JOURNAL OF VIROLOGY, Nov. 1991, p. 6061–6070
0022-538X/91/116061-10$02.00/0

Vol. 65, No. 11

Infectious Entry Pathway of Adenovirus Type 2

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Received 19 June 1991/Accepted 16 August 1991

Internalization of the infectious fraction of human adenovirus type 2 into HeLa cells was followed by a quantitative internalization assay. Treatments known to selectively block receptor-mediated endocytosis reduced the internalization of infectious virus to an extent close to the reduction of endocytosis of transferrin. This suggests that one of the first steps in the infectious cycle of adenovirus type 2 is internalization by the coated-pit and -vesicle pathway.

An obligate step for animal viruses to initiate a successful infection is the penetration of the host cell plasma membrane. Enveloped viruses have been described to gain access to the cell by fusion of the viral membrane and the plasma membrane with an accompanying release of the nucleocapsid into the cytoplasm (8, 19). The membrane fusion is mediated by certain glycoproteins on the virus surface, which act either directly on the cell surface, as in the case of, e.g., the members of the *Paramyxoviridae* (2, 40), or after activation by the low pH inside endosomes, as demonstrated for the family *Ortomyxoviridae* (23, 31, 43). In contrast, the mechanisms by which naked viruses enter the cell are much less clear. However, a number of naked viruses appear to employ the method of receptor-mediated endocytosis as the first step in the entry process (1, 28, 32, 42, 51).

Adenoviruses attach to specific receptors on the plasma membrane of HeLa cells (36). The attachment step can easily be separated from the following steps in the process of infection by performing the attachment at a low temperature (45). Subsequent warming of the virus-cell mixture will initiate a synchronous infection. Within 15 min after such a treatment, apparently intact virus particles are found free in the cytoplasm (45). Subsequently, these are vectorially transported to the cell nucleus, where the final uncoating of the particles occurs (9).

The adenovirus penetration of the lipid bilayer has been described to occur according to two alternative pathways: one is by direct penetration of the plasma membrane (4, 33), and in the other the particles are transported to acid vesicles from which the penetration occurs (5, 12, 42, 44, 45). The important role that receptor-mediated endocytosis and acid vesicles play during the entry of adenovirus has been shown indirectly by the adenovirus-mediated enhancement of the toxicity of *Pseudomonas* toxin conjugated to epidermal growth factor or anti-transferrin receptor antibodies (15, 16). Other investigations have shown that chemical cross-linking of the virus and antihexon-mediated neutralization all cause entrapment of virions inside cytoplasmic vesicles (44, 45, 50). However, one of the major obstacles of characterizing the adenovirus entry into HeLa cells is the high particle-to-infectious-unit ratio. Only about 10% of the attached virions are capable of initiating a productive infection (references 27 and 49 and the present investigation). Because biochemical and electron microscopic approaches deal with the bulk population of the virus inoculum, these methods suffer from the obvious limitation that the infectious fraction of the virus may behave differently compared with the observed majority of the virions.

The aim of the present investigation was to study the infectious fraction of the adenovirus inoculum and to monitor its entry into cells and to further establish the role of receptor-mediated endocytosis in a productive virus infection. This was done by combining the capacity of antihexon antibodies to neutralize virions already attached to the cell surface at a low temperature (47) with certain established treatments of cells interfering with receptor-mediated endocytosis, e.g., acidification of the cytosol (7, 11, 37–39) and hypertonic treatment (10, 22). As a marker for the latter event, transferrin was employed. Our results suggest that the infectious fraction of adenovirus type 2 (Ad2), responsible for the progeny virus production, was rapidly internalized by receptor-mediated endocytosis.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells were grown in suspension culture at densities of 2.8×10^5 to 6×10^5 cells per ml in Eagle minimal essential medium (EMEM) (Biological Industries, Beth Haemek, Israel) fortified with 3.5% fetal bovine serum (Flow Laboratories, Irvine, Scotland) and 20 μg of gentamicin (Biological Industries) per ml. The cells were routinely assayed for *Mycoplasma* infections by a Mycoplasma T-C II kit (Gen-Probe, San Diego, Calif.).

Human Ad2 was propagated in HeLa cells maintained in suspension culture. The virions were purified by two successive ultracentrifugations in CsCl. Purified virus was dialyzed, passed through a 0.22-μm-pore-size Millex GV filter, spectrophotometrically quantified (29), and stored frozen at −75°C (14).

Antihexon serum. A monospecific polyclonal antihexon serum was produced in rabbits as described earlier (48) and subsequently heat inactivated before use.

Attachment studies. Virus attachment to HeLa cells was quantified as described earlier (34). Briefly, [^{35}S]methionine-labeled virions at different multiplicities of infection (MOIs) were added to suspensions of cells at a density of 3×10^7 cells per ml in buffers containing 1% bovine serum albumin (BSA). After incubation for 30 min at 4°C, samples were withdrawn, diluted about five times in ice-cold 0.015 M phosphate buffer (pH 7.35)–0.15 M NaCl (PBS), and sedimented. The distribution of radioactivity associated with pellets and supernatants was measured and was used to determine the level of virion attachment.

Quantification of virus infectivity by a hexon immunotitration assay. Determination of virus infectivity was performed

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by a modification of a previously described immunotitration assay (47, 49). Virus-infected HeLa cells (10^7 cells) were transferred from tubes to 100-ml bottles by two washes of 500 μl of EMEM containing 25 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) (Sigma Chemical Co., St. Louis, Mo.) (pH 7.0), 3.5% fetal bovine serum, and 50 μg of gentamicin per ml, and finally 25 ml of EMEM, supplemented as described above, was added to each bottle. After incubation on an orbital shaker platform for 39 h at 37°C, the cells were harvested by centrifugation and ultrasonically disintegrated. The cell homogenates were centrifuged at 12,000 × g_{av} for 15 min to sediment cell debris, and the supernatants were analyzed for hexon antigen by rocket immunoelectrophoresis.

The hexon production was taken as a measure of virus production, since it has been shown that there is a true correlation between the production of progeny virus and the size of the accumulated excess pool of hexons in infected cells (49). Moreover, none of the cell treatments used to inhibit endocytosis affected the ratio between hexons and virions. The maximal hexon production of infected control cells was in the range of 150 to 175 μg/10^7 cells and was in each series of experiments set at 100%. In this system we define one experimental infectious unit as the lowest number of attached virions producing a maximal amount of hexon. However, it should be stressed that this definition of one experimental infectious unit is an operational one, since a maximal hexon yield will require a total input of more than one infectious unit per cell following the Poisson distribution.

