

Adenovirus Antigens Detectable Using Passive Hemagglutination Methods.* (32501)

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The sensitivity of the passive hemagglutination (PHA) technique for detection of antibody to certain viruses is well documented. Probably the adenoviruses have been studied more extensively utilizing this procedure than have any other viral agent. To date, insufficient data are available on the nature of the viral antigen responsible for this activity. Friedman and Bennett(1) reported that tanned erythrocytes coated with adenovirus antigens would agglutinate in the presence of immune sera. They found that the PHA test was considerably more type-specific than the complement fixation (CF) test but that the various adenovirus antisera would cross-react with heterotypic adenoviruses. Ross and Ginsberg(2) reported that the antigen which was apparently coated on the tanned erythrocytes was not pelleted by forces sufficient to sediment intact virus particles, but remained in the supernatant indicating that it was one of the viral subunits as opposed to intact virus. They also showed that the PHA technique could be made type-specific by using a pool of heterotypic adenoviruses to absorb antisera. It was suggested(3) that the heterotypic enhancement phenomena associated with adenovirus hemagglutination was comparable to the indirect or PHA method, and that the latter may be due primarily to the group specific or A antigen. Using chromatography on DEAE cellulose(4,5) 3 antigens associated with the adenovirus type 5 particle were demonstrated. This antigenic activity, detectable by complement fixation and gel diffusion, has been correlated with the morphological structures identifiable under the electron microscope(6). Utilizing the technique of polyacrylamide gel electrophoresis, it has recently been shown that the adenovirus particle may actually be made up of at least 10 different polypeptide com-

ponents(7). Recently the PHA test has been used to study a number of different viruses (8).

It was the purpose of this study to determine the principal antigen or antigens which are coated on the tanned erythrocytes causing their agglutination in the presence of specific immune serum; and furthermore, to identify the antigen cross-reacting with heterotypic sera.

Materials and methods. Virus. Adenovirus type 5 was grown in HeLa cells which were harvested after 4+ CPE. The cells were lysed by multiple cycles of freezing and thawing followed by centrifugation at $800 \times g$ for 15 minutes. The preparation was further clarified at $6,600 \times g$. The supernatant was then extracted twice with Gensolv-D†(9).

Preparations of extracted virus were dialyzed against the appropriate buffer and chromatographed on DEAE cellulose(4). The viral antigens were eluted with increasing concentrations of NaCl using both gradient and stepwise procedures. Peaks with antigenic activity were pooled, concentrated by dialysis against carbowax and recycled on DEAE. In certain experiments, freon (Gensolv-D) extracted antigens were purified by centrifugation in CsCl(9) prior to chromatography.

Antisera. Antisera were prepared by injecting extracted viral preparations intravenously into rabbits. Usually 3 or 4 injections spaced one or two weeks apart were used. Animals were bled one week following the last injection. Widely separated and rechromatographed fractions representing areas of antigenic activity separated on DEAE cellulose were injected into rabbits as described above to prepare antisera against the specific antigenic components of adenovirus 5.

Test methods. Fractions eluted from DEAE cellulose columns were tested by PHA

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and CF against antisera to: (1) freon extracted adenovirus 5 (2) antigens from DEAE eluates of adenovirus 5 and (3) heterotypic adenoviruses including types 2, 3, 7 and 12. Identification of these antigens was further complemented with the use of rat erythrocytes for detection of viral hemagglutinins and toxin titrations according to methods used by others(5). The antigenic spectra of adenovirus type 5 fractions eluting from DEAE were elucidated using micro-CF(10) and micro-PHA methods. The latter procedure was similar to that reported previously(10) with the following modifications: Five one-hundredths ml of a 0.625% suspension of antigen coated sheep erythrocytes were added to 0.1 ml serum dilutions contained in microtiter dishes. The mixture was shaken and allowed to settle and after 2 or 3 hours incubation at room temperature agglutination patterns were read as described previously.

Early experiments were concerned with whether or not the antigenic activity was separable from the infectious particle. Partially purified viral preparations were centrifuged at $105,000 \times g$ for 4 hours to remove infectious virus particles. Both the pellet and the supernatant were coated on the tanned erythrocytes and tested in the presence of specific immune serum. The effect of filtration on these preparations was also tested. This was accomplished by passing the supernatant through a 0.45 and a 0.22 μ filter. In order to break up possible aggregations of antigenic particles, these preparations were sonified for 15 to 30 seconds at 6.5 amperes with a Branson sonifier model no. LS-75.

