Production of Adenovirus Antiserums in Horses

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REFERENCE and control adenovirus immune serums have been produced mainly in rabbits (*1–3*). Our studies were undertaken to develop an economical method for the production of sizable volumes of reference serums in horses with the first 18 adenovirus strains. We have summarized the production methods and results obtained in the evaluation of these serums by serum neutralization and hemagglutination inhibition tests.

Materials and Methods

Viruses. The history of the prototype viruses used and the titer of the immunizing antigens determined by complement fixation test with human immune serums (*4*) are summarized in table 1.

Preparation of immunizing and serologic antigens. Thirty-two ounce prescription bottles containing KB tissue culture monolayers were inoculated with adenovirus strains 1 through 18 to provide sufficient volumes for immunization and serologic testing. The growth medium was mixture 199 containing 10 percent calf serum. The maintenance medium was prepared with mixture 199, 25 percent tryptose phosphate broth, and 5 percent chicken serum. Both media contained 200 units of penicillin and 100 μg. of streptomycin per milliliter. The inoculated bottles were incubated at 37° C. until a complete or nearly complete cytopathic effect was observed. This occurred, with few exceptions, within 5 days. The contents of the bottles were then alternately frozen and thawed five times, pooled, and finally centrifuged at 2,000 rpm for 20 minutes.

The supernatant fluid was divided into 45-ml. aliquots for immunizing purposes and into smaller aliquots for serologic testing. The material was stored at −65° C. The potency and specificity were determined by complement fixation testing with human serum and by serum neutralization testing in rhesus monkey kidney tissue cultures with specific reference rabbit antiserums. The serum neutralization test was essentially that of Rowe and associates (*5*), except that rhesus monkey kidney tissue cultures were used.

Immunization schedule. Pre-immunization blood samples from all horses used in the study were shown to be free of adenovirus antibodies by complement fixation testing with adenovirus type 4 antigen; by serum neutralization testing (*5*) with adenovirus types 1, 2, 4, 5, 6, 12, and 18; and by hemagglutination inhibition testing (*6*) with adenovirus types 3, 7*a*, 8–11, and 13–17. Identical immunization schedules were followed for all horses (table 1). On days 0, 7, 14, 28, and 35, serum samples were collected and the horses inoculated intravenously with 40 ml. of the immunizing antigen.

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Serum was again collected on day 45, at which time homologous titers were determined for all six serums by serum neutralization and hemagglutination inhibition tests.

Exsanguination of the animal was performed, under light surgical anesthesia by carotid artery canulation on day 49 if acceptable serologic titer was demonstrated. However, if titers were found to be less than 1:80 by hemagglutination inhibition or less than 1:320 by serum neutralization on day 45, booster inoculations of 40 ml. immunizing antigen were injected intravenously at weekly intervals, and exsanguination was performed 4 days after the test blood samples showed acceptable titers. All serums were stored in the lyophilized state at 4° C.

Serologic test. The complement fixation, hemagglutination inhibition, and serum neutralization tests were performed according to the following methods: Adenovirus type 4 antigen was used for the complement fixation tests, adenovirus types 1 through 11 and 13 through 17 for the hemagglutination inhibition tests, and adenovirus types 1 through 18 for the serum neutralization tests. Titers in all three serologic tests were expressed as reciprocals of the initial serum dilutions exhibiting the end point of the bioassay procedure.

These tests were used to check the preinoculation blood samples and all subsequent blood samples from each horse. The antigens had been tested for potency—the complement fixation antigen with known human immune serums and the other types of antigen with hyperimmune rabbit serums in the appropriate test—before using them in evaluating the horse serums.

Results

Table 1 gives the specifications of antigens that were produced and the schedule of immunization for each type of adenovirus. For all but two types, 12 and 13, the complement fixation titer of the immunizing antigen was equal to or greater than 1:8, and several attempts to produce additional lots of immunizing antigen with higher complement fixation titers were unsuccessful. Although a complement fixation antigen titer of 1:4 was considered low for use, no difficulty was encountered in producing high-titer antiserums in horses. Titration in KB tissue culture cells showed infectivity titers to be generally on the order of 3 logs. Tests of pre-exsanguination blood samples did not indicate the necessity of more than the five doses of immunizing antigen to produce the desired titers in 12 of the 18 serum preparations.