Assay for internalization of infectious virus. Virions at different MOIs, as indicated in the figure legends, were added to 1 × 10^7 HeLa cells at a concentration of 3 × 10^7 cells per ml in the appropriate buffers containing 1% BSA. After attachment for 30 min at 4°C, the cells were diluted 10-fold with buffers and unattached virus was removed by centrifugation. The virus-cell mixtures were resuspended and further transferred to 37°C to allow for internalization of the cell-bound virus. At various times, as indicated in the figure legends, 20 volumes of ice-cold PBS were added to stop the process of internalization. The cells were sedimented and resuspended in 300 μl of ice-cold PBS. Different volumes of heat-inactivated antihexon serum, as indicated in the figure legends, were added to the virus-cell mixtures. The mixtures were incubated for 30 min at 4°C and subsequently transferred to 37°C for 10 min. Then the virus-cell mixtures were incubated in 100-ml bottles for 39 h as described above and eventually processed for the quantification of hexon production.

Methods to inhibit endocytosis. Three different methods to inhibit endocytosis were used: two methods which acidify the cytosol of cells (20, 21, 39) and hypertonic treatment of the cells (10, 22). In the first acidification method, cells were exposed to a pulse of NH_4Cl followed by a chase in a sodium-free medium without NH_4Cl. Upon removal of extracellular NH_4Cl, intracellular NH_4^+ dissociates into NH_3, which is membrane permeable and rapidly leaves the cell, and protons, which cannot penetrate the membrane, thereby acidifying the cytosol. Under normal conditions, the Na^+-H^+ exchanger is activated and the cytosolic pH is brought back to normal (41). However, the low pH inside the cell can be maintained by inhibiting the Na^+-H^+ exchanger through the presence of amiloride or by blocking the exchanger simply by excluding sodium ions from the medium (20, 25, 41). We also directly acidified the cytosol via application of a weak acid. Certain weak acids, such as acetic acid, promptly penetrate the plasma membrane in their undissociated forms. In the cytosol, the acid dissociates and thereby decreases the cytoplasmic pH. To provide a high concentration of the membrane-penetrable form of acetic acid, cells were incubated in Ringer's acetate medium [consisting of 80 mM NaCl, 70 mM Na-acetate, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM NaH_2PO_4, 10 mM glucose, 1% BSA, and 25 mM HEPES (pH 6.9) or 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma) (pH 6.5 and 6.3)], which contains a high concentration of acetate. By varying the pH in the medium, it is possible to impose a change upon the intracellular pH (20, 39).

A third way to inhibit the process of endocytosis is by hypertonic treatment of cells (10, 22). This treatment induces several changes in the cell (e.g., causes an increase in protein and divalent-cation concentrations and a lowering of the cytoplasmic pH), but the actual factor that induces the blocking of endocytosis remains uncertain.

Acidification treatments. (i) Ammonia prepulse method. For the ammonia prepulse method (39), cells were washed once with PBS and resuspended to give a density of 10^7 cells per ml in PBS containing different concentrations of NH_4Cl. After incubation for 30 min at 37°C, the cells were sedimented and resuspended in a sodium-free medium, composed of 0.14 M KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM amiloride (Sigma), 1% BSA, and 20 mM HEPES-Tris (pH 7.0), to a concentration of 3 × 10^7 cells per ml. The cells were incubated in the sodium-free medium for 5 min at 37°C before being chilled to 4°C during 5 min.

(ii) Direct acidification method. For the method of direct acidification via application of a weak acid (20, 39), cells were washed once in PBS and resuspended to give a density of 3 × 10^7 cells per ml in Ringer's acetate medium. After incubation for 10 min at 37°C, the cells were chilled to 4°C during 5 min.

Hypertonic treatment. Cells were washed once in PBS and resuspended to give a concentration of 3 × 10^7 cells per ml in Earle's balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) buffered with 20 mM HEPES (pH 7.5), containing 1% BSA and different total concentrations of NaCl. After incubation for 10 min at 37°C, the cells were chilled to 4°C for 5 min.

Cells in all series were further incubated for 30 min at 4°C in a shaking water bath (for virus attachment in the internalization assay). Then the cells were washed once and resuspended in their appropriate media to a concentration of 3 × 10^7 cells per ml and further incubated at 37°C for the purpose of assessment of transferrin, sucrose, and Ad2 internalization.

Assessment of receptor-mediated endocytosis of [^{125}I]transferrin. Iron-free human transferrin (Sigma) was saturated with iron (24), was iodinated with ^{125}I according to the method of Fraker and Speck (17), and yielded a specific radioactivity of 40,000 cpm per μg. The amounts of surface-bound and internalized transferrin were quantified essentially as described by Ciechanover et al. (6). Transferrin (1 to 3 μg) was added to 10^7 cells which had been treated as described above. After incubation for 10 min at 37°C, the cells were washed twice in ice-cold PBS and treated with 1 ml of PBS containing 0.3% (wt/vol) pronase (Boehringer Mannheim Scandinavia AB, Bromma, Sweden) for 1 h on ice. Then the cells were sedimented, and the radioactivity distributed between the pellet and the supernatant was measured.

In reversal experiments, the treated cells were incubated for 15 min at 37°C before being washed once in PBS and

resuspended to give a concentration of 10^6 cells per ml in PBS or bicarbonate-buffered EMEM for treatments with Ringer's acetate and hypertonic media or ammonia prepulsing, respectively. After incubation for 30 min at 37°C, the cells were sedimented and resuspended in PBS to give a density of 3×10^7 cells per ml. Transferrin was added to the cells, and the protocol described above was followed for quantification of surface-bound and internalized transferrin.

Nonspecific binding of [^{125}I]transferrin to the cells, which amounted to 10% of the total binding, was determined by performing the binding assay in the presence of a 250-fold excess of unlabeled transferrin. The background (4°C) and the maximal (37°C) levels of transferrin internalization, measured as pronase-resistant radioactivity, were typically 15 to 20% and 75 to 85%, respectively.

