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Reference Equine Antisera to 33 Human Adenovirus Types: Homologous and Heterologous Titers

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Equine antisera to human adenovirus types 1 to 33 were prepared and evaluated by hemagglutination-inhibition and serum neutralization tests. Detailed data on the potency and purity of the immunizing antigens were tabulated as one means of evaluating the antisera. Most of the 52 hemagglutination-inhibition and 25 serum neutralization major or minor heterotypic responses among the equine antisera were observed at similar levels in previous studies with rabbit antisera and appeared to represent genuine antigenic relationships among the human adenoviruses. Equine antisera to human adenoviruses 1 to 33 and a similarly packaged normal horse serum served as lots of fully tested sera for definitive typing of isolates and as reference standards for evaluating other antisera.

The adenoviruses (AV) were first described in 1953 as cytopathic agents in tissue cultures of human adenoids (49). As knowledge about them has grown, their importance in human disease has increasingly been recognized. Some AV serotypes, especially those of groups I and III and types 8 and 19 of group II (22, 45), are associated with ocular, aural, oral, respiratory, enteric, urinary, or generalized disease conditions (1, 2, 5, 6, 9, 11, 12, 16, 17, 19, 26, 27, 32, 34, 35, 37-40, 42, 51, 54, 56, 57, 60; H. L. S. Knopf and J. C. Hierholzer, Amer. J. Ophthalmol., in press). Adenovirus infections account for some of the most severe illnesses in infants and children (5, 6, 8-11, 17, 21, 38-40, 55, 56, 60, 62, 63), military trainees (15, 27, 30, 50, 61), and, occasionally, adults (4, 13, 26, 32, 35, 42, 44, 56, 57, 60; Knopf and Hierholzer, in press).

As epidemiological, genetic, and biophysical knowledge of the adenoviruses has developed, new serotypes and antigenic hybrids have been described until the present number of 33 serotypes and an unknown number of complex intermediate strains have made serotyping an ambitious task. (Types 32 and 33 were officially accepted as new types at the London meeting of Directors of World Health Organization Respiratory Virus and Enterovirus Reference Centres, 16-19 April 1973.) Yet, with the increasing association of adenoviruses with human illness, the positive identification of isolates is more important than ever. To facilitate typing of AV isolates and evaluation of independently prepared antisera, the Center for Disease Control has been producing reference antisera in horses since 1962. The first 18 of these type-specific sera were described in an earlier report (36). Antisera to all 33 currently accepted serotypes are described in the present report, and particular attention is given to heterologous reactions.

MATERIALS AND METHODS

Production of immunizing antigen (IA). Prototype strains of AV 1 to 33 (Table 1) were obtained from the American Type Culture Collection, Rockville, Md., or from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. The absence of mycoplasmas in the seed virus cultures was ensured by initially treating most strains with 20% diethyl ether for 18 h at 4 C, titrating to infectivity end points, and passaging from the highest dilution containing infectious virus. Stock viruses and IA were prepared for types 1 to 3 and 5 to 18 in KB cell cultures infected, harvested, and clarified as described by Lucas et al. (36), and for types 4 and 19 to 33 in KB or HEp-2 cultures handled as described by Hierholzer et al. (25). The working stocks and IAs were comprehensively tested for bacterial, fungal, and mycoplasmal sterility as previously described (25), for contamination with other complete viruses by the serum neutralization breakthrough test (20, 23), and for contamination with adeno-associated viruses (AAV), types 1 to 4, by type-specific complement fixation (CF) tests (31).

Evaluation of IA. Infectivity titrations were performed in HEp-2 cells and in primary human embryonic kidney (HEK) cells as previously described (25). CF tests were performed by the standardized microtiter methods with 5 units of complement and overnight fixation at 4 C (7). The group-specific CF (hexon) activity of the IA was measured by block titration against a human convalescent-phase serum (from an AV 4 infection) and against mouse immune ascitic fluid prepared with purified AV 2 hexon antigen (14).

