Hemagglutination Properties of Adenovirus Types 20, 25, and 28 (34817)

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The human adenoviruses are separated into convenient groups based on the ability of viruses to agglutinate erythrocytes of various animal species. Adenoviruses in Group I agglutinate rhesus monkey erythrocytes; those in Group II agglutinate rat erythrocytes; those in Group III partially or incompletely agglutinate rat erythrocytes but exhibit complete HA patterns in the presence of heterotypic antiserum; and those in Group IV fail to hemagglutinate (1). This scheme has also been successfully applied to the simian adenoviruses (2).

Three of the newer adenovirus types, 20, 25, and 28, were placed in Group I because they agglutinated rhesus cells and could be identified in the HI test using type-specific rabbit antiserum (3, 4). However, in recent years many laboratories, in addition to our own, have been unable to demonstrate rhesus hemagglutination with these types (5–9).

We found in the course of our studies that types 20, 25, and 28 yielded higher and more consistent HA titers with rat cells than with rhesus. In addition, whereas the early HI antibody (IgM) to type 20 was detected with both rat and rhesus cells, the IgG antibody produced later in the immunization series was detected only with rat cells. In this report we present data showing that adenovirus types 20, 25, and 28 are not typical Group I viruses but are more closely related to viruses in Group II.

Materials and Methods. Virus strains. Prototype strains of adenovirus 20 (931), 25 (BP-1), and 28 (BP-5) were obtained from the Research Reference Reagents Branch (RRRB) of the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health, Bethesda, Maryland (10). Wild strains of type 25 (No. 201089 and No. 201091) and 28 (No. 90420 and No. 96012) and earlier passages of the prototype strains were obtained from Dr. Leon Rosen, Pacific Research Section, NIAID, Honolulu, Hawaii, and from our own laboratory. Virus seed stocks were prepared in KB or HEp-2 cell lines as described by Stevens *et al.* (7). Growth medium consisted of Eagle's MEM (Hanks') with 10% newborn calf serum and maintenance medium consisted of MEM with 2% serum.

Hemagglutination (HA) and hemagglutination-inhibition (HI) tests. The standardized micro HA-HI tests (11, 12) using 0.4% red blood cells (RBC) were employed. Red blood cells were collected from rhesus monkeys (*Macaca mulatta*), African green or vervet monkeys (*Cercopithecus aethiops sabaeus*), and Sprague-Dawley albino rats. Sera to be tested by HI were heat-treated at 56° for 30 min and absorbed with the same type of red cells to be used in the test.

Serum neutralization (SN) test. The SN test was performed in primary rhesus monkey kidney (MK) cell cultures as described by Stevens *et al.* (7) except that the MK titrations and test proper were read at 3–4 days.

Strain purification. The NIAID prototype strains of adenoviruses 20, 25, and 28 were purified by serial triple terminal dilution passages in HEp-2 cells using a 2-fold dilution series of virus, followed by triple plaque purifications. Plaquing procedures using HEp-2 cells in plastic dishes were those of Coates *et al.* (13). The final plaque harvest was passaged in tubes and bottles with serum-free, antibiotic-free maintenance medium.

Determination of purity. Comprehensive tests for the presence of bacterial or mycoplasmal contaminants were performed on the seed virus stocks and on all final bulk passages. Strain-specific complement-fixation (CF) tests for the known adenoassociated viruses (AAV) (14) were performed by Dr. M. David Hoggan, NIAID, NIH, Bethesda, Maryland. In addition, a diligent electron-microscope search for AAV was undertaken on viral material concentrated by high-speed centrifugation at 144,000*g* for 5 hr.

Breakthrough neutralization tests designed to detect possible contamination by another adenovirus or other viral agents were performed using essentially the same procedure as described by Hampil and Melnick (15). The culture was considered pure if all breakthrough viruses were neutralized by serum dilutions equivalent to the known homologous serum titers.

Production of antisera. Antisera for all three adenovirus types were produced in New Zealand white rabbits, Hartley strain guinea pigs, and horses of mixed breed. The immunizing antigens consisted of bulk harvests of purified viruses clarified by centrifugation at 1060*g* for 30 min. Rabbits and guinea pigs received 3 or 5 biweekly subcutaneous injections of equal parts of virus emulsified in Freund's incomplete adjuvant. Rabbits were given 2.5 ml and guinea pigs 1.5 ml of the emulsion with each injection. Horses received five intravenous inoculations of 50 ml of virus each, at biweekly intervals. All animals were test-bled periodically during the immunization schedule and were exsanguinated 9 days after the final inoculation.

Test for early antibody. HI and SN activity related to 19S macroglobulins was detected by the mercaptoethanol-reduction procedure described by Svehag and Mandel (16).

