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Role of Vesicles During Adenovirus 2 Internalization into HeLa Cells

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In this investigation, the early period of adenovirus type 2 (Ad2)-HeLa cell interaction was analyzed by electron microscopy and biochemical techniques. Events observed in this period ranged from the disappearance of virions from the cell surface to their subsequent association with the cell nucleus. Destabilization of the virions attached to the intact cell was necessary for virions to escape from intracellular vesicles. Strong temperature dependence and rapid escape from a vesicular compartment were shown in temporal kinetic experiments. These vesicles appeared to be acidic, since lysosomotropic agents partly inhibited the release of virions from vesicles. Studies of Ad2 binding to cells in buffers of different pH values suggested that adenovirus binds to cells by two different mechanisms. At low pH the binding was most probably mediated by the penton base and at neutral pH by the fiber protein. The number of receptor sites per cell was 25,000 and 6,000 at low and neutral pH, respectively. This study suggests that the low-pH affinity between the penton base and a vesicular membrane is important inside acid vesicles when Ad2 quickly enters the cytoplasm. However, a significant fraction of the virions was possibly internalized by a pathway not requiring a passage through such vesicles.

The early interaction between animal viruses and host cells has been the subject of several investigations in the past 20 years. The conclusions of these investigations are continually brought together in review articles (4, 6, 8, 19, 23). Many viruses are internalized by a pathway referred to as receptor-mediated endocytosis (4, 8), which is also utilized, for example, by hormones and plasma proteins (14). This process of internalization has been defined by Goldstein et al. (14). It commences with the attachment of ligands to specific receptor sites on the cell surface; the receptor-ligand complexes accumulate in coated pits and are subsequently seen inside coated vesicles and endosomes. From the endosomes the ligands are further released into the cytoplasm or transported to different compartments within the cell, such as the lysosomes or the nucleus.

Receptor-mediated endocytosis has been suggested as one mechanism of adenovirus type 2 (Ad2) internalization (12, 41). It is well established that Ad2 attaches to specific saturable proteins on the plasma membrane of HeLa cells (34, 42). The virions are internalized via coated pits and coated vesicles and are subsequently observed within endosomes (5, 12, 41). The importance of an acidic pH inside vesicles has been shown for the Ad2-mediated enhancement of the toxicity of *Pseudomonas* toxin conjugated to epidermal growth factor (12) and has also been indicated for the sole Ad2 infection of cells (41). Most Ad2 virions leave the endosomes (41), and a suggestion of penton base involvement in this event has recently appeared (38).

A partial characterization was previously made of the early events of Ad2 destabilization and penetration that take place in association with the plasma membrane (41). The effect of various reagents on those steps was compared with the effect on early virus polypeptide production (41). In the present investigation the intervening events were analyzed; i.e., the transfer of virions from the plasma membrane to the nucleus, the involvement of vesicles during this transfer, and the prerequisites for Ad2 to leave an endocytotic vesicle.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at densities of 2×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μg of gentamicin per ml. Ad2 was propagated, radioisotope ([^{3}H]thymidine) labeled, and purified as described previously (10).

EM. Cells were sedimented, washed once, and suspended in phosphate-buffered saline (PBS) at a concentration of 5×10^7 cells per ml. Chemicals were added, as specified, and the cells were incubated under slow agitation for 30 min at 37°C. After equilibration for 5 min at 3°C, Ad2 was added at a multiplicity of infection (MOI) of 4,000 particles per cell and allowed to attach for 30 min. Unattached viruses were removed by sedimentation of the cells, which were resuspended in PBS containing the appropriate reagent. Samples were incubated at 37°C for 60 min, at 37°C for 10, 20, 40, and 60 min, and at different temperatures for 60 min in the chemical, kinetic, and temperature series, respectively. After the appropriate incubations, the cells were processed for electron microscopy (EM) as previously described (37, 41).

Isolation of nuclei. Cells were sedimented, washed with PBS, and incubated in the presence of different reagents as described above. The cells were equilibrated at 3°C for 5 min, and [^{3}H]thymidine-labeled Ad2 was added at an MOI of 340. Virus particles were allowed to attach for 30 min at 3°C. Unattached virions were removed, and the cells were resuspended in PBS, at a concentration of 10^7 cells per ml, containing the appropriate reagent. The cells with attached virus were incubated at 37°C for 45 min, and samples were withdrawn at intervals and kept on ice. In all experiments, the cells were subsequently pelleted, and the nuclei were isolated essentially as described by Penman (30). The nuclear pellet was dissolved in Protosol, and radioactivity in the nuclear and extranuclear fraction was measured. For each series, a background value for nonspecific nuclear association of radioactivity was obtained after 1 min of

incubation. These values were subtracted from the amount of radioactivity located in the nuclear fraction of all subsequent samples. Phase-contrast microscopic examination showed intact nuclei without contaminating whole cells or membrane fragments.

