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Production and Evaluation of a Purified Adenovirus Group-Specific (Hexon) Antigen for Use in the Diagnostic Complement Fixation Test

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A simple procedure for the production of large volumes of purified adenovirus group-specific complement-fixing (CF) (hexon) antigen by selective adsorption to and elution from $CaHPO_4$ is described. Results of immunodiffusion tests, electrophoresis, electron microscopy, and tests for hemagglutination and infectivity indicate that the purified antigen consisted of a single virus component (hexon). The purified product contained little host materials. Unlike the crude virus harvest usually employed for serodiagnostic CF tests, the purified antigen demonstrated no anticomplementary activity and did not develop such activity during storage. The purified antigen was equal to or slightly more sensitive than crude virus harvests for serodiagnosis of adenovirus infections.

All known adenoviruses of human origin share a common complement-fixing (CF) antigen (2). This fact has highly practical significance in the diagnostic laboratory since an infection with any of the dozen or so types associated with human disease theoretically may be detected by a CF test using only a single adenovirus type. In practice, however, the sensitivity of the CF test for diagnosis of adenovirus infections, particularly among children, leaves much to be desired (7, 9).

The possible reasons for failure of the group-specific CF test to detect an antibody rise despite evidence of an acute infection by type-specific neutralization or hemagglutination-inhibition (HI) tests may be related to the host or to the antigen. Among those which relate to the host are temporal differences in the appearance of group-specific and type-specific antibody and the unknown influence of previous adenovirus infections on antibody response. The possible reasons for failure which may relate to the antigen are the quality and the heterogeneity of the reagent employed for the test. The antigen commonly used in the CF test is a crude product which includes the group-specific (hexon) antigen as well as type-specific hemagglutinins (HA). The HA antigens are also reactive by CF and therefore potentially competitive.

Host factors which may contribute to the poor serodiagnostic performance of the CF test have been the subject of much discussion (7-9, 13), but little attention has been paid to the quality of the CF antigen used to detect antibody response. This paper reports the development of a simple procedure for the production of a purified group-specific (hexon) adenovirus antigen and the evaluation of its use in the CF test.

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MATERIALS AND METHODS

Virus. Adenovirus type 2 (strain Adenoid 6) was used for all purification studies. Type 4 (strain RI-67), prepared as a CF reference reagent by Biological Reagents Section, Center for Disease Control, was used for control purposes.

Antigen production. Monolayers of HEp-2 cells were grown on Eagle's minimal essential medium (MEM) containing 10% calf serum. Before virus inoculation the cells were washed and the medium was replaced with serum-free Eagle's MEM. The adenovirus type 2 inoculum was allowed to adsorb for 6 hr at 35 C, the medium was again replaced with serum-free MEM, and incubation continued. Forty-eight hours after maximum cytopathic effect, which usually occurred after 5 days of incubation, the infected cells were harvested by freezing and thawing six times. Cellular debris was removed by low-speed centrifugation (500 × *g* for 15 min). The supernatant fluid served as the crude adenovirus antigen. Control tissue antigens (HEp-2 cells without virus) were prepared under identical conditions.

Buffers. Stock 1.0 M phosphate buffer, *p*H 7.3, was prepared by dissolving 106.26 g of $NaH_2PO_4 \times H_2O$ and 32.66 g of Na_2HPO_4 (anhydrous) per liter of distilled water. Solutions of lower molarities were pre-

pared from this stock by diluting with distilled water.

Treatment with SLS. One volume of 5% sodium lauryl sulfate (SLS) in distilled water was added to 100 volumes of crude virus suspension (11). The mixture was incubated at room temperature for 1 hr and then dialyzed against the appropriate phosphate buffer, *p*H 7.3, with frequent changes for 24 hr.

Chromatography. The brushite form of calcium phosphate ($CaHPO_4 \times 2H_2O$) was prepared as described by Taverne and associates (12). The precipitate was washed five times with distilled water and stored at 4 C as a suspension in distilled water. Just before use the precipitate was washed three times with the buffer solution that was to be used for adsorption of the antigens.

Pilot studies on the separation of adenovirus antigens by the batch method were based on the column chromatography method (10) which used stepwise increasing concentrations of phosphate buffer for elution of the antigen. Calcium phosphate, washed and resuspended in the appropriate buffer solution, was packed by centrifugation, and the supernatant was discarded. To one part of packed calcium phosphate was added 25 parts of SLS-treated tissue harvest dialyzed against the appropriate buffer. The mixture was stirred gently at room temperature for 1 hr. The calcium phosphate was packed by centrifugation and washed twice with the same concentration of buffer used for antigen adsorption. Antigens were eluted by resuspending the calcium phosphate in buffers of varying concentrations and stirring for 1 hr at room temperature. The calcium phosphate was then removed by centrifugation, and the supernatant fluid containing the antigen was decanted and dialyzed against 0.85% sodium chloride for final testing and storage.

