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Cytotoxicity of Adenovirus-Antibody Aggregates: Sensitivity to Different Cell Strains, and Inhibition by Hexon Antiserum and by Complement

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Adenovirus-antibody aggregates under defined conditions are cytotoxic in vitro. All members of adenovirus groups I, II, and III caused toxicity upon aggregation. The toxicity of the clusters is exerted by the virions. Toxicity is temperature dependent and may be caused by a mechanism similar to that used in viral penetration. Cells permitting direct viral penetration were all sensitive to the toxic aggregates. The toxicity seems to be related to hexon antigens on the surface of the virions since antihexon sera neutralized the toxicity. No evidence was obtained showing that pentons are required for this kind of cytotoxicity. Adenovirus types 3, 5, and 9 were used in the experiment. Cytotoxicity was estimated by the ^{51}Cr release assay. Complement factors could be excluded as mediators of the cytolytic reactions. Instead, complement was shown to prevent the formation of toxic aggregates or to neutralize the toxicity of preformed ones.

Aggregates formed by adenovirus type 5 and specific antibodies against whole virions are under specified conditions cytotoxic for human cells in vitro. This finding was first observed by electron microscopy (7) and was subsequently analyzed in more details by ^{51}Cr release cytotoxic tests (8, 9). Evidence was presented indicating that the cytotoxic aggregates were formed only when virus and antibody were mixed in limited proportions. It was also shown that the cytotoxicity was not associated with soluble viral antigens, that cytotoxic aggregates were not formed by heat-inactivated virions, and that cytolytic complement activity was not required for induction of cytolysis. In a subsequent report (1) the influence of the cytotoxicity of type-specific anti-capsomere sera was described, and it was demonstrated that mixtures of virus and type-specific antifiber serum were as cytotoxic as the aggregates formed by antiserum against complete virion. The antihexon serum did not produce toxic aggregates. When administered to the virus prior to the antifiber serum it efficiently prevented the formation of toxic aggregates.

The observed capacity of the type 5 hexon antiserum to neutralize infectivity and to inhibit toxicity (9) raises the question to what extent penetration of virus through the cell membrane and cytotoxicity of virion aggregates are related. Presumably both interactions rest with the hexon or a hexon-associated polypeptide. This report deals with this question.

The failure of the homotypic type 5 hexon antiserum to produce toxicity may depend on an inability of the antibody to aggregate adenovirus type 5 (16). Furthermore, the antifiber sera of adenoviruses belonging to group III (15), one of which is adeno 5, may not neutralize the "cell-detaching factor" (13). It can therefore be argued that the toxicity of the aggregates produced by type 5 antifiber serum may still be due to the cell-detaching factor which resides in the penton bases. To solve these problems we have taken advantage of the characteristics of adeno type 3 virus. Serotype 3 is a member of adenovirus group I and differs from group III in relevant respects. The anti-type 3 hexon serum does aggregate type 3 virions (12). Therefore, aggregates produced by type 3 antihexon serum can be tested on toxicity. Secondly, the type 3 fiber antiserum efficiently neutralizes the penton's "toxin" or "cell-detaching factor" (17). Production of toxic aggregates by antifiber serum

should therefore be of significance. To complete the picture, assays with adenovirus type 9, a group II member, were also performed. It is of interest that adenoviruses of group II appear to be free from the "toxin" or cell-detaching factor of the pentons (4).

This report also deals with the question to what extent complement may be involved in the cytotoxic reactions. The problem seems of importance since complement has been assigned a crucial role in the development of immune-complex diseases.

MATERIALS AND METHODS

Cells. HeLa-S3 (Flow Laboratories) were grown in Eagle MEM with 1% fetal calf serum in roller bottles. SIRC cells, a rabbit corneal cell strain, and PB1, a rat fibroblast strain, were grown in Roux bottles and maintained as HeLa-S3. Cells from grey seal (Hg) and mongoose (He) were kindly supplied by K. Fredga (Institute of Genetics, Lund). They were grown and maintained as the SIRC cells.

Virus. Adenovirus type 5r, the prototype 3, and 9 were used in this study. Virus was harvested after a single cycle of growth in HeLa cells. Extracts of infected cells were obtained by the techniques of Gessler et al. (5) by using fluorocarbon compound Freon 113. The extracts were further purified by two cycles of density gradient centrifugations in solutions of CsCl. Batches were diluted and dialyzed against 0.02 M Tris buffer at pH 7.4 containing 0.25 M sucrose and 0.001 M $MgCl_2$ and stored at −20 C. The virus batches were filtered through 220-nm membranes (Millipore) before use.

