Adenovirus Neutralizing Antibody Determination by Colorimetric Assay.* (22937)

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The newly recognized group of adenoviruses(1) isolated primarily from the respiratory tract(2,3) has been shown to be made up of at least 14 types separated on the basis of neutralizing antibody(4). To facilitate our study of this group of viruses a more simplified procedure for determining adenovirus neutralizing antibody in HeLa cell tissue culture was developed(5). In this test serum, virus antigen, and HeLa cell suspension are added to test tube at one time. If the virus antigen is neutralized by the serum, normal growth of HeLa cells occurs. If the serum does not contain sufficient antibody cytopathogenesis of the HeLa cells takes place and can be seen after 24 to 48 hours incubation. In our experience this procedure has proven somewhat more accurate, considerably easier and more rapid to perform than the customary method of inoculating the serum virus combination into previously prepared tissue cultures.

The present report describes a still more simplified method by colorimetric assay for determining adenovirus antibody. This procedure depends on the previously reported phenomenon that growth of adenovirus in HeLa cell cultures causes lowering of pH of medium below that of uninfected cultures and that specific immune serum prevents this pH effect(6). By quantitating the pH lowering effect of adenovirus growth in HeLa cells in relation to serum dilution the amount of specific antibody can be determined. The pH lowering effect occurs in 3 days and the serum neutralizing end points are determined by gross inspection of color of phenol red indicator. For further ease of performance the entire reaction has been carried out in cups of a disposable plastic panel.

Materials and methods. HeLa cells. The HeLa cell line employed was obtained from Dr. Jerome T. Syverton in 1953 and has been maintained in this laboratory. This line now grows readily in culture medium to which either human or horse serum has been added. Stock cultures of HeLa cells were grown in Eagle's basal medium(7) to which 10% human serum was added. Recently it has been found that the S3 clone HeLa cell line developed by Puck and associates(8) may be employed in the test with equally satisfactory results. *Adenovirus prototypes* 1 through 10 were obtained from Dr. R. J. Huebner. Type 4 (RI-67) was supplied by Dr. M. R. Hilleman. Stock antigens were prepared in HeLa cell cultures grown in 32 ounce prescription bottles. Type specific rabbit antiserums were prepared by inoculating intravenously rabbits with 10 ml of a given type of adenovirus followed by intraperitoneal inoculation of 10 ml of the same antigen one week later. The rabbits were then bled one month after the first injection. Paired serum samples from persons suffering acute respiratory disease and from whose throat washing an adenovirus was isolated were employed in the evaluation of the test. These were the same serum samples previously used in the comparative study of "conventional" and "improved" neutralization technics(5,9). Each serum sample was heat inactivated (56°C for 30 minutes) and twofold dilutions from 1:4 to 1:32 and then fourfold dilutions to 1:2048 were prepared. *Medium.* For all components of the test and for all dilutions Eagle's basal medium with 10% horse serum§ was employed. Hanks' balanced salt solution(10) with .035% sodium bicarbonate was substituted for Earle's salt solution called for in Eagle's formula(7).

* This investigation was supported in part by grants from Eli Lilly and Co., Common Cold Foundation, and University of Chicago Seymour Coman Fellowship Fund.

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§ Obtained from Microbiological Associates, Washington, D.C.

Phenol red‖ pH indicator was used at concentration of 0.002% in Hanks' solution. After adding the rest of the constituents of the basal medium and the horse serum, the final indicator concentration was 0.0016%. To all media 100 units of penicillin, 20 units of mycostatin and 0.1 mg of streptomycin per ml were added. Plastic panels¶ used have been described(11). They were molded from white opaque styrene sheets and contained 96 cups in 12 rows of 8 each. Each cup has maximum capacity of 2.2 ml. Mineral seal oil** was used as a seal for the cultures. It is a light oil with viscosity of 43 Saybolt seconds Universal at 100°F and with flash point of 260°F. The oil was used as received since it was always sterile when cultured on blood agar. Each of 4 separate lots have proven to be satisfactory.

