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# Role of a Low-pH Environment in Adenovirus Enhancement of the Toxicity of a *Pseudomonas* Exotoxin-Epidermal Growth Factor Conjugate

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**A conjugate of *Pseudomonas* exotoxin and epidermal growth factor (PE-EGF) inhibits proteins synthesis in KB cells, and this inhibition is increased by adenovirus. Protein synthesis inhibiton is dependent on the amount of adenovirus and PE-EGF used and the time of incubation of cells with these agents. With 1 μg of adenovirus and 0.5 μg of PE-EGF per ml, protein synthesis is inhibited about 80% in a 60-min experiment. Under these conditions neither adenovirus nor PE-EGF alone has any effect. In the presence of several weak bases or monensin, the enhancement of toxicity was substantially inhibited; half-maximal inhibiton was achieved with 40 μM chloroquine, 10 mM ammonium chloride, 5 mM methylamine, 0.1 mM *N*-hexylamine and 1 μM monensin. At the concentrations employed, none of the inhibitors affected the amount of virus taken up or bound to the cell surface, and chloroquine had no effect on the amount of EGF taken up in 60 min. Chloroquine did not prevent the toxicity of the PE-EGF (5 μg/ml) alone. Because these compounds are known to elevate the pH in receptosomes, it seems likely that the acidification of the receptosome either enhances the lysis of the membrane by adenovirus or enhances some other step in the release of PE-EGF.**

Many hormones, viruses, and toxins are internalized by a process termed receptor-mediated endocytosis (for review, see references 7 and 19). The process involves binding of ligands to specific receptors on the cell surface, the accumulation or clustering of ligand-receptor complexes in coated pits on the cell surface, and the subsequent internalization and transfer of the ligands from coated pits to a specialized organelle which we have termed a receptosome (22) and which has also been termed an endosome (15).

Recently, we have compared the organelles involved in the entry into KB cells of human adenovirus (type 2) with those involved in epidermal growth factor (EGF) entry or the entry of a hybrid toxin of *Pseudomonas* exotoxin (PE) and EGF. It was shown that adenovirus, EGF, and PE-EGF entered the cells through the same coated pits and receptosomes. It was also shown that adenovirus (1 μg/ml) was able to protentiate the ability of PE-EGF to inhibit protein synthesis of the host cells (5). This appeared to occur because some of the adenovirus particles and PE-EGF molecules were internalized in the same receptosomes. When adenovirus disrupted the membrane of the receptosomes, PE-EGF was released into the cytoplasm in greater amounts than would have occurred in the absence of virus.

One characteristic feature of receptosomes is that they are acidic vesicles with a pH less than 5 (16). We report here studies designed to examine whether the acidic environment of this vesicle plays any role in adenovirus disruption of receptosomes. To do this we have assayed for adenovirus-mediated enhancement of PE-EGF toxicity in the presence of several compounds known to elevate the pH of receptosomes (16).

## MATERIALS AND METHODS

**Cells.** KB cells (American Type Culture Collection, CCL-17) were grown as monolayers in Dulbecco modified Eagle medium (DMEM) supplemented with 10% calf serum, penicillin, and streptomycin.

**Virus.** Adenovirus type 2 was purified by the procedures described previously (8). KB cells grown in suspension were used for propagation of virus. Purified adenovirus was stored in Tris–saline–30% glycerol solution at −70°C.

**PE-EGF.** PE-EGF hybrid toxin was constructed by a disulfide exchange reaction as described previously (5).

**Assay for enhancement of PE-EGF toxicity by adenovirus.** KB cells were planted at $5 \times 10^5$ cells per 35-mm dish and used 24 h later at 80 to 90% confluency. The cells were washed twice with DMEM containing 2 mg of bovine serum albumin per ml, and then 1.5 ml of fresh medium was added containing either adenovirus (0.1 to 10 μg/ml) or PE-EGF (0.01 to 0.5 μg/ml) or both. A suspension of adenovirus at a concentration of 0.1 μg/ml contained, on the average, $9 \times 10^2$ particles per cell. Incubations were done at 37°C for 1 h unless otherwise specified. At the end of the incubation, the medium was removed and replaced with 2 ml of DMEM-[$^3$H]leucine (2 to 5 μCi/ml) for another 45 min. The monolayers were rinsed with phosphate-buffered saline (PBS), solubilized by the addition of 1 ml of 0.1 N NaOH, and transferred into tubes. Proteins were precipitated by addition of 2.5 ml of 12.5% trichloroacetic acid. The level of protein synthesis was determined by the number of counts per minute in trichloroacetic acid-insoluble material from each dish. Experiments were done in duplicate dishes, and the counts were averaged. Variation between dishes was always less than 5% of the mean value.

**Effect of weak bases and monensin.** Chloroquine, $NH_4Cl$, methylamine, *N*-hexylamine, and monensin were dissolved in PBS, and the pH was adjusted to 7.0. To study the effect of weak bases on the potentiation of PE-EGF toxicity by adenovirus, cells were pretreated with the respective drug for 15 min at the concentration required. Adenovirus and PE-EGF were added to the cells in the presence or absence of the drugs, and the cells were incubated at 37°C for 45 min. At the end of this period, the cells were washed, [$^3$H]leucine

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was added for 30 min, and the level of protein synthesis was determined as described above.

