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Neutralization of Adenoviruses: Kinetics, Stoichiometry, and Mechanisms

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Kinetic curves for neutralization of adenovirus type 2 with anti-hexon serum revealed no lag periods even when the serum was highly diluted or when the temperature was lowered to 4°C, thus indicating a single-hit mechanism. Multiplicity curves determined with anti-hexon serum displayed a linear correlation between the degree of neutralization and dilution of antiserum. Neutralization values experimentally obtained under steady-state conditions fully fitted a single-hit model based on Poisson calculations. Quantitation of the amount of ^{125}I-labeled type-specific anti-hexon antibodies needed for full neutralization of adenovirus showed that 1.4 antibodies were attached per virion under such conditions. Virions already attached to HeLa cells at 4°C were, to a large extent, neutralizable by anti-hexon serum, whereas anti-fiber and anti-penton base antisera were negative. It is suggested that adenovirus may be neutralized by two pathways: aggregation of the virions (extracellular neutralization) as performed by anti-fiber antibodies and blocking of virion entrance from the acidic endosomes into the cytoplasm (intracellular neutralization). The latter effect could be obtained by (i) covering of the penton bases, as performed by anti-penton base antibodies, thereby preventing interaction between the penton bases and the endosomal membrane, which results in trapping of virions within endosomes, and (ii) inhibition of the low-pH-induced conformational change of the viral capsid, which seems to occur in the endosomes and is necessary for proper exposure of the penton bases, as performed by anti-hexon antibodies.

Adenovirus has been reported to enter cells by two alternative mechanisms: direct penetration (2, 35) and an endocytotic process (4, 16, 45). The penton base has been shown to play a crucial role for proper virion entry from the acidic endosomes into the cytoplasmic compartment by interaction between the penton base and the endosomal membrane (40–42, 45). Recently we have shown that the viral capsid of adenovirus undergoes a conformational change when the pH is lowered, as judged by different protease sensitivities of adenovirus at pHs 5 and 7.5. Dispase cleaves hexons from the excess pool of antigens into a few discrete fragments at pH 5. At pH 7.5, degradation of hexons is negligible (13).

In a previous report on the interaction between HeLa cells and adenovirus neutralized by various antisera, it was shown that anti-hexon antibody-neutralized and anti-penton base antibody-neutralized virions attach to cells to the same extent as do untreated control viruses (53). Moreover, the neutralized virions become destabilized on the cell surface and penetrate the plasma membrane to the same extent as do untreated virions. Untreated virions are found free in the cytoplasmic compartment, whereas antibody-treated virions are trapped within intracellular vesicles. Anti-fiber antibodies efficiently aggregate virions, and such aggregates are able to attach to cells. Of attached anti-fiber antibody-treated virions, about 15% are subsequently found in intracellular vesicles (53).

The aim of the present study was to analyze the neutralization of adenovirus with regard to kinetics and stoichiometry, as well as to shed some light on the different mechanisms that act in the neutralization processes.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were maintained in suspension cultures at densities of 2.5×10^5 to 5×10^5 cells per ml in Eagle minimal essential medium supplemented with 5% fetal bovine serum and 5 μg of gentamicin per ml. Adenovirus type 2 (Ad2) was propagated and purified as previously described (12, 14).

Production of monospecific antisera. The soluble antigens that remained after virus isolation by preparative ultracentrifugation on CsCl gradients were separated by DEAE-cellulose chromatography as previously described (51). The hexon antigen was further purified by gel exclusion chromatography on Sepharose 6B and used in line immunoelectrophoresis (27). Immunoelectrophoretic lines formed between the hexon antigen and an anti-hexon antibody-containing serum in agarose gels were cut out and compressed into dry films. One milliliter of phosphate-buffered saline (PBS) was added to the dried precipitate-containing agarose films, which thereafter were ultrasonically treated and injected intramuscularly into a rabbit. Three immunizations were performed within 4 weeks (20). Serum was collected 8 days after the last immunization and then weekly for 1 month. The material for each immunization contained about 3 μg of hexon antigen. The anti-hexon serum was heat inactivated at 56°C for 30 min before use. Production of anti-fiber, anti-penton base, and anti-Ad2 sera was described in a previous report (53).

Isolation of adenovirus serotype-specific immunoglobulins from anti-hexon serum. The type-specific immunoglobulins of an anti-hexon serum were isolated by a three-step procedure consisting of affinity chromatography on immobilized adenovirion–AH-Sepharose 4B prepared as previously described (46), affinity chromatography on immobilized Ad5 hexon-Sepharose 4B prepared essentially as described before (53), and affinity chromatography on protein A-Sepharose 4B. The type-specific immunoglobulins were labeled with ^{125}I as described by Fraker and Speck (17). The specific

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activity of the immunoglobulins was $2.68 \cdot 10^8$ cpm/mg of protein, and the molecular weight used in the calculations was 150,000 for the immunoglobulins.

Protein determinations. Protein concentrations were determined as described by Hartree (21), with bovine serum albumin (BSA) as the standard.

Neutralization assay. Quantitative determination of neutralization of virus infectivity was performed by a progeny virus immunotitration method as previously described (52). Briefly, 3×10^{10} virions in 25-μl volumes were added to equal volumes of appropriate antisera or type-specific immunoglobulins, and when necessary these were diluted in PBS containing 1% BSA to yield final serum or immunoglobulin dilutions of 1/2 to 1/4,000. In some instances, 100 or 250 μl of serum was added to 25 μl of virus, and these series are referred to as 1/1.25 and 1/1.1 samples, respectively. After incubation 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 of 25 μl from such virus-antibody mixtures were removed and added to 3 ml of Eagle minimal essential medium containing 1.52×10^7 cells, yielding a multiplicity of infection of 32 particles per cell, which is equivalent to 1 infectious unit per cell. The cells were incubated at 37°C in suspension culture, and at 39 h postinfection, progeny virus was quantitatively isolated by a one-step CsCl gradient centrifugation procedure. The progeny virus was disrupted in the presence of 5 M urea and subsequently quantified by rocket immunoelectrophoresis against anti-hexon serum in a standardized system. Neutralized virus samples were compared with a separate control in which the neutralizing agent was replaced by PBS containing 1% BSA. The working range in the standardized assay was 0 to 94% neutralization because of the detection limit of progeny virus when few cells were infected. For this reason, full neutralization was manifested as ≥94%.

