MOLECULAR AND CELLULAR BIOLOGY, Aug. 1984, p. 1528–1533
0270-7306/84/081528-06$02.00/0
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Vol. 4, No. 8

Evidence that the Penton Base of Adenovirus Is Involved in Potentiation of Toxicity of *Pseudomonas* Exotoxin Conjugated to Epidermal Growth Factor

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Received 5 April 1984/Accepted 25 May 1984

When KB cells are incubated for 1 h with human adenovirus type 2 or type 5 (1 μg/ml) and a conjugate of epidermal growth factor and *Pseudomonas* exotoxin (EGF-PE), protein synthesis is inhibited by 80 to 90%. Under these conditions, neither adenovirus nor EGF-PE alone has any effect on host protein synthesis. Thus, adenovirus enhances the toxicity of EGF-PE. A number of antibodies to intact virus and capsid components were tested for their ability to block the enhancing activity and virus uptake. At appropriate dilutions, antibodies prepared against intact virus and penton base blocked the enhancing activity without affecting virus uptake. Antibodies against hexon and fiber blocked virus uptake and enhancing activity in parallel. These studies suggest that the penton base is important in lysis of the vesicles which contain adenovirus and EGF-PE, and this base allows virus and toxin to enter the cytoplasm.

Many viruses, toxins, plasma proteins, and hormones are brought into cells by receptor-mediated endocytosis (for reviews, see references 10 and 16). The process involves the binding of these ligands to specific receptors on the cell surface, accumulation of ligand-receptor complexes in coated pits of the cell surface, and movement of these ligands into endocytic vesicles termed receptosomes (18, 31) or endosomes (12). From these vesicles the ligands are directed on to their ultimate cellular target which can be, for example, the cytoplasm, the Golgi system, or lysosomes. In the case of transferrin, the ligand is carried back to the cell surface (4, 17, 18, 30).

It was recently demonstrated that short-term incubation of KB cells with adenovirus potentiated the toxicity of a conjugate of epidermal growth factor and *Pseudomonas* exotoxin (EGF-PE) by about 10,000-fold (7). Because adenovirus and EGF-PE were internalized in the same endocytic vesicle and both were released into the cytoplasm, it was suggested that the enhancing effect of adenovirus was due to a biological activity present in adenovirus which disrupts the membrane of the vesicle. As a result, adenovirus and EGF-PE are released into the cytoplasm where EGF-PE ADP-ribosylates elongation factor 2 and inhibits protein synthesis in the host cells. Because what is measured is the enhanced ability of EGF-PE to inhibit protein synthesis, this activity of adenovirus is defined as enhancing activity. The enhancing activity of adenovirus appears to function better in the acidic environment of the receptosome than it does at a higher pH (unpublished data).

The enhancing activity of adenovirus is probably due to an external viral protein (7). The major external proteins of adenovirus are hexon, penton base, and fiber (8, 9, 21). Penton base and fiber are tightly bound together to form pentons, although penton bases can be isolated from the extracts of adenovirus-infected cells. Fibers appear to be the proteins responsible for the binding of adenovirus to the cell surface (8, 9). When added back to cultured cells, pentons have been shown to have a cytopathic activity, apparently a function of the penton bases (8, 9). The current study was initiated to investigate which viral components are necessary for the enhancing activity.

MATERIALS AND METHODS

Cells and cell culture. KB cells were maintained as monolayers in Dulbecco modified Eagle medium (DMEM) containing Pen-Strep and 10% bovine calf serum.

Virus. Human adenovirus type 2 and type 5 were grown as described earlier (11). Adenovirus was purified by banding it twice in CsCl gradients. Virus was UV inactivated by exposure to UV light for 15 min.

EGF-PE. A conjugate of *Pseudomonas* endotoxin and epidermal growth factor was constructed as described earlier (7).