Assays. Virus assays were performed in MAS cells using the PFU method in petri dishes.

Antiserum. Rabbits were inoculated intravenously with ~10^9 PFU of purified adenovirus type 5r. Additional injections were given once a week for 4 weeks followed by booster doses every fourth week. They were bled 10 days after each booster. The antiserum batches used were pools from several bleedings. The pools were from single rabbits. Fresh serum or serum inactivated at 56 C for 1 h was used as described in the text. Antisera against adenovirus type 3 and type 9 were prepared according to a similar schedule. Rabbit antisera against adenovirus type 3 hexon, penton, and fibers were kindly supplied by E. Norrby, Stockholm.

Isolation of immunoglobulin G (IgG). Each batch of rabbit hyperimmune serum was diluted with three parts of 0.1 M NaCl. The solution was made 1.84 M with respect to ammonium sulfate, and the pH was adjusted to 7.0. The precipitate was dissolved and fractionated on DEAE-cellulose and on Sephadex G-200 (Pharmacia, Uppsala) as described by Hansson et al. (6).

Tests for cytotoxicity. A modification of the earlier described (2) ^{51}Cr-release cytotoxicity tests was used. HeLa-S3 cells were labeled with sodium chromate (New England Nuclear, Boston, Mass.) as monolayers growing in culture bottles. Two hundred to 500 μCi of ^{51}Cr was added to 10 ml of culture medium, and the bottles were incubated for 14 to 16 h at 37 C. After this incubation the monolayers were washed five times with phosphate-buffered saline (PBS), once with a mixture of 0.1% trypsin and 0.02% EDTA, and treated again with 1 ml of 0.1% trypsin and 0.02% EDTA for 5 to 10 min. Dispersed cells were suspended and washed with culture medium and centrifuged at 700 × *g*. After the centrifugation the cells were resuspended in culture medium and adjust to a concentration of 2.5 × 10^5 cells/ml. The cell suspension was continuously mixed, and 0.4-ml samples of the suspension were distributed to test tubes. To each tube 0.4 ml each of virus, virus-antibody, and substitute was added. Two tubes were used in parallel for each sample. The tubes were shaken and incubated at room temperature for 4 h. After 4 h of incubation the samples were centrifuged at 1,500 × *g* for 5 min and incubated for an additional 2 h. Subsequently 0.4 ml of culture medium was added to each tube, and the cells were spun down by centrifugation at 1,500 × *g*. Two samples of 0.3 ml each of the supernatant fluid from each tube were removed by an automatic pipette and measured for radioactivity in a scintillation counter (Selektronik, Horsholm, Denmark). The percentage isotope release was calculated as: (average radioactivity in 0.3 ml of supernatant × 4 × 100)/(total radioactivity/tube). The average percentage release per tube was calculated on the basis of the release in two parallel tubes. The significance of differences in release of ^{51}Cr as compared to controls was calculated according to Student's *t* tests.

Virus-antibody mixtures. Samples (0.9 ml) of purified virus preparations were mixed with 0.1 ml of antisera, 2 h was allowed for virus-antibody interaction, and mixtures were added to tubes with cell suspensions. In some experiments virus preparations were incubated with two different types of antisera or with guinea pig sera. In such experiments 0.9 ml of virus preparation was incubated with 0.1 ml of the first antiserum. Simultaneously or after 1 h of incubation the second type of serum was admixed in amounts described in the text, and the tubes were incubated for an additional hour. All of the incubations were performed at room temperature. The difference in amount of serum or protein concentration in diluted antiserum was corrected by addition of preimmune rabbit sera.

Viral multiplication. Petri dishes containing ~10^6 cells of the strains described in the text were inoculated with purified adeno type 5r virus at a multiplicity of infection of 50 to 100. After an adsorption period of 2 h the cells were washed three times with PBS before 5 ml of Eagle MEM with 1% calf serum was added to each dish. After incubation for different periods at 37 C in a humidified CO_2 incubator, samples were taken, the cells were disrupted with a blendor, and the total amount of virus was titrated.

Electron microscopy. Monolayers of cells described in the text were fixed and embedded for preparation of ultrathin sections in accordance with methods described in detail previously (7). Sections were obtained in an LKB-ultratome microtome and post-stained with magnesium uranyl acetate and lead citrate. Negative staining of virus preparations was done as described previously (7). Electron microscopy was carried out on a Philips EM 300.