Electron microscopy. HeLa cells in suspension were treated as described in "Methods to inhibit endocytosis." Virions were added to the cells at a MOI of 20,000 particles per cell and incubated at 4°C for 30 min. Then the cells were washed in the appropriate buffers and further incubated for 15 min at 37°C before being washed twice in ice-cold PBS. Samples for electron microscopy were fixed overnight at 4°C with 1% osmium tetroxide in PBS, dehydrated with acetone, and embedded in epoxy resin (agar 100; Agar Scientific, Stansted, United Kingdom). Thin sections were stained with 5% uranyl acetate at room temperature for 45 min and studied with a Philips EM 300 electron microscope working at 60 kV.

Assessment of fluid-phase endocytosis of [^{14}C]sucrose. To examine the effects of the different treatments on the uptake of bulk fluid, we used [^{14}C]sucrose as a fluid-phase marker (10, 30, 46). Cell samples containing 10^7 cells were treated as described above, and 2 μCi of [^{14}C]sucrose (540 mCi/mmol) (Amersham, Aylesbury, England) was added to each sample before being transferred to 37°C. At various times, as indicated in the figures, samples containing 2×10^6 cells were withdrawn from the cell-sucrose mixture and diluted and sedimented twice in ice-cold PBS, and then the radioactivity confined to the cells was measured. For each series, a background value for nonspecific cellular association of radioactivity was obtained immediately after the mixing of cells with [^{14}C]sucrose. For control cells, such background values never exceeded 10% of the radioactivity associated with cells incubated for 5 min at 37°C.

Assessment of cellular viability. The tetrazolium salt (MTT) method as described by Edmondson et al. (13) was employed for the assessment of cellular proliferation rates and the cellular ability to reestablish monolayer cultures after the various treatments influencing endocytosis. In the proliferation rate assays, 2,200 cells in 50 μl were seeded into six wells per sample series in four separate plates. Medium containing 5% fetal bovine serum and fortified with nonessential amino acids was added to give final volumes of 200 μl per well. The plates were incubated at 37°C in an atmosphere of 5% CO_2. One plate was removed every 24 h and assayed for the production of purple formazan. Readings were made at 570 nm in a Dynatech MR 600 microplate reader (Dynatech Instruments Inc., Torrance, Calif.). To monitor the viability status of cells within 1 h after the various treatments, 5×10^5 cells per series were removed, diluted with 5 ml of PBS, and sedimented at $150 \times g$ for 5 min; the supernatants were aspirated, and the cellular pellets were suspended in 100 μl of PBS containing 5 mg of MTT per ml. The cells were incubated in the dark at 37°C for 3 h. Five milliliters of PBS was subsequently added, and the cells were sedimented at $500 \times g$ for 5 min. The supernatants were removed, and the pellets were dissolved in 1 ml of acidified isopropanol and read at 570 nm.

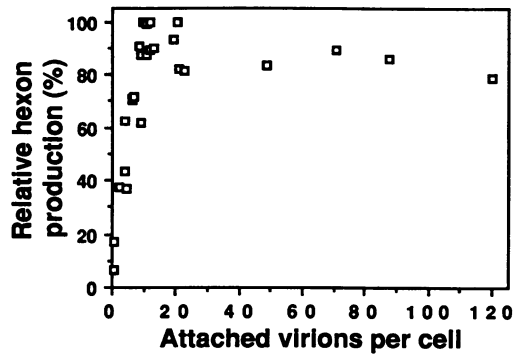

FIG. 1. Hexon production in response to the number of attached virions. HeLa cells were incubated with ^{35}S-labeled virions at different MOIs ranging from 5 to 1,200 virions. After attachment, the cells were cultivated for 39 h at 37°C and subsequently harvested and analyzed for hexon production by the immunotitration method as described in Materials and Methods. The actual number of virions attached per cell was monitored by measuring the radioactivity associated with cells removed from samples destined for infectivity titration. Maximal hexon production was set at 100%.

RESULTS

Assay for internalization of infectious virus. To quantitatively monitor the internalization of the infectious fraction of Ad2 into HeLa cells, we developed a virus internalization assay. The assay takes advantage of the facts that antihexon antibodies can neutralize already-attached virions at the cell surface and that the cell membrane prevents antibodies from entering the interior of the cell, thereby protecting virions which already have been internalized. Then by allowing one infectious cycle to proceed, the virions which have been internalized before the addition of neutralizing antibodies produce progeny virus as well as viral antigens. The amount of hexons produced by such virus-infected cells mirrors the actual amount of internalized virus at the time of antibody addition under the specified conditions, in which not more than about one infectious unit is attached to the cells. To establish the amount of virus needed to reach one experimental attached infectious unit, cells were incubated with virions at different MOIs at 4°C to minimize internalization. After 30 min an aliquot was removed to measure the level of virion attachment, and the rest of the cells were washed and further incubated for hexon production. Attached virions in the interval of 1 to 10 particles per cell gradually increased the production of hexon, and at 10 to 12 attached virions (corresponding to an added MOI of 70 to 85 virions) a plateau was reached in the hexon production, indicating that all cells were infected (Fig. 1).

The addition of 30 μl or more of an antihexon serum to cells with 9 to 10 attached virions (i.e., about one experimental infectious unit) was highly efficient in neutralizing the infectivity of 90 to 95% of the cell surface-bound virions (Fig. 2).

To study the internalization kinetics of cell-bound Ad2, cells with 9 to 10 attached virions were incubated at 37°C to allow internalization of virions. After different periods of time, the internalization of virions was interrupted and 80 μl of heat-inactivated antihexon serum was added to each mixture to neutralize virus still remaining at the cell surface.

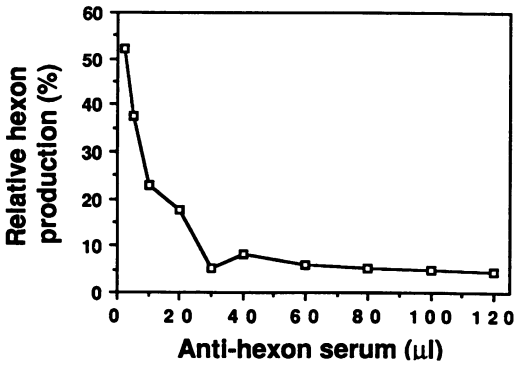

FIG. 2. Neutralization of virions attached to HeLa cells. Virions were allowed to attach at 4°C to yield 9 to 10 attached particles per cell. Unattached virions were removed by sedimentation of the cells, and different volumes of a neutralizing monospecific polyclonal rabbit antihexon serum (ranging from 2.5 to 120 μl) were added in a final volume of 380 μl of PBS. Finally, complete cell culture medium was added to each sample, and at 39 h postinfection, the amount of synthesized hexons was quantified by the immunotitration method described in Materials and Methods. The hexon production of infected control cells without administration of antihexon serum was set at 100%.