**Preparation of $^{35}$S-labeled virus.** $^{35}$S-labeled adenovirus was produced in KB cells by a procedure decribed earlier (20), with slight modifications. KB cells were seeded as monolayers 12 to 18 h before infection with human adenovirus type 2. To $5 \times 10^6$ cells in a 75-cm tissue culture flask were added 6 μg (protein) of virus ($7.2 \times 10^3$ particles per cell) in DMEM, penicillin, streptomycin, and 2 mg of bovine serum albumin per ml. The virus suspension was passed through a 0.45-μm filter before the start of the infection. The infection proceeded uninterrupted for 15 h, after which time the medium was removed and replaced by fresh medium containing methionine-free DMEM, 1% calf serum, and 5 mCi of [$^{35}$S]methionine for a further period of 16 h. Cells were removed from the plastic surface by virgorous pipetting. The cell suspension was centrifuged at 2,000 × *g* for 5 min, washed, and recentrifuged three times. The cell pellet was suspended in 0.5 ml of PBS and sonicated for 5 min at 4°C. Cell extracts were centrifuged at 15,000 × *g* for 15 min. The supernatants were collected and centrifuged (50,000 × *g* for 1 h) into 0.8 ml of 50% glycerol over 0.5 ml of 80% sucrose. The virus was collected at the interface between the glycerol and sucrose and banded twice in CsCl gradients.

**Binding and uptake of $^{35}$S-virus.** To measure binding of adenovirus to the cell surface, monolayers of KB cells grown in 35-mm dishes were incubated with $^{35}$S-labeled adenovirus (specific activity, 33,580 cpm/μg of protein) at a concentration of 1 μg/ml ($6 \times 10^3$ particles per cell) at 4°C. The volume of the incubation mixture was 0.5 ml, and the cells were rocked during the incubation. At the end of the incubation period (usually 60 min), cells were washed five times with PBS and treated with 1 ml of trypsin for 10 min at 37°C. Cells were then collected and centrifuged at 2,000 × *g* for 5 min. Supernatants were collected and counted, and the cell pellets were dissolved in 1.0 ml of 0.1 N NaOH and counted. In parallel experiments, the total amount of cell-associated virus was determined by dissolving the washed untrypsinized cells in 1 ml of 0.1 N NaOH added directly to the dishes. Usually more than 80% of the total virus bound to the cell surface was in a trypsin-releasable form.

To measure adenovirus uptake, cells were incubated with $^{35}$S-adenovirus as described in the above section except the incubations were done at 37°C. At various times, the cells were washed five times with PBS and treated with trypsin as described above. The total amount of cell-associated virus, the amount of virus bound to the cell surface, and the amount within the cells were determined as described above.

**Uptake of $^{125}$I-EGF.** EGF was iodinated by a publisher procedure (2). The amount of EGF taken up by the cells and bound to the cell surface was determined by the procedure described earlier (9).

Mouse EGF was purchased from Bethesda Research Laboratories (Gaithersburg, Md.). All chemicals were bought from Sigma Chemical Co. (St. Louis, Mo.). $^3$H-labeled leucine was purchased from New England Nuclear Corp. (Boston, Mass.).

## RESULTS

**Minimum time required to study the effect of adenovirus on enhancement of PE-EGF toxicity.** It was previously shown that readily detectable amounts of adenovirus are internalized and appear in the cytosol in less than 30 min (5, 12). Thus, if adenovirus is responsible for the release of PE-EGF into the cytoplasm, one should be able to show that, after a short incubation of KB cells with PE-EGF and adenovirus, there will be a substantial reduction of protein synthesis of the host cells. When the cells were treated with adenovirus (1 μg/ml) and various concentrations of PE-EGF at 37°C for 30 min to 2 h, followed by a 45-min pulse of [$^3$H]leucine, it was found that protein synthesis of host cells was diminished. The degree of inhibition of protein synthesis increased with the length of the incubation (Fig. 1A). Even after only a 30-min incubation of cells with adenovirus and PE-EGF, there was a significant inhibition of protein synthesis (Fig. 1A). Using a shortened assay in which cells were incubated with PE-EGF and adenovirus for 45 min, we next investigated the effect of increasing concentrations of virus at a fixed concentration of PE-EGF (0.5 μg/ml). It was found that with increasing amounts of adenovirus there was a corresponding