RESULTS

Viral entry into different cell strains. In Table 1 are shown the results of adenovirus type 5 growth experiments with five different cell cultures. Total viral yields were estimated 2, 24, and 48 h after infection at a multiplicity of about 10^2 PFU/cell. When HeLa cells were infected, the harvests 24 and 48 h later indicated a significant viral replication. In contrast, the yields obtained from the rabbit cells (SIRC), rat cells (PB1), grey seal cells (Hg), and mongoose cells (He) were maintained unaltered at the level remaining after washing of the cultures at 2 h.

There may be many different causes for the failure of adenovirus replication. The restriction displayed by the rabbit, the rat, the grey seal, and the mongoose cells was not dependent upon a failure of viral entrance. Entrance of virions by *direct penetration* of the cell membranes (11) could be observed by electron microscopy on all cell lines. The appearance of the entering virions was identical to that presented previously on HeLa cells (7).

Cytotoxicity induced by virus-antibody aggregates on different cell strains. When preparations of adenovirus type 5 at a concentration of about 10^9 PFU/ml, in the presence of homologous heat-inactivated antiserum at different dilutions, were tested in a ^{51}Cr release assay, it was found that they were strongly cytotoxic and damaged HeLa cells when tested with antiserum in dilutions varying from 1:10 to 1:100 (Table 2). In confirmation of previous results (9) the toxicity disappeared or diminished when antiserum was added in higher or lower dilutions.

Virus-antibody mixtures which produced a severe damage on HeLa cells were also toxic to rabbit, rat, grey seal, and mongoose cells (Table 2). As mentioned above, all of these cell lines permitted entrance of virus by a direct penetration although no complete viral replication ensued.

TABLE 1. *Virus production in different cell strains*

Cell strain for virus production	Virus yields at different times (PFU/ml)[a]		
	2 h	24 h	48 h
HeLa human	2.6×10^6	2.6×10^7	1.3×10^8
SIRC rabbit	2.9×10^6	1.9×10^6	1.2×10^6
PB1 rat	3.0×10^6	1.1×10^6	1.2×10^6
He mongoose	6.8×10^5	6.3×10^5	8.1×10^5
Hg grey seal	4.3×10^6	1.2×10^6	3.9×10^6

[a] The harvests were assayed on HeLa cells; four petri dishes per dilution step.

TABLE 2. *Cytotoxicity of aggregates of adenovirus type 5 heat-inactivated antibody induced in different cell strains*

Composition of mixtures (~10^9 PFU of virus/ml in 0.9 ml and 0.1 ml of:)	Test cells	Percentage of ^{51}Cr release ±S.D.	Difference from pre-immune serum control (%)
Antiserum L^{10}			
Undiluted	HeLa	4.84 ± 0.11	−2.04
Diluted 1:10	HeLa	5.56 ± 0.13	−1.32
Diluted 1:50	HeLa	61.72 ± 1.99	54.84[a]
Diluted 1:100	HeLa	10.82 ± 0.19	3.94
Preimmune serum			
Diluted 1:50	HeLa	6.88 ± 0.04	
Antiserum L^{10}			
Undiluted	SIRC	13.88 ± 1.78	−10.86
Diluted 1:10	SIRC	18.61 ± 0.16	−6.13
Diluted 1:50	SIRC	59.90 ± 0.34	35.16[b]
Diluted 1:100	SIRC	21.94 ± 0.80	−2.80
Preimmune serum			
Diluted 1:50	SIRC	24.74 ± 3.12	
Antiserum L^{10}			
Undiluted	PB1	5.10 ± 0.17	−1.63
Diluted 1:10	PB1	21.49 ± 1.87	14.76[b]
Diluted 1:50	PB1	57.06 ± 5.54	50.33[b]
Diluted 1:100	PB1	10.43 ± 0.01	3.70
Preimmune serum			
Diluted 1:50	PB1	6.73 ± 0.06	
Antiserum L^{11}			
Undiluted	Hg	29.64 ± 0.23	26.45[a]
Diluted 1:10	Hg	40.74 ± 0.09	37.55[a]
Diluted 1:50	Hg	26.11 ± 0.01	22.92[a]
Diluted 1:100	Hg	23.90 ± 0.40	20.71[a]
Preimmune serum			
Diluted 1:10	Hg	3.19 ± 0.02	
Antiserum L^{11}			
Undiluted	He	36.13 ± 4.96	31.40[c]
Diluted 1:10	He	61.11 ± 4.24	56.38[b]
Diluted 1:50	He	12.79 ± 0.01	8.06[a]
Diluted 1:100	He	12.19 ± 0.01	7.46[a]
Preimmune serum			
Diluted 1:10	He	4.73 ± 0.04	

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$.

