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Interaction Between HeLa Cells and Adenovirus Type 2 Virions Neutralized by Different Antisera

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Three adenovirus type 2-specified immunogens elicited neutralizing antibodies when injected into rabbits; these were the fiber, the hexon, and the penton base. Adenovirus type 2 virions, neutralized by antihexon- or anti-penton base antisera, attached to HeLa cells to the same extent as untreated control virus, and after attachment, neutralized viruses also became sensitive to DNase treatment. A fraction of 75 to 80% of the attached antibody-treated virions penetrated the plasma membrane, which should be compared with an 84 to 88% penetration level in the control series. A majority of the antihexon-neutralized virions was found in intracellular vesicles, as revealed with an electron microscope, but in the case of anti-penton base neutralization, a maximum of 50% of the virions was retained within vesicles, and ca. 30% was free in the cytoplasmic compartment. A value greater than 45% was never obtained for neutralization with a monospecific anti-penton base antiserum, which could imply the existence of alternative pathways for virus penetration into HeLa cells—one of these being sensitive to treatment with anti-penton base antiserum. Antisera containing antifiber specificities efficiently aggregated virions, and the aggregation data mirrored the degree of neutralization. Antifiber-neutralized virions attached to cells to a three- to five times greater extent than untreated control virus, but the former virions had a reduced ability to become sensitive to DNase treatment. Around 15% of the attached antifiber-treated virions was found as large aggregates inside multivesicular bodies or lysosomes.

Antibody-mediated neutralization of viruses has been extensively studied and has been reviewed by Mandel (20, 21) and Dimmock (4). In the adenovirus system, the hexon antigen from serotypes 1 to 5 has been shown to elicit neutralizing antibodies when immunized into different host species (1, 9, 14, 22, 24, 25, 35, 40, 42, 43), although the neutralizing potency of those antisera varies among the serotypes. Antisera against the fiber antigen of serotypes 1, 2, and 5 have been reported both to contain (1, 22, 42) and to lack (14, 27, 28, 40) significant amounts of neutralizing antibodies. One possible explanation for these conflicting data could be the employment of different neutralization assays by these investigators. An antifiber antiserum effectively aggregates virions (5, 31, 39), and aggregation of virions in vivo could be an obvious way to abolish virus infectivity, but this is not always considered as being equivalent to a true neutralization (20). Neutralization of adenovirus is a serotype-specific phenomenon; i.e., different serotypes are neutralized by homologous antisera (22, 24, 41, 42). Heterologous neutralization is achieved among some serotypes, provided that the antibodies are allowed to attach to virions at low pH values (14); however, isolated type-specific antihexon antibodies exert a strong neutralizing effect (9, 43, 44), whereas the group-specific antihexon antibodies do not (9, 43). Antisera against the penton base and protein IIIa have been reported not to be involved in neutralization of adenovirus type 2 (Ad2) (2, 16).

The aims of the present investigation were to evaluate the role of different viral immunogens in eliciting neutralizing antibodies and to study the subsequent interaction between neutralized virions and HeLa cells. For this purpose, two series of antisera were used. One was a total anti-Ad2 antiserum from which antifiber or antihexon antibodies or both were removed, and the other was a series of monospecific antisera produced in response to extensively purified Ad2 structural proteins. Virions neutralized by various antisera were subsequently studied with regard to patterns of attachment, penetration, uncoating, and cellular distribution.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at densities between 2.5×10^5 to 5×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal calf serum and 5 μg of gentamicin per ml. For attachment studies, cells were grown in petri dishes (60-mm diameter) in Eagle minimal essential medium formulated for monolayer cultures and supplemented with 10% fetal calf serum and gentamicin as above.

The wild type of human Ad2 was propagated and purified as previously described (6, 8). [^{3}H]thymidine-labeled virus was prepared by the method of Svensson et al. (38).

Production of antisera. (i) Polyspecific antisera. Antisera against Ad2 were obtained after three successive intramuscular immunizations in rabbits over a 4-week period (10) with 1.4×10^{12} highly purified virions each time, corresponding to 0.36 mg of total protein (17). The immunogens were mixed with equal volumes of Freund complete adjuvant at the first and second immunization. The rabbits were bled 1 week after the last immunization and thereafter weekly over a 4-week period. Two separately prepared antisera against intact Ad2 particles were used in this investigation, and these will be referred to as no. 111 and no. 113.

(ii) Monospecific antisera. The major soluble antigens (i.e., hexon, fiber, and penton base) remaining after virus isolation by preparative ultracentrifugation on CsCl gradients were separated by DEAE-cellulose chromatography as described by Pettersson et al. (26) and modified as recently described (45). The hexon antigen was further purified by quaternary aminoethyl-Sephadex chromatography and negative affinity on a column of antipenton-immunoglobulin G-Sepharose 4B (see below). The fiber antigen was further purified by

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carboxymethyl-Sephadex chromatography, and the penton base was purified by quaternary aminoethyl-Sephadex chromatography, followed by negative affinity on antifiber-immunoglobulin G-Sepharose 4B and antihexon-immunoglobulin G-Sepharose 4B. Purified protein IIIa, prepared as described by Everitt and Philipson (7), was obtained from L. Philipson.

