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MICROPRECIPITATION TEST FOR THE DETECTION OF ADENOVIRUS ANTIBODY

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Summary

A microprecipitation test (MPT) for the detection of adenovirus antibody has been developed. The new procedure combines precipitation of virus particles with specific antibody, separation of unreacted components from the resulting electroneutral virus-antibody complexes by electrophoresis, and detection of these complexes with a protein stain. Type-specific antibody was detected in rabbit sera but, under similar conditions, antibody in convalescent human sera reacted with adenovirus antigens of types 4 and 7. Paired sera from 57 patients with suspected adenovirus infection were examined for significant rises in antibody activity by the microprecipitation test and by complement fixation.

The definitive diagnosis of diseases of virus etiology depends upon virus isolation or demonstration of the presence of antibody specific to a known virus antigen.

We recently described a procedure (1) for detection of antibody to tobacco mosaic virus which utilized microprecipitation and electrophoresis. These studies have been extended to determine the applicability of this microprecipitation test (MPT) to the assay of human antibody to selected viruses. This report describes the detection of antibody in the sera of patients subsequent to adenovirus infection.

Methods

Antigen preparation. The adenovirus antigens used in this study were prepared by a modification of the method of Green and P... (2). Roller bottle (Bellico Glass Inc., Vineland, NJ) cultures of the human epithelial cells, strain Kb (Flow Laboratories, Rockville, MD) were inoculated with 10 ml of

medium 190 with Earle's balanced salts containing approximately  $10^3$  to  $10^4$  TCD<sub>50</sub>/ml of either adenovirus type 4 or type 7. After adsorption for 2 hr, an additional 90 ml of medium were added; the culture was then rotated at 8-1/3 rev/min at 37 C. After incubation for 20 to 24 hr, and prior to cell detachment from the glass surface, the medium was decanted and replaced with 10 ml of 0.01 M tris-HCl buffer, pH 8.1. Intracellular virus was liberated from the cells by freezing and thawing three times. Medium and cellular debris were pooled and, after stirring at 4 C for 30 min, was centrifuged at 1,000 x g for 20 min. The supernatant fluid was decanted and homogenized with an equal volume of fluorocarbon (Genetron-113) (Allied Chemical Co., Morristown, NJ) in a blender for 2 min at 4 C. The homogenate was then centrifuged at 1,000 x g for 10 min to separate aqueous and organic phases. The top aqueous phase was collected and the extraction with Genetron repeated until three distinct layers were visible: a top aqueous layer and a bottom organic layer separated by a thin milky, gelatinous interface. The virus in the aqueous layer was banded onto a CsCl cushion (density = 1.43) by centrifugation at 60,000 x g for 90 min at 4 C in a SW 25.1 rotor (Beckman Instruments, Inc., Palo Alto, CA). Human serum albumin was added to a final concentration of 0.1 g/ml to 10 ml of banded virus and the mixture dialyzed overnight against 400 volumes of 0.01 M tris-HCl, 0.15 M NaCl buffer, pH 8.1. The concentrated virus preparation was dispensed in 1.0-ml volumes and stored at -70 C. Immediately before use, the virus preparation was filtered through a 1.2  $\mu$ m pore size Swinnex filter (Millipore Corp., Bedford, MA). Dilutions of each virus lot were tested against both normal and immune rabbit and human sera in the MPT. Since zones of inhibition were not detected, we chose for routine assay the greatest dilution of virus resulting in an undiminished precipitate when reacted with immune serum in effective excess. A control antigen was prepared from uninfected KB cells as described above for adenovirus antigens.

Sera. The acute and convalescent sera used were from suspected adenovirus patients and had been assayed for antibody activity in complement

