Turbidimetric Method for the Assay of Antiviral Antibodies

W. B. DANDLIKER, V. A. DE SAUSSURE, AND T. E. GROW

Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037

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A rapid, simple assay method has been developed for antiviral antibodies. The technique has been applied to antisera, immune γ -globulins, and immunospecifically purified antibody for two strains of influenza virus, Asian 305 and PR8, and to antisera to tobacco mosaic virus. Turbidity changes due to the specific interaction of a virus with its antibody were measured by the increase in optical density in a sensitive wavelength region, e.g., 436 nm. Successful application of the method required that nonspecific effects which give rise to turbidity changes be eliminated. This was accomplished by proper choice of ionic strength (0.3 M) and pH (5.5), and by the addition of normal serum or serum albumin to the virus before contact with the antibody. Sensitivity of the method allowed quantitation of antibody down to the level of 10 μ g of antibody protein per ml. The specificity of the reaction causing the turbidity change was established by experiments which showed that precipitation of virus-antibody complexes removed the reactive component in the serum, and by the absence of turbidity changes for nonspecific pairs (virus plus unrelated antisera).

Antibodies against viruses may be quantitatively characterized by several classical methods (2). These include the neutralization of viral activity by antibody, the inhibition of the agglutination produced by hemagglutinating viruses, complement fixation, and the quantitative precipitin test. Precipitates in the quantitative test are detectable in submicrogram quantities by fluorometric determination (8). Immuno-osmophoresis (9) probably also could be employed for semi-quantitative determinations. Although most of the methods enumerated above are highly sensitive, they are somewhat time-consuming and may require labile accessory reagents such as erythrocytes, complement components, or tissue cultures.

A light-scattering study (1) of the interaction of antibody with tobacco mosaic virus (TMV) and with tomato bushy stunt virus has been reported, but the results were not interpreted in terms of antibody assay procedures. In addition, the conditions of reaction were not suitable for the study of influenza viruses.

This paper describes a rapid quantitative method for measuring anti-influenza antibody, using only virus, antibody, and normal serum as the reagents. The method is based upon the change in optical density (OD) due to light scattering which arises from the aggregation of the virus particles by the specific reaction with antibody. Conditions have been found to eliminate or

largely suppress nonspecific reactions between the virus and serum proteins.

MATERIALS AND METHODS

Viruses. Two influenza virus strains were used: (i) formaldehyde-treated Asian 305, prepared by B. A. Rubin, Wyeth Laboratories (Philadelphia, Pa.), and obtained through collaboration with Cordis Laboratories (Miami, Fla.); and (ii) live PR8, obtained from Eli Lilly and Co. (Indianapolis, Ind.) through the courtesy of Richard Lerner. Both viruses were propagated in the allantoic sacs of chick embryos, and concentrated and purified by high-speed centrifugation. Tests for the absence of allantoic protein in the Asian 305 preparation are shown in Fig. 10. The PR8 virus was purified by zonal centrifugation.

The influenza virus suspensions as received were centrifuged at $68,000 \times g$ for 14 hr, and the resulting pellets were resuspended in the desired buffer. The Asian 305 virus contained about 1.6 mg of virus/ml, as determined by Kjeldahl nitrogen analysis, assuming that the virus is 10% N. The hemagglutination titer of this virus was 1:1,280. The PR8 virus contained about 1.5 mg/ml, as determined by OD₂₆₀ and OD₂₈₀, assuming the extinction coefficient to be the same for both viruses.

TMV, obtained from H. Fraenkel-Conrat, University of California at Berkeley, was used as a suspension in water (7.2 mg/ml).

Antisera. Antisera to Asian 305 influenza virus were prepared by Cordis Laboratories by immunizing rabbits with a series of three once-weekly intradermal injections of the virus in complete Freund's adjuvant.

