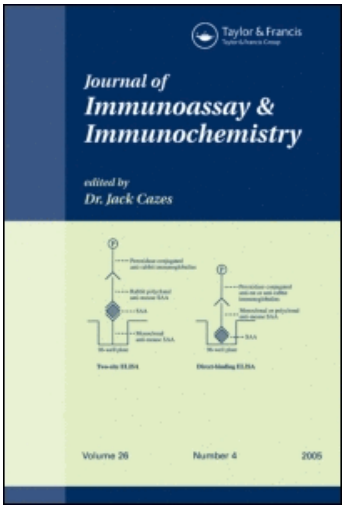

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AN ULTRASENSITIVE RADIOLIGAND ASSAY FOR IgG USING THE PROTEIN A ON STAPHYLOCOCCUS AUREUS BACTERIA

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ABSTRACT

The purpose of this study was to develop a simple and sensitive assay to measure IgG. Human IgG was radiolabelled with ^{125}Iodine and 7.5 ng was incubated with heat-killed Staphylococcus aureus bacteria (Cowan 1 strain). To replicate sets of tubes, increasing amounts of a standard IgG preparation were added. The samples were incubated at room temperature for two hours and separated by centrifugation. Using this assay it was found that the IgG concentration could readily be determined in one nanoliter or less of human serum. There was no significant cross-reactivity with IgA, IgE and IgM or the F(ab')$_2$ fragment of IgG. Serial dilutions of normal human or SLE sera, rabbit or guinea pig sera, the Fc fragment of human IgG and a mouse monoclonal anti-human DNA antibody parallelled the dose response curve obtained with standard human IgG. The method correlated well (r=0.89) with a routinely used nephelometric method. The mean (±SD) IgG concentration in 20 normal subjects measured by this assay was 10 ± 3.6 g/L.

INTRODUCTION

The measurement of immunoglobulin levels in biological fluids is a frequently used technique. Several methods are presently available for the estimation of immunoglobulin G (IgG) but they require relatively large amounts of assay material. It has been shown by several authors that Staphylococcus aureus of the Cowan 1 strain contains Fc receptors on its cell wall that bind IgG (1-4). The specificity and high affinity of protein A makes it an ideal agent for the development of an assay that would allow the measurement of small amounts of IgG.

METHODS

Heat-killed Staphylococcus aureus bacteria (Cowan 1 strain) were used as a source of protein A and were obtained from The Enzyme Centre, Inc. (Malden, MA, USA) as a preparation known as IgGSorb. Monomeric human IgG was obtained from Cappel Laboratories, (Cochranville, PA, USA).

PREPARATION OF LABELLED IgG

IgG was labelled with ^{125}Iodine using the chloramine T method described by Greenwood and Hunter (5). One mg of monomeric human IgG was dissolved in 25 µl of 0.5M

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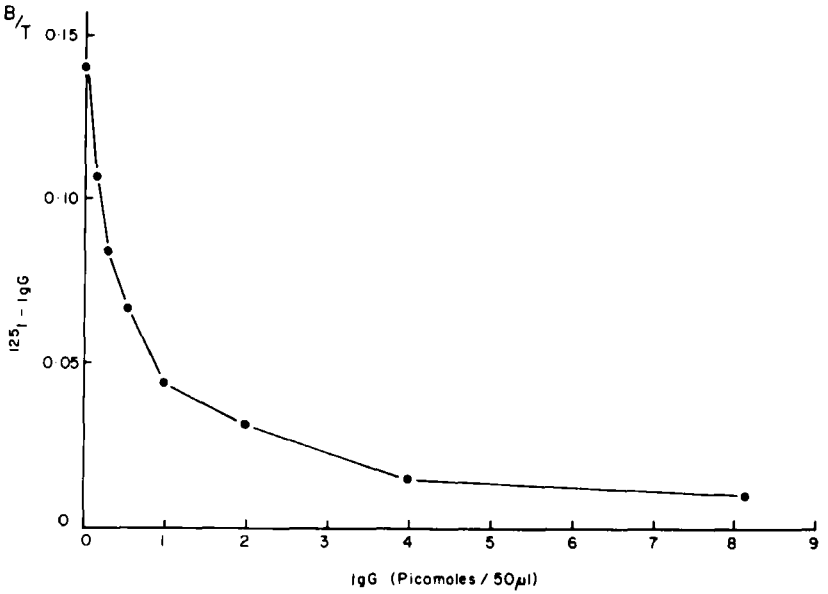

