with thin section techniques were unsuccessful. Although virions could sometimes be found in a position partly inside and partly outside the cell, the classical appearance of the membrane bilayer was not preserved. Therefore, it was impossible to determine whether the virion occupied this position fortuitously as a result of an accidental breakage of the plasma membrane during preparative handling or if the penetrating virion had altered the continuity of the membrane.

The penetration of the host cell membrane by the adenovirion subsequent to adsorption by the penton fiber was studied in replicas of freeze-etched KB cells infected with Ad 2. Figure 3 shows an area of the outermost surface of a KB cell which was fixed at 10 min after infection and then cleaved and deep-etched as described in Materials and Methods. Adenovirions were found on the surface of these cells and apparently were embedded to varying depths in the plasma membrane. Virions attached to the cell surface by penton fibers (Fig. 2) were not seen on deep-etched cell surfaces. It is likely that these particles were removed from the cell surface during the etching or sublimation process. The virions which were embedded in the outer surface of the cell (Fig. 3) were possibly so tightly bound to the cell surface that they were not removed by etching.

One of the particles (Fig. 3a) is the most superficial. This virion has the regular shape seen in negatively stained preparations of adenovirions (Fig. 1) and casts an angular shadow expected of an icosahedron. A second particle (Fig. 3b) appears to be more deeply embedded in the cell surface. The subunits on this particle are more distinct, and the organization of the subunits suggests that the virion is viewed on its fivefold axis of symmetry. The shadow cast by this particle, although shorter than the first particle (3a), is also angular in appearance. A third and a fourth virion (3c) cast short angular shadows and seem deeply embedded in the cell surface.

Two explanations may account for the buried appearance of the penetrating adenovirions. (i) The virions have broken the continuity of the membrane bilayer and are protruding into or through it, or (ii) the membrane bilayer is intact and continuous but has engulfed the virions by microphagocytosis. To distinguish between these alternatives, replicas of the intramembrane faces of plasma membranes were studied (18).

Figure 4a shows an area of the outwardly facing surface of the inner leaflet of the plasma membrane. Fig. 4b shows at low magnification the cell from which this area (outlined) was photographed. The surface is identified as the outwardly facing surface of the inner leaflet of the membrane bilayer from well-established criteria. The exposed surface possesses the 7- to

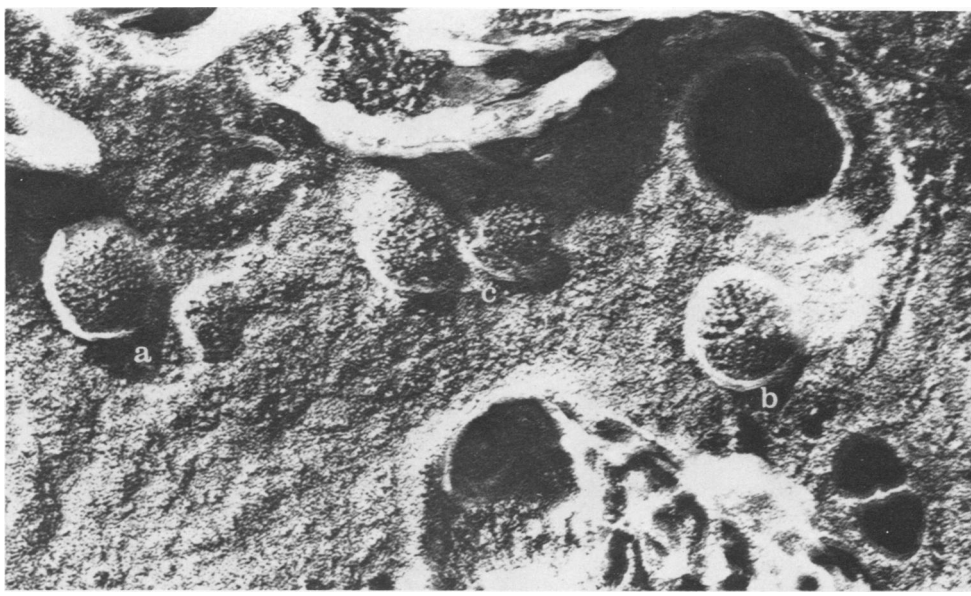

FIG. 3. *Electron micrograph of a replica of a freeze-etched KB cell. The cell had been infected with Ad 2 for 10 min before cleaving and 30 min of deep-etching. a, b, and c point out adenovirions which are embedded in the plasma membrane. Magnification ×200,000.*

10-nm granules which are characteristic of the interior membrane surfaces and are not found on the outer surface of the cell (18). This surface faces away from the interior of the cell (Fig. 4b) and possesses the 7- to 10-nm intramembrane granules in high density. The inwardly facing surface of the outer leaflet possesses fewer such intramembrane granules (18, Fig. 5B).

