Solid-Phase Radioimmunoassay of Total and Influenza-Specific Immunoglobulin G

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An antigen-antibody system of polystyrene tubes coated with immunoglobulin antibody was used for quantitating immunoglobulins. A similar radioimmunoassay method was adapted for a viral antigen-antibody system. The viral system can be used for quantitating viruses and for measuring virus-specific antibodies by reacting with 125I-labeled anti-immunoglobulin G (IgG). Optimal conditions for coating the solid phase, specificity of the immune reaction, and other kinetics and sensitivities of the assay method were investigated. Comparison of direct and indirect methods of assaying for immunoglobulins or viral antibody indicates that the indirect method is more sensitive and can quantitate a minimum of 0.037 μg of IgG per ml. Results of solid-phase radioimmunoassay for influenza antibody correlate well with hemagglutinin antibody titers but not with complement-fixing antibody titers. Radioimmunoassay results for influenza antibody by solid phase are likewise in agreement with results by the carrier precipitate radioimmunoassay method. The simplicity, reproducibility, and versatility of the solid-phase procedure make it diagnostically useful.

Solid-phase radioimmunoassay is a rapid and reproducible method for quantitating either total immunoglobulin G (IgG) concentrations or influenza-specific antibody. Its usefulness as a diagnostic tool is attributed to the versatility and sensitivity of the solid-phase procedure.

Berson and Yalow reviewed the general principles of radioimmunoassay (5), and many others have made modifications to meet specific requirements. The critical step common to all of the modifications is separation of the unbound isotopically labeled reactants, whether antigen or antibody, from the bound, labeled reagent. This is accomplished either by filtering (12) antigen-antibody complexes or by using an insoluble phase for one of the reagents. The insoluble phase may be in the form of sedimenting particles such as bromoacetyl cellulose (19, 21, 23), polystyrene (7, 17), or bentonite (8). The polymerized or cross-linked antibody itself can also be used as the insoluble solid phase by treating it with ethyl chloroform (2) or glutaraldehyde (3). A simplified procedure is to allow reactants to bind in situ with other reagents linked by a charge phenomenon (17) to the inner wall of polystyrene test tubes (1, 22). The versatility of the solid-phase procedure has allowed workers to quantitate various peptides and hormones (5) or immunoglobulins themselves as antigens (1, 7, 8, 17, 19, 22, 23). The solid-phase method was effectively used in our study for measuring both serum immunoglobulins and virus-specific antibody.

By methods other than solid-phase radioimmunoassay, Daniels et al. used virus-antibody complexes to quantitate the IgG antibody (9). Conversely, our previous studies used an IgG antibody to measure a specifically bound virus antibody by radioimmunoassay (10). In this procedure, unbound 125I-labeled anti-IgG was eliminated by carrier coprecipitation or by sucrose density gradient centrifugation. Others have used solid-phase attachment of influenza virus to glass for the immune reaction (6). The method described here uses the attachment of influenza virus to polystyrene test tubes as the solid-phase medium for radioimmunoassay. The sensitivity of the method, the kinetics of the reactions, and the correlation of viral radioimmunoassay procedures with conventional viral serological techniques are presented in this report.

MATERIALS AND METHODS

Influenza antigen. Influenza seed virus, strain A2/Japan 170/62, was propagated in 10-day-old
embryonated eggs. The allantioic fluid harvest was absorbed with chicken red blood cells after which the virus was eluted with receptor destroying enzyme (RDE) from *Vibrio cholerae*. The virus was then pelleted at 70,000 × g for 1 hr, suspended in borate saline buffer (BSB; 0.15 M NaCl, 0.002 M H₂BO₃, pH 7.4), and stored at −70°C. It was used at 50 hemagglutinin units per 0.06 ml in the solid-phase radioimmunoassay for influenza antibody, unless the variation in virus concentration is explicitly noted for an experiment.

**Antiserum and globulin preparations.** Antiserum against strain A2/J170/62 was prepared in rabbits by weekly intravenous injections and bleedings. Sera were preserved by adding 1% Merthiolate to a final 1:10,000 dilution and storing at −20°C. The end-point dilution by the hemagglutination inhibition (HI) test as performed by the microtitration technique was 1:1,000.