Assay of adenovirus enhancement of EGF-PE toxicity. KB cells were seeded in 35-mm dishes at a density of 5×10^5 per dish and were used the next day. Just before the experiment, cells were washed with DMEM containing Pen-Strep and bovine serum albumin (BSA) (2 mg/ml). For assaying the enhancing activity of adenovirus, 1 μg of UV-inactivated adenovirus per ml (9×10^3 particles per cell) was incubated with or without EGF-PE (0.5 μg/ml) for 60 min in a total volume of 1 ml. The medium was then replaced by DMEM–Pen-Strep–BSA (2 mg/ml) containing 2 to 5 μCi of [^{3}H]leucine per ml and was incubated for an additional 30 min. After this, the monolayers were washed with phosphate-buffered saline, and the cells were dissolved in 0.1 N NaOH. Proteins were precipitated by trichloroacetic acid, and trichloroacetic acid-insoluble material was counted to determine the level of protein synthesis. Under these assay conditions, there was no inhibition of protein synthesis of host cells by either adenovirus or EGF-PE alone. However, when cells were incubated with UV-inactivated adenovirus and EGF-PE together, the inhibition of protein synthesis was usually between 80 and 90%. This degree of inhibition of protein

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synthesis was arbitrarily defined as 100% enhancing activity of adenovirus.

Preparation of ^{35}S-labeled virus and viral components. KB cells were seeded as monolayers 12 to 18 h before they were infected with human adenovirus type 2. To 5×10^6 cells in a 75-cm tissue culture flask was added 6 μg (protein) of virus (~50 PFU per cell) in DMEM-Pen-Strep-BSA (2 mg/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, at 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, and infection continued for 16 h more. Cells were removed from the plastic surface by vigorous 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 phosphate-buffered saline 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 was banded twice in CsCl gradients. ^{35}S-labeled viral proteins were obtained as the supernatants above the first sucrose-glycerol gradient.

Uptake of ^{35}S-labeled adenovirus. KB cells were incubated with ^{35}S-labeled UV-inactivated adenovirus at 1 μg of protein per ml (specific activity, 3,000 to 15,000 cpm/μg of viral protein). Cells were incubated at 37°C for 60 min, after which they were washed with phosphate-buffered saline and trypsinized for 15 min at 37°C to remove the virus bound to the cell surface. Cells were harvested and dissolved in 0.1 N NaOH, and their ^{35}S content was determined. The number of counts obtained represented the amount of virus taken up by the cells.

Antisera. Antisera were raised against the purified subunits of hexon, penton base, and fiber. Purified adenovirus type 2 was subjected to sodium dodecyl sulfate (SDS) gel electrophoresis with 3-mm-thick slab gels. Slices containing the individual polypeptide bands of hexon (molecular weight, 120,000 [120K]), penton base (85K), and fiber (61K) were cut out, and the proteins were electroeluted. After dialysis against water to remove SDS, the samples were lyophilized, suspended in 10 mM borate (pH 7.0), and used to raise antisera in rabbits. Three injections of 200 μg each were given. The first was with complete adjuvant, and the next two were with incomplete adjuvant. Serum was collected 3 weeks after the last injection and weekly thereafter.

Antisera to adenovirus type 2 and adenovirus type 5 were prepared by injecting purified virus into rabbits as described above. Antisera against purified native hexon and native fiber from adenovirus type 5 were prepared as described earlier (5, 23, 24).

Immunoprecipitations. Samples of the ^{35}S-labeled viral components were used to check the specificity of the various antisera. The details of the protocol of immunoprecipitation are described in the legend to Fig. 1. As shown in Fig. 1, antisera to adenovirus type 2 precipitated several viral components, including hexon, penton base, and fiber; antisera prepared against hexon, penton base, and fiber of type 2 each mainly immunoprecipitated their respective proteins. Small amounts of hexon were brought down by the control serum (Fig. 1).