Kinetic analyses. Prewarmed virions, in the volumes and quantities described above, were mixed with prewarmed antisera diluted to result in either about 50% or full neutralization. The mixtures were incubated at 37°C for 0 to 65 min. At indicated times, cold 1% BSA in PBS was added to the mixtures to a final volume of 1,550 μl. Samples of 25 μl were immediately withdrawn and added to 1.52×10^7 cells. Further incubations and assays of progeny virus production were as described above.

Neutralization of virions attached to HeLa cells. Virions were added to HeLa cells at a multiplicity of 100 particles per cell, and the virus-cell mixtures were incubated at 4°C for 45 min. Under these conditions, a final attachment of about 30 virus particles per cell was anticipated (36). Unattached virus was removed by centrifugation, and the cell suspension was divided into several flasks in each experiment, yielding 1.52×10^7 infected cells per bottle in a total volume of 1 ml. Antisera, as indicated in the text, were added to each virus-cell mixture. The mixtures were incubated for another 30 min at 4°C and then transferred to 37°C. After incubation for 30 min, 34 ml of medium was added to the cells, and at 39 h postinfection the samples were analyzed for progeny virus production as described above.

Hydrophobicity analyses of adenoviral structural proteins. Hydrophobicity analyses were performed in a Triton X-114 phase system essentially as described by Seth et al. (43). ^{35}S-labeled structural proteins (51) in 5-μl volumes in 50 mM Tris hydrochloride buffer, pH 7.5, were incubated in 250 μl of 50 mM morpholineethanesulfonic acid buffer adjusted to the required pH. After incubation for 10 min at 4°C, 50 μl of 5% Triton X-114 in the buffers described above was added to the samples, which thereafter were incubated for another 10 min at 4°C. Phase separation was achieved by incubation for 5 min at 37°C, followed by low-speed centrifugation at 37°C. The aqueous upper phase (260 μl) was removed, and the lower detergent phase was diluted in 200 μl of ice-cold 10 mM Tris hydrochloride buffer, pH 7.5. The upper and lower phases were dissolved in Ready-Solve HP/b and assayed for radioactivity.

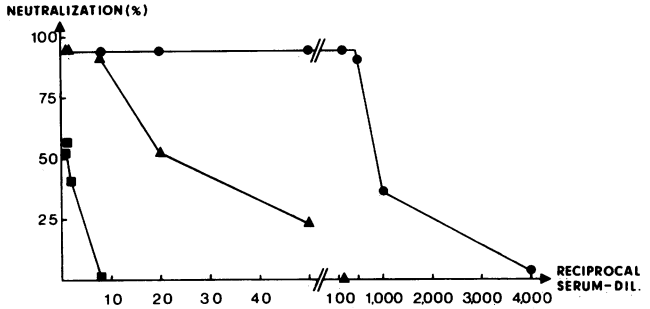

FIG. 1. Virus neutralization by different antisera. Virions were mixed with different antisera and assayed for neutralization by the progeny virus immunotitration method. Neutralization is expressed as the percentage of reduction in progeny virus yield compared with that of untreated control infections. Symbols: ●, anti-fiber serum; ▲, anti-hexon serum; ■, anti-penton base serum. Note the change in scales on the abscissa.

Dispase digestion of anti-hexon-neutralized virions. Anti-hexon or preimmune serum was added to 4×10^{10} ^{35}S-labeled virions to give final serum dilutions of 1/4. The adenovirus-antibody mixtures were incubated at 37°C for 30 min and then dialyzed for 24 h against either 0.01 M acetic acid buffer, pH 5.0, containing 0.15 M NaCl or PBS at 4°C. $CaCl_2$ was added to the samples to a final concentration of 2.5 mM, whereafter 10 μg of dispase was added to each sample. After incubation at 37°C for 30 min, the digested samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described earlier (51).

Chemicals. Eagle minimal essential medium, fetal bovine serum, and gentamicin were obtained from Flow Laboratories Ltd., Irvine, Scotland. Agarose (type I), BSA, Triton X-114, and morpholineethanesulfonic acid buffer were from Sigma Chemical Co., St. Louis, Mo. AH-Sepharose 4B was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Anti-rabbit immunoglobulin G (IgG) serum was obtained from Organon Teknika, Malvern, Pa. Ready Solve HP/b was purchased from Beckman Instruments AB, Bromma, Sweden. Dispase was obtained from Boehringer Mannheim Scandinavia AB, Bromma, Sweden.