Human serum IgG was obtained from pooled plasma which was successively precipitated three times with 18, 14, and 12.5% sodium sulfate (16). Each precipitate was suspended in 0.15 M BSB for the succeeding precipitation with sodium sulfate. After a final dialysis against 0.0175 M phosphate buffer (pH 6.9), the sodium sulfate-precipitated globulin was further purified by diethylaminoethyl cellulose (DEAE) chromatography (Whatman DE32). Elution was carried out with the same buffer; the fractions that represented the IgG chromatographic peak were pooled, concentrated by pervaporation, dialyzed against BSB, and then quantitated as antigen in the immunoglobulin assay system. Protein concentrations were determined from optical density readings at 280 nm, with the extinction coefficient E₁%280 = 15.0.

Antiserum, specific for human IgG heavy chain, was prepared by dialyzing the chromatographic IgG eluate against 0.3 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at pH 8.5. The IgG was then reduced with 0.3 M 2-mercaptoethanol, and the heavy- and light-chain mixture was alkylated with iodoacetate. The heavy-chain fraction was then isolated chromatographically on Sephadex G100 in 1.0 M propionic acid (15).

The purified IgG heavy-chain fraction was used for immunizing 2- to 3-month-old female rabbits to make anti-human IgG heavy-chain antiserum. Initial injections consisted of 2 mg of protein per rabbit, injected subcutaneously as a homogenate with Freund's adjuvant. Each successive week the rabbits were bled and intravenously injected with 2 mg of protein. Antiserum specific for gamma heavy-chain determinants did not cross-react with other classes of immunoglobulins by the Ouchterlony technique of double diffusion in agar or by immunoelectrophoresis when whole serum was used as antigen.

Chromatographically purified human IgG was also used in the preparation of Fc fragment as an antigen (20) for injecting rabbits; the IgG heavy-chain schedule for immunization was used. The IgG was dialyzed against 0.15 M phosphate-buffered saline (pH 7.2) and brought to 0.002 M with ethylenediaminetetraacetate and 0.01 M with cysteine. In a nitrogen atmosphere, the globulin was then digested with 1% (w/w) mercuripapain (Whatrington Biochemical Corp). After being incubated at 37°C for 5 min, the digest was chilled in an ice bath slurry and chromatographed on Sephadex G25 at 4°C. The globulin peak that was free of papain was then applied to a DEAE column equilibrated with 0.01 M NaH₂PO₄, pH 8.0 (24). The Fab fragment was eluted first with 0.01 M NaH₂PO₄ (pH 8.0), and then the Fc fragment was eluted with 0.3 M NaH₂PO₄. After additional purification on Sephadex G100 in BSB, the Fc fragment was electrophoretically pure for use as antigen.

**Iodination of protein.** Globulins obtained by chromatographic or precipitation methods were labeled with the ¹²⁵I isotope of sodium iodide, and 50 µCi per mg of protein was added. Iodine monochloride (ICl) was used as an isotope carrier with an approximate 4:1 molar ratio of carrier to protein (18). Unbound ¹²⁵I was removed from the mixture by anion-exchange resin chromatography with Amberlite IRA-400 (Mallinckrodt Chemical Works). Specific binding of the radioactive iodine to protein in all preparations was demonstrated; 95 to 98% of the radioactivity was precipitable with 10% trichloroacetic acid.

**Solid-phase radioimmunoassay.** The solid-phase radioimmunoassay procedure consisted of layering the reactants on Falcon plastic (polystyrene, 10 by 75 mm) tubes by the "sandwich" technique (22) as diagrammed in Fig. 1. The tubes were initially coated for 1 hr at 37°C by adding 1 ml of the globulin fraction of rabbit anti-influenza antiserum. This left a coat of electrostatically bound antibody on the wall of the tube. Except for the volume of radioactive indicator, subsequent reactants were added separately in 1-ml fractions for sequential attachment to the tube under these same conditions. The sandwich layer thus consisted of nonhuman anti-influenza antibody, influenza virus, and specimen antibody which is virus-specific. As an indicator, 25 to 50 µCi (4 × 10⁴ counts/min) of ¹²⁵I-anti-human IgG, containing 150 to 300 µg of antibody protein, was added to each tube to quanitate variations in any one of the previously bound reactants. After incubation, each reactant was aspirated, and the tubes were rinsed twice by filling with BSB. The washed