In addition to the intramembrane granules, particles about 75 nm in diameter are seen on this interior-membrane surface. These structures could not be found in replicas of uninfected cells and we assumed that they were adenovirions. One of these particles has sub-

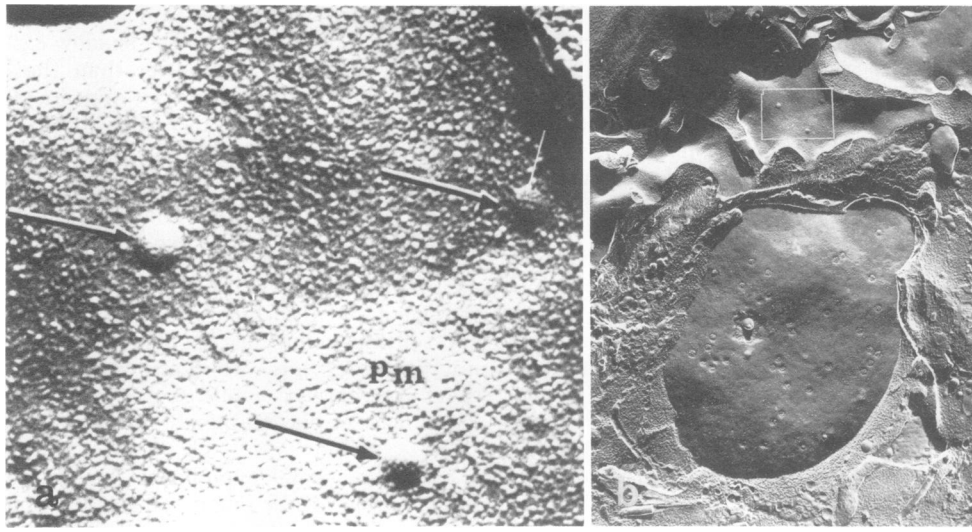

FIG. 4. *Freeze-etched replica of the external surface of the inner leaflet of the cleaved plasma membrane (Pm) of a KB cell. The black and white arrows point to adenovirions penetrating the membrane (a). The white arrow points to subunits on one of the particles. Magnification ×80,000. b, Low-magnification electron micrograph showing the cell from which the area shown in a was photographed (white square).*

