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A Quantitative Antibody-Binding Method for the Determination of Specific Antibody within Different Immunoglobulin Classes

APPLICATION TO FOUR Ig CLASSES IN THE MOUSE

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Summary. A quantitative method for determining specific antibody in the different classes of immunoglobulins is described. The technique is based on the binding of all antibody present in an immune serum with antigen, and the subsequent precipitation of the bound immunoglobulins with a heterologous antiserum specific for the antigen employed. The concentration of each immunoglobulin class is determined by means of single radial immunodiffusion, before and after removal of its antibody-active portion. The accuracy of the measurements is essentially that of the immunodiffusion technique, and the whole analysis, applied to four immunoglobulin classes, requires only 0·06 ml of serum.

The applicability of the technique has been tested using sera from mice immunized with ferritin by different routes. Antibody activity was predominant in the IgG_1 and IgG_2 immunoglobulin classes. However, measurable anti-ferritin activity in the IgM and IgA classes was also found in a limited number of sera.

INTRODUCTION

In many types of experiments it is desirable to obtain information of a quantitative nature on the distribution of antibody activities among the different classes of immunoglobulins. This may, for instance, be achieved by first purifying each immunoglobulin class by physico-chemical means and assessing its antibody activity separately (Schultze, 1959). Alternatively, one may determine the nature of immunoglobulins released by dissociation of antigen–antibody complexes (Kunkel and Rockey, 1963; Kabat and Mayer, 1964). The former procedure is limited by the difficulty of quantitatively isolating each immunoglobulin class, whereas the latter is dependent upon the possibility of obtaining complete dissociation of the complex. Yet a different approach is based on the combination of antigen with antibody which is itself precipitated by a heterologous antiserum directed against a specific immunoglobulin class. To this category belong a variety of immunodiffusion methods such as radioimmunoelectrophoresis (Yagi, Maier, Pressman, Arbesman and Reisman, 1963) and related procedures (Bazin, 1967), all of which are, by nature, qualitative. The quantitative antigen-binding method of Skom and Talmadge (1958) and its recent modification, radioimmunoprecipitation (Gleich and Stankievic, 1969), are

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based on the same principle. In this case, antibody is indirectly quantified by determining the amount of antigen bound in immune precipitates formed after the addition of heterologous antisera directed against specific immunoglobulin classes. This method is applicable only with antigens forming soluble antigen–antibody complexes.

The method presented in this communication is designed to alleviate many of the difficulties encountered in existing techniques. Besides avoiding the use of radioactive isotopes, it is both simple and accurate, and should be adaptable to any kind of antigen. In addition, analysis of the antibody content, whether precipitating or not, of the different immunoglobulin classes can be carried out simultaneously on as little as a total of 60 μl of a serum sample.

MATERIALS AND METHODS

Animals

The animals used in this study were C3H mice which had originally been raised germ-free but had been contaminated with an unknown strain of spore-forming bacillus. They were considered more suitable than conventional animals because of the low immunoglobulin concentrations in their serum.

Immunization procedures

Horse spleen ferritin was administered in four different ways:

(1) Two mice received three subcutaneous (s.c.) injections of 1 mg of antigen at 10-day intervals.

(2) Four mice received three intraperitoneal (i.p.) injections of 1 mg of antigen at 10-day intervals.

(3) Two mice received one subcutaneous (s.c.) injection and two intraperitoneal (i.p.) injections of 1 mg of antigen at 10-day intervals.

(4) Two mice had access to drinking water containing 1 mg ferritin/ml, for a period of 38 days prior to killing.

For the first injection in Groups 1–3, the antigen was emulsified in Freund's incomplete adjuvant. The animals receiving antigen by the parenteral route were killed 10 days following the last injection.

Four conventional C3H mice aged 36–88 days received no antigen and were used as controls.

Method for the differential determination of the antibody content of separate immunoglobulin classes

The procedure here described is based on the differential determination of each immunoglobulin class before and after removal of the specific antibody which it contains. The elimination of specific antibody, both precipitating and non-precipitating, is achieved by first combining it with an excess of antigen and then removing the antigen–antibody complexes by precipitating them with an excess of heterologous antiserum directed against the antigen.