![Wall of test tube](image-url)

**Fig. 1. Solid-phase radioimmunoassay.** Reactants as they are sandwiched on inner wall of polystyrene test tube: A = antibody (nonhuman) against virus; V = virus; A' = antibody (human) against virus; A' = anti-human antibody.
tubes were finally placed in a Packard autogamma scintillation counter for measuring $^{125}$I-labeled anti-IgG that was bound to the sandwiched reactants on the side of the test tube.

A solid-phase radioimmunoassay system for measuring total IgG has essentially the same sandwich reactants as the virus system, except that viral antigen is omitted and the initial coat is nonhuman antibody with specificity for IgG. Unlike the antibody-antigen-antibody sequence of reactants for measuring viral antibody, the antibody-antigen order of reagents was used for quantitating immunoglobulins.

**Virus antibody titrations by other methods.** Solid-phase radioimmunoassay for influenza antibody was compared with an alternate radioimmunoassay procedure (10). This alternate procedure involves an immune carrier precipitate of bovine serum albumin (BSA) and its specific antibody at equivalence ratios for maximum precipitate. This coprecipitatin technique is similar to the solid-phase technique; the virus-antibody complex in the carrier precipitate method of radioimmunoassay reacts with a radioisotope indicator, namely, $^{125}$I-anti-IgG. All radioimmunoassays followed the solid-phase procedure unless otherwise indicated.

Human serum HI titers for influenza virus were determined by the Respiratory Virology Unit at the Center for Disease Control, Atlanta, Ga., with the standard microtiter techniques (11). Sera were treated overnight with RDE to remove nonspecific inhibitors.

**RESULTS**

**Solid-phase kinetics.** We evaluated the sequential adsorption of reactants onto the side of polystyrene test tubes for solid-phase radioimmunoassay of virus-specific antibody. The initial coating of globulin onto the wall of the test tube was accomplished most efficiently at a globulin concentration of approximately 0.5 to 1.0 mg/ml (Fig. 2). This concentration permitted maximum binding of $^{125}$I-anti-IgG to the walls of the polystyrene test tubes. The concentration levels at which the binding to the solid-phase medium was most efficient were almost identical for the labeled globulins of both rabbit and horse origin. As determined from its specific activity (counts per min per microgram), only about 1% of the optimal rabbit or horse globulin concentration of 0.5 mg/ml actually bound to the wall of the tubes. Concentrations of either species of globulin greater than 0.5 to 1.0 mg/ml resulted in negligible increases in amount (counts per minute) of isotopically labeled globulin bound to the tubes. Neither the species differences in globulin preparations nor the specificity of the globulin influenced the optimal concentration for coating the polystyrene solid phase.

To determine the appropriate filler sub-

stance, we added various proteins after the binding of the globulin coat. The filler substance presumably binds itself to the tube between globulin molecules and reduces nonspecific binding of the anti-IgG isotopic indicator. Ovalbumin acted similarly to BSA in a solid-phase virus dilution assay (Fig. 3). In the virus assay system, an initial tube coat of virus-specific globulin was followed sequentially by filler, virus dilution, and constant amounts of virus-specific human antibody and $^{125}$I-anti-human IgG. Greater isotope binding was observed in the presence of higher virus concentrations regardless of the filler coating used. Data for radioimmunoassay of IgG by the solid-phase assay (Table 1) indicate that 1% BSA, with or without heat treatment, permitted the greatest ratio of specific to nonspecific binding after 1 hr of incubation with anti-IgG. The use of filler substances other than BSA (Table 1) resulted in increased nonspecific binding of $^{125}$I-anti-IgG; they were not considered further. Consequently, 1% BSA has been used routinely in our solid-phase radioimmunoassays.

