A Method for the Quantitation of Immunoglobulin Class-Specific Antibodies[1] (35510)

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(Introduced by Allan Granoff)

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Experiments designed to determine the role of immunoglobulins in the local or secretory antibody response have been hampered by the lack of a strictly quantitative and highly sensitive procedure for the measurement of immunoglobulin class-specific antibodies. This report describes the application of a highly sensitive radioimmunoassay (1, 2) to the quantitative measurement of antibodies belonging to the three major immunoglobulin classes: IgG, IgA, and IgM. For demonstration purposes, the determination of class-specific antibodies to sheep red blood cells and of class-specific antibodies to influenza virus are presented.

Materials and Methods. Source, purification, and labeling of myeloma proteins. BALB/c mice harboring plasma cell tumors were kindly donated by Dr. J. F. Albright, Oak Ridge, Tenn. (IgG-myeloma protein producers and IgA-myeloma protein producers) and by Dr. R. K. McIntire, National Institutes of Health, Bethesda, Md. (IgM-myeloma protein producers) and were transplanted through a series of BALB/c mice (3). IgG, IgA, and IgM myeloma proteins were purified by preparative electrophoresis, Sephadex G-200 gel filtration and DEAE-cellulose chromatography (4, 5). A Tris–boric acid–EDTA buffer, pH 8.5, was substituted for Veronal buffer in electrophoresis (6). Purified immunoglobulins were labeled with carrier-free $Na^{125}I$ (Schwarz BioResearch, Inc., Orangeburg, N. Y.) (10). After repeated dialysis (20 changes of normal saline), 99% of the radioactivity was acid precipitable. The antigens retained their antigenicity as evidenced by their ability to combine with monospecific antisera. The specific activity varied between 20 and 60 μCi/mg of protein.

Preparation of monospecific antisera to mouse immunoglobulins. Groups of three young adult male New Zealand white rabbits were inoculated in each hind footpad with a 1:1 mixture of purified immunoglobulin (about 1 mg/rabbit) and Freund's complete adjuvant, followed 3 weeks later with a single injection intraperitoneally of 1–2 mg of purified immunoglobulin in an aqueous mixture. On the seventh day, the animals were exsanguinated, the serum was collected and stored at $-20°$ until processed as follows. After clarification of each serum by ultracentrifugation at 30,000*g* for 30 min to remove lipids, each serum was absorbed to remove antibodies to the light chains in the following manner. First, purified IgG and IgM were attached to bromoacetylcellulose (BAC) (Miles Laboratories, Elkhart, Ind.) (7). Second, the rabbit antimouse IgG serum was absorbed with the BAC-IgM preparation, while the rabbit antimouse IgA serum and the rabbit antimouse IgM serum were absorbed with the BAC-IgG preparation. After absorption, the BAC-immunoglobulin preparations were regenerated by removal of absorbed antibodies with acid treatment (pH 2.8) (7). In this manner, 1 ml of antiserum could be made monospecific for a single

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heavy chain by five absorptions with the same BAC-immunoglobulin preparation consisting of 100 mg of BAC and 1 mg of immunoglobulin. Therefore, the amount of immunoglobulin needed for the absorption procedures was considerably reduced.

Analysis of purity of immunoglobulins and of monospecificity of antisera. Identification of immunoglobulins, the determination of their purity, and the determination of the monospecificity of the absorbed antisera were accomplished by means of immunoelectrophoresis (8) and double diffusion in agar (Ouchterlony). Protein concentrations were measured by optical density determinations at 280 nm and by the Lowry modification of the Folin reaction (9).

Preparation of antisheep red blood cell antisera. BALB/c female mice, 6 weeks old, were inoculated intraperitoneally with 0.5 ml of a 5% sheep red blood cell (SRBC) suspension, and blood was collected by axillary incisions 4 and 12 days later. The hemagglutination titer of each serum was determined by the microhemagglutination method (11). Mercaptoethanol sensitivity was also determined (12) and exclusion chromatography was performed (4).