Choice of the appropriate amount of antigen (ferritin). Since the method is based on the combination of all antibody present in the serum samples, whether precipitating or not, and

regardless of its affinity, it was necessary that antigen be added in excess. For that purpose a preliminary determination of the precipitating antibodies in the mouse sera was made by means of a reversed version of the radial immunodiffusion method (Vaerman, Lebacq-Verheyden, Scolari and Heremans, 1969). Briefly, 3-μl amounts of the individual serum samples were allowed to diffuse from circular wells (2·5 mm in diameter) punched out in 1·5 mm thick agar gels (2 per cent in buffered saline) containing ferritin at different concentrations. The mouse sera containing the highest concentrations of precipitating antibodies, as judged from the size of their precipitin rings, were submitted to graded absorptions with ferritin and tested again for their residual precipitating capacity.

From this pilot study it was concluded that the addition of 1 μl of a 1 per cent ferritin solution to 30 μl of mouse serum would remove all precipitating antibodies. In the actual experiment this proportion was doubled in order to allow for the possible presence of antigen binding to non-precipitating antibodies.

Choice of the appropriate amount of heterologous (rabbit) antibody. In order to remove soluble as well as insoluble antigen–antibody complexes, it was necessary to add heterologous anti-ferritin in excess to the ferritin, whether combined or free, present in the test mixture. Graded amounts of rabbit antiserum against horse spleen ferritin were added to constant volumes of ferritin at a concentration of 20 μg in 33 μl (similar to the test system: see below), and after overnight incubation at 4°, the residual free antigen was determined by diffusion in antibody-containing agar gel. It was found that 2 μl of rabbit antiserum would eliminate all detectable antigen from the test system.

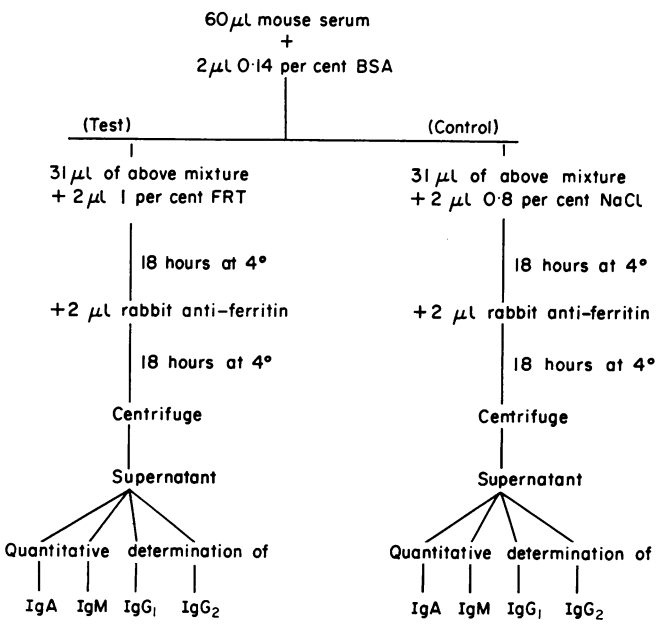

FIG. 1. Flow chart describing the method used for the quantitative antibody-binding technique.

Description of the test and control systems (Fig. 1). Of each serum sample, 60 μl was introduced into a 4·5×0·8 cm test-tube and mixed with 2 μl of a 0·14 per cent. solution of bovine serum albumin (BSA) in saline (as an internal control: see below). The mixture

was divided into two equal portions by transferring half of it to a separate test tube to serve as a control. To the test sample was added 2 μl of a 1 per cent ferritin solution in saline, whereas the control sample received 2 μl of 0·8 per cent NaCl. Both the test sample and the control were kept at 4° for 18 hours, after which 2 μl of rabbit anti-ferritin were added to each. The resulting precipitates were removed by centrifugation after a further 18 hours of incubation at 4°.