![Fig. 2. Affinity of isotopically labeled globulins for determining optimum polystyrene coating concentration.](image)

After adding the $^{125}$I-anti-IgG, we tested the effect of various incubation periods. When all test reagents were included, the isotope-binding curves increased up to 18 hr of incubation at 37 C (Fig. 4). However, when the ratios of specific to nonspecific binding were calculated for these values, there was no indication that a prolonged incubation time significantly enhanced the specific binding of anti-IgG. Ratios for 1-, 4-, 6-, and 18-hr incubation intervals were 2.77, 3.28, 3.28, and 2.50, respectively (also shown in Fig. 4). All subsequent radioassays were routinely incubated for 1 hr.
with anti-IgG. In addition, controls without human IgG or those with only the filler coat present gave parallel curves which indicated that bound radioactivity was indeed nonspecifically bound anti-IgG. Approximately equal binding affinities were observed in each instance. In controls for subsequent tests, we omitted human IgG, thus providing for maximum nonspecific binding of the label.

**Sensitivity measurements.** Variations in the solid-phase radioimmunoassay with 125I-anti-IgG were made for quantitating IgG concentrations or for measuring virus-specific antibody. For IgG measurements, an initial coat of IgG-specific antibody must be applied to the solid-phase medium before it will react with IgG as the antigen. However, when virus-specific antibody is measured, the virus must be bound to an initial nonhuman antiviral antibody layer before it can react with human virus-specific antibody (Fig. 1). The intermediary action of an influenza-specific immuno-

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**Table 1. Effect of "filler" coat upon isotope binding**

| Rabbit anti-IgG* | "filler" coating material | Human IgG coating | 125I-anti-IgG bound (counts/min)* |
|------------------|--------------------------|-------------------|----------------------------------|
| + 1% BSA         | +                        | 43,941            |
| + 1% BSA         | -                        | 952               |
| + 1% Heated BSA  | +                        | 40,858            |
| + 1% Heated BSA  | -                        | 709               |
| + 1% Fetal calf serum | +                 | 38,409            |
| + 1% Fetal calf serum | -                    | 3,731             |
| + 1% Heated fetal calf serum | +             | 41,822            |
| + 1% Heated fetal calf serum | -               | 2,322             |
| + 1% Gelatin     | +                        | 40,638            |
| + 1% Gelatin     | -                        | 3,853             |

*Symbols: +, present; −, absent.
*Average of duplicates.

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**FIG. 3.** Dependence of the solid-phase method of virus antibody assay upon influenza virus concentration with either BSA or ovalbumin as an inert filler coat added subsequent to virus but before human viral antibody and 125I-anti-IgG.

**FIG. 4.** Optimum 125I-anti-IgG incubation time as determined by ratios of specific to nonspecific isotope binding in the final step of solid-phase radioimmunoassay (i.e., ratio = ○:●). For (○), reagents present were rabbit anti-Fc (human), BSA filler, human IgG, and 125I-anti-IgG. For (●), reagents present were rabbit anti-Fc (human), BSA filler, and 125I-anti-IgG. For (△), reagents present were for BSA filler and 125I-anti-IgG.

globulin binds the virus antigen to the solid-phase medium. The globulin is obtained from a species different from that of the viral antibody to be quantitated.

We compared direct and indirect methodologies for sensitivities in measuring immunoglobulin and virus antibody specimens. End points were taken as the lowest dilution of the immunoglobulin or viral antibody specimen at which radioactive binding of anti-IgG (Fig. 5 and 6) had plateaued and was equal to radioactive binding for controls without specimen. Either radiolabeled human IgG or influenza antibody was used as the specimen in the direct methods. For the indirect method, the specimen globulin was not tagged with isotope but was quantitated by binding 125I-labeled anti-IgG. The direct immunoglobulin assay method (Table 2, Fig. 5) bound 0.18 μg of IgG as determined from the specific activity (counts per min per mg) of the assayed specimen. The end-point concentration of IgG was 2.9 μg/ml. By the indirect method, the specimen end-point concentration was 0.037 μg/ml. Thus, the indirect method measured approximately 1/80th of the minimum protein detectable by the direct method.