RESULTS

Neutralization of virions by different antisera. Antisera at final dilutions of 1/1.1 to 1/4,000 were incubated with a constant amount of virions for 30 min at 37°C. The extent of virus neutralization was analyzed as described in Materials and Methods. A final anti-hexon serum dilution of 1/2 fully neutralized virus infectivity (Fig. 1). At serum dilutions of 1/8 to 1/200, the extent of neutralization gradually decreased. Anti-fiber serum fully neutralized the virus up to a final serum dilution of 1/200. As reported previously (53), anti-penton base serum caused a maximum of around 50% neutralization at final serum dilutions of 1/1.1 and 1/1.25. If the concentration of the anti-penton base serum was in-

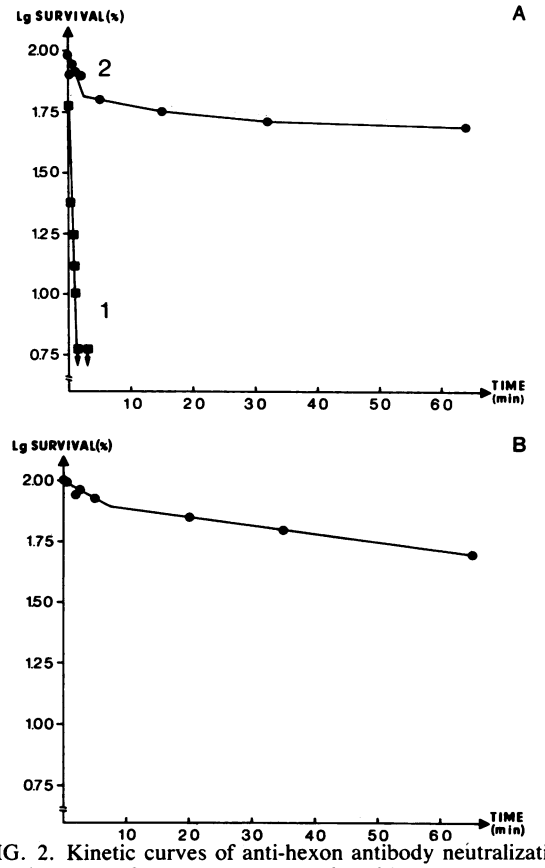

FIG. 2. Kinetic curves of anti-hexon antibody neutralization of adenovirus. (A) Constant amounts of anti-hexon serum at final dilutions of 1/1.25 (curve 1) and 1/20 (curve 2) were mixed with virions and incubated for the indicated periods at 37°C, whereafter remaining virus infectivity was determined by the immunotitration method. (B) Anti-hexon serum at a final dilution of 1/20 was mixed with virions, incubated at 4°C, and further processed as described above. Lg, Log_{10}.

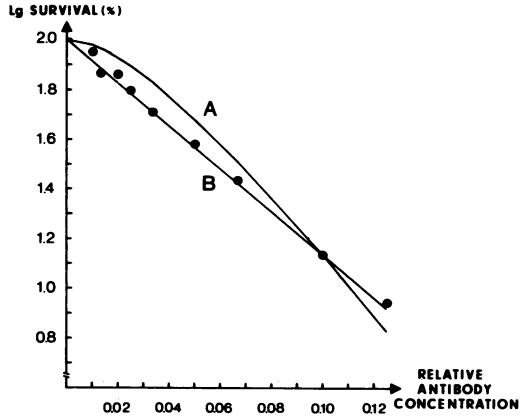

FIG. 3. Steady-state neutralization with increasing antibody concentrations. Poisson calculations were based on an anti-hexon serum dilution of 1/10 and $e^{-\lambda} = 0.136$ and $e^{-\lambda} + \lambda \cdot e^{-\lambda} = 0.136$ for determinations of the multiplicities of antibodies (λ) needed to yield a surviving fraction of 0.136 in the single-hit (B) and two-hit (A) models, respectively. The amounts of antibodies in the different serum dilutions were subsequently determined on the basis of that in the 1/10 dilution, and the corresponding survival value was calculated from the formulas above. The filled circles represent the experimental data. Lg, Log_{10}.

creased six times as compared with the 1/1.1 sample, no further increase in the degree of neutralization was observed.

Kinetic analyses of virus neutralization. Anti-hexon serum, at the two final dilutions of 1/1.25 and 1/20, yielding ≥94 and 50% neutralization, respectively (Fig. 1), was analyzed regarding the kinetics of neutralization. Virions were mixed with either of the two serum dilutions and incubated for 0 and 65 min, whereafter samples were analyzed for remaining infectious virus. For the high anti-hexon concentration, plots of infectivity data against time revealed a straight line (curve 1, Fig. 2A); i.e., a constant fraction of the virus was neutralized per unit of time. After 1 to 2 min, a maximal degree of neutralization was reached, and it was maintained upon prolonged incubation. Curve 2 in Fig. 2A (dilution, 1/20) followed a linear or curvilinear course for about 3 min, whereafter the rate of neutralization leveled off, finally to reach the expected 50% plateau of survival. An interesting feature of this curve was the fact that, even after 60 min of incubation, no steady state was reached and obviously a continuous process of neutralization still occurred. Both curves were indicative of a single-hit mechanism, since neither of them was preceded by a lag period before commencement of the exponential decrease of infectivity. However, the possibility still exists that the association between viruses and antibodies was too rapid to be measured accurately by this technique, and therefore a kinetic experiment of the 1/20 dilution of anti-hexon serum was performed at 4°C. The initial rate of neutralization appeared to be lower at this temperature, but the final level of neutralization reached after prolonged incubation was almost the same as that at 37°C (Fig. 2A and B). No lag period appeared at the lower temperature. Anti-hexon serum at a final dilution of 1/30, yielding around 35% neutralization (Fig. 1), revealed no lag period when tested in the same way at 37°C (data not shown).

