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Immunological Basis of the Adenovirus 8-9 Cross-Reaction

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The dodecon and hexon components of adenovirus types 8 and 9 have been extensively purified for use in establishing the basis of the cross-reaction between these types. Dodecons, the complete hemagglutinins, were purified 304- to 362-fold by fluorocarbon extraction, calcium phosphate batch chromatography, and ion-exchange column chromatography. Hexons, the group complement-fixation (CF) antigens, were purified 230- to 240-fold by erythrocyte adsorption, ion-exchange chromatography, and exclusion chromatography. Component antisera prepared in rabbits were tested in reciprocal fashion with crude virus and dodecon and hexon components. By hemagglutination-inhibition (HI), the dodecons of types 8 and 9 demonstrated the same predominantly one-sided relationship characteristic of the crude antigens. Some neutralizing activity was associated with both dodecons and hexons of each type. However, combining anti-dodecon and anti-hexon sera or producing antisera against the combined dodecon-hexon components resulted in neutralizing titers which were identical to titers obtained with antisera against the crude virus harvests. Dodecons of each type appear to share at least one antigenic determinant with hexons of the same type, and this determinant may reside on the vertex capsomere. Hexons possess group- and type-specific determinants, as shown by CF, neutralization, and immunodiffusion tests, and may exhibit some minor relationship between types 8 and 9. The results with the purified components are consistent with the predominantly one-sided antigenic relationship between types 8 and 9 in the conventional HI tests and the largely type-specific relationship by neutralization tests.

Recent improvements in techniques have made possible the separation and high degree of purification of the major adenovirus components, and have provided the opportunity for investigation of some of the serological quandaries encountered in adenovirus identification. The nature of the reciprocal cross-reactions between types 8 and 9 has been particularly intriguing, since these two types, which are distinct by neutralization tests, cannot be differentiated by hemagglutination-inhibition (HI) tests in many laboratories (8, 18, 21, 22).

The two major soluble components of types 8 and 9 have been shown in preliminary experiments to be the complete hemagglutinin (dodecon) and the group-specific complement-fixation (CF) antigen (hexon). In this paper, we present the results of reciprocal serological tests performed with purified dodecon and hexon components and component-specific antisera and discuss the basis of the cross-reactions between the two virus types.

MATERIALS AND METHODS

Virus production. Prototype strains of adenovirus 8 (Trim) and adenovirus 9 (Hicks), both obtained from the Research Reference Reagents Branch of the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, were passaged several times in HEp-2 tissue culture. Virus and viral components were harvested 2 days after complete cytopathology and were clarified by centrifugation at 1,060 × *g* for 30 min at 4 C. The supernatant fluid is referred to as the "crude" virus preparation.

Purification of dodecons. Portions of the crude virus preparations were first extracted with the fluorocarbon trifluorotrichloroethane to remove cellular protein material (4). Dodecons were then partially purified by chromatography with brushite $CaHPO_4$ gel (20). The viral antigen suspension, dialyzed against 0.001 M phosphate buffer (*p*H 7.2), was then mixed with equal volumes of packed gel and 0.001 M phosphate buffer. The mixture was incubated at 24 C for 1 hr and at 0 C for an additional 1 hr and was stirred frequently. The gel was packed by centrifugation, washed three times in the 0.001 M buffer, and then successively extracted with single volumes of

0.005, 0.01, and 0.05 M phosphate buffers (*p*H 7.2) to remove extraneous proteins. The hemagglutinating (HA) activity was eluted from the gel with 0.10 M phosphate buffer (*p*H 7.2) at 35 C.

Final purification of the dodecons was achieved by ion-exchange chromatography performed in columns (5 by 70 cm) packed with diethylaminoethyl (DEAE)-Sephadex A-25 resin (Pharmacia, New York, N.Y.) with tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (0.04 M, *p*H 8.4) as the equilibrating buffer (13). The column was loaded with 15 to 20 ml of concentrated, dialyzed, $CaHPO_4$ eluate. A convex sodium chloride gradient, from 0 to 0.3 M NaCl in the Tris buffer, was used for elution. The gradient curve (see Fig. 1) was designed to provide a very gradual salt increase between 0.2 and 0.3 M NaCl while providing a large eluant volume. Fractions (12 ml) were collected at a flow rate of 4 to 8 ml per cm^2 per hr. All chromatography was performed at 24 C.