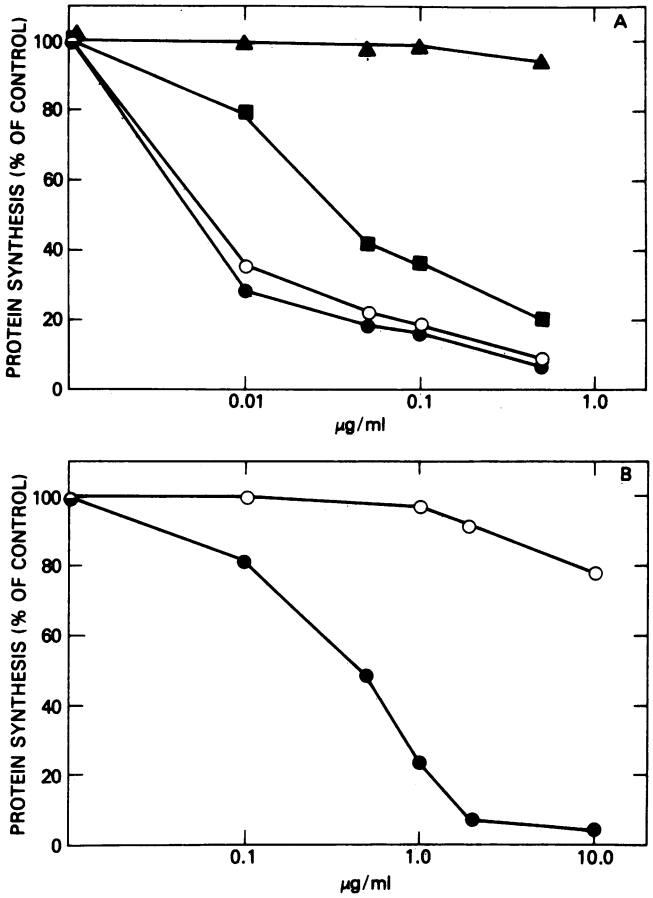


FIG. 1. Inhibition of protein synthesis in KB cells by PE-EGF and adenovirus. (A) KB cells were incubated with various concentrations of PE-EGF and a fixed concentration of adenovirus (1 μg/ml). Incubations were done for either 2 h (●), 1 h (○), or 30 min (■) in separate experiments. At the end of these periods, cells were replaced with medium containing [$^3$H]leucine (2 μCi/ml) for 45 min. The level of protein synthesis was determined as described in the text. The value obtained for controls which received PE-EGF alone in a 2-h incubation experiment is represented by (▲). In a 2-h incubation experiment, 100% was 9,443 cpm; in a 1-h incubation experiment, 100% was 9,311 cpm; and in the 30-min incubation, 100% was 7,762 cpm. (B) KB cells were incubated with various concentrations of adenovirus and a fixed concentration of PE-EGF (0.5 μg/ml). Controls did not receive PE-EGF. After the incubation at 37°C for 1 h, medium was removed and replaced by medium containing [$^3$H]leucine (2 μCi/ml). After 45 min the level of protein synthesis was determined as described in the text. Symbols: (●) adenovirus and PE-EGF, (○) control; 100% was 7,150 cpm.

increase in the inhibition of protein synthesis of host cells (Fig. 1B).

**Effect of weak bases and monensin on the potentiation activity of adenovirus.** Several chemicals which are known to raise the pH of intact receptosomes (16) were tested for their effects on adenovirus-mediated enhancement of toxicity. If the activity present in the virus functions better at low pH, then one should be able to demonstrate that adenovirus does not potentiate the toxic action of PE-EGF to the same degree in the presence of these drugs.

KB cells were pretreated for 15 min with various concentrations of chloroquine, $NH_4Cl$, methylamine, *N*-hexylamine, and monensin. Then the medium was removed and replaced with fresh medium containing the inhibitors, adenovirus, and PE-EGF at various concentrations. The cells were incubated at 37°C for 45 min, after which virus, PE-EGF, and drugs were removed and protein synthesis was assayed by adding [$^3$H]leucine for 30 min.

Chloroquine, ammonium chloride, methylamine, and *N*-hexylamine all decreased the ability of adenovirus to potentiate the toxicity of PE-EGF (Fig. 2). This effect was dependent on their concentrations. Under the conditions used, half-maximal inhibition was achieved with 40 μM chloroquine, 10 mM ammonium chloride, 5 mM methylamine, 0.1 mM *N*-hexylamine, and 1 μM monensin (data not shown).

TABLE 1. Concentrations required for half-maximal effect on adenovirus enhancement of PE-EGF toxicity and increase in 490-nm fluorescence

| Weak base | PE-EGF toxicity: $C_{0.5}$[a] (M) | Increase m 490-nm fluorescence: $C_{0.5}$[b] (M) |
|---|---|---|
| Chloroquine | $0.4 \times 10^{-4}$ | $1.4 \times 10^{-4}$ |
| $NH_4Cl$ | $1 \times 10^{-2}$ | $5 \times 10^{-3}$ |
| Methylamine | $0.5 \times 10^{-2}$ | $1 \times 10^{-2}$ |
| *N*-Hexylamine | $1 \times 10^{-4}$ | $1 \times 10^{-2}$ |
| Monensin | $1 \times 10^{-6}$ | $6 \times 10^{-6}$ |

[a] Values were calculated from Fig. 2.
[b] From reference 16.

Maximum effect of these drugs occurred at 60 μM chloroquine, 40 mM $NH_4Cl$, 10 mM methylamine, and 1 mM *N*-hexylamine. The concentrations of drugs required to inhibit the adenovirus effect are in the range found previously to raise the pH of receptosomes to pH 7 (reference 16 and Table 1), with the exception of *N*-hexylamine. One possible explanation for this difference is the fact that the previous experiments were performed in BALB/c 3T3 mouse fibroblasts for 1 to 10 min, whereas the experiments reported here were performed with KB cells for 60 min (16).