[This portion of the project was supported under a contract between the Office of the Surgeon General and Cordis Laboratories, Miami, Fla. (contract no. DA-49-193-MD-2829), under the supervision of Seymour P. Halbert.] Each dose was divided into injections at five widely separated intradermal sites. Bleedings were taken 1 and 2 weeks after the third injection. Booster doses were begun 2 weeks after the last immunization, and continued at monthly intervals. After each booster dose, sample bleedings were obtained 1 and 2 weeks after the injection. Antibody titers were followed by immuno-osmophoresis (8) and by inhibition of hemagglutination (6). The latter titer was usually of the order of 1:640. The titers of normal serum varied between 1:16 and 1:80. Antisera to TMV were similarly prepared.

Antisera to PR8 virus were obtained from R. Lerner and were prepared by immunizing rabbits with a series of eight once-weekly injections; two injections were intravenous (iv), two in Freund's adjuvant in the footpad, and then four iv. Bleedings were taken 1 and 2 weeks after the last injection.

Immunospecifically purified antibody. Antibody was obtained from the anti-Asian 305 antiserum by immunospecific purification with sodium thiocyanate (NaSCN) by the method of Dandliker et al. (4). A 5-ml portion of anti-Asian 305 antiserum was added to 10 ml of purified virus suspended in diluent buffer, pH 6.8, slowly with mixing. The precipitate, which formed rapidly, was incubated for 30 min at 37 C, centrifuged twice for 15 min at $16,000 \times g$, washed with 0.15 M NaCl, and suspended in 10 ml of 3 M NaSCN for 1 hr at 0 C. Virus was removed by centrifugation at $68,000 \times g$ for 4 hr. The supernatant fluid was dialyzed against two changes of diluent and filtered through a 0.45 μ m membrane filter (Millipore Corp., Bedford, Mass.). The purified antibody contained about 1.0 mg of protein/ml, based on OD measurements at 280 nm.

Normal γ -globulin fractions. The normal γ -globulin was made from rabbit serum (Pel-Freez Biologicals, Inc., Rogers, Ark.) by two successive precipitations with 0.75 volumes of $(\text{NH}_4)_2\text{SO}_4$ adjusted to pH 8.5. The second precipitate was dissolved in 0.25 serum volumes of 0.15 M NaCl and chromatographed on diethylaminoethyl (DEAE)-cellulose by a column modification of the method of Campbell et al. (3).

Allantoic membrane and fluid. Allantoic membranes were removed from 11-day incubated eggs, suspended in 0.15 M NaCl, washed in several changes of 0.15 M NaCl, and homogenized in saline with a tissue homogenizer. The suspension was centrifuged at $1,400 \times g$, and the supernatant fluid was centrifuged at $68,000 \times g$. The precipitate was resuspended in 0.15 M NaCl at a concentration of 1.3 mg/ml. Allantoic fluid was obtained from Eli Lilly and Company.

Buffer solutions. Buffer solutions of the compositions shown in Table 1 were prepared to test the effect of varying ionic strength and pH. Buffer 3 is the buffer described in the text and in figure legends as "0.055 M PO_4 buffer, pH 5.5." "Diluent" buffer refers to a buffer containing 0.15 M NaCl, 0.01 M K_2HPO_4 , and 0.005 M KH_2PO_4 , pH 6.8.

TABLE 1. *Composition and pH of buffer solutions*

Buffer	NaCl ^a	Na ₂ HPO ₄ ^a	NaH ₂ PO ₄ ^a	pH
1		0.005	0.125	5.5
2	0.15	0.005	0.065	5.5
3	0.30	0.005	0.050	5.5
4	0.30	0.005	0.017	6.0
5	0.30	0.005	0.002	7.0

^a Concentrations in moles per liter.

Turbidity measurements. Turbidity changes effected by the virus-antibody reaction were followed by OD measurements at 436 nm, by means of a Zeiss PMQ II spectrophotometer. The wavelength used is not critical, but was chosen to afford simultaneously high scattering and negligible absorption.