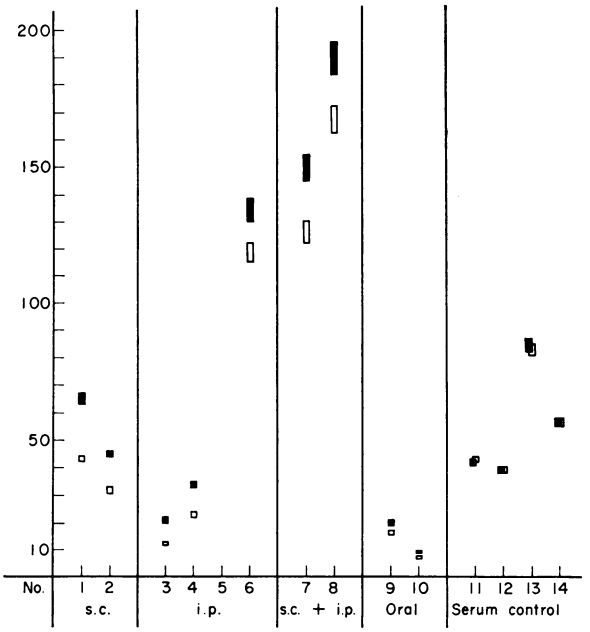

FIG. 2. Results of quantification of total immunoglobulin and anti-ferritin antibody in the IgG_1 class of mouse immunoglobulins. Units for the vertical axis represent the percentage of the corresponding immunoglobulin in a pool of sera from conventional C3H mice. Rectangles indicate immunoglobulin levels before (solid) and after (open) removal of anti-ferritin antibody. S.C., 3 × 1 mg ferritin subcutaneously; i.p., 3 × 1 mg ferritin intraperitoneally; S.C.+i.p., ferritin given subcutaneously (once) and intraperitoneally (twice); oral, 1 mg ferritin/ml drinking water for 38 days; serum control, conventional C3H mice of different ages: No. 11, 36 days; No. 12, 52 days; No. 13, 68 days; No. 14, 88 days.

Quantification of immunoglobulins. The concentrations of mouse IgA, IgM, IgG_1 and IgG_2 in the supernatants of test and control mixtures were determined using the single radial immunodiffusion method described by Mancini, Carbonara and Heremans (1965).

The specific antisera against mouse α-, μ-, γ_1- and γ_2- polypeptide chains used for this purpose have been described in an earlier publication (Bazin, 1966). All concentrations were expressed as percentages of the concentration of the corresponding immunoglobulins in a pool of sera from normal adult C3H mice. This use of relative units was necessary because of the unavailability of purified standard immunoglobulin preparations.

Since a preliminary study had shown that the methodological error of single measurements by the immunodiffusion method amounted to about 3 per cent of the mean, throughout the range of concentrations studied and for all four immunoglobulins, each value illustrated in Figs. 2–5 is represented by a rectangle covering the ± 3 per cent range about the actual measurement.

BSA-internal control. Because the value of the method is dependent on the accuracy with which a difference in protein concentration between two equally diluted samples can be measured, it was important to have a control which would allow for the detection of errors due to pipetting. For this purpose, each sample received an internal marker consisting of BSA (see above) prior to being separated into a test and control half. Following the final centrifugation, the supernatants of all tubes were evaluated for their individual concentrations of BSA by the single radial diffusion technique. The resultant precipitin rings developing from the test and control halves of each serum sample were found to be virtually identical indicating that no dilution errors of any significance had occurred. Had such not been the case, it would have been possible to adjust each value of the immunoglobulin concentrations by multiplying it with an appropriate correction factor.