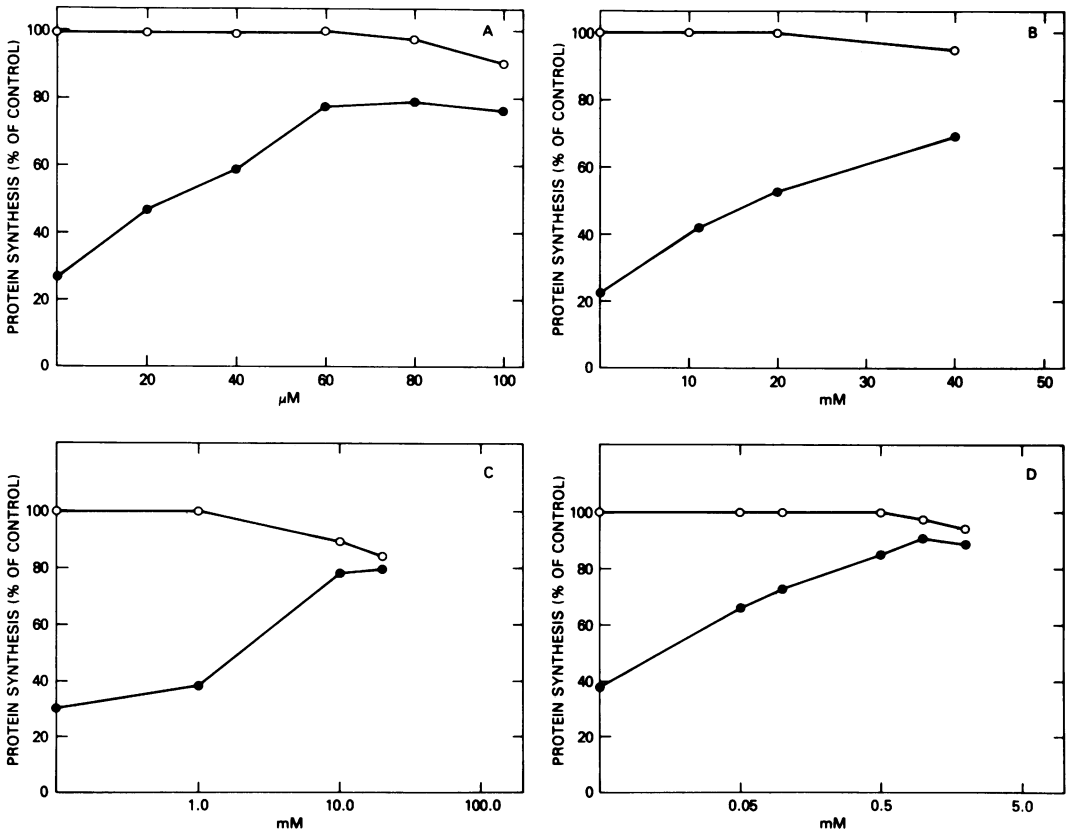


FIG. 2. Effect of weak bases on the inhibition of protein synthesis by adenovirus and PE-EGF. KB cells were incubated with the appropriate concentration of chloroquine (A), $NH_4Cl$ (B), methylamine (C), and *N*-hexylamine (D) for 15 min at 37°C. Medium was removed and replaced by medium containing adenovirus (1.0 μg/ml) and PE-EGF (0.5 μg/ml) with or without appropriate concentrations of the respective weak base. The incubations were for 45 min at 37°C. The medium was removed, and [$^3$H]leucine was added to the cells for 30 min. The level of protein synthesis was determined as described in the text. Half-minimal values reported in Table 2 were obtained by calculating the difference in the percent protein synthesis in the absence and presence of the drug giving maximum inhibition of enhancement. This difference was divided by two, calculating the corresponding concentration of the drug. Symbols: (●) protein synthesis in the presence of adenovirus, PE-EGF, and the respective drug at the concentrations shown; (○) level of protein synthesis in controls without adenovirus. In these experiments 100% was 7,448 cpm for chloroquine, 4,082 cpm for $NH_4Cl$, 3,834 cpm for methylamine, and 3,345 cpm for hexylamine.