CF test. Micro-CF tests based on 50% fixation of 5 units of complement were performed as previously described (1). Antigen titers were expressed in terms of optimal dilution, i.e., that dilution of antigen which gives the highest CF antibody titer with the standard serum. The standard serum consisted of a pool of human sera from patients infected with adenoviruses other than type 2 whose sera did not contain neutralizing or HI antibodies against type 2.

HA test. Microtiter HA tests for AV 2 fractions were performed with Sprague-Dawley rat erythrocytes and with 0.01 M phosphate-buffered saline (PBS) diluent, *p*H 7.2, containing 1% adenovirus type 6 equine antiserum (4).

Virus infectivity titrations. Infectivity titrations were performed in tubes of HEp-2 monolayers incubated at 35 C for 14 days.

Protein determinations. Protein concentrations were determined by the method of Lowry et al. (5) with absorption readings at 750 nm and bovine serum albumin standard curves.

Tests of purity. Polyacetate strip electrophoresis and immunodiffusion tests were modified from a previous description (3). Strip electrophoresis was carried out in 0.05 M barbital buffer, *p*H 8.6, at 300 v (0.6 ma) for 7 hr at room temperature. Ouchterlony double-diffusion tests were performed on 25 by 75 mm slides containing 5 ml of 0.7% agarose in 0.01 M PBS, *p*H 7.2, with 0.01% Merthiolate as preservative. Wells were 3 mm in diameter and 9 mm apart, center to center. Immunoelectrophoresis was carried out on slides containing 5 ml of 1% agarose dissolved in 0.05 M barbital buffer, *p*H 8.6. Antigens were electrophoresed at 355 v (35 ma) for 1 hr at room temperature with 0.10 M barbital buffer in the reservoirs. Antiserum was then added to the troughs. All immunodiffusion tests were read after 2 days of incubation at room temperature.

RESULTS

In preliminary chromatographic studies, infectious virus particles were detected in all fractions tested. Infectivity was found to be effectively destroyed by pretreatment of the crude virus harvest with SLS in a final concentration of 0.05% for 1 hr at room temperature. Although neither CF nor HA titers were increased by SLS treatment, the effectiveness of SLS was evident by the complete loss of infectivity and by the absence of intact virus when the treated material was viewed under electron microscopy.

In our initial attempts to purify the group-specific CF antigen, $CaHPO_4$ batch chromatography was used in which all antigens were adsorbed at a low molarity of buffer followed by selective elution of specific antigens at increasingly high molarities of buffer. Crude SLS-treated adenovirus harvests were dialyzed against 0.001 M phosphate buffer and adsorbed with $CaHPO_4$. The gel was then washed twice in the same buffer and extracted with 0.05 M increments of phosphate buffer ranging from 0.005 to 0.5 M. The HA antigen eluted first at a buffer concentration of 0.1 M. The CF antigen eluted later with the 0.3 M buffer. Despite numerous washings of the $CaHPO_4$ with 0.1 and 0.2 M buffers, the CF eluates remained contaminated with HA antigens. For this reason the method of selective elution was abandoned.

To avoid the problem of HA contamination and to further simplify the purification procedure, we attempted to selectively adsorb the hexon antigen onto $CaHPO_4$. Crude SLS-treated virus harvests dialyzed against phosphate buffers of various molarities were adsorbed with $CaHPO_4$ equilibrated in the same buffers. After adsorption for 1 hr, the supernatant fluids were assayed for the presence of CF and HA antigens (Fig. 1). All CF and HA activities were removed from the crude virus suspension by $CaHPO_4$ at molarities of 0.001 and 0.01. At molarities of 0.05 to 0.15 only the CF antigen was adsorbed; the HA titers in the supernatant were undiminished. The CF antigen was also selectively adsorbed at buffer concentrations greater than 0.15 M, although with less efficiency. The 0.1 M buffer was chosen for

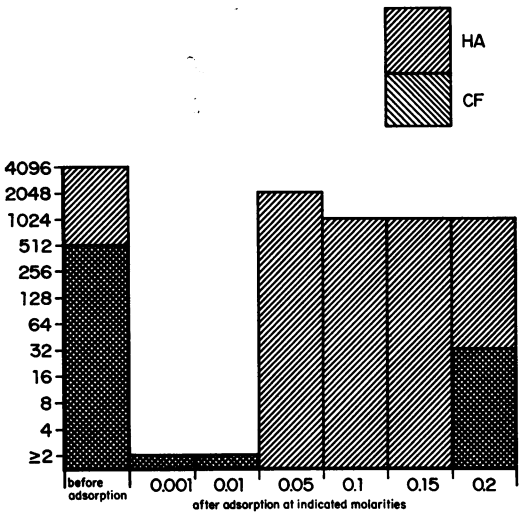

FIG. 1. *Concentrations of adenovirus group-specific complement-fixing (CF) (hexon) and hemagglutinating (HA) antigens remaining in the supernatant after selective adsorption with $CaHPO_4$ at various molarities of phosphate buffer.*