Procedure. Before each test the plastic panels are rinsed in distilled water, dried in air and irradiated for about 15 minutes with ultraviolet light. 0.5 ml of mineral seal oil is first added to each cup with 2 ml Cornwall pipette. The serum, virus antigen and HeLa cells then are added consecutively through the oil. 0.1 ml of appropriately diluted serum is added to each cup with a mouth pipette. One cup per serum dilution is used.†† The adenovirus antigen (4 units, see below) in 0.2 ml amounts is next added to each cup with 1 ml Cornwall pipette. The serum-virus mixture is allowed to stand at room temperature for at least 30 minutes before HeLa cells are added. 0.5 ml of newly harvested HeLa cell suspension (trypsin method) containing approximately 100,000 cells, by hemocytometer chamber counts, is then added rapidly to each cup with a Brewer automatic pipette. After addition of all materials each cup then contains a total volume of 1.3 ml. The following controls are included in each run: (a) Cell controls: HeLa cells alone; (b) Serum controls: HeLa cells plus serum (0.1 ml of the lowest dilution of each serum tested along with 100,000 HeLa cells); (3) Virus controls: HeLa cells plus virus (0.2 ml of each virus antigen with 100,000 HeLa cells). For each antigen type employed a test against its type specific rabbit antiserum also is included. The plastic panels containing above ingredients are then incubated at 36°C and observed periodically for pH color change.

The final reading of a test is made when the medium of virus controls becomes yellow in color. This is usually after 2½ to 3 days of incubation. An occasional test had a sharper end point in 3½ days. The end point was usually still clear after 4 and 5 days incubation. During incubation the purple color of the medium of HeLa cell control (pH 7.8-7.6) changes only to red orange to orange (pH 7.5-7.3) while that of virus controls become yellow to lemon yellow (pH 7.0-6.8). The color of medium in test cups containing serum, virus antigen and cells varies from red to orange to yellow as the end point of serum dilution is reached. To give a numerical value to color changes, the pH of cups is determined by comparison with a set of phenol red standards with 0.2 pH intervals ranging from 6.8 to 7.8. In actual practice the serum neutralizing end points can be determined rapidly by direct inspection of color in the cups. In cups containing serum, virus and cells, a full orange color indicates neutralization while a full yellow color signifies no neutralization. An intermediate yellow-orange color seen at end point in a series of 2-fold dilutions of serum is considered to show neutralization if the pH in the cup containing this dilution is about 0.2 unit above pH of the virus control. The antibody titer in a given serum is expressed as the reciprocal of the highest serum dilution showing evidence of neutralization. An example of colorimetric neutralization test is shown in Fig. 1.

‖ Phenol red stock solution was prepared from 0.4% aqueous suspension, dissolved by careful neutralization (to avoid excess) with sodium hydroxide.

¶ Obtained from Linbro Chemical Co., New Haven, Conn.

**Trade name of Standard Oil Co. (Indiana) who kindly supplied the mineral oils tested. Other heavier mineral oils with higher viscosities were less satisfactory since their use slowed the lowering of pH of the virus culture system.

†† It was found that plates containing the oil and serum could stand for at least one week in icebox at 4°C before the virus antigen and HeLa cells are added without altering results of the test.