Purification of hexons. A crude separation of the hexon antigens from the virus was effected by differential centrifugation at 26,400 × *g* (9). Residual HA activity in the resulting supernatant fluid was removed by five cycles of adsorption for 1 hr at 30 C with 20% human "O" erythrocytes (final concentration, v/v). The absorbed supernatant was chromatographed on DEAE-Sephadex A-25 columns (2.5 by 35 cm) equilibrated with the *p*H 8.4 Tris-hydrochloride buffer above. Fractions (10 ml) were collected at a flow rate of 12 to 14 ml per cm^2 per hr.

The appropriate fractions were concentrated, dialyzed, and applied to Sephadex G-200 columns (2.5 by 35 cm) equilibrated with 0.01 M Tris-0.10 M glycine buffer, *p*H 8.0 (17). Fractions (4 ml) were collected at a flow rate of 7 to 11 ml per cm^2 per hr and at 24 C.

Property tests. HA tests for complete and incomplete hemagglutinins were performed in microtiter with 0.4% Sprague-Dawley rat red blood cells, by use of 0.01 M phosphate-buffered saline (PBS), *p*H 7.2, and PBS containing 1% adenovirus type 6, 10, 15, or 17 antiserum as diluents (5). HI antibody consumption tests for fiber antigen were performed as described by Norrby and Skaaret (14). CF tests were performed by the standardized microtiter method (1). Infectivity and toxicity titrations were performed in primary human embryonic kidney tissue culture (17).

Protein. Protein determinations were made by the method of Lowry et al. (7), with absorbance measured at 500 nm.

Electron microscopy. Antigens were mixed with 2% sodium tungstosilicate at *p*H 7.0 and applied to carbon-coated grids by the drop technique. The grids were examined in a Philips EM-300 electron microscope operated at 80 kv.

Electrophoresis and immunoelectrophoresis. Analytical electrophoresis was performed on cellulose polyacetate strips (2.5 by 15 cm, Sepraphore III; Gelman Instrument Co., Ann Arbor, Mich.) at 400 v and 0.9 ma of current for 12 hr at 20 C. Electrophoresis buffers included: Tris-glycine, *p*H 8.0 and 8.4; barbital, *p*H 8.6 and 8.8; and carbonate, *p*H 10.0. Immunoelectrophoresis was carried out in 1% agarose on slides precoated with 0.1% Noble agar. The antigens were subjected to electrophoresis at 250 v, 17 ma, for 4 hr at 4 C in a 0.1 M barbital buffer (*p*H 8.6), and then were reacted with antiserum for 1 to 2 days at 24 C.

Zonal centrifugation. Density gradient centrifugation in linear 10 to 30% sucrose gradients was performed at 60,000 × *g* for 6 hr by use of a Spinco SW 39L rotor. Fractions were collected dropwise through a hole punctured in the bottom of the tube.

Ouchterlony tests. Radial double-diffusion tests for antigen purity and identification were carried out on slides containing 0.8% agarose in 0.01 M PBS (*p*H 7.2). Wells were 2 mm in diameter by 1 mm deep and 7 mm apart, center to center. Tests were held at room temperature for 1 to 3 days.

Antiserum production. Component-specific and crude virus antisera were prepared in New Zealand white rabbits by three biweekly subcutaneous injections of 1.5 ml of the component or crude virus preparation emulsified in Freund's incomplete adjuvant. Final bleedings were taken 7 days after the third inoculation.

RESULTS

Purification of dodecons and hexons. DEAE-Sephadex ion-exchange chromatography of adenovirus 8 and 9 supernatant fluids effected the best separation of soluble antigens. An elution curve typical of both virus types is shown in Fig. 1. The incomplete hemagglutinin (penton) was eluted first, the group CF component (hexon) next, and the complete hemagglutinin (dodecon) last. The overlap in elution peaks and the severe contamination with host protein prevented complete separation by this method alone.