GA fixation of cells and viruses. HeLa cells were washed and fixed with 0.015% glutaraldehyde (GA) as previously described (31). Purified Ad2 was dialyzed overnight against 0.1 M sodium phosphate buffer (pH 7.3). The virus particles, at a concentration of 2 OD (at 257 nm) per ml and corresponding to ca. 2×10^{12} particles per ml (25), were fixed with 0.1% GA at 3°C overnight under slow agitation. The particles were separated from free GA by gel filtration on a PD-10 column equilibrated in PBS. Neutralized ethanolamine was added to the virus at a final concentration of 1 M. The samples were incubated at 3°C for 40 min under slow agitation, and then Ad2 was reisolated on a CsCl cushion (11). Virus recovery was 50%, and the aldehyde-stabilized virions attached to cells with 60% of the efficiency of untreated virions.

DNase sensitivity of the viral genome. The procedure for determination of DNase sensitivity was as described by Joklik (18) and modified by Svensson and Persson (41), except that the cells were disrupted by 0.5% sodium deoxycholate (21).

Radioisotope-labeled virus structural proteins. Virus structural proteins, obtained from an Ad2-infected cell culture labeled 15 h postinfection with [^{35}S]methionine, were isolated by DEAE-cellulose chromatography essentially as described by Pettersson et al. (32).

Attachment studies. HeLa cells were washed once in PBS and once in the appropriate buffer and were finally suspended in the latter buffer at a density of 5×10^7 cells per ml. [^{3}H]thymidine-labeled virus at an MOI of 340 or [^{35}S]methionine-labeled virus structural proteins at a ratio of 100,000 molecules per cell were added to the cells and attached at 37°C for 45 and 30 min, respectively. After incubation, the samples were diluted five times, and the cells were pelleted. The percentage of attached virus or virus structural proteins was calculated after radioactivity measurements. In one experimental series with different buffers, Ad2 was attached to cells pretreated at 3°C with 250,000 fiber molecules per cell for 120 min at pH 7.0. In another experimental series, different MOIs of Ad2 were added to cells to determine the number of cellular receptor sites at pH 4.8 and 7.0. The buffer systems used were 6 mM universal buffer (pH 3.5 to 8.5) (2), 10 mM sodium acetate buffer (pH 3.5 to 5.5), 10 mM sodium phosphate buffer (pH 5.5 to 8.0), and 10 mM glycine-sodium hydroxide (pH 8.5 to 9.0), all containing 150 mM NaCl. The pH values of the different buffers were obtained in the presence of cells.

Preparation of pentonless virions. Pentonless virions were prepared by dialysis against double-distilled water as described by Laver et al. (20). Viruses without pentons were separated from released material by centrifugation at 50,000 $\times$ *g* for 30 min (35). The concentration of pentonless virus was estimated by UV absorption at 280 nm (25).

The hydrophobicity of pentonless and intact virus was analyzed by incubation for 30 min at room temperature of 10^{12} virus particles with 0.05% Triton X-100 containing 30 μCi of ^{3}H-labeled Triton X-100 in a total volume of 250 μl. The particles were separated from free detergent in linear sucrose gradients (10 to 25% [wt/vol] in 50 mM Tris hydrochloride [pH 7.5]) formed on a cushion of CsCl (ρ = 1.45 g/ml). The gradients were centrifuged at 100,000 $\times$ *g* for 30 min and fractionated by puncturing.

Protein determination. Protein was determined by the method of Hartree (16) with bovine serum albumin as the standard.

Liquid scintillation spectrometry. Radioactivity measurements in the nuclear isolation and DNase sensitivity assays were performed in a cocktail of toluene-methanol (1:1) containing 0.4% Omnifluor. All other radioactive samples were analyzed in Ready Solv HP/b.

Chemicals. Eagle minimal essential medium, fetal calf serum, gentamicin, and L-glutamine were obtained from Flow Laboratories Ltd., Irvine, Scotland. [^{35}S]methionine (>800 Ci/mmol) was from Amersham International Ltd., Buckinghamshire, England. Omnifluor, Protosol, [^{3}H]thymidine (75 Ci/mmol), and ^{3}H-labeled Triton X-100 (1.58 mCi/mg) were purchased from New England Nuclear Chemicals GmbH, Dreieich, Federal Republic of Germany. Bovine serum albumin, chloroquine, colchicine, cytochalasin B, 2-deoxyglucose, DNase I (from bovine pancreas), dithiothreitol (DTT), ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), monodansylcadaverine, sodium azide, and trifluoperazine were from Sigma Chemical Co., St. Louis, Mo. Ammonium chloride, EDTA, and methylamine were from E. Merck AG, Darmstadt, Federal Republic of Germany. PD-10 columns with Sephadex G-25M were obtained from Pharmacia, Uppsala, Sweden. Triton X-100 (scintillation grade) and sodium deoxycholate were from BDH, Poole, England. Ready Solv HP/b was obtained from Beckman Instruments AB, Bromma, Sweden.