Chemicals. All media constituents were purchased from GIBCO Laboratories, Grand Island, N.Y. [^{3}H]leucine was from New England Nuclear Corp., Boston, Mass. Epidermal growth factor was from Bethesda Research Laboratory, Gaithersburg, Md. Chemicals for SDS gel electrophoresis were from Bio-Rad Laboratories, Richmond, Calif., X-ray films were from Eastman Kodak Co., Rochester, N.Y. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Potentiation of EGF-PE toxicity by adenovirus type 2 and type 5. KB cells were treated with various concentrations of UV-inactivated adenovirus type 2 or type 5 in the absence and presence of EGF-PE (0.5 μg/ml) for 60 min at 37°C. When either adenovirus type 2 or type 5 was used alone at 0.1 or 1 μg/ml, there was no inhibition of protein synthesis of host cells (Fig. 2). At 10 μg/ml, there was about 10% inhibition of protein synthesis (unpublished data). EGF-PE alone at 0.5 μg/ml for 1 h did not inhibit protein synthesis.

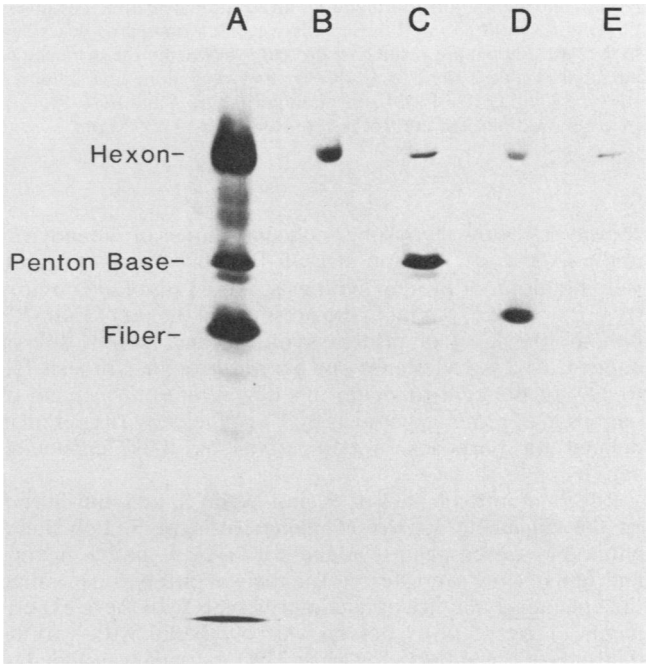

FIG. 1. Immunoprecipitations of ^{35}S-labeled viral proteins. Five microliters of ^{35}S-labeled adenovirus type 2 components was incubated with a 1:50 dilution of anti-adenovirus 2, anti-hexon 2, anti-penton base 2, anti-fiber 2, and preimmune serum in a final volume of 1 ml with buffer A (Tris-buffered saline, 0.5% Nonidet P-40, 0.02% SDS, 0.5% tyrasylol). Incubations were done at 4°C for 30 min, after which 50 μl of *Staphylococcus aureus* was added and incubations were continued for an additional 30 min. The solutions were centrifuged at 5,000 × *g* for 5 min, and the pellets obtained were successively washed with buffer A, buffer B (buffer A plus 1 M NaCl), and buffer A again. One hundred microliters of SDS-gel sample buffer was added and boiled for 5 min at 100°C to elute the proteins from the immunoadsorbent. The tubes were centrifuged again at 5,000 × *g* for 5 min to remove *S. aureus*, and 50 μl of the supernatant was loaded on 7.5% SDS gel (13). The gel was stained by 0.25% Coomassie blue in 50% trichloroacetic acid for 30 min and was destained overnight in 10% acetic acid and 10% methanol. The gel was fluorographed using 20% 2,5-diphenyloxazole in dimethyl sulfoxide. The gel was dried and autoradiographed by exposing the gels to X-ray film for 4 h. Shown are proteins brought down by anti-adenovirus 2 (lane A), by anti-hexon 2 (lane B), by anti-penton base 2 (lane C), by anti-fiber 2 (lane D), and by preimmune serum (lane E).