Fig. 1. Dose response curve of IgG with the protein A on the surface of Staph aureus bacteria. Fifty femtomoles of ^{125}I-IgG were added to 20 µl of a 0.02% suspension of the heat-killed bacteria and made up with 1.0% BSA phosphosaline buffer to a final volume of 50 µl. After two hours incubation at RT the tubes were centrifuged, the supernatants discarded, and the radioactivity determined in the precipitates. To replicate set of tubes unlabelled IgG was added in the concentrations indicated.

phosphosaline buffer, pH 7.4. One mCi of Na ^{125}I (Amersham, Oakville, Ont., Canada) in 10 µl was added and mixed well. Freshly prepared chloramine T, 200 µg in 20 µl of buffer, was then added and allowed to react for one minute. The oxidation reaction was stopped by adding 400 µg sodium metabisulphite in 500 µl buffer. After mixing for 10 sec. 500 µg of BSA in 20 µl of buffer was added. The products were separated on a 50 x 1 cm Sephadex G50 column using assay buffer. The performance of the tracer was checked by incubating it with protein A by itself to determine its binding and with unlabelled IgG to determine inhibition of specific tracer binding.

BINDING STUDIES OF ^{125}I-IgG WITH PROTEIN A

Fifty femtomoles (7.5 ng) of ^{125}I-IgG was incubated at room temperature (RT) for 2 hours with sufficient protein A to result in a bound to free ratio (B/F) of approximately 0.3. The final incubation volume was 50 µl. At the end of the incubation period 1 ml of 0.9% saline containing 1% Tween was added to each tube and the tubes were centrifuged for 30 min. at 2000 g. The supernatant was decanted and the radioactivity in the precipitate counted in a gamma counter. A dose response curve (Fig. 1) was obtained by adding increasing amounts of unlabelled IgG to a set of tubes prepared as described above.

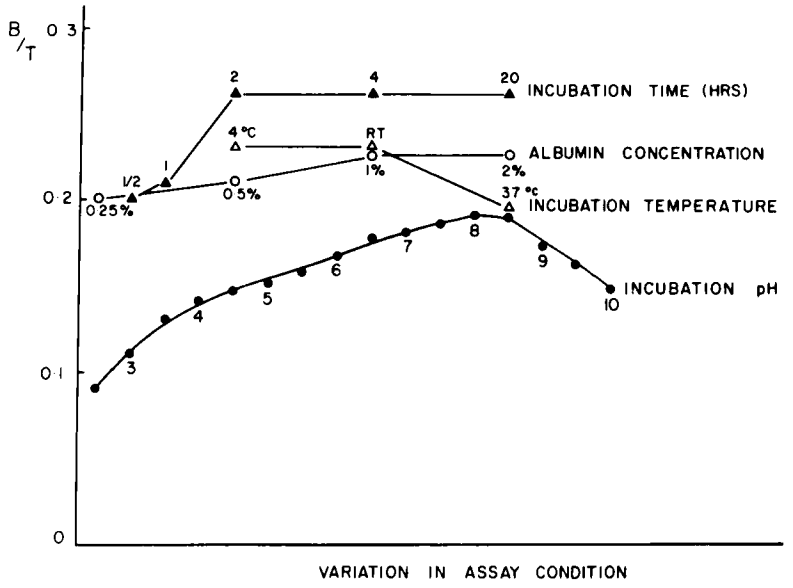

Fig. 2. Effect on IgG binding to protein A of incubation time, temperature, pH and albumin concentration. The variation in maximum binding reached is due to the experiments being done at different times. Maximum binding was reached after 2 hours incubation at an albumin concentration of at least 1%, a temperature of 4°C to room temperature and at a pH of 8 to 8.5.

