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Adenovirus Uncoating and Nuclear Establishment Are Not Affected by Weak Base Amines

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We have used four established lysosomotropic agents, ammonium chloride, amantadine, chloroquine, and methylamine, to monitor the possible interference with an early low-pH-dependent step during adenovirus replication. Two concentrations of each of the different agents were selected; one was essentially nontoxic to uninfected HeLa cells, and the other resulted in some toxicity as measured by trypan blue staining and by interference with cell monolayer establishment, cell proliferation, and radioisotope labelling. It was separately determined that these concentrations displayed pH-raising effects of the same magnitude as higher concentrations previously used in similar studies. Adenovirus uncoating in vivo, normally reaching its maximum within 1 h after infection, was not affected by any of the agents. The subsequent levels of successful nuclear entry events by the parental genomes were monitored by measuring the extent of transcription of an mRNA species coding for the early 72-kDa DNA-binding protein at 10 to 12 h postinfection. In HeLa, KB, HEp-2, and A549 cells, none of the agents were able to affect the levels of early transcription after administration at the point of infection or at 3 h after infection. The cumulative synthesis of the hexon antigen was assessed late in infection, and inhibitory effects were revealed upon administration of 10, 20, and 40 mM ammonium chloride, 10 mM methylamine, and 0.5 mM amantadine, irrespective of the time point of addition. Ammonium chloride at 5 mM reduced the hexon yield by 20% at the most when added within 50 min after infection. Chloroquine at concentrations of 2.5 and 5 μM specifically reduced the hexon yields by 30 to 40% when administered within the first 50 min of infection. On the basis of the lack of effects of nontoxic concentrations of the four agents on the early virus-cell interactive event of uncoating and the early virus-specified transcription, we conclude that a low-pH-dependent step early in the adenovirus replication cycle is not mandatory for a successful infection.

Animal viruses display an impressive versatility of mechanisms by which the virions are internalized and eventually gain access to the cytoplasm. A number of enveloped viruses may initiate a pH-independent external fusion process at the plasma membrane level, as exemplified by members of the families *Paramyxoviridae*, *Coronaviridae*, *Retroviridae*, and *Herpesviridae* (26, 31, 40, 41). On the other hand, viruses of the families *Togaviridae* and *Orthomyxoviridae* will, upon endocytotic uptake, penetrate intracellular endosomal membranes first when triggered by the low-pH conditions of these vesicular organelles (26, 31, 40). Treatment of cells with a number of weak-base primary amines will cause an immediate increase of the endosomal and lysosomal pH primarily as a consequence of proton trapping (33, 38, 44). Such agents have been used to unequivocally demonstrate that the enveloped viruses, e.g., Semliki Forest virus (25), vesicular stomatitis virus (35), arenaviruses (7, 14, 20), and rabies virus (61), all need low-pH conditions early in infection in order to force the endosomal membrane and thereby become translocated into the cytoplasmic compartment.

The receptor-mediated endocytotic uptake of the naked viruses of the families *Picornaviridae* and *Adenoviridae* is also followed by a step of endosomal membrane traverse (26, 31, 40). However, the mechanisms by which these processes occur are less well understood. A wealth of observations in studies using lysosomotropic agents have indicated a low-pH-dependent step early during poliovirus (28, 29, 69), human rhinovirus 2 (30), and foot-and-mouth disease virus infections (4, 10). The lysosome-mediated uncoating step of reoviruses (57) and an early stage during the replication of the birnavirus infectious pancreatic necrosis virus (18) are also affected by the pH-raising agents. However, recent studies using the antibiotic bafilomycin A1, an inhibitor of vacuolar proton-ATPases (8), have revealed that the replication of polioviruses is not inhibited when treatment with this agent is applied early in infection (42). Also, the process of poliovirus uncoating does not seem to rely on acidic pH conditions early in infection (22). Thus, the view has become increasingly attractive that a retained energy-requiring pH gradient and/or membrane potential, rather than a low-pH endosomal environment per se, is important for the subsequent endosomal release of a number of viruses (9, 43).

The naked and icosahedrally shaped adenovirus types 2 and 5 (Ad2 and Ad5), with a diameter of 70 nm, are composed of 12 to 13 unique polypeptides (62). The topography and neighbor relationships of these structural units are continuously elucidated at an increasingly higher resolution (55, 56). Such findings together with the recent identification of the pentavalent Arg-Gly-Asp (RGD) sequence of the penton base (3), responsible for the interaction with cell surface integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (27, 32, 66) of the host cell, now offer new challenges in creating better hypotheses as to how these viruses carry out the final step of entrance into the cytoplasmic compartment. The bulk population of the physical particles together with the actual infectious fraction of adenoviruses are internalized by means of receptor-mediated endocytosis (11, 19, 60, 64). Once inside the cell, but still confined to endosomal vesicles, the virions encounter the next obstacle of how to traverse the endosomal membrane. A number of adenovirion-vesicle and virion-cell interactive studies (6, 49, 52, 53) together with coinfection experiments using Ad2 virions and an epidermal

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growth factor-*Pseudomonas* exotoxin (EGF-PE) conjugate (19, 50, 51) have implicated the low-pH-induced conformational change of the penton base (54) as the mechanism responsible for the low-pH-dependent puncturing process leading to the release of virions into the cytoplasm (39).