Preparation of mouse anti-influenza virus lung washings. BALB/c female mice, 6 weeks old, were inoculated intranasally with active, mouse-adapted A_2/Jap 305 strain of influenza virus at 2-week intervals with increasing doses equal to 0.01, 1.0, and 100 MLD_{50}. Two weeks after the last inoculation, lung washings were obtained (13, 14). Neutralization tests were done in the allantois-on-shell system using $10^{2.0}$ EID_{50} of influenza virus (15). Hemagglutination and hemagglutination-inhibition assays were performed in plastic trays (WHO type) using chicken erythrocytes (16).

Influenza virus attachment to formalinized human red blood cells. The A_2/Jap 305 strain of influenza virus, concentrated and purified by differential centrifugation followed by centrifugation in a sucrose gradient (17), was attached to formalinized human erythrocytes (18, 19). Specifically, 10^4 HA units of virus were added to 10 ml of a 10% suspension of formalinized cells (about 10^{10} cells). Twenty ml of a 20% solution of 1-ethyl-3-(3-dimethylaninopropyl)-carbodiimide HCl (Ott Chemical Co., Muskegon, Mich.) in PBS, pH 7.2, was added to that mixture, and the whole was mixed gently for 1 hr at room temperature. The mixture was spun at 1500 rpm for 10 min, and the supernatant fluids were tested for unattached virus. Ninety-eight percent of the input virus was attached, representing an approximate ratio of 10 virus particles/cell. The pellet was washed twice with 2% EDTA (disodium ethylenediamine tetraacetate, Fisher Scientific Co., Fair Lawn, N. J.) in PBS, pH 7.2, followed by three washes in PBS containing 1% fetal calf serum.

The radioimmunoassay. The radioimmunoassay employed in this study is based on the property of cellulose membranes to retain antigen–antibody complexes but to pass unbound antigen (1, 2). Since the antigen in this system is labeled with ^{125}I, the radioactivity on the filter is a measure of antigen bound. When an excess of antiserum (*e.g.*, anti-IgM) is reacted with a standard amount of radiolabeled antigen (*e.g.*, ^{125}I-labeled IgM), all of the antigen is retained on the filter. Below the equivalence zone, the amount of specifically-retained radiolabeled antigen decreases in direct proportion to the decreasing concentration of antibody. When increasing amounts of unlabeled antigen (*e.g.*, IgM) are added to a mixture of ^{125}I-labeled antigen (^{125}IgM) and antibody (anti-IgM) in equivalent proportions (the zone of equivalence), there is a linear inhibition of the radioactivity on the filter (Fig. 1). The amounts of radiolabeled antigen and antibody in the equivalence zone are defined as the standard amount of antigen (1.6 μg of ^{125}I-IgG, 0.2 μg of ^{125}I-IgA, and 1.4 μg of ^{125}I-labeled IgM) and the equivalent unit of antiserum (1.0 ml of a 1:100 dilution of each monospecific antiserum). This competitive-inhibition radioimmunoassay was used to quantitate each class of immunoglobulin antibody as described below.

The quantitation of immunoglobulin class-specific antibodies. The procedure for the quantitative determination of antibodies belonging to each immunoglobulin class consists

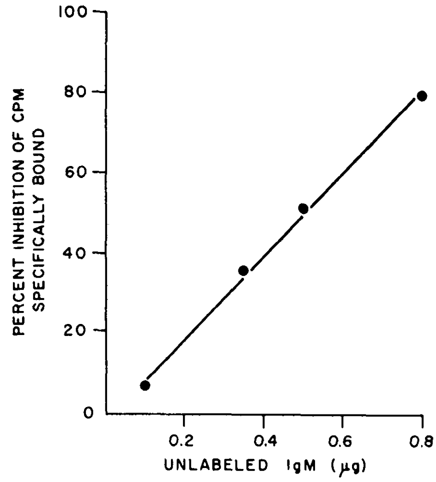

FIG. 1. The linear relationship between percentage inhibition of ^{125}I-labeled IgM specifically bound to anti-IgM and the addition of increasing amounts of unlabeled IgM.

