Capsid Mosaics of Intermediate Strains of Human Adenoviruses

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Antisera against isolated capsid components of intermediate adenovirus strains, types 3-16 (the San Carlos agent) and 15-9 and of "parental" prototype strains were compared in neutralization tests, hemagglutination-inhibition (HI) tests employing soluble and virion-associated hemagglutinin as antigens, and by electron microscopy. Hexons of the intermediate strains were found to be similar to, but not identical to, those of the prototype strains with which a cross-reaction occurred in neutralization tests (types 3 and 15). In contrast, fibers of intermediate strains displayed characteristics relating them to the corresponding components of prototype strains to which a relationship has been found in HI tests. Fibers (and possibly even pentons) of types 9 and 15-9 appeared to be identical, whereas fibers of types 3-16 and 16 displayed antigen specificities of both common and unique nature.

Adenoviruses are separated into serotypes on the basis of their behavior in neutralization and hemagglutination-inhibition (HI) tests. These two tests are based on the presence of two distinct type-specific antigens carried by hexons and fibers, respectively (3, 8a, 13). The results of these two tests do not always parallel each other. Some isolates of adenoviruses have been found to behave like one serotype in neutralization tests but a completely different one in HI tests. Isolates of this kind have been referred to as intermediate strains. They have been repeatedly encountered within Rosen's subgroup II of human adenoviruses, as first described by Cramblett et al. (1) and later studied by Wigand and co-workers (16, 18). An intermediate strain related to two prototypes of subgroup I has also been described. This strain was represented among the San Carlos agents isolated from cases of hepatitis occurring among children living in an Indian reservation in Arizona. It was found to behave similar to type 3 in neutralization tests and type 16 in HI tests (2).

The aim of the present investigation was to explain the characteristics of intermediate strains by reference to immunological properties of different structural components occurring in their capsid. For this purpose two different strains were studied. These are referred to as types 3-16 and 15-9. In this hyphenated expression the first and second figure denote the prototypes to which the intermediate strain is related in neutralization and HI tests, respectively. This descriptive formula

deviates from that proposed by Wigand and Flidner (17), for reasons given in a recent review (8a). To study the relationship of different structural components of the intermediate strains to the corresponding components of "parental" strains, immunological techniques were employed. Specific antisera against purified components of types 3, 3-16, and 16 (9), 15-9, and 15 (6) were prepared and then characterized by different serological tests and by electron microscopy. The results obtained demonstrated that intermediate strains carry hexons and fibers (or pentons) similar to or identical with those of the prototype strains, with which they cross-react in neutralization and HI tests.

MATERIALS AND METHODS

Virus and cell cultures. The prototype strains of types 3, 9 ("Hicks"), 15 (Ch 38), and 16 (CH 79) and the intermediate strains, type 3-16 (isolates SC-8 and SC-49; reference 2) and 15-9 (strain 5399, isolated by J. van der Veen, Nijmegen, Holland) were used. The latter two strains were kindly provided by G. J. Love, National Communicable Disease Center, Phoenix Field Station, Ariz., and R. Wigand, Homburg (Saar), Germany, respectively. Virus materials were prepared in a human embryonic lung cell line (Lu 106 cells) or a human bone-marrow cell line (MAS-A cells) by techniques previously described (4, 5). Infected cultures were harvested at an advanced stage of virus-induced cell degeneration. The medium and cells were concentrated by forced dialysis against polyethylene glycol (Carbowax 6000, Union Carbide Corp., New York, N.Y.) and were frozen and thawed three times. After low-speed centrifugation to remove cell debris,

the concentrates were used for preparation of virus products.