OPTIMIZATION OF ASSAY CONDITIONS

The effect of temperature, incubation time, pH and albumin concentration of the assay buffer on binding at equilibrium was studied by incubating the assay components in the usual manner but varying any one of the above (see Fig. 2).

DETERMINATION OF CROSS REACTIVITY OF IgA, IgE, IgM and the Fc and $F(ab')_2$ FRAGMENTS OF IgG

A dose response curve, similar to the one described for IgG, was performed with IgA, IgE and IgM and the Fc and $F(ab')_2$ fragments of IgG. The procedure was identical to that used for IgG in every respect except that equimolar amounts of one of the above were substituted for the IgG.

EFFECT OF SERIAL DILUTION OF NORMAL HUMAN AND SLE SERA AND RABBIT AND GUINEA PIG SERA ON THE AMOUNT OF IgG DETECTED

Serum samples from the above sources were assayed at various dilutions and the results compared after a correction had been made for the dilution.

EFFECT OF MOUSE MONOCLONAL ANTI-HUMAN-DNA ANTIBODY ON BINDING OF ^{125}I-IgG.

Equimolar amounts of a mouse IgG antibody to human DNA, prepared by E.G. Tron (Paris, France) using a hybrid cell line, were assayed and the effect on inhibiting ^{125}I-IgG binding compared to that of the human IgG standard.

ASSAY PRECISION

The intra-assay precision was determined by assaying 36 replicate 20 µl aliquots of a 1:20,000 dilution of human serum with an IgG concentration in the mid-normal range. Inter-assay precision was based on measuring aliquots from the same serum in 12 consecutive assays. The coefficients of variation (CV) were then calculated.

MEASUREMENT OF IgG LEVELS IN HUMAN SERUM

Serum was collected from 20 normal volunteers ranging in age 23 to 45 years of age. The samples were diluted 1:20,000 with assay buffer and 20 µl aliquots (containing one nanoliter of neat serum) were used in the assay.

CORRELATION WITH NEPHELOMETRIC ASSAY USING A RABBIT ANTI-HUMAN IgG ANTIBODY

Thirty-seven clinical samples from patients with varying IgG abnormalities were assayed by a nephelometric assay, based on a rabbit anti-human IgG antibody (6). They were then assayed by the protein A assay using a 1 nanoliter volume of neat serum added as 20 µl of a 1:20,000 dilution. The results of the two assays were plotted against each other and their coefficient of correlation calculated.

RESULTS

It was found that maximum binding of ^{125}I-IgG occurred after two hours incubation (Fig. 2). Binding was similar at 4^{0}C and room temperature but significantly less at 37^{0}C. Maximum binding occurred at pH 8 to 8.5 but the increase in binding over that occurring at physiologic pH was minimal. Figure 2 also demonstrates the effect of albumin concentration in the assay buffer: binding is reduced at a BSA concentration of less than 1%. Based on these findings assays were performed at room temperature, using an incubation time of 2 hours and an assay buffer of pH 7.4 containing 1% BSA. Non-specific binding varied between 1.5 and 4%.

Figure 1 shows the dose response curve obtained with a human IgG standard. The initial part of the curve shows that unlabelled IgG in the femtomole range causes significant inhibition of labelled IgG binding. Scatchard analysis of the curve revealed an initial affinity constant of 1.4 x 10^{9} L/mol (7).

Figure 3 shows that serial dilutions of pooled normal human serum, human SLE serum, rabbit serum and guinea pig serum parallel the dose response curve obtained with the standard human IgG preparation.