Rabbits were immunized with each protein (20 to 50 μg) according to the schedule described above, and preimmune sera were collected before all immunizations. Each monospecific antiserum was titrated by rocket immunoelectrophoresis against the anti-Ad2 antiserum no. 113 and subsequently diluted with phosphate-buffered saline (PBS) to contain the same concentration of precipitating antibody against the appropriate antigen. All antisera were heat inactivated at 56°C for 30 min before use.

Cyanogen bromide activation of Sepharose and coupling of antigens. Sepharose 4B in portions of 3 g was activated by addition of CNBr dissolved in bidistilled water. Either 14 mg of hexon or 4 mg of fiber antigen, both purified as described above, was added to the activated gel. Incubations and blocking of remaining reactive groups were performed as described by the manufacturer of Sepharose 4B. The immunoglobulin fractions from monospecific antisera were precipitated in $(NH_4)_2SO_4$ at a concentration of 33% relative saturation and were coupled in an analogous way.

Removal of selected antibody specificities from an anti-Ad2 antiserum. Portions (200 μl) of an anti-Ad2 antiserum (no. 111) were passed through columns of fiber-Sepharose 4B or hexon-Sepharose 4B. All UV-absorbing material above 0.01 at 280 nm not retained by the Sepharose column was collected and dialyzed extensively against bidistilled water. After lyophilization, the absorbed antisera were resuspended in PBS to give four times the original volume. The specificities of the immunoabsorbed antisera were tested by rocket immunoelectrophoresis (RIE) (15).

RIE. Appropriate amounts of antisera, as indicated in Fig. 1, were mixed with 1% agarose in 73 mM Tris–24 mM barbitone buffer (pH 8.6) containing 0.45 mM calcium lactate. Portions (5 μl) were applied in each sample well, and electrophoreses were performed at 75 V for 16 to 18 h at 10°C. After electrophoreses, the gels were compressed, dried, and stained in 0.2% Coomassie brilliant blue R dissolved in methanol-acetic acid-water (45:10:45).

Immunoaggregation of virions by different antisera. Samples (25 μl) of [^{3}H]thymidine-labeled virions (4.4×10^{10} particles and 125,000 cpm of radioactivity) were added to an equal volume of the appropriate antiserum, and when necessary, these were diluted in PBS containing 1% bovine serum albumin (BSA) to yield final antiserum dilutions ranging from 1/2 to 1/4,000. Higher concentrations of antisera were obtained by adding 100 μl of the appropriate antiserum to 25 μl of virus, and these series will be referred to as the 1/1.25 samples.

After incubations at 37°C for 30 min, 50 μl of PBS was added to the virus-antibody mixtures, and after thorough mixing, the samples were layered onto sucrose gradients formed on top of a cushion of CsCl (1.4 g/ml) and consisting of 2 × 1.5 ml of 10 to 25% (wt/vol) sucrose in 5 mM Tris hydrochloride–0.2 mM EDTA (pH 7.5). Centrifugations were performed at 50,000 × *g* for 10 min at 5°C. The tubes were fractionated from the bottom into fractions of 5 drops each, and portions were assayed for radioactivity in Ready-Solv (Beckman Instruments AB) after precipitation with trichloroacetic acid on paper filter disks. For each sample, the immunoaggregated radioactivity recovered on the CsCl cushion was monitored relative to the rest of the radioactivity in the tube.

Neutralization of virus infectivity measured by progeny virus immunotitration. The neutralization tests were performed by a progeny virus immunotitration assay as recently described (46). Briefly, 4.4×10^{10} virions in volumes of 25 μl were added to appropriate antisera at final dilutions as described above. After incubations at 37°C for 30 min, PBS containing 1% BSA was added to each sample to give a final volume of 1,550 μl. Samples (25 μl) of antibody-virus complexes were added to 3 ml of Eagle minimal essential medium containing 1.52×10^7 cells in 100-ml screw-cap Schott Glaswerke laboratory bottles, yielding a multiplicity of infection of 46 particles per cell, equivalent to one infectious unit per cell. After attachment for 30 min at 37°C, 34 ml of Eagle minimal essential medium containing serum was added to each bottle, yielding a concentration of around 4.1×10^5 cells per ml. At 39 h postinfection, progeny virus was prepared by a one-step CsCl gradient centrifugation procedure. An equal volume of 10 M urea was added to the virus material, and progeny virus was quantified by RIE against an antihexon antiserum. Neutralized virus samples were compared with separate controls in which the neutralizing antisera were replaced by PBS containing 1% BSA or preimmune serum.