The lysosomotropic (acidotropic) agents ammonium chloride, amantadine, chloroquine, and methylamine all function by the same mechanism but may display different side effects and toxicity profiles (12, 48). However, these substances are established as pH-raising compounds, and their function as such is well documented as inhibitors of endosomal release of a number of enveloped and naked viruses. We therefore wanted to examine the possible effects of these substances at controlled concentrations of toxicity on the process of adenovirus uncoating and also on adenovirus-specified early transcriptional and late translational events as a means to assess the interference with the efficiency of parental virion establishment in the nuclear compartment.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells were grown and maintained in suspension culture at densities of 2.8×10^5 to 6×10^5 cells per ml in Eagle's minimal essential medium designed for suspension cultures (S-MEM; Flow Laboratories, Irvine, Scotland) fortified with 3.5% fetal bovine serum (FBS; HyClone, Logan, Utah) and 20 μg of gentamicin (Biological Industries, Beth Haemek, Israel) per ml. The cells were routinely assayed for *Mycoplasma* infections by a Mycoplasma T-C II kit (Gen-probe, San Diego, Calif.). KB cells (Flow Laboratories), HEp-2 cells (kindly provided by B. Sjögren, Department of Tumor Immunology, University of Lund), and A549 cells (kindly provided by M. Oberg, Department of Virology, University of Lund) were grown as monolayers at 37°C in Eagle's minimal essential medium supplemented with 10% FBS. Human Ad2 was propagated in HeLa cells maintained in suspension culture. $^{32}P_i$-labelled and unlabelled virions were prepared and purified as described earlier (64).

Lysosomotropic agents. Stock solutions of ammonium chloride (1 M) and chloroquine diphosphate (100 mM) (Sigma Chemical Co., St. Louis, Mo.) were prepared in phosphate-buffered saline (PBS; 0.14 M NaCl and 3 mM KCl in 0.01 M phosphate buffer [pH 7.4]), 100 mM amantadine-HCl (Sigma) in bidistilled water, and 1 M methylamine-HCl in 0.15 M NaCl, titrated with HCl to give pH 6.3. All solutions were passed through 0.22-μm-pore-size Millex-GV filters (Millipore, Molsheim, France) and stored at 4°C.

Assay of toxicity by cell proliferation in suspension culture and trypan blue exclusion. A calculated number of HeLa cells was sedimented and transferred to a volume of 50 ml of S-MEM with 3.5% FBS. From this stock suspension, 10-ml samples (yielding 8.25×10^7 cells) were removed and added to 500-ml spinner flasks containing 240 ml of S-MEM, 3.5% FBS, and different concentrations of the agent under investigation. A positive control series without agent addition was always included. The cell densities were measured every 24 h in a Bürker counting chamber in the presence of trypan blue, and all spinner cultures were subsequently diluted by the same factor as the one used for the positive control that gave a final cell concentration of 3.3×10^5 cells per ml. The appropriate agents were all added to maintain the desired final concentrations. According to this procedure, the negative effect of any agent addition would be enhanced since the density of growth-retarded cells would rapidly drop below the level needed for an optimal density-dependent proliferation. The cultures were monitored for 5 days, and the recovered cell densities at each day of counting were divided by the densities obtained the previous day after dilution, thus yielding proliferation factors (doubling factors) based on 24-h growth. The relative concentration of trypan blue-stained cells was also monitored as a function of agent concentration and total incubation time.

Assay of toxicity by monolayer culture establishment and cell proliferation. A calculated number of HeLa cells was sedimented and then brought to a density of 7.5×10^6/ml in S-MEM containing 3.5% FBS and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.0). From such starting suspensions of cells, 200 μl were removed and added to 10 ml containing S-MEM, 3.5% FBS, and the appropriate concentration of the agent under investigation. Five-milliliter portions were further diluted in twofold series (in medium containing the agent) to yield a range of densities from 1.5×10^5 to 2.3×10^3 cells per ml. From each dilution, six parallel samples of 200 μl each were transferred to 96-well tissue culture plates, which subsequently were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cells were allowed to adhere and establish growth for 48 h, and then the first plate was removed, followed by one plate every 24 h for a total incubation period of 96 h counted from the day of cell seeding.

The growth medium of the plates was removed, and the monolayers were fixed with 150 μl of 1% formaldehyde in PBS per well at room temperature (RT) for 10 min. The fixative was removed, and each well subsequently received 100 μl of freshly made crystal violet (5% in bidistilled water). After incubation at RT for 10 min, the plates were emptied and rinsed four times with cold tap water. The plates were dried, and finally each well received 50 μl of 20% sodium dodecyl sulfate in acidified 50% formamide as a dye solubilizer (23). The plates were read at 630 nm in a Dynatech MR 600 microplate reader (Dynatech Laboratories Inc., Chantilly, Va.). The mean values for each series of six wells were calculated and used for scoring of cell proliferation. Proliferation rates were calculated as a change in absorbance per day derived from the three daily measurement points. Negative values indicate a lack of monolayer establishment and subsequent cell growth.

Effects of agents on macromolecular syntheses. A radioisotope incorporation assay was performed to determine the immediate effects of the different agents on uninfected HeLa cells. Three parallel series were set up in 2 ml of S-MEM (supplemented with 3.5% FBS and 25 mM HEPES [pH 7.0]) containing approximately 6×10^5 cells, which then were incubated separately with 10 μCi of [^{3}H]thymidine, [^{3}H]uridine, or ^{3}H-amino acids (Amersham, Aylesbury, England) in the absence or in the presence of different concentrations of the various agents. Samples of 50 μl were withdrawn at 1-h intervals over a total incubation period of 6 h and put onto Whatman 3 MM paper discs, which were immersed in ice-cold 10% trichloroacetic acid and stored at 4°C overnight. The discs were washed twice in 5% trichloroacetic acid and twice in 70% ethanol and then allowed to dry at RT. The samples were assayed for radioactivity in Ready Safe liquid scintillation cocktail (Beckman Inc., Galway, Ireland) by using a 1214 Rackbeta liquid scintillation spectrometer (LKB/Wallac, Turku, Finland). The data were treated to produce mean incorporation rates (counts per minute per hour per 10^5 cells), which were related to rates for untreated controls.