essentially of two parts: (a) the binding of all specific antibody in the serum to an excess of the homologous antigen, *e.g.*, SRBC, and the separation of this antigen–antibody complex (SRBC–anti-SRBC) from all other serum immunoglobulins; and (b) the application of a simple and highly sensitive radioimmunoassay to quantitate IgG, IgA, and IgM antibodies (anti-SRBC) bound to the antigen (SRBC). The basic steps in the procedure are shown schematically in Fig. 2 and are detailed in Table I.

In Part A, an excess of antigen (SRBC) was added to the serum to bind all the antibodies (anti-SRBC). Separation of this antigen–antibody complex from the other serum immunoglobulins was accomplished by centrifugation and thorough washing of the pellet. If the antigen was not sedimentable (as in the case of influenza virus), it was attached to a sedimentable carrier, formalinized erythrocytes.

In Part B, an equivalent unit of rabbit antimouse IgG was added to the washed pellet from step 3. After a period of absorption, the complex (antigen-IgG antibody–anti-IgG) was spun down and the supernatant fluids, containing any unreacted anti-IgG, were collected.

In Part C, these supernatant fluids from step 6 were added to a standard amount of ^{125}I-labeled IgG. After a period of incubation, the mixture was filtered and the radioactivity retained on the filter was counted.

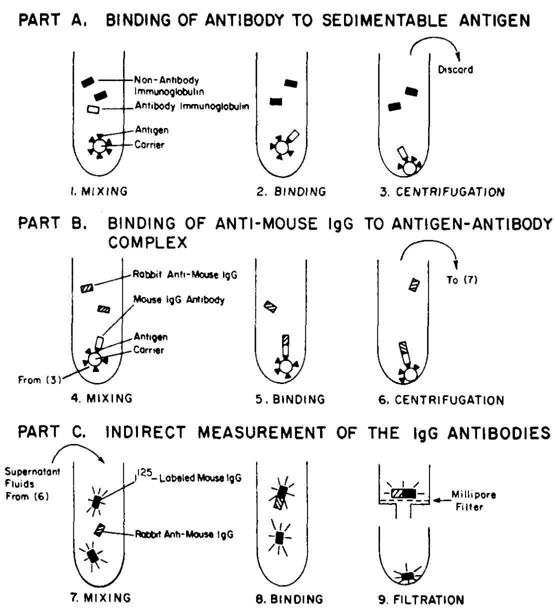

FIG. 2. Schematic outline of the procedures for the quantitative determination of antibodies belonging to a single immunoglobulin class. The procedure is demonstrated for IgG antibody determination, but the same steps hold for IgA or IgM antibody determinations.

TABLE I. Outline of Procedures Employed to Determine Quantitatively Mouse-Immunoglobulin Class-Specific Antibodies to Sheep Red Blood Cells.

Part A. Binding of antibody (anti-SRBC) to antigen (SRBC).

1. Varying amounts (0.01–0.1 ml) of mouse antiserum to SRBC were added to 0.5–1.0 ml of packed cell volumes of SRBC.
2. The mixture was shaken 1 hr at room temperature.
3. The mixture was centrifuged at 1500 rpm for 10 min: the supernatant fluids were tested for unabsorbed antibody; the pellet was washed five times with 5–10 vol of PBS.

Part B. Binding of rabbit antimouse immunoglobulin (*e.g.*, antimouse IgG) to the antigen–antibody complex (SRBC–anti-SRBC).