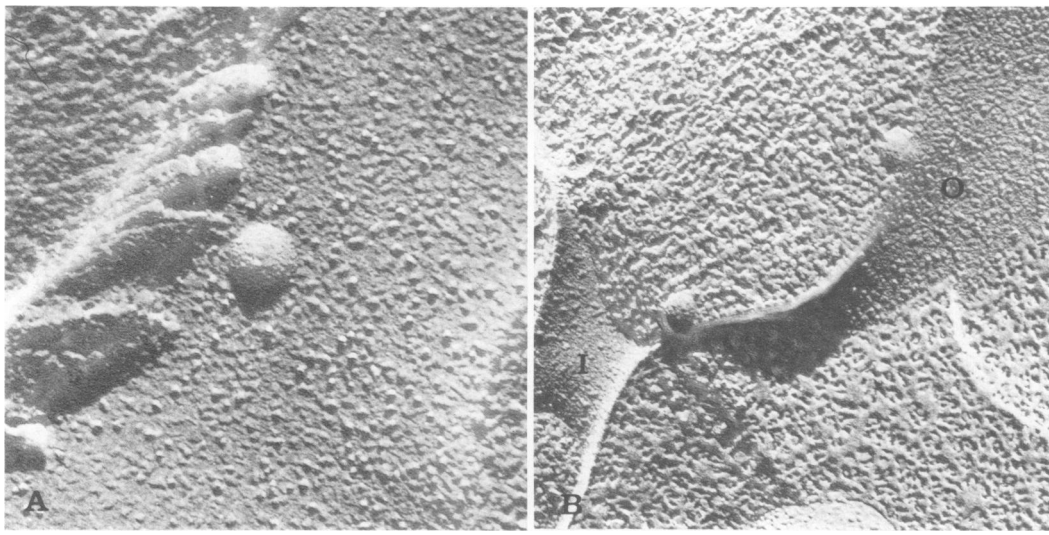

FIG. 5. *Replicas of freeze-etched KB cells at 10 min after infection with Ad 2. A, An adenovirion embedded in the outwardly facing surface of the inner leaflet of the plasma membrane bilayer. The virion is surrounded by the 6- to 10-nm interior-membrane particles. B, Two virions seen in the cell cytoplasm. Both are closely associated with the inner surface of the plasma membrane as though they had just penetrated the membrane bilayer. This cell demonstrates the characteristic differences in numbers of interior-membrane particles seen on the outwardly facing surface of the inner leaflet (O) and the inwardly facing surface of the outer leaflet (I). Magnification: A, ×120,000; B ×88,000.*

units (Fig. 4, white arrow) about 7 nm in diameter similar to the subunits seen in negatively stained adenovirions (Fig. 1).

A single virion embedded in an interior membrane surface of the plasma membrane is shown at higher magnification in Fig. 5A. The virus particle is surrounded by many of the 6- to 10-nm interior-membrane granules. There is no evidence of the presence of any fragment of the other member of the bilayer surrounding this particle. Although rounded in appearance (similar to the virions in Fig. 4a), the shadow cast by this particle has an angular appearance, suggesting that the outermost part of this particle still possesses the shape characteristic of an icosahedron.

The presence of virus particles on the intramembrane surface of the plasma membrane favors the first of the above two alternatives, i.e., direct penetration. If the membrane bilayer had been continuous and simply wrapped around the virion, the cleavage plane would have followed this contour, removing the outer leaflet of the plasma membrane and the associated virus particles. Therefore the viral structures would not have remained on the exposed outwardly facing surface of the inner leaflet of the membrane bilayer. The removal of membrane-bound arbovirus nucleocapsids from depressions in the host plasma membrane by cleavage of the membrane has been demonstrated by Brown et al. (3). These data strongly suggest that the virions have broken the continuity of the outer membrane bilayer. Thus the cleavage plane which split the membrane and removed the perforated outer leaflet from around the partially penetrated virion left the adenovirion associated with the inner leaflet of the membrane bilayer.

The virions embedded in the exterior surface of the inner leaflet (Fig. 4, 5A) were distinctly rounder in appearance and cast less angular shadows than those adsorbed to the outer surface of the plasma membrane (Fig. 3). Freeze-etching also revealed virus particles just inside the cell closely associated with the plasma membrane (Fig. 5B). These virions which may have just penetrated are also rounded in appearance. These observations suggest that the configurational change in the virion described by Morgan et al. (17) and Lonberg-Holm and Philipson (14) takes place during the penetration of the plasma membrane.

At 20 min after infection, large numbers of virions were found free in the cell cytoplasm close to the plasma membrane in thin sections, suggesting that the virions had just penetrated the membrane (Fig. 6). Many of the virions in the cytoplasm showed the rounded appearance described by Morgan et al. (17), whereas others had a more hexagonal shape (Fig. 6, arrows). The reason for this morphological variation is not clear. If the configurational change occurred during passage through the plasma membrane, one would anticipate that all virions would be structurally similar. The difference in appearance could result simply from physical stress imparted to the individual virions by the surrounding cytoplasm or during the embedding process.

A replica of a freeze-etched KB cell at 20 min after infection is shown in Fig. 7. Spherical structures about 75 to 80 nm in diameter are seen free in the cytoplasm of these cells. These particles were not found in mock-infected control cells. These particles were consistently rounded in appearance and morphologically similar to the particles seen on the interior membrane surface (Fig. 5). The rounded appearance of these virions agrees with the observation of Morgan et al. (17) and suggests that the virions which appear hexagonal in thin-sectioned preparations may have had their original shape restored to some degree by the physical stresses of embedding.

Attachment of adenovirions to isolated cell membranes. We attempted to determine whether the direct penetration of cell membranes described above could be duplicated in vitro by using isolated KB cell membranes. Smooth membranes from 4×10^6 KB cells were prepared by the procedure described by Caliguiri and Tamm (7), mixed with 10^{10} PFU of radioactively labeled Ad 2, and incubated in PBS for 5 min at 37 C. The reaction mixture was placed in discontinuous sucrose gradients as described by Caliguiri and Tamm (7) and centrifuged to equilibrium. The membrane-virus complex was located by assaying collected fractions for radioactivity. These complexes were observed in the electron microscope after negative staining with phosphotungstic acid (PTA) (Fig. 8).