Tests on unstabilized virus to measure nonspecific antibody reactions. From 10 to 100 μ liters of the serum or antibody fraction to be tested was made up to 2 ml of the desired buffer, and the solution was mixed with a footed Teflon rod. An initial reading of OD was made at 436 nm. Influenza virus (generally 50 μ liters, equivalent to about 80 μ g) was rapidly added and stirred. The OD₄₃₆ was measured at short intervals beginning 5 to 10 sec after mixing; readings continued for at least 250 sec. Virus blanks were obtained by reading OD₄₃₆ for solutions containing all components except the antiserum; serum blanks were obtained by reading OD₄₃₆ for solutions containing all components except the virus. The sum of the virus blank and the serum blank was subtracted from the OD₄₃₆ of the reaction mixture to give ΔOD_{436} .

Tests on prestabilized virus to measure antibody reaction. From 50 to 100 μ liters of virus (~ 80 to 160 μ g) was added to 2 ml of buffer containing 0.3 M NaCl and 0.2 ml of normal serum. The solution was mixed with a footed Teflon rod. When no further changes in OD₄₃₆ were noted, 10 to 100 μ liters of the serum or antibody fraction to be assayed was added. OD₄₃₆ readings were recorded at specified intervals after mixing. Virus and serum blanks were subtracted as described above.

RESULTS

Turbidity changes (ΔOD_{436}) which occurred upon mixing Asian 305 virus with immune serum or with immunospecifically purified antibody, together with controls with normal serum, are shown in Fig. 1 and 2. Figure 3 presents similar data for PR8 virus. The amount of virus used ($\sim 75 \mu$ g) was chosen to produce convenient ranges of ΔOD_{436} . Larger amounts of virus resulted in greater values of ΔOD_{436} . Conditions shown in these figures represent the optimal ones found for assaying anti-influenza virus antisera, and were established after consideration of the various factors illustrated in Fig. 4-10 and in Table 2.

Test conditions were selected to minimize the

nonspecific reaction between virus and normal serum or normal γ -globulin, which could also cause turbidity changes (Fig. 4-6). For the Asian 305 virus system, the nonspecific reaction was minimal at pH 5.5 and 0.3 M NaCl, a small negative ΔOD being observed with *n*-serum (Fig. 4) or serum albumin, and no ΔOD with normal γ -globulin (Fig. 5). For the PR8 virus, these con-

FIG. 1. Turbidity changes resulting from the immune reaction of Asian 305 virus with anti-Asian 305 antiserum. Conditions: 100 μ liters of Asian 305 virus ($\sim 160 \mu$ g) was stabilized by suspending it in 2 ml of 0.055 M PO_4 buffer (pH 5.5) containing 0.3 M NaCl and 0.2 ml of normal serum. (These conditions were found to be optimal for minimizing the nonspecific reaction.) Antiserum added: ∇ , 10 μ liters; $\square$, 25 μ liters; $\circ$, 50 μ liters; Δ , 100 μ liters; Control: $\blacktriangle$, 100 μ liters of normal serum added.

FIG. 2. Turbidity changes resulting from the immune reaction of Asian 305 virus with immunospecifically purified anti-Asian 305 antibody. Conditions: 100 μ liters of Asian 305 virus ($\sim 160 \mu$ g) was stabilized by suspending it in 2 ml of 0.055 M PO_4 buffer, pH 5.5, containing 0.3 M NaCl and 0.2 ml of normal serum. Immunospecifically purified antibody added: ∇ , 10 μ liters; $\square$, 25 μ liters; $\circ$, 50 μ liters; Δ , 100 μ liters. (This antibody preparation contained 1.0 mg of protein/ml.) Control: $\blacktriangle$, 100 μ liters of normal γ -globulin (2 mg) added to the stabilized virus.

FIG. 3. Turbidity changes resulting from the immune reaction of PR8 virus with anti-PR8 antiserum. Conditions: 50 μ liters of PR8 virus ($\sim 75 \mu$ g) stabilized by suspending in 2 ml of 0.055 M PO_4 buffer, pH 5.5, containing 0.3 M NaCl and 0.2 ml of normal serum. Antiserum added: $\square$, 25 μ liters; $\circ$, 50 μ liters; Δ , 100 μ liters. Control: $\blacktriangle$, 100 μ liters of normal serum added to stabilized virus.

ditions reduced but did not eliminate the nonspecific reaction with normal γ -globulin.