RESULTS

Kinetics of virus internalization. EM was used to measure the frequency of virus particles in various compartments of the cell at intervals from early attachment to 1 h postinfection. Virions disappeared from the cell surface within 20 min (Fig. 1). During hour 1 of incubation, a small fraction of virus particles was seen inside vesicles at the observation times selected. However, the transfer of virions through vesicles was rapid; 10 to 20 min after attachment most of the virus particles were free inside the cytoplasmic compartment of the cell. The number of virions near the nucleus and, in most cases, adjacent to clearly visible nuclear pores increased continuously during the first 30 min and reached 50% after 45 min (Fig. 1).

The vesicles which contained virus particles were almost exclusively small (100 to 200 nm) and located near the plasma membrane. Some vesicles were coated. A small fraction of the scored vesicles possibly were invaginations of the plasma membrane (see Fig. 4A).

When nuclei from [^{3}H]thymidine-labeled virus-infected cells were prepared, the amount of viral nucleic acid inside the nucleus reached a maximum level of 50% after 60 min (data not shown). At this time such a level corresponded to the amount of free virions near the nucleus as determined by EM (Fig. 1). The amount of viral nucleic acid in the cell nucleus did not further increase during 5 h of incubation (data not shown).

Temperature dependence of Ad2 internalization. The distribution of virus particles between various compartments of the cell at different temperatures was studied by EM. Based on the outcome of the kinetic EM studies of virus localization within the cells, a 60-min incubation period was chosen for the temperature analyses. A temperature above 15°C was required for virus to disappear from the cell surface (Fig. 2A). Virions did not accumulate in vesicles at any of the temperatures used. At 17°C, about 6% of the scored virions

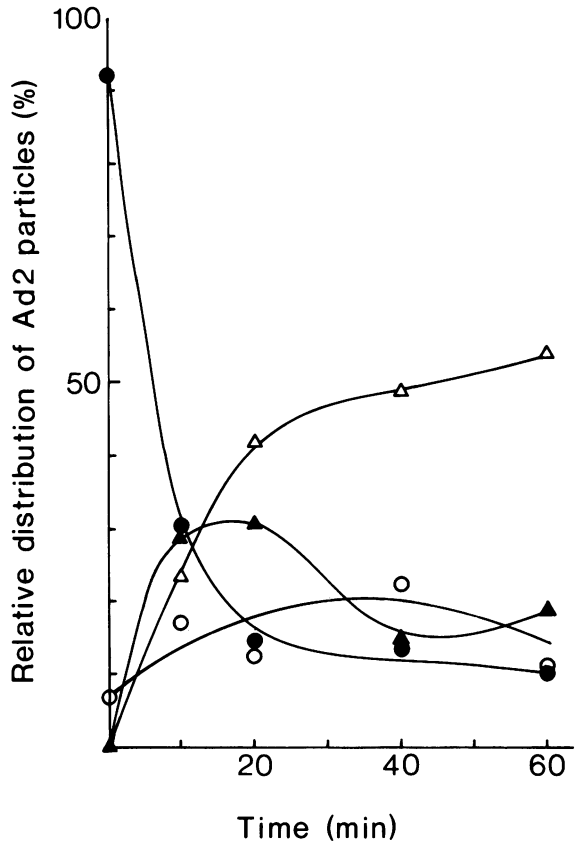

FIG. 1. Temporal distribution of Ad2 in cells. Cells with virions attached at 3°C were shifted to 37°C, and samples were continually withdrawn and studied by EM as described in the text. The frequency of Ad2 in different compartments of the cell was scored. Symbols: ●, Ad2 at the cell surface; ○, Ad2 inside vesicles; ▲, Ad2 free in the cytoplasm; △, Ad2 free near the nucleus.

were free in the cytoplasm. The amount of free virions in the cytoplasm reached a maximum at 25°C, and the number of free virions near the nucleus continuously increased until the temperature reached 37°C (Fig. 2A). The temperature dependence of virus transport to the nuclear compartment was also studied biochemically by nuclear isolations (Fig. 2B). These studies revealed a similar temperature dependence pattern for virion transport to the nucleus as was shown by EM.

Influence of various reagents on virus internalization. Internalization in the presence of 12 different reagents was analyzed by EM at 60 min postinfection and by the nuclear isolation technique at 45 min postinfection.