Many of the membranes seen in this preparation were free of virions, whereas a few had many associated virions. It is likely that only a small portion of the smooth membranes in this fraction represented plasma membranes and that only these membranes contained adenovirus receptors. The virions associated with membranes in this fraction are probably bound, because unbound virions were recovered in a region of the gradient with a higher buoyant density. The attached virions are morphologically similar to unadsorbed particles shown in Fig. 1. No configurational change is apparent and there is no obvious loss of subunits from the vertexes (Fig. 8, insert). Virions near the edge of

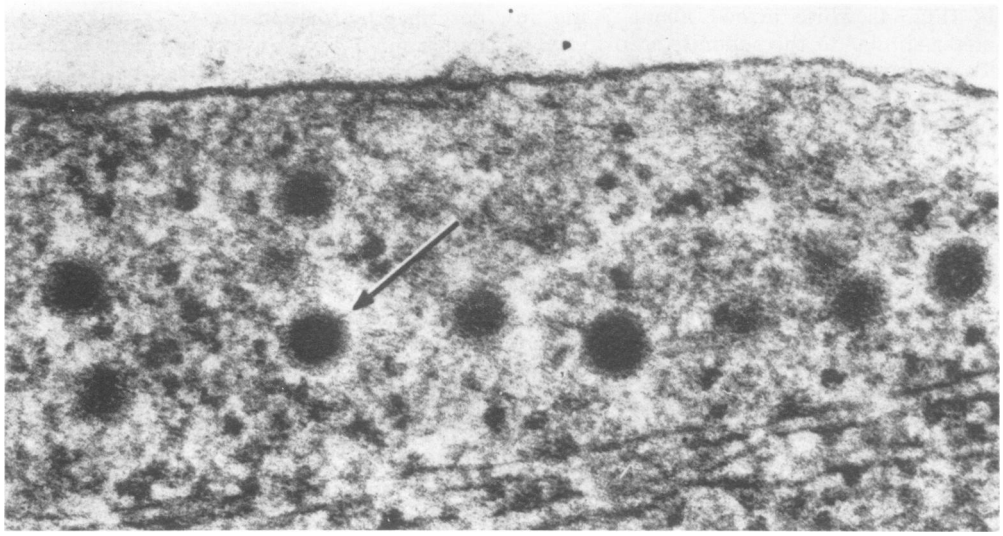

FIG. 6. *Ultrathin section of KB cell 20 min after infection with Ad 2. Virions are seen free in the cell cytoplasm. At least one particle (arrow) has hexagonal symmetry. Magnification ×120,000.*