Assessment of the relative pH-raising effects of weak-base amines. To obtain an estimate of the relative pH-raising effects of the selected amines used at the indicated concentrations, and in the present context with HeLa cells in suspension culture at a density of 3×10^5 cells per ml, we designed a modification of the procedure described by Ohkuma and Poole (38). Cells were detached from monolayer cultures and seeded into small spinner suspension cultures as described above. Between 6 and 8 h after seeding, the cells received fluorescein isothiocyanate-dextran (FD; average molecular weight, 71,200; Sigma) at a final concentration of 1 mg/ml. After 20 h in culture and growth to a 50% higher cell density, the cells were harvested and washed once in S-MEM and once in Earle's balanced salts without phenol red and with glucose and sodium bicarbonate (pH 7.3) (Sigma). The cells were resuspended in Earle's balanced salts to give 3×10^5 cells per ml, and 3-ml portions were transferred to tubes at 37°C containing the different agents to yield the indicated concentrations. FD-labelled control cells without agent and unlabelled blank cells were included. Between 5 and 10 min after exposure to the agents, the fluorescence emission at 519 nm was measured upon excitation at 450 and 495 nm in a Shimadzu RF-1501 spectrofluorometer. The ratios of fluorescence intensity at 495 nm to that at 450 nm (495/450 ratios) were calculated, and thereby data from separate experimental series could be compared (38).

Adenovirus uncoating. Calculated numbers of HeLa cells, removed from a spinner culture, were distributed into tubes and sedimented. The cell pellets were brought to final densities of 3×10^5 cells per ml in PBS or S-MEM without serum. The cells were pretreated with the different lysosomotropic agents at the indicated concentrations at 37°C for 10 min. The cells were sedimented at RT and resuspended in agent-containing PBS or S-MEM to yield a density of 1.2×10^7 cells per ml. The cells were chilled on ice, and then $^{32}P^i$-labeled virions (25,800 cpm/10^{10} virions) were added at a multiplicity of infection (MOI) of 2,800. Virions were allowed to attach at 4°C in a shaking water bath for 45 min. The cells were diluted 10-fold with agent-containing PBS or medium at 4°C and then sedimented to remove unattached virions. The pellets were subsequently resuspended in 0.5 ml of ice-cold PBS or medium with the different agents and then transferred to a shaking water bath at 37°C. Samples of 6×10^5 cells were removed at intervals and assayed for uncoating by measuring the levels of DNase-sensitive radioactivity as described earlier (5). A control series, kept on ice throughout the entire incubation, was also included.

Assay of late adenovirus-specified translation. HeLa cells (3.3×10^7 to 5.5×10^7) were kept in 100 ml of S-MEM at 4°C for 15 to 30 min and then were infected at an MOI of 96 at 4°C for 30 min. After attachment, a calculated number of cells was removed by centrifugation at 1,500 rpm for 10 min, and then the cells were resuspended in stationary suspension culture medium (SS-MEM; S-MEM containing 0.1 mM nonessential amino acids and 25 mM HEPES buffer [pH 7.0]) supplemented with 3% FBS and distributed into the required number of round-bottom cell culture tubes (17 by 100 mm; Becton Dickinson, Lincoln Park, N.J.). Samples used for hexon analyses thus consisted of 1 ml of SS-MEM containing 1.5×10^5 virus-infected cells, which were incubated at 37°C for 45 h. Two series of incubations were performed. In one series of experiments, the agents were added at different times postinfection (p.i.) and then allowed to remain throughout the entire incubation period (45 h). In another series, the cells were preincubated with one of the agents at different concentrations for 30 min, and then the cells were infected as described above. The agent was present until removed at different times p.i., at which time the cells were sedimented, washed, resuspended with SS-MEM containing 3% FBS, and incubated further. Finally, the cells of all series were harvested at 45 h p.i., and the samples were directly sonicated for 10 s at RT (using a Branson Ultrasonic disintegrator at a duty cycle of 0.5 and output control of 3) and diluted 40 times in PBS containing 1% bovine serum albumin and 0.5% Triton X-100. The samples were quantita-

tively assayed for their hexon content by using a capture enzyme-linked immunosorbent assay as previously described (63).

Northern (RNA) and dot blot analyses. Total mRNA from infected cells was purified by using a Dynabeads mRNA purification kit (Dynal A.S., Skøyen, Norway) and then separated in 1% agarose-formaldehyde gels for 2.5 h at 100 V according to the Amersham protocol (1). The mRNA was transferred onto a Hybond-N membrane (Amersham) by capillary blotting overnight.

Dot blotting was done according to standard procedures (47). Membranes were prehybridized as described previously (47) and then hybridized overnight at 42°C with the same prehybridization solution plus a radiolabelled probe corresponding to a selected fragment within the E2 region of Ad2. Final washes of the hybridized membranes were performed according to the Amersham hybridization procedure (1).

The E2 probe was a *Bst*EII fragment of 391 bp from plasmid pAL/E2 (58) (kindly provided by C. Svensson, Department of Microbial Genetics, Karolinska Institute, Stockholm, Sweden) containing Ad2 *Eco*RI fragments B and F (nucleotides 21338 to 27372) (46). The E2 probe was labelled with [α-^{32}P]dCTP by using a random-primed labelling kit (Boehringer Mannheim, Mannheim, Germany).

Assay of early adenovirus-specified transcription. For the purpose of determining the lowest MOI needed to achieve a positive signal in the dot blot analyses, infections for Northern blot analyses were carried out in sterile round-bottom cell culture tubes (17 by 100 m, with caps) containing 10^6 cells in 2 ml of SS-MEM. Virions were added at MOIs of 500, 1,000, 1,500, and 2,000, which remained throughout the infections. The samples were incubated for 10 h at 37°C on a shaking water bath under orbital agitation, and then the cells were removed for mRNA extraction as described above. To check labelling intensities obtained with the probe and also to corroborate the expected size distribution of the probed mRNA, the total mRNA was separated by agarose gel electrophoresis and analyzed by Northern blotting (47).