TABLE 1. *Steps in the purification of adenovirus type 2 hexon*[a]

Step in purification process	Titers[b]		Infectivity	Protein (μg/ml)	
	HA	CF	(log_{10} $TCID_{50}$/0.1 ml)	Type 2	Tissue culture
Crude harvest	1,024	128	4.4	1,710	2,470
SLS treated and dialyzed[c]	1,024	128	0.3	1,320	2,165
$CaHPO_4$ supernate (0.1 M buffer)	1,024	≤4	0.0		
$CaHPO_4$ eluate (0.5 M buffer)	≤4	256	0.0	72	78

[a] All values are averages of five runs.
[b] Titers expressed as reciprocal of the final dilution producing complete hemagglutination (HA) and reciprocal of antigen dilution giving the highest titer with reference antiserum by complement fixation (CF).
[c] Sodium lauryl sulfate.

adsorption of the hexon antigen in all of the following studies.

The final method selected for preparation of the purified hexon antigen was as follows: crude virus harvests were clarified by low-speed centrifugation to remove tissue debris, dissolved in 0.05% SLS (final concentration w/v), and dialyzed for 24 hr against several changes of 0.1 M phosphate buffer, *p*H 7.3. Twenty-five volumes of the virus harvest were mixed with 1 volume of packed $CaHPO_4$ gel and adsorbed for 1 hr at room temperature. The gel was then exhaustively washed in the 0.1 M buffer. The hexon antigen was eluted with 6 volumes of 0.5 M buffer. The final product was dialyzed against saline for 12 hr at 4 C and stored at 4 or −20 C.

The above procedure resulted in a clean separation of the HA and CF antigens. No infective virus was detected. The HA titers in the supernatant fluids were not decreased by adsorption with $CaHPO_4$, and the HA activities of the eluates averaged less than 1:8 (Table 1). The hexon antigens were quantitatively removed from the supernatant fluids by $CaHPO_4$. The CF titers of the hexon antigen eluate in all five runs were 1:256. The final product represented a 24-fold reduction in total protein.

Additional evidence of purity of the hexon antigen was obtained by electrophoretic and gel diffusion techniques. Strip electrophoresis of crude virus and hexon antigens of equal concentrations showed that the bulk of the contaminating proteins were removed by the purification process (Fig. 2). Immunoelectrophoresis showed a single arc between the hexon antigen and type 2 antiserum, and this arc matched one of the three

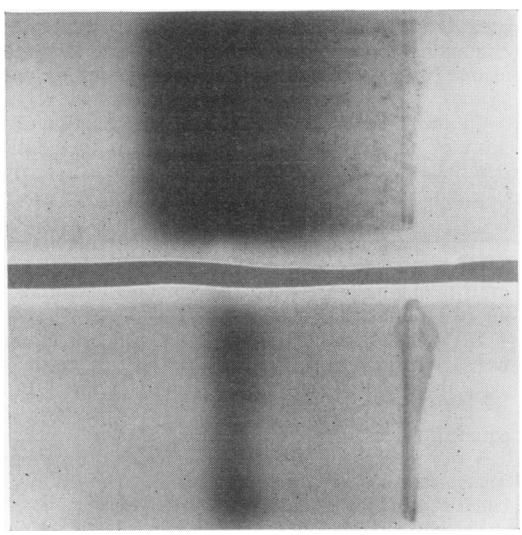

FIG. 2. *Cellulose polyacetate strip electrophoresis of crude adenovirus type 2 (top) and purified hexon component (bottom). Samples were applied at the vertical bar (right) and were electrophoresed for 7 hr at 300 v, 0.6 ma, using 0.05 M barbital buffer (pH 8.6). Migration was from cathode (right) to anode (left). Strips were stained with Ponceau S in 5% trichloroacetic acid (aqueous).*

or more arcs seen with the crude virus (Fig. 3). The diffuse zone which appeared around the crude antigen was probably nonmovable whole virus. Ouchterlony tests showed three lines of precipitate between crude virus and type 2 antiserum but only a single line of identity between crude and hexon antigens when tested with either a human convalescent serum pool (non-type 2) or equine type 2 antiserum. The Ouchterlony results support the immunoelectrophoresis patterns, and all three procedures provide evidence of the immunological purity and identification of the hexon antigen.