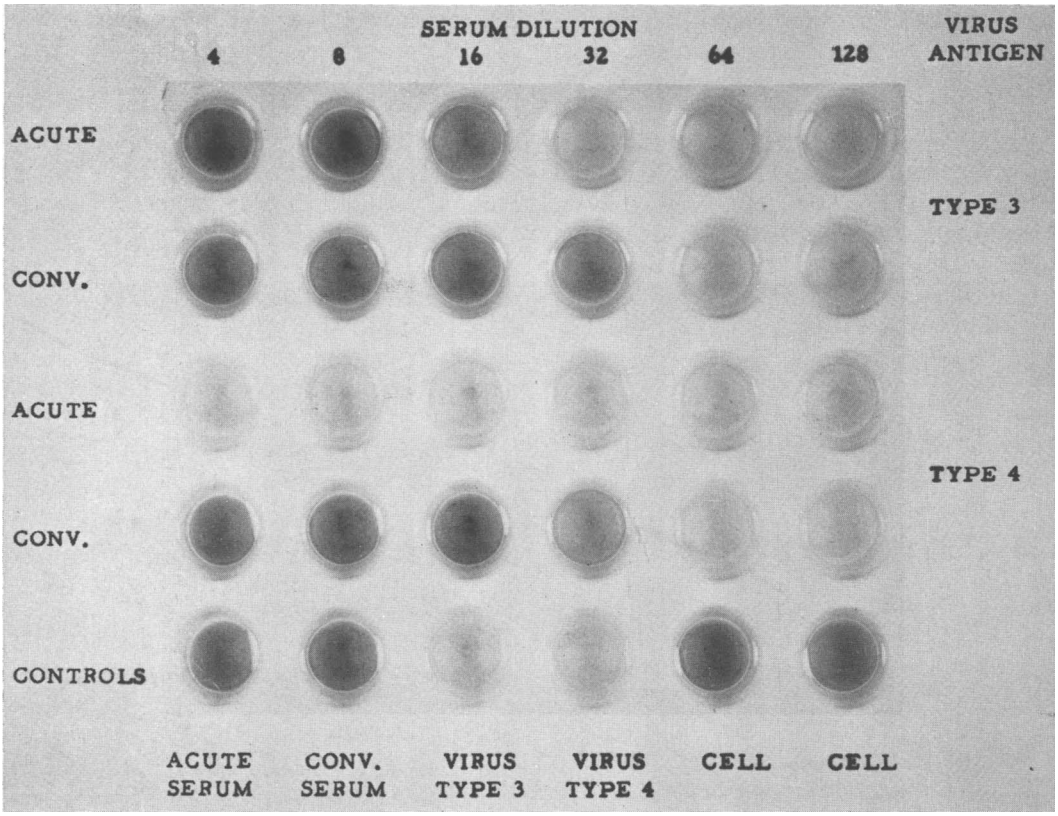

FIG. 1. Photograph of plastic panel showing final reading (3 days) of adenovirus-HeLa cell colorimetric neutralization test with phenol red indicator. (Conv. = convalescent serum.)

The paired serums were from an individual who had a febrile respiratory disease and from whom a type 4 adenovirus had been isolated from the throat washing in the acute phase of illness. The convalescent blood was taken approximately 3 weeks after onset of illness. In the test shown in Fig. 1 serum antibodies for adenovirus antigens, types 3 and 4, are determined. The photograph of the plastic panel with the various ingredients of the test was taken after 3 days of incubation. The dark cups, such as cell and serum controls, have an orange red color and a pH of approximately 7.4 while the light cups such as virus controls have a lemon yellow color and a pH of 6.8. An intermediate color at end point of the 2-fold serum dilutions can be seen particularly at the 1:32 dilution of the convalescent serum against type 4 antigen. In the test shown in Fig. 1 the neutralizing titers in acute and convalescent serum against type 3 antigen were read respectively as 16 and 32 while those for type 4 antigen were less than 4 and 32.

Adenovirus antigen titration. Twofold dilutions to 2048 of stock adenovirus types 1 through 10 are placed in plastic panel cups as described for virus controls and incubated for 3 days. The highest viral dilution showing a definite color change, compared to that of the cell control during this period of time, is considered the virus titer (one unit). A fourfold more concentrated suspension (4 units) is then employed in the neutralization test. This amount corresponds closely to that used in the previously reported simplified test tube microscopic procedure(5). In Table I are shown examples of titrations of the 10 adenoviruses employed. The end points of titrations are underscored and varied from 1:8 for type 8 to 1:256 for types 1, 2, 3 and 6. It can be noted that the end

TABLE I. Examples of Adenovirus Titrations* of Each of 10 Types in Plastic Cups Read by Inspection of Color Change of Phenol Red pH Indicator. The virus titer underscored is considered one unit.