fixation (CF) (3) and neutralization (4) tests. These sera together with virus isolation and serological test results, were generously supplied by Fourth U. S. Army Medical Laboratory, St. Louis, MO and Walter Reed Army Institute of Research, Washington, DC. The type-specific rabbit antisera employed in the initial experiments (Grand Island Biological Company, Grand Island, NY) were prepared by serial immunization with virus grown in primary cultures of human embryonic kidney (HEK) cells. In the initial attempts to employ human sera in the MPT, it was demonstrated that a significant number of sera contained an electroneutral component which resulted in "false" positive reactions. In attempts to eliminate these substances a variety of procedures were evaluated. These included treatment with heparin- $MnSO_4$ , kaolin and Genetron-113. Only Genetron-113 eliminated the "false" reactions without significant loss in antibody titer and was employed routinely. The following describes the procedure used. The lowest dilution of serum being tested (usually 1:10 or 1:20) was extracted for 10 or 15 sec with constant stirring in 5 volumes of Genetron-113. The mixture was centrifuged at 1,000 x g for 5 min and the top aqueous phase (serum) aspirated and passed through a 0.22  $\mu$ m pore size Swinney filter to remove particulate material. No decrease in levels of IgM, IgG, IgA or complement was detected by the Immunoplate assay (Hyland, Costa Mesa, CA) after treatment of serum in this manner.

Detection of precipitating antibody. The microprecipitation test has been described (1). Equal volumes of adenovirus antigen and serum dilutions, prepared in 0.1 M HEPES (Pierce Chemical Co., Rockford, IL) buffered normal saline, pH 7.2, were mixed in a Microtiter plate (Microbiological Associates, Bethesda, MD). The plate was placed at 37 C for 1 hr followed by overnight incubation at 4 C. After incubation, 10  $\mu$ l of each antigen-serum mixture was applied to the cathode end of a 1 x 3 inch cellulose acetate plate (Helena Laboratories, Beaumont, TX) previously soaked in tris-barbital buffer (pH 8.4, ionic strength 0.05). The plates were electrophoresed for 15 min at 220 v in a chamber (Shandon Scientific Co., Sewickly, PA) containing tris-barbital

buffer solution and were subsequently fixed and stained for 15 min in Ponceau-S stain dissolved in 7.5% trichloroacetic acid. The plates were rinsed in 2.0% acetic acid to remove excess stain, air dried and examined for elliptical precipitates at the site of sample application. Controls consisted of: (1) antigen plus saline, positive and negative control sera; (2) test serum plus saline; (3) test serum plus normal cell antigen. All controls were tested concurrently with the antigen-test sera mixtures.

#### Results

Specificity of the precipitation reaction. To determine the role of contaminating cell antigens in the MPT, the antigen prepared from uninfected KB cells was tested along with supernatant fluids from infected and uninfected cells. Adenovirus 7 (ADV-7) antigen served as a positive control.

No precipitation was observed when cell culture fluid or concentrated antigen from uninfected cells was reacted with rabbit ADV-7 antiserum. In addition, no reaction was seen when supernatant fluids from infected cells were used. The reaction of ADV-7 antigen and antiserum, however, gave a visible precipitate at serum dilution up to 1:160. It thus appeared that the antigen-antibody reaction depended on the presence of virus particles and was independent of host cell proteins.

Type specificity with rabbit antiserum. The adenovirus group possesses several antigenic structural components which can be used in appropriate tests to detect antibody to either group or type-specific antigens. We determined MPT activities for ADV-4 and -7 antigens reacting independently with 2-fold dilutions of ADV-4, -7 and -21 rabbit antisera.

The results of this experiment are shown in Table I. The activities of ADV-4 and ADV-7 antisera were 1:320 and 1:160, respectively in tests with homologous antigens and 1:10 in tests with heterologous antigens or in tests with the same antigens against heterologous or normal sera.

TABLE I

Type Specificity of Adenovirus Antigen-antibody  
Precipitin Reaction Using Rabbit Antisera

Antigen	Reciprocal Titer			NRS <sup>1</sup>
	ADV-4	ADV-7	ADV-21	
ADV-4	320	<10	<10	<10
ADV-7	<10	160	<10	<10

1. NRS - Normal rabbit serum control

In contrast, an experiment employing human sera in the MPT demonstrated that either antigen (ADV-4 or -7) could be used to detect a significant antibody rise in sera from patients recently infected with adenovirus types 4 or 7 (Table II).

TABLE II

Cross-reactivity in human sera of ADV-4 and -7  
using both antigens in the MPT

Paired Sera	Virus Isolated	24-fold Rise in MPT Titer against ADV-4	24-fold Rise in MPT Titer against ADV-7
006 009	ADV-4 ADV-7	+	
111 116	ADV-4 ADV-7		+

Comparison of the MPT and the complement fixation (CF) test. Paired acute and convalescent sera (taken 16 days apart) from 57 patients were diluted 1:10 with buffered saline and treated with Genatron-113. Serial 2-fold dilutions of each serum were tested for precipitating antibody against ADV-7 antigen. Sera (1:10 dilution) which were mixed with saline in place of antigen served as controls.

