A Solid Phase Radioimmunoassay for the Detection of Anti-Insulin Antibodies

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Abstract

A solid phase radioimmunoassay for the detection of anti-insulin antibodies has been developed and evaluated. After coating round-bottomed wells of polyvinyl chloride with porcine monocomponent insulin, 50 μ l of serum from either insulin-treated diabetics or non diabetic controls was added to the wells, followed by the addition of ¹²⁵I-protein A, and then the radioactivity of the bound ¹²⁵I-protein A on the wells was counted. This solid phase radioimmunoassay is very simple, does not require centrifugal steps and the results correlated well with those of previously published immune precipitation methods ($r=0.68$, $p<0.01$).

Our present method requires only 50 μ l of serum and 4 hours for the assay, if the wells are coated with insulin in advance. Therefore, this procedure is thought to be suitable for clinical use.

It is well known that nearly all insulin-treated diabetic patients develop antibodies to insulin. Although the clinical significance of these antibodies regarding the extent of their influence on diabetic complications is not fully understood, there is no doubt that they play a role in some forms of insulin resistance.

Several techniques have been introduced for the measurement of antibodies to insulin (Feldman *et al.*, 1963; Hebert *et al.*, 1965; Hedding, 1965; Welborn *et al.*, 1967; Dixon, 1974; Gerbitz and Kemmler, 1978). These procedures involve the addition of radiolabeled insulin to serum and separation of free and bound insulin by precipitating either free insulin (Feldman *et al.*, 1963; Hebert *et al.*, 1965) or antigen-antibody complexes with ethanol (Hedding, 1965; Welborn *et al.*, 1967) or with polyethylene

glycol (Dixon, 1974; Gerbitz, 1978).

We wish to report here a simplified assay for the detection of anti-insulin antibodies using immobilized insulin. This assay, in contrast to the methods previously reported, does not need centrifugation and is very easy to operate, requiring only a relatively short time. Also only 50 μ l of serum is necessary for the detection of anti-insulin antibodies.

Materials and Methods

Solid phase radioimmunoassay with ¹²⁵I-protein A.
Determination of the optimum concentration of insulin to be used in coating the wells.

Porcine monocomponent insulin (NOVO industry, Denmark) was dissolved in phosphate-buffered saline (PBS), pH 7.4, $\mu=0.16$ from concentrations of 10^{-1} mg/ml to 10^{-5} mg/ml and 100 μ l of insulin solution thus prepared were poured into round-bottomed wells of polyvinyl chloride. The wells were left for 2 hours at 25°C and then the insulin solution was

removed from them. Residual binding sites on the wells were blocked by incubation with 0.1% equine myoglobin dissolved in PBS for one hour at 25°C. After washing the wells with PBS three times, 50 μ l of serum from either insulin-treated diabetics or non diabetic controls was added and incubated for 2 hours at 25°C. Unbound serum was washed off with PBS three times, followed by incubation with constant amounts of 125 I-protein A (10 μ g; 30×10^3 c.p.m. and 20 μ g; 60×10^3 c.p.m.). Two hours later, wells were washed with PBS three times and each well was cut off and radioactivity of the bound 125 I-protein A on each well was counted using a Searl γ -counter.

Determination of the incubation time of 125 I-protein A.

Using two sera from insulin-treated diabetic patients (one had a relatively high titer, the other had a low titer of anti-insulin antibodies), the experiment was carried out exactly as shown above, except for the incubation time with 125 I-protein A. After washing unbound antibodies from the wells, constant amounts of 125 I-protein A (20 μ g; 60×10^3 c.p.m.) were added to each well at 25°C and the effect of the incubation time on the binding of 125 I-protein A to the anti-insulin antibodies was examined.

Intra- and interassay variations

To check the reproducibility and stability of the solid phase radioimmunoassay using 125 I-protein A, coefficients of variations (C.V.) of intraassay as well as interassay were determined using two sera obtained from insulin-treated diabetics.

Immune precipitation of 125 I-insulin with polyethylene glycol.