Antihexon serum added after 10 to 15 min was essentially unable to protect the cells against the attached virus, indicating that all infectious particles were internalized by this time (Fig. 3).

Consequently, in all of the following internalization assays, in which interference with endocytosis was anticipated, virions were added to obtain 9 to 10 attached virions per cell and the virions were allowed to be internalized for 15 min before the addition of antibodies.

Significance of receptor-mediated endocytosis in the entry pathway of Ad2. In order to investigate whether the productive infection by Ad2 requires endocytosis from coated pits, HeLa cells were treated according to different methods known to inhibit the endocytotic pathway. The different experiments were done in parallel with Ad2 and transferrin, the latter being a well established marker for endocytosis by the coated-pit and -vesicle pathway. Thus, the conditions required for the different treatments to inhibit the endocytotic process in HeLa cells were monitored with transferrin as a marker.

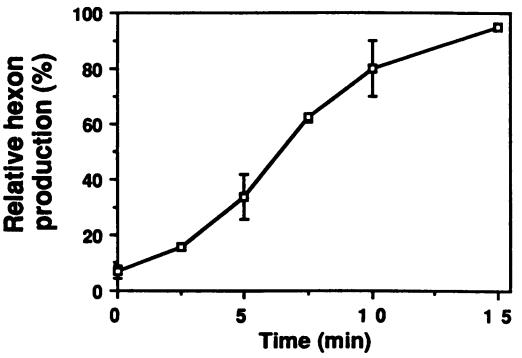

FIG. 3. Kinetics of infectious virus internalization. HeLa cells were incubated with virions, yielding 9 to 10 attached particles per cell. After removal of unattached virions, the virus-cell mixtures were transferred to 37°C. At different times (ranging from 0 to 15 min), the internalization process was stopped by the addition of ice-cold PBS. After sedimentation of the cells, 80 μl of a neutralizing antihexon serum was added to the mixtures in a final volume of 380 μl of PBS. Finally, complete cell culture medium was added to each sample, and at 39 h postinfection, the amount of synthesized hexons was quantified by the immunotitration method described in Materials and Methods. The hexon production of infected cells without the administration of antihexon serum was set at 100%. The mean values from three separate experiments are shown, with vertical bars indicating the standard deviations.

In one series of experiments, cells were prepulsed with different concentrations of NH_4Cl and then chased in a medium containing amiloride but lacking NH_4Cl and sodium ions. These treatments cause an acidification of the cell cytosol, thus inhibiting endocytosis. It was shown that preincubation of the cells in PBS containing concentrations of NH_4Cl above 20 mM reduced the actual endocytosis of transferrin by 75% or more (Fig. 4A); this is in good correlation with other studies (39). Treatments with only NH_4Cl or sodium-free buffer containing amiloride alone had no effect on the endocytosis of transferrin (data not shown). For further studies, an NH_4Cl concentration of 30 mM was chosen. At this concentration, endocytosis of transferrin was reduced to 18% of that of controls, and upon reversal for 30 min at 37°C in bicarbonate-buffered EMEM, the endocytosis recovered to 70% ± 20% (standard deviation) ($n = 3$) of the actual control value. However, treated cells recovered poorly in only PBS (data not shown), indicating that the cells were dependent on the carbonate buffer to stabilize the intracellular pH (26). This suggested that amiloride displayed irreversible effects on the Na^+-H^+ exchanger. The internalization of the infectious fraction of Ad2 was strongly reduced by the ammonia prepulse method (Fig. 4B). In treated cells, in which surface-bound virions were neutralized by the antihexon serum, a reduction by 76% of virus internalization was demonstrated. Treated control cells without addition of the antihexon serum revealed a reduction in hexon production by 45% compared with production in untreated control cells. This also indicates that the treatment per se affected the ability of the cells to produce progeny virus.

In other experiments, HeLa cells were exposed to Ringer's acetate medium at different pHs, which induce different degrees of acidification of the cytosol. The endocytosis of transferrin was essentially unaffected at pH 6.9 (Fig. 5A). Upon lowering the pH to 6.5 and 6.3, the endocytosis decreased and revealed a reduction to 51 and 27%, respectively, compared with the level of endocytosis in control cells. The effects on endocytosis of transferrin were fully reversible for cells treated at pH 6.5 when transferred to PBS. In contrast, cells treated at pH 6.3 recovered only to 55% ± 10% (standard deviation) ($n = 3$) of the actual control level of endocytosis after reversal in PBS for 30 min. The internalization of Ad2 in the acetate-treated cells mainly mirrored the behavior of transferrin, displaying a gradual decrease as the pH in the medium was lowered (Fig. 5B). There was an 18% reduction in hexon production at pH 6.9, and when the pH of the medium was further decreased to 6.5 and 6.3, the hexon yield dropped by 40 and 52%, respectively. The true reduction was probably greater, since the results depend on the amount of actually attached virus. In the two low-pH buffers, the attachment level increased by 20 to 25% compared with that in control cells in PBS, thus causing more than one experimental infectious unit per cell to become attached. This increase in virus input might affect the hexon production in cells in which the virus internalization is partly blocked but not the hexon yield in control cells already maximally infected. Virus-infected cells treated at