Similarly, by the direct assay for viral antibody (Table 2, Fig. 6), a minimum concentration of 7.0 μg/ml was detected as compared to 2.8 μg/ml by the indirect method. This indicates that the indirect virus method is approximately three-times more sensitive than the
direct method for measuring virus-specific antibody. Only a very small percentage of available globulin actually binds to the solid-phase medium. By the direct method for measuring either immunoglobulin or virus antibody, the amount of bound specimen represented only about 6% of the total globulin present (Table 2).

Solid-phase correlations with other assay procedures. The amount of \(^{125}\text{I}\) bound by the solid-phase procedure for measuring influenza antibody was compared with titers for the same sera obtained by hemagglutination inhibition (Table 3). A significant percentage of sera had positive HI titers as well as radioactivity binding values greater than the sera with negative HI titers \((P = <0.01, >0.001)\). Sera with negative HI titers consistently had anti-IgG radioactivity of \(\leq 15 \times 10^3\) counts/min bound.

Radioimmunoassay by solid-phase and carrier precipitates were compared by using the same sera and antigens. Results of \(^{125}\text{I}\) radioactivity bound as anti-IgG are presented in Fig. 7. The correlation coefficient was highly significant, 0.890 \((P = <0.001)\).

**DISCUSSION**

Kinetics and sensitivities of solid-phase radioimmunoassay were studied. Our results with direct and indirect variations of the assay procedure indicate that it is a very rapid and highly versatile assay procedure. It represents a potentially useful diagnostic tool for quantitating immunoglobulin proteins and viral antigens.

An initial coat of virus-specific antibody was necessary to mediate virus binding to the wall of the polystyrene solid phase. A globulin concentration of 0.5 mg/ml was used, but only 3 to 7 \(\mu\)g of globulin was actually bound to the solid-phase medium. Because of the low percentage of globulin actually bound, the globulin reagents could be used several times before there was a significant reduction in coating efficiency. Since neither the host species nor the antibody specificity of globulins significantly affected the coating capacity, it is

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**Table 2. Immunoglobulin and virus antibody solid-phase radioimmunoassay**

| Method                | Immunoglobulin assay | Influenza antibody assay |
|-----------------------|----------------------|-------------------------|
|                       | Undilute specimen concn (mg/ml) | End-point concn (µg/ml) | Globulin bound (%) | End-point concn (µg/ml) | Globulin bound (%) |
| Direct                | 7.5                  | 1:2,560                  | 2.9               | 0.18                     | 18.2                | 1:2,560                  | 7.0               | 0.40                     | 5.7               |
| Indirect              | 7.5                  | 1:2 (2 x 10)^5          | 0.037             | 18.2                     | 16,400              | 2.8                        |
| Sensitivity ratio (direct/indirect) | 78                    | 78                       |               | 2.5                        | 2.5                    |
Having CPB bound serum negative probable solid phase isoelectric point obtained the 6% avidity solid-phase percentage similar point qualitative methods is.

TABLE 3. Influenza antibody detected by hemagglutination inhibition (HI) and solid-phase radioimmunoassay (RIA)

|                | No. tested | Percentage of sera having |
|----------------|------------|---------------------------|
|                |            | HI > 10 | HI < 10 |
| Having > 15 x 10^8 counts/ min by RIA | 22 | 91 | 9 |
| Having < 15 x 10^8 counts/ min by RIA | 5 | 20 | 80 |

* Value 15 x 10^8 = Counts/min bound by control serum negative by HI; x, P < 0.01, > 0.001.

Fig. 7. Correlation of counts per minute bound by solid-phase and carrier precipitate radioimmunoassay procedures.

probable that the coating process does not necessarily involve the active site of the antibody molecule. It could be a nonspecific characteristic pertaining perhaps to the general isoelectric properties of globulins.

One advantage of the solid-phase system is the use of the direct assay method to quantitate the amount of immunoglobulin bound to the solid phase. Conventional serological methods only give comparative end-point dilutions. By the precise quantitative results obtained by radioimmunoassay, one can assess qualitative aspects of antibody activity such as avidity or binding affinity. In any case, the percentage of total globulin bound at the end-point dilutions is very small (approximately 6% of the total globulin). The sensitivity measurement of actual globulin bound at end points is in the nanogram range as indicated by similar solid-phase methods (1, 7, 22).