Results. Strain purification. The purified seed stocks of prototype strains of types 20, 25, and 28 were free of bacterial, fungal, and mycoplasmal contamination. All three stocks were also negative for seven strains of AAV types 1–4 by strain-specific CF tests and by electron-microscopic examination. Breakthrough neutralization tests failed to detect the presence of other viruses. No difference in behavior was detected between the original and the purified stocks of each virus type when tested by HA at 4, 24, and 37° using rat, rhesus, vervet, human "O," and guinea pig erythrocytes and by SN using reference rabbit antisera prepared for the NIH (10).

Hemagglutination properties. The hemagglutination properties of types 20, 25, and 28 are shown in Table I. HA titers of low and intermediate passages and purified lots of types 20, 25, and 28 were consistently 4-fold greater with rat cells than with monkey cells using the standard 0.01 *M* phosphate-buffered saline (PBS) diluent (12). PBS containing RBC-absorbed AV 1, 2, 4, 6, 11, or 15 equine antiserum in a 1% v/v final concentration failed to enhance agglutination. All prototype and wild strains of the viruses reacted identically. Agglutination titers were determined using RBC from a minimum of 10 animals of each species. Rat, rhesus, and vervet cell titers varied somewhat from one animal to another, with rat cells giving the most consistent results. In general, the variation in titer between lots of cells was never greater than 4-fold. AV 20 titered 1:32 to 1:128 with different rat cells and 1:2 to 1:16 with different rhesus cells. AV 25 and 28 had similar but slightly lower ranges.

HI antibody response in laboratory animals. The development of rat- and rhesus-erythrocyte-detectable HI antibody was followed during the immunization of rabbits, guinea pigs, and horses (Table II). In all of these animals, a clear progression of HI titer was detectable with rat cells. HI antibody detectable with rhesus cells was seen only in the early sera from the AV 20 equine immunization series, suggesting the formation and disappearance of early antibody or 19S macroglobulin. This was further supported when treatment of the sera with mercaptoethanol destroyed all rhesus HI antibody (Table III). The rat HI activity of the 7- and 14-day test bleedings was removed or reduced by mercaptoethanol but the titer was unaffected thereafter. The SN activity of only the 7-day serum was removed by mercaptoethanol.

The observation that AV 20, 25, and 28 elicited HI antibody detectable with rat cells but not a persistent titer detectable with rhesus cells prompted an investigation into other

TABLE I. HA Titers of Prototype Strains of AV 20, 25, and 28 with Various RBC Types.

	HA titer[a]										
	PBS and PBS-HS diluents[b]						PBS only				
	Rat	Rhesus	Vervet	Human "O"			Guinea pig		Mouse	Sheep	Chicken
Strain and passage	37°	37°	37°	4°	24°	37°	24°	37°	37°	37°	37°
AV 20 (931) original[c] ChangC$_8$HeLa$_1$KB$_2$	16	4	2	<1	<1	<1					
AV 20 (931) purified[d] ChangC$_8$HeLa$_1$KB$_2$HEp$_{15}$	64	8	4	<1	<1	<1	1	1	8	1	<1
AV 25 (BP-1) original[c] LAC$_2$HeLa$_1$KB$_4$HEK$_1$KB$_8$	16	8	8	<1	1	1					
AV 25 (BP-1) purified[d] LAC$_2$HeLa$_1$KB$_4$HEK$_1$KB$_8$HEp$_9$	32	8	8	<1	1	1	1	2	2	1	<1
AV 28 (BP-5) original[c] HEK$_2$KB$_9$	8	1	1	<1	<1	<1					
AV 28 (BP-5) purified[d] HEK$_2$KB$_9$HEp$_{10}$	32	4	4	<1	<1	<1	1	1	4	1	<1

[a] Titer expressed as reciprocal of endpoint dilution.

[b] Various PBS-1% heterotypic serum diluents (PBS containing rat RBC-absorbed AV 1, 2, 4, 6, or 15 equine antiserum or rhesus RBC-absorbed AV 11 equine antiserum) were tried, all without any enhancement effect.

[c] Produced by the Public Health Research Institute under NIH contract no. AI-04161, and certified as prototype seed virus by the NIAID (7). This passage of AV 20 prototype is RRRB catalog No. V-220-002-014, of AV 25 is V-225-002-014, and of AV 28 is V-228-002-014.

[d] The prototype strain purified by serial triple-terminal dilution and plaque-passage procedures.

TABLE II. Development of HI Antibody Response in Laboratory Animals.