Studies were undertaken to determine the effect of trypsin on the antigens responsible for the PHA reaction. Freon-extracted viral preparations were treated with trypsin and assayed by PHA before and after chromatography. After separation on DEAE, eluates were assayed by PHA and CF.

Results. Following centrifugation of the freon-extracted virus at $105,000 \times g$ the antigenic activity detectable by the PHA test remained in the supernatant (Table I). These results were compatible with those

TABLE I. Antibody Titers* to Genesolv-D Extracted Adenovirus Type 5 Viral Antigen Treated by Various Procedures Prior to Coating on Tanned Erythrocytes.

Antigen preparation	Rabbit sera	
	2185/14	2885/14
Untreated	10240	163840
Filtered	<40	<40
Sonified	10240	163840
Sonified (filtered)	<40	<40
Supernatant	10240	163840
Supernatant (filtered)	<10	<10
Pellet	<10	<10

* Represented as a reciprocal of the dilution.

obtained by others. It can be seen from Table I that after filtration through a 0.45 and a 0.22 μ filter the antigenic activity was removed. To eliminate the possibility of the viral antigens being aggregated they were exposed to sonification. This treatment did not affect the viral titer. However, if the sonified virus was filtered, similar results were noted as with the non-sonified filtered viral preparations, *i.e.*, all of the PHA, as well as CF reactive antigens were removed. Little, if any, PHA activity could be detected in the material sedimented at $105,000 \times g$, which contained the bulk of the infectious virus particles.

Three peaks of antigenic activity could be discerned from the preparations of freon-extracted adenovirus 5 after chromatography on DEAE cellulose (Fig. 1). These 3 peaks were recognized using CF methods with a reference serum prepared against the homologous preparations. Only the first 2 peaks eluting with 0.05 to 0.075 and 0.1 to 0.2 M NaCl, respectively, could be detected by PHA. The third antigen eluting with 0.25 to 0.3 M NaCl was detectable only by CF. The second peak (0.1 M to 0.2 M) which reacted in both the CF and the PHA tests also caused the agglutination of rat erythrocytes. This agglutination was enhanced in the presence of heterotypic antisera as was agglutination of rat erythrocytes by the antigen eluting with 0.05 to 0.075 M NaCl. The second antigenic peak was also responsible for the toxin activity associated with the adenovirus 5 as evidenced by rapid removal of HeLa cells from the glass surface. Purified viral preparations centrifuged in CsCl prior to chromatography on DEAE cellulose were generally associated with

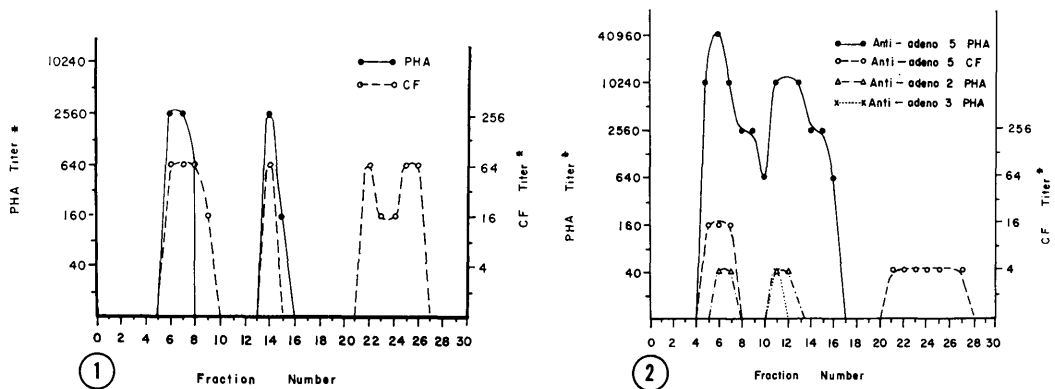


FIG. 1. Antibody levels of homotypic rabbit antisera to adenovirus type 5 antigens separated on DEAE cellulose.

* Expressed as a reciprocal of the dilution.

FIG. 2. Antibody levels of homotypic and heterotypic antisera to adenovirus type 5 antigens separated on DEAE cellulose.

* Expressed as a reciprocal of the dilution.

a single peak of PHA activity occurring at about 0.05 to 0.1 M sodium chloride. The above antigens probably represent the fiber, penton and hexon antigens in order of their elution from DEAE cellulose, and will be referred to as such.