The number of virus isolations and the number of persons for whom a significant increase in antibody was demonstrated by CF tests and/or MPT are presented in Table III. A neutralization test was performed only when

TABLE III

Comparison of the MPT with the CF Test for the Detection of Adenovirus Antibodies

No. Patients	Virus Isolation	No. of paired sera showing >4-fold rise in antibody titer by:	
		CF	MPT
39	39	30	37
18	None	4	5
Total 57	39	34	42

adenovirus was isolated without a concomitant rise in CF titer. The 57 cases were divided into two groups: (1) virus isolated; (2) no virus isolated. Adenovirus was isolated from 30 patients each demonstrating a significant rise in CF titer. Sera from 29 of these patients were positive by the MPT. An additional 9 patients from whom virus was isolated had no rise in CF titer. However, 8 of the 9 patients had demonstrable rises by neutralization test and MPT; no significant rise in titer was noted in the remaining case by either test. Four patients from whom no virus was isolated showed a rise in both CF and precipitating antibody. In addition, sera from one of the remaining 14 patients who would be considered negative for recent adenovirus infection by CF results, was positive by the MPT. It can be seen that the CF test detected an antibody rise in sera of 14 patients; while the MPT showed a rise in titer in 42.

#### Discussion

In a previous report (1), we introduced an immunoelectrophoretic technique which combined virus precipitation, electrophoresis, and staining for the detection of antibody to tobacco mosaic virus. The present study illustrates the application of this immunological test for human antibody screening against a selected virus, in this case, adenovirus.

We observed that human sera contain, in contrast to most animal sera, lipoprotein substances which remain at the origin after electrophoresis.

In the procedure described, false positive reactions result if these substances are not removed from the serum prior to its interaction with viral antigen. To accomplish this, we developed a relatively simple and efficient procedure for extracting the serum with Genetron-113, a fluorocarbon, which removes the nonmigrating substances without observable effects on immunoglobulins.

The specificity of the adenovirus antigen-antibody reaction was demonstrated in initial studies in which an antigen prepared from normal KB cells failed to react with adenovirus immune serum. It was considered advisable to grow the virus to be used as test antigen and for antiserum production in different human cell lines (KB and HEK, respectively), thus diminishing the possibility of reactions resulting from host cell antibody. The requirement for concentrated antigen was also evident from this experiment; unconcentrated tissue culture supernatant from virus-infected cells did not contain sufficient antigen to elicit a positive reaction with immune serum.

Virus type specificity was observed when ADV-4 and ADV-7 antigens were reacted with homotypic and heterotypic rabbit immune sera. This finding is in agreement with other investigators (5), who demonstrated adenovirus type specificity in a microscopic agglutination test employing rabbit antisera. These workers suggested that type specificity indicated the presence of type-specific antigens intimately associated with the surface of intact adenovirus particles and that disruption of these particles increased their group reactivity in a CF test. Interestingly, we were unable to demonstrate adenovirus type specificity when human serum was employed in the MPT. The different antibody responses of rabbits and humans observed here may be a reflection of the virus susceptibility of these hosts. Since adenoviruses generally do not replicate to any appreciable extent in rabbits (6), it is possible that insufficient group antigen was available for the stimulation of antibody production. The situation is probably more complex since the infected humans may have had experience with antigens of other adenoviruses prior to the



infection investigated in the current study, whereas the rabbits which were immunized were exposed to only one adenovirus serotype.

When precipitin and CF titers of paired sera from 57 patients with suspected adenovirus infection were compared, the MPT appeared more sensitive than the routinely used CF test. Of the 39 patients from whom virus was isolated, a rise in CF titer was demonstrated in only 30, and a rise in neutralizing antibody in 8 of the remaining 9. The MPT detected a specific antibody rise in 37 of these 39 patients. We believe that these results indicate that the MPT can be used successfully as a diagnostic procedure and should be a worthwhile adjunct to standard techniques employed in adenovirus diagnosis.

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