The data in Table 2 indicate that the direct methods for measuring both immunoglobulins and virus antibody have about equal end-point dilutions (1:2,560). However, both indirect methods are more sensitive than direct methods for detecting minimal concentrations of antigen or antibody by the immunoglobulin or influenza antibody assay procedures (78 and 2.5 times more sensitive, respectively; Table 2). The sensitivity of the indirect immunoglobulin assay method over that of its direct method is approximately 30 times greater than the sensitivity of the indirect virus antibody assay method over its direct method (Table 2; 78/2.5 = 31.2). Presumably this greater increase in sensitivity for the indirect immunoglobulin assay method is largely spatial (22). Much less steric hinderance is probably involved in the indirect method for the immunoglobulin assay than in the indirect virus method for anti-IgG binding. In the latter case, the close proximity of virus-specific antibody on the virus surface would make many of their antigenic determinants inaccessible for combing with anti-IgG. This is entirely plausible considering the large number of antibody molecules which might bind to a single virion (13).

The indirect virus antibody assay required a higher concentration of globulin specimen at the end-point reaction than did the indirect immunoglobulin assay. This may be due to the fact that the specific virus antibody to be quantitated represents only a very small fraction of the total globulin specimen. On the other hand, the IgG fraction to be quantitated is much greater. De St. Groth and Webster (13) found approximately 4% of the total globulin to be influenza antibody, whereas we found 5 to 6% (Table 2) to be influenza-specific globulin. Consequently the dilutions for quantitating IgG were 30-fold greater than dilutions for measuring viral antibody (Table 2; 200,000/6,400 = 31.2). This was also evident when the total globulin concentration at the end-point dilution for virus antibody assay was 75- to 80-times the globulin concentration for immunoglobulin assay (Table 2).

The amount of anti-IgG radioactivity bound in the virus antibody radioimmunoassays was about twice the radioactivity bound by the comparable method for measuring immunoglobulins. This may reflect the greater surface area for virus as a template for both the direct and indirect method, whereas the template for both the direct or indirect immunoglobulin assay method is globulin (anti-Fc fragment). This may also account for more globulin being
bound by the direct virus antibody assay than by the direct immunoglobulin assay method (0.40 and 0.18 μg, respectively). However, the direct methods for both the immunoglobulin and the virus antibody assays have practically negligible nonspecific binding where the curves (Fig. 5 and 6) plateau at binding values for negative controls. The relatively high background binding by the indirect methods indicates that there may be nonspecific adherence of 125I-labeled globulin to the bound specimen globulin (i.e., globulin to globulin) by the indirect method. This nonspecific binding of globulin to globulin was not evident by the direct methods of either assay system. Presumably the filler of BSA serves to inhibit nonspecific binding in the direct method.

Isotope binding by either of the radioimmunoassay methods, solid-phase or carrier precipitate, had high correlation values when compared to titers by the HI test. When this relationship is extended even further, a greater percentage of sera having higher HI titers were also positive by the solid-phase method (Table 3). However, the correlation may be due to the greater contribution of IgG as the class of influenza antibody at peak HI titers of the immune response (14). Since HI titers are usually elevated in convalescent sera, this would be compatible with the prevailing theories on sequential development of antibody in the immune response (4, 25, 26). There was definitely no correlation between 125I-IgG binding by the solid-phase assay system and serum titers determined by complement fixation. However, correlation of solid-phase isotope binding with the comparable carrier precipitate radioassay procedure was highly significant (P < 0.001; Fig. 7).

The versatility of radioimmunoassay procedures, especially the solid-phase method, contributes to its diagnostic capabilities. Varying the reactants sandwiched as antibody-antigen or antigen-antibody onto the solid-phase medium allows one to choose the system for quantitating class-specific immunoglobulins or virus-specific antibody concentrations. The virus antibody radioimmunoassay is reproducible and sensitive and further allows one to quantitate virus specimens and to determine virus-specific antibody concentrations.

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