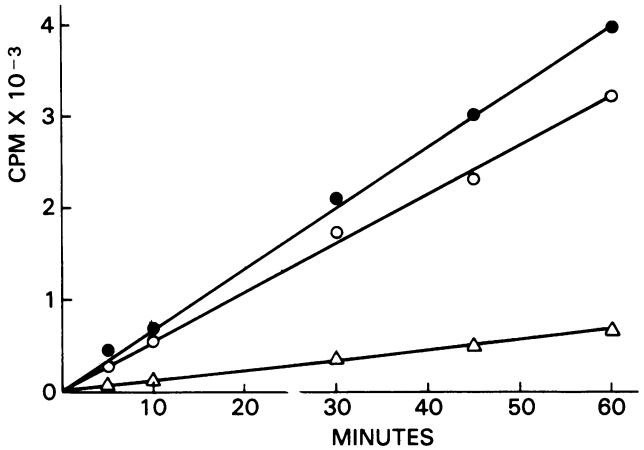


FIG. 3. Uptake of $^{35}$S-labeled adenovirus by KB cells. KB cells were incubated with $^{35}$S-labeled adenovirus (33,580 cpm/μg of protein) at 37°C as described in the text. At various time intervals, cells were washed five times with PBS and treated with 1 ml of trypsin at 37°C for 10 min to remove the surface-bound counts. After trypsin treatment, the cells were centrifuged and the cell pellet was dissolved in 1 ml of 0.1 N NaOH. In parallel experiments, the cells were not treated with trypsin, but instead were dissolved in 1 ml of 0.1 N NaOH to determine the total amount of adenovirus both bound and taken up by the cells. Symbols: (△) counts released by trypsin; (○) counts taken up by the cells; (●) total counts bound to the cell surface and taken up by the cells.

The above results show that chloroquine and other weak bases decrease virus-mediated enhancement of toxicity, but do not show which step is being inhibited. For example, the effect of inhibitors could be at the level of binding of adenovirus or PE-EGF, at the level of internalization, or at the point of escape from the receptosome. To distinguish among these possibilities, the following experiments were performed.

**Effect of chloroquine and other weak bases on virus uptake.** The uptake of adenovirus was studied by the procedure described in Materials and Methods. The data show that the uptake of adenovirus under the conditions of assay was linear for 60 min (Fig. 3). Within a few minutes at 37°C, most of the virus was in a form that could not be released by trypsin, suggesting that it was inside the cells. The compounds which were shown to inhibit the potentiating activity of adenovirus were also tested for their effect on virus uptake of KB cells. Cells were incubated with $^{35}$S-labeled virus in the presence and absence of the various compounds. At the end of the incubation, the cells were washed, the surface-bound adenovirus was removed by trypsin treatment and counted, and the amount of virus internalized in the cells was determined by counting the trypsinized cells after dissolving them in 0.1 N NaOH. None of the inhibitors used affected the amount of virus taken up by the cells or bound to the cell surface at the concentration tested (Table 2).

TABLE 2. Effect of chloroquine and other weak bases on $^{35}$S-adenovirus uptake[a]

| Addition (concn) | $^{35}$S-virus uptake (cpm) | $^{35}$S-virus on cell surface (cpm) |
|---|---|---|
| None | 957 | 394 |
| Chloroquine (50 μM) | 1,027 | 362 |
| Chloroquine (100 μM) | 1,012 | 428 |
| $NH_4Cl$ (40 mM) | 1,090 | 360 |
| Methylamine (10 mM) | 1,097 | 468 |
| Hexylamine (1 mM) | 1,057 | 442 |

[a] KB cells were incubated with $^{35}$S-labeled adenovirus (16, 202 cpm/μg of protein) at 37°C for 60 min. The concentration of adenovirus used was 1 μg/ml, and the volume of incubating medium was 0.5 ml as described in the text. The incubations were done in the absence and presence of the various compounds listed in the table. At the end of the incubations the amount of $^{35}$S-adenovirus bound to the cell surface and taken up by the cells was determined by trypsin treatment methods as described in the text.

**Effect of chloroquine on the binding of adenovirus and PE-EGF.** To examine whether chloroquine could act after adenovirus and PE-EGF were bound to KB cells, the cells were exposed to adenovirus and PE-EGF for 30 min at 4°C. At the end of this period, cells were washed twice with a PBS-bovine serum albumin solution. Then the cells were incubated at 37°C in the presence and absence of chloroquine (100 μM), and at the end of 1 h, protein synthesis was measured. Figure 4 shows that 50 or 100 μM chloroquine was able to prevent the potentiating activity of adenovirus. This result indicates that chloroquine probably acts after the initial binding step.

**Effect of chloroquine on EGF uptake.** Since PE-EGF has been shown to enter cells by the EFG receptors, we studied the effect of chloroquine on the uptake of $^{125}$I-EGF itself. To assess the effect of chloroquine on the uptake of EGF by KB