A series of procedures was required to achieve the desired high degree of purity of the two major components. By fluorocarbon extraction, $CaHPO_4$ batch chromatography, and ion-exchange column chromatography, the dodecons were purified 304- to 362-fold with a 20 to 21% yield. The final product was serologically pure: no incomplete hemagglutinin (penton, fiber) was detected, either by adsorbing out the complete hemagglutinin and retesting with heterotypic sera, or by the HI antibody consumption test; no group CF activity was detected by testing with type 4 human or type 5 rabbit antisera; and no infective virus or toxic factors were detectable. In addition, electron microscopy of the highly concentrated product confirmed the complete hemagglutinin of adenovirus 8 to be a dodecon structure identical to that of adenovirus 9, which has been previously described (9, 13).

By use of erythrocyte adsorption, ion-exchange chromatography, and exclusion chromatography, the hexons were purified 230- to 240-fold with a 50 to 60% yield. The final preparation was serologically pure as determined by HA tests for

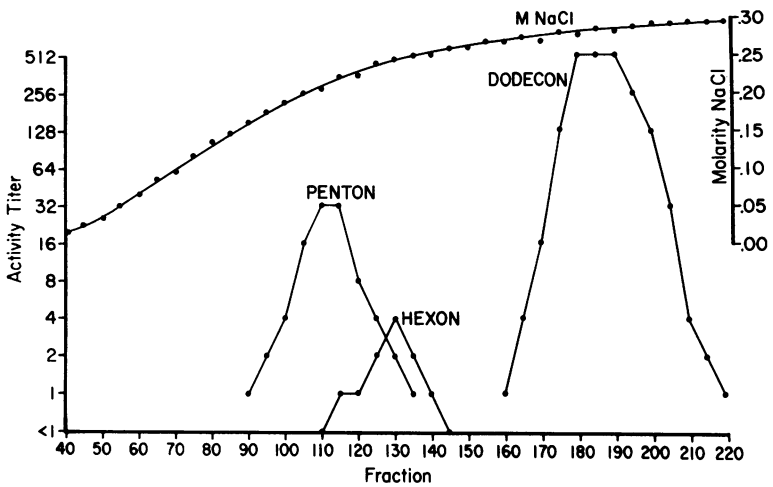

FIG. 1. *Separation of adenovirus 8 soluble components on DEAE-Sephadex A-25.*

complete and incomplete hemagglutinins, HI antibody consumption tests for fiber component, and infectivity/toxicity tests for whole virus and vertex capsomeres.

Other tests of purity on the final dodecon and hexon preparations included strip electrophoresis in different buffer systems, zonal centrifugation in 10 to 30% linear sucrose gradients, and immunoelectrophoresis and Ouchterlony double-diffusion tests performed with crude virus antisera. In each case only single bands were obtained, indicating component purity.

Serological results with purified components and component-specific antisera. Antisera prepared against crude antigens, dodecons, hexons, or combined dodecon-hexon components exhibited nearly identical activity in HI tests with crude harvests of types 8 and 9 (Table 1). In each instance, type 9 antisera reacted equally as well against type 8 or type 9 antigens, whereas all type 8 antisera gave titers which were 8- to 64-fold lower against type 9 antigens than with the homologous antigen. This same degree of bilateral but predominantly one-sided cross-relationship between 8 and 9 was also observed in HI tests with the purified dodecons. Although types 8 and 9 antihexon sera inhibited crude antigen hemagglutination, both failed to inhibit dodecon hemagglutination.

Neutralization by antisera prepared against crude antigen and combined dodecon-hexon antigens was highly type-specific (1:1,280) with only low-order (1:10) cross-reactions with the heterotypic viruses (Table 1). The hexon and dodecon antisera were entirely specific for their respective virus types, but neutralization titers did not exceed 1:80. Homologous neutralization titers were increased 8- to 16-fold when these same hexon and dodecon antisera were combined. The resulting titers were essentially identical to those obtained with antisera against the crude virus or antisera against the combined dodecon-hexon components.

A bilateral but predominantly one-sided type-specific relationship between types 8 and 9 was also observed by reciprocal CF tests with hexon as well as dodecon components (Table 2). In the CF, as in the HI tests, the antihexon sera produced against either virus type failed to react with the dodecon antigens.

Ouchterlony tests supported the antigenic relationships between the dodecons of types 8 and 9 as determined by HI and CF, and the relationship between the hexons as determined by neutralization tests. Type 8 dodecon formed a loop of identity with type 9 dodecon against type 8 antidodecon serum, whereas type 9 dodecon showed only partial identity to type 8 dodecon against type 9 antidodecon serum. Lines of reciprocal partial identity were obtained with hexons of both types when tested against types 8 and 9 antihexon sera. Dodecons of either type failed to react with antihexon sera, and hexons did not react with antidodecon sera.