Preparation of different virus products. Virions were prepared by centrifugation of stock materials in a discontinuous CsCl gradient by the technique described recently (12a). Soluble components remaining on top of the gradients were dialyzed against phosphate-buffered (0.067 M, pH 7.2) physiological saline and then separated further. The separation techniques employed were designed to give a distinct separation of fiber-associated structures (isolated fibers, fiber oligomers, pentons, penton oligomers, or dodecons) from hexons, to prepare specific antisera against these two capsid components. Absolute biochemical purification was not strived for. Techniques for separation of different type 3 components were already described (8). Dodecons of types 9 and 15-9 were isolated by adsorption-elution on red cells (6, 11), whereas dodecons and fibers of type 15 were prepared by zonal centrifugation and anion-exchange chromatography on diethylaminoethyl (DEAE)-Sephadex A 25 (Pharmacia Fine Chemicals, Uppsala, Sweden), respectively (6). Monomers and oligomers (mostly dimers) of type 16 fibers, and pentons of type 3-16 were prepared by zonal centrifugation and exclusion chromatography on spherical agarose (Bio-Gel A-15m; BioRad Laboratories, Richmond, Calif.), respectively (9). None of these preparations contained demonstrable amounts of hexon complement-fixing (CF) antigen. Hexons of all serotypes, except type 3-16, were isolated by anion-exchange chromatography on DEAE-Sephadex A25 (5, 6, 9). Exclusion chromatography (Sephadex G200) was employed for preparation of hexons of type 3-16 (9). All hexon preparations were devoid of any complete or incomplete hemagglutinin activity.

Preparation and absorption of hyperimmune sera. Rabbits were immunized with 4 ml of material mixed with Freund's complete adjuvant. After 5 weeks an intravenous booster injection of 1 to 2 ml was administered. At 1 week later, the animals were exsanguinated and serum was collected.

Absorption of sera was performed by mixing them with purified antigen in an amount calculated to be two to four times higher than that required to remove all antibody. After incubation at room temperature for 1 hr and at 4°C overnight, the samples were centrifuged at (P_i) a performance index of 16.2 (81,000 $\times g$, rotor 40, Spinco Division, Beckman Instruments Inc., Calif.; reference 10) for 2 hr to remove antigen-antibody complexes.

Tests for biological activities. Serological techniques for demonstration of incomplete and complete hemagglutinins and CF antigen were already presented (5, 11). The microtechnique of Takatsy (equipment supplied by Flow Laboratories, Irvine, Scotland) was employed. Antibody activities were measured in neutralization tests in tubes of diploid human lung cells, and in HI and in CF tests, by use of previously described techniques (8).

Electron microscopy. The procedure for preparation of virion-antibody complexes was recently described (12). All sera used were obtained by hyperimmuniza-

tions and, thus, contained mainly immunoglobulin G antibodies. All sera carried high titers of antibodies and, therefore, could be employed at dilutions of 1 to 100 or higher. This precluded the necessity for purification of virion-antibody complexes.

RESULTS

Neutralizing and HI antibody activities of sera against isolated soluble components (hexons and fiber-carrying structures) of different serotypes. In previous studies (8, 10), it was found that antibodies against hexons of members of subgroups I and II, which carry the major fraction of all neutralizing activity, displayed a marked capacity to inhibit the HA activity of homotypic virions, but not soluble components. This finding provided a convenient technique as a supplement to neutralization tests for determination of antibodies against type-specific parts of hexons.

The capacity of some selected antisera against hexons and fiber-associated soluble components of types 3, 3-16, and 16 to inhibit homotypic and heterotypic virions in neutralization and HI tests and soluble hemagglutinin in HI tests have been summarized in Table 1.

Sera against type 3 hexons were equally capable of inhibiting infectivity and hemagglutinin activity of virions of both types 3 and 3-16. Antisera against type 3-16 hexons behaved similarly, although titers against homotypic antigens in this case consistently were two to four times higher than against heterotypic antigens. Sera against type 16 hexons only inhibited homotypic activities.