(i) EM. The effect of some of the reagents on virus internalization after 20 min of virus-cell incubation at 37°C has previously been studied (41). However, in those studies the virus infections were not synchronized, and the present kinetic investigation showed that 60 min of incubation was an optimal time. The inhibitor of oxidative phosphorylation, sodium azide, almost completely prevented the internalization of Ad2, and consequently 80% of the virions were left in association with the plasma membrane, and no virions at all reached the nucleus (Fig. 3). In the presence of DTT, 32% of the virions could be seen inside large vesicles. Such vesicles, referred to as multivesicular bodies (MVB), had a diameter of 300 to 700 nm and usually contained smaller vesicles of different sizes (Fig. 4B). These structures were located anywhere in the cytoplasm and appeared to contain one to five virions. Only 30% of the virions became free in the cytoplasm and associated with the nucleus when DTT was present, compared with ca. 80% in the control series. In the presence of EDTA and EGTA, most of the virions were left on the cell surface. When dansylcadaverine was used, 44% of the particles were seen inside vesicles with small diameters (100 to 200 nm) and near the plasma membrane (Fig. 3B). Colchicine, which interferes with microtubular polymerization, almost fully inhibited the transport of free virus particles to the nucleus, according to the EM analyses. In this case virions appeared to accumulate free in the cytoplasm (Fig. 3C). In the presence of lysosomotropic agents, which previously were shown to interfere only slightly with the virus early gene expression (41), 15 to 30% of the virions remained inside the vesicles, compared with ca. 10% in the control series (Fig. 3). When lysosomotropic agents were used, about half of the virus-containing vesicles were MVB, whereas virions inside MVB were extremely rare in the control series. In the control series, 50% of the virions reached the nucleus, but in the presence of the most potent lysosomotropic agent, ammonium chloride, only 15% reached this compartment.

(ii) Nuclear isolation. The pattern of reagent interference with virion transport to the cell nucleus closely resembled the results from the EM studies. However, in the presence of colchicine, ca. 30% of the nucleic acid-labeled material appeared to reach the nucleus (Fig. 3E).

Effect of DTT and GA fixation on viral uncoating. Initial uncoating of Ad2 is believed to take place on the outside of the plasma membrane of the intact cell, and DTT was previously shown to inhibit this process by 50 to 60% compared with the control situation (41). It was therefore of interest to know whether this inhibition was due to an effect on the virions or interference with the plasma membrane (17). It was clearly shown that only when cells were preincubated with DTT and only when DTT was present during the virus-cell incubation was the DNase sensitivity maximally reduced (Table 1). Virions stabilized by GA fixation were totally inert to the cell-mediated destabilization process. A similar lack of destabilization was obtained when the cells were GA fixed and subsequently exposed to untreated virions (data not shown).

Internalization of GA-fixed Ad2. Because it was shown that DTT exerted its function on the cell and significantly impaired the uncoating process but allowed endocytosis, it was of interest to learn about the behavior of virions which were rendered insensitive to the destabilization process by treatment with GA. The EM studies revealed that the GA-fixed virions disappeared from the cell surface at the same frequency as control virions. All virions that were internalized appeared to be trapped inside vesicles, the majority of them

TABLE 1. DNase sensitivity of the Ad2 genome[a]

Cells preincubated with DTT	Virus preincubated with DTT	DTT present during the virus-cell incubation	DNase sensitivity (%)
−	−	−	76
+	−	+	36
−	+	+	60
−	+	−	77

[a] Incubation was at 37°C for 40 min. Preincubation was at 37°C for 30 min. The DNase sensitivity analysis was as described in the text.