TABLE 1. *Potency and specificity of immunizing antigens*

Type	Strain	Passage history[a]		Infectivity titer[b]		HA titers[c]			CF[d] titer
				HEp-2	MK	Rhesus	Human	Rat	
1	Adenoid-71	H_5KB_{10}	5-25-61	4.6	32	–[e]	–	512	32
2	Adenoid-6	$Ad_1HES_2H_{15}KB_{11}$	5-9-61	4.1	64	–	–	1024	32
3	G.B.	H_7KB_4	5-9-61	4.1	16	512	–	–	16
4	RI-67	$HT_4H_{19}KB_8$	2-6-67	3.5	256	–	–	256	32
5	Adenoid-75	H_4KB_{11}	5-9-61	6.1	32	–	–	512	16
6	Tonsil-99	H_5KB_7	5-15-61	3.5	64	–	–	256	16
7a	S-1058	$H_1KB_1H_1KB_3H_1KB_7$	4-8-65	4.6	64	2048	–	–	16
8	Trim	$H_{11}KB_8$	4-8-65	2.8	16	8	2048	2048	16
9	Hicks	H_3KB_9	5-31-61	3.8	16	8	4096	4096	8
10	J.J.	H_8KB_6	9-19-61	3.8	32	2	512	4096	16
11	Slobitski	H_3KB_8	9-12-61	3.5	16	2048	–	–	16
12	Huie	$HEK_xMK_xH_{12}KB_7$	2-13-62	2.0	8	–	–	2	4
13	A.A.	$H_3KB_1H_1KB_6$	1-11-62	3.2	32	16	64	1024	4
14	deWit	H_9KB_{13}	2-14-62	3.0	8	128	–	–	16
15	305[f]	$LN_5H_2KB_8$	12-2-62	2.7	4	8	–	4096	8
16	Ch.79	$ChangC_{15}H_1KB_1H_1KB_6$	3-14-62	3.7	128	2048	–	–	16
17	Ch.22	$ChangC_{15}H_1KB_1H_1KB_9$	2-20-63	3.6	16	–	–	1024	16
18	D.C.	H_4KB_{19}	1-8-63	2.8	4	–	–	2	8
19	587	$ChangC_6KB_2HEK_2HEp_4$	6-21-68	2.8	64	4	512	4096	64
20	931	$ChangC_8H_1KB_{10}$	4-14-67	3.4	64	16	–	128	64
21	1645	$ChangC_8KB_3$	12-27-62	3.3	32	256	–	–	32
22	2711	$ChangC_7KB_3HEp_3$	9-29-67	3.8	32	8	–	4096	64
23	2732	$LN_7KB_2HEK_2HEp_6$	8-2-68	2.5	8	8	–	4096	16
24	3153	$H_3LN_3ChangC_3KB_2HEp_9$	5-13-68	3.4	64	2	–	4096	64
25	BP-1	$LAC_2H_1KB_4HEK_1KB_8HEK_5HEp_1$	5-17-71	2.7	8	2	–	16	32
26	BP-2	$LAC_2H_4KB_1HEK_1KB_2HEp_5$	9-2-69	3.9	32	–	256	1024	32
27	BP-4	$HEK_2KB_4HEK_1KB_5HEp_5$	9-5-69	3.7	128	4	512	1024	64
28	BP-5	$HEK_1KB_6HEK_1KB_3HEK_5HEp_1$	5-17-71	3.0	32	2	–	8	32
29	BP-6	$KB_7HEp_1HEK_1KB_6HEK_1HEp_7$	11-3-70	3.0	8	2	–	64	64
30	BP-7	$KB_4HEK_1KB_5HEK_2HEp_2$	4-11-72	3.2	8	32	–	4096	32
31	1315	$HEK_7KB_5HEp_4$	9-16-69	2.5	4	–	–	4	64
32	H.H.	HEK_5HEp_3	8-20-71	2.5	2	–	–	2048	32
33	D.J.	HEK_5HEp_3	8-27-71	3.5	16	2	–	8192	32

[a] Abbreviations: H, HeLa cervical carcinoma cell line; KB, KB nasopharyngeal epidermoid carcinoma cell line; Ad, Adenoid culture; HES, human embryonic skin; HT, human trachea; HEK, primary human embryonic kidney; MK, primary rhesus monkey kidney; LN, human lymph node cells; Chang C, Chang conjunctiva cells; HEp, HEp-2 laryngeal epidermoid carcinoma cell line; LAC, human lung adenocarcinoma cells.