Infections for dot blot analyses were subsequently done on cells preincubated at 4°C for 30 min. Virions were added to HeLa, KB, HEp-2, or A549 cells at an MOI of 8,000. The cells were contained in spinner flasks with 100 ml of S-MEM at a density of 5×10^5, and virions were allowed to attach at 4°C for 30 min. The cells were then washed and resuspended in S-MEM at 4°C to give a concentration of 10^6 cells per ml. After appropriate dilution, 1 ml of the infected cell suspension was seeded per well in six-well culture plates (Costar Europe Ltd., Badhoevedorp, The Netherlands) with medium containing calculated concentrations of the different lysosomotropic agents in such a way that the final cell density was 1.5×10^5/ml. For the dot blot analyses, the lysosomotropic agents were added at time zero (i.e., at the point of the temperature shift and seeding into the plates) and at 3 h p.i. and then allowed to remain during the entire incubation period of 10 h at 37°C in an atmosphere of 5% CO_2.

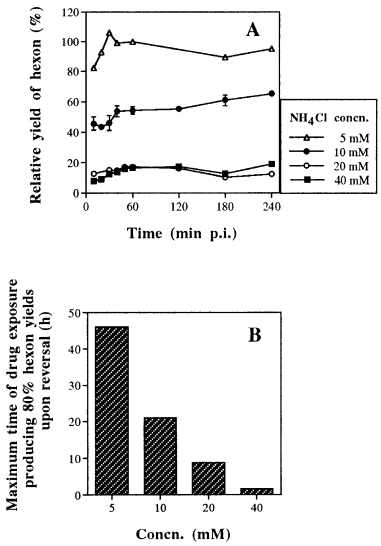

FIG. 1. Hexon antigen yields in Ad2-infected cells treated with different concentrations of ammonium chloride during infection. (A) HeLa cells were virus infected in the cold as described in Materials and Methods. Ammonium chloride was added to the 1-ml cultures, to yield the indicated concentrations, at 10-min intervals during the first hour p.i. and then at every hour until 3 h p.i. The agent remained until harvest at 45 h p.i. The samples were subsequently assayed for total hexon production as described in Materials and Methods. The hexon yields relative an untreated control series are represented versus the time of agent addition. For the 10 mM series, the mean values from three separate experiments are shown, with vertical bars indicating the standard deviation. The other plots represent single and typical experiments. (B) Cells were pretreated with ammonium chloride and then virus infected as described in Materials and Methods. The ammonium chloride was removed at different intervals p.i., and then the cells were cultivated further for a total period of 45 h. The lengths of the periods of ammonium chloride treatment allowing an 80% hexon yield after reversal are plotted versus the ammonium chloride concentration.

RESULTS

Effect of ammonium chloride added or removed at different times during infection. To test the possible effect of ammonium chloride early during Ad2 infection of HeLa cells, two series of infections were done. In one series, ammonium chloride was added at different times p.i. and then allowed to remain throughout the entire infection. It was shown that high concentrations (10 to 40 mM) of ammonium chloride added at 1 h p.i., which is the time it takes for Ad2 to reach the nucleus (59), had essentially the same effect as when added earlier in infection (Fig. 1A). When the agent was administered as late as 3 h p.i., the reduction of the hexon yield was of the same magnitude as when it was added early in infection, therefore indicating a general toxic effect. The low concentration of 5 mM revealed a possible early effect during infection, however, decreasing the hexon yield by 20% at the most.

To study the possible reversibility of the effect, another series of cells was pretreated with different concentrations of ammonium chloride for 30 min before addition of the virions. The ammonium chloride was subsequently removed at different times p.i., and then the cells were incubated further for a total period of 45 h. The data are presented as the longest time of incubation tolerated in the presence of different concentrations of ammonium chloride that upon subsequent reversal yielded a final hexon production corresponding to 80% of the value for the untreated control series. Thus, 5 mM ammonium chloride could be present throughout the entire incubation period and still support an 80% translational level (Fig. 1B). At the other extreme, it was evident that 40 mM ammonium chloride could not be present for more than a few hours before the hexon yield upon reversal was severely impaired. This result indicates a serious irreversible damage to the cells upon exposure to excessive concentrations of this established lysosomotropic agent.

Assays of agent toxicity. The initial studies revealing a possible effect of ammonium chloride on adenovirus replication, mirrored at the level of late translation, raised the following questions: is the low level of inhibition early in infection with 5 mM ammonium chloride together with the general toxic effect of higher concentrations enough to warrant a statement of a mandatory dependence of acid vesicles early during adenovirus infection?

To further investigate this problem, we decided to study the replication of Ad2 in the presence of four different agents displaying the same mechanisms of action (12) and further to rule out general toxic effects of these substances. On the basis of documentation in the available literature, initial concentrations of the agents were chosen as indicated, and three different assays for toxicity assessments were designed.

Cell proliferation studies of suspension cultures over a maximum period of 4 days clearly demonstrated toxic effects of low concentrations of all agents within the first 24 h. This was evident from decreasing proliferation factors that fell below the level of the control series and the concomitantly increasing fraction of trypan blue-positive cells (Table 1). The levels of cell monolayer culture establishment and the subsequent rates of proliferation also became a sensitive means to monitor the toxicities of the different agents. Thus, as exemplified for the cell seeding density of 7.5×10^3 per well, it became apparent