Immunoglobulins A, E and M had no inhibitory effect on the binding of radiolabelled IgG (Fig. 4). IgE showed some inhibitory effect at very high concentrations while both IgA and IgM showed an increase in the precipitation of radiolabelled IgG. The

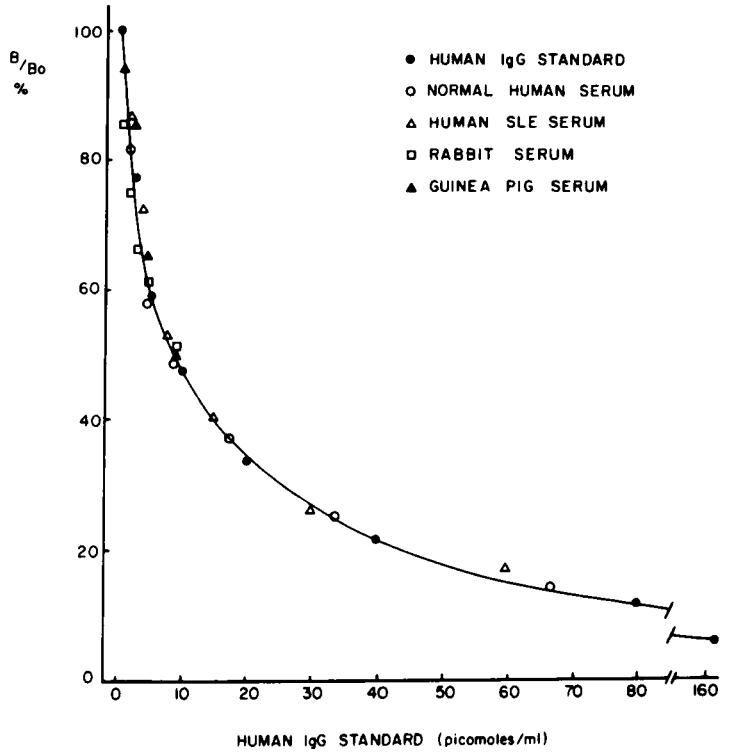

Fig. 3. Measurement of serum IgG from various species in serially diluted samples. Serum samples from a pool of normal human subjects, a patient with severe systemic lupus erythematosis (SLE), a rabbit and a guinea pig were diluted serially and assayed for the IgG concentration. The results were plotted by placing the intermediate results on the standard curve while the remaining values were plotted by serially doubling the less diluted samples and serially halving the more diluted ones. In order to be able to compare assays performed at different times the results were expressed as B/B_0. All four serum samples parallelled the curve obtained with the standard human IgG preparation.

$F(ab')_2$ fragments had no effect on IgG binding while the Fc fragments parallelled the effect of the standard human IgG preparation at low concentrations and was somewhat less effective at high concentrations. A monoclonal mouse IgG raised against human DNA showed a greater effectiveness in inhibiting the binding of labelled IgG than did the standard human IgG preparation. This effect was confined to the low concentration (Fig. 4).

The intra-assay CV for a human serum sample with an IgG concentration in the mid-normal range was 2.6 percent while the interassay CV was 6.5%.

