NONPRECIPITATING INSULIN ANTIBODIES[1, 2]

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(Submitted for publication November 13, 1957; accepted February 6, 1958)

Most immunologic tests currently in use depend upon the detection and quantitation of some result of the union of antigen and antibody. Such consequences of union include precipitation, agglutination, complement fixation and hemolysis. It is evident that the union of antigen and antibody precedes and is a necessary condition for the occurrence of these and other secondary phenomena. There have always been excellent reasons to believe that antigen could combine with antibody without the occurrence of such secondary reactions. A major difficulty in certain types of immunologic work in the past has been the necessity of determining the presence or the amount of antibody by measurement of the result of its union with the antigen rather than by the direct measurement of such a combination itself. A method which permits direct measurement of the union of antigen and antibody has obvious value for the study of systems where secondary reactions are either nonexistent or difficult to measure. The antibody which combines with insulin and which is the subject of this and the following paper is such an antibody. Its existence has long been suspected because of occasional local reactions to insulin and because of some of the biologic effects which the plasma of insulin treated patients has been known to possess. Until electrophoretic studies (1) demonstrated the combination of I^{131} insulin with gamma globulin of selected patients, there was no direct evidence of the existence of an antibody which combines with insulin in any human plasma. The recent work of Farr (2) demonstrating antibody with binding capacity for bovine serum albumin (BSA) stimulated the search for a similar method of demonstrating antibodies to insulin. A method of precipitating bound insulin with antihuman gamma globulin was finally selected. A similar method was used by Feinberg in a rabbit system (3).

MATERIALS AND METHODS

Antihuman gamma globulin (AHGG) was prepared in rabbits by repeated injections of human globulin either as a solution in 0.85 per cent NaCl or in a water-in-oil emulsion. The human gamma globulin (HGG) was obtained from The Cutter Laboratories and labeled with I^{131} (4). The titer of AHGG was determined either by estimating the equivalence point of the precipitin curve or more accurately by the labeled antigen precipitation method (5).

To 0.5 ml. of a 1:25 dilution of human serum was added 0.5 ml. of 1:40,000 dilution of I^{131} insulin containing 0.1 μg. insulin per ml. (from Abbott Laboratories). The specific activity of the I^{131} insulin was 0.5 mc. per mg. The mixture of serum and insulin was allowed to incubate two or more hours and AHGG was added. After standing in the cold overnight, the mixture was centrifuged for 30 minutes at 2,000 rpm, decanted, washed once, and the radioactivity of the precipitate and supernatant counted. All dilutions and washings were performed with a nine parts normal saline, one part borate buffer (pH 8.4) solution. All procedures were done in duplicate at 4° C.

Human anti-insulin serum was obtained from a patient receiving injections of insulin. This serum and I^{131} insulin were mixed in concentrations known to bind approximately 80 per cent of the insulin. Identical mixtures were prepared in two tubes. After incubation for two hours, sufficient rabbit AHGG was added to one tube to precipitate all the HGG. To the other tube was added an equal volume of normal rabbit serum. The mixtures were then centrifuged for one hour at 600 × G, and the supernatants fractionated electrophoretically on a starch block (6).

RESULTS AND DISCUSSION

If AHGG is added to HGG in a molecular ratio of 3:1, there is approximately 100 per cent precipitation of HGG (Figure 1, a). Insulin tagged with I^{131} was found to be soluble in AHGG, in any HGG, and in the supernatant of an AHGG and HGG mixture (Figures 1, b and c). When

[1] Presented in part before the Central Society for Clinical Research, November 9–10, 1956, Chicago, Ill.

[2] This work was done with the support of Grant E-1378 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health.

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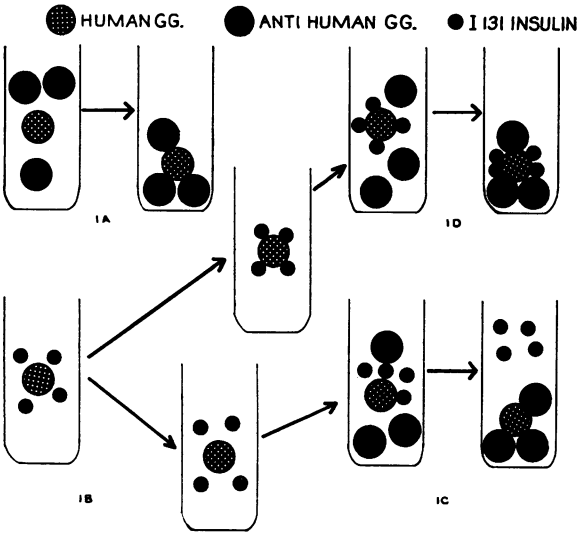