TABLE 1. Toxicities of lysosomotropic agents

Agent	Concn	Proliferation factors after 24, 48, and 72 h of exposure[a]	Trypan blue-positive cells (%) after 24, 48, and 72 h of exposure[a]	Relative rate of growth and establishment as monolayer cell cultures[b]	Relative rate of radioisotope incorporation (%)[c]		
					TdR	Urd	aa
Ammonium chloride	0	1.36, 1.40, 1.50	0	1	100	100	100
	5 mM	1.27, 1.19, 1.12	1.2, 5.0, 8.8	0.53	128	63	97
	10 mM	1.21, 0.92, 0.74	2.4, 9.1, 22.2	−0.1	76	57	113
	20 mM	0.97, 0.74, 0.77	3.6, 16.7, 46.0	−0.09	46	50	91
	40 mM	0.49, 0.29, 0.17	16.7, 73.1, 96.8	−0.03	35	25	59
Amantadine	0	1.50, 1.30, 1.39	0	1	100	100	100
	0.02 mM	1.44, 1.34, 1.48	0.6, 2.8, 2.4	1.1	93	110	140
	0.1 mM	1.41, 1.29, 1.30	3.0, 5.7, 5.7	0.95	100	96	134
	0.5 mM	1.31, 1.12, 1.06	3.7, 7.5, 15.6	0.18	104	84	110
	1.0 mM	1.06, 0.87, 0.18	8.1, 30.0, 82.5	0.07	96	64	59
Chloroquine	0	1.57, 1.32, 1.50	0	1	100	100	100
	2.5 μM	ND[d]	ND	0.62	62	85	82
	5 μM	ND	ND	−0.18	65	83	99
	10 μM	ND	ND	−0.18	66	90	102
	20 μM	1.39, 0.65, 0.28	2.5, 17.4, 73.7	−0.05	75	82	92
	50 μM	0.76, 0.25, 0	8.7, 64.9, —[e]	ND	ND	ND	ND
	100 μM	0.24, 0.31, 0	25.9, 86.0, —	ND	ND	ND	ND
Methylamine	0	1.45, 1.47, 1.53	0	1	100	100	100
	2.5 mM	1.45, 1.35, 1.20	1.2, 3.1, 4.2	1.06	71	87	65
	5 mM	1.39, 1.28, 1.10	2.5, 4.3, 9.1	0.49	104	83	102
	10 mM	1.27, 1.12, 1.01	3.7, 8.0, 15.4	−0.21	99	81	122
	20 mM	1.03, 0.95, 0.40	6.6, 14.0, 55.9	−0.04	106	110	94

[a] The growth of HeLa cells in suspension culture with and without the indicated agents was determined as described in Materials and Methods.
[b] Monolayer culture establishment and subsequent proliferation rates of HeLa cells over 96 h were monitored as described in Materials and Methods. A negative value indicates a detachment of cells.
[c] The total DNA (TdR [thymidine]), RNA (Urd [uridine]), and protein (aa [amino acids]) synthesis in HeLa cells was monitored over a period of 6 h, and radioisotope incorporation rates (counts per minute per hour per 10^5 cells) were determined as described in Materials and Methods.
[d] ND, not done.
[e] —, all cells were lysed.

that the second-lowest concentrations of all agents, except for amantadine, interfered with the attachment and subsequent growth of the cells (Table 1). Incorporation of radiolabelled macromolecular precursors also revealed some impairment of the corresponding synthetic events within a few hours of exposure (Table 1). Amantadine was the least toxic substance, displaying a stimulatory effect on total protein synthesis, which was mirrored by only weakly toxic effects of a concentration of 0.1 mM in the cell proliferation studies.

The following pairs of agent concentrations were chosen for further studies on the possible impairment on the early phase of adenovirus infection: 0.1 and 0.5 mM for amantadine, 5 and 10 mM for ammonium chloride, 2.5 and 5 μM for chloroquine, and 5 and 10 mM for methylamine.

Effects of lysosomotropic agents on endosomal pH. Upon determination of the toxicity profiles, it also became essential to monitor the actual pH-raising effects of the selected agents, especially at the chosen nontoxic and mildly toxic concentrations and in relation to the cell densities used throughout this investigation. The two established lysosomotropic agents NH_4Cl and chloroquine revealed significant pH-raising effects under the conditions used (Fig. 2). Interestingly, the nontoxic and mildly toxic concentrations of 5 and 10 mM NH_4Cl, respectively, yielded 495/450 ratios equivalent to the values obtained at the 10-times-higher concentrations commonly used in similar studies. We do not elaborate further on the actual pH values that the 495/450 ratios would correspond to, since the data obtained by Ohkuma and Poole (38) are derived from assays of highly specialized macrophages in monolayer culture and furthermore are compared with fluorescence intensity studies performed in pure buffer solutions. However, the mean 495/450 ratios of the untreated control cells would, according to Ohkuma and Poole (38), correspond to a pH of approximately 5.7, thus putting the FD-containing cytoplasmic vesicles in a class somewhere between early and late endosomes (31). The maximum ratios obtained after excessive lysosomotropic treatment were converted to a pH of 6.3, which is identical with to the maximum effects described by Ohkuma and Poole (38). In the case of chloroquine, it was also shown that the selected nontoxic and mildly toxic concentrations in the low-micromolar region affected the cells to the same extent as did the highly toxic concentrations in the range of 0.1 and 0.4 mM (Fig. 2). It has previously been shown that chloroquine displays pH-rais-

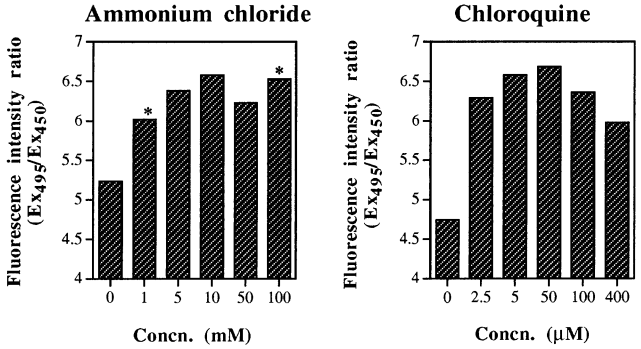

FIG. 2. The pH-elevating effects of ammonium chloride and chloroquine on cytoplasmic vesicles of HeLa cells in suspension. HeLa cells were allowed to incorporate FD for 12 to 14 h. The cells were washed and incubated at 37°C in the presence of the indicated concentrations of ammonium chloride and chloroquine as described in Materials and Methods. Fluorescence emission at 519 nm was measured upon excitation at 450 and 495 nm (Ex_{450} and Ex_{495}). Mean 495/450 ratios from two separate experiments are shown. Asterisks indicate values from one experiment. The minimum and maximum values of the 495/450 ratios correspond to intravesicular pH values of approximately 5.7 and 6.3, respectively (38).