These nonspecific effects for both virus systems were virtually eliminated, without suppression of the immune reaction, by prestabilizing the virus with normal serum or serum albumin until no further turbidity change occurred (Fig. 6); 0.2 ml of normal serum was found sufficient to stabilize 80 to 160 μ g of virus.

Conditions for the suppression of nonspecific reactions may not be the same for all viral systems. As an alternative to seeking ideal conditions for each new system, we have found that the changes due to the immune reaction could be calculated by obtaining a ΔOD curve for a normal control, and subtracting these values from the total ΔOD .

Figures 7 and 8 show for the Asian 305 and PR8 virus systems, respectively, that the γ -globulin fraction of antiserum contained the substance responsible for turbidity changes when added to virus. The material remaining after chromatographic separation of the γ -globulin fraction was also tested, and showed the same lack of turbidity change as did the normal controls shown in these two figures.

The immunologically active substance could be depleted from antiserum by precipitation with the virus (Fig. 9). The active substance could be recovered from the precipitate by dissociation with thiocyanate to yield immunospecifically purified antibody (see Fig. 2).

Tests for the specificity of the reaction are shown in Fig. 10, 11, and Table 2. Virtually no turbidity changes occurred with a virus and an unrelated antiserum; a small amount of cross-

TABLE 2. *Test for the specificity of the virus-antibody reaction^a*

Component 1	Component 2 (antiserum)	OD ₄₃₆ × 1,000 ^c				
		Before mixing components	Time after mixing (sec)			
			10 ^b	60	120	240
Asian 305 virus (50 μliters, ~80 μg)	Anti-Asian 305 (50 μliters)	35	122	150	165	195
	Anti-PR8 (100 μliters)	35	72	70	70	71
	Anti-allantoic fluid (50 μliters)	32	100	98	96	96
	Anti-A ₂ -J170 (50 μliters)	38	150	145	147	150
Supernatant fluid after centrifugation of Asian 305 virus at 68,000 × g (100 μliters)	Anti-Asian 305 (100 μliters)	88	198	198	199	200
PR8 virus (50 μliters, ~75 μg)	Anti-PR8 (50 μliters)	180	215	225	235	250
	Anti-Asian 305 (50 μliters)	160	211	211	213	214
	Anti-A ₂ -J170 (50 μliters)	171	284	289	290	291
	Anti-allantoic membrane (100 μliters)	210	350	344	340	340
Allantoic fluid (100 μliters, 1.4 mg)	Anti-Asian 305 (100 μliters)	108	219	214	212	211
	Anti-PR8 (100 μliters)	110	150	148	145	145

^a Component 2 was added to a suspension of component 1 in 2 ml of 0.055 M PO₄ buffer (pH 5.5) containing 0.3 M NaCl and 0.2 ml of normal serum as stabilizer.

^b This initial increase represents the OD₄₃₆ contribution of component 2 itself, in systems showing no further Δ OD with time.

^c Increasing OD with time indicates reaction.

FIG. 4. Effect of salt on the turbidity changes resulting from the specific or nonspecific reactions of immune or normal serum with Asian 305 virus. Conditions: 50 μ liters of Asian 305 virus ($\sim$ 80 μ g), not prestabilized with normal serum, was added to 50 μ liters of anti-Asian 305 antiserum (open symbols) or normal serum (filled symbols) in 2 ml of 0.055 M PO₄ buffer, pH 5.5, containing NaCl in the following concentrations: $\circ$, none; $\square$, 0.15 M; $\triangle$, 0.3 M. (In the final choice of test conditions, the negative Δ OD readings which occurred upon contact of the virus with normal serum at 0.3 M NaCl were eliminated by prestabilizing the Asian 305 virus with normal serum. See legends of Fig. 1-3.)

FIG. 5. Effect of salt and pH on the nonspecific interaction between Asian 305 virus and normal γ -globulin. Conditions: 50 μ liters ($\sim$ 80 μ g) of Asian 305 virus (not prestabilized with normal serum) was added to 80 μ liters of normal γ -globulin ($\sim$ 0.7 mg) in 2 ml of PO₄ buffer, pH 5.5 (open symbols) or pH 7 (filled symbols). See Materials and Methods section for buffer compositions. NaCl concentrations in buffers: $\circ$, none; $\square$, 0.15 M; $\triangle$, 0.3 M. Based on these results and those in Fig. 4, buffers at pH 5.5 containing 0.3 M NaCl were chosen for final test conditions to minimize the non-specific reaction.