FIG. 1. DIAGRAMMATIC REPRESENTATION OF THE PRINCIPLE BY WHICH ANTIHUMAN GAMMA GLOBULIN (AHGG) DEMONSTRATES THE PREEXISTING UNION BETWEEN I^{131} INSULIN AND HUMAN GAMMA GLOBULIN (HGG)

HGG is precipitated by AHGG, insulin bound by HGG is found in the precipitate (Figure 1, d).

An experiment was performed to determine the relationship between the precipitation of gamma globulin and the demonstration of the insulin gamma globulin complex. Varying amounts of AHGG were added to constant mixtures of : 1) I^{131} insulin and a human anti-insulin serum from a patient treated with insulin; 2) trace amounts of I^{131} HGG and the same anti-insulin serum; and 3) I^{131} insulin and a normal human serum. The results in Figure 2 show that the identification of the gamma globulin-insulin complex depends upon the quantitative precipitation of the gamma globulin. The precipitation of globulin does not increase the binding of insulin; it merely demonstrates the already existing union.

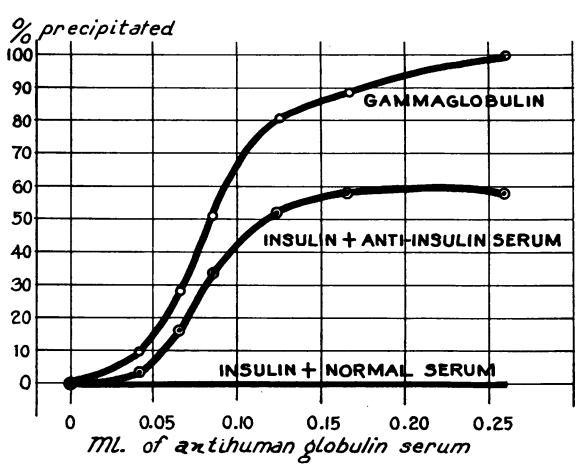

FIG. 2. DEMONSTRATION THAT MEASUREMENT AND IDENTIFICATION OF INSULIN-GAMMA GLOBULIN COMPLEXES DEPEND UPON THE QUANTITATIVE PRECIPITATION OF GAMMA GLOBULINS

The curve labeled gamma globulin refers to the per cent of labeled gamma globulin precipitated with each concentration of the antihuman gamma globulin added. The other two curves refer to the per cent of insulin demonstrated in the precipitate.

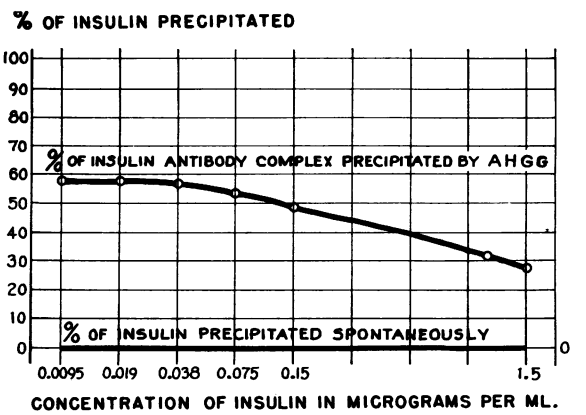

FIG. 3. EFFECT OF INSULIN EXCESS ON PER CENT BINDING

The percentage of insulin which is bound to gamma globulin is decreased although the total amount bound is increased.

In another experiment, varying amounts of I^{131} insulin were mixed with a constant amount of an anti-insulin serum before the addition of AHGG. The amount of AHGG added was that amount which was previously determined to precipitate 100 per cent of the gamma globulin in this serum. The time of incubation before addition of AHGG was two hours; longer incubation did not yield higher percentages of insulin bound. The results of this experiment as shown in Figure 3 indicate that the reaction between insulin and globulin behaves according to the mass action law. A fivefold increase in the amount of insulin added reduced the fraction of insulin precipitated by a factor of only two. The total amount of insulin bound increased, therefore, by a factor of two and one-half. Since the total quantity of insulin bound

was increased by increasing the insulin concentration, it is evident that there was incomplete saturation of binding sites at the lower concentration. Such an incomplete saturation of binding sites could occur at equilibrium in the presence of excess insulin only if the bond between insulin and globulin were reversible. This conclusion is in agreement with that of Berson, Yalow, Bauman, Rothschild, and Newerly (1), who demonstrated the reversibility of the insulin-globulin bond directly by an exchange between labeled and unlabeled insulin.