Antibody-virion ratio required for neutralization. The kinetic curves indicated that one virion was neutralized by a single antibody in the case of anti-hexon neutralization. To test this hypothesis further, the anti-hexon serum was analyzed at various dilutions for its ability to neutralize virions. Since steady-state values were not reached after 65 min of incubation, as shown above, the virus-antibody mixtures were incubated at 37°C for 3 h and further at 4°C for 21 h, whereafter the remaining virus infectivity was determined. Repeated determinations of the extent of virus neutralization caused by anti-hexon serum at a final dilution of 1/10 revealed a mean survival value of 13.6%, with a coefficient of variation of 3% ($n = 4$). On the basis of this survival level, the multiplicity of antibodies to virions was calculated by using the Poissonian distribution and assuming a single- or two-hit model (Fig. 3). Theoretical survival values were subsequently calculated for a number of anti-hexon serum dilutions. The single- and two-hit survival values for anti-hexon neutralization at a final dilution of 1/20 were 36.9 and 47.8%, respectively, which should be compared with the corresponding experimental value of 38.1%. At higher serum dilutions, the discrepancies between the two models became more evident; e.g., at a final dilution of 1/75, the theoretical values for the single- and two-hit neutralization models were 76.6 and 92%, respectively, with the former figure approaching the experimentally obtained value of 73.3%. It is obvious that the experimentally obtained values closely fit the theoretical values for a single-hit model (Fig. 3). An equation for

the experimental data in Fig. 3 revealed a straight line originating at 2.01 with a regression coefficient of −0.998.

Essentially the same result as that presented in Fig. 3 was obtained when type-specific anti-hexon antibodies were added to virions at different multiplicities; i.e., the experimental survival data fitted very well with theoretical values based on a Poisson-calculated single-hit model (data not shown).

For direct quantitation of the amount of antibodies needed for neutralization, ^{125}I-labeled serotype-specific anti-hexon antibodies were used. Virions were mixed with ^{125}I-labeled antibodies, and after incubation for 30 min at 37°C, the virions were separated from free antibodies by centrifugation in a 10 to 25% sucrose gradient under conditions described before (53). Recovery of virions from sucrose gradients was 87%, and these virions were fully neutralized. On the basis of the specific radioactivity of the anti-hexon antibody, it was calculated that the neutralized virions obtained from the sucrose gradients contained 1.4 antibodies per virion, thus substantiating a single-hit mechanism for neutralization by anti-hexon antibodies.

Involvement of accessory factors in the neutralization and sensitization of virions. The involvement of thermolabile components in anti-hexon or anti-Ad2 antiserum in neutralization was examined. Heat-inactivated and untreated antisera, both at different dilutions, were run in parallel. No differences in the ability to neutralize virions were found, indicating that thermolabile components do not enhance neutralization of adenovirus by anti-hexon or anti-Ad2 antibodies.

To investigate whether other classes of anti-hexon antibodies besides the type-specific ones were able to neutralize virus infectivity, the following experiment was done. Anti-hexon serum at a dilution of 1/20, yielding around 50% neutralization, was mixed with virions at 37°C. After incubation for 30 min, anti-rabbit IgG serum was added and the mixture was incubated for another 30 min. At 39 h postinfection, the progeny virus was quantified. The degree of neutralization was enhanced to ≥94% by addition of anti-rabbit IgG antibodies. The obvious interpretation of these results is that antibodies with different hexon specificities exist within a polyclonal anti-hexon serum and that only type-specific antibodies are able to neutralize virions directly. The mechanism of neutralization by addition of anti-rabbit IgG antibodies is probably aggregation of virions, since 96% of the virions in this reaction mixture were found as large aggregates on a CsCl cushion after sucrose gradient centrifugation on 10 to 25% sucrose, performed as previously described (53).

Neutralization of virions attached to HeLa cells. Virions were mixed with HeLa cells at 4°C, a condition under which no penetration of virus occurs (45). After removal of unattached virions, different volumes of various antisera were added to the virus-cell mixtures. After incubations (see Materials and Methods), progeny virus production in each series was determined by the immunotitration method. Addition of anti-fiber serum or anti-penton base serum did not influence production of progeny virus in a dose-response manner as compared with control infections (Table 1). However, anti-hexon serum decreased virus production at all serum dilutions tested.

Mechanism behind anti-penton base neutralization of virions. Adenovirus enters cells via receptor-mediated endocytosis and leaves the endosomes by an interaction between the endosomal membrane and penton bases (41). This hypothesis is strengthened by the fact that the penton base reveals a drastic increase in hydrophobicity at low pH values (Fig. 4). Seth et al. have demonstrated that adenoviruses show a gradual increase in hydrophobicity upon lowering of the pH, and at pH 5 about 60% of the virions were associated with the detergent phase (43). It is plausible that this effect is based mainly on the properties of the penton base, since around 65% of this protein was associated with the detergent phase under the same experimental conditions (Fig. 4). Since the maximum degree of viral neutralization by anti-penton base antibodies is around 50% and neutralized virions are found in intracellular vesicles (53) and since anti-penton base antibodies were not able to neutralize virions already attached to cells, it was of interest to investigate whether a higher degree of neutralization was obtainable if antibodies were allowed to react with virions at pH 5. For this purpose, the anti-penton base serum was dialyzed against 50 mM morpholineethanesulfonic acid buffer, pH 5.0, containing 0.15 M NaCl and mixed with virions at a final serum dilution of 1/1.1. It was found that virions were fully neutralized. Virions kept at low pH did not differ in their capacities to produce progeny virus when compared with that of virus kept at pH 7.0. It thus appears as if lowering of the pH results in exposure of hidden antigenic sites of the penton bases. Binding of anti-penton base antibodies to these sites results in full neutralization. At pH 7.0, these sites are not exposed to anti-penton base antibodies, thereby rendering the virus only 50% neutralizable.

TABLE 1. Neutralization of virions attached to HeLa cells

Antiserum treatment	% Neutralization when the following amt (μl) of antiserum was added to the virus-cell mixture[a]			
	500	250	50	25
Anti-hexon	≥94	≥94	82	77
Anti-penton base	0	2	15	13
Anti-fiber	13	8	8	8
Preimmune	NT	0	0	NT

[a] Theoretically, 30 virus particles were attached to each cell after incubation for 45 min at 4°C; further incubations were as described in Materials and Methods. NT, Not tested.