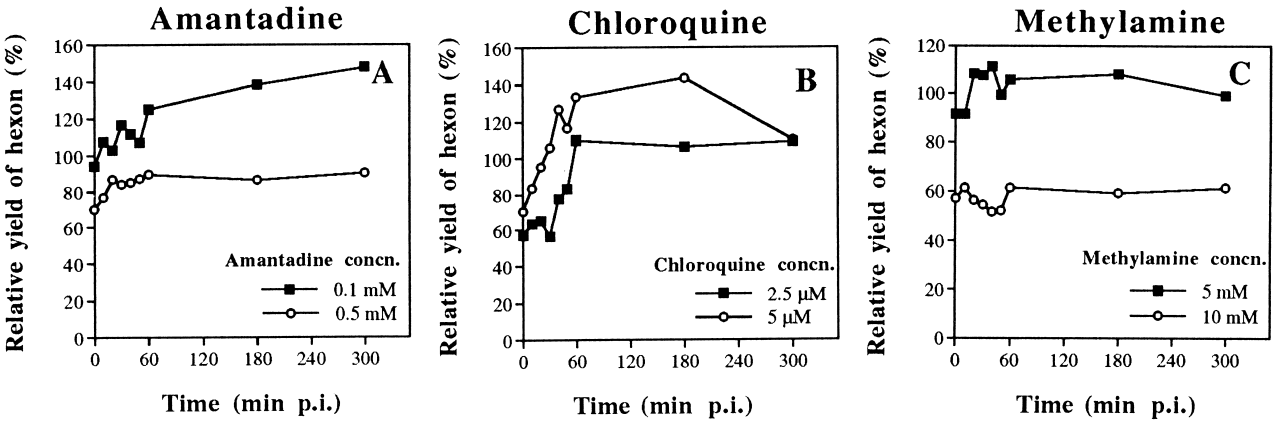

FIG. 3. Hexon production of Ad2-infected cells in the presence of amantadine, chloroquine, or methylamine. HeLa cells were virus infected in the cold as described in Materials and Methods. The lysosomotropic agents were added at the indicated times p.i. and remained throughout the entire incubation of 45 h. Relative hexon yields are shown versus the time of agent addition. Data from individual series are shown since the relative deviation between separate experiments was of the same magnitude as that shown in Fig. 1.

ing effects at concentrations 1,000 to 5,000 times lower than those needed for the other amines to achieve the same increase of the intracellular pH (44). For both amantadine and methylamine, increasing but less drastic 495/450 ratios were obtained from one experiment. But when only the 495-nm values within each series were considered, the former agent displayed a continuous increase in fluorescence intensity compared with the untreated control, from 26% (at 0.1 mM) to 66% (at 10 mM). On the other hand, methylamine showed a 38% increase at 5 mM, with a maximum effect of 40% at 100 mM.

Effects of lysosomotropic agents on late adenovirus-specified translation. The initial studies of the possible effect of ammonium chloride added early during adenovirus infection (Fig. 1) were repeated with the remaining lysosomotropic agents, but only at the two selected concentrations. Thus, infected HeLa cells received the three different weak-base amines amantadine, chloroquine, and methylamine at different times p.i. The agents remained throughout the entire infection period of 45 h. Nontoxic concentrations of chloroquine had an inhibitory effect on hexon synthesis when administered within the first hour of infection (Fig. 3). However, the level was reduced by only by 40% at most. Such a qualitative effect was previously also seen in the case of ammonium chloride (Fig. 1A). Both amantadine and methylamine at nontoxic concentrations were not able to depress late translation but rather displayed stimulatory effects which were particularly apparent for the former agent. Toxic concentrations of ammonium chloride (Fig. 1A) and methylamine (Fig. 3) led to inhibitory patterns indicative of nonspecific and general toxicity. This was also the case, but less prominently, for amantadine. On the other hand, the highest concentrations of chloroquine reduced hexon synthesis by only 30% at the most when added within the first 30 to 40 min of infection and revealed a stimulatory effect when administered later than 1 h p.i. Taking into account the established multiple effects of chloroquine (48), it became important to measure the level of an earlier adenovirus-specific synthetic event as an indicator of the possible interference by the pH-raising agents early during infection.

Effects of lysosomotropic agents on early adenovirus-specified transcription. For these studies, the transcripts of the early 72-kDa DNA-binding protein, detectable 10 h p.i., were chosen as early virus-specified synthetic products, thereby hopefully minimizing possible long-term side effects of the panel of agents used. Again the two previously selected agent concentrations were used together with a total of four different cell lines permissive for productive Ad2 infections. The agents were added at time zero and at 3 h p.i. as described in Materials and Methods. It was shown that none of the agents, at any concentration or administered at any time to any of the different cell lines, displayed any significant effect on early adenovirus transcription (Fig. 4). However, we observed slight variations not consistent with an early agent effect and also within the technical variation of the assay per se, which was separately determined to work with a coefficient of variation of 4%. Such quantitative assessments were done by piercing out the dot areas of the membranes and counting the radioactivity of the pieces in a liquid scintillation spectrometer (data not shown).

Adenovirus uncoating in the presence of lysosomotropic agents. Since an early phase during adenovirus-cell interaction, monitored as an early transcriptional activity of Ad2, was not affected by any of the selected lysosomotropic agents, we wanted to reexamine (60) the possible effects of these four agents on the process of virion uncoating. Therefore, an experiment was performed with series of cells that were pretreated at 37°C with all four agents as described in Materials and Methods. To ensure any possible effect, only the highest of the two previously selected agent concentrations were used. It was shown that none of the agents displayed any effect on virion uncoating (Table 2), nor were the levels of virion attachment affected (data not shown). The reproducibility and the absolute levels of uncoating in S-MEM at 37 and 0°C were reassessed in two triplicate control series processed in such a way that the excessive centrifugation steps used in the drug series were omitted. The mean absolute uncoating level for samples maintained at 37°C for 1 h was 53.0% ± 1.11% (standard deviation) ($n = 3$); for those that were incubated between 0 and 4°C for 1 h, the uncoating level was 6.4% ± 0.65% (standard deviation) ($n = 3$). The higher background levels of uncoating seen in the drug experiment (Table 2) could be a consequence of the stress applied to the cells following the repeated centrifugations. Thus, the minor differences in levels of uncoating seen between the two series performed with different media were not consistent with the idea of a unique effect on virion uncoating. We therefore conclude that this early step during infection is not dependent on an acidic pH in such a way that pH-raising weak-base amines will display any effects of interference.