[b] Listed for HEp-2 as $\log_{10}$ mean tissue culture infective doses per 0.1 ml at 14 days, and for MK as the dilution factor of the highest dilution of virus showing 2^+ CPE in 3 or 4 days.

[c] Hemagglutination titers expressed as the dilution factor of the highest dilution of antigen showing complete hemagglutination with rhesus, human "O", and rat erythrocytes in PBS diluent. HA titers with rat erythrocytes for AV 1, 2, 4, 5, 6, 12, 18, and 31 were determined in PBS with 1% heterotypic serum diluent.

[d] Complement-fixation titer of group-specific hexon component, expressed as the dilution factor of the optimal dilution of antigen as determined by the optimal dilution of anti-AV 2 hexon mouse immune ascitic fluid tested in block titrations.

[e] –, <2.

[f] Often incorrectly listed as strain Ch.38.

Hemagglutination (HA) tests were performed by the microtiter method using 0.01 M phosphate-buffered saline (PBS) diluent, pH 7.2, and spectrophotometrically standardized 0.4% suspensions of rat, rhesus, vervet, and human "O" erythrocytes (28). HA tests with group III AV 1, 2, 4, 5, 12, 18, and 31, which show "partial" agglutination of rat erythrocytes, were performed in PBS diluent containing 1% AV 6 equine antiserum absorbed with rat erythrocytes. HA tests with AV 6 similarly were carried out in 1% AV 2 heterotypic serum diluent (22, 29, 45). All HA tests were incubated at 37 C for 1 h.

Production of antiserum. Large healthy horses with no detectable (titer <10) hemagglutination-inhibition (HI) antibody to any of the 33 human AV types were selected for immunization. For production of antisera to types 1 to 24, a schedule (no. 1) was used which required multiple intravenous (i.v.) injections

of 40-ml volumes of IA (25, 36). For production of antisera to types 25 to 33, a schedule (no. 3) was used which required a single 40-ml intramuscular (i.m.) injection of a 50:50 emulsion of IA in Freund incomplete adjuvant, followed by 20 ml of IA injected i.m. at 14 days and again at 21 days, and exsanguination 35 to 38 days after primary inoculation (25). Details of the inoculation sites for schedules no. 1 and 3 have been described (25).

Upon completion of the immunization schedule, the horses were fasted for 24 to 48 h, tranquilized by intravenous injection of 0.2 to 0.5 mg of promazine-hydrochloride per lb (ca. 373 g) of body weight, and exsanguinated from the right carotid artery by a cut-down catheterization with local anesthesia (procaine-hydrochloride). During exsanguination, 8 to 10 ml of epinephrine was administered in the jugular vein to increase the blood flow. A volume of 22 to 30 liters of blood was collected in sterile cylinders and transported on ice to the laboratory.

Packaging of antiserum. After the blood was stored for 3 h at 4 C, the clots were broken up and the serum was expressed from the macerated clots by adding lead weights for an overnight period at 4 C. The serum was collected by decanting and clarified by centrifugation at 10,000 rpm through an RC2-B Sorvall centrifuge equipped with a type KSB Szent-Gyorgi/Blum continuous-flow system with a two-channel distributor operating at an adjusted rate of 400 ml/min. The clarified product was filtered through 0.22-μm membrane filters (Millipore Corp.) and tested for sterility as described above. The final product was dispensed in 2-ml volumes, frozen at −65 C, and lyophilized for 48 h at ambient shelf temperature under a chamber vacuum of 20 to 30 μm and a condenser temperature of −40 to −45 C.

Evaluation of antiserum. Sera were tested for type-specific HI and serum neutralization (SN) antibody levels throughout the immunization schedule and before and after lyophilization (25, 36). Lyophilized equine antisera were reconstituted with sterile distilled water. All antisera were prepared as 1:10 starting dilutions in sterile PBS and absorbed with 0.1 ml of 50% rat red blood cell (RBC) suspension for HI tests with groups II and III adenoviruses and with 0.1 ml of 50% rhesus erythrocytes for HI tests with group I viruses (22, 29). The sera were not heat inactivated or treated in any other way (25). HI tests were performed by the standardized microtiter method, with 0.01 M PBS diluent or PBS modified with heterotypic serum as described above and with 4 HA units of antigen per 0.025 ml (29). Rat and rhesus erythrocyte suspensions were standardized to 0.4% (28). SN tests were performed by the 3-day test in primary rhesus monkey kidney (MK) cell cultures (25, 58). As in the HI test sera for SN testing were initially diluted 1:10 in sterile PBS and were not heat inactivated or otherwise treated. Heterotypic reactions were measured by these same procedures. Sterility checks on the final lyophilized products were performed as for the IAs.