DISCUSSION

Purification data on dodecons of adenovirus types 8 and 9 or other adenovirus types are not available in the current literature, although some inferences may be drawn from Pettersson's work on purified components of adenovirus type 2 (16, 17). Our 304- to 362-fold purification of the hemagglutinins (dodecon) and our 230- to 240-fold purification of the hexon compare favorably

TABLE 1. *Serological relations between type 8 and type 9 by hemagglutination-inhibition (HI) and neutralization (SN) tests with antisera against crude and purified antigens*

Test antigen	Antiserum									
	Type 8					Type 9				
	Crude	Dodecon	Hexon	Dodecon + hexon[a]	Dodecon + hexon[b]	Crude	Dodecon	Hexon	Dodecon + hexon[a]	Dodecon + hexon[b]
HI										
Crude										
Adenovirus 8	2,560[c]	2,560	640	2,560	—	640	1,280	640	640	—
Adenovirus 9	320	40	10	40	—	1,280	1,280	1,280	1,280	—
Dodecon										
Adenovirus 8	2,560	2,560	0[d]	1,280	—	1,280	2,560	0	640	—
Adenovirus 9	320	40	0	40	—	1,280	2,560	0	1,280	—
SN										
Virion										
Adenovirus 8	1,280	80	80	1,280	640	10	0	0	10	5
Adenovirus 9	10	0	0	10	0	1,280	80	40	1,280	640

[a] A single antiserum prepared against a pool of equal volumes of purified dodecon and hexon components.
[b] A pooled serum composed of equal volumes of antidodecon serum and antihexon serum.
[c] Titer expressed as reciprocal of end-point dilution.
[d] Values given as 0 were <5.

TABLE 2. *Serological relations among type 8 and type 9 antigens by complement-fixation tests with antisera against crude and purified antigens*[a]

Test antigen	Antiserum								
	Type 8				Type 9				Type 4 (control)[c]
	Crude	Dodecon	Hexon	Dodecon + hexon[b]	Crude	Dodecon	Hexon	Dodecon + hexon[b]	
Crude									
Adenovirus 8	1,280[d]	80	2,560	1,280	320	40	1,280	640	80
Adenovirus 9	640	20	1,280	640	1,280	80	5,120	1,280	80
Dodecon									
Adenovirus 8	40	80	0[e]	40	40	40	0	40	0
Adenovirus 9	10	10	0	5	40	80	0	40	0
Hexon									
Adenovirus 8	1,280	10	2,560	1,280	320	0	640	640	80
Adenovirus 9	40	0	80	40	640	10	2,560	1,280	80
Control[c]									
Adenovirus 5	160	0	320	160	160	0	320	160	80

[a] All tests performed by block titration.
[b] Antiserum prepared against a pool of equal volumes of purified dodecon and hexon components.
[c] Indicative of soluble, group-specific antibody levels.
[d] Titer expressed as reciprocal of end-point dilution.
[e] Values given as 0 were <5.

with Pettersson's 228-fold and 196-fold purification for the type 2 hemagglutinin (penton) and hexon, respectively. Our purification factors were, therefore, somewhat higher for types 8 and 9 (dodecon, 61 and 76; hexon, 114 and 144) than reported (16, 17) for type 2 (penton, 20; hexon, 46).

The soluble components of types 8 and 9 were

virtually identical in their elution patterns from DEAE-Sephadex, in their purification patterns, and in their morphological appearance. Such similar biophysical characteristics have also been reported for other group II viruses (2, 12). In addition, the dodecons, as well as the crude virus preparations, of types 8 and 9 have nearly identical rat, mouse, and human "O" hemagglutination properties. They differ, however, in their ability to agglutinate guinea pig and chicken erythrocytes. Both types agglutinate guinea pig red cells to high titers at 4 C, but only type 8 hemagglutinates to similar titers at 20 to 37 C. Adenovirus 8 also agglutinates chicken erythrocytes at 4 to 20 C.

The complex interaction between type specificity of the dodecons and group and type specificity of the hexons may be summarized as follows.