Virus-antibody aggregates innocuous to human red blood cells. When preparations of adenovirus type 3 were mixed with homologous anti-complete virion antiserum, aggregates formed which were toxic to HeLa cells. In the controls containing virus and preimmune serum the ^{51}Cr release was 3% only. In the mixtures containing antiserum diluted 1:50 and 1:100 the ^{51}Cr release was 65 and 71%, respectively.

Human red blood cells exposed to the same mixtures were completely unaffected. The ^{51}Cr release did not rise above the 1% level in any mixture.

Temperature dependence of cytotoxicity. As shown by Morgan's group (11), as well as by others, entry of adenovirus into susceptible cells is not dependent upon the synthesis of new enzymes. The process is energy dependent, however. Attachment of adenovirus occurs at 0 C, but penetration does not ensue unless the temperature is raised (14). Similar conditions may apply for the cytotoxicity, as indicated by the results presented in Table 3. When mixtures of adenovirus and heat-inactivated antiserum, after formation of aggregates at room temperature for 2 h, were cooled to 4 C and incubated at 4 C in a suspension of HeLa cells, no cytotoxicity was revealed by the ^{51}Cr release assay. Identical mixtures run at room temperature or at 37 C were highly toxic (Table 3).

It is also shown in Table 3 that a dose of 5 μg of actinomycin D per ml—reported to bring RNA synthesis down to a 1% level (14)—added to cells 4 h ahead of the toxic mixtures did not affect the cytolysis, suggesting that a de novo synthesis of enzymes was not necessary to bring about cell damage.

Cytotoxicity of adenovirus type 3-antibody aggregates. Since the discrepancies between adeno type 5 and type 3 should be helpful for the further analysis of the mechanism behind the cytotoxicity of virus-antibody aggregates, we assayed adenovirus type 3 in mixtures containing various homotypic antisera. The results of the experiments (Fig. 1) confirm the findings obtained with adenovirus type 5 (9). In conclusion, within narrow zones of antibody concentrations toxic aggregates were produced not only by type 3 complete virion antiserum, but also by homotypic fiber antiserum and by homotypic penton antiserum. As seen by electron microscopy as well as by direct microscopy, aggregates were produced by homotypic hexon antiserum. These aggregates were always innocuous to the cells. Furthermore, it could be demonstrated that the formation of toxic aggregates by antifiber serum could be prevented by the hexon antiserum (Table 4).

Cytotoxicity of adenovirus type 9-antibody aggregates. The type 9 virus is a group II member of adenoviruses. Group II members appear to be free from the cell-detaching factor or "toxin" which resides in the pentons of the virions belonging to groups I and III (4). The lack of early cytopathic activity of the pentons had no influence on the capacity of adenovirus type 9 aggregates to exert cell damage. The results of ^{51}Cr release assays obtained with different type 9 virus-antibody mixtures are documented in Table 5. They show that, whereas the undiluted and the 1:10 dilution of antiserum protected the cells from a reasonable toxicity exercised by the virus alone, the same serum diluted 1:50, 1:100, and 1:200 produced aggregates which caused severe damage of the cells. The toxic activity of the aggregates was of a completely different order of magnitude compared to that of virus alone.

TABLE 3. *Cytotoxicity of aggregates of adenovirus type 5 heat-inactivated antibody at different temperatures*

Composition of mixtures (10^9 PFU of virus/ml in 0.9 ml and 0.1 ml of:)	Test temp	Percentage of ^{51}Cr release	Difference from controls
Antiserum			
Undiluted	37 C	7.60 ± 0.29	−3.17
Diluted 1:50	37 C	66.96 ± 0.11	56.19[a]
Diluted 1:1,000	37 C	10.76 ± 0.07	−0.01
Preimmune serum			
Diluted 1:50	37 C	10.77 ± 0.20	
Antiserum			
Undiluted	4 C	3.33 ± 0.11	−1.29
Diluted 1:50	4 C	3.40 ± 0.04	−1.22
Diluted 1:1,000	4 C	4.40 ± 0.06	−0.22
Preimmune serum			
Diluted 1:50	4 C	4.62 ± 0.03	
		Actinomycin-treated cells	
Antiserum			
Undiluted	22 C	6.25 ± 0.12	−0.32
Diluted 1:50	22 C	69.33 ± 2.41	62.76[a]
Diluted 1:1,000	22 C	6.61 ± 0.11	0.04
Preimmune serum			
Diluted 1:50	22 C	6.57 ± 0.21	

[a] $P < 0.001$.