RESULTS

Immunizing antigens for the 33 prototype adenovirus strains were prepared and thoroughly evaluated as described in Materials and Methods. The background information and potency data of the IA are summarized in Table 1. The infectivity titers in HEp-2 cells ranged from 10^2 to 10^5 and in HEK from 10^3 to 10^7 mean tissue culture infective doses per 0.1 ml at 14 days. These titers were somewhat lower than those which could be obtained from cultures harvested before cytopathology (CPE) was complete. Similarly, HA and CF titers were slightly lower than could be obtained from cultures left 3 to 5 days after complete CPE.

All IA cultures were free of contamination by common aerobic and anaerobic bacteria, yeasts, and mycoplasmas detectable in the systems used. Three IAs were shown by CF tests to be contaminated with adeno-associated viruses: AV 18 and AV 19 stocks contained AAV-1 and AV 20 had AAV-3. None of the cultures appeared to contain contaminating complete viruses which would have been detected by breakthrough neutralization tests.

For several serotypes more than one lot of serum was prepared. This was necessitated by a variety of problems, such as premature death of the animal, development of disease which made continued immunization of the horse impractical, failure of the horse to give a suitable serological response, or some accident during the handling and packaging processes. In other cases two lots of antisera (i.e., serum from two horses immunized with different IAs) were of suitable potency and specificity, but only the one with highest homologous titers or lowest heterologous titers was included in this study. For these reasons, the lot number of each serum is included at the bottom of Table 2. Two lot numbers are listed for serotypes 25, 28, and 31; for each type, these represent the same antiserum (1 horse) packaged in two samples. Both samples were fully tested and gave identical results.

The HI antibody titers of the equine antisera for each of the AV types 1 to 33 are shown in Table 2. An HI titer is defined as the dilution factor of the highest dilution of antiserum completely inhibiting hemagglutination by 4 HA units of antigen per 0.025 ml. (One HA unit is the dilution factor of the highest dilution of antigen producing complete agglutination of 0.4% rat or rhesus RBC suspensions in 1 h at 37 C.) HI titers for antiserum types 3, 7a, 11, 14, 16, and 21 were measured in PBS diluent with rhesus erythrocytes; HI titers for types 1, 2, 4, 5, 6, 12, 18, and 31 were measured in heterotypic serum diluent with rat erythrocytes; and HI titers for all remaining types were measured in PBS with rat erythrocytes. Each titer listed

TABLE 2. *Homologous and heterologous HI antibody titers of the reference equine antisera*[a]

Virus type	Antiserum type																														
	1	2	3	4	5	6	7a	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	80																																
2		160																															
3			160				40																										
4				160																													
5					160																												
6						160																											
7a			10				160				20			10																			
8								320	80	20									20														
9								80	320	20									10														
10								20	20	320									640														
11			10				10				160			10							10												
12																																	
13													320		40					20						10		10					
14							20				10			40							10												
15															320							40											
16																40																	
17								20		20					10		320					20											
18																																	
19								10		80									1280				20							10			10
20																				320													
21																					40												
22															40							320											
23																				20			2560							20			
24																								1280								640	160
25																									320								
26								10	40	20																2560	10	80					
27																										10	1280	10					
28																												80					
29																									80				2560	80			
30																														640			
31																																	
32																								320								640	320
33																								640								640	640
Lot no.	1	1	1	8	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3,4	1	1	3,4	1	4	1,2	1	1

[a] Blank spaces equal titers of <10. See text for full definition of titers.

represents the inferred mode of at least five determinations carried out with different lots of buffer, RBC suspensions, and antigen dilutions to minimize the effect of any one reagent at one time (22, 29, 45). This is particularly critical since variability in HI titers may be caused by variation of the HA titer of the test antigen under different conditions, even though the same antigen dose may always be used (29, 36, 45). The variability normally observed in HI titers throughout five determinations was no more than plus or minus one dilution from the listed titer.