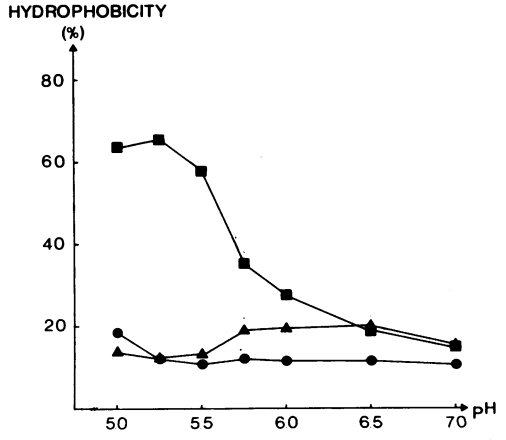

FIG. 4. Hydrophobicity properties of adenoviral structural proteins at different pHs. ^{35}S-labeled adenoviral structural proteins were partitioned between an aqueous phase and a Triton X-114 detergent phase at different pH values as described in Materials and Methods. The hydrophobicity is expressed as the percentage of radioactivity recovered in the detergent phase. Symbols: ●, fiber; ▲, hexon; ■, penton base.

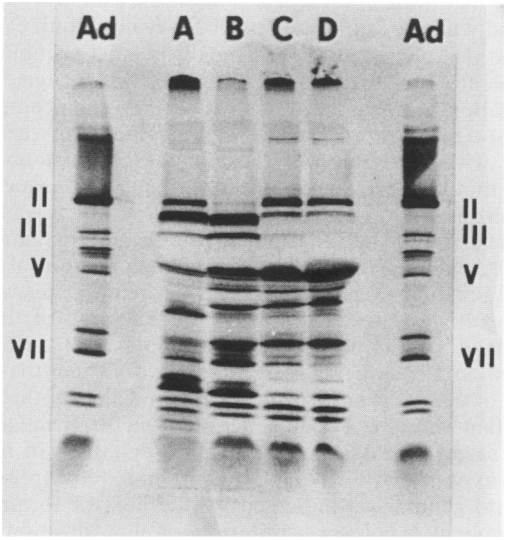

FIG. 5. Dispase treatment of anti-hexon antibody-neutralized virions at pHs 5 and 7.5. ^{35}S-labeled adenovirions were mixed with anti-hexon serum or preimmune serum, and after dispase treatment they were run in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An autoradiograph of the gel is shown. Lanes: A, anti-hexon antibody-neutralized virions digested at pH 5; B, preimmune serum-treated virions digested at pH 5; C, anti-hexon antibody-neutralized virions digested at pH 7.5; D, preimmune serum-treated virions digested at pH 7.5; Ad, ^{35}S-labeled adenovirus polypeptide marker. The roman numerals indicate polypeptides. The hexon protein corresponds to polypeptide II.

Mechanism behind anti-hexon neutralization of adenovirus. We have recently shown that adenovirus undergoes a conformational change when transferred from pH 7.5 to pH 5.0. As judged by dispase digestion of virions at pH 5 compared with that at pH 7.5, this structural change appears in the hexon antigen (13). It was therefore of interest to determine whether anti-hexon antibodies affect the digestion pattern at pH 5. ^{35}S-labeled adenovirions were mixed with neutralizing amounts of the anti-hexon serum or preimmune serum, and after incubation the mixtures were dialyzed against acetic acid buffer, pH 5.0. The dialyzed virus-antibody mixtures were treated with dispase and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Dispase digestion of the virion-associated hexon antigen was negligible at pH 7.5, whereas at pH 5 and in the presence of preimmune serum the virion hexons were totally degraded (Fig. 5). However, at pH 5 and after anti-hexon antibody treatment of the virions, digestion of hexons was markedly reduced. The results indicate that anti-hexon antibodies partially inhibit the low-pH-induced conformational change of the virions.

DISCUSSION

An immediate decline of remaining infectious virus, as judged from neutralization kinetic curves, has often been taken as an indication that one antibody per virion is needed for successful neutralization (8, 18, 30, 39). On the other hand, when a lag period is observed before the inactivation phase begins, this is often interpreted as the result of cooperation of several antibodies in the process of neutralizing one virion (33). Recently, this way of dealing with kinetic data has been challenged by Daniels (6) and Della-Porta and Westaway (7). In the view of these researchers, the association between virions and antibodies is too rapid to be measurable by conventional techniques, and therefore the absence of a lag period does not necessarily imply that one antibody inactivates one virion. In the western equine encephalitis virus system, revealing a single-hit mechanism at 37°C, a lag period is observed if the temperature is lowered to 4°C (5, 8), thus demonstrating the rapidity of the union between antibodies and virions at higher temperatures. Lafferty (28) showed that a dilution of antiserum was accompanied by a shift from single-hit to multihit curves in the influenza virus system. Thus, it is important not to judge whether neutralization is a single-hit or multihit phenomenon solely on the basis of kinetic studies. By constructing multiplicity curves, i.e., plotting the fraction of remaining virus infectivity versus antibody concentration, a straight line originating from the origin of the plot is often obtained (8, 18, 19, 30). Since the virus-antibody mixtures are incubated for an extended period, often for several hours or days, such steady-state neutralization values would be preferable to deal with in judging whether the neutralization process obeys the law of a single-hit or a multihit model.

In this study, no lag period was detected in kinetic experiments, even when the anti-hexon serum was highly diluted or the temperature was lowered to 4°C, thus indicating a single-hit mechanism. Poisson calculations of the steady-state neutralization values also supported a single-hit model. However, this requires that the antibodies attach to the virus particles according to the Poissonian distribution. The validity of this assumption has been demonstrated in the poliovirus system, in which radioactively labeled antibodies attach to virions according to this distribution (22).