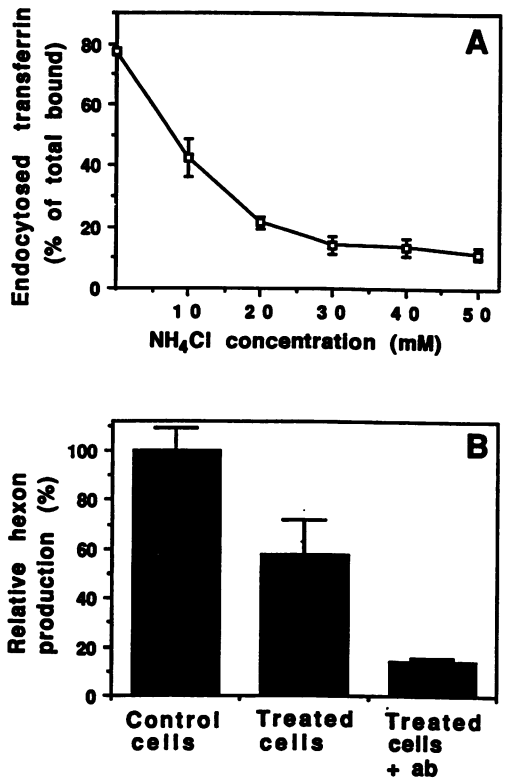

FIG. 4. Internalization of transferrin and Ad2 in ammonia-prepulsed cells. (A) HeLa cells in suspension were treated with different concentrations of NH_4Cl (ranging from 10 to 50 mM) as described in Materials and Methods. Samples containing 10^7 treated cells were incubated for 10 min at 37°C in the presence of 2 to 3 μg of [^{125}I]transferrin in the appropriate buffers. Then the cells were washed and incubated with pronase to release transferrin still remaining at the cell surface. The radioactivity associated with the cell pellet after pronase treatment was documented as internalized transferrin. The plot shows the relative distribution of 3,000 to 4,000 cpm per sample. Mean values from four separate experiments are shown, and bars indicate the standard deviations. (B) Cells were prepulsed with 30 mM NH_4Cl, and virions were allowed to attach, yielding 9 to 10 attached virions per cell. The virions were internalized for 15 min, and then the culture was divided into two parts. To one of these, 80 μl of an antihexon serum was added, as described in Materials and Methods, to neutralize virions remaining at the cell surface. The other part of the culture received only PBS and was used as a control of the ability of the treated cells to produce hexons. After one infectious cycle, the amount of hexon was quantified as described in Materials and Methods. ab indicates the sample which received neutralizing antihexon antibodies. Mean values from four separate experiments are shown, and bars indicate the corresponding standard deviations. The hexon production of infected control cells was set at 100%. Addition of neutralizing antibodies to untreated control cells at 15 min postinfection never provoked a neutralization greater than 5%.

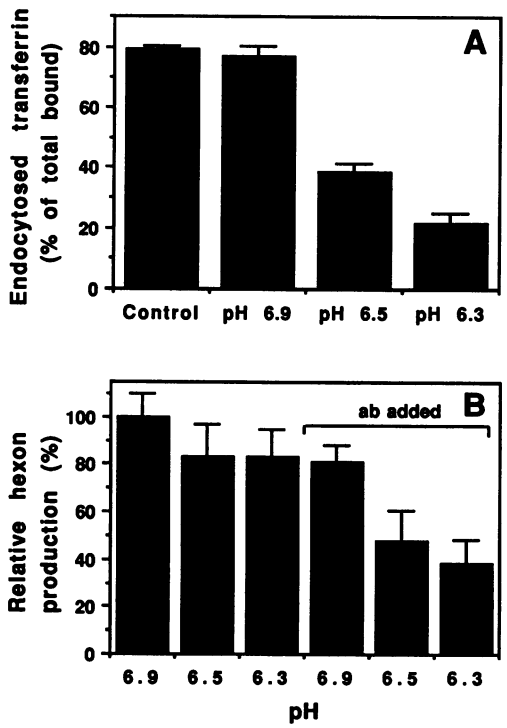

FIG. 5. Internalization of transferrin and Ad2 in acetate-treated cells. (A) HeLa cells in suspension were treated with Ringer's acetate medium of different pHs (6.9, 6.5, and 6.3) as described in Materials and Methods. Samples containing 10^7 treated cells were incubated in the presence of 2 to 3 μg of [^{125}I]transferrin in the appropriate buffers. The cells were further processed for the quantification of internalized transferrin as described in Materials and Methods. The plot shows the relative distribution of 3,000 to 4,000 cpm per sample. Mean values from four separate experiments are shown, and bars indicate the standard deviations. (B) Virions were allowed to attach to acetate-treated cells, yielding 9 to 10 attached virions per cell. Virus internalization was interrupted after 15 min, and then each culture of the three-pH series was divided into two parts. To one of these, 80 μl of an antihexon serum was added as described in Materials and Methods. The other part of the culture received only PBS and was used as a control of the ability of the treated cells to produce hexons. After one infectious cycle, the amount of hexon was quantified as described in Materials and Methods. ab indicates those samples which received neutralizing antibodies. Mean values from four separate experiments are shown, and bars indicate the corresponding standard deviations. The hexon production of untreated, infected control cells was set at 100% and is not included in the figure. Addition of neutralizing antibodies to untreated control cells at 15 min postinfection never provoked a neutralization greater than 5%.

pH 6.9 but without the addition of antibodies showed no reduction in hexon production compared with that in control cells in PBS (data not shown).

Cells treated with hypertonic media containing a total concentration of 0.33 and 0.45 M NaCl revealed, compared with control cells, a reduction of endocytosis of transferrin by 69 and 72%, respectively (Fig. 6A). Upon removal of the salt, the endocytosis returned to normal within 30 min at 37°C. Although recovering after the hypertonic treatments, cells which were exposed to the highest salt concentration had a tendency to form some aggregates during the incubations at 4°C. These aggregates disappeared when the excessive salt was removed or when the cells were resuspended during the experimental handling. The internalization of the infectious fraction of Ad2 decreased by 46 and 77% for the 0.33 and 0.45 M NaCl series, respectively, compared with that for the appropriate salt-treated control series (Fig. 6B). The reduced levels of hexon production due to the mere salt treatments were 45 and 81% for the 0.33 and 0.45 M NaCl series, respectively. This blockage in antigen production was not due to a lower degree of virus attachment (data not shown), nor did the hypertonicity affect the attached virions,