Negative (titer <10) virus-serum reactions were tested at least twice. In instances where cross-reactions with rabbit antisera were reported by other investigators but no reaction was seen with the equine antisera, the tests were repeated five times with different reagents. Antisera to types 12, 18, and 31 contained no detectable homologous or heterologous HI titers when tested with either rat or rhesus erythrocytes in either PBS or heterotypic serum diluent. However, low-level HA activity in the IAs could be demonstrated with all three virus types.

Throughout the course of immunization, homologous and heterologous (including anticipated heterologous) HI titers were determined in parallel tests with unheated and heat-inactivated sera (56 C, 30 min). The titers were consistently identical. Also, homologous HI titers of antisera types 1 to 21 were determined in parallel tests with unheated sera, sera unheated but absorbed with 12.5% acid-washed kaolin (24 C, 20 min), and sera heat inactivated and absorbed with kaolin. Since serum controls, in all instances, did not reveal nonspecific inhibition, there was no need for serum treatment. Furthermore, titers in the kaolin-absorbed sera (heated or unheated) were generally one dilution lower than in the untreated sera.

The SN antibody titers of the equine antisera are shown in Table 3. An SN titer is defined as the dilution factor of the highest dilution of antiserum causing a 2+ reduction in CPE from that observed in the working dilution (virus control) in 3 or 4 days (58). Each of the homologous and heterologous titers listed is the inferred mode of at least three tests performed with different lots of MK tissue culture. Although the sensitivity of MK cells has been reported to vary considerably and thus influence the titer (36, 58), little variability was observed in repeated tests in this study. Variation was never more than plus or minus one dilution from the listed titer. All negative (titer <10) virus-serum reactions were tested twice.

SN homologous and selected heterologous titers were determined in parallel with unheated sera and with sera heat inactivated at 56 C for 30 min. No difference in SN titers was observed and in none of the 33 equine antisera were nonspecific inhibitors found.

The 33 reference adenovirus antisera prepared in horses are complemented by normal horse serum (lot 2, 9-25-70), which like the antisera was lyophilized in 2-ml volumes. This control serum was obtained from a horse raised as the immunized horses were, but with no injections given. The lot 2 normal serum has been thoroughly tested and contains no measureable HI or SN antibodies to AV 1 to 33.

Most of the cross-reactions observed by HI (Table 4) and SN (Table 5) among the 33 serotypes were anticipated on the basis of published data with rabbit antisera (3, 18, 23, 24, 33, 41, 43, 45, 46–48, 53, 58, 59, 64, 66, 67). Where cross-reactions had been reported with rabbit sera and were also observed with equine sera, they occurred at approximately the same level in relation to the homologous serum titer. Nineteen of the 52 major and minor cross-reactions found by HI with the equine sera have not been previously reported. At least 35 other cross-reactions, mostly minor ones, which have been reported at least once among rabbit sera were not found with the horse sera even after repeated testing with heated and unheated sera.

All but two of the 25 SN cross-reactions with equine sera had been found previously in rabbit antisera (Table 5). Both of those were in antiserum type 30 which has been evaluated (in rabbits) three times (47, 58, 67). Additional 65 SN cross-reactions, both major and minor, which had been reported for rabbit antisera were not found in the horse sera.

AV 32 AV 33 are recently described types, and no cross-typing information other than the original report (3) is available. Accordingly, rabbit antisera were prepared to confirm both the horse antisera data and the data of Blacklow et al. (3). The rabbit antisera were prepared by three biweekly subcutaneous injections in Freund incomplete adjuvant (24). Homologous and heterologous HI and SN titers of AV 32 and AV 33 rabbit antisera and AV 24 equine antiserum with all 33 serotypes are shown in Table 6. The data with our rabbit antisera confirm the reciprocal high-level HI cross-reactions among types 24, 32, and 33, which were reported by Blacklow et al. (3) and which we found in the equine antisera (Table 2) as well.