The sensitivity of the purified hexon antigen for serodiagnosis of adenovirus infection was slightly better than the crude adenovirus type 2 or the crude adenovirus type 4 CF antigen (Table 2). The total fourfold or greater antibody rises detected by the crude type 4, crude type 2, and purified type 2 antigens in percentages were 62, 66, and 72%, respectively. Geometric mean antibody titers on the human sera tested were essentially the same for all three antigens.

No anticomplementary activity was observed with any of the purified hexon antigen preparations. The purified antigen also appeared to be stable; the optimal antigen dilution was undiminished after 3 yr of storage at either 4 or −20 C.

DISCUSSION

A simple procedure for the production of large volumes of purified adenovirus group-specific CF antigen has been presented. By immunoelectrophoresis, radial double diffusion (Ouchterlony) tests, electrophoresis, electron microscopy, and tests for HA and infectivity, the purified group-specific CF antigen consisted of only a single (hexon) component of the virus. The purified product contained little host materials.

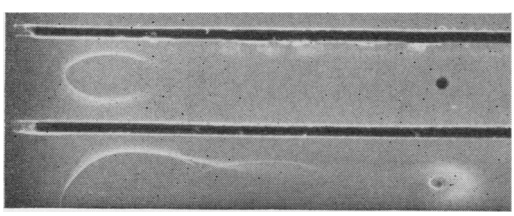

FIG. 3. *Immunoelectrophoresis of purified hexon component (top) and crude adenovirus type 2 harvest (bottom). Samples were separated though 1% agarose in 0.05 M barbital buffer (pH 8.6) at 350 v for 1 hr, with 0.1 M buffer in the reservoirs (cathode on right). Antigens were then reacted with a human convalescent type 2 antiserum for 2 days at room temperature. Purity of the hexon component is indicated by a single arc with type 2 antiserum.*

TABLE 2. *Relative sensitivity of crude type 4, crude type 2, and purified type 2 hexon antigen for serodiagnosis of adenovirus infections by complement fixation tests*

Adenovirus type isolated from illness	No. of patients	Fourfold or greater rise in antibody titer		
		Type 4	Type 2	Hexon
2	4	1	1	1
3	5	3	3	4
4	24	19	23	24
5	4	2	2	2
7	15	10	11	11
8	8	2	2	3
11	1	1	1	1
21	13	7	4	5
No isolation	12	8	10	11
Totals				
With isolations	74	45 (61%)	47 (64%)	51 (69%)
All patients	86	53 (62%)	57 (66%)	62 (72%)

Unlike the crude virus harvest usually employed for serodiagnostic CF tests, the purified antigens demonstrated no anticomplementary activity and did not develop such activity on storage.

Although the purified antigen contains only the hexon component, recent evidence suggests that this antigen may exhibit low level type-specific as well as group-specific characteristics when tested by CF (6). What effect, if any, this dual activity may have on the measurement of group-specific antibody in human sera is not known. As a precaution, any determination of optimal dilution of the purified type 2 hexon antigen should be performed by testing with human sera that are free of type 2 antibodies.

For the purified hexon antigen as well as the crude type 2 and type 4 antigens, the per cent serodiagnosis among patients from whom an adenovirus was isolated was somewhat higher than that reported by others. Rosen (8) found that serodiagnosis of infection by CF could be made in only 37% of children with adenovirus isolates. Vargosko et al. (13) found significant antibody rises among only 19% of the 225 infants and children yielding adenovirus isolates. Schmidt, Lennette, and King (9) reported that only 38% of the patients from whom an adenovirus had been isolated showed significant CF antibody increases, and most of these occurred in patients infected with type 4, the same virus used for the CF antigen. Neutralization tests revealed significant antibody increases among 52% of these same subjects. Portnoy et al. (7) also found the CF test to be less sensitive than the neutralization for serodiagnosis of infection

among infants and children. Of 45 patients with adenovirus infection documented by neutralizing antibody rises and virus isolation, only 39% were seropositive by CF. Our rate of seropositives with the purified hexon antigen was 69% among those patients from whom an adenovirus was recovered. Our apparently higher rate may reflect a higher percentage of adults in our study or differences in sensitivity of CF tests.

Although the trend of the CF results suggested that the purified type 2 hexon antigen was more sensitive for serodiagnosis, numerical increases over the rates for the crude CF antigen were not statistically significant. Further proof of increased sensitivity must await continued diagnostic experience. However, this in no way detracts from the advantages of the reagent. The purified group specific antigen is simple to prepare; it is free from contaminating tissue fluids and from anticomplementary activity. It represents a known reagent of high titer containing a single structural component, and it is stable during storage. We strongly recommend use of this reagent in the diagnostic laboratory setting.

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