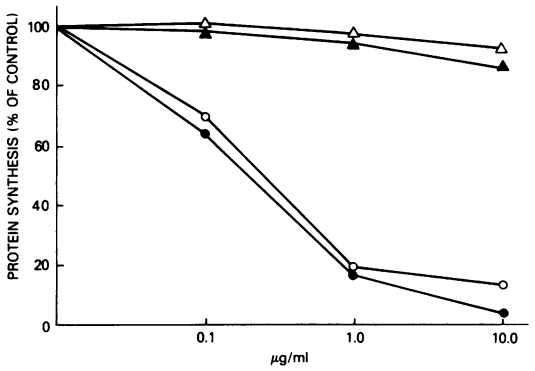

FIG. 2. Inhibition of protein synthesis by adenovirus type 2 and adenovirus type 5 in the absence and presence of EGF-PE. KB cells were incubated with various concentrations of UV-inactivated adenovirus type 2 and type 5 with and without EGF-PE (0.5 μg/ml) for 60 min at 37°C. At the end of this incubation, the medium was replaced by DMEM-BSA (2 mg/ml) containing 2 μCi of [^{3}H]leucine per ml, and incubation continued for an additional 30 min. The levels of protein synthesis in all these cells were determined as described in the text. Shown are results for protein synthesis in the presence of adenovirus type 2 alone (▲), adenovirus type 5 alone (△), adenovirus type 2 and EGF-PE (●), and adenovirus type 5 and EGF-PE (○). One hundred percent control is represented by 11,392 cpm.

However, with increasing concentrations of adenovirus and a fixed concentration of EGF-PE, there was a progressive inhibition of protein synthesis. At 0.1 μg of adenovirus (type 2 or type 5) per ml in the presence of 0.5 μg of EGF-PE per ml, the level of protein synthesis was about 70% of control. At 1 μg of adenovirus per ml, protein synthesis fell to 20% of the control value; this degree of enhancement of inhibition of protein synthesis by 1 μg of adenovirus per ml is defined for purposes of comparison as 100% enhancing activity.

Effects of anti-adenovirus 5, anti-hexon 5, and anti-fiber 5 on the enhancing activity of adenovirus type 5. Polyclonal antibodies raised against adenovirus type 5, native hexon, and native fiber were tested for their abilities to neutralize the enhancing activity of adenovirus type 5. In these experiments, 1 μg of virus per ml was incubated with various dilutions of the antisera for 1 h at 23°C and then was added to the cells with 0.5 μg of EGF-PE per ml and incubated for 1 h. All three antisera neutralized the enhancing activity, but the antiserum against hexon was somewhat less potent (Fig. 3).

The above results indicate that antisera against adenovirus type 5 and two of its components, hexon and fiber, neutralized the enhancing ability of adenovirus. There are at least two ways by which this might have happened. One is that all these antisera aggregated the virus so that it could not bind to the cell surface and hence was not internalized. The other is that the antisera did not prevent the binding and internalization of adenovirus but specifically neutralized the viral component involved in the potentiation of the toxicity of EGF-PE. To distinguish between these possibilities, the effects of the antisera on the uptake of the adenovirus and enhancing activity were measured under similar conditions. ^{35}S-labeled adenovirus type 5 was incubated with various concentrations of anti-adenovirus type 5, anti-hexon, and anti-fiber. At the end of the incubation, EGF-PE was added to all the samples, and portions of each were used to study adenovirus uptake and enhancing activity of adenovirus. The results of these experiments are shown in Fig. 4. Anti-adenovirus 5 antiserum at 1:100 neutralized the enhancing activity by about 90%, and the uptake of adenovirus was almost completely blocked, indicating that adenovirus probably did not bind to the cells (Fig. 4A). However, at a dilution of 1:1,000, the antiserum neutralized the enhancing activity by about 90%, and the uptake of virus was reduced only by about 15%. At a dilution of 1:10,000, the enhancing ability of adenovirus was reduced by about 40%, and there was no inhibition of virus uptake (Fig. 4A). Thus, although adenovirus is internalized by the cells, it apparently is not able to deliver EGF-PE into the cytoplasm.