4. One equivalent unit[a] of the rabbit antimouse immunoglobulin (*e.g.*, antimouse IgG) was added to the antigen–antibody complex (SRBC–anti-SRBC) from step 3.
5. The mixtures were shaken 16 hr at 4°.
6. The mixtures were centrifuged at 1500 rpm for 10 min: the supernatant fluids were saved; the pellets were washed once with 1% fetal calf serum in PBS and this wash was added to the supernatant fluids; this combined mixture was clarified by centrifugation at 2000 rpm for 20 min.

Part C. Indirect measurement of immunoglobulin class-specific antibodies (*e.g.*, IgG-antibodies to SRBC) bound to the antigen (SRBC).

7. The clarified supernatant fluids from step 6 were added to 1.0-ml aliquots of a standard amount[b] of each ^{125}I-labeled mouse immunoglobulin (*e.g.*, ^{125}I-labeled IgG). The following controls were also set up:
   a. 1.0 ml of 1% normal rabbit serum in PBS + 1.0 ml of the standard amount of each radiolabeled mouse immunoglobulin (*e.g.*, ^{125}I-labeled IgG).
   b. One equivalent unit of each rabbit antiserum (*e.g.*, rabbit antimouse IgG) + 1.0 ml of the standard amount of each labeled mouse immunoglobulin (*e.g.*, ^{125}I-labeled IgG).
8. The mixtures were incubated at 36° for 24 hr.
9. The mixtures were filtered through a 220-mμ Millipore filter previously soaked in 10–50% fetal calf serum in PBS and washed twice with 2–3 ml of PBS; the filters were placed in polyethylene tubes and counted in a Packard gamma counter.

[a] One equivalent unit of antiserum is defined as 1.0 ml of the highest dilution of antiserum which retained 80–100% of the radioactivity of a standard amount of the homologous antigen.

[b] The standard amounts of radiolabeled immunoglobulins of each class were 1.6 μg of ^{125}I-IgG, 0.2 μg of ^{125}I-IgA, and 1.4 μg of ^{125}I-IgM.

At the same time, control mixtures consisting of a standard amount of radiolabeled IgG and an unreacted equivalent unit of anti-IgG were also filtered. Any decrease in amount of labeled IgG retained on the filters compared with the filtered control mixtures was due to a decrease in anti-IgG in the experimental mixtures because of binding to IgG-antibody. Using standard inhibition curves, it was possible to determine the amount of mouse IgG antibody specifically bound to the antigen.

Results. Specificity of the radioimmunoassay for quantitation of mouse immunoglobulins. The specificity of the reactions was tested in several ways: (a) there was no cross-reactivity between the three immunoglobulin systems as determined by immunodiffusion and immunoelectrophoresis; (b) retention of the radioactivity of any one of the radiolabeled immunoglobulin classes was less than 10% in samples containing only diluent and in those incubated with antiserum specific for one of the other immunoglobulin classes; and (c) inhibition of retention of radioactivity was possible only with unlabeled immunoglobulin of the same class as that being tested. Investigation of the correlation between concentrations of each purified, unlabeled immunoglobulin added, respectively, to each of the monospecific rabbit antisera and the inhibition of specific retention of the ^{125}I-labeled mouse immunoglobulin of each class showed in each

TABLE II. Binding of Days 4 and 12 Mouse Anti-SRBC Antibody to Excess Sheep Red Blood Cells and the Quantitation of Each Immunoglobulin Class of Antibody Bound.[a]

Serum	Vol added (ml)	% Inhibition (cpm) specifically retained			Anti-SRBC (μg/0.1 ml)		
		^{125}I-labeled IgG-Anti-IgG	^{125}I-labeled IgA-Anti-IgA	^{125}I-labeled IgM-Anti-IgM	IgG	IgA	IgM
Normal	0.10	0	0	30	<1.0	<1.0	<0.5
Day 4 antiserum	0.10	19	1	98			
	0.04	3	N.D.	96	<1.0	<1.0	3.0
	0.02	4	N.D.	68			
	0.01	1	N.D.	6			
Day 12 antiserum	0.10	97	2	99			
	0.06	74	N.D.	94	3.4	<1.0	1.0
	0.04	48	N.D.	20			
	0.02	8	N.D.	3			
	0.01	0	N.D.	0			

[a] The experiments were carried out as described in Materials and Methods and in Table I.

instance a direct linear relationship between inhibition and the concentration of each unlabeled mouse immunoglobulin. This is shown in Fig. 1 using the IgM system as an example.