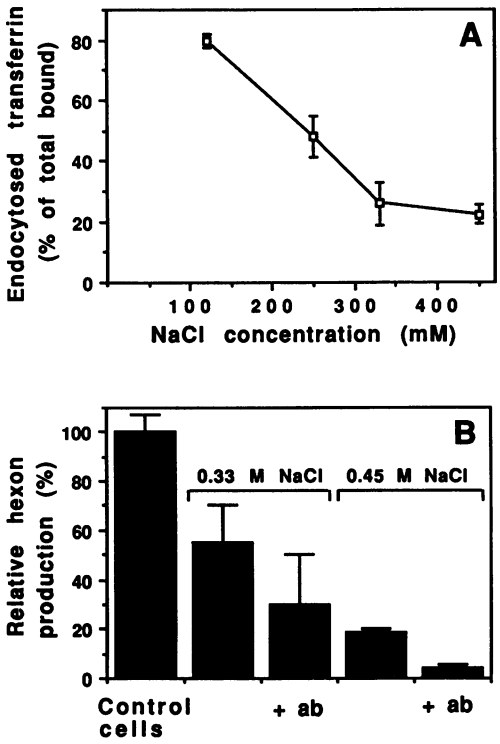

FIG. 6. Internalization of transferrin and Ad2 in hypertonically treated cells. (A) HeLa cells in suspension culture were treated with different total concentrations of NaCl (0.12, 0.25, 0.33, and 0.45 M) as described in Materials and Methods. Samples containing 10^7 treated cells were incubated in the presence of 2 to 3 μg of [^{125}I]transferrin in the appropriate buffers, and the cells were further processed for the quantification of internalized transferrin as described in Materials and Methods. The plot shows the relative distribution of 3,000 to 4,000 cpm per sample. Mean values from four separate experiments are shown, and bars indicate the standard deviations. (B) Virions were attached to salt-treated (0.33 and 0.45 M NaCl) cells to yield 9 to 10 attached particles per cell. Virus internalization was interrupted after 15 min, and then each culture of the two-salt series was divided into two parts. To one of these, 80 μl of an antihexon serum was added, as described in Materials and Methods. The other part of the culture received only PBS and was used as a control of the ability of the treated cells to produce hexons. After one infectious cycle, the amount of hexon was quantified as described in Materials and Methods. ab indicates those samples which received neutralizing antibodies. Mean values from four separate experiments are shown, and bars indicate the corresponding standard deviations. The hexon production of infected control cells was set at 100%. Addition of neutralizing antibodies to untreated control cells never provoked a neutralization greater than 5%.

since cells reverted for 30 min at 37°C in PBS prior to infection still showed a similar reduction in virus production when infected with virus (data not shown).

Distribution of cell-associated virions. To monitor the cellular distribution of virions attached to HeLa cells and corroborate the infectivity data, an electron microscopic investigation was performed (Table 1 and Fig. 7). Virions were allowed to attach to pretreated cells at 4°C, and the virus-cell mixtures were further incubated for 15 min at 37°C before being processed for electron microscopy.

Treatments of cells by a 30 mM NH_4Cl prepulse or 0.33 M NaCl yielded a large fraction of the virions remaining at the cell surface (Fig. 7B and D). For both treatments, about 30 to

TABLE 1. Cellular distribution of Ad2 particles in HeLa cells

Treatment	Relative distribution of virus particles (%)[a]		
	On surface	In vesicles	Free in the cytoplasm
Control	12	10	78
Hypertonic (NaCl)			
0.33 M	65	24	11
0.45 M	30	61	9
Ammonia prepulsing (30 mM NH_4Cl)	85	5	10
Ringer's acetate medium			
pH 6.9	46	40	14
pH 6.5	35	53	12
pH 6.3	55	39	6

[a] In each series, ca. 10 cells were examined and the relative distribution of ca. 110 counted virions was determined.

40% of the virions associated with the plasma membrane were found in structures that seemed to be coated pits. However, the coated pits of the hypertonically treated cells were smaller and less pronounced compared with those found on the ammonia-prepulsed cells. In the former cells, virions were often found in pits without coating. In cells acidified by the ammonia prepulse method, only 15% of the virions were internalized, and of this population, about one-third was found in vesicles that appeared coated. Cells treated with the high-salt media displayed a large fraction of the virions within vesicles (Fig. 7D and E). In the 0.33 M NaCl series, about half of the virion-containing vesicles were coated, the rest being large vesicles in the vicinity of the cell surface. Surprisingly, most of the virions in cells treated with medium containing 0.45 M NaCl were found in such vesicles. Occasionally, virions found in the large vesiclelike structures appeared to be associated with coated pits.

Cells incubated in Ringer's acetate medium revealed virions mainly on the cell surface and enclosed within vesicles. Although this pattern was independent of the pH of the medium, the fraction of virions residing within coated pits or coated vesicles seemed to increase with decreasing pH (Fig. 7C).

Bulk fluid-phase endocytosis of sucrose. To investigate whether the different treatments were able to selectively block either receptor-mediated endocytosis, which is exclusively derived from coated regions of the cell membrane, or bulk fluid uptake, which seems to occur mainly from uncoated regions, we monitored the endocytosis of sucrose—a bulk fluid-phase marker. Prepulsing with 30 mM NH_4Cl, which inhibited receptor-mediated endocytosis of transferrin and Ad2, still allowed a significant level of fluid-phase endocytosis (Fig. 8). All other treatments inhibited the uptake of [^{14}C]sucrose, as shown by assays carried out under conditions that repressed receptor-mediated endocytosis of transferrin. Interestingly, Ringer's acetate medium at pH 6.9 inhibited to some extent the bulk fluid uptake but apparently allowed the internalization of transferrin as well as the infectious fraction of Ad2.

Assessment of cellular viability. To monitor the immediate viability status of the cells after the various treatments, cells were removed and assayed according the MTT method. Cells obtained from the different series were also seeded onto microtiter plates, and their proliferative properties were monitored for 4 days. Cells treated according to the ammonia prepulsing method including amiloride displayed a reduced response in the MTT assay within 1 h after treatment (Table 2). The proliferative properties were also impaired, and the growth rate was only 25% of that of the control