Phenol red indicator		Adenovirus type									
pH	Color†	1	2	3	4	5	6	7	8	9	10
6.8	lemon yellow	16 32	32 64	32 64	8	16	32 64	8	2	8	16
6.9		64	128		16	32	128	16		16	32
7.0	yellow	128		128		64	256		4	32	
7.1		256	256	256	32		512	32	8		64
7.2	yel-or					128	CC		16		
7.3	orange	512	512	512 CC	64	256 CC		64	32 CC	64 CC	128 CC
7.4	red-or	CC	CC		CC			CC			

* Reciprocal of stock virus dilution. † yel-or = yellow-orange; red-or = red-orange. CC = Cell control.

points represent color change brought about by lowering of pH approximately 0.2 unit compared to pH of the corresponding cell control (CC). After 3 full days incubation the dilution of virus 4-fold more concentrated than the end point dilution produced in each case a yellow color. The development of a full yellow color never occurred in less than 2 days even with undiluted virus.

Results. Rabbit type specific antiserums had been prepared against adenovirus types 1 through 10. Each of these serums had been previously titered with the test tube microscopic neutralization technic. Since these serums were included as positive controls of each antigen in each test several repeat runs utilizing the colorimetric assay method in plastic cups are available to study repeatability and to compare with results obtained by the prior methods. Table II summarizes the results obtained with these rabbit serums. As many as 21 repeat runs have been performed with type 3 antiserum. Repeatability of end points was close with each antigen. Only one determination (with type 7) fell out of a 4-fold range. The average titer obtained with each type was that reported with the previous method(5) except in the case of type 5 which was 2 to 4 fold higher. Results with 2 different type 9 rabbit antiserum are shown in Table II because the serum of rabbit "P" had been previously reported(5),

TABLE II. Studies of Repeatability of Neutralization Titers in Type Specific Rabbit Antiserums, Types 1 through 10, with Colorimetric Method and Comparison of These Titers with Those Previously Obtained with the Test Tube Microscopic Method.*

Adenovirus antigen type	Microscopic method, avg titer	Colorimetric method: No. of tests with indicated neutralizing titer						
		32†	64	128	256	512	1024	2048
1	128		1	3	2			
2	512				1	1	2	
3	512					16	3	2
4	512					6	1	2
5	256					2	2	
6	512					2	3	
7	512			1		7	3	1
8	128			2	1			
9 "P"	64		1					
9 "R"	512					2	1	
10	128		2	1	1			

* Reference (5). † Titer expressed as reciprocal of serum dilution.

while the higher titered serum of rabbit "R" has been employed as positive control in recent tests.

Each of type 1 to 10 rabbit antiserums has been titered against types 1 to 10 virus to prove type specificity of neutralizations by the color test. The same high specificity resulted as reported with these serums in the microscopic test(5). Newly isolated types 3, 4 and 7 adenoviruses have been successfully typed with this colorimetric method by using undiluted virus and 1:30 dilution of these rabbit antiserums.

Human serum. The neutralization titers present in paired serum from 53 persons suffering acute respiratory illness and from whom adenovirus had been isolated had been determined by the tube microscopic method against adenovirus antigen types 1 through 10(5,9). Serums were available on 47 of these persons for comparative testing in the colorimetric assay method. Table III presents a comparison of results between the two methods with both acute and convalescent serum titers against all 10 antigens tabulated together. A total of 812 comparisons were made with almost half showing no titer with either test. There was in general close repetition of results. In 10 tests the titer obtained with the colorimetric method was 8-fold higher and one test 8-fold less. Once the colorimetric test showed no titer against type 6 antigen in a serum previously read as having a titer of 32. Except for these 12 tests the other 800 repeat tests had a titer within fourfold of the tube microscopic determination.