Quantitation of immunoglobulin class-specific antibody to sheep red blood cells in days 4 and 12 mouse antisera. To demonstrate the technique for quantitation of immunoglobulin class-specific antibodies, days 4 and 12 mouse antisera to sheep red blood cells were used (Table II). The day 4 antiserum had mainly IgM antibodies while most of the antibodies to SRBC in the day 12 antiserum were IgG, as determined by mercaptoethanol sensitivity and by exclusion chromatography.

The day 4 antiserum contained 30 μg/ml of IgM anti-SRBC antibody. There was no detectable IgA anti-SRBC antibody. IgG anti-SRBC antibody was detectable (19% inhibition at 0.1 ml of serum) but was not quantitated. If larger amounts of sera had been tested, quantitation would have been possible. The day 12 antiserum contained 34 μg/ml of IgG anti-SRBC antibody and 10 μg/ml of IgM anti-SRBC antibody. Again, IgA anti-SRBC antibody was not detected.

Normal mouse serum controls demonstrated the absence of nonantibody immunoglobulins belonging to either the IgG or IgA classes on the sheep red blood cells after washing five times with PBS. However, a small, but significant amount of inhibition of the ^{125}I-labeled IgM-anti-IgM reaction (30% on the average) indicated the presence of IgM attached to the sheep red blood cells even after thorough washing. This IgM is believed to represent "natural" antibodies to SRBC known to be present in normal mouse serum.

The results indicate the feasibility of this procedure to quantitate in absolute terms the amount of antibody in a specimen belonging to each of the three major immunoglobulin classes.

Detection of immunoglobulin class-specific antibody to influenza virus in mouse lung washings. Using influenza virus (A_2/Jap 305) attached covalently to formalinized human red blood cells by carbodiimide treatment, antibodies to the virus were absorbed out of lung washings obtained from hyperimmune BALB/c mice. These antibodies were then tested for their ability to inhibit the ^{125}I-labeled IgG-anti-IgG reaction and the ^{125}I-labeled IgA-anti-IgA reaction (Table III).

One ml of the lung washings from hyperimmune mice inhibited the ^{125}I-labeled IgG-anti-IgG reaction and the ^{125}I-labeled IgA-anti-IgA reaction 28 and 91%, respectively. Serological tests on the hyperimmune lung washings indicated a low titer of neutralizing antibody (1:100)/ml), but no detectable hemagglutination-inhibiting antibody. Lung washings from nonimmunized mice treated in

TABLE III. The Demonstration of IgG and IgA Anti-A_2/Jap 305 Antibodies in Lung Washings of Immunized Mice by Their Inhibition of the Specific Reactions ^{125}I-Labeled IgG–Anti-IgG and ^{125}I-Labeled IgA–Anti-IgA, Respectively.[a]

		% Inhibition (cpm) specifically retained	
Lung wash	Vol added (ml)	^{125}I-labeled IgG-Anti-IgG	^{125}I-labeled IgA-Anti-IgA
Not immunized	1.0	0	0
Immunized	1.0	28	91

[a] The experiments were carried out as described in Materials and Methods and in Table I.

the same manner as the lung washings from hyperimmune mice had no effect on either reaction, indicating the absence of nonspecific absorption of immunoglobulins by the virus–red blood cell complex. These results indicate the sensitivity of the procedure and its applicability to the detection and quantitation of class-specific antibodies to viruses.