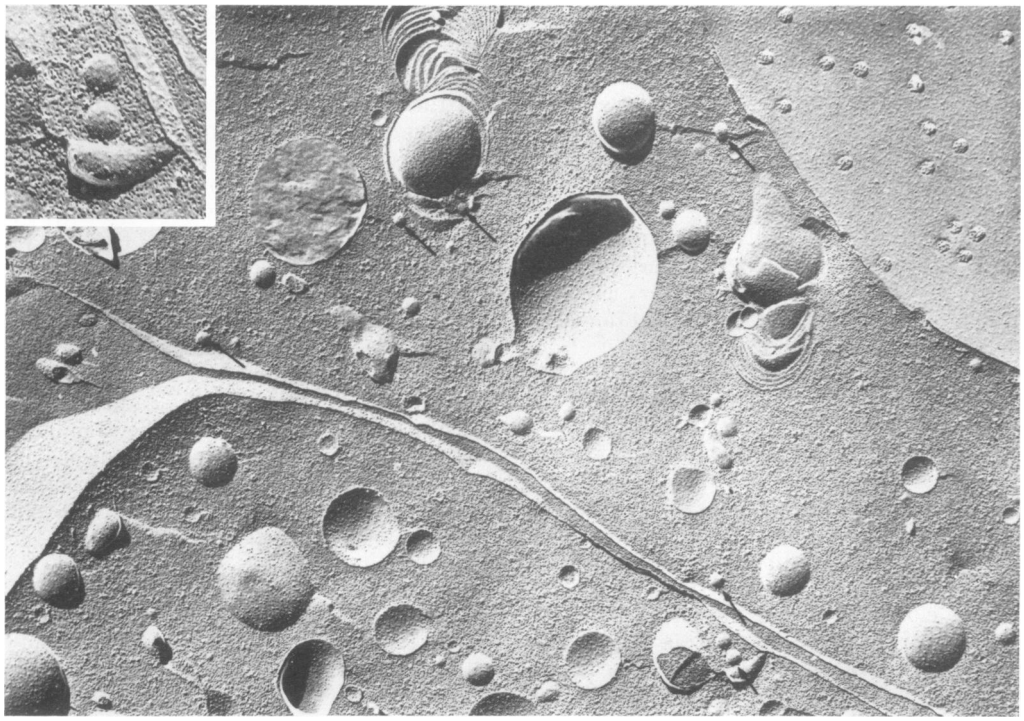

FIG. 7. *Freeze-etching of a KB cell 20 min after infection with Ad 2. Arrows point out some of the cytoplasmic structures which may be adenovirions. Magnification ×34,000. Two of the particles are enlarged in the insert (magnification ×67,000).*

the membrane seemed to be attached at a distance equivalent to that seen in the first stage of adsorption (Fig. 2, Morgan et al. [17]) in thin sections. Some of the virions in the central areas of the membrane were surrounded by rings as though the membrane had been drawn around the particle. The distance between the membranes and the capsids was again about the length of the penton fiber. This suggests that the virions are attached to the

membrane by the fiber. The partial envelopment of the virion by the membrane probably resulted from the multiple attachment of penton fibers to the free membrane surface. We concluded that, in this in vitro system, only the adsorption of the virion to the plasma membrane occurred. Conditions essential for penetration of the plasma membrane and the configurational change in the virus have apparently not been provided here, although the reaction mixture would have allowed this process to occur with intact cells. The process of membrane penetration and configurational change may therefore require the active participation of the intact cell.

Penetration of cells by inactivated adenovirions. Russell et al. (19) have shown that heating adenovirions to temperatures of 50 C and above for extended periods of time causes the particles to become noninfectious. Virions heated to 56 C undergo a morphological change, losing the penton subunits with the associated fiber and peripentonal hexons. The inactivation of these virions probably results from the loss of the organelles of attachment. Virions heated at 45 C lose infectivity (Table 1); however, electron microscopy of such heated particles revealed them to be morphologically indistinguishable from the virions seen in Fig. 1. These particles have the same buoyant density as do infectious particles (Table 1), and therefore the chemical composition does not seem to be altered.

KB cells exposed to adenovirions which had been partially inactivated by heat or antibody treatment were examined morphologically in an attempt to determine at what stage in host cell penetration these treatments block infection. Samples of a purified adenovirus preparation were (i) treated with normal serum (control) at 37 C, (ii) treated with anti-Ad 2 serum, (iii) heated at 45 C for 30 min, and (iv) heated at 60 C for 15 min. The reduction in infectivity resulting from these treatments is shown in Table 1. Equal numbers of the treated particles were added to monolayers of KB cells and incubated for 20 min. The cells were then fixed and embedded for electron microscopy. Ultrathin sections were examined, and the number of