In order to compare our present solid phase radioimmunoassay with other published methods, the sera were measured for the activity of anti-insulin antibodies by immune precipitation with 125 I-insulin (Table 1). Dextran coated charcoal (DCC) was prepared by mixing activated 5.0 g Norit A charcoal and 0.5 g dextran T 70 in 200 ml of 0.05 M Glycine-HCl buffer, pH 3.0 while constantly stirring with a magnetic stirrer to prevent settling during the procedure. Two hundred microliters of sera were added to 200 μ l of 0.10 M HCl, followed by the addition of 600 μ l of DCC solution. Then they were mixed with a vortex mixer. The mixture was left for ten minutes at 25°C. After centrifugation, 800 μ l of the supernatant was transferred into two tubes (400 μ l each) and neutralized by the addition of 400 μ l of 0.10 M phosphate buffer (pH 7.4) to each tube, followed by the addition of 0.10 M NaOH. This was followed by further adding 100 μ l of 125 I-insulin dissolved in 0.1% equine myoglobin solution and then it was incubated at 37°C for an hour and at 4°C for 12 to 16 hours. After incubation, 1.0 ml of 25% polyethylene glycol (PEG, MW 6,000) dissolved in PBS was added to each tube, mixed well and centrifuged at 1,800 G for 20 minutes at 4°C. The supernatant was aspirated and the precipitated radioactivity was counted with a searl γ -counter.

Quantitation of anti-insulin antibody titers

Quantitation of anti-insulin antibody titers in the solid phase radioimmunoassay using 125 I-protein A was expressed as either bound c.p.m. or the percent-

Fig. 1. Solid phase radioimmunoassay procedures for the detection of anti-insulin antibodies.

tage of ^{125}I -protein A bound to the wells. In the immune precipitation methods, counted radioactivity was expressed quantitatively as percentage binding, that is the percentage of ^{125}I -insulin precipitated with immunoglobulins.

Protein A and equine myoglobin were purchased from Sigma Chemicals (St. Louis, MO). ^{125}I Na was obtained from Amersham (England) and radioiodination of protein A and insulin was done according to the method of Hunter and Greenwood (1962). ^{125}I -protein A thus prepared was kept in 0.1% equine myoglobin solution at 4°C until used. PEG with an average molecular weight of 6,000 was obtained from Nakarai Chemicals (Kyoto). Other chemicals employed were all reagent grade.

Results

Determination of the optimum concentration of the insulin to be coated on the wells

Fig. 2. Determination of the optimum concentration of the porcine insulin to be coated on the wells of polyvinyl chloride. After incubation with serum from an insulin-treated diabetic patient, anti-insulin antibodies were detected either by 30×10^3 c.p.m (○) or 60×10^3 c.p.m (Δ) of ^{125}I -protein A.

As shown in Fig. 2, the bound radioactivity of ^{125}I -protein A was almost on a plateau at a porcine insulin coating concentration of 10^{-2} mg/ml. Therefore, in the following experiments, coating of the wells with porcine insulin was done at a concentration of 10^{-2} mg/ml.

Determination of the incubation time of ^{125}I -protein A

The results shown in Fig. 3 indicate that the incubation of ^{125}I -protein A was enough after one hour at 25°C . Based on these results, all the steps of this method are summarized in Fig. 1. If the wells of polyvinyl chloride were coated with porcine

Fig. 3. Time course studies of the binding of ^{125}I -protein A with human anti-insulin antibodies at 25°C , using sera either from relatively high titers of (○) or low titers of (Δ) anti-insulin antibody activity. A constant amount of ^{125}I -protein A (58×10^3 c.p.m) was added to each well and binding with anti-insulin antibodies was examined every hour for up to 6 hours. (□) indicates non specific binding using bovine albumin coated polyvinyl chloride wells and represents 5% or less of the total ^{125}I -protein A added.

Fig. 4. Correlation between solid phase radioimmunoassay using ^{125}I -protein A and the immune precipitation method. The same sera either from insulin-treated diabetic patients ($\circ$) or non diabetic normal controls ($\bullet$) were measured for their antibody activity using the two methods. The correlation coefficient between these two methods was 0.68 ($p < 0.01$).

Table 1. Immune precipitation method for detection of anti-insulin antibodies.

Serum 200 μl + 200 μl of 0.10 M HCl
Addition of 600 μl of DCC suspension (pH 3.0)
Centrifugation at 1,800 G, 20 minutes, at 4°C
↓
400 μl of supernatant
+ 400 μl of 0.1 M phosphate buffer (pH 7.4)
+ 100 μl of 0.10 M NaOH
+ 100 μl of ^{125}I -insulin ($30\text{--}40 \times 10^3$ c.p.m.)
One hour at 37°C, followed by 12–16 hours at 4°C
↓
Addition of 1.0 ml of 25% PEG
Mixed well and centrifuged at 1,800 G for 30 minutes at 4°C
↓
Aspiration of supernatant and counting radioactivity

Table 2. Intra- and interassay variations of solid phase radioimmunoassay using ^{125}I -protein A

	Intraassay (Mean $\pm$ SD)	Interassay (Mean $\pm$ SD)
Case 1	15.34 $\pm$ 15.0% CV = 9.8%	14.98 $\pm$ 1.42% CV = 9.5%
Case 2	21.28 $\pm$ 2.02% CV = 9.5%	20.01 $\pm$ 1.84% CV = 9.2%

In order to check the stability of the ^{125}I -protein A method, sera obtained from two insulin-treated diabetics (case 1 and case 2) were measured ten times for their anti-insulin antibody activity in the same assay and CV values were calculated. Reproducibility of ^{125}I -protein A method was quantitated by CV values obtained by measuring the anti-insulin antibody activity by six different assays, respectively.

insulin in advance, the time necessary for the whole procedure was only 4 hours at most.