FIG. 6. Stabilization of PR8 virus by addition of normal serum. Conditions: ∇ , 50 μ liters of PR8 virus ($\sim 75 \mu$ g) was added to 2 ml of 0.055 M PO_4 buffer (pH 5.5) containing 0.3 M NaCl. The solid line indicates actual OD_{436} readings, and the broken line indicates ΔOD_{436} readings obtained by subtracting from the actual value the contribution which the added component would have in the absence of virus. Successive additions were made as follows: O, 100 μ liters of normal serum; $\bullet$, additional 100 μ liters of normal serum; Δ , 100 μ liters of immune serum; $\square$, 100 μ liters of serum albumin; $\blacktriangle$, additional 100 μ liters of immune serum. After initial nonspecific reaction with normal serum (O), immune serum gave further increases in OD (Δ , $\blacktriangle$), whereas normal serum ($\bullet$) or albumin ($\square$) did not.

FIG. 7. Experiments showing that the material responsible for the turbidity changes is contained in the γ -globulin fraction of the antiserum. Conditions: 50 μ liters of Asian 305 virus ($\sim 80 \mu$ g) was stabilized by suspending in 2 ml of 0.055 M PO_4 buffer (pH 5.5) containing 0.3 M NaCl and 0.2 ml of normal serum. γ -Globulin added: O and $\bullet$ (duplicates), 100 μ liters of γ -globulin (0.7 mg) obtained by DEAE-cellulose chromatography of anti-Asian 305 antiserum; Δ , control consisting of 100 μ liters (2 mg) of normal γ -globulin obtained similarly.

FIG. 8. Turbidity changes resulting from the reaction between PR8 virus and normal or immune γ -globulin. Conditions: 50 μ liters of PR8 virus ($\sim 75 \mu$ g) was stabilized by suspending in 2 ml of 0.055 M PO_4 buffer (pH 5.5) containing 0.3 M NaCl and 0.2 ml of normal serum. γ -Globulin added: Δ , 100 μ liters (0.7 mg) of γ -globulin fraction from DEAE-cellulose chromatography of anti-PR8 antiserum; O, 300 μ liters (2.1 mg) of same; $\square$, control consisting of 100 μ liters (2 mg) of normal γ -globulin obtained similarly.

FIG. 9. Depletion of specific activity in immune serum by precipitation of antibody with virus. Conditions: 50 μ liters of Asian 305 virus ($\sim 80 \mu$ g) reacted with: O, 60 μ liters of original immune serum (equivalent to 100 μ liters of either supernatant below); Δ , 100 μ liters of supernatant from reaction of 1 ml of Asian 305 virus (1.6 g) with 1.5 ml of immune serum; ∇ , 100 μ liters of supernatant from reaction of 1 ml of virus with first supernatant (2.5 ml concentrated to 1.5 ml).

reaction was noted between PR8 virus and anti- $\text{A}_2/\text{J170}$ antiserum, in agreement with the findings of Neurath, Rubin, and Fontes by fluorometric analysis of precipitates (8).

Figure 11 also shows the applicability of the present method to TMV. Substantial turbidity changes occurred with this virus and its antiserum. The plant virus differed from the influenza virus in that it did not show any nonspecific interaction with serum components.

FIG. 10. Absence of turbidity change between homogenized normal allantoic membrane and anti-Asian 305 antiserum. (See Materials and Methods section for preparation of homogenate.) Anti-Asian 305 antiserum (50 μ liters) was added to: ○, 15 μ liters of homogenized allantoic membrane suspension (~ 80 μ g); □, 50 μ liters of Asian 305 virus (~ 80 μ g); Δ, 15 μ liters of allantoic membrane suspension and 50 μ liters of Asian 305 virus. All suspensions were in 2 ml of 0.055 M PO_4 buffer (pH 5.5) containing 0.3 M NaCl and 0.2 ml of normal serum.