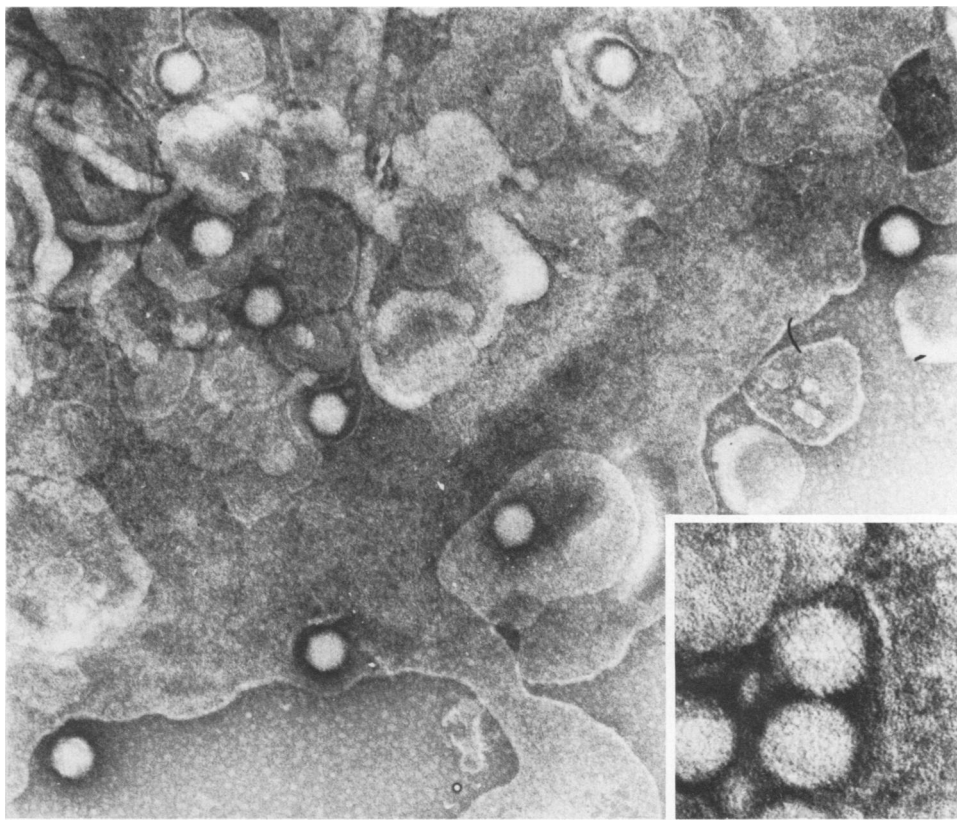

FIG. 8. *Adenovirus-cell membrane complex prepared in vitro as described in the text and negatively stained with PTA (see text for detailed explanation). Magnification ×75,000; insert magnification ×170,000.*

TABLE 1. *Penetration of KB cells by adenovirions which have been pretreated with specific antiserum or heat*[a]

Characteristics	Type of pretreatment			
	Normal serum control	Specific antiserum	Heated at 45 C	Heated at 60 C
Titer after treatment (PFU/ml)	4.7×10^{10}	2.4×10^{8}	2.6×10^{9}	2×10^{2}
Buoyant density in CsCl[b] (g/cm^3)	1.334 ± 0.00147		1.334 ± 0.00157	1.377 ± 0.0026
Number of particles attached to cell surface	126	180[c]	245	6
Number of particles in vacuoles	46	36	98	83
Number of particles free in cytoplasm	284	0	7	0

[a] Monolayers of KB cells were infected with an equal number of normal or treated Ad 2. In the case of the control preparation this represented a multiplicity of infection of 5,000 to 10,000 PFU/cell. The cells were incubated for 20 min after the addition of virus and then fixed and embedded as described in Materials and Methods. Virions were counted in each case from approximately the same number of cell profiles.

[b] Conditions of centrifugation were described previously (5).

[c] Large clumps of virions were found near the cell surfaces.

virus particles on the cell membrane, in cytoplasmic vacuoles, and free in the cytoplasm were counted in an equal number of representative cells from each preparation (Table 1).

The cells incubated with virions treated with normal serum presented a typical picture of virus infection. Virions were found attached to the surface of the cell, in cytoplasmic vacuoles, and free in the cytoplasm, as expected. Virions inactivated by specific antiserum were clumped together near the surface of the cell and in vacuoles. Virions partially inactivated at 45 C were primarily attached to the cell surface and in vacuoles or pinocytotic vesicles. Virions inactivated at 60 C were found in very small quantities, probably because of their inability to adsorb to the cell membrane. Those few virions seen were associated with the cells on the cell membrane or in cytoplasmic vacuoles.

Only in the control preparation incubated with nonimmune serum were significant numbers of virions detected in the cell cytoplasm. These results suggest that the loss of infectivity of the virions heated at 45 C does not result from an inability of these virions to attach to the cell or to be enclosed in vacuoles, but rather results from an inability of such particles to penetrate plasma or vacuolar membranes. The virions inactivated at 60 C have lost their pentons (20) and cannot adsorb to cell membranes (as evidenced by few attached particles), but are randomly taken up into cytoplasmic vacuoles by the cell. This random uptake of particles is also evident with the antibody-treated virions seen in vacuoles.

DISCUSSION

Two mechanisms have been suggested to explain how adenovirions may penetrate host cells. Dales (10) has suggested that the route of adenovirus penetration is by engulfment by pinocytosis (viropexis). Morgan et al. (17) have shown that although many virions are indeed enclosed in vacuoles resulting from pinocytosis, virions may also penetrate host cells by passing directly through plasma membranes. Our study supports the hypothesis of Morgan et al. that adenovirions may enter host cells by a process of direct membrane penetration.