DISCUSSION

The equine antisera were prepared with immunizing antigens which had been thoroughly

TABLE 3. *Homologous and heterologous SN antibody titers of the reference equine antisera*[a]

Virus type	Antiserum type																														
	1	2	3	4	5	6	7a	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
1	160																																
2		320																															
3			160				10																										
4				320												20																	
5					320																												
6						320																											
7a							320				10																						
8								160	10																								
9								10	160																								
10										160									20														
11							10				160			20																			
12												160						20												20	40		
13													160																	10			
14							10				10			160																			
15															80								10						20				
16				20												160																	
17																	320																
18												20						160												10	20		
19																			320														
20																				160													
21																					160												
22																						80											
23															10								640						10				
24																								320									
25																									160								
26																										80							
27																											160						
28																												160					
29															20								20						80				
30																														80			
31																														10	640		
32																																160	
33																																	160

[a] Blank spaces equal titers of <10. See text for full definition of titers.

TABLE 4. *HI cross-reactions among the reference equine antisera*

Antiserum type	Heterologous titers with virus types	Previously observed in rabbit antisera[a]
Group I		
3	7a, 11	
7a	3	
	11, 14	43, 45, 47, 53, 58
11	7a, 14	43, 45, 47, 58
14	7a, 11	43, 45, 47, 53, 58
21	11, 14	
Group II		
8	9	24, 33, 43, 45, 47, 58, 53, 64, 67
	10, 17	33, 58, 64
	19, 26	
9	8, 10	24, 33, 43, 45, 47, 58, 53, 64, 67
	26	33
10	8, 9, 19	33, 47, 53, 58, 64
	17, 26	
15	13, 22	33, 47, 53, 58, 64, 67
	17	
19	8, 9	64
	10	33, 47, 53, 58, 64, 67
20	13, 23	
22	15	33, 47, 53, 64, 67
	17	
23	19	
24	32, 33	3
25	29	23, 58, 64, 67
26	13	47, 58
	27	
27	26	33, 64
28	13, 26	64
	27	23, 58, 64, 67
30	19, 23, 29	
32	24, 33	3
33	19	
	24, 32	3

[a] Reference number.

studied for correct total passage history, virus infectivity titrations, HA and CF antigen titrations, AAV contamination, virus purity, and nonviral sterility. Additionally, many IAs were prepared in washed monolayers under serum-free maintenance medium to minimize the elicitation of anti-albumin antibodies (25). Contamination of some IA by AAV serotypes was a problem which was never fully resolved. Many of the original seed cultures of adenoviruses in various repositories contain AAV (M. D. Hoggan, personal communication). Titers of infective AAV tend to be highest in multiple-passage HEK cultures.

The possible existence of AAV in the IAs was not recognized during the early stages of serum production. However, our routine procedures for preparing IA apparently served to minimize the titer of AAV, if it was present. Initial ether treatment of seed stocks and passage of treated virus from the end-point dilution as well as routine passage of stocks in KB or HEp-2 cell lines may have eliminated or further reduced the opportunity for high titers of AAV. Despite

TABLE 5. *SN cross-reactions among the reference equine antisera*

Antiserum type	Heterologous titers with virus types	Previously observed in rabbit antisera[a]
4	16	43, 58, 66
7a	3, 11, 14	18, 43, 48, 58, 59, 66
8	9	24, 43
9	8	18, 24, 58, 59
11	7a, 14	43, 48, 58, 59, 66
12	18	41, 43, 58
14	11	43, 58, 59, 66
15	23	58
	29	47, 58, 67
16	4	43, 48, 58, 66
18	12	41, 43, 58
19	10	58
23	15, 29	58
29	15	47, 58, 67
	23	58
30	12, 31	
	13	58, 67
	18	47
31	12, 18	41, 58

[a] Reference number.

TABLE 6. *Evaluation of AV types 24, 32, and 33 in reciprocal cross tests with all serotypes and type 32 and type 33 rabbit antisera*

Test	Virus type	Equine antisera[a]				Rabbit antisera	
		Normal horse lot 2, 9-25-70	1-23	24	25-31	32	33
HI	1-23	<10	—[b]	<10	—	<10	<10
	24	<10	<10	1280	<10	80	160
	25-31	<10	—	<10	—	<10	<10
	32	<10	<10	320	<10	80	320
	33	<10	<10	640	<10	80	1280
SN	1-23	<10	—	<10	—	<10	<10
	24	<10	<10	320	<10	<10	<10
	25-31	<10	—	<10	—	<10	<10
	32	<10	<10	<10	<10	640	<10
	33	<10	<10	<10	<10	<10	640

[a] HI and SN titers in body of table are defined as for Tables 2 and 3 (see text).