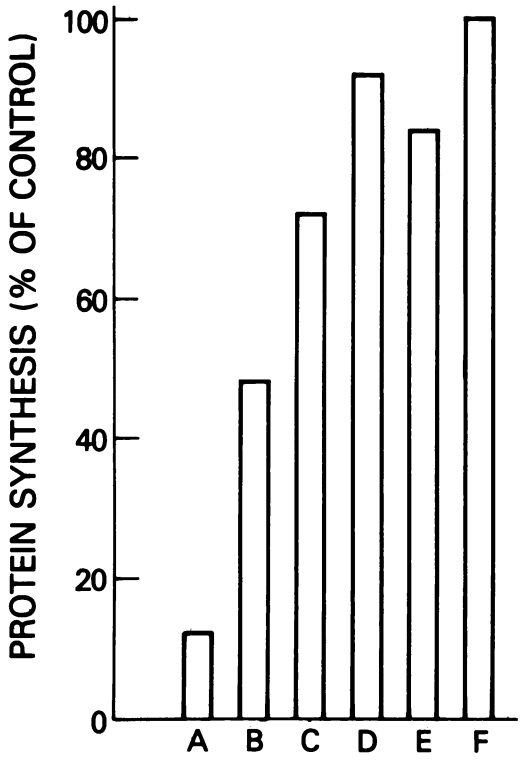


FIG. 4. Effect of chloroquine on the inhibition of protein synthesis by adenovirus and PE-EGF. Adenovirus (3 μg/ml) and PE-EGF (1 μg/ml) were bound to cells at 4°C for 30 min. Cells were washed three times with cold PBS-bovine serum albumin and incubated with 50 or 100 μM chloroquine at 37°C for 1 h. Medium was then replaced by [$^3$H]leucine (5 μCi/ml) and the cells were incubated for another 30 min at 37°C. (A) Protein synthesis in cells incubated with adenovirus and PE-EGF; (B) protein synthesis in cells incubated with adenovirus, PE-EGF, and 50 μM chloroquine; (C) protein synthesis in cells incubated with adenovirus, PE-EGF, and 100 μM chloroquine; (D and E) 50 or 100 μM chloroquine without adenovirus; (F) protein synthesis in cells with no additions; 100% represents 7,375 cpm.

cells, cells were incubated with $^{125}$I-EGF (0.05 μg/ml; specific activity, $10^7$ cpm/μg) in the absence and presence of chloroquine (100 μM) at 37°C. At each time interval, surface-bound EGF and EGF taken up by the cells were determined as described in Materials and Methods. In control cells the amount of EGF taken up by the cells increased with time up to about 30 min (Fig. 5). At 60 min the cell-associated EGF fell, probably due to its degradation within lysosomes. The rate of EGF uptake was unchanged in the presence of chloroquine. However, the decline of EGF seen at the 60-min point in control cells was prevented by chloroquine. This is probably due to inhibition of EGF degradation (2). In fact, lysosomotropic amines have been shown to inhibit mitogenesis induced by growth factors (10, 11).

**Effect of chloroquine on the toxicity of PE-EGF alone.** To inhibit protein synthesis, PE-EGF must enter cells and escape across a membrane to reach the cytosol where EF-2 is found. To examine whether chloroquine had an effect on this process, cells were incubated with PE-EGF in the absence of adenovirus. Usually assays with adenovirus employ a PE-EGF concentration of 0.5 μg/ml. At this concentration PE-EGF by itself does not inhibit protein synthesis in 2 h. Therefore, cells were exposed to 5 μg of PE-EGF per ml, which by itself inhibits protein synthesis by about 80% in 2 h. Chloroquine (100 μM) did not prevent the ability of PE-EGF (5 μg/ml) to inhibit protein synthesis (Table 3). This experiment makes it unlikely that chloroquine is acting on PE-EGF entry directly. Hence, we conclude that the effect of chloroquine is to prevent some activity present in the virus that facilitates the release of PE-EGF into the cytosol.

**Effect of chloroquine on the enhancement of PE-EGF toxicity by higher concentrations of adenovirus.** For the results presented above we usually used a single concentration of adenovirus (1 μg/ml) to enhance the toxicity of PE-EGF. Since we have used higher concentrations of adenovirus for ultrastructural studies (5), it was important to know whether

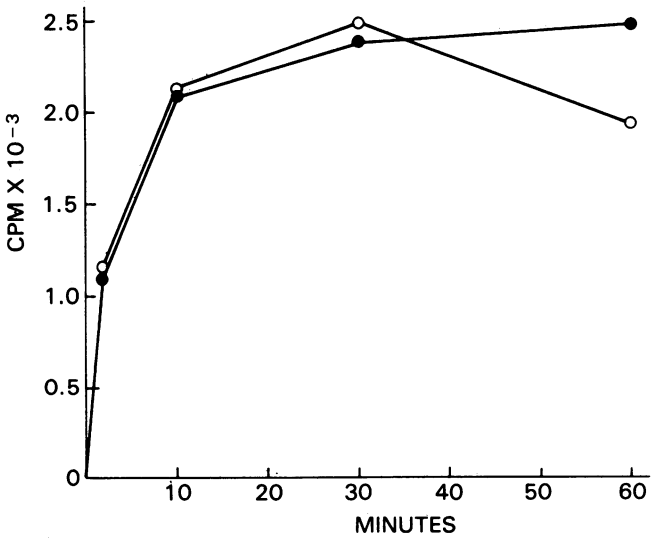


FIG. 5. Effect of chloroquine on EGF uptake. KB cells were incubated with $^{125}$I-EGF in the absence and presence of chloroquine (100 μM) as described in the text. At each time interval, the cells were washed five times with PBS and treated with 0.6 ml of 0.5 N NaCl in 0.2 N acetic acid. This wash was collected, and the cells on the dish were dissolved in 1 ml of 0.1 N NaOH. Symbols: (○) counts taken up by the control cells; (●) counts taken up by the chloroquine-treated cells.