Intra- and interassay variations

The results of intra- and interassay of two sera are shown in Table 2. The coefficients of variation of intraassay in case 1 and 2 were 9.8% and 9.5% and those of

the interassay were 9.5% and 9.2%, respectively.

Comparison of the present solid phase radioimmunoassay with the immune precipitation method

Sera from 17 insulin-treated diabetics and 6 non-diabetic controls were measured simultaneously to determine their anti-insulin

antibody activity using present solid phase radioimmunoassay and the immune precipitation method. The titer of anti-insulin antibodies as measured by ^{125}I -protein A correlated well with percent binding ($r=0.68$, $p<0.01$) obtained using the immune precipitation method (Gerboth and Kemmler, 1978) (Fig. 4).

Discussion

Previously, one of the present authors (S. Sakata) reported a very sensitive radioimmunoassay for the detection of the autoantibodies to rabbit serum albumin in the rabbit immunized with the rabbit's own serum albumin, human serum albumin, or bovine serum albumin. In these experiments, ^{125}I -protein A and ^{125}I -goat anti-rabbit IgG were employed as a second antibody and we found ^{125}I -protein A to be the better one (Sakata and Atassi, 1981). This result led us to extend to other immunoassay systems for the detection of the antibodies. Anti-insulin antibodies were selected next, because of their clinical importance in the care of insulin-treated diabetic patients. As our present results indicate, this solid phase immunoassay worked well in the detection of anti-insulin antibodies.

Protein A is a protein isolated from the wall of the staphylococcus aureus. The characteristic property of protein A is its ability to interact and to precipitate with a wide variety of IgG molecules from several species via the Fc part of the IgG molecule. Kronvall and Williams (1969) found that the precipitation reaction is specific for sub-class 1, 2 and 4 of human IgG. It has also been reported that IgA 2 binds to protein A while IgA 1 does not (Saltvedt and Harboe, 1976). A minority of IgM is also reported to bind to protein A (Harboe and Folling, 1976). Although anti-insulin antibody activity in sera has been demonstrated in all 5 major classes of

immunoglobulins, most of the activity has been shown in the IgG class (Andersen, 1976). IgG 3 accounts for only 1-3% of total human IgG, but may have anti-insulin activity. In addition, the presence of IgM class anti-insulin antibodies has been reported in insulin-treated patients, especially early in the course of insulin treatment and in patients with insulin resistance (Reisman *et al*, 1969). Moreover, IgA (Kniker, 1967), IgD (Devey *et al.*, 1970) as well as IgE (Dolovich *et al.*, 1970) class anti-insulin antibodies have been found in patients with insulin resistance and allergies. Such anti-insulin antibody activity might not be detected in this assay. Therefore, compared with other reported methods such as immune precipitation, our present method might have a disadvantage regarding the assayable immunoglobulins and their subclasses. However, in our examination of the sera from 17 insulin-treated diabetic patients, this was hardly a problem, for if high titers of anti-insulin immunoglobulins are present in other than IgG class, high percent binding of ^{125}I -insulin and very low or negative radioactivity of the ^{125}I -protein A could be expected in the same serum, but there wasn't such a case.

The reason why the correlation coefficient between two methods stayed on the level of 0.68 may arise from the difference of the treatment of sera employed. Sera used for the immune precipitation method were treated with acid charcoal to remove endogeneous and/or exogeneous insulin, while in solid phase radioimmunoassay, sera were used without charcoal treatment, because our primary aim was to develop a simple and convenient method for detection of anti-insulin antibodies. Thus acid charcoal treatment makes possible to detect all the activities of anti-insulin antibody regardless it is bound with insulin or not, while solid phase radioimmunoassay may not have detected all the anti-insulin antibodies, particularly if it is occupied with

insulin.

In summary insulin-coated solid phase radioimmunoassay is sensitive enough to detect anti-insulin antibodies with only 50 μ l of serum. Also the whole procedure are very simple and easy to operate. If the wells are coated in advance, only 4 hours is required for the assay. It is therefore a convenient method to use in detection of anti-insulin antibodies in insulin-treated diabetic patients.

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