Prevention of cytotoxicity. In the experiments reported, heat-inactivated antisera were used for the formation of virus aggregates. Cell lysis was apparently not mediated by complement factors. This conclusion is strengthened by the findings reported previously (9).

A further investigation of the role played by complement was, however, indicated for two reasons. First, when we compared the effect on formation of toxic aggregates between fresh and heat-inactivated rabbit antiserum, we found the heat-inactivated more efficient. Second, complement factors have been ascribed a crucial role as a mediator during development of immune-complex diseases in vivo.

Formation of toxic aggregates by purified IgG. In order to remove a possible influence of

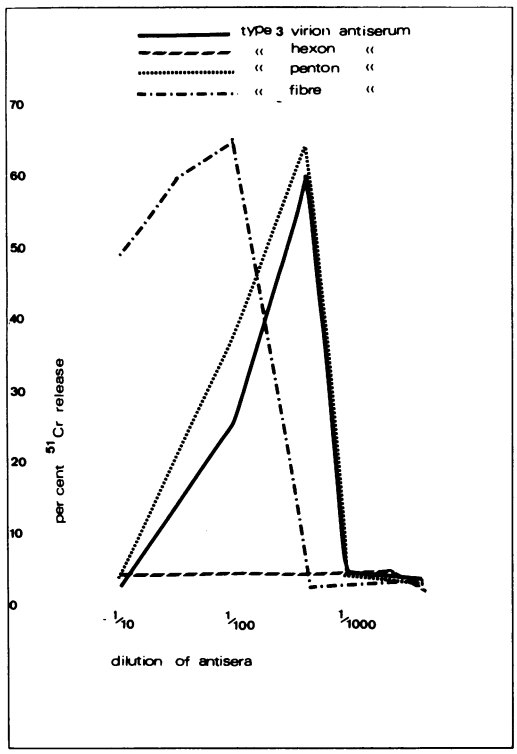

FIG. 1. *Production of cytotoxic aggregates by different type 3 adenovirus capsomere antisera. A 0.9-ml amount of virus was mixed with 0.1 ml of heat-inactivated antiserum. The serum dilutions of the figure refer to final dilutions of the mixtures. The antisera used were kindly supplied by E. Norrby, Stockholm. Data for the sera are as follows: hexon antiserum had a titer of 640 in complement fixation (CF) against type 2 hexon, <8 in hemagglutination enhancement test (HE), <8 in hemagglutination inhibition tests (HI). The penton antiserum had <10 in CF against type 2 hexon, 12,800 in HE, and 6,400 in HI tests. The fiber antiserum had titers <4 in CF against type 2 hexon, <4 in HE, and 12,800 in HI tests.*

lytic complement activity, the anti-adenovirus type 5 serum was fractionated and the IgG was purified (see Materials and Methods). When the IgG was mixed with virus, toxic aggregates were formed. In agreement with the results obtained with unfractionated antisera, toxic aggregates were formed only within a narrow zone of IgG concentration.

Inhibition of cytotoxicity by addition of fresh guinea pig serum. Addition of fresh guinea pig serum to toxic aggregates neutralized the toxicity. Fresh or inactivated guinea pig serum was added in different amounts to preformed toxic adenovirus-antibody aggregates. The mixtures were allowed an incubation of 30 min before addition to the cell suspension in ^{51}Cr release tests. The results are given in Table 6. It can be seen that 0.2 ml of fresh guinea pig serum significantly reduced cytotoxicity. That the addition of 0.3 ml of fresh guinea pig serum completely extinguished toxicity is also shown in Table 6. In the controls incubated with 0.3 ml of heat-inactivated guinea pig serum, no reduction in ^{51}Cr release occurred. The guinea pig serum was harmless to the cells when inoculated alone.

It is thus conceivable that complement factors are involved in the detoxification of the aggregates. If they are, the experimental set-up may not be proper for displaying the role

TABLE 4. *Addition of hexon type 3 antiserum to virus prior to type 3 fiber antiserum prevents formation of toxic aggregates*

Amt of type 3 hexon antiserum added to 0.9 ml of virus suspension (ml)	Amt of type 3 fiber antiserum added to the mixture for aggregate production (ml)	Percentage of ^{51}Cr release ± S.D.	Difference from controls
0.01	0.1	22.42 ± 0.18	26.78[a]
0.01	0.01	58.62 ± 0.94	6.78
0.1	0.1	2.88 ± 0.08	46.32[a]
0.1	0.01	18.51 ± 22.05	46.89
0.2	0.1	3.55 ± 0.24	45.65[a]
0.2	0.01	3.41 ± 0.13	61.99[a]
Preimmune serum 0.2	0.1	49.20 ± 1.03	
Preimmune serum 0.2	0.01	65.40 ± 0.41	

[a] $P < 0.001$.