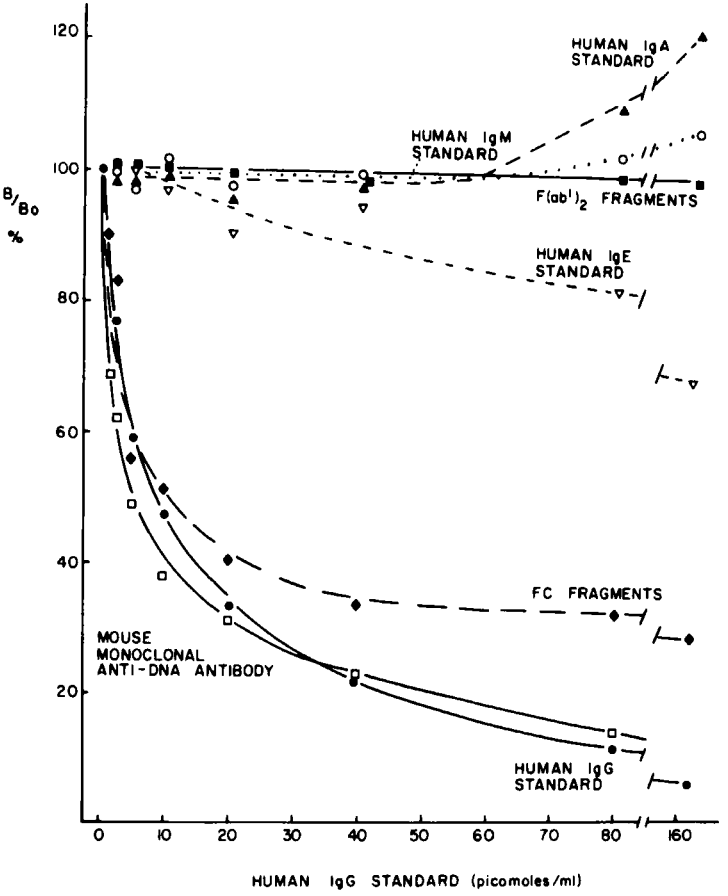

Fig. 4. Comparison of the binding of various immunoglobulins and IgG fragments to the Protein A of Staph aureus. Equimolar concentrations of IgA and IgM and the $F(ab')_2$ fragment of IgG had no inhibitory effect on the binding of ^{125}I-IgG while IgE had a minimal effect (possibly due to IgG contamination of the sample). The effect of the Fc fragment of IgG was similar to intact IgG at low concentrations but somewhat less at higher concentrations. A mouse monoclonal anti-DNA antibody preparation competed more effectively for the binding sites than the purified IgG standard at low but not high concentrations.

The mean (±SD) serum concentration of IgG in 10 normal males was found to be 10.4 ± 3.4 g/L while in 10 normal females it was found to be 9.6 ± 4.1 g/L. The results obtained in 37 patients with widely varying IgG levels were compared to a nephelometric assay and the correlation coefficient was found to be 0.89.

DISCUSSION

The radioligand assay described here is based on the ability of protein A to bind IgG with great specificity and high affinity. The results show that IgG can be measured in very small quantities, for example 1 nanoliter of human serum, using Staphylococcus aureus protein A as an IgG-specific receptor. There was no cross-reactivity with either IgA or IgM while the small amount of cross-reactivity observed with the IgE preparation may have been due to contamination of the specimen with IgG (Fig. 3). The increased precipitation of ^{125}I-IgG at high concentrations of both IgA and IgM is unexplained but may have been due to an increase in viscosity. There was nearly complete cross-reactivity with the Fc fragment of human IgG but none with the $F(ab')_2$ fragment. The IgG from normal subjects, patients with SLE or from a guinea pig, rabbit or mouse exhibited complete cross-reactivity with the standard human IgG preparation. The mean (±SD) concentration of IgG in twenty normal subjects was 10 ± 3.6 g/L, a value similar to that found by other methods (8). Similarly, the results obtained with this assay compared well with a nephelometric method (6).

Protein A was discovered in 1940 by Verwey (1). It is produced by most coagulase positive strains of staphylococcus and in particular by the Cowan 1 strain (9). It readily binds the IgG of various species, including man, rabbit, guinea pig and mouse (4). This fact has led to its use as a separating agent in immunoassays (10). In the human species it does not bind IgG_3, one of the four subclasses (3). However, IgG_3 represents less than 10% of the total IgG in man. Binding is via a specific and unique region on the Fc part of IgG. As IgA, IgE, IgM and IgG_3 do not possess such a specific Fc part they are not bound by protein A (3, 4, 7). This feature has been used to separate IgG from the remaining immunoglobulins.

The ability to detect very small amounts of IgG with the assay described here is primarily a function of the high affinity of IgG for protein A. The fact that IgG molecules are readily labelled with ^{125}Iodine to a high specific activity is an added advantage while the large size of the Staph aureus bacteria obviates the need for a separating agent. The assay in its present configuration was readily able to detect amounts smaller than 50 femtomoles of IgG, the amount contained in approximately one nanoliter of human serum. Advantages of the assay include its sensitivity and specificity, its simplicity and its low cost. Disadvantages include the fact that subclass IgG_3 is not measured and there is a need to newly prepare radioiodinated IgG every two to three months.

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