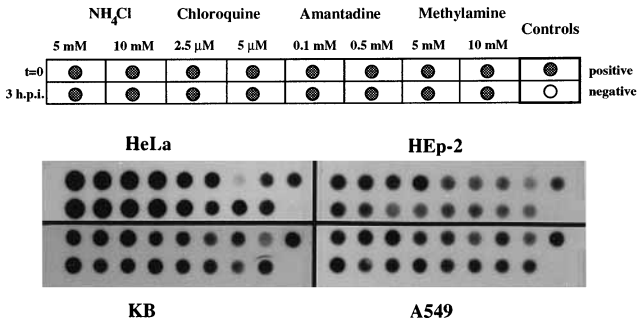

FIG. 4. Early effects of lysosomotropic agents on adenovirus replication as revealed by dot blot analysis of the synthesis of mRNA encoding the early 72-kDa DNA-binding protein. HeLa, KB, HEp-2, and A549 cells were virus infected as described in Materials and Methods. The cells were subsequently exposed to two different concentrations of the four agents, which were added at time zero and 3 h p.i. mRNA was extracted and probed as described in Materials and Methods. (The sample corresponding to the dot of HeLa cells treated with 5 mM methylamine at time zero was not quantitatively recovered.)

DISCUSSION

To understand the mechanism of early adenovirus-cell interaction, a number of observations on virion structure and stability together with cell-dependent processes must be considered simultaneously. Thus, we need to discuss the full spectrum of findings regarding (i) the complex structure of the virion (37, 55, 56, 62), (ii) the low-pH destabilization of the virion in vitro (16), (iii) the nonspecific protective actions of salt and protein on the low-pH-mediated uncoating of adenovirions in vitro together with the destabilizing effect of antifiber antibodies upon treatment of detergent-protected virions at low pH (15), (iv) the low-pH-triggered and penton base-mediated puncturing of plasma membranes, liposomes, and plasma membrane vesicles (6, 49, 52, 53), (v) the association of the penton base with integrins early in infection (3, 24, 65, 66), and finally (vi) the various aspects of adenovirus uncoating in vivo and previous studies of this virus system that used the established pH-raising lysosomotropic agents (5, 21, 36, 60).

Adenovirions easily become destabilized (i.e., uncoated) in vitro at low pH under low-ionic-strength conditions and thereby release structural components of the vertex region such as the pentons, protein IIIa, and some of the peripentonal hexons (17, 45). This phenomenon was first extrapolated to be valid also in vivo, and it could possibly mimic some situation within the cell early in infection. This idea was based on electron microscopic visualization of more rounded virions, i.e., presumably pentonless, in the cytoplasmic compartment early upon infection (11) and also on the fact that virions in vivo, under true virus-cell interactive conditions, within 30 to 60 min also become destabilized to fully expose the DNA to exogenously added DNase (60). It therefore at this point seemed plausible that a low-pH condition might be a crucial factor in common between the systems of uncoating in vitro and in vivo. The subsequent observation that the penton base of the virion upon transfer to pH 5.0 conditions displays hydrophobic properties (54), together with the fact that intact virions at low pH will puncture the plasma membrane of intact cells to the extent that radiolabelled chromium will be released (53) and likewise affect artificially prepared liposomes (6) and plasma membrane vesicles (49), led to the notion that the penton may have a major part in the process of virus internalization, in this case the release from endosomes into the cytoplasmic compartment. Studies of cells infected by adenovirus in the presence of an EGF-PE conjugate suggest that the penton base is involved in the process of endosomal release of the virus, since antipenton antibodies administered at the point of virus/EGF-PE infection blocks the virus-mediated release of the toxin into the cytoplasm (50). Intrinsic neutralization by antipenton base antibodies also strongly implicate the involvement of this structure at an early stage of infection (67). The significance of a low pH within endosomes to support endosomal release processes was suggested by the observation that lysosomotropic agents to some extent inhibit the process of EGF-PE release mediated by adenoviruses; however, even excessive concentrations of chloroquine (40 μM) and ammonium chloride (30 mM) still are not able to restore protein synthesis to more than 60% of its maximum level, i.e., to prevent the release of the exotoxin into the cytoplasm (51). Thus, these indirect observations and far from fully efficient effects led to the hypothesis that a low pH within endosomes is needed for viral release into the cytoplasm (39). However, since at this stage no experiments were performed to determine any synthetic activities actually displayed by the infectious fraction of the parental adenoviruses, and also considering the fact that only approximately 3% of the physical particles of this virus system are infectious, the conclusion is premature because one does not know if it is the infectious, the noninfectious, or both classes of particles that participate in the process of toxin release. Indeed, the same argument regarding the low specific infectivity of adenoviruses may be applied to electron microscopic data suggesting inhibitory effects of weak-base amines on the transportation of adenovirions to the nucleus (59).

In this study, we have investigated the possible existence of an early low-pH-dependent step in the adenovirus replicative cycle from the point of view that a priori, generally toxic concentrations of established lysosomotropic agents should be avoided. The chosen concentrations of the agents were separately demonstrated to cause pH-raising effects in cytoplasmic vesicles of the same magnitude as significantly higher concentrations previously used by others. Furthermore, we have studied an early virus-cell interactive event, i.e., the process of uncoating which is believed to occur at the very point of endosomal release (5). Early transcriptional and late translational biosynthetic events were also monitored as a means to assess the actual level of successful virion hits into the cell nucleus.