When specific antiserum against hexon protein or fiber was tested at various dilutions, inhibition of virus uptake and inhibition of enhancing activity changed in parallel (Fig. 4B and C). These results indicate that antisera to hexon and fiber proteins can prevent the ability of adenovirus to potentiate the toxicity of EGF-PE by preventing the internalization of adenovirus, but this does not explain the results with antibody to whole virus.

The above results suggest that the hexon and fiber are not the proteins in adenovirus directly responsible for release of EGF-PE into the cytoplasm. The third major component present in the adenovirus is penton base. Since most of the penton base exists in the adenovirus-infected cell bound to fiber in the form of pentons (8), we purified penton base from adenovirus type 2 by separating it from other viral proteins on SDS gels and made antibodies to this form of the protein. Antisera were also raised against the polypeptides of hexon and fiber purified from adenovirus type 2 in a similar way. All these antisera were used to study the neutralization of the enhancing ability of adenovirus type 2.

Neutralization of enhancing activity of adenovirus type 2 by various antisera against adenovirus type 2 and its proteins. UV-inactivated adenovirus type 2 was incubated with polyvalent antisera to total adenovirus type 2 and to the subunits

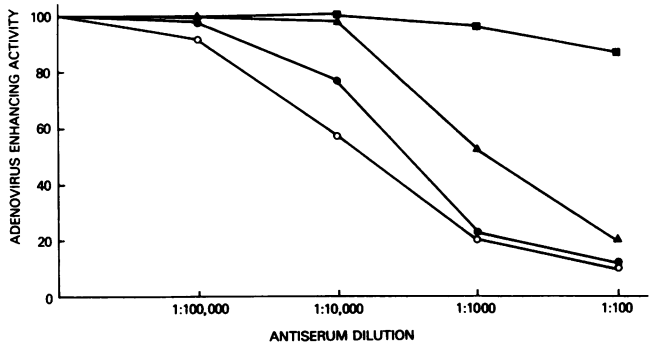

FIG. 3. Neutralization of adenovirus type 5 enhancing activity by anti-adenovirus 5, anti-hexon 5, and anti-fiber 5. UV-inactivated adenovirus type 5 (1 μg/ml) was incubated with various concentrations of the respective antiserum and preimmune serum in a final volume of 3 ml. The dilutions were made with DMEM-BSA. The incubations were done at room temperature for 60 min. At the end of this incubation, EGF-PE was added at a final concentration of 0.5 μg/ml. This medium was immediately added to monolayers of KB cells. The cells were incubated with this medium for 60 min, after which the medium was replaced by DMEM-BSA containing 2 μCi of [^{3}H]leucine per ml and incubation continued for an additional 30 min. The level of protein synthesis was determined as described in the text. From these values, enhancing activity of adenovirus was calculated. Shown are the enhancing activites of adenovirus type 5 when the virus was preincubated with preimmune serum (■), with anti-hexon 5 (▲), with anti-adenovirus 5 (●), and with anti-fiber (○).