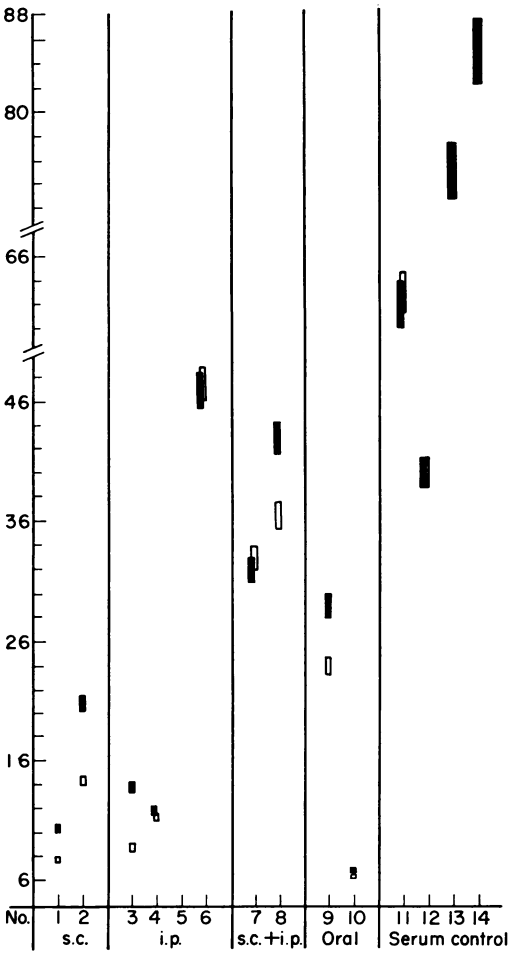

FIG. 3. Results of quantification of total immunoglobulin and anti-ferritin antibody in the IgG_2 class of mouse immunoglobulins. For key see Fig. 2.

RESULTS

The concentrations of the different immunoglobulin classes in the sera of the immunized and non-immunized animals, before and after elimination of specific anti-ferritin antibodies,

TABLE 1

PRECIPITATING CAPACITY OF INDIVIDUAL MOUSE ANTISERA AND NUMERICAL REPRESENTATION OF ANTIBODY MEASURED WITHIN FOUR CLASSES OF MOUSE IMMUNOGLOBULINS

Animal No. (see Figs. 2–5)	Precipitation with ferritin in gel_1*	Distribution of anti-ferritin antibody among different immunoglobulin classes							
		IgG_1		IgG_2		IgM		IgA	
		Anti-ferritin (units/ml)†	Anti-ferritin × 100 / Total IgG_1	Anti-ferritin (units/ml)	Anti-ferritin × 100 / Total IgG_2	Anti-ferritin (units/ml)	Anti-ferritin × 100 / Total IgM	Anti-ferritin (units/ml)	Anti-ferritin × 100 / Total IgA
1	++	22	33·8	2·5	23·9	n.s.	n.s.	n.s.	n.s.
2	+	13	28·9	6·5	31·0	9·0	6·8	n.s.	n.s.
3	±	8	38·1	5·0	35·7	n.s.	n.s.	n.s.	n.s.
4	+	11	32·3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
5	+++±	——‡	——	——	——	n.s.	n.s.	n.s.	n.s.
6	+++	16	11·9	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
7	++±	23	15·4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
8	++++	22	11·6	6·5	15·1	12·1	6·5	11·1	29·0
9	–	4	20·0	5·0	17·2	35·0	17·5	3·6	17·5
10	–	2	20·0	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
11	–	n.s.§	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
12	–	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
13	–	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
14	–	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

* Refers to the presence or absence, and size of the precipitin rings developed by the mouse antisera upon diffusion through a gel containing ferritin.
† All concentrations are expressed as percentages of the concentration of the corresponding immunoglobulins in a pool of sera from normal adult C3H mice.
‡ Not determined.
§ No significant antibody detected.