The direct penetration of the cell surface by adenovirions could not be clearly demonstrated by the technique of ultrathin sectioning. The explanation for this may reside in the suggestion of Morgan et al. (17) and Lonberg-Holm and Philipson (14) that this early step in virus infection occurs very rapidly. The statistical probability of finding virions in the process of passing through the cell membrane is very low in thin sections where only a small cross-sectional area of cell surface is seen. Furthermore, the chemical and physical stresses imparted to the cell membrane during the procedures of dehydration and embedding for ultrathin sections may alter the classical membrane morphology in areas where the continuity of the bilayer is interrupted. This may explain why virions were not found protruding through a clearly defined plasma membrane. Similarly, Morgan et al. (17) and Dunnebecke et al. (12) could not clearly demonstrate virions transversing the membrane bilayer in thin sections.

Deep-etching of freeze-cleaved adenovirus cells revealed virions embedded in the cell surface at 10 min after infection. When the membrane bilayers of the cells were split by the cleaving process, virus particles could be seen embedded in the interior membrane space. This observation could only be explained if the

virions had transversed the continuity of the plasma membrane. Most of the virions seen on the interior membrane surfaces as well as those seen in the cytoplasm by freeze-etching were rounded in appearance. The rounded shape of these virions supports the observation of Morgan et al. (17) and Lonberg-Holm and Philipson (14) that a configurational change takes place in the virion during cell penetration. Our observation further suggests that this configurational change occurs as the virions pass through the membrane bilayer. Morgan et al. (17) have suggested that this configurational change may result from a loss of capsomeres from the vertexes of the icosahedral capsid. Our data suggest that such a loss of structural protein may occur as the virus particle passes through the plasma membrane. The loss of capsomeres from the vertexes of the icosahedral capsid may relax the rigid icosahedral structure of the particle, allowing it to assume a spherical shape.

The configurational change of the adenovirions found in the cytoplasm of thin-sectioned and freeze-etched cells was not detected when purified virions were incubated in vitro with isolated cell membranes. The virions attached to the isolated membranes by their penton fibers; however, no change in appearance or loss of capsomeres was detected. This suggests that the process of membrane penetration results in this configurational change and is dependent upon some function with the intact cell.

Burlingham and Doefler have shown that an endonuclease is associated with intact adenovirions (6). This endonuclease is part of the penton base and can be recovered in the nucleus of infected KB cells (5, 6). Therefore, if the configurational change which occurs as the virions pass through the plasma membranes follows the loss of penton fiber from the viral capsid, a significant number of these penton fiber complexes must enter the cell with the infecting virion. Alternatively, the release of the penton subunit from the virus capsid during membrane penetration may leave a peptide with endonuclease activity attached to the modified virus particle.

As in the study of Morgan et al. (17), we found a large proportion of virions free in the cytoplasm of cells at 20 min after infection relative to virions seen enclosed in vacuoles and in pinocytotic vesicles (a ratio of about 5:1; Table 1). By comparison, Dales (10) and Chardonnet and Dales (9) found a small number of free cytoplasmic virions and a large number of virions in vacuoles at these early times. The differences in these studies may reflect a more favorable particle-to-PFU ratio obtained through improved procedures in virus purification. We have shown here that when plaque-forming ability of a virus suspension is reduced by heating at 45 C, the net result is a drastic reduction in the number of virions seen in the cytoplasm at 20 min after infection. This treatment did not reduce the number of virions seen on the cell surface or in pinocytotic vesicles. Indeed, the numbers of virions disposed in the vesicles were larger than was seen in the control experiment. It would appear that these inactivated virions were unable to penetrate the cell membranes and were simply accumulating on the surface and in vacuoles. In their study, Morgan et al. (17) tested the effects of formaldehyde treatment of adenovirions on the penetration and appearance of free cytoplasmic virus. Although this treatment reduced the number of attached invacuolated particles, it also eliminated the cytoplasmic virions.

It seems that heating at 45 C produces a subtle alteration in the adenovirus particle. The fine structure of the virion at the electron microscopy level is unchanged and the buoyant density of the virion is unaltered, yet these particles have lost the ability to transverse cell membranes. The concurrent reduction in numbers of free cytoplasmic virions with the loss in plaque-forming ability suggests that the direct penetration of plasma and vacuolar membranes is an essential step in the successful infection of host cells.

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