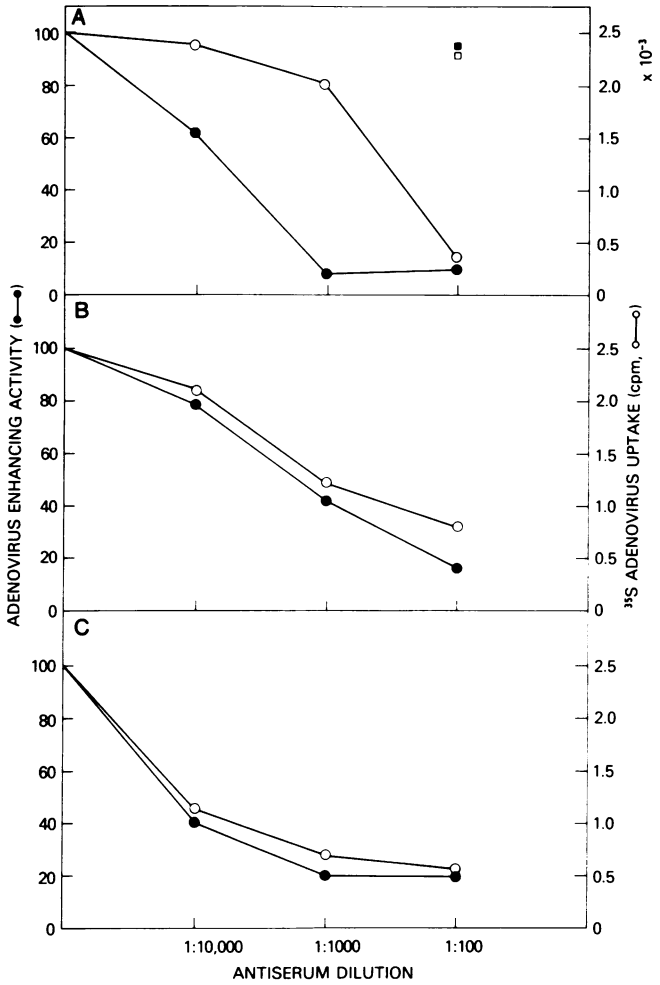

FIG. 4. Effect of anti-adenovirus 5, anti-hexon 5, and anti-fiber 5 on the enhancing activity and uptake of adenovirus type 5. UV-inactivated, ^{35}S-labeled adenovirus type 5 (1 μg/ml; specific activity, 15,000 cpm/μg of protein) was incubated with various concentrations of anti-adenovirus 5 (A), anti-hexon 5 (B), anti-fiber 5 (C), and perimmune serum as shown in the figure. DMEM-BSA was used for the dilutions. Incubations were done for 60 min at room temperature, after which EGF-PE was added at a final concentration of 0.5 μg/ml. Incubating solutions were immediately split in two portions. One half was used to determine the enhancing activity of adenovirus as described in the legend to Fig. 3, and the other portion was used to study the uptake of ^{35}S-labeled adenovirus on KB cells as described in the text. Enhancing activity of adenovirus (■) and uptake of ^{35}S-labeled adenovirus type 5 (□) in the presence of preimmune are also shown.

of hexon, penton base, and fiber of adenovirus type 2. Anti-adenovirus 2 completely neutralized the enhancing activity at dilutions of 1:10 and 1:100 (Fig. 5). At 1:1,000, there was about 80% inhibition and at 1:10,000, 30% inhibition (Fig. 5 and 6). With anti-penton base, enhancing activity was completely neutralized at an antiserum dilution of 1:10, 70% neutralized at 1:100, and 20% neutralized at 1:1,000 (Fig. 5). Anti-fiber showed only about 30% inhibition of enhancing activity at a 1:10 dilution; at dilutions of 1:100 and 1:1,000, no inhibition was detected. Anti-hexon did not neutralize the enhancing activity of adenovirus type 2, indicating either that the antibody against hexon subunit does not immunoprecipitate the native virus or that there is very little antihexon in the antiserum.

Effects of anti-adenovirus 2 and anti-penton base 2 on the uptake of adenovirus type 2. Earlier experiments with adenovirus type 5 raised the possibility that penton base might be the protein responsible for the enhanced release of EGF-PE into the cytoplasm. Since the antiserum against penton base of adenovirus type 2 neutralized the enhancing ability of adenovirus type 2 (Fig. 5), we compared the effect of anti-penton base and anti-adenovirus 2 on the uptake of adenovirus type 2, along with the enhancing activity of the virus (Fig. 6). With antiserum against the native adenovirus type 2 at 1:10, the enhancing activity and uptake of the virus were blocked, perhaps owing to aggregation of the virus (Fig. 6A). However, at a dilution of 1:100, there was about 90% inhibition of the enhancing activity of adenovirus, but the inhibition of uptake was only about 30%. At a dilution of 1:1,000, enhancing activity was inhibited by about 90%, whereas the uptake of adenovirus was normal. At a 1:10,000 dilution, virus uptake was unaffected, but there was still about 30% inhibition of enhancing activity. These results indicate that the antiserum against whole adenovirus might contain antibodies which do not interfere with adenovirus internalization but do prevent its ability to deliver EGF-PE into the cytoplasm.