TABLE 3. Effect of chloroquine on PE-EGF toxicity[a]

| Addition (concn) | Protein synthesis (% of control) |
|---|---|
| PE-EGF ( μg/ml) | 22 |
| PE-EGF (5 μg/ml) + chloroquine (100 μM) | 12 |
| Chloroquine (100 μM) | 90 |

[a] Cells were incubated with PE-EGF or PE-EGF + chloroquine for 4 h at 37°C. The medium was then removed and replaced for a further 1 h by DMEM containing 2 μCi of [$^3$H]leucine per ml.

chloroquine also prevents the enhancement of PE-EGF by higher concentrations of adenovirus. Cells were incubated with various amounts of adenovirus, and a fixed concentration of PE-EGF (0.5 μg/ml) (Fig. 6). Experiments were done both in the absence and presence of chloroquine (100 μM). Controls which received various concentrations of adenovirus alone were also performed. The results (Fig. 6) show that chloroquine at 100 μM reduced the adenovirus-dependent inhibition of protein synthesis at all the concentrations of adenovirus used up to 10 μg/ml. Thus the potentiating effect of adenovirus could be decreased by chloroquine. Adenovirus at 10 μg/ml or more inhibited protein synthesis by itself. This toxic effect of adenovirus alone was not prevented by chloroquine.

## DISCUSSION

The results reported in this paper show that the agents which raise the pH of endocytic vesicles decrease the ability of adenovirus to potentiate the toxic action of PE-EGF. None of the drugs tested blocked the cellular uptake of adenovirus or PE-EGF. Thus, it is a step beyond the binding and uptake of adenovirus and PE-EGF which is affected by raising the pH of the vesicles. On the basis of these findings it seems likely that the acidification of the receptosome enhances either the lysis of the receptosome membrane by adenovirus or some other step in the release of PE-EGF, and a rise in pH inhibits this process. The rapidity of the response (Fig. 1), the fact that UV treatment of the virus does not diminish the effect (unpublished data), and the fact that morphologically intact virus appears in the cytosol suggest that one of the external proteins of the virus is responsible for this effect. The three major external proteins are those that make up the fiber, the penton base, and the hexon (6).

Preliminary experiments using electron microscopy to examine the release of intact virus into the cytosol have not yet demonstrated a quantitative difference in release of virus from receptosomes due to chloroquine treatment, using high concentrations of virus. Because in these experiments individual virus particles must be counted, such experiments require large amounts of virus (10 μg/ml or greater). At high virus concentrations the action of chloroquine is diminished (Fig. 6). Nevertheless these preliminary results raise the possibility that the inhibition of viral enhancement of toxicity may not result from slowing of the lysis of receptosomes by the virus. For example, viral escape may proceed at a normal rate but the release of the toxin conjugate from the EFG receptor may be interfered with by the higher pH of the receptosome, or the inhibition may depend upon some other effect of chloroquine. It is noteworthy that the effects of chloroquine on enhancement of toxicity are never absolute and that at 100 μM chloroquine, where the effect on pH of receptosomes is maximal, the virus still shows some ability to enhance toxicity.

Several lipophilic amines have been found to inhibit the

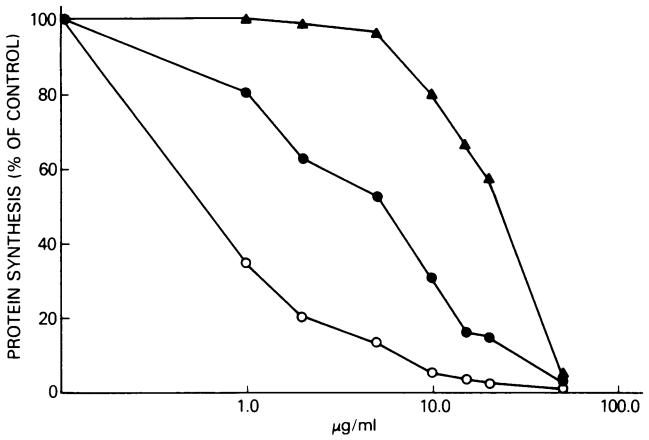


FIG. 6. Effect of chloroquine on the potentiation of PE-EGF toxicity by adenovirus. Cells were incubated with various concentrations of adenovirus and a fixed concentration of PE-EGF (0.5 μg/ml) in the absence (○) and presence (●) of chloroquine (100 μM). A suspension of adenovirus at a concentration of 1 μg/ml was sufficient to expose each cell to $9 \times 10^3$ adenovirus particles. Control cells received adenovirus alone (▲). The incubation was done for 45 min. At the end of this period, cells were replaced with medium containing [$^3$H]leucine (5 μCi/ml) for 30 min, and the level of protein synthesis was determined.

infection of cells by Sendai, influenza, and Semliki Forest viruses (17). One site of action of these agents is probably in the receptosome (endosome). Penetration of Semliki Forest virus into the cytoplasm requires a pH of less than 6.0 (14). These membrane-limited viruses do not appear to disrupt the receptosome, but instead the membrane of the virus fuses with that of the vesicle, allowing the nucleocapsid to escape into the cytosol. It has also been postulated that certain bacterial toxins such as diphtheria and tetanus toxins also require an acidic environment within an endocytic vesicle before they can be released into the cytoplasm (1, 3, 13, 21). Thus in certain instances, a low pH appears to promote three different biochemical effects: disruption of a vesicle by adenovirus, fusion of the membrane of some viruses with that of the vesicle, and translocation of a toxin across a membrane.