Activities of antifiber sera demonstrated a pattern of relationship between the three serotypes which was different from that of antihexon sera. Sera against fibers of type 3 displayed activity in tests with homotypic antigens only, whereas sera against the corresponding components of types 3-16 and 16 revealed a reciprocal cross-reactivity. HI titers of antisera against type 3-16 pentons were about four times higher in tests with homotypic soluble hemagglutinin and virions than with type 16 antigens. The same kind of homotypic preference was displayed by type 16 antifiber sera. A relationship between type 3-16 and 16 fibers could also be inferred from the finding that some sera against type 16 fibers (Table 1) carried a low-titer neutralizing activity against homotypic and also, although in an even lower titer, against type 3-16 virions. Some type 16 antifiber sera containing homotypic HI antibodies in high titers were found also to display a slight HI activity against type 3 antigens.

Sera against type 9, 15-9, and 15 components carried antibody activities of a pattern basically similar to the one described above. Thus, a rela-

tionship between hexons of types 15 and 15-9 was suggested from the fact that antibodies against both of them could neutralize or inhibit the HI activity of either type of virions. Sera against type 15 hexons were equally active against type 15 and 15-9 antigens, whereas sera against type 15-9 hexons gave distinctly higher titers with homotypic than with heterotypic antigens (Table 2). Antisera against type 9 hexons neutralized homotypic virions only. The behavior of antifiber sera, in contrast to antihexon sera, demonstrated a relationship between serotypes 9 and 15-9 analogous to that found for types 3-16 and 16. However, HI antibodies against fibers of types 9 and 15-9 did not display any preference for inhibition of homotypic antigen of the kind displayed by 3-16 and 16 antifiber sera. It should be mentioned that antisera against type 15 fibers carried a low-titer homotypic-neutralizing activity, but did not neutralize type 15-9 virions.

Further analysis of the relationship between hexons of different serotypes. To evaluate further the degree of relationship between hexons of intermediate and "parental" strains, absorption experiments were performed. The results of one experiment, in which neutralizing and CF antibody activity of sera against hexons of types 9, 15-9, and 15 were determined before and after absorption with homotypic and heterotypic hexons, are summarized in Table 2. The serum against type 9 hexons neutralized only homotypic virions. Absorption with hexons of types 15-9 or 15 changed neither this neutralizing activity nor homotypic (type 9) hexon CF antibody activity, although all CF antibody activity against both hexons of type 15-9 and 15 were removed by either antigen. A similarity of behavior of type 15 and 15-9 hexons was apparent also from the results of absorption of antisera against these components. Type 15-9 hexons were capable of re-

TABLE 1. *Antibody titers of sera against different components of types 3, 3-16, and 16 in neutralization tests and in HI tests employing soluble and virion-associated hemagglutinins (HA) as antigens*

Sera against type	Neutralizing activity in tests with virions of type			HI activity in tests with					
				Type 3		Type 3-16		Type 16	
	3	3-16	16	Soluble HA	Virions	Soluble HA	Virions	Soluble HA	Virions
3 hexons	12,800	6,400	<4	<4	12,800	<4	12,800	<4	<4
3 fibers	64	<4	<4	6,400	6,400	<4	<4	<4	<4
3-16 hexons	180	512	<4	<4	640	<4	320	<4	<4
3-16 pentons	<4	<4	<4	<4	<4	640	640	160	320
16 hexons	<4	<4	512	<4	<4	<4	<4	<4	1,280
16 fibers	<4	8	32	16	16	160	160	640	1,280

TABLE 2. *Neutralization and CF antibody activities of antisera against hexons of types 9, 15-9, and 15 before and after absorption with homotypic and heterotypic hexons*

Sera against hexons of type	Absorbed with hexons of type	Neutralizing activity against virions of type			CF antibody activity in tests with hexons of type		
		9	15-9	15	9	15-9	15
9	0	320	<4	<4	160	160	80
	9	<10	NT ^a	NT	<10	<10	<10
	15-9	450	NT	NT	160	<10	<10
	15	320	NT	NT	160	<10	<10
15-9	0	<4	1,280	220	320	640	320
	9	NT	1,800	320	<20	640	320
	15-9	NT	<20	<20	<20	<20	<20
	15	NT	<20	<20	<20	<20	<20
15	0	<4	1,280	1,280	320	640	640
	9	NT	1,280	1,800	<20	320	640
	15-9	NT	<20	<20	<20	<20	<20
	15	NT	<20	<20	<20	<20	<20

^a Not tested.

moving all neutralizing and CF antibody activity of sera against type 15 hexons and vice versa. Type 9 hexons, in contrast, only absorbed CF antibodies against the same type.