The effects of an antiserum to penton base on the uptake and enhancing activity of adenovirus 2 are shown in Fig. 6B. At an antiserum dilution of 1:10, the uptake of adenovirus was only 10% of the control, and the enhancing activity was also only 10%. However, at a 1:100 antiserum dilution the uptake of adenovirus was completely normal, but adenovirus did not enhance the toxicity of EGF-PE. At dilutions of 1:1,000 and 1:10,000, the uptake of adenovirus was not affected, whereas the enhancing activities of virus were still reduced by about 30 and 15%, respectively. These results indicate that the antibody to penton base can inactivate the enhancing function of adenovirus without interfering with the uptake of the virus.

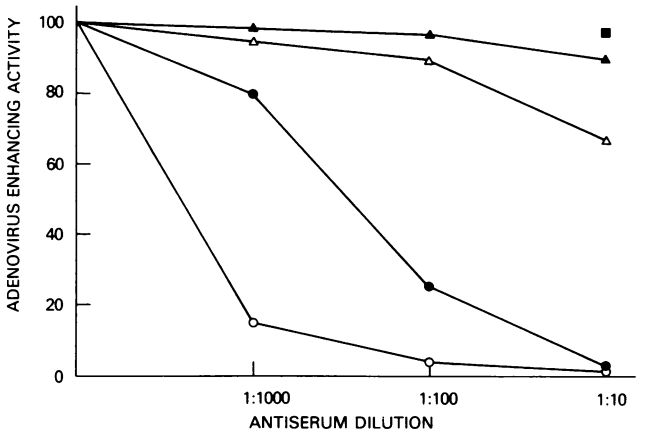

FIG. 5. Neutralization of adenovirus type 2 enhancing activity by anti-adenovirus 2, anti-hexon 2, anti-penton base 2, and anti-fiber 2. UV-inactivated adenovirus type 2 (1 μg/ml) was incubated with antisera against native adenovirus type 2 (○), against the hexon subunit of adenovirus type 2 (▲), against the penton base subunit of adenovirus type 2 (●), and against the subunit of fiber of adenovirus type 2 (△) and with preimmune serum (■) for 60 min at room temperature. The effects of these preincubations on the enhancing activity of adenovirus type 2 was determined as described in legend to Fig. 3 for adenovirus type 5.

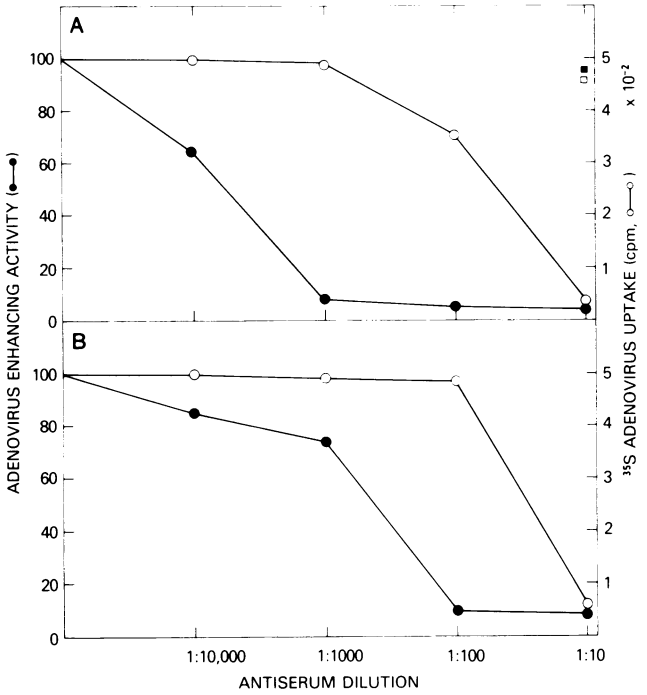

FIG. 6. Effect of anti-adenovirus 2 and anti-penton base 2 on the enhancing activity and uptake of adenovirus type 2. UV-inactivated, ^{35}S-labeled adenovirus type 2 (1 μg/ml; specific activity, 3,000 cpm/μg of protein) was incubated with various concentrations of anti-adenovirus 2 (A), anti-penton base 2 (B), and preimmune serum. Incubations were done for 60 min at room temperature. The enhancing activity of adenovirus type 2 and uptake of ^{35}S-labeled adenovirus type 2 were determined as described in legend to Fig. 4 for adenovirus type 5. Enhancing activity of adenovirus (■) and uptake of ^{35}S-labeled adenovirus (□) in the presence of preimmune serum are also shown.