Chloroquine did not alter the toxicity of PE-EGF by itself for KB cells, making it possible to assess directly the effect of chloroquine on adenovirus enhancing activity. Previously it was reported that chloroquine, methylamine, and ammonium chloride inhibited the toxicity of PE for mouse fibroblasts (4, 18). In preliminary experiments, not presented here, PE was substituted for PE-EGF and the effect of chloroquine on adenovirus-mediated enhancement of PE toxicity was determined; the virus-mediated enhancement of toxicity appeared to be decreased. We believe this was a combined effect in which chloroquine was active against both PE and adenovirus. Thus, it appears that chloroquine has a specific inhibitory effect on PE toxicity when PE uptake is mediated by its own receptor, but not when it is mediated by the EGF receptor.

## ACKNOWLEDGMENTS

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## LITERATURE CITED

1. **Boguet, P., and E. Duflot.** 1982. Tetanus toxin fragment forms channels in lipid vesicles at low pH. Proc. Natl. Acad. Sci. U.S.A. **79**:7614–7618.
2. **Carpenter, G., and S. Cohen.** 1976. $^{125}$I-Labeled human epidermal growth factors. Binding, internalization, and degradation in human fibroblasts. J. Cell Biol. **71**:159–171.
3. **Donovan, J. J., M. I. Simon, R. K. Draper, and M. Montal.** 1981. Diphtheria toxin forms transmembrane channels in plasma lipid bilayers. Proc. Natl. Acad. Sci. U.S.A. **78**:172–176.
4. **FitzGerald, D., R. E. Morris, and C. B. Saelinger.** 1980. Receptor-mediated internalization of *Pseudomonas* toxin by mouse fibroblasts. Cell **21**:867–873.
5. **FitzGerald, D. J. P., R. Padmanabhan, I. Pastan, and M. C. Willingham.** 1983. Adenovirus-induced release of epidermal growth factor and pseudomonas toxin into the cytosol of KB cells during receptor-mediated endocytosis. Cell **32**:607–617.
6. **Ginsberg, H. S.** 1979. Adenovirus structural proteins. Compr. Virol. **13**:409–457.
7. **Goldstein, J. L., R. G. Anderson, and M. S. Brown.** 1979. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature (London) **279**:679–685.
8. **Green, M., and M. Pina.** 1963. Biochemical studies on adenovirus multiplication. IV. Isolations, purification, and chemical analysis of adenovirus. Virology **20**:199–207.
9. **Haigler, H. T., F. R. Maxfield, M. C. Willingham, and I. Pastan.** 1980. Dansylcadaverine inhibits internalization of $^{125}$I-epidermal growth factor in Balb 3T3 cells. J. Biol. Chem. **255**:1239–1241.
10. **King, A. C., and P. Cuatrecasas.** 1982. Exposure of cells to an acidic environment reverses the inhibition of methylamine of the mitogenic response to epidermal growth factor. Biochem. Biophys. Res. Commun. **106**:479–485.
11. **King, A. C., L. Hernaez-Davis, and P. Cuatrecasas.** 1981. Lysosomotropic amines inhibit mitogenesis induced by growth factors. Proc. Natl. Acad. Sci. U.S.A. **78**:717–721.
12. **Lyon, M., Y. Chardonnet, and S. Dales.** 1978. Early events in the interaction of adenovirus with HeLa cells. V. Polypeptides associated with the penetrating inoculum. Virology **87**:81–88.
13. **Marnell, M. H., M. Stookey, and R. K. Draper.** 1982. Monensin blocks the transport of diphtheria toxin to the cell cytoplasm. J. Cell Biol. **93**:57–62.
14. **Marsh, M., E. Bolzau, and A. Helenius.** 1983. Penetration of Semliki forest virus from acidic prelysosomal vacuoles. Cell **32**:931–940.
15. **Marsh, M., and A. Helenius.** 1980. Adsorptive endocytosis of Semliki forest virus. J. Mol. Biol. **142**:439–454.
16. **Maxfield, F. R.** 1982. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. J. Cell Biol. **95**:676–681.
17. **Miller, D. K., and J. Lenard.** 1981. Antihistamines, local anesthetics, and other amines as antiviral agents. Proc. Natl. Acad. Sci. U.S.A. **78**:3605–3609.
18. **Morris, R. E., M. D. Manhart, and C. B. Saelinger.** 1983. Receptor-mediated entry of *Pseudomonas* toxin: methylamine blocks clustering step. Infect. Immun. **40**:806–811.
19. **Pastan, I. H., and M. C. Willingham.** 1981. Receptor-mediated endocytosis of hormones in cultured cells. Annu. Rev. Physiol. **43**:239–250.
20. **Philipson, L., K. Lonberg-Holn, and V. Peterson.** 1968. Virus-receptors interaction in an adenovirus system. J. Virol. **2**:1064–1075.
21. **Sandvig, K., and S. Olsnes.** 1980. Diptheria toxin entry into cells is facilitated by low pH. J. Cell Biol. **87**:828–832.
22. **Willingham, M. C., and I. Pastan.** 1980. The receptosome: an intermediate organelle of receptor-mediated endocytosis in cultured fibroblasts. Cell **21**:67–77.