The kinetic curves in Fig. 2 show a marked biphasic rate of neutralization. In the first part of the curves, an exponential decline in surviving virus was observed, followed by a second part with a decreased rate of neutralization. In other virus systems, it has been proposed that the second part of such kinetic curves and the occurrence of a persistent fraction is due to interference of noncritical antibodies with critical neutralizing antibodies and the latter are thereby hindered from reaching the critical sites, leading to lack of neutralization. Addition of anti-IgG antibodies to such virus-antibody mixtures neutralizes virions by covering the unreacted critical sites (19, 29). In the adenovirus system, it seems as if noncritical antibodies may attach to virions and neutralization is achieved upon addition of anti-rabbit IgG antibodies (26, 49). This could be a reflection of aggregation, since virions were found on a CsCl cushion upon sucrose gradient centrifugation in the present investigation. Kjellén has shown that the second part of the kinetic curve is due not to combination of more antibodies with virions but rather to reactions that occur within the already formed virus-antibody complex (25). Moreover, kinetic studies with neutralizing monoclonal antibodies reveal persistent fractions in various virus systems (23, 48). In such preparations, the antibodies are homogeneous with regard to specificity, and interfering noncritical antibodies are not available, yet a persistent fraction exists. It thus appears that the occurrence of a persistent fraction is due to an inherent variation in binding specificity of the neutralizing antibodies, thus leading to incorrect combination of some antibodies with the virions. In the adenovirus system, the first part of the kinetic curve could represent a successful combination of antibodies with virions, thus directly rendering the virus noninfectious. Because of incorrect combinations of some antibodies with virions, the rate of neutralization is retarded and this second

part of the kinetic curve would then represent neutralization of infectious virus-antibody complexes due to rearrangements of the antibodies within these complexes.

Anti-hexon neutralization appears to be a single-hit phenomenon, and since anti-hexon antibodies partially inhibit dispase digestion of hexon at pH 5, it is possible that the major mechanism of neutralization by these antibodies is through inhibition of the low-pH-induced conformational change of the viral capsid. Such a conformational change of virions is necessary for proper exposure of hidden antigenic sites of the penton bases, which subsequently leads to release of virions from endosomes into the cytoplasmic compartment. This hypothesis fits very well with earlier findings that anti-hexon antibody-neutralized virions and glutaraldehyde-stabilized virions are trapped within endocytic vesicles (15). The fact that anti-hexon serum is able to neutralize virions already attached to cells supports this idea. The same fact also supports a single-hit mechanism for anti-hexon-mediated neutralization. The observation that anti-hexon serum only partially inhibited dispase digestion of virion hexons at low pH does not contradict the above hypothesis if one considers the possibility that inhibition of dispase digestion involves only, e.g., the peripentonal hexons. However, anti-hexon-mediated inhibition of dispase digestion at low pH values could also be explained by blocking of proteolytic cleavage sites by the antibodies. The conformational neutralization pathway has been demonstrated for a variety of virus systems, such as poliovirus (9, 10, 31, 32), influenza virus (38), and bovine enterovirus (3).

The penton base has been shown to play a crucial role in the proper entry of virions from the acidic endosomes into the cytoplasmic compartment (40–42, 45). The experimentally demonstrated gradual increase in hydrophobicity of the penton base upon lowering of the pH substantiated this hypothesis. The increase in hydrophobicity was not due to aggregation of the protein (data not shown). A maximal level of 50% neutralization was obtained with an anti-penton base serum at physiological conditions and since the neutralized virions are found in intracellular vesicles, it seems plausible that anti-penton base antibodies play a stoichiometrical role in the neutralization process; i.e., the antibodies cover the penton bases and thereby prevent them from interacting with the endosomal membrane. When the antibodies were allowed to interact with virions at pH 5, all virions were neutralizable, which suggests low-pH-induced exposure of antigenic sites of the penton bases normally not accessible to interaction with anti-penton base antibodies at pHs around neutrality. This model for anti-penton base neutralization also fits very well with the fact that virions already attached to cells were not neutralizable by an anti-penton base serum. The model thus obeys a multihit mechanism.

Antisera against the fiber antigen have been reported both to contain (1, 24, 34, 50) and to lack (26, 37, 49) significant amounts of neutralizing antibodies. Most probably this is a reflection of various degrees of aggregation of viruses.

In vivo studies have shown that immunization with purified fiber protects experimental animals against the challenge of a lethal dose of Ad5 (34). In a study with humans it was demonstrated that fiber-immunized volunteers were almost totally protected against ocular challenge with homologous Ad1 as compared with unimmunized control persons (24). Thus, even though several investigators have demonstrated that anti-fiber sera are devoid of neutralizing antibodies, the above-mentioned in vivo experiments clearly show the protective role of the fiber as an immunogen.

An anti-fiber serum efficiently aggregates virions (11, 47, 53), and working with total anti-Ad2 serum, Smith et al. (44) showed that aggregation of virions was most pronounced at equivalent concentrations of antibodies and virions, and no aggregation was observed at high antibody concentrations. In most neutralization studies, small amounts of viruses are mixed with antibodies in great excess, conditions under which minor or no aggregation occurs and subsequently no neutralization is observed. In a previous study, it was shown that the degree of aggregation mirrors the neutralization data (53). Attempts in this study to avoid aggregation of virions by addition of a great excess of anti-fiber antibodies were not successful. Most probably this is a reflection of the large amount of virions used in the immunotitration assay (data not shown). Although several investigators feel that aggregation of virions is not true neutralization and for this reason claim that anti-fiber serum is devoid of neutralizing antibodies, it seems appropriate to refer to a definition of neutralization as stated by G. Wadell: "Only inhibited expression of the viral genome as indicated by the lack of formation of viral progeny is taken as a criterion for neutralization of viral infectivity" (49). By this definition, an anti-fiber serum contains neutralizing activities. To be able to distinguish between the mechanism that acts in the aggregation of virions and those that act in anti-hexon-mediated and anti-penton base-mediated neutralization, it could be preferable to refer to extracellular neutralization in the former and intracellular neutralization in the latter case.

