Factors Affecting Enzyme-Linked Immunosorbent Assay (ELISA) for Insulin Antibodies in Serum

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We have evaluated the factors affecting an enzyme-linked immunosorbent assay (ELISA) for circulating insulin antibodies. Dilutions of patients' sera were incubated in polystyrene tubes coated with porcine insulin. The second incubation was with alkaline phosphatase-conjugated anti-human Fab. Each of these reactions was complete after 4 h. Specificity of the reaction for insulin antibodies was demonstrated by removal of anti-insulin activity after preincubation with insulin, but not with glucagon at similar concentrations. Sensitivity of the ELISA system, assessed by performing the reaction with affinity-column purified insulin antibodies, was 10 μg of specific antibody per liter. Using this system, we examined sera from 22 patients who had been determined by radioimmunoassay to have insulin antibodies, and sera from 23 normal individuals. The ELISA results correlated reasonably well with those of RIA (r = +0.84). Besides detecting insulin antibodies in diabetic patients who are being treated with insulin, we use this ELISA test as a screening procedure to be certain insulin antibodies are not present when we use indirect immunofluorescence methods on fixed pancreatic substrate to detect islet-cell antibodies.

Additional Keyphrases: screening for insulin antibodies • monitoring therapy • diabetes

Since 1938, when Banting et al. (1) described insulin-neutralizing substances in the serum of an insulin-treated patient, several investigators have studied these antibodies. The prevalence of circulating insulin antibodies (1) after the institution of insulin therapy is high, although there is some disagreement as to the actual percentage (2, 3, 3a). Also, the clinical significance of these antibodies is a matter of considerable debate. Some groups claim that they are related to immunopathological injury, including retinopathy and glomerulosclerosis (3–7); others found no relationship of insulin antibodies to small-vessel injury (8–10).

Recently another circulating antibody, islet-cell antibody, has been found early in the course of juvenile-onset diabetes (11, 12). It is still unclear whether it plays a role in the pathogenesis of diabetes mellitus or is merely a concomitant event that serves as a convenient marker of the disease. Islet-cell antibodies are detected in our laboratory by an indirect immunofluorescence technique on Bouin's-fixed substrate (13). Because the presence of insulin antibodies may interfere with this technique, a sensitive, specific screening test for insulin antibodies is of great importance for correctly assessing the presence of islet-cell antibodies.

We have assessed several factors affecting an enzyme-linked immunosorbent assay (ELISA) technique for measuring insulin antibodies and compared this method with a standard radioimmunoassay (RIA). The ELISA affords the following advantages: the enzyme conjugates and the substrate reaction products are stable; results can be read visually or with a spectrophotometer; the assay is inexpensive to perform; and it lacks the potential hazards and problems of disposal of radioactivity in radioimmunoassay.

Materials and Methods

ELISA was performed as previously described (14), with the following modifications. Disposable polystyrene tubes (12 X 75 mm; Falcon Plastics, Oxnard, CA) were coated with 0.5 mL of antigen solution (1 mg of purified porcine insulin per liter, except where stated) in 50 mmol/L carbonate buffer, pH 9.6 (coating buffer), at 37 °C for 4 h (except where noted). Tubes containing the antigen solution were covered with Parafilm (to prevent evaporation) and stored at 4 °C until use. Immediately before testing, the antigen solution was aspirated and the tubes were washed four times (5 min, with agitation, per wash) with PTA: phosphate-buffered isotonic saline containing, per liter, 50 mL of Tween 20 (Fisher Scientific Co., Pittsburgh, PA 15219) and 20 mg of sodium azide.

We diluted the patients' sera 10- and 100-fold (or as stated) in PTA, and added 0.5 mL to the antigen-coated tubes and to uncoated tubes (as a control for nonspecific adsorption). The tubes were incubated at room temperature 4 h (or as stated) on a horizontal rotary shaker and then again washed four times with PTA. We then added 0.5 mL of alkaline phosphatase-conjugated anti-human Fab (AP-AHF) to each tube. The reaction mixtures were incubated for 18 h (except where otherwise stated) at room temperature, with agitation.

After washing the tubes four times with PTA again, we added to each tube 1 mg of the substrate, disodium p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO 63178) in 1 mL of 50 mmol/L carbonate buffer (pH 9.8) containing 1 mmol of magnesium chloride per liter. The reaction proceeded at room temperature (with agitation) until the absorbance at 400 nm was approximately 1.0 [the enzyme-substrate reaction is linear to this level (15)], and stopped at 25, 50, or 100 min by the addition of 1 mL of 0.2 mol/L NaOH. All results were extrapolated to 100 min (15). The absorbances of the uncoated tubes at 400 nm were subtracted from those of the coated tubes containing the same solutions. All reactions were performed in duplicate.