Neutralization of enhancement of adenovirus type 5 by antisera against adenovirus type 2 and its components. Antisera against adenovirus type 2 and its components hexon, penton base, and fiber were tested for their ability to neutralize the potentiation of EGF-PE toxicity by adenovirus type 5. Even at the highest concentration of anti-adenovirus 2, there was no neutralization of the enhancing activity of adenovirus type 5. This result is not surprising since these two neutralizing antibodies are not cross-reactive (26, 28). However, the antiserum against penton base neutralized the enhancing activity of adenovirus type 5. At a 1:10 dilution, the inhibition of enhancing activity was about 90%; at 1:100, it was about 50%; and at 1:1,000, it did not neutralize enhancing activity (data not shown). It seems likely, therefore, that the penton bases of adenovirus 2 and type 5 are antigenically and functionally similar. Antisera against hexon and fiber protein also did not neutralize the enhancing activity of adenovirus type 5. This result is consistent with the previous observations that both hexon and fiber of adenovirus type 2 and type 5 contain immunologically distinct determinants, although hexons and pentons also cross-react immunologically (9, 29).

DISCUSSION

Human adenovirus type 2 and type 5 enhance the ability of a hybrid toxin (EGF-PE) to inhibit protein synthesis in KB cells. Apparently, both have similar mechanisms for delivering EGF-PE into the cytoplasm. The rapidity of this process, along with the facts that UV-inactivated adenovirus and adenovirus devoid of DNA enhance the toxicity of EGF-PE (unpublished observations) and that intact virus appears in cytoplasm within 15 to 30 min after binding to the cell surface (7, 15), suggests that one of the external proteins of adenovirus is responsible for the enhancing activity of the virus. The data in this paper implicate the penton base as having an important role in this process. Antisera prepared against penton base block the enhancing activity of virus without affecting virus uptake. Although it seems unlikely, there is a formal possibility that anti-penton base does not directly interfere with adenovirus enhancement of the toxicity of EGF-PE, but rather that the antibody indirectly blocks enhancement by changing the route of cellular uptake of the toxin. Antisera to other prepared components (fiber and hexon) only block enhancing activity in proportion to their ability to inhibit virus uptake. It is interesting that penton bases of both adenovirus type 2 and type 5 conserve their functional and antigenic properties. In fact, antibodies to penton base of adenovirus type 2 and type 5 are cross-reactive (1).

These findings also suggest the mechanism by which adenoviruses enter cells to initiate uncoating of the virion in preparation for its nuclear replication (14). Thus, virions attached to susceptible cells by interaction of the fibers with specific cell receptors, which is rapidly followed by envelopment of the viral particles in endocytic vesicles, receptosomes. However, even after the virions are incorporated into the receptosomes, they remain outside the cytoplasm relative to the cellular plasma membrane. The data presented imply that the penton base serves to disrupt the membrane of the receptosome to permit actual entry of the virion into the cytoplasmic matrix.

The protein penton base of adenovirus has been shown to be cytopathic to the cells (1, 6, 19, 20, 22, 25). It is an open question whether this cytopathic action of penton base of adenovirus is due to a similar kind of activity which gives adenovirus its ability to enhance the toxicity of EGF-PE and to enter the susceptible host cell. It will be interesting to investigate this problem by making use of the genetic mutants of adenovirus defective in the production of penton and fiber (2, 3).

ACKNOWLEDGMENTS

We thank M. Gallo for preparing various conjugates and E. Lovelace and A. Harris for propagating various cell lines.

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