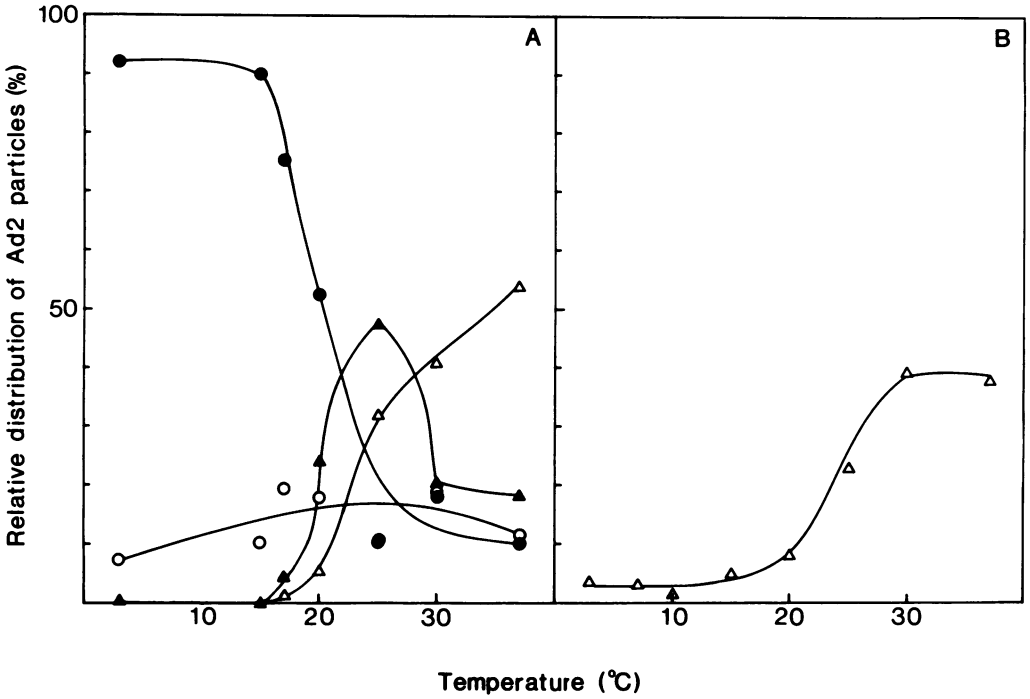

FIG. 2. Temperature dependence of Ad2 compartmentalization in cells. Cells with virions attached at 3°C were further incubated at various temperatures and subsequently studied by EM or the nuclear isolation technique as described in the text. The frequency of Ad2 in different compartments of the cell was assessed by EM after 60 min of incubation (panel A). Symbols: ●, Ad2 at the cell surface; ○, Ad2 in vesicles; ▲, Ad2 free in the cytoplasm; △, Ad2 free near the nucleus. The frequency of Ad2 [^{3}H]thymidine-labeled parental nucleic acid association with the cell nucleus was monitored as revealed by the nuclear isolation technique after 45 min of incubation (panel B).

being MVB (Fig. 3B and 4B). A low percentage of virions was located inside electron-dense vesicles, probably lysosomes. GA-fixed virions were not able to reach the nuclear compartment, as shown by both EM and the nuclear preparation method (Fig. 3D and 3E).

Attachment of Ad2 and virus structural proteins at different pH values. To mimic the situation when Ad2 interacts with the membranes of presumably acidic endosomes, studies of Ad2 binding to cells at different pH values were performed. The pH-dependent attachment of Ad2 to HeLa cells displayed two noticeable pH optima, one at 4.7 to 5.0 and the other at 6.0 to 6.5 (Fig. 5). Different overlapping buffer systems were used, and the data suggested that the attachment process was affected only by the change in pH and not by the actual buffering ions. Attachment of the fiber protein was optimal in the pH range 6.0 to 6.7, whereas attachment of the penton base drastically increased in the pH range 4.7 to 5.1. The hexon attachment was slightly enhanced below pH 5.5 (Fig. 5). Preincubation of cells with fiber at pH 7.0 inhibited subsequent virus attachment at a pH above 5.2 (Fig. 5). It was further shown that fibers attached at pH 7.0 were released upon acidification below pH 5.2. Under the same experimental conditions, it was also shown that virions were not released at any pH after attachment. Cells preincubated at pH 4.8 did not allow attachment of virions at a pH higher than 5.5, indicating that the attachment site for virions was destroyed at the lower pH (data not shown). It was possible to saturate the cellular binding sites at both pH 7.0 and 4.8 when Ad2 was attached to cells at different MOIs. Binding data transformed into Scatchard plots revealed 6,000 and 25,000 receptor sites per cell at pH 7.0 and 4.8, respectively (data not shown). In a control series all the structural proteins and the virions were exposed to the different buffers to show whether any of the ligands precipitated. No such pH-dependent precipitation was discernible, and none of the buffers rendered the viral genome sensitive to DNase treatment (data not shown).

Hydrophobicity of intact and pentonless virus particles. Adenovirus is destabilized at an early stage in the infectious cycle—possibly at the cell surface (21, 41). If this process mediates the transfer of virions through the plasma membrane, this may be a consequence of exposure of viral hydrophobic regions. Thus, the possible difference in hydrophobicity between pentonless and intact virus was studied. Pentonless virions were obtained by dialysis against bidistilled water, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24) showed that the particles quantitatively lost the antigens of the vertex region (data not shown). ^{3}H-labeled Triton X-100 binding was used to estimate the level of hydrophobicity. The ratio of the amount of ^{3}H-labeled Triton X-100 bound to intact virions and pentonless particles was 5.4:1, which indicated that intact virions were significantly more hydrophobic than pentonless virions.

DISCUSSION

In this study, which is an extension of a previous investigation on the mechanisms of Ad2 entry into cells (41), the further steps necessary for Ad2 to reach the cell nucleus were examined.

It was shown that virions disappeared from the cell surface very rapidly at 37°C. The maximum level of 50% for nuclear membrane association of virions and parental viral DNA confinement to the nucleus was obtained at 45 and 60 min after attachment, respectively. These observations are in good agreement with previous data on Ad2 and Ad5