TABLE 5. *Cytotoxicity of mixtures of adenovirus type 9 heat-inactivated antibody*

Composition of mixture (0.9 ml of purified type 9 virus and 0.1 ml of)	Percentage of ^{51}Cr release ± S.D.	Difference from preimmune serum control
Whole virion antiserum		
Undiluted	3.59 ± 0.01	−9.16
Diluted 1:10	3.91 ± 0.00	−8.84
Diluted 1:50	55.25 ± 2.99	42.50[a]
Diluted 1:100	64.94 ± 0.17	52.19[b]
Diluted 1:200	68.00 ± 0.32	55.25[b]
Diluted 1:500	38.04 ± 42.23	25.29
Diluted 1:1,000	7.71 ± 0.16	−5.04
Preimmune serum		
Diluted 1:50	12.75 ± 0.04	

[a] $P < 0.01$.
[b] $P < 0.001$.

TABLE 6. *Neutralization of cytotoxic type 5-antibody mixtures by fresh guinea pig serum*

Serum added to the toxic mixture[a]	Percentage ^{51}Cr release ± S.D.	Difference from control (%)
0.3 ml of heat-inactivated preimmune	55.85 ± 0.57	53.49[b]
0.3 ml of heat-inactivated guinea pig	52.58 ± 4.85	50.22[c]
0.1 ml fresh guinea pig	55.83 ± 1.06	53.47[b]
0.2 ml fresh guinea pig	15.62 ± 5.16	13.26
0.3 ml fresh guinea pig	2.35 ± 0.02	−0.01
Control: 0.3 ml fresh guinea pig added to virus alone	2.36 ± 0.06	

[a] The toxic mixture consisted of ~10^9 type 5 virus in 0.9 ml to which 0.1 ml of a whole virion heat-inactivated antiserum diluted 1:50 was added. The mixture was incubated for 1 h before addition of the guinea pig serum.

[b] $P < 0.001$.

[c] $P < 0.01$.

played. It seems unlikely that the complement under conditions in vivo would not be involved until the formation of aggregates is finished. The complement may instead participate during the formation of virus-antibody interactions. To mimic such interaction fresh guinea pig serum was added to each dilution of antiserum before mixing with virus. As usual, 2 h was subsequently allowed for reactions before the samples were assayed in a ^{51}Cr release test. The results are given in Fig. 2 and permit two conclusions. First, fresh but not heat-inactivated guinea pig serum in 0.2-ml amounts prevented the formation of toxic aggregates. Second, it is apparent that the presence of fresh guinea pig serum in those mixtures which were atoxic due to too high or too low contents of antibody did not generate toxicity.

Evidence for the participation of complement was brought forth in the following experiment. In one of our anti-type 5 sera, measurable amounts of anti-cell antibody could be detected. When this antiserum was heat inactivated and added in 0.1 ml to a suspension of HeLa cells without virus but together with fresh guinea pig serum a significant ^{51}Cr release was obtained. The cell lysis was apparently mediated by complement since samples with heat-inactivated guinea pig serum were harmless to the cells. When the same antiserum was used undiluted to form virus aggregates and fresh guinea pig serum was added, no cell lysis occurred upon addition to cell suspension. Apparently no complement was available to the cells, indicating a consumption of the complement by the aggregates.

DISCUSSION

The aim of the present study was twofold: first, a further analysis of the mechanism of the cytotoxicity exhibited by the virus aggregates; second, a further analysis of the role played by complement factors during development of toxic aggregates.

The new information presented in this report demonstrates that the cytotoxicity of the virus-antibody aggregates is exerted by the virions. The following findings indicate that the cytolytic reactions are not mediated by complement:

(i) Heat-inactivated antisera not only induced the production of cytotoxic aggregates, but they were also more efficient than fresh sera.