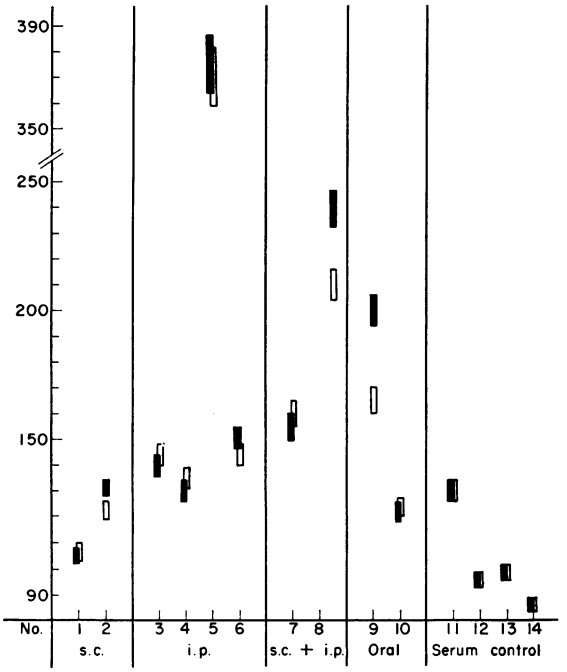

FIG. 4. Results of quantification of total immunoglobulin and anti-ferritin antibody in the IgM class of mouse immunoglobulins. For key see Fig. 2.

are illustrated in Figs. 2–5. Wherever, in an individual sample, the two rectangles representing the test (open) and control (solid) halves of the samples do not overlap, it may be considered that the distance between them is a measure of the anti-ferritin antibody belonging to that particular class of immunoglobulins studied. Numerical data concerning the distribution of anti-ferritin among the different immunoglobulin classes are given in Table 1.

Every immunized mouse was found to have developed anti-ferritin antibodies of the IgG_1 class (Fig. 2), however, this type of response was very weak in the orally immunized animals. The amounts of antibody synthesized by the parenterally immunized mice were fairly similar in different animals and did not correlate with their individual IgG_1 levels.

For the IgG_2 class of immunoglobulins (Fig. 3), significant anti-ferritin response could be demonstrated in four out of seven parenterally stimulated animals, as well as in one of the two orally immunized mice. As with IgG_1 the antibody response, when present, was independent of the IgG_2 level in the serum.

Only three out of ten animals showed a definite IgM antibody response. One of these animals belonged to the orally immunized group (Fig. 4).

Two mice were found to have anti-ferritin antibodies of the IgA class. One of these had received the antigen by the oral route (Fig. 5).

DISCUSSION

CONCERNING THE METHOD

The method proposed here has several advantages over existing techniques for the allocation of antibody activities to individual immunoglobulin classes.

The total analysis can be carried out on 60 μl of serum. With a sample of this size, the

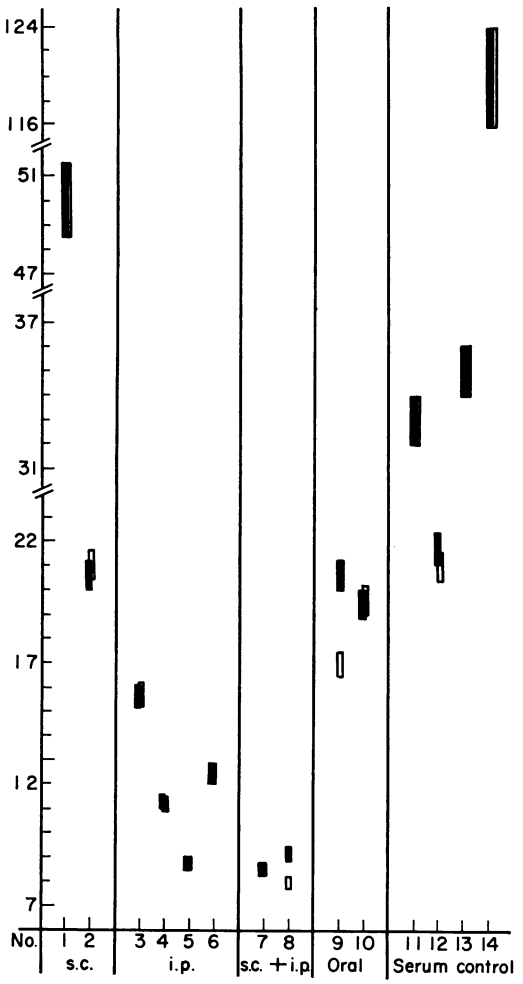

FIG. 5. Results of quantification of total immunoglobulin and anti-ferritin antibody in the IgA class of mouse immunoglobulins. For key see Fig 2.

method will provide data on the specific antibody content of several immunoglobulin classes, as well as on the concentration of each of these immunoglobulins in the serum.