In vitro DNase sensitivity of virions after incubation with different antisera. The DNase sensitivity analyses, both in vitro and of virions attached to cells (see below), were performed essentially as described by Joklik (11). Appropriate antisera at the dilutions described above were added, in volumes of 25 μl, to 4.4×10^{10} [^{3}H]thymidine-labeled virions. After incubations at 37°C for 30 min, 1 volume of 1% BSA in PBS was added to the sample, whereafter 7.5×10^9 particles were withdrawn and incubated for 30 min at 37°C in the presence of 0.2 mg of DNase I and 7.5 mM $MgCl_2$ in PBS in a final volume of 60 μl. The samples were subsequently diluted with 400 μl of PBS containing 0.1% BSA, and undegraded [^{3}H]DNA was precipitated with trichloroacetic acid at a final concentration of 10%. Trichloroacetic acid precipitates were pelleted, washed with ethanol, and dissolved in Protosol (New England Nuclear). The supernatants and the precipitates were subsequently assayed for radioactivity in 0.4% Omnifluor (New England Nuclear) in toluene-methanol (1:1).

Attachment of virus-antiserum mixtures. Confluent monolayers of HeLa cells were washed with PBS containing 1% BSA. [^{3}H]thymidine-labeled virions were preincubated with the appropriate antiserum for 30 min at 37°C as described above and added to the monolayer cells at multiplicities of infection of 2,000 in total volumes of 500 μl of Eagle minimal essential medium formulated for monolayer cultures. Incubations were performed for 60 min at 37°C on a rocking platform. Unattached virions were aspirated, and the cells were washed once with 1% BSA in PBS and once with 0.02% EDTA in PBS. The monolayers were incubated in the presence of the latter solution at 37°C until the cells were released. The radioactivity of the cellular fraction and the washing solutions was measured, and the percentage of virus attached was calculated.

DNase sensitivity of virus-antiserum complexes attached to cells. [^{3}H]thymidine-labeled virions, preincubated with different antisera, were allowed to attach to cells in monolayer cultures for 60 min at 37°C as described above. The cells were released from the petri dishes as described above, transferred to centrifuge tubes, and sedimented. Suspension of cells was done in 10 mM sodium phosphate buffer (pH 7.3)

containing 10 mM $MgCl_2$. The cells were disrupted by addition of sodium deoxycholate to give a concentration of 0.5%. After addition of 1 mg of DNase I and incubation at 37°C for 30 min, the undegraded DNA was precipitated with trichloroacetic acid at a final concentration of 10%. Precipitates were pelleted, washed with 70% ethanol, dissolved in Protosol, and assayed for radioactivity.

Electron microscopy. Cells in suspension cultures were sedimented, washed once, and suspended in PBS at a concentration of 5×10^7 cells per ml. Virions, pretreated with the appropriate antisera at concentrations indicated in Table 1, were added to the cells at calculated multiplicities of infection of 5,000. The samples were incubated for 60 min at 37°C, whereafter the cells were washed twice in 10 ml of PBS each time. The cells were resuspended in PBS and kept at 3°C at a density of 10^6 cells per ml. Cells were fixed in 2% glutaraldehyde for 30 min at 3°C in a roller bottle, after which the cells were washed three times in PBS. Postfixation with osmium tetroxide and dehydrations were performed essentially as described by Ryter and Kellenberger (32), and finally the cells were embedded in Epon.

Chemicals. Eagle minimal essential medium for suspension cultures and for monolayer cultures, fetal calf serum, and gentamicin were obtained from Flow Laboratories Ltd., Irvine, Scotland. Freund complete adjuvant was from Difco Laboratories, Detroit, Mich. DEAE-cellulose (DE 52) was obtained from Whatman Inc., Clifton, N.J., and Sepharose 4B was from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Agarose (type 1) and DNase I were obtained from Sigma Chemical Co., St. Louis, Mo. Ready-Solv HP/b was purchased from Beckman Instruments AB, Bromma, Sweden. Omnifluor and Protosol were obtained from New England Nuclear, Dreieich, Federal Republic of Germany. Sodium deoxycholate was from BDH Chemicals Ltd., Poole, England, and the laboratory bottles were from Schott Glaswerke, Mainz, Federal Republic of Germany.

RESULTS

Specificity of the immunoabsorbed antisera. Columns of fiber-Sepharose 4B and hexon-Sepharose 4B were used to selectively remove antifiber or antihexon antibodies from an anti-Ad2 serum (no. 111). The remaining specificities of the antisera were analyzed by RIE against different virion-specific antigens (Fig. 1). As compared with the control serum (Fig. 1A and B, gel 1), it was shown that the antibody titers against the penton base (Fig. 1A) and protein IIIa (Fig. 1B) were not significantly affected by the removal of antihexon antibodies (gel 2), antifiber antibodies (gel 3) or both (gel 4). Moreover, no precipitates against the homologous antigens could be detected among the absorbed antisera when they were tested by RIE against a wide range of concentrations of both antigens and antisera as exemplified in Fig. 1.