		HI titer of antiserum[a]							
		Reference[b]		Rabbit		Guinea pig		Horse	
Type	Days after primary inoculation	Rat	Rhesus	Rat	Rhesus	Rat	Rhesus	Rat	Rhesus
20	0			0[c]	0	0	0	0	0
	7			10	0	10	0	20	40
	14			20	10	80	0	80	40
	21			80	10	320	10	80	20
	28			160	0	1280	0	160	0
	35			*320*	*0*	*1280*	*0*	320	0
	65	*320*	*0*					*320*	*0*
25	0			0	0	0	0	0	0
	7			10	0	0	0	10	0
	14			20	0	0	0	20	0
	21			40	0	40	0	80	0
	28			40	0	320	0	320	0
	35			80	0	640	0	640	0
	65	*320*	*0*	*640*	*0*	*1280*	*0*	*640*	*0*
28	0			0	0	0	0	0	0
	7			0	0	0	0	0	0
	14			20	0	0	0	10	0
	21			40	0	40	10	80	0
	28			80	0	160	0	80	0
	35			160	0	320	0	320	0
	65	*40*	*0*	*80*	*0*	*320*	*0*	*320*	*0*

[a] Titer expressed as reciprocal of endpoint dilution.
[b] NIH rabbit antiserum, produced and certified through the NIAID (7).
[c] 0 = <10.

TABLE III. HI and SN Titers of the Test Bleedings of the Adenovirus 20 Equine Immunization Series Before and After Mercaptoethanol Treatment.

		HI titer[a]				SN titer[a]	
		Rat		Rhesus			
Serum	Days after primary inoculation	PBS[b]	2-ME[c]	PBS	2-ME	PBS	2-ME
S1	0	0[d]	0	0	0	0	0
S2	7	20	0	40	0	40	0
S3	14	80	40	40	0	80	80
S4	21	80	80	10	0	80	80
S5	28	160	80	0	0	160	160
S6	35	320	320	0	0	320	320

[a] Titer expressed as reciprocal of endpoint dilution.
[b] Untreated control, prepared by diluting and incubating the serum with plain PBS in place of mercaptoethanol.
[c] Mercaptoethanol-treated serum, prepared as described in Materials and Methods.
[d] 0 = <5.

TABLE IV. Homologous and Heterologous HI Titers of Reference Rabbit Antisera Tested with Rat Cells.[a]

Virus type	Titers of antisera[b]									
	20	25	28	6	10	15	19	22	27	29
20	*320*	0[c]	0							
25	0	*640*	0		0	0	0			10
28	0	0	*80*	0				0	0	
6			0	*1280*						
10		0			*640*					
15		0				*320*				
19		10					*320*			
22			0					*320*		
27			10						*2560*	
29		20								*1280*

[a] This same block of titrations was entirely negative (<1:5) with rhesus cells.

[b] Titer expressed as reciprocal of endpoint dilution.

[c] 0 = <5.

adenoviruses (types 9, 13, 15) which agglutinate both rat (HA titer 1:2048) and rhesus (HA titer 1:8) cells. As with the 20, 25, and 28 group, horse and rabbit antisera to adenovirus types 9, 13, and 15 inhibited homologous rat RBC agglutination but failed to inhibit homologous rhesus cell agglutination.

Cross-reactions. Because of the observations that HI tests with 20, 25, and 28 were far more sensitive with rat cells than with rhesus cells, cross-reactions by HI or SN previously observed to involve this group were reinvestigated by HI. These reported (3, 4, 7–9, 17, 18) cross-reactions were thoroughly examined in the HI test using both rat and rhesus agglutination systems and reference rabbit antisera.

Reciprocal HI cross-testing was carried out with types 20–25, 20–28; 25–10, 25–15, 25–19, 25–28, 25–29; 28–6, 28–22, and 28–27 (Table IV). When rat erythrocytes were used for the HI test, antisera to members of the 20–25–28 subgroup exhibited homologous titers greater or equal to 1:80 and were not cross-reactive among themselves. When rhesus cells were used neither homologous nor heterologous titers could be demonstrated. Several heterotypic reactions in the rat cell HI test were observed among Group II virus types: a 1:10 cross with AV 19 and type 25 serum, a 1:20 cross with AV 29 and type 25 serum (also a reciprocal cross of 1:10), and a 1:5 to 1:10 reaction with AV 27 and type 28 serum. All of these cross-reactions were at least 32-fold lower in titer than the homologous reactions. There were no unilateral or bilateral cross-reactions between members of the 20–25–28 subgroup and types 6, 10, 15, and 22 in the rat RBC HI test. Again, no serum titers were found when the viruses were tested with rhesus cells.

Homologous and heterologous neutralizing titers also were determined for the 20–25–28 antisera listed in Table II. All of the homologous SN titers, like the homologous HI titers, are within the usual range for adenovirus antisera (1:80–1:1280). The low-level SN cross between AV 28 and type 20 serum reported by Stevens *et al.* (7) was not found in any of the four sera.