When the viral fractions eluting from DEAE and representing the 3 antigenic peaks were pooled separately, concentrated and re-cycled on DEAE, the fiber antigen re-eluted with the same concentration of NaCl. The second antigen detectable by PHA frequently lost considerable activity. However, the CF reactivity to this antigen persisted, indicating the possibility that the penton antigen eluting from DEAE may consist of two components, one of which is unstable and detectable by PHA only for a short period. It was subsequently found that after storage of viral fractions at -20°C , the second PHA antigen invariably could not be detected emphasizing this possibility, whereas the CF detectable component eluting with the 0.1 to 0.2 M NaCl did persist. In contrast to the above, the hexon antigen was stable and could be re-eluted with 0.25 M NaCl as assayed by CF.

When freon extracted adenovirus 5 preparations were exposed to trypsin prior to chromatography, the PHA activity could not be detected after elution from the DEAE. It was subsequently found that in the presence of trypsin, the partially purified antigen preparations would not cause agglutination of

erythrocytes in the presence of homotypic sera. This inhibition of the PHA reaction did not carry over with the CF test and the presence of trypsin in partially purified viral preparations did not affect the demonstration of antigens by CF.

Antisera prepared against the fiber antigen cross-reacted by PHA with the penton antigen. These antigens have also been shown to cross react by CF as well as by gel diffusion(4). The fiber and penton antigens reacted with the antisera prepared against the fiber antigen. This was repeated on 3 occasions using different antisera inoculated with fractions from different columns. Antisera prepared against the hexon antigen reacted by CF only with the homologous antigen and not with the fiber or penton antigens.

Attempts were made to determine which of the antigens reacting by PHA with homotypic sera also cross-reacted with heterotypic sera. Column fractionated adenovirus 5 was assayed with antisera to the following adenovirus types: 2, 3, 5, 7 and 12. It was found that the fiber and/or penton antigens associated with adenovirus type 5 would cross-react with the heterotypic antisera of types 2 and 3 but not with 7 and 12 (Fig. 2). This cross-reactivity was at an extremely low level compared to the homotypic response. There appeared to be a greater cross-reactivity of adenovirus 5 antigens by PHA with adenovirus type 2 antisera than with the other sera tested. It should be pointed out that the

penton antigen, which was not detected by CF in the original fractions of this column (Fig. 2), was detected by this method after fractions 11 through 15 were concentrated 10-fold by dialysis against carbowax.

Discussion. It is apparent from these results that the antigens responsible for the PHA activity were viral antigens separable from the infectious particles. The fact that they were removed by passage through a 0.45 and a 0.22 μ filter suggests that they may be in an aggregated or clumped form. Another explanation may be that a factor in virus harvests which affects the passage and absorption of virus particles is removed by passage through millipore filters(11).

These data tend to support the idea that the antigens responsible for the activity demonstrable by PHA are the same antigens which agglutinate erythrocytes either directly or in the presence of heterotypic sera. These are the type specific fiber and toxin or penton antigens of adenovirus type 5. Corresponding activity could also be demonstrated in the same fractions using CF methods. Because of the instability of the penton antigen detectable by PHA, it is possible that the antigenic site demonstrable by this method is different from the antigenic site detectable by CF. This is evidenced by the comparative stability of its reactivity detectable by CF methods. The presence of different sites associated with the fiber antigen has already been postulated(6). It is clear, however, that the hexon or group antigen does not participate in the PHA reaction.

The inhibition of the PHA reaction in the presence of trypsin was unexpected. It is difficult to say on the basis of this interference whether or not a basic difference in mechanism exists between the direct and the indirect hemagglutination. It is more likely that trypsin interferes with attachment of the antigen to the tanned erythrocytes, particularly in the light of its inhibitory effect on the PHA without incubation to allow sufficient time for digestion of proteins.

The heterotypic responses observed by passive hemagglutination seem to be caused by both antigens detectable by this test. Since it is known that both these antigens

cross-react using various serologic assays, the fact that they cross-react by PHA is not unexpected. This reactivity may be a quantitative phenomenon or simply represent contamination of one antigen with the other through breakdown of the penton antigen, etc. In either event, cross-reactions were observed only with high titered antigenic preparations and primarily with the penton antigens. The responses obtained using adenovirus 5 viral antigens against sera prepared to heterotypic adenoviruses were extremely low. This is not unusual since adenovirus 5 is not as reactive with heterotypic sera as are certain other adenoviruses(1). Further elucidation of this point is needed.

Summary. Adenovirus type 5 viral antigens were separated on DEAE cellulose. These antigens were reacted with various homotypic and heterotypic adenovirus antisera in order to determine which of the viral antigens were reactive in the passive hemagglutination test. It was found that the fiber antigen was the principal antigen responsible for this activity. Some activity was also associated with the penton antigen. The hexon antigen could not be assayed by this method. Heterotypic responses were due primarily to the penton and fiber antigens.

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