Absorption experiments employing sera against hexons of types 3, 3-16, and 16 gave results basically identical with those presented above.

Further analysis of the relationship between fibers of different serotypes. Antisera against "whole" virus material of types 3-16 and 16 were absorbed with purified pentons (monomers and oligomers) of type 3-16 or fibers (monomers and oligomers) of type 16, after which neutralizing and HI activities against the two serotypes were determined. The corresponding type of experiment was performed with sera against "whole" virus material of types 9 and 15-9, although, in this case, dodecons were used for absorption (Table 3). Neutralizing activity of all sera was not affected by any of the absorptions. Similarly, HI activity against homotypic virions (carried by both antihexon and antifiber antibodies) remained unchanged or displayed a minor reduction. HI activities against soluble hemagglutinins of types 3-16 and 16 were completely removed by absorption with homotypic antigen. Absorption with heterotypic antigen, in contrast, did not remove all HI antibodies reacting with homotypic antigen, indicating the occurrence of antigenic specificities which are either absent or hidden in heterotypic soluble components. No similar heterogeneity

among HI antibodies reacting with soluble hemagglutinins of types 9 and 15-9, could be demonstrated.

Immune electron microscopy. In previous studies of adenovirus type 3 (12), it was found that the type-specific parts of fibers and hexons could be demonstrated at the surface of virions by use of electron microscopy. The same technique was employed in the present study as a means of revealing antigen specificities available in virions of intermediate strains. Specific antisera against hexons and fiber-associated structures of the different serotypes were used. The results obtained gave a morphological confirmation of the immunological findings presented above. Thus fibers of two types which cross-reacted in HI tests employing soluble components as antigen could associate with immunoglobulin G antibodies of either type (Fig. 1). Correspondingly, antibodies against hexons of two types which behaved similarly in neutralization tests displayed a capacity to interact not only with their own virions but also those of the cross-reacting serotype (Fig. 1). In some combinations, results of testing of various serum dilutions suggested that attachment of antibodies to the capsid of homotypic virions occurred more readily than attachment to virions of the cross-reacting serotype. No such difference was seen in the case of type 9 and 15-9 antidodecon sera and type 3 and 15 antihexon sera.

TABLE 3. *Neutralizing and HI antibody activities of sera against "whole" virus material before and after absorption with homotypic and heterotypic hemagglutinins (HA)*

Sera against "whole" virus material of type	Absorbed with		Neutralizing activity against		HI activity against			
	Type	Component	Homotypic virions	Heterotypic ^a virions	Homotypic		Heterotypic ^a	
					Virions	Soluble HA	Virions	Soluble HA
3-16		0	2,560	32	2,560	1,280	320	160
	3-16	Pentons ^b	2,560	— ^d	2,560	<40	<40	<40
	16	Fibers ^c	3,600	—	2,560	320	<40	<40
16		0	1,280	8	1,280	640	160	320
	3-16	Pentons	1,800	—	640	320	<40	<40
	16	Fibers	1,280	—	640	<40	<40	<40
9		0	640	<20	6,400	3,200	6,400	6,400
	9	Dodecons	900	—	1,600	<200	<200	<200
	15-9	Dodecons	640	—	1,600	<200	<200	<200
15-9		0	1,280	<20	6,400	25,600	12,800	12,800
	9	Dodecons	900	—	1,600	<200	<200	<200
	15-9	Dodecons	900	—	3,200	<200	<200	<200

^a Heterotypic refers to the serotype employed in the comparative analysis, viz., type 16 in the case of type 3-16, type 15-9 in the case of type 9, and vice versa.

^b Prepared by exclusion chromatography; contained monomers and dimers of pentons.