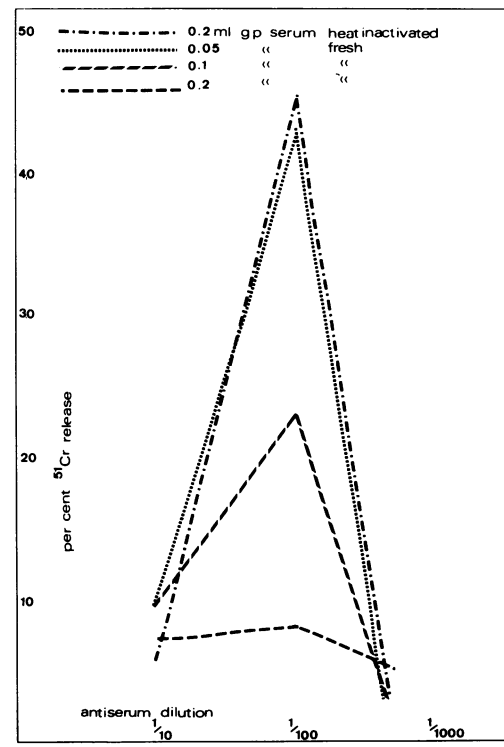

FIG. 2. *Prevention by the addition of fresh guinea pig serum of the formation of cytotoxic aggregates by adenovirus type 5-virion antiserum. Fresh guinea pig serum (gp) in different amounts was added to the antiserum dilutions before mixing with virus. Two hours was allowed for aggregate formation before addition to the test cells. Antiserum dilutions refer to final concentration in the mixtures.*

(ii) Purified IgG was as capable as unfractionated antiserum of producing toxic complexes.

(iii) It could be shown that the virus-antibody complexes consumed complement, but fresh guinea pig serum added to *atoxic* virus-antibody aggregates did not generate cytotoxicity.

(iv) Fresh guinea pig serum added to preformed *toxic* virus-antibody mixtures suppressed the toxicity.

(v) Fresh guinea pig serum added to virus and antibody prevented the formation of toxic aggregates.

The viral structures responsible for the toxic effects are most likely located on the hexons. The following data support these conclusions:

(i) Type 3 hexon antisera did aggregate type 3 virions. These aggregates which were formed over a wide range of antibody concentrations were always innocuous to the test cells.

(ii) The serotype 3 fiber and penton antisera produced toxic aggregates. Both sera neutralize the "cell-detaching factor" located in the pentons.

(iii) The production of cytotoxic aggregates by fiber and penton antisera could be prevented after addition of hexon antiserum.

(iv) Type 9 adenovirus belongs to the adenovirus group II. Members of this group appear to be free from the cell-detaching factor causing early cytopathic effects. Type 9 antibody aggregates were, however, as toxic to the cells as aggregates produced by members belonging to group I and III adenoviruses.

From what has been recorded under Results as well as from previous reports (2) it is obvious that high concentrations of penton and fiber antisera produced aggregates which were not toxic. In order to make them toxic, diluted sera could be used which produced complexes of a toxic nature. It might be argued therefore that the capacity of serotype 3 hexon antisera to prevent formation of toxic aggregates in accordance to point iii above can be explained by the known ability of the hexon antiserum to aggregate virions. It should be remembered, however, that type 5 hexon antiserum similarly prevented the production of cytotoxic aggregates by type 5 fiber antiserum. Since the type 5 hexon antisera do not cause aggregation, the inhibition most likely depends on a coating of the structures responsible for the toxic effects.

It should be mentioned in this connection that the possibility that virion fiber and penton antibodies release some soluble toxic principle seems unlikely, as the toxicity was held back by filtering the toxic mixtures through a 220-nm Millipore membrane (9).

Thus, of the capsomere antisera the hexon antiserum is the only one inhibiting cytotoxicity. We have previously demonstrated (9) that the type 5 hexon antiserum was also the only one capable of neutralizing virus infectivity. The fiber antiserum caused a reduction of infectious units only apparent when conditions for viral aggregation were at hand.

The structure responsible for the adenoviral penetration is therefore most likely the hexons. In the present report we have shown that cell strains permitting direct viral penetration (11) are also sensitive to the toxicity of the aggregates. Like the penetration procedure, the cytotoxicity was temperature dependent, but a de novo synthesis of enzymes may not be necessary. We have not been able to find a cell line resistant to adenovirus penetration, and we have therefore not been able to prove the thesis that impenetrability implies resistance to toxic action by aggregates. It might be noted, however, that human O blood cells were resistant.

The cytotoxic tests described may be a useful method for a detailed study of early virus-cell interactions. A precise knowledge of the viral penetration mechanism should facilitate arrangements to prevent this process.