TABLE 2. Adenovirus uncoating in the presence of lysosomotropic agents[a]

Agent, concn	Relative uncoating 45 min after transfer of cells to 37°C (%)	
	PBS	S-MEM
Amantadine-HCl, 0.5 mM	112.1	104.6
Ammonium chloride, 10 mM	96.3	108.3
Chloroquine, 10 μM	107.7	102.1
Methylamine-HCl, 10 mM	113.9	93.6
Control, no agent	100	100
Control, no agent, cells kept at 0°C	21.0	23.8

[a] HeLa cells were pretreated with the indicated agents at 37°C for 10 min at a cell density of 3×10^5/ml. The cells were sedimented, resuspended in 1/40 of the original volume of medium containing the agents, and chilled on ice, and then $^{32}P_i$-labelled virions were added at an MOI of 2,800. After virion attachment for 45 min at 4°C, the cells were freed of unattached virions by sedimentation. The cells were resuspended at a density of 1.2×10^7/ml in medium with the corresponding agent and subsequently transferred to 37°C. Samples were removed after 5, 20, and 45 min of incubation and processed for uncoating as described in Materials and Methods. Only the data obtained after 45 min of incubation are shown. The absolute uncoating levels of the controls in PBS and S-MEM were 48 and 52%, respectively.

This investigation commenced by looking at a late translational process, i.e., the synthesis of the hexon antigen in the virus-infected cell, in response to the temporal administration of different concentrations of ammonium chloride. The weak interference demonstrated by this established lysosomotropic agent early in infection indicated some interference with a step possibly relying on an acidic pH. However, the blockage was only by 20% and does not support the notion of a mandatory need for low-pH dependence early in infection, since higher concentrations of ammonium chloride, amantadine, and methylamine all ruled out an early specific interference but rather demonstrated general toxic effects throughout the infection. Interestingly, a low concentration of amantadine demonstrated a stimulation of the cumulative hexon synthesis which simply could be a function of stimulatory effects on translation per se, as also mirrored by the enhanced incorporation of radiolabelled amino acids in the toxicity tests of uninfected cells. The fact that chloroquine had inhibitory effects when administered within the first hour of infection could lend some support to the involvement of acid vesicles early in infection. However, chloroquine, apart from its pH-elevating properties, also displays a vast number of other established effects such as binding to cell surfaces, binding to phospholipids, reduction of membrane fluidity properties (12, 48), and possibly capsid-stabilizing effects (22). Therefore, at this point, its effect on late translation after early administration is not easily interpreted.

In a pretreatment and reversal experiment, it was shown that, for instance, a 20 mM concentration of ammonium chloride could be present for 10 h from the point of infection, plus 30 min before the infection, and upon subsequent reversal the cells were still able to maintain the ability of synthesizing the hexon antigen to a level 80% of that of the untreated control cells. These findings again demonstrate the toxicity of the agent upon prolonged exposure and also the lack of a unique effect early in the infection. The present data are further corroborated by the recent finding that bafilomycin A1, by blocking the H^+-ATPase-driven acidification of endosomes, does not inhibit adenovirus infection as assessed by late translational events (43).

Since the analyses of the late synthesis of a structural protein were done well after one complete cycle of replication, we then focused on an early transcriptional event 10 to 12 h p.i. To further rule out that our Ad2-HeLa system was unique because of some laboratory adaptation, we also used KB, HEp-2, and A549 cells for these studies. In none of the agent examples, agent concentrations, time points of agent addition, and cell lines could a significant interference with early transcription be detected. Thus, since the agents act within 1 min after administration (38), it is safe to claim that no delay of the delivery of the infectious parental genome to the nucleus could be established. A lack of interference with the early step of virion uncoating was also demonstrated, again supporting the idea that none of these established lysosomotropic agents affect a mandatory low-pH-dependent step early in infection.

It has recently become apparent that animal viruses interact not only with cellular receptors during the process of attachment. For instance, in the mouse hepatitis virus system, a cell surface component apart from the actual receptor has been implicated in the process of virus internalization (2, 68). The establishment of the second-receptor concept in the adenovirus system now offers an intriguing model for a preliminary understanding not only of the mechanism of internalization but also of the process of virus release into the cytoplasmic compartment (24, 65). Thus, the interaction between the penton base and $\alpha_v\beta_3/\alpha_v\beta_5$ integrins not only may mediate the initial step of endocytosis but also could apply a constraint on the virion to such an extent that hydrophobic regions of the penton base may further interact with the endosomal membrane, thereby triggering a translocation over the membrane. Alternatively, the penton base may not at all be actively involved in the penetration step since the α/β integrin may display pore formation activities of its own (65). The significance of intact pentons of the adenovirion in mediating the step of translocation to the cytoplasm has been demonstrated in gene transfer systems using adenovirions incapable of cell attachment by antifiber antibodies (34). Such virions still possess the capacity to traverse the endosomal membrane, thereby delivering their genetic material into the cytoplasm for further processing. These observations together with our previous studies on adenovirion uncoating in vitro demonstrate that although virions are destabilized at pH 5 under low-ionic-strength conditions, they become nonspecifically resistant to uncoating by the presence of any protein or by hydrophobic interaction with nonionic detergents (15). However, this protection is overcome by the highly specific cross-linking of virions to form aggregates by antifiber antibodies (15). We believe that this situation in vitro to some extent may reflect other unique and specific protein-protein interactions within the cell needed for the subsequent step of endosomal release and uncoating (24). Uncoating of adenovirions in vitro is not provoked by isolated preparations of plasma membranes (60), again indicating the importance of exact steric arrangements of all components involved in the sequence of steps from attachment to internalization and uncoating. A somewhat similar situation has been described for polioviruses that become irreversibly destabilized by monoclonal antibodies under low-ionic-strength conditions in vitro (13). This observation, together with the notion that polioviruses during the cell-mediated uncoating do not rely on intracellular low-pH conditions, again lends support to the existence of other and more complex means by which a number of viruses become intracellularly destabilized (24).

Having analyzed the fidelity by which adenovirions become uncoated together with the subsequent levels of successfully nuclearized virions measured as their early transcriptional activities in the presence of nontoxic concentrations of a number of established lysosomotropic agents, and having analyzed the latter processes in four different cell lines, we suggest that an endosomal low-pH exposure per se is not important for any event early during adenovirus replication.

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