Virions already attached to cells were not neutralizable by anti-fiber serum, demonstrating that aggregation of virions is the major cause of neutralization by these antibodies. It was previously shown that anti-fiber antibody-neutralized virions attached to HeLa cells and around 15% of the attached virions were able to penetrate the cell membrane and were found in intracellular vesicles, usually in aggregates of 10 to 20 particles (53). This implies that, although some aggregated virus particles are capable of penetrating the cell membrane, these virions are not able to initiate replication. This may be due to the aggregational status of the virus and a subsequent lack of the proper conformational change of the viral capsid.

It is suggested that adenovirus may be neutralized by two pathways: (i) aggregation as performed by anti-fiber antibodies and (ii) blocking virion entrance from endosomes into the cytoplasm. The latter effect could be obtained by either covering of the penton bases as performed by anti-penton base antibodies or by inhibition of the low-pH-induced conformational change necessary for proper exposure of the penton bases as performed by anti-hexon antibodies.

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LITERATURE CITED

1. **Banks, P. A., J. A. Kasel, M. Huber, R. H. Alford, and V. Knight.** 1966. Persistence of neutralizing antibody in adult volunteers immunized with adenovirus soluble antigens. Proc. Soc. Exp. Biol. Med. **121**:240–243.
2. **Brown, D. T., and B. T. Burlingham.** 1973. Penetration of host cell membranes by adenovirus 2. J. Virol. **12**:386–396.
3. **Carthew, P.** 1976. The surface nature of proteins of a bovine enterovirus before and after neutralization. J. Gen. Virol. **32**:17–23.
4. **Chardonnet, Y., and S. Dales.** 1970. Early events in the interac-