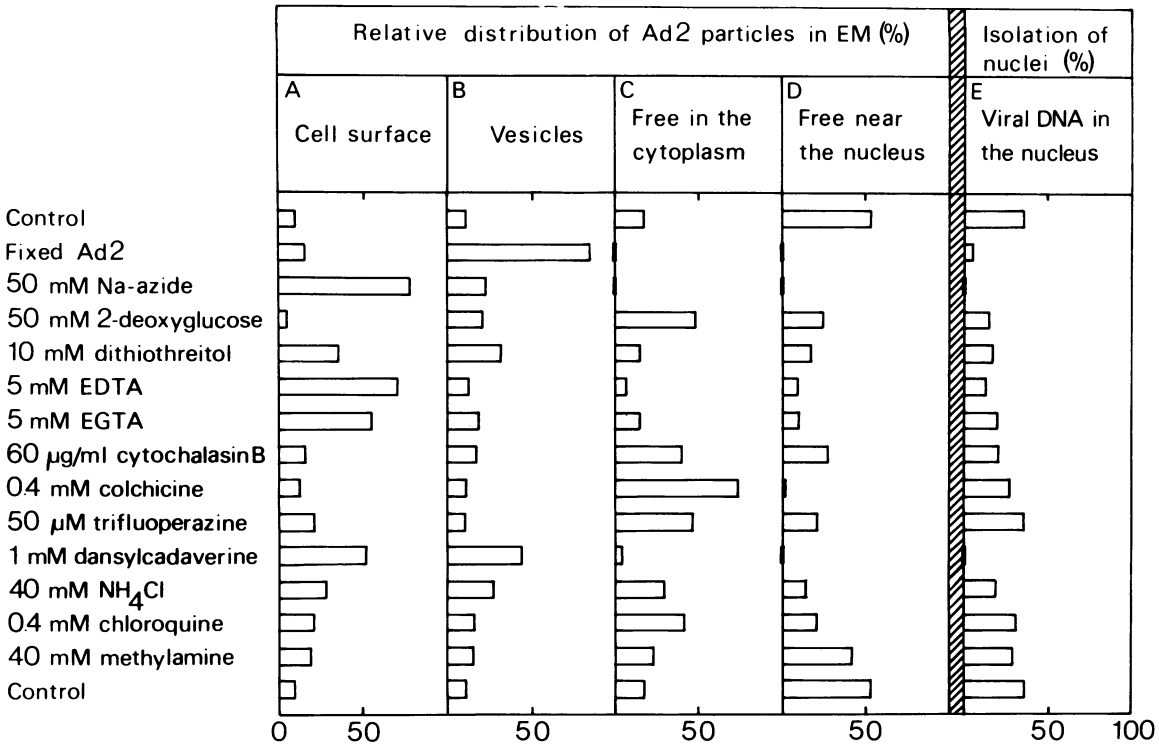

FIG. 3. Influence of added reagents on the cellular distribution of Ad2 particles as revealed by EM and the nuclear isolation procedure. Ad2 was attached for 30 min at 3°C to cells which were pretreated with the indicated reagents as described in the text. Cells with attached virions were subsequently incubated at 37°C for 60 min in the EM procedure, in which the relative distribution of ca. 150 counted virions per sample series was calculated (panels A to D). Samples for the nuclear isolation series were incubated at 37°C for 45 min, and the amount of radioactive parental DNA associated with the cell nucleus, relative to the totally attached Ad2, was determined (panel E).

transport to the cell nucleus (5, 22). Chardonnet and Dales (5) found that maximum levels of Ad5 occur in vacuoles 10 min after attachment. For Ad2 this process seemed to be less synchronized; i.e., ca. 15% of the virions were seen in vesicles at all times within 1 h after attachment.

The internalization of Ad2 was strongly temperature dependent, as has also been shown for the endocytotic uptake of Semliki Forest virus (26), asialoglycoprotein (45), and plant toxin (36). At 15°C no virions appeared free in the cytoplasm, but at 17°C a few percent had already escaped into the cytoplasmic compartment. A similar temperature dependence was previously shown for the destabilization of virions on the cell surface (41). The present study clearly showed that it was necessary for virions to become destabilized if they are to subsequently escape from endocytotic vesicles (see below). Chardonnet and Dales (5) found that Ad5 was retarded on the cell surface or in vacuoles at 25°C. At this temperature, most Ad2 particles were already free in the cytoplasmic compartment. This indicates that the mechanism of internalization for the two viruses belonging to the same subgroup of adenoviridae is not necessarily identical.

In a previous investigation, the effect of 12 reagents on the early steps of viral interaction with the plasma membrane and on viral polypeptide production was studied (41). The same reagents were used to obtain more information about the steps taking place after the virus leaves the cell surface but before entry of the viral nucleic acid into the cell nucleus. Several observations of the previous and present investigations are in good agreement. However, reversion of inhibition was a problem when polypeptide production was measured (41). This problem was eliminated when transport to the nucleus was analyzed, because chemicals were allowed to remain throughout the study. Thus, all the reagents used had a more pronounced effect on this event than on polypeptide production. Inhibition of nuclear association was at the most 70% of the control level when lysosomotropic agents were used. This indicated that a maximum of about 70% of all virions predestined to reach the nucleus were dependent on vesicular structures of low pH. Favoring this idea, Seth et al. (39) showed in a recent investigation that lysosomotropic agents completely blocked the Ad2-enhanced toxicity of an epidermal growth factor-*Pseudomonas* exotoxin conjugate.