The results of serum neutralization and hemagglutination inhibition tests to determine the specificity of reactivity are given in tables 2

Table 1. Specifications of antigen pools and schedule of immunization

Antigen type	Strain	Passage history [1]	Complement fixation titer	Number doses	Day of exsanguination
1	Adenoid 71	H_5KB_{10}	1:32	5	49
2	Adenoid 6	$AD,HES_2H_{15}KB_{11}$	1:32	5	49
3	G.B	H_7KB_4	1:16	7	66
4	RI-67	$HT_4H_{17}KB_{10}$	1:16	5	49
5	Adenoid 75	H_4KB_{11}	1:16	5	49
6	Tonsil 99	H_5KB_7	1:16	5	49
7a	S-1058	$H_1KB_1H_1KB_3H_1KB_5$	1:16	5	49
8	Trim	$H_{11}KB_7$	1:8	7	63
9	Hicks	H_3KB_9	1:8	7	65
10	J.J	(H_7KB_1 and KB_2H_1 pool)KB_3	1:16	5	49
11	Slobitski	H_3KB_8	1:12	7	70
12	Huie	H or $KB_{12}KB_7$	1:4	5	49
13	A.A	$H_3KB_1H_1KB_6$	1:4	5	50
14	de Wit	$H_9(KB_{5,6}$ pool)KB_7	1:16	5	49
15	Ch. 38	H_2KB_8	1:8	7	69
16	Ch. 79	$H_1KB_1H_1KB_8$	1:16	7	102
17	Ch. 22	$H_1KB_1H_1KB_9$	1:16	5	49
18	D.C	H_4KB_{19}	1:8	5	49

[1] H—HeLa, KB—KB tissue culture, AD—adenoid, HES—human embryo skin, HT—human trachea.

and 3. By serum neutralization testing, all serums had titers ranging from 1 : 160 to 1 : 1,280 (table 2). It was deemed essential for purposes of typing adenovirus isolates that there be at least an eightfold differential between homologous and any heterologous serum neutralization or hemagglutination inhibition titers. On the basis of this criterion, serums prepared against adenovirus types 14 and 18 were unacceptable.

At the onset of this study, the serum neutralization test was planned as the bioassay method of choice for measuring the acceptability of horse antiserums. Subsequently, the hemagglutination inhibition test came into general use. For this reason as well as for its economy in per-

Table 2. Cross reactivity of adenovirus antiserums measured by serum neutralization

Adenovirus-type antigen	Adenovirus-type antiserum																	
	1	2	3	4	5	6	7a	8	9	10	11	12	13	14	15	16	17	18
1	[1] 320																	
2		640																
3			160															
4				320														
5					1, 280													
6						640												
7a							640				10			40				
8								320	10			20						
9								10	160			10						
10										160								
11							10				160			20				
12												320						40
13													320					
14														160				
15	10	10		10											160			
16				40												320		
17																	640	
18												40						160

[1] Figures represent reciprocals of serum dilutions.

Table 3. Cross reactivity of adenovirus antiserums measured by hemagglutination inhibition

Adenovirus-type antigen	Adenovirus-type antiserum																	
	1	2	3	4	5	6	7a	8	9	10	11	12	13	14	15	16	17	18
1	[1] 160																	
2		80																
3			80				80											
4				320														
5					160													
6						160												
7a							640				20			10				
8								120	80									
9								80	160	10								
10									20	320								
11							10				120							
12												----						
13									10				320		20			
14							160				40			40				
15															160			
16														0		<10		
17							20		10						20		640	
18																		----

[1] Figures represent reciprocals of serum dilutions.

formance, the hemagglutination inhibition test method also was used for evaluation. The hemagglutination inhibition titers are listed in table 3. The results were less satisfactory than those obtained by the serum neutralization test. Titers ranged from negative (adenovirus type 16) to 1:640. Types 12 and 18 are nonhemagglutinating adenoviruses.