Figures 4 and 5 give the results of an electrophoretic separation of an insulin anti-insulin mixture before and after precipitation of the HGG with AHGG. The fact that precipitation of the I^{131} insulin-HGG complex by AHGG did not result in an increase in the amount of free insulin indicates that precipitation of the complex does not result in dissociation of the insulin from the globulin.

The precipitation of an antigen-antibody complex with an antiglobulin serum is a relatively simple method of demonstrating a nonprecipitating antibody. The same principle has been used to study the binding by selected human sera of

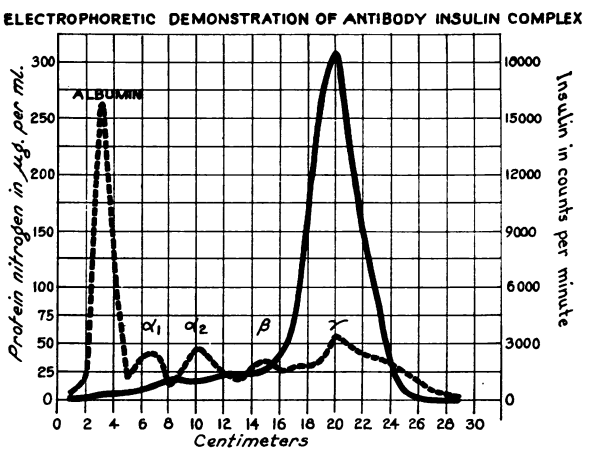

FIG. 4. ELECTROPHORETIC PATTERN OF I^{131} INSULIN ADDED TO SERUM CONTAINING INSULIN BINDING ANTIBODIES

The dotted line shows the electrophoretic migration pattern of this patient's serum protein. The solid line shows the migration pattern of I^{131} insulin with this serum on starch block. With a normal serum, unbound insulin migrates at approximately the same speed as albumin.

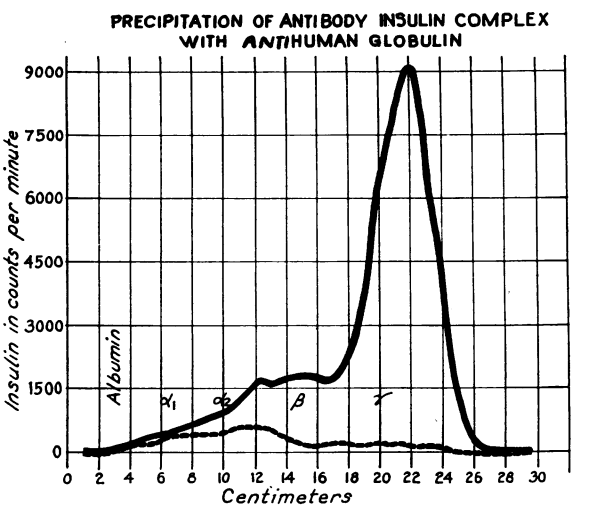

FIG. 5. ELECTROPHORETIC MIGRATION PATTERN BEFORE AND AFTER PRECIPITATION OF THE I^{131} INSULIN HUMAN GAMMA GLOBULIN COMPLEX

For the purpose of simplicity the serum protein curve is omitted. The solid line shows, as in Figure 4, the migration pattern of insulin bound to another patient's serum. The dotted line shows the electrophoretic distribution of the radioactivity remaining after the insulin-gamma globulin complex in this serum is precipitated by antihuman gamma globulin.

I^{131} thyroxine (7) and C^{14} labeled progesterone (8). The absence of detectable binding in the latter case confirms the electrophoretic studies of Westphal (9). In addition, AHGG has been used to separate gamma globulin from Bence Jones protein in tissue culture in order to study the differential incorporation of C^{14} lysine into these products (10).

SUMMARY

A method is presented which measures the binding of insulin by human serum. The method is similar to that previously described by Feinberg in the use of an anti-gamma globulin serum to precipitate a soluble complex of antigen and gamma globulin. The method may be useful in demonstrating antibodies to other hormones.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Dr. Peter Stelos for performing the electrophoretic studies, and to Mr. William Cheng for his technical assistance.

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