When colchicine was present during infection, a result emerged from the EM studies that was puzzling when compared with the data obtained by the nuclear isolation procedure. About 2 and 30% of the virions reached the nucleus in the two procedures, respectively. In cells treated with colchicine, Dales and Chardonnet (7) previously showed that virions appear to be retarded on the route to the nucleus because of association with so-called annulate lamellae. An earlier study showed that colchicine displays no effect on the synthesis of early or late virus-specified proteins (41). These contradictory results, together with the present EM data, may be explained by one or more of the following possible interpretations. (i) Noninfectious particles were preferentially trapped in the cytoplasmic compartment; (ii) the destabilized virions released the DNA or core structures, which subsequently managed to reach the nucleus; or (iii) any one or more of the techniques used produced artifactual results in the presence of colchicine.

When virion destabilization was blocked after treatment of cells with DTT or when GA-fixed virions were used, most of

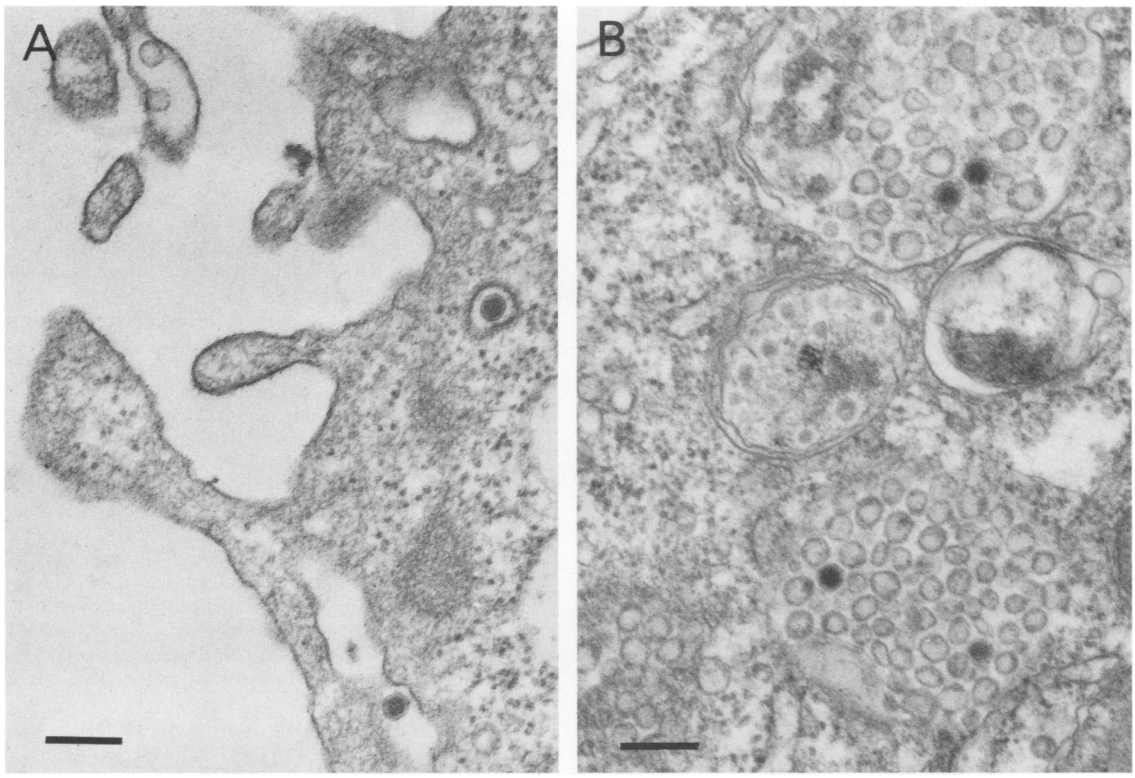

FIG. 4. Electron micrographs of Ad2-infected HeLa cells. Cellular distribution of virions 60 min after attachment. Panels: A, incubation at 17°C; B, incubation with GA-fixed virions at 37°C. Bar, 200 nm.

the virus particles were trapped inside MVB, but some could also be seen within more dense vacuoles—possibly lysosomes. In cells pretreated with DTT, one-third of the virions became free in the cytoplasm, compared with 80% in the control series. These values corresponded very well with the 36 and 76% of the virions which became DNase sensitive in the DTT and control series, respectively (Table 1). These findings indicated that virion destabilization was a prerequisite for the virus to merge from vesicles into the cytoplasmic compartment.