Since 4-fold serum dilutions had been employed above 1:8 in the tube microscopic technic and 4-fold dilutions were used in the colorimetric assay method above 1:32 these 4-fold variations frequently meant the repeat was within one tube dilution. A definite tendency can be seen in Table III for the colorimetric assay method to give slightly higher titer result. There were 44 repeat tests that were 2- or 4-fold lower with the colorimetric method but there were 200 repeats that were 2- or 4-fold higher.

The changes in titer that were observed did on occasion result in 4-fold rises being present between acute and convalescent serums in one test procedure but not the other. There were 5 rises observed with the tube microscopic technic alone and 15 only with the colorimetric method. None of these 4-fold rises seen in only one test were with the adenovirus type isolated from the patient.

TABLE III. Comparison of Neutralizing Titers with Adenovirus Antigens Types 1 through 10 Using 2 Technics with the Same Paired Human Serum Samples from Persons Suffering Acute Respiratory Illness and Having an Adenovirus Isolated from Their Throat.

Titer with colorimetric method	Neutralizing titer with test tube microscopic observation method*									
	0	2	4	8	16	32	64	128	256	512
0	386	29	16	6		1				
4	31	16	10	1	1	1				
8	5	10	13	29	5	4				
16			2	6	2	2	1			
32			4	31	24	41	5	3		
128						38	13	24		
512							1	11	2	10

* Reference (5,9).

Discussion. The use of a colorimetric assay method has allowed large scale determination of poliomyelitis neutralizing antibodies(12). With the numerous adenovirus types a simple technic is essential to determination of neutralizing antibody in large epidemiological studies. The colorimetric assay method in plastic panel cups has allowed many serum samples to be tested simultaneously for neutralizing antibody with 10 different adenovirus antigens. In addition to paired serum in association with infection neutralizing antibody response to adenovirus vaccine has been successfully studied with this colorimetric technic(13). The colorimetric procedure has also provided rapid accurate typing of newly isolated adenoviruses.

The color reaction employed in this test is the opposite of that used in poliomyelitis neutralization test(11,14-17) where inhibition of cellular metabolism by the poliovirus results in a higher pH than in uninfected or neutralized virus cultures. The pH lowering effect associated with adenovirus growth in HeLa cell tissue culture allows more rapid pH indicator changes than metabolic inhibi-

tion. This more rapid (3 day) determination of neutralizing effect is particularly valuable with the adenovirus group due to their tendency to readily "break-through" the neutralizing effect of antiserums.

Summary. A colorimetric assay method for measuring neutralizing antibodies against adenovirus in HeLa cell tissue culture in plastic panel cups is described. Advantage is taken of the fact that adenovirus growth in HeLa cell cultures lowers the pH of the medium below that of uninfected cultures. The reaction is standardized so that in 3 days the phenol red indicator has turned yellow by unneutralized virus while the indicator has turned only to red orange by the metabolism of the HeLa cells when the virus antigen is neutralized. It has been shown that this colorimetric test gives titers in paired serums from human adenovirus infections with antigen types 1 through 10 which are similar or slightly higher than those obtained with a test tube method depending for reading on microscopic observation of cytopathogenesis. This method has also been found suitable for typing adenoviruses.

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Received December 10, 1956. P.S.E.B.M., 1957, v94.

Dibenamine Blockade of Epinephrine and Glucagon Hyperkalemias.* (22938)

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Although the liver is the major source(2,3) of increased plasma potassium which follows intravenous injection of epinephrine(1), the mechanism by which hepatic release of potassium occurs is still unknown(4). The hyperkalemic response to epinephrine has not been correlated definitively with either vasopressor or hyperglycemic response to epinephrine (2,5,6,7). Nonetheless, some findings suggest an association between potassium and

* This investigation was supported in part by a research grant (B-501) from Natl. Inst. of Neurological Diseases and Blindness, of N.I.H., Public Health Service.

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