^c Prepared by zonal centrifugation. Contained monomers and dimers of fibers.

^d No test was performed.

FIG. 1. Electron microscopy of virion-antibody interactions. (a) type 15-9 virions + antiadenovirus type 9 fiber serum, (b) type 15-9 virions + antiadenovirus type 9 hexon serum, (c) type 9 virions + antiadenovirus type 9 hexon serum, (d) type 15-9 virions + antiadenovirus type 15 hexon serum, (e) type 15-9 virions + antiadenovirus type 15 fiber serum, (f) type 15 virions + antiadenovirus type 15 fiber serum. Sera were diluted 1:200 in (a), (c), (d), and (f) and 1:75 in (b) and (e); magnifications: (a) and (c) $\times 125,000$ and (d)-(f) $\times 100,000$.

DISCUSSION

Hexons of types 3 and 3-16 and also 15 and 15-9 behaved pairwise identically in absorption tests. However, the neutralization tests with unabsorbed sera did not give identical titers in all cases. Thus a preference for homotypic neutralization was found in tests of type 3-16 and, in particular, type 15-9 sera. Earlier studies have demonstrated a slight difference in position in the elution diagrams obtained after anion-exchange chromatography of hexons of types 3 and 3-16 (9) and types 15 and 15-9 (6). Similar differences can be shown by use of the isoelectric focusing technique (Norrby, unpublished data). Taken together, these data indicate that hexons of two serotypes cross-reacting in neutralization tests are similar, but not identical, and that they both carry the antigen specificities which are responsible for interaction

with neutralizing antibody. Since there appeared to be some differences in the capacity of sera to neutralize homotypic and heterotypic virions, it seems likely that the above-mentioned common antigen specificities are available to a varying extent at the surface of virions of different serotypes. This conclusion was also reached from studies on the structural basis for the cross-neutralization observed between types 4 and 16 (10).

Fibers of types 9 and 15-9 behaved identically in cross-absorption HI tests. Previous studies have revealed that not only these components but also pentons of the two types display identical physicochemical characteristics. Thus, it seems likely that the pentons of these two types are identical, although a reservation has to be made regarding immunological characteristics of vertex cap-

somers, since it has not as yet been technically feasible to demonstrate possible type-specific characteristics of this type of component (8, 15). In contrast to this situation, fibers of types 16 and 3-16 were found by cross-absorption HI tests both to share and to carry unique antigen specificities. This difference also appeared to be recognizable in electron microscopy experiments. Fewer immunoglobulin G molecules attached to heterotypic than to homotypic fibers. The behavior of pentons of types 16 and 3-16 in anion-exchange chromatography experiments were previously shown to be different (9). This finding might reflect the immunological differences between fibers of the two serotypes, but, possibly, differences unrecognizable at present between the vertex capsomers might be of some importance.

The appearance of intermediate adenovirus strains in nature is a matter for speculation. One possible origin might be a recombination between "parental" strains. To test this hypothesis, current plans include attempts to perform recombination experiments in this laboratory. The fact that the corresponding components of the "parental" and intermediate type exhibit some different characteristics (the only exception being pentons of types 9 and 15-9, which probably are identical) indicates that mutational changes must have occurred after the possible event of recombination. An alternative explanation for the occurrence of intermediate strains could be that they represent an intermediate form in the evolutionary differentiation of human adenoviruses into the different distinct serotypes which are presently recognized.

The practical importance of intermediate strains for infection of man has not been fully evaluated. The immunological specificity of antibodies carrying protection against different types of adenovirus infections in vivo has not been clearly established. However, it seems likely that antihexon antibodies are of paramount importance. If this is the case, the capacity of intermediate strains to disseminate in nature should reflect the similarity between their hexons and those of the "parental" types. Against this background the present results may suggest that previous infections with types 3 and 15 possibly could protect against subsequent infections with types 3-16 and 15-9, respectively, whereas this must not necessarily be true when the infections occur in the reversed order. Direct challenge experiments in man appears to be the only way of analyzing this problem.

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