It is conceivable that conditions favoring formation of toxic antigen complexes may appear during viral infections in vivo. As the aggregated viral particles are to be blamed for the toxicity, it implies a way by which the immune response may lead to tissue damage. There is only vague information to pinpoint the factors responsible for producing virus-associated immune-complex disease (10). Such diseases have been attributed to deposition of aggregates on the intima of small vessels. Complement is fixed which presumably elicits an Arthus type of inflammatory reactions. While this may happen in vivo, we found another function of the complement in our in vitro study. The addition of fresh guinea pig serum to the virus-antibody mixtures either prevented the formation of toxic aggregates or detoxified the preformed ones. Thus, far from generating cell lysis the complement inhibited cytotoxicity. A similar function of complement is described by Taniguchi and Yoshino (19), who found that immunization of rabbits with herpes simplex virus elicited early complement-requiring antibody for infectivity neutralization. The piling up of complement components on the surface of herpes simplex virions is a plausible cause of neutralization of infectivity of sensitized herpes virus, which was shown by Daniel et al. (3). A similar interpretation may be valid for the detoxifying ability of complement en-

countered in the present investigation. Enhancement of immunoaggregation by complement, similar to that described for herpes virus by Wallis and Melnick (18), could be another possibility. In our system, however, no toxic aggregates were generated by complement.

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LITERATURE CITED

1. Ankerst, J., and L. Kjellén. 1973. Cytotoxicity induced by mixtures of adenovirus and specific antibodies. Arch. Ges. Virusforsch. In press.
2. Ankerst, J., and H. O. Sjögren. 1969. Cross reacting TSTA:s in adeno 7 and 12 tumors demonstrated by ^{51}Cr-cytotoxicity and isograft rejection test. Int. J. Cancer **4**:279–287.
3. Daniels, C. A., T. Borsos, H. J. Rapp, R. Snydeman, and A. L. Notkins. 1970. Neutralization of sensitized virus by purified components of complement. Proc. Nat. Acad. Sci. U.S.A. **65**:528–535.
4. Gelderblom, H., H. Bauer, H. Frank, and R. Wigand. 1967. The structure of group II adenoviruses. J. Gen. Virol. **1**:553–560.
5. Gessler, A. E., C. E. Bender, and N. C. Parkinsson. 1956. A new rapid method for isolating viruses by selective fluorocarbon deproteinization. Trans. N.Y. Acad. Sci. **18**:701–703.
6. Hansson, U.-B., C. B. Laurell, and R. Bachmann. 1966. Sedimentation constants of IgG and IgD myeloma proteins compared with those of normal IgG. Acta Med. Scand. Suppl. **445**:89–92.
7. Kjellén, L. 1972. Cytotoxicity by antigen aggregation. Arch. Ges. Virusforsch. **39**:1–12.
8. Kjellén, L. 1972. Cytotoxic virus-antibody complexes. J. Infect. Dis. **126**:682.
9. Kjellén, L., and J. Ankerst. 1973. Cytotoxicity of adenovirus-antibody complexes. Specificity of antibody and virus-antibody ratio at which cytotoxicity is induced. Eur. J. Immunol. **3**:78–84.
10. Memoranda prepared by 12 signatories. 1972. Virus-associated immunopathology. Bull. WHO **47**:257–274.
11. Morgan, C., H. S. Rosenkranz, and B. Mednis. 1969. Structure and development of viruses observed in the electron microscope. J. Virol. **4**:777–796.
12. Norrby, E., H. Marusyk, and M.-L. Hammarskjöld. 1969. The relationship between the soluble antigens and the virion of adenovirus type 3. Virology **38**:477–482.
13. Pettersson, U., L. Philipson, and S. Höglund. 1969. Structural protein of adenoviruses. II. Purification and characterization of the adenovirus type 2 fibre antigen. Virology **35**:204–215.
14. Philipson, L. 1967. Attachment and eclipse of adenovirus. J. Virol. **1**:868–875.
15. Rosen, L. 1960. A hemagglutination-inhibition technique for typing adenoviruses. Amer. J. Hyg. **71**:120.
16. Wadell, G. 1972. Sensitization and neutralization of adenovirus by specific sera against capsid subunits. J. Virol. **3**:622–632.
17. Wadell, G., and E. Norrby. 1969. Immunological and other biological characteristics of pentons of human adenoviruses. J. Virol. **5**:671–680.
18. Wallis, C., and J. L. Melnick. 1971. Herpesvirus neutralization: the role of complement. J. Immunol. **107**:1235–1242.
19. Yoshino, K., and S. Taniguchi. 1965. Studies on neutralization of herpes simplex virus. Virology **26**:44–53.