Immunoaggregation and neutralization of virions by different antisera. Two series of antisera were used. One was a total anti-Ad2 serum (no. 111) together with the absorbed antisera derived from the same serum pool (described above), and the other was a series of monospecific antisera, titrated in RIE and diluted to contain the same concentration of precipitating antibody as a reference anti-Ad2 serum (no. 113). Such immunoelectrophoretic titrations were necessary to compare the degree of aggregation and neutralization in the subsequent studies.

Virions were mixed with appropriate heat-inactivated antisera as described in Materials and Methods. Such mixtures were both analyzed for possible aggregation by centrifugational rate separation in sucrose gradients and assayed for neutralization as described. The aggregation patterns (Fig. 2, solid bars) mirrored the neutralization data (Fig. 2, open bars); i.e., a high degree of aggregation yielded a correspondingly high degree of neutralization. The most striking effects were observed with antisera containing antifiber specificities, i.e., the two anti-Ad2 sera (no. 111 and 113), the total anti-Ad2 serum (no. 111) minus antihexon antibodies, and the monospecific antifiber serum. For these four antisera, a final dilution of 1/200 displayed almost 100% neutralization and 100% aggregation (Fig. 2). The total anti-Ad2 serum minus antifiber antibodies revealed close to 100% neutralization at a final serum dilution of 1/8, and the antihexon serum revealed 80% neutralization and 30% aggregation at a final dilution of 1/2. When both antifiber and antihexon antibodies were removed from the total anti-Ad2 serum, neutralization of around 85% was observed at a final dilution of 1/1.25. This effect was probably due to the anti-penton base antibodies, since a monospecific anti-penton base serum displayed 45% neutralization at the same final dilution. A notable feature of the neutralization tests with the anti-penton base serum was the fact that no further increase in neutralization was achieved at higher serum concentrations; thus, a final serum dilution of 1/1.1 gave the same result as a 1/1.25 dilution (not shown). Preimmune sera and an anti-protein IIIa serum were both totally devoid of antibodies causing aggregation or neutralization.

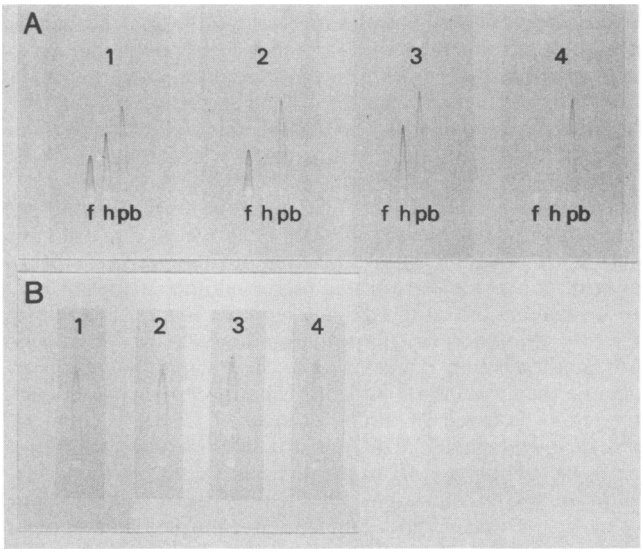

FIG. 1. RIE showing the specificities of the immunoabsorbed antisera. The agarose gels contained the following antisera: 1, Anti-Ad2 serum (no. 111); 2, Anti-Ad2 serum minus antihexon antibodies; 3, Anti-Ad2 serum minus antifiber antibodies; 4, Anti-Ad2 serum minus antihexon and antifiber antibodies. (A) Gels containing 0.5% of each antiserum. f, Fiber antigen; h, hexon antigen, and pb, penton base antigen. (B) Gels containing 2% of each antiserum. Protein IIIa was applied in all wells.

In summary, three classes of antibodies showed inhibitory effects on virus infectivity; these were antifiber, antihexon, and anti-penton base antibodies.

To exclude the possibility that the results above were due to formation of cytotoxic antibody-virion complexes (12, 13), with subsequent killing of the cells, the viability of the cells was studied by trypan blue exclusion. It was shown that the anti-Ad2 serum no. 113 at final dilutions ranging from 1/2 to 1/200 and the antihexon serum at a dilution of 1/1.25, i.e.,