Applying these criteria of antiserum acceptability, antiserums prepared with adenovirus types 7*a*, 8, 9, 11, 14, and 16 were considered unacceptable for identification of isolates, although adenovirus types 7*a* and 14 could be used with adequate controls since there was a fourfold difference between homologous and heterologous titers. No booster inoculations were given to horses immunized with these two viruses since a high degree of cross reactivity was evident by the hemagglutination inhibition test after the first five inoculations.

The results of antigen and antiserum titrations with six lots of rhesus erythrocyte suspensions prepared by the same technique are given in table 4. The antigen titers of adenovirus types 3, 7*a*, and 14 varied markedly with the six erythrocyte suspensions, as previously reported by Rosen (*3*). The variation in antigen titer was found to be as high as 64-fold when adenovirus type 7*a* was used. The variation in antigen titers, therefore, directly affected the homologous or heterologous titers measurable with antigen, requiring the use of rhesus erythrocytes with serum titers directly proportional to the antigen titers. In subsequent studies, 48 lots of rhesus monkey erythrocytes were collected and standard suspensions tested in titrations with the same passage of adenovirus type 3. In this experiment, 90 percent of the erythrocyte lots were found to be less sensitive than might be expected in terms of the maximum measurable titers obtained.

Discussion

The horse was found to be suitable for the production of large volumes of antiserums directed against adenoviruses.

Acceptable levels of homologous antibodies were measured by serum neutralization testing in all cases. Heterotypic serum neutralization antibody titers were encountered in 10 of the 18 antiserums produced. With two exceptions, all were at least eightfold lower than the homologous titer. Many of the heterotypic reactions were anticipated on the basis of studies published by others and may represent antigenic relationships (*6*). In only two antiserums were homologous titers found to be less than eightfold higher than the heterotypic titers. Antiserums prepared with adenovirus types 14 and 18 showed fourfold lower heterologous titers with adenovirus types 7*a* and 12, respectively. Both heterotypic titers were anticipated on the basis of published data (*6*). If such heterotypic reactions represent antigenic relationships, adequate controls must be applied in the tests in which these serums are used for identification of new isolates.

The hemagglutination inhibition test revealed that 10 of the 16 antiserums prepared with hemagglutinating viruses had acceptable homologous titers, and all heterologous titers measured were at least eightfold lower. Five antiserums showed higher levels of cross reactivity (types 7*a*, 8, 9, 11, and 14) and one (type 16) had no detectable titer.

By our methods of preparation, all anti-

Table 4. Variability of antigen and antiserum titers with different lots of erythrocytes

Erythrocyte lot (rhesus)	Adenovirus type 3		Adenovirus type 7*a*		Adenovirus type 14	
	Antigen	Antiserum	Antigen	Antiserum	Antigen	Antiserum
1	[1] 320	120	1, 280	1, 280	240	60
2	160	80	640	1, 280	160	40
3	80	40	640	1, 280	160	40
4	40	20	320	960	40	20
5	<10	10	20	240	10	<10
6	<10	10	40	320	15	<10

[1] Figures represent reciprocals of dilutions of antigen or antiserum.

serums proved to be adequate for use in the serum neutralization test although two (types 14 and 18) had undesirable heterotypic titers that might limit their usefulness. We do not know whether it is possible to eliminate these undesirable titers since similar relationships have been observed by others. Further studies with more potent immunizing antigens and fewer immunizing doses could prove to be effective in increasing the difference between homologous and heterologous titers.

Generally, these serums were less specific for use in the hemagglutination inhibition test, and wider variability in titers was noted with repeated testing. However, most antiserums are satisfactory for use with this technique, and several of the heterotypic reactions were anticipated (*6*, *7*). Further attempts to produce certain antiserums with immunizing antigens of high potency, in terms of hemagglutinin content, and a shorter course of immunization are indicated to reduce heterotypic titers if possible. Reduced specificity and variability of titers obtained in the hemagglutination inhibition test suggest a need for more precise definition of this procedure.