Membranes of cell endosomes closely resemble the plasma membrane (28) and, further, the pH level within an endosome varies between 4.8 and 5.5 (43, 44). Taking these facts into account, this study used intact HeLa cells as an inverted model system for an endosome. Cells were incubated in different buffers with final pH values between 4.7 and 7.2. The pH-dependent attachment studies revealed two pH regions at which the attachment was enhanced, one between 4.7 and 5.1 and the other at ca. 6.5. From the present investigation it became apparent that virion attachment at low pH was mediated by the penton base and, at a more physiological pH, the fiber antigen was the structure responsible for attachment. Supporting the role of the penton base in virion association with membranous structures at low pH, Seth et al. showed that anti-penton base antibodies efficiently inhibited the Ad2-enhanced toxicity of *Pseudomonas* exotoxin conjugated to epidermal growth factor (38) and also inhibited the Ad2 induction of ^{51}Cr release from KB cells at low pH (40). The penton base mediates the established cytopathic effect (33) and also appeared to affect the degree of hydrophobicity of the virion. In this laboratory we recently showed that Ad2 infectivity is not neutralized to an extent greater than 50% with anti-penton base antibodies (C. Wohlfart, unpublished data). This finding and the fact that the effect of lysosomotropic agents never inhibited virion transport to the nucleus by more than 70% suggest the existence of an alternative way for Ad2 internalization besides receptor-mediated endocytosis coupled to passage through acidic vesicles. The other pathway could possibly be direct penetration as described by Morgan et al. (29) and Brown and Burlingham (3).

The fiber antigen is recognized as the virus attachment protein in the adenovirus system (34), and it attaches well to cells under physiological conditions. Fiber structures attached to cells under such conditions are readily detached at a pH below 5.2. Likewise, preincubation of cells at an acidic pH showed that the cellular receptor site for this protein was destroyed. Ligand-receptor dissociations at low pH are well-known phenomena in many systems of receptor-mediated endocytosis (1, 15, 27). In the case of epidermal growth factor- and asialoglycoprotein-receptor dissociation, this is believed to be due to conformational changes of the receptor site (9).

For the Ad2 system the number of binding sites per cell at pH 4.8 was estimated to be ca. 25,000, whereas at a physiological pH of 7.0 it was ca. 6,000. These results suggest that the structure(s) recognized by the virus at low pH is not the same as under neutral pH conditions. It has been proposed that Semliki Forest virus is attached to a plasma membrane protein at a neutral pH but also reveals affinity for membrane lipids in an acidic environment (13).

In a previous communication, it was suggested that Ad2 is internalized by receptor-mediated endocytosis (41). Based on the present investigation, it is appropriate to supplement this view with the following observations. (i) Destabilization of the virions rendering the viral genome sensitive to DNase

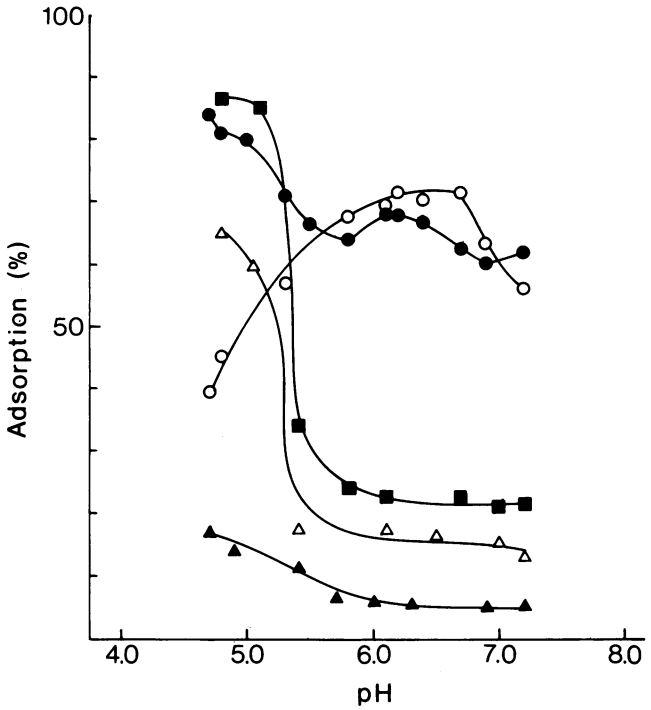

FIG. 5. pH dependence of Ad2 and structural protein adsorption. Cells were incubated with Ad2 or virus structural proteins at the indicated pH values. The amounts of cell-adsorbed virions and structural proteins were measured after 45 and 30 min, respectively. Symbols indicate adsorption of Ad2 (●), fiber protein (○), hexon protein (▲), penton base protein (△), and Ad2 to cells pretreated with fiber protein at pH 7.0 (■).

treatment is a prerequisite for the escape of virions from intracellular vesicles. (ii) It is also obvious that rapid passage through acidic vesicles is included in one route for Ad2 internalization. (iii) Inside the acid vesicles the affinity between the penton base and the membrane seems important for virions to leave these organelles. (iv) It is possible that a significant fraction of the virions is internalized by a pathway in which low pH inside a vesicle and interaction between the penton base and the vesicular membrane are not required.

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