[b] —, See Table 2 for HI titers and Table 3 for SN titers.

these procedures, three IAs were found to contain high levels of AAV-1 (CF titers of 512 of AV 18 and 1024 of AV 19 against a 1:320 to 1:640 optimal dilution of AAV-1 antiserum) or AAV-3 (CF titer of 64 of AV 20 against a 1:640 optimal dilution of AAV-3 antiserum). Passage of the adenovirus through AAV antiserum was not attempted because we have not found this to be effective. Antibodies to these AAV contaminants are assumed to be present in these three antisera, but this has not been confirmed. Horse sera are often unreactive or anticomplementary in CF tests, and other AAV antibody assays are not yet available.

The homologous and heterologous HI antibody titers for adenoviruses are highly dependent on the sensitivity and accuracy of the red blood cell suspension, buffer lot, antigen dilution, HA titer of original antigen suspension, and normal variation in microtiter manipulations (22, 28, 29, 36, 43, 45). Similarly, variation in SN titers may be caused by differing sensitivities of MK tissue, virus dose, normal variation in pipetting techniques, and interpretation of serum end point (36, 43, 48, 58). With all of these sources of variation considered, the HI and SN titers listed in Tables 2 and 3 are genuine measurements of the antibody status of these sera. These titers (within one dilution either side of the mean) have been consistently found in tests with prototype strains and, although with more variation in titer, in tests with wild strains and current isolates.

Heat inactivation of equine antisera is not required for either HI or SN tests with prototype or currently isolated strains. Inactivation had no effect on homologous or heterologous titer or in "readability" of the test. This confirms our recent findings (25), but remains in contrast to Zolotarskaya and Dreizin's report of high-titered, nonspecific HI inhibitors in horse sera (68). We have also confirmed that kaolin is unnecessary for serum absorption in the HI test. Its use is also inadvisable since it may reduce antibody titers (25, 29, 65). RBC absorption is the only serum treatment required for HI. No treatment is needed for SN.

It was not unexpected to find that the equine antisera to types 12, 18, and 31, the group IIIB viruses, did not contain detectable homologous antibodies (22). Homologous HI antibodies have been measured in rabbit antiserum only with AV 12 (52). Possibly, higher titered HA antigens for these types would increase the sensitivity of the HI test. A similar argument can be made for the relatively low (i.e., 40) homologous HI titers of types 14 and 21 (group IB) antisera, but it cannot be made for the HI titer of 40 of type 16 (group IA) antiserum. Regardless of the cause of low or absent HI titers with these serotypes, such findings are the rule rather than the exception (36, 43, 53, 58).

The SN titers determined by the 3-day test in MK compared well with those determined in our laboratory by a 7-day test in HEK microcultures. This supports the original findings of Rowe et al. in their use of the 3-day test in preference to a 7-day test in HeLa cells (48). The ease of completing a neutralization test in so short a time has made the test in MK an indispensable tool in adenovirus research.

Comparing even in general terms the heterotypic responses found among the equine antisera with those found among rabbit antisera by a number of investigators is difficult. No other study has tested all 33 antisera against all 33 viruses. Stevens et al. performed complete testing with 30 types (58), Rafajko with 18 (43), and Lucas et al. with 18 (36). Other studies dealt with selected types, and often only one test procedure was used (3, 18, 23, 24, 33, 41, 45–48, 53, 59, 64, 66, 67). In addition to variations in immunizing antigens, test procedures, interpretations, etc., rabbits may respond differently than horses to adenovirus immunization. In fact, some of the low-level (i.e., 10 or 20) heterologous titers among the equine antisera could be heterotypic anamnestic responses resulting from prior natural infection with unknown cross-reacting equine adenoviruses. Hence, random, low-level or even major crosses found only once or twice are perhaps more the response of an individual animal than a genuine cross-reaction. On the other hand, major or minor heterotypic titers which are repeatedly observed by many laboratories and with different animals (Tables 4 and 5) must be assumed to represent genuine antigenic relationships.

The reference equine antisera to adenovirus types 1 to 33 were prepared with a twofold purpose: to make available a stock of fully tested, type-specific antisera for definitive typing of isolates and detailed study of antigenic hybrids, and to serve as reference standards for the evaluation of other antisera prepared for routine use. In the present report, both of these objectives have been met.

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