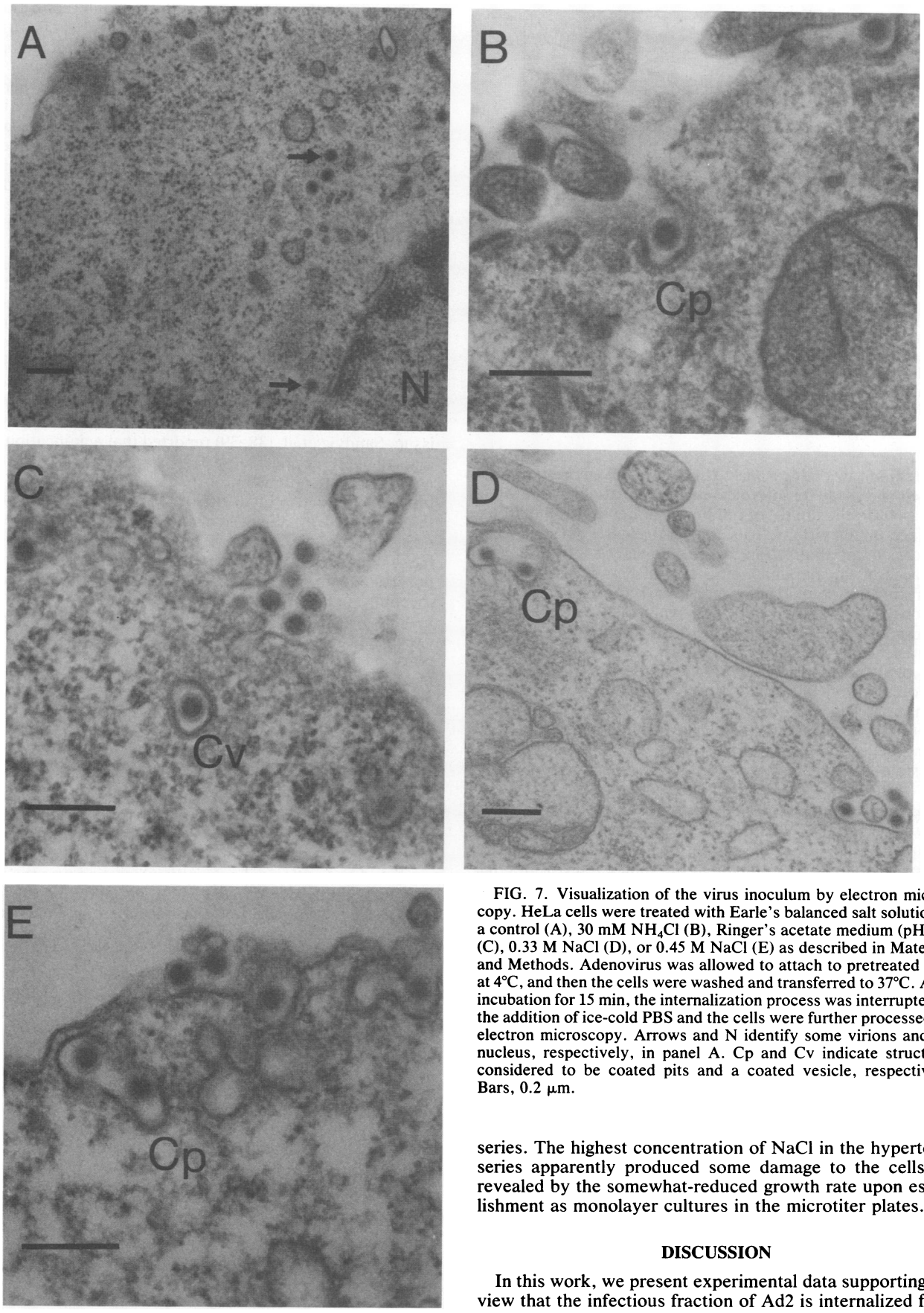

FIG. 7. Visualization of the virus inoculum by electron microscopy. HeLa cells were treated with Earle's balanced salt solution as a control (A), 30 mM NH_4Cl (B), Ringer's acetate medium (pH 6.3) (C), 0.33 M NaCl (D), or 0.45 M NaCl (E) as described in Materials and Methods. Adenovirus was allowed to attach to pretreated cells at 4°C, and then the cells were washed and transferred to 37°C. After incubation for 15 min, the internalization process was interrupted by the addition of ice-cold PBS and the cells were further processed for electron microscopy. Arrows and N identify some virions and the nucleus, respectively, in panel A. Cp and Cv indicate structures considered to be coated pits and a coated vesicle, respectively. Bars, 0.2 μm.

series. The highest concentration of NaCl in the hypertonic series apparently produced some damage to the cells, as revealed by the somewhat-reduced growth rate upon establishment as monolayer cultures in the microtiter plates.

DISCUSSION

In this work, we present experimental data supporting the view that the infectious fraction of Ad2 is internalized from

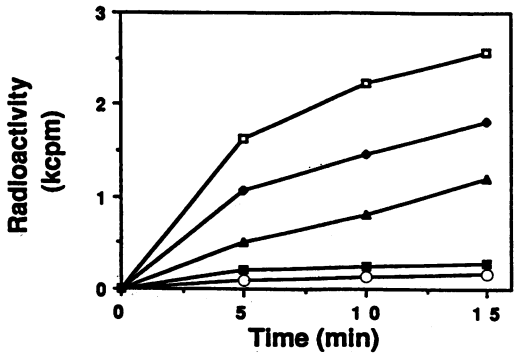

FIG. 8. Fluid-phase uptake of [^{14}C]sucrose. Cells were treated with 30 mM NH_4Cl (◆), Ringer's acetate medium (pH 6.9 [▲] or pH 6.5 [■]), or 0.33 M NaCl (○) as described in Materials and Methods. To each sample containing 10^7 cells, 2 μCi of [^{14}C]sucrose was added. At the indicated times, 2×10^6 cells were withdrawn and washed before the radioactivity associated with the cell pellet was monitored. Open squares represent the sucrose uptake of untreated control cells.

coated pits by endocytosis. This result heavily relies on the developed assay for internalization of infectious virus, which is based on the ability of antihexon antibodies to neutralize surface-bound virions. This assay has several benefits: (i) the process of internalization can be studied at a low virus input because of the amplification accomplished by virus replication, (ii) the internalization of the infectious fraction of the virions can be monitored, (iii) when cells are treated to accomplish a blockage of endocytosis, the treatments can be limited to the time before antibody addition, thus avoiding the need for nonphysiological conditions during viral replication, and (iv) by always comparing the hexon production from treated cells with or without the addition of antibodies, the obtained results will be derived from living and metabolizing cells, thereby avoiding the risk of overestimating a possible lack of internalization due to impaired viability of the cells following the various treatments.

After attachment to the cell, the virions were rapidly cleared from the cell surface, and after 6 to 7 min, half the amount of the surface-bound virions was internalized. This time schedule is very similar to the time course of internalization presented for Semliki Forest virus, which is a well-studied model for an enveloped animal virus that enters the host cell by an endocytosis-dependent mechanism (18).