Dodecon component. Types 8 and 9 dodecons are clearly related. The degree of relatedness is uniformly greater with type 8 measured by type 9 antisera than with type 9 measured by type 8 antisera (HI, CF, immunodiffusion). In addition, dodecons share some type-specific determinants with hexons (CF).

Hexon component. The hexons of types 8 and 9 contain group-specific (CF) and type-specific (CF, neutralization, and immunodiffusion) determinants, and may exhibit some minor antigenic relationship (neutralization). The finding that hexons contain both group and type specificity confirms similar reports of other investigators (3, 6, 11, 15, 19, 24).

The overall analysis of our data suggests that the predominant adenovirus 8 HA determinants are entirely incorporated within the capsid of adenovirus 9, whereas adenovirus 9 possesses distinctive determinants not found in adenovirus 8. Intermediate adenovirus strains, often referred to as hybrid virus types (e.g., types 3-16 and 9-15), also may reflect closely related determinants located on the hexon or fiber components (10, 12, 23).

If only findings with the purified components are considered, the basis of the predominantly one-sided antigenic relationship between types 8 and 9 in the conventional HI tests and the largely type-specific relationship by neutralization tests may be readily explained. The results would suggest (i) that the antigens measured by HI tests with types 8 and 9 are located entirely on the dodecon (presumably the fiber) and (ii) that the type-specific antigens measured in the neutralization tests are located entirely on the hexons.

These two simple hypotheses, however, fail to take into account two major inconsistencies in the test results: (i) the pattern of HI titers of the antihexon sera with the crude antigens, and (ii) the 8- to 16-fold increase in neutralization titers obtained by combining antihexon and antidodecon sera. Inhibition of hemagglutination by antihexon sera is well recognized (3, 10, 15, 23), and has been attributed to the removal of available fiber agglutinins through aggregation of the virion (12). The inconsistency lies in the apparent one-sided cross exhibited by 8 and 9 hexon antisera which paralleled the HI results with the dodecon antigens and antisera. This cross should not occur if the nongroup hexon components were rigidly type-specific as indicated in the neutralization test. The question then is whether the hexons may possess additional (nongroup) antigenic determinants which are not expressed in the neutralization tests. The question becomes more pertinent in light of the CF test results with types 8 and 9 hexon components: all sera suggested a one-sided cross-relationship, similar in direction and magnitude to that seen by HI.

The crude antigen is a complex mixture containing unknown quantities of intact virions, dodecons, pentons, fibers, and hexons in all combinations. At present, the complex interaction of antigen-antibody combinations and the contribution of minor antigenic components in test systems that use crude antigens and antisera prepared against crude antigen cannot be fully appreciated. We do not know, for example, what role the vertex capsomeres may play either in eliciting antibody or in acting as a free component in the HI tests with crude antigen mixtures. The evidence suggesting that the hexons and dodecons (presumably the vertex capsomere portion) share antigenic determinants is only circumstantial and has been largely supported by observations that dodecons elicit neutralizing antibody as do hexons (3, 6, 23, 24). Our inability to demonstrate serological activity with hexon antisera and dodecon antigen by either HI or CF does not conflict with the concept of shared determinants and might be anticipated through steric hindrance of antibody by the structural configuration of the dodecons.

The second inconsistency in test results, the 8- to 16-fold increase in neutralization titers obtained by combining antihexon and antidodecon sera or by producing antisera against the combined dodecon-hexon components, suggests that the mechanism of neutralization is more complex than it might appear. Our data indicate that maximal neutralization titers may involve antigen-antibody reactions at more than one site—the hexon and possibly some antigenic component of the dodecon (penton). The penton site involved in neutralization may not be

the same as that measured by HI, since neutralizing antisera remain strain-specific.

Despite what would appear to be a simple arrangement of hexon and penton components, the adenovirus capsid may be a far more complicated structure. Shortridge and Biddle (19), in fact, have proposed the existence of three species of hexon capsomeres based on differences in bonding potentials. How these findings may relate to minor protein or antigenic differences, if any, remains to be seen. Until more is understood of the nature of each of the adenovirus structural species and until more is understood of the interaction of antibody in a mixture of these species, antigenic relationships based on studies with only purified components must be interpreted with caution.

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