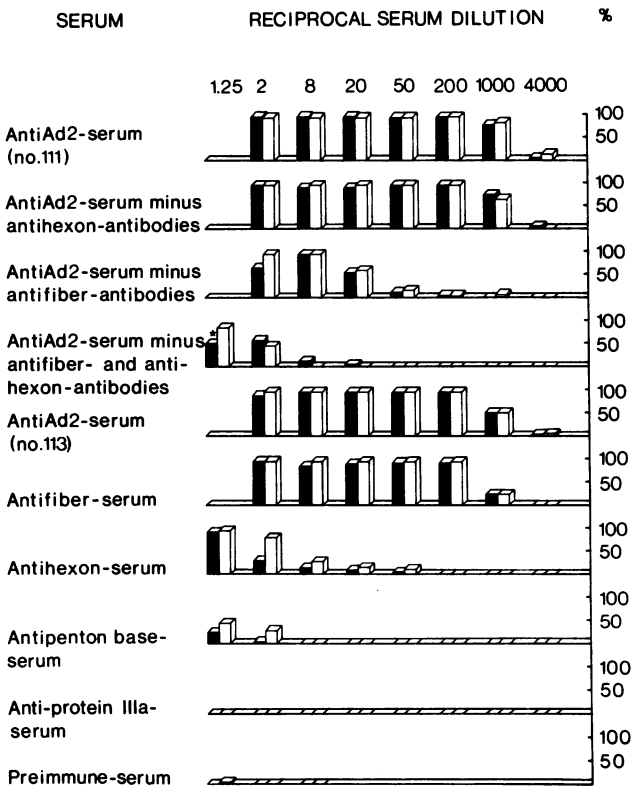

FIG. 2. Neutralization and aggregation of virions by different antisera. Ad2 virions were mixed with different antisera as indicated in the figure, and the mixtures were analyzed for aggregation in sucrose gradients (solid bars) and for neutralization by the progeny virus immunotitration method (open bars). The figures refer to the percentage of reduction in progeny virus yield as compared with that in the untreated control and to the percentage of the total applied radioactivity, subsequently recovered on a cushion of CsCl in sucrose gradients, for the neutralization and aggregation studies, respectively. In one aggregation sample (*), the reciprocal serum dilution was 1.5.

dilutions with maximal neutralization, neither caused increased trypan blue uptake of the cells, measured at 40 h postinfection, nor affected the proliferation of the cells during the infection period. However, at final anti-Ad2 serum dilutions of 1/1,000 to 1/4,000, in which the degree of neutralization is diminished, the frequency of stained cells at 40 h postinfection was 11 and 27%, respectively. In the control infection without neutralizing antiserum, 35% of the cells did not exclude the stain at 40 h postinfection. Antipenton base serum at final dilutions of 1/1.25 and 1/1.1 revealed intermediate effects with around 12% trypan blue-stained cells, which is in good agreement with the maximal neutralizing capacity of 45% displayed by this antiserum.

Antibody-mediated destabilization of virions in vitro. The possibility that incubations of Ad2 with various antisera could destabilize the virions in vitro, thus rendering the genome sensitive to DNase treatment, was investigated. Virions were mixed with the appropriate antisera at the same final concentrations as described above, and samples were subsequently analyzed for DNase sensitivity. After virus treatment with antisera containing antifiber specificities, i.e., total anti-Ad2 serum, anti-Ad2 serum minus antihexon antibodies, and antifiber serum, 15 to 20% of the labeled virion material became sensitive to DNase treatment at final serum dilutions of 1/8 (data not shown). A less pronounced tendency to destabilize was observed at both higher and lower dilutions of all antifiber-containing antisera. These observations could reflect the existence of a critical ratio between antifiber antibodies and virions. However, it seems reasonable to believe that these effects are not of any major significance for the capacity of these antisera to neutralize virus infectivity. No other antisera or preimmune serum displayed an effect on viral DNase sensitivity.

Attachment of virus-antibody complexes to HeLa cells. Attachment of the total mixtures of virus particles incubated with appropriate antisera was analyzed on monolayer cells and compared with a control in which virus was preincubated with either 1% BSA in PBS or preimmune serum. When virions were treated with any of the antifiber-containing antisera at various dilutions in a range of 1/8 to 1/1,000, an increase of a factor of three to five was observed in virion attachment, as compared with the control series (Fig. 3). The amount of virions attached in the presence of these antisera decreased and approached that of the control level when the serum concentrations were lowered from 1/1,000 to 1/4,000 (Fig. 3). The attachment of virions preincubated with antihexon serum, anti-penton base serum, or anti-Ad2 serum minus both antifiber and antihexon antibodies did not significantly differ from the results obtained after incubations with preimmune serum (Fig. 3). The anti-Ad2 serum minus antifiber antibodies did not influence the attachment except at the highest serum concentration.