Discussion. The investigations described in this report illustrate the problems encountered in classifying adenovirus types 20, 25, and 28 as members of adenovirus hemagglutination Group I. Repeated tests on a multitude of early and later passages of the prototypes and on wild isolates showed these viruses to have a greater HA titer with rat cells than with rhesus or vervet cells, consistent with viruses of Group II. Furthermore, HI antibody to types 20, 25, and 28 were detectable in rabbit and guinea pig antisera with rat cells but not with rhesus cells. The only rhesus-detectable HI antibody which was observed—in the AV 20 horse serum—proved to be macroglobulin and was present only in the early test bleedings.

Cross-reactions by HI test with the 20–25–28 subgroup were observed with members of Group II. In other studies, Wigand (8) using rat-cell HI tests on rabbit antisera reported crosses of 1:80 with AV 27 against type 28 serum and 1:80 with AV 29 against type 25 serum, and Stevens *et al.* (7) found crosses of 1:10 with AV 19 and AV 29 against type 25 serum and 1:10 with AV 22 and AV 27 against type 28 serum. All of these reactions were at least 16-fold less than the homologous titers. The reciprocal cross between types 25 and 15 by SN test is well established (3, 17, 18). Low-level and predominantly unilateral heterologous neutralizations have been observed between types 25 and 10 (3, 17), 28 and 6 (17); and between types 20 and 26, 20 and 28, 25 and 9, 25 and 10, 28 and 17, and 28 and 19 (7). In our studies, however, the 20–28 cross previously reported (7) could not be confirmed. All of the HI and SN cross-reactions listed above, with the exception of type 6, are with Group II types, further suggesting a closer relationship of the 20–25–28 subgroup to hemagglutination Group II than to Group I.[1]

While our studies were in progress, Wigand and Fliedner (9) suggested that AV 20, 25, and 28 appeared to be atypical Group II types although devoid of rat HA activity. They also noted the relationship to many Group II viruses. Our results agree in principle with their findings except that we find this subgroup to possess definite rat HA activity which is useful in a diagnostic HI test.

By other criteria as well, types 20, 25, and 28 are more closely related to hemagglutination Group II than to Group I. For example, on the basis of plaque size and time of appearance in KB monolayers, they are placed with Group II viruses and not with Group I (19). On the basis of guanine-plus-cytosine content, calculated from buoyant density and inactivation temperature, types 20, 25, and 28 fall in the same category as the hemagglutination Group II viruses (20). Otherwise, this classification scheme closely parallels Rosen's hemagglutination groups.

The same relationship holds true when adenoviruses are classified based on the group-specific T antigen produced by the induction of tumors in hamsters or by *in vitro* transformation in rat embryo cells (21). The four categories (A, B, C, D) correspond very closely to the HA groups with the exception of adenovirus types 20, 25, and 28, whose T antigens were found to be identical to those of the HA Group II viruses.

A summation of the pertinent data strongly suggests that adenovirus types 20, 25, and 28 should not be considered as Group I viruses but as a distinct subgroup of Rosen's hemagglutination Group II. (1) These types agglutinate rat erythrocytes (1:16 to 1:128) to consistently higher titers than they do rhesus or vervet cells (1:2 to 1:8), and rat agglutination is not enhanced by the presence of heterotypic serum from Groups I, II, or III. (2) Adequate rhesus HA titers cannot be demonstrated under any conditions. (3) HI antibody to laboratory animals is measurable only with rat cells; this subgroup is, therefore, similar to the AV 9–13–15 subgroup which also agglutinates both rat and rhesus cells and which exhibits HI titers with rat cells only. (4) All cross-reactions with 20, 25, or 28 are with Group II viruses only. (5) The CPE in HEp-2 tissue culture progresses at a decidedly slower rate than that of the other Group I viruses. (6) Various biochemical and oncological classifications of the adenoviruses show that 20, 25, and 28 are more closely related to members of Group II than to those of Group I.

Summary. Adenovirus types 20, 25, and 28 are presently included in Rosen's hemagglutination Group I, although they are generally considered as atypical members. Reexamination of these three types in our laboratory showed that (1) hemagglutination titers were consistently higher with rat than with

[1] Most cross-reactions among adenoviruses are found to be within-group: 3–7–11–14–21 (all Group I); 1–2–5–6 (all Group III); 12–18–31 (Group IV); 8–9–10–15, 8–17, 8–30, 9–19, 10–19, 10–22–23, 13–15, 13–26, 13–30, 15–22–23–24–29, 19–29, 22–30 (all Group II). Exceptions are 2–12, 4–16, 4–21, and 15–21.

rhesus or vervet erythrocytes, (2) hemagglutination-inhibition titers may be readily demonstrated with rat but not with rhesus erythrocytes, and (3) antigenic relationships occur with members of Group II and not with members of other groups. On the basis of these findings we propose that adenovirus types 20, 25, and 28 be classified as members of Group II rather than atypical members of Group I.

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