Discussion. Methods available and used by others to study immunoglobulin class-specific antibodies are either qualitative (20–22) or insufficiently sensitive (23). The radioimmunoprecipitation method (24, 25), while quantitative and sensitive, is restricted in its application to antigens forming soluble antigen–antibody complexes. A new method has been described which makes use of a simple and highly sensitive radioimmunoassay (1, 2) to quantitatively measure antibodies belonging to each immunoglobulin class. The advantages of this procedure over other reported methods are: (a) it is strictly quantitative; (b) it is highly sensitive, detecting concentrations of 1.0 μg/ml; (c) it indirectly quantitates the antibody itself; (d) there are no technically difficult operations; and (e) it is not dependent on special considerations, such as size of antigen.

Figure 2 shows schematically the basic steps followed. The object of the first three steps was to absorb out all the specific antibody from the specimen by absorption with an excess of antigen. There are three critical points in these steps: (a) all the antibody must be absorbed; (b) nonantibody immunoglobulins must not be absorbed; and (c) the antigen–antibody complex must be sedimentable, and preferably, at low speed.

The first point was accomplished by absorbing with at least four times as much antigen as would be required to give a positive reaction in the hemagglutination test. Also, supernatant fluids after absorption were tested for unabsorbed antibody. The second point was assured by washing the antigen–antibody complex thoroughly and by testing for nonantibody immunoglobulins on antigens or antigen-carrier system after treatment with nonimmune serum or lung washings. In the SRBC–anti-SRBC system, the antigen itself was sedimentable, which was, of course, one of the reasons for using this system to demonstrate the feasibility of measuring immunoglobulin class-specific antibodies. In the case of the influenza–anti-influenza system, the antigen was made sedimentable at low speed by attachment of the virus particles to formalinized human erythrocytes by carbodiimide treatment (19).

The object of the last six steps was to quantitate the antibody belonging to a single immunoglobulin class by means of the competitive-inhibition radioimmunoassay. The critical points are: (i) the specificity of the reaction, and (ii) the sensitivity of the reaction. The specificity of the reaction was demonstrated by a lack of cross-reactivity in (a) immunodiffusion tests, (b) reaction of radiolabeled immunoglobulins with antisera specific for the other immunoglobulins, and (c) inhibition of each reaction with nonspecific unlabeled immunoglobulins. The sensitivity of the procedure was demonstrated by its ability to detect quantities of immunoglobulins at levels of 0.5–1.5 μg/ml. However, by increasing the specific activity, a proportionately higher sensitivity may be achieved.

Using the procedure described in this report, the antibody to SRBC in days 4 and 12 mouse antisera has been quantitated according to immunoglobulin class and IgA and IgG antibodies to influenza virus have been detected in lung washings from immunized mice. Only IgM antibodies could be measured quantitatively in the day 4 antiserum (30 μg/ml), while both IgG and IgM antibodies were quantitated in the day 12 antiserum (34) and 10 μg/ml, respectively). No IgA antibodies were detected in either antiserum (<10 μg/ml). The results were consistent with qualitative results obtained using the techniques of mercaptoethanol sensitivity and exclusion chromatography. Moreover, the technique has been shown to be applicable to the detection of antiviral antibodies in lung washings of immunized mice.

With this procedure, therefore, quantitative studies of the kinetics of the production of antibody belonging to each immunoglobulin class are now possible, and the implications in host resistance of such antibodies in secretions can be better assessed.

Summary A new method for the quantitative determination of antibodies according to the immunoglobulin class to which they belong has been described. It consists of two parts: (a) the removal of all antibody in the serum or secretions by absorption with excess antigen; and (b) the quantitation of a single immunoglobulin class of antibody bound to the sedimented antigen by means of a competitive-inhibition radioimmunoassay. The method is specific, quantitative, and sensitive to at least 1 μg/ml of specific antibody. The procedure is demonstrated by the quantitation of antibodies to sheep red blood cells according to immunoglobulin class in days 4 and 12 mouse antisera and by the detection of immunoglobulin class-specific antibodies to influenza virus in mouse lung washings.

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