DNase sensitivity of virus-antibody complexes attached to cells. In a previous report, it was suggested that virion destabilization takes place at the cell surface (37). To exam-

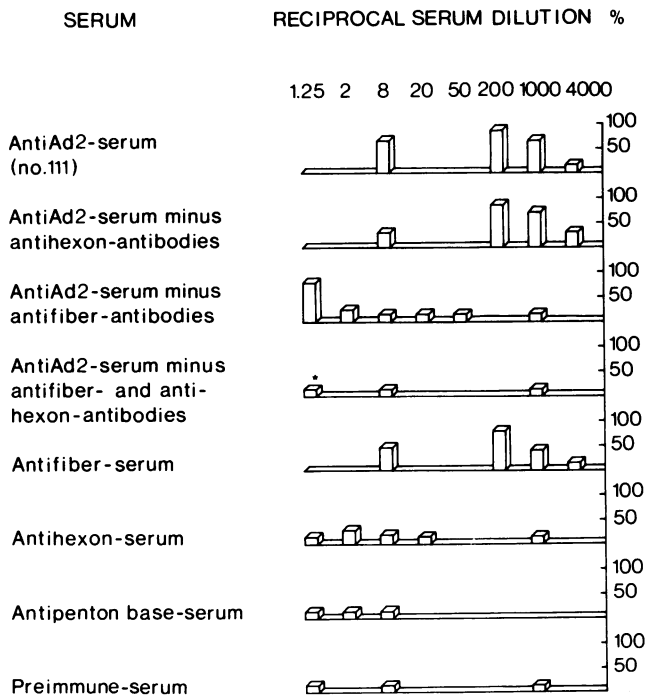

FIG. 3. Attachment of Ad2 virion-antibody complexes to cells. [^{3}H]thymidine-labeled virions were preincubated with different antisera and allowed to attach to monolayer cells for 60 min at 37°C. Cells were harvested, and the relative amount of attached virions was calculated after radioactivity measurement as described in the text. The absence of a box indicates that the relevant serum dilution was not tested. The figures refer to the percentage of attached virions. In one sample (*), the reciprocal serum dilution was 1.5.

ine whether pretreatment of virions with various antibodies affected the normal uncoating in any way, virus incubated with various antisera were allowed to attach to monolayer cells, and attached virions were subjected to DNase treatment as described in Materials and Methods. The background level of DNase sensitivity in vitro, as discussed above, was subtracted from the values obtained after the interaction of virus-antibody complexes with cells. All antifiber-containing antisera displayed a pronounced inhibitory effect on the cell-mediated destabilization of the virus particles (Fig. 4), whereas antihexon- or anti-penton base-neutralized virions became destabilized to the same extent as the untreated control. However, virions pretreated with anti-Ad2 serum minus antifiber antibodies at a reciprocal serum dilution of 1.5 were not destabilized to the same extent as the control.

Fate of antibody-treated virions attached to cells. In analogy with a previous study on virion internalization (37), ^{125}I-labeled anti-rabbit immunoglobulin G was used to quantify the degree of internalization of attached virion-antibody complexes. In the presence of either antihexon or anti-penton base sera (diluted 1/1.25), ca. 85% of the virions were internalized. This figure was reduced by half when an antifiber serum (diluted 1/200) was tested (data not shown). To follow further the cellular distribution of virions treated with various antisera, an electron microscopic investigation was performed (Table 1). After treatment of virions with a total anti-Ad2 serum or an antifiber serum, ca. 85% of the virions remained on the surface. The 15% of the virions which were internalized appeared in large aggregates and were located in vesicular structures, such as multivesicular bodies or lysosomes (Fig. 5A). Virions preincubated with an antihexon serum or an anti-penton base serum entered the cells nearly as efficiently as virions treated with a preimmune serum (Table 1). In the case of the antihexon serum, ca. 70% of the virions were accumulated inside vesicles, and only 8% were found near the nucleus. After pretreatment with anti-penton base serum, around 50% of the virions were left inside vesicles, which usually contained only a few virus particles, as also was the case for vesicles harboring antihexon-treated virions (Fig. 5B and C). However, 25% of the anti-penton base-treated virions appeared near the nuclear membrane, compared with the control series in which ca. 55% of the virions reached the nucleus (Table 1 and Fig. 5D).