tion of adenoviruses with HeLa cells. I. Penetration of type 5 and intracellular release of the DNA genome. Virology **40**:462–477.
5. **Cremer, N. E., J. L. Riggs, and E. H. Lennette.** 1975. Neutralization kinetics of western equine encephalitis virus by antibody fragments. Immunochemistry **12**:597–601.
6. **Daniels, C. A.** 1975. Mechanisms of viral neutralization, p. 79–97. *In* A. L. Notkins (ed.), Viral immunology and immunopathology. Academic Press, Inc., New York.
7. **Della-Porta, A. J., and E. G. Westaway.** 1977. A multi-hit model for the neutralization of animal viruses. J. Gen. Virol. **38**:1–19.
8. **Dulbecco, R., M. Vogt, and A. G. R. Strickland.** 1956. A study of the basic aspects of neutralization of two animal viruses, western equine encephalitis virus and poliomyelitis virus. Virology **2**:162–205.
9. **Emini, E. A., S.-Y. Kao, A. J. Lewis, R. Crainic, and E. Wimmer.** 1983. Functional basis of poliovirus neutralization determined with monospecific neutralizing antibodies. J. Virol. **46**:466–474.
10. **Emini, E. A., P. Ostapchuk, and E. Wimmer.** 1983. Bivalent attachment of antibody onto poliovirus leads to conformational alteration and neutralization. J. Virol. **48**:547–550.
11. **Everitt, E., L. Lutter, and L. Philipson.** 1975. Structural proteins of adenoviruses. XII. Location and neighbor relationship among proteins of adenovirion type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking. Virology **67**:197–208.
12. **Everitt, E., S. A. Meador, and A. S. Levine.** 1977. Synthesis and processing of the precursor to the major core protein of adenovirus type 2. J. Virol. **21**:199–214.
13. **Everitt, E., M. J. Persson, and C. Wohlfart.** 1988. pH-dependent exposure of endoproteolytic cleavage sites of the adenovirus 2 hexon protein. FEMS Microbiol. Lett. **49**:229–233.
14. **Everitt, E., B. Sundquist, and L. Philipson.** 1971. Mechanism of the arginine requirement for adenovirus synthesis. I. Synthesis of structural proteins. J. Virol. **8**:742–753.
15. **Everitt, E., U. Svensson, C. Wohlfart, and R. Persson.** 1986. Requirements for initial adenovirus uncoating and internalization, p. 196–204. *In* R. L. Crowell and K. Lonberg-Holm (ed.), Virus attachment and entry into cells. American Society for Microbiology, Washington, D.C.
16. **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.
17. **Fraker, P. J., and J. C. Speck, Jr.** 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. Biochem. Biophys. Res. Commun. **80**:849–857.
18. **Granoff, A.** 1965. The interaction of Newcastle disease virus and neutralizing antibody. Virology **25**:38–47.
19. **Hahon, N.** 1970. Neutralization of residual infectivity of Venezuelan equine encephalomyelitis virus by anti-gammaglobulin. J. Gen. Virol. **6**:361–372.
20. **Harboe, N., and A. Ingild.** 1973. Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand. J. Immunol. **2**(Suppl. 1):161–164.
21. **Hartree, E. F.** 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem. **48**:422–427.
22. **Icenogle, J., H. Shiwen, G. Duke, S. Gilbert, R. Rueckert, and J. Anderegg.** 1983. Neutralization of poliovirus by a monoclonal antibody: kinetics and stoichiometry. Virology **127**:412–425.
23. **Iorio, R. M., and M. A. Bratt.** 1984. Neutralization of Newcastle disease virus by monoclonal antibodies to the hemagglutinin-neuraminidase glycoprotein: requirement for antibodies to four sites for complete neutralization. J. Virol. **51**:445–451.
24. **Kasel, J. A., M. Huber, F. Loda, P. A. Banks, and V. Knight.** 1964. Immunization of volunteers with soluble antigens of adenovirus type 1. Proc. Soc. Exp. Biol. Med. **117**:186–190.
25. **Kjellén, L.** 1962. Studies on the interactions of adenovirus, antibody, and host cells in vitro. Virology **18**:448–456.
26. **Kjellén, L., and H. G. Pereira.** 1968. Role of adenovirus antigens in the induction of virus neutralizing antibody. J. Gen. Virol. **2**:177–185.
27. **Kröll, J.** 1973. Line immunoelectrophoresis. Scand. J. Immunol. **2**(Suppl. 1):61–67.
28. **Lafferty, K. J.** 1963. The interaction between virus and antibody. Virology **21**:61–75.
29. **Majer, M.** 1972. Virus sensitization. Curr. Top. Microbiol. Immunol. **58**:69–84.
30. **Mandel, B.** 1960. Neutralization of viral infectivity: characterization of the virus-antibody complex including association, dissociation, and host-cell interaction. Ann. N.Y. Acad. Sci. **83**:515–527.
31. **Mandel, B.** 1971. Characterization of type 1 poliovirus by electrophoretic analysis. Virology **44**:554–568.
32. **Mandel, B.** 1976. Neutralization of poliovirus: a hypothesis to explain the mechanism and the one-hit character of the neutralization reaction. Virology **69**:500–510.
33. **Mandel, B.** 1979. Interaction of viruses with neutralizing antibodies, p. 37–121. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 15. Plenum Publishing Corp., New York.
34. **Mautner, V., and H. N. A. Willcox.** 1974. Adenovirus antigens: a model system in mice for subunit vaccination. J. Gen. Virol. **25**:325–336.
35. **Morgan, C., H. S. Rosenkranz, and B. Mednis.** 1969. Structure and development of viruses as observed in the electron microscope. X. Entry and uncoating of adenovirus. J. Virol. **4**:777–796.
36. **Persson, R., U. Svensson, and E. Everitt.** 1983. Virus receptor interaction in the adenovirus system. II. Capping and cooperative binding of virions on HeLa cells. J. Virol. **46**:956–963.
37. **Pettersson, U., and G. Wadell.** 1985. Antigenic structure of the adenoviruses, p. 295–323. *In* M. H. V. van Regenmortel and A. R. Neurath (ed.), Immunochemistry of viruses. The basis for serodiagnosis and vaccines. Elsevier Science Publishing, Inc., New York.
38. **Possee, R. D., G. C. Schild, and N. J. Dimmock.** 1982. Studies on the mechanism of neutralization of influenza virus by antibody: evidence that neutralizing antibody (anti-haemagglutinin) inactivates influenza virus in vivo by inhibiting virion transcriptase activity. J. Gen. Virol. **58**:373–386.
39. **Rubin, H., and R. M. Franklin.** 1957. On the mechanism of Newcastle disease virus neutralization by immune serum. Virology **3**:84–95.
40. **Seth, P., D. FitzGerald, H. Ginsberg, M. Willingham, and I. Pastan.** 1984. Evidence that the penton base of adenovirus is involved in potentiation of toxicity of *Pseudomonas* exotoxin conjugated to epidermal growth factor. Mol. Cell. Biol. **4**:1528–1533.
41. **Seth, P., D. FitzGerald, M. Willingham, and I. Pastan.** 1986. Pathway of adenovirus entry into cells, p. 191–195. *In* R. L. Crowell and K. Lonberg-Holm (ed.), Virus attachment and entry into cells. American Society for Microbiology, Washington, D.C.
42. **Seth, P., M. C. Willingham, and I. Pastan.** 1984. Adenovirus-dependent release of ^{51}Cr from KB cells at an acidic pH. J. Biol. Chem. **259**:14350–14353.
43. **Seth, P., M. C. Willingham, and I. Pastan.** 1985. Binding of adenovirus and its external proteins to Triton X-114. J. Biol. Chem. **260**:14431–14434.
44. **Smith, K. O., M. Trousdale, and W. Gehle.** 1965. Adenovirus-antibody agglutination reactions. J. Immunol. **95**:810–817.
45. **Svensson, U.** 1985. Role of vesicles during adenovirus 2 internalization into HeLa cells. J. Virol. **55**:442–449.
46. **Svensson, U., R. Persson, and E. Everitt.** 1981. Virus-receptor interaction in the adenovirus system. I. Identification of virion attachment proteins of the HeLa cell plasma membrane. J. Virol. **38**:70–81.
47. **Valentine, R. C., and H. G. Pereira.** 1965. Antigens and structure of the adenovirus. J. Mol. Biol. **13**:13–20.
48. **Volk, W. A., R. M. Snyder, D. C. Benjamin, and R. R. Wagner.** 1982. Monoclonal antibodies to the glycoprotein of vesicular stomatitis virus: comparative neutralizing activity. J. Virol.

42:220–227.
49. **Wadell, G.** 1972. Sensitization and neutralization of adenovirus by specific sera against capsid subunits. J. Immunol. **108**:622–632.
50. **Wilcox, W. C., and H. S. Ginsberg.** 1963. Production of specific neutralizing antibody with soluble antigens of type 5 adenovirus. Proc. Soc. Exp. Biol. Med. **114**:37–42.
51. **Wohlfart, C., and E. Everitt.** 1985. Human adenovirus 2 as immunogen in rabbits yields antisera with high titers of antibodies against the nonstructural 72K DNA-binding protein. Virus Res. **3**:77–85.
52. **Wohlfart, C. E. G., and E. Everitt.** 1985. Assessment of specific virus infectivity and virus neutralization by a progeny virus immunotitration method as exemplified in an adenovirus system. J. Virol. Methods **11**:241–251.
53. **Wohlfart, C. E. G., U. K. Svensson, and E. Everitt.** 1985. Interaction between HeLa cells and adenovirus type 2 virions neutralized by different antisera. J. Virol. **56**:896–903.