The allantoic membrane suspension neither induced a turbidity change with the antiserum nor inhibited that caused by the virus plus antiserum.

FIG. 11. Immune reaction between TMV and its antiserum; lack of reaction between TMV and antiserum to Asian 305 virus. Conditions: 50 μ liters of TMV was added to 2 ml of 0.055 M PO_4 buffer (pH 5.5) containing 0.3 M NaCl. Antiserum added: ○, 50 μ liters of anti-Asian 305; Δ, 50 μ liters of anti-TMV; □, 100 μ liters of anti-TMV. TMV did not require prestabilization with normal serum; it did not show any nonspecific reaction with serum components.

DISCUSSION

The general utility of the turbidity method for assaying antiviral antibody is demonstrated in Fig. 1 and 3 for whole antisera, in Fig. 2 for immunospecifically purified antibody, and in Fig. 6 and 7 for immune γ -globulins. The nonspecific reaction between the virus and the serum

components other than antibody is probably electrostatic in nature, since it is profoundly affected either by changes in ionic strength or in pH (Fig. 4 and 5). By prestabilizing the virus with normal serum at pH 5.5 and with 0.3 M NaCl before contacting it with the antiserum, the nonspecific reaction was virtually suppressed, whereas the immune reaction was still substantial. Higher salt concentrations (about 1 M) were undesirable because they partially suppressed the immune reaction as well.

Assaying under conditions where the nonspecific reaction is suppressed obviates the necessity for running normal controls. For a system being studied at other than optimal conditions, essentially similar information on the specific reaction can be obtained by measuring, and subtracting from the total ΔOD , the ΔOD due to the nonspecific reaction.

The specific nature of the reaction of virus with antibody, as followed by turbidity changes, was confirmed by Fig. 10 and Table 2 which show the absence of nonspecific reactions, by Fig. 7 and 8 which show that the active component was contained in the γ -globulin fraction, and by Fig. 9 which shows that the active component was removed from antiserum by precipitation with the corresponding virus.

The sensitivity range for the turbidity method can be estimated from Fig. 1 and 2. If the assumption is made that the immunospecifically purified antibody assayed in Fig. 2 was all antibody, then 10 μ g of antibody per ml would produce a ΔOD_{436} of about 0.01 at 250 sec. This is approximately equivalent to 5 μ liters of immune serum per ml of reaction mixture; the hemagglutination titer obtained at Cordis Laboratories for this serum was 1:640.

The method of antibody assay presented here depends upon the increase in light scattering occurring when virus particles become aggregated by interaction with antibody. Viral systems are uniquely suited to such a method because of the large size of the virus particle, the large difference in size between the virus and the antibody molecule, and the fact that the aggregation proceeds both at a rate and to a degree convenient for experimental measurement. The quantitation of antigen-antibody reactions by light scattering is usually complicated by the presence of three factors: the unknown distribution of aggregates of different sizes, the interference effects present in the scattering from large particles (1, 5, 7), and the rapid settling of precipitates. However, the scattering from a reacting system of a large virus and antiviral antibody should possess some particularly simple features. The primary reaction be-

tween one or more antibody molecules and a virus particle should not produce any substantial change in scattering because of the large size of the virus. However, the next stage of the reaction in which two virus particles combine through an antibody molecule results in approximately a twofold increase in scattering. An important conclusion to be drawn from these considerations is that the initial rate of increase in scattering (0 to ~30 sec) should contain information about the rate of dimer formation (two virus particles and one antibody molecule), and merits further study with fast reaction measurement.

The principal value of this method is that it provides an extremely simple and rapid quantitative measure of antiviral antibody for systems where relatively large quantities of materials are available, a situation likely to become more common now that advanced techniques of zonal centrifugation of viruses are available (C. B. Reimer, Federation Proc., p. 365, 1968). The method should be useful in assays of antisera and purified antibodies and in studies of the effects of specific ions or other environmental variables on virus aggregation and on the immune reaction between virus and antibody (*unpublished data*).

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