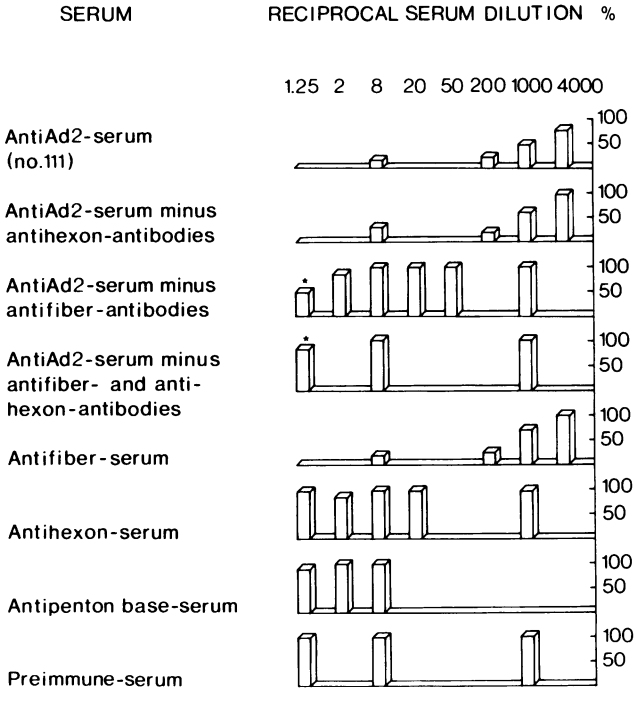

FIG. 4. DNase sensitivity of virion-antibody complexes attached to cells. [^{3}H]thymidine-labeled virions treated with different antisera were attached to monolayer cells for 60 min at 37°C. The cells were harvested, and the DNase sensitivity of the parental virion DNA was estimated as described in the text. The absence of a box indicates that the relevant serum dilution was not tested. The figures refer to the relative DNase sensitivity of the viral genome compared with the maximum uncoating efficiency of ca. 80% in a control sample of untreated virions and cells. In two samples (*), the reciprocal serum dilution was 1.5.

TABLE 1. Cellular distribution of Ad2 particles after incubation with various antisera

Serum treatment[a]	Relative distribution of Ad2 particles (%)[b]			
	Cell surface	Vesicles	Free	
			In cytoplasm	Near nucleus
Anti-Ad2 no. 113 (1/200)	88	12	0	0
Antifiber (1/200)	85	15	0	0
Antihexon (1/1.25)	21	69	2	8
Anti-penton base (1/1.25)	25	47	3	25
Preimmune (1/1.25)	16	16	12	56
PBS	12	14	20	54

[a] The figures within parentheses refer to the final dilution of antiserum in the reaction mixture.

[b] The relative distribution of ca. 200 counted virions within ca. 40 cells was calculated.

DISCUSSION

In this communication, we have demonstrated that three different viral immunogens elicit neutralizing antibodies as judged by reduction in progeny virus yield. A monospecific antifiber serum displayed a neutralizing capacity of the same magnitude as a total anti-Ad2 serum. When the antifiber antibodies were removed from an anti-Ad2 serum, the remaining neutralizing potential was of the same order as that of an antihexon serum, and when the anti-Ad2 serum was depleted of both antifiber and antihexon antibodies, the residual neutralizing activity equalled that of an anti-penton base serum.

A notable feature of the neutralization assays was the observation, especially among the antifiber-containing antisera, that the extent of neutralization paralleled the aggregation patterns. This raises the question whether the present neutralization data simply reflect the degrees of aggregation of virions, i.e., aggregation of several infectious units into a single infectious entity, which subsequently leads to a lower number of cells being infected than expected in the immunotitration assay. If this were true, addition of more than one infectious unit per cell would increase the total virus yield in the immunotitration assays. We tried this hypothesis among the different antisera by adding up to 250 neutralized infectious units per cell, but no increase in virus yield was observed.

Despite the aggregation of virions with antifiber-containing antisera, such complexes had a capacity to attach to cells at an even greater extent than untreated virions. An