It also presents some especially attractive features. Firstly, the data produced by this method are of a quantitative nature, unlike those obtained by radioimmunoelectrophoresis or related techniques. The accuracy of these measurements is essentially that inherent in the method of single radial immunodiffusion. As applied here, this technique was found to produce methodological errors not exceeding 3 per cent of the estimates. Any errors that might arise from volumetric inaccuracies during the processing of the samples can be detected and corrected by means of an internal dilution marker. Such a marker (e.g. BSA) may be added to the system at the outset, as was done in the experiments described here. Alternatively, a built-in marker might be provided by some natural component of the mixture (e.g. mouse albumin or transferrin), if a specific antibody to such a substance be available.

Owing to the fact that antigen is added in excess and subsequently completely precipitated by a heterologous antiserum, it may be assumed that the method effectively disposes of the problems created by non-precipitating antibodies, antibodies of low affinity and soluble antigen–antibody complexes. The first of these points is well illustrated by animals Nos. 8 and 9, both of which had appreciable amounts of anti-ferritin antibodies belonging to all four immunoglobulin classes although only the first of these mice was found to have developed precipitating antibodies (Table 1).

One potential difficulty which has not been taken into account here might arise from the presence of rheumatoid factor in the sample. In such cases it is to be expected that rheumatoid factor will co-precipitate with the antigen–antibody complexes on whose formation the method is founded. This might conceivably lead to wrong assumptions about the antibody activity within the IgM class. It should be possible, however, to eliminate this source of error by an appropriate control, consisting of some unrelated antigen–antibody system (for instance, BSA+rabbit anti-BSA). Alternatively, rheumatoid factor might be removed from the system by adsorbing it onto a suitable insoluble reagent such as heat aggregated heterologous IgG. Besides the specific combination of rheumatoid factor with antigen-bound IgG, one must also make a reservation for the possible non-specific adsorption of immunoglobulins onto protein precipitates. The controls proposed to account for the presence of rheumatoid factor will presumably effectively deal with the last-mentioned difficulty as well. That the latter problem should not be overemphasized is illustrated by the completely negative findings obtained on the serum from non-immunized animals (Figs. 2–5).

CONCERNING THE RESULTS

In mice immunized with a mixture of ferritin and haemocyanin, Barth, McLaughlin and Fahey (1965), on the basis of electrophoretic observations, assumed antibody activity to be present in the IgG_1 and IgG_2 classes. Bazin (1967) studied the antibody response of mice injected with horse ferritin and human serum albumin. Using an adaptation of the immunoelectrophoretic technique, he was able to show that precipitin lines, obtained with specific antisera against mouse IgG_1 and IgG_2, contained antibodies reacting with both antigens. In addition, it was shown that rabbits injected with human albumin or ferritin precipitated by mouse antisera would develop antibodies against mouse IgG_1, IgG_2 and IgM, indicating that antibody activity also occurred in the last-mentioned class. Radioimmunoelectrophoretic studies by Fahey, Wunderlich and Mishell (1964), demonstrated that antibody activity against haemocyanin could be elicited in all four mouse immunoglobulin classes, including IgA.

The findings presented here, of a quantitative nature, while confirming the qualitative data obtained by previous investigators, also indicate that most of the anti-ferritin antibody developed by parenterally immunized mice belongs to the IgG_1 and IgG_2 classes, with the accessory and inconstant participation of IgM and IgA.

In one animal immunized by the oral route antibody activity was measurable in all four immunoglobulin classes. Studies are currently in progress to quantify, by the same method, the amount of specific antibody present in IgA from intestinal secretions of mice immunized by oral or parenteral routes.

The well-known individual variation between animals of the same species responding to an antigen is further exemplified in the present study. The data from Table 1

demonstrate that such variability is not only of a quantitative nature but also bears upon the allocation of antibody activity to different immunoglobulin classes.

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