In limited studies with adenovirus types 3, 7*a*, and 14, we learned that serum titers are not always reproducible with the same antigen preparation but different lots of rhesus erythrocytes. Furthermore, because of this erythrocytic variability, the antigen requirement also varies from one hemagglutination inhibition test to another. Therefore, hemagglutination inhibition serum titers would be related to the antigen hemagglutinating titers that are obtained, and variations in antigen titers may occur despite the use of carefully prepared standard suspensions of rhesus cells. Alternately, it was found that serum titers could be anticipated on the basis of erythrocyte sensitivity. Highly sensitive erythrocytes, when used, give high values to the serum tested, and vice versa. For this reason, a precise definition of serum titers should be expressed in terms of the hemagglutinin titration. Similar variability was not encountered in different lots of rat erythrocytes, nor was variability encountered with types 11 and 16 antigens, with which rhesus erythrocytes are conventionally used in the test procedure.

Summary

Studies were conducted to determine the suitability of using horses for the economical large-volume production of adenovirus antiserums. Antiserums were produced with adenovirus types 1 through 18. Methods of antigen production and control and immunization schedules are described. All serums were tested for homologous and heterotypic crosses in serum neutralization and hemagglutination inhibition tests. Most serums were found to be satisfactory for use as reference or working reagents for the identification of adenovirus isolates with either the serum neutralization or hemagglutination inhibition tests or both. Generally, serums were found to be less specific in the hemagglutination inhibition test, and greater variability with repeated testing was noted.

The extent of heterologous cross reactivity among these 18 serotypes is reported, and suggestions are made that might minimize them in future production schedules. Studies with adenovirus types 3, 7*a*, and 14 disclosed that hemagglutination inhibition serum titers were not consistently reproducible with the same antigen preparation but different lots of rhesus erythrocytes. This variability in erythrocyte sensitivity causes a variation in antigen requirement for separate hemagglutination inhibition tests, which directly affects the titer obtained for the serum. For this reason it has been suggested that, with these three serotypes, a precise definition of serum titers should be expressed in terms of the hemagglutinin titration.

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PUBLIC HEALTH MONOGRAPH

Standardized Diagnostic Complement Fixation Method And Adaptation to Micro Test

A standardized complement fixation (CF) procedure, useful with all diagnostic CF antigens, has long been needed. Until such a procedure is established, CF results from one laboratory cannot be compared with those of another. Results in laboratory evaluations and standardization of reagents also can proceed much more easily and rapidly when a standardized procedure is used.

This monograph has two parts, both concerned with standardized CF procedure. In Part I a reasonably small volume CF technique, based largely on the work of Mayer, Osler, and other investigators, is discussed. This technique was in the developmental stage for several years at the Communicable Disease Center and was thoroughly evaluated by several independent laboratories. Having been developed by a task force in the Laboratory Branch of the CDC, the name "Laboratory Branch Complement Fixation" (LBCF) was chosen for the technique.

The standard diagnostic LBCF procedure is useful for bacterial, fungal, rickettsial, and viral antigens. This method uses the 50 percent hemolytic complement techniques, adheres to the theoretical principles set forth by previous workers, and serves well as a practical day-to-day working tool with all antigens tested. Certain of the pertinent experimental data accumulated during the several years of development of this procedure are presented. Detailed explanation of many features of the procedure is offered to help the reader understand the rationale behind the technique.

Adaptation of the standard LBCF procedure to a micro technique is described in Part II. The adaptation produces comparable results with minute volumes of reagents by using commercial equipment designed for micro CF techniques. Since there is an eightfold savings in reagent volumes, the micro technique for performing complement fixation tests is particularly useful for large-scale serologic testing or when reagent volumes such as experimental antigens and sera are in short supply. It should also be mentioned that an approximate fourfold savings in man-hours is possible during large-scale serologic testing with no loss of accuracy.

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Standardized Diagnostic Complement Fixation Method and Adaptation to Micro Test. I. Laboratory Branch Complement Fixation Method. *By Laboratory Branch Task Force.* II. Adaptation of LBCF method to Micro Technique. *By Helen L. Casey.* *Public Health Monograph No. 74 (PHS Publication No. 1228), 36 pages. U.S. Government Printing Office, Washington, D.C., 1965, price $0.30.*

The accompanying article summarizes the contents of Public Health Monograph No. 74. All the authors are with the Communicable Disease Center, Atlanta, Ga.

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