TABLE 2. Viability of the treated cells

Treatment	Relative MTT reading 1 h after treatment (%)	Relative rate of cellular proliferation (%)[a]
Control	100	100
Hypertonic (NaCl)		
0.33 M	88	96
0.45 M	72	83
Ammonia prepulsing (30 mM NH_4Cl)	59	25
Ringer's acetate medium		
pH 6.9	67	87
pH 6.5	84	100
pH 6.3	78	100

[a] The rates of cellular proliferation were calculated from four measurements performed over a 93-h period postseeding onto microtiter plates.

To examine the mechanism of the Ad2 internalization into HeLa cells, we studied the virus entrance in cells treated according to different methods that have been developed to selectively block receptor-mediated endocytosis. These treatments utilize cytoplasmic acidification and hypertonicity (7, 10, 11, 21, 22, 38, 39). They have been described to produce changes in the distribution of clathrin, the protein which forms the coat around coated pits and coated vesicles (3). Acidification has been suggested to induce precipitation of clathrin, which causes a stabilization of coated pits, leading to a subsequent paralysis of the receptor-mediated endocytosis (21, 39). Because of this, ligands are entrapped within the coated pit as normal but the pit is unable to proceed further and deliver the ligand to the subsequent compartments. Hypertonicity appears to arrest the formation of the coated pits by a mechanism similar to intracellular potassium depletion that leads to polymerization of the clathrin into abnormal microcages, rendering the coated pits smaller and more flattened than normal and unable to support further endocytosis (22). However, whether the fluid-phase uptake is inhibited under the same conditions as receptor-mediated endocytosis seems to be a controversial issue. Sandvig et al. (38, 39) reported that acidification of the cytosol of HeLa cells below pH 6.5 by the ammonia prepulse method inhibits the receptor-mediated endocytosis of transferrin, epidermal growth factor, and Shiga toxin, whereas the fluid phase uptake of ricin and lucifer yellow proceeds as normal. Moreover, Daukas and Zigmond (10) found that leukocytes treated with hypertonic medium still display a normal level of the bulk fluid uptake of sucrose although the receptor-mediated endocytosis is inhibited. On the other hand, when a fibroblast cell line, mutated in the Na^+-H^+ exchanger, is acidified, both receptor-mediated and fluid-phase endocytosis are affected (7, 11), and hypertonically treated fibroblasts reveal both inhibition of receptor-mediated and fluid-phase endocytosis (22). In our hands, acidification by the ammonia prepulse method selectively inhibited only the receptor-mediated endocytosis whereas acidification by acetate and hypertonicity inhibited both endocytotic pathways. To obtain the anticipated inhibition, these results may suggest that not only the degree of cytosolic acidification but also the means to achieve this effect are critical.

All three methods inhibited the infectious entry of Ad2 when assayed under conditions that inhibited receptor-mediated endocytosis. Especially interesting was the finding that the ammonia prepulse method inhibited the entry, suggesting that the virions were dependent on the coated-pit and -vesicle pathway for proper internalization. This finding was also supported by the localization of the virions when visualized by electron microscopy. Most of the virions were left at the cell surface, and a large fraction of these was entrapped within coated pits which evidently were unable to pinch off from the plasma membrane. The ammonia treatment was the only procedure causing severe damage to the cells, as revealed by the MTT assay. However, this adverse effect seemed to occur only after a rather long time span after treatment, since the cells could at least partly resume the endocytotic activity as well as partly support the virus infection.

Both the acetate and the high-salt (0.45 M NaCl) treatments produced somewhat contradictory results when assayed by electron microscopy as compared with the assay of internalization. In the case of the high-salt treatment, virions were located within large vesicles in the vicinity of the cell surface. Some of these virions were found associated with coated-pit-like structures. This could indicate that the larger

vesicles were salt-induced invaginations of the plasma membrane. Perhaps the large vesicles represent a dead end in the infectious pathway, since virions attached at hypertonically treated cells, but without addition of antihexon serum, were unable to support a normal infection in spite of an almost-normal viability of the cells in the MTT assay. This could obviously also be an effect of the combined stress of salt treatment and virus infection. In the case of acetate treatment, direct visualization by electron microscopy revealed no essential differences in the distribution of virions in cells treated at the different pHs. These contradictory results may be explained by the following interpretations. (i) Possibly the pH 6.9 treatment to some extent affected the endocytosis, as revealed by the slightly reduced internalization of transferrin. This somewhat-lessened endocytotic activity allowed the small amount of virus particles used in the internalization assay to become internalized. However, the high MOI employed in the electron microscopy series might have overloaded the system, leaving a substantial amount of virions at the cell surface. (ii) Virus observed inside cytoplasmic vesicles in the pH 6.5 and 6.3 series may represent a noninfectious pathway or a cellular compartment which still has contact with the cell surface. Both the high-salt and acetate treatments seemed to accomplish a vesicularization of the area below the cell surface. When studying these vesicles by electron microscopy, it can be difficult to determine whether they are true vesicles or invaginations of the plasma membrane. This has been exemplified by the finding that most coated vesicles residing close to the cell surface in fact are coated pits when examined by serial section analysis (35).

In this investigation, we have monitored both the fate of infectious adenovirus, as measured by the assay of internalization of infectivity, and the bulk population of virions, as observed by electron microscopy of the incoming virus in HeLa cells. After attachment both the bulk population and the infectious fraction of the virions rapidly became internalized, apparently by the coated-pit and -vesicle pathway, suggesting a further delivery of the virions to the endosomal compartment. In this acidic environment, the hypothetical pH-dependent conformational change of the capsid may occur, which has been suggested to mediate the penetration of the membrane and the subsequent delivery of the destabilized virus into the cell cytoplasm.

ACKNOWLEDGMENTS

The excellent technical assistance performed by Susanna Ekholm-Jensen, who participated in this work for the partial fulfillment of the requirements for a B.S., is appreciated. The competent maintenance of the cell cultures by Blanka Boberg is also acknowledged. Preparation of the samples for electron microscopy was kindly performed by Ulla Wulf.

This work was financially supported by grants to E.E. from the Swedish Natural Science Research Council; Anders Otto Swärd's Foundation, Stockholm, Sweden; Carl Trygger's Foundation, Stockholm; The Crafoord Foundation, Lund, Sweden; and the Royal Physiographic Foundation, Lund.

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