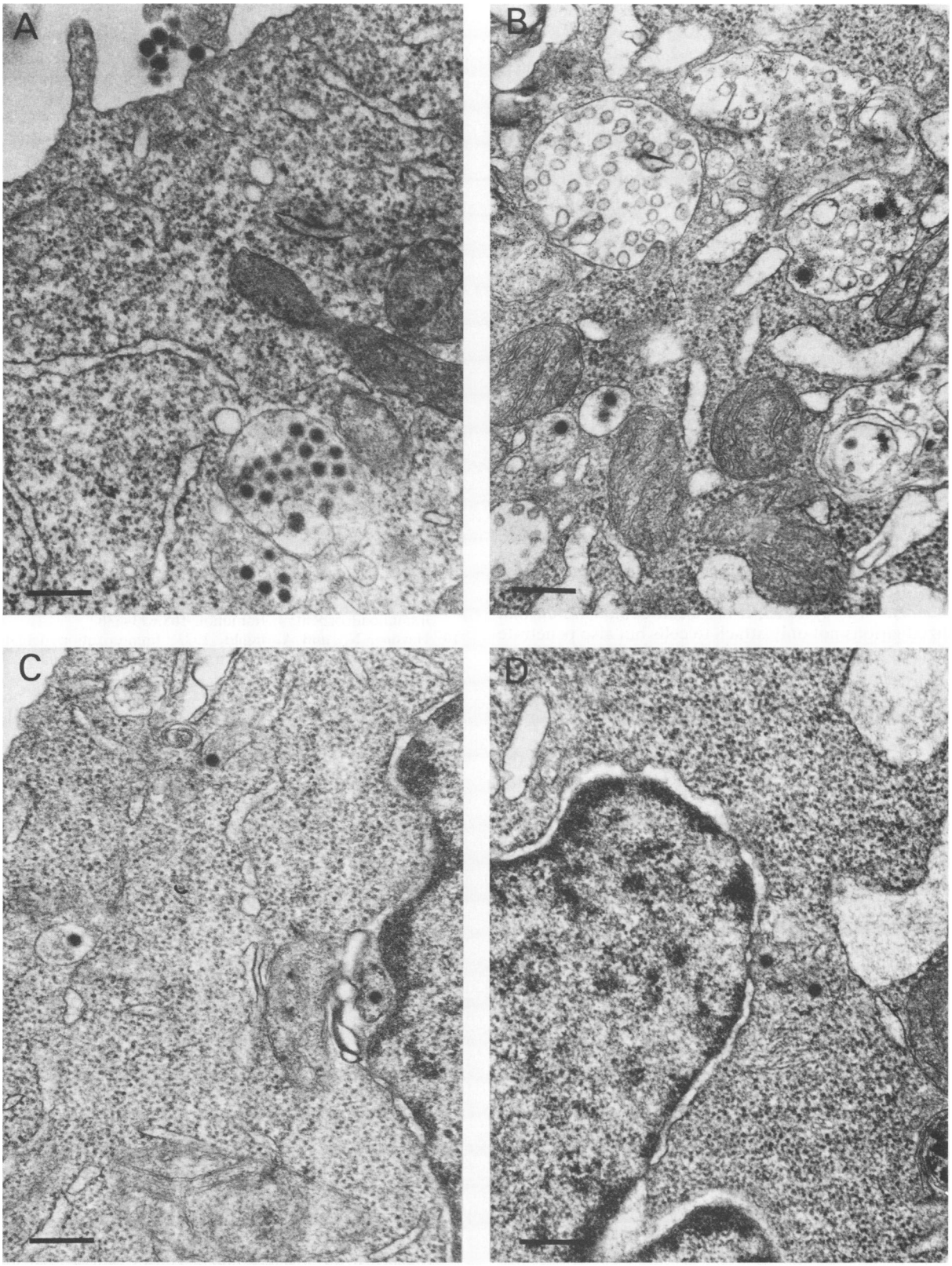

FIG. 5. Cellular distribution of antibody-treated adenovirus as observed with the electron microscope. Virions, pretreated with different antisera, were incubated with cells for 60 min at 37°C. Cells had virions pretreated with antifiber serum, dilution 1/200 (A); antihexon serum, dilution 1/1.25 (B); anti-penton base serum, dilution 1/1.25 (C); preimmune serum, dilution 1/1.25 (D). Bar, 200 nm.

unaltered or enhanced attachment efficiency after treatment with neutralizing antisera has been described for adenovirus (29) and other virus systems, e.g., poliovirus (18) and influenza virus (30). The attached Ad2 virions treated with anti-Ad2 or antifiber serum did not become sensitive to DNase treatment to the same extent as the controls; this could possibly be explained by steric hindrance, leading to an inability of virions within complexes to properly reach the plasma membrane and thereby avoiding the cell-mediated destabilization process. Antifiber-treated virions remained on the cell surface (85%) or were located inside vesicles (15%). This cellular localization is in good agreement with the observations obtained by Brown and Burlingham (3), who used KB cells and an anti-Ad2 serum. From the results above, it is possible to suggest that the reduction in virus yield in the immunotitration assays when virions were incubated with antifiber-containing antisera is not just a reflection of aggregation but is also dependent on a neutralizing mechanism apart from a mere aggregation.

When the data from antihexon and anti-penton base neutralization were analyzed, it was found that the percentage of neutralization was always higher than the percentage of aggregated virions. Virions incubated with either of these antisera attached to cells and became sensitive to DNase treatment to the same extent as the control. It was previously shown that glutaraldehyde-stabilized virions become internalized but are unable to leave an endocytotic vesicle (36). When antihexon and anti-penton base sera were used at neutralizing concentrations, the majority of the virions were internalized but confined within vesicles. In the poliovirus and influenza virus systems, it has been demonstrated that neutralized virions not only attach to cells but also penetrate the cell membrane (19, 30), and in the case of the influenza virus, uncoating also occurs (30). The interaction between the penton base and the endosome membrane has been suggested to be important during adenovirus entry from an acidic vesicle into the cytoplasmic compartment (33, 34, 36). Possibly this interaction was blocked as a result of the reaction between virus and anti-penton base antibodies. However, 25% of the virions incubated with this antiserum succeeded in reaching the nuclear membrane. This agrees well with the result that the maximum level of anti-penton base neutralization of adenovirus never exceeded 45%. These observations also support the earlier proposal that not all virions appear dependent on an acidic vesicle and the penton base for a proper entry into the cytoplasmic compartment (36). This entry pathway is at present uncharacterized; however, an alternative entry mechanism of direct penetration has previously been described (3, 23).

It is obvious that when the three monospecific neutralizing antisera were used, the cellular distribution of neutralized virions was not the same; thus, an antifiber serum generally inhibited the relative virus internalization, and the antihexon and anti-penton base sera prevented the entry of virions from acidic vesicles into the cytoplasmic compartment. Besides the mere neutralization data presented in this communication, topological alteration of virions by attachment of different classes of antibodies could be a valuable tool in further studying the viral penetration and uncoating processes.

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