Detection of insulin antibodies by RIA was a modification of a previously described procedure for detecting insulin (16). Briefly, 100 μL of plasma was incubated with 700 μL of barbital–saline buffer, 100 mmol/L, pH 8.6, containing, per liter, 0.1 mL of thimerosal, 2.5 g of human serum albumin, and 1 milli-int. unit of 125I-labeled insulin (porcine insulin, lot 818194; Eli Lilly and Co., Indianapolis, IN 46225) for three days at 4 °C. Then we added 2 mg of rabbit gamma-globulin (Cohn Fraction II, lot 81; Miles Labs., Elkhart, IN 46515) in 100 μL of assay buffer. After agitating the samples we added 1 mL of 250 g/L polyethylene glycol 8000 (Carbowax; Union Carbide, Rye, NY) in distilled water to each tube. After vortex-mixing for 5 s, we centrifuged the tubes at 2000 × g for 30 min at 4 °C. The supernate was decanted and the radioactivity...
of the precipitate was measured with a gamma counter (Model 1285; Searle Radiographers, Des Plaines, IL 60018). The percent antibody bound was calculated as counts in the unknown precipitate divided by the mean total counts. The samples were considered positive for insulin antibodies if the binding exceeded the mean +3 SD of sera from healthy subjects who had never received insulin.

Results

Optimal antigen coating of tubes. To determine the optimal concentration of insulin to coat the tubes, we incubated various concentrations of porcine insulin in coating buffer for 3 h at 37 °C (Figure 1). These tubes were tested with a 10-fold diluted patient's serum that had been shown by RIA to contain insulin antibodies (control positive serum). Although 10 mg of insulin per liter gave slightly stronger sensitization than 1 mg/L, a considerable decrease in sensitization occurred with a coating solution concentration of 100 mg/L. The most consistent reactivity was seen between 1 and 10 mg/L, and we therefore used this coating concentration for all future studies.

Kinetics of primary antibody binding. Using 100-, 1000-, and 10 000-fold dilutions of the control positive serum, we investigated the rate at which insulin antibodies were bound to the porcine insulin with which the tubes were coated, to determine whether the kinetics of primary antibody binding was related to antibody concentration. The antibody-positive sera were allowed to react with the antigen-coated tubes for various intervals (Figure 2). After the tubes were washed, AP-AHF was added and allowed to react for 18 h, followed by the substrate reaction. As shown in Figure 2, the primary antibody was completely bound after 3 h of incubation with coated tubes, for all concentrations tested. In fact, most of the binding was completed by the end of the first hour.

Kinetics of secondary antibody binding. Using tubes coated with porcine insulin by use of a 1 mg/mL solution, we investigated the rate of binding of AP-AHF at two concentrations of primary antibody (100- and 10 000-fold dilutions). The

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**Fig. 1.** Determination of optimal insulin concentration for coating tubes

Tubes were incubated for 3 h at 37 °C with the indicated concentrations of porcine insulin, then incubated with serum from a patient having circulating insulin antibodies. Enzyme-anti-human Fab conjugate was diluted 100-fold.

**Fig. 2.** Kinetics of the binding of primary antibodies to tubes coated with porcine insulin, 1 mg/L

Control antisera to insulin were diluted 1/10^2 (●), 1/10^3 (○), and 1/10^4 (△), incubated for the interval indicated, then incubated for 18 h with AP-AHF.

**Fig. 3.** Kinetics of the binding of secondary antibody (AP-AHF)

Tubes were coated by use of porcine insulin, 1 mg/L solution, reacted for 4 h with 100- or 10 000-fold diluted control antisera to insulin diluted 1/10^2 (●) or 1/10^4 (○), and then with AP-AHF for the intervals indicated.
primary antibodies were allowed to react for 4 h, followed by reaction with AP-AHF (diluted 100-fold in PTA) for various periods. The conjugate reaction was completed by 4 h for the two concentrations of primary antibody used (Figure 3). Extending the reactions for 28 h resulted in no increase in reactivity.

Reproducibility. Using the optimal conditions described above, we assayed 10 replicate samples of a 100-fold dilution of the control positive serum. The mean change in absorbance at 400 nm was 3.109 (SEM 0.120) after 100 min. The coefficient of variation was 12.15.

Sensitivity of ELISA. Human insulin antibody was purified from a serum with strong reactivity (both by ELISA and RIA) by loading 2 mL of the serum on an insulin-conjugated CH-Sepharose (Pharmacia, Piscataway, NJ 08854) affinity column (17). After extensive washing, the insulin antibody was eluted with 10 mmol/L HCl in isotonic saline and immediately neutralized. The eluate was tested by radioimmunoassay with 125I-labeled insulin, and a Scatchard plot was used to determine the amount of eluted insulin antibody. The eluate was assayed by ELISA as described above. As shown in Figure 4, a specific antibody concentration of 10 μg/L gave a mean change in absorbance of 0.170 at 400 nm in 100 min (approximately three times background).

Antigen specificity of ELISA. We used glucagon (lot no. 258-234 B-167-1; Eli Lilly and Co.) and porcine insulin (lot no. 818194; Eli Lilly and Co.) in absorption studies to ensure that ELISA reactions represented specific responses to the insulin coating the tube wall. The positive control serum was mixed with log dilutions of either glucagon or insulin and then reacted in the ELISA system in tubes coated with porcine insulin. The insulin antibody activity was completely removed by pre-incubation with 1 mg of porcine insulin per liter. In contrast, no activity was lost by preincubation with 1 mg of glucagon per liter (Table 1). Even when 100 mg of glucagon was added per liter, more activity remained than when only 10 μg of insulin was added per liter.

Correlation between insulin antibodies values as measured by ELISA and RIA. ELISA results were compared with the radioimmunoassay procedure for 45 samples (Figure 5). The correlation coefficient (r) was 0.84 and the coefficient index (r²) 0.71 (r=0.71, df=43). Thus, there was a significant positive correlation between ELISA and RIA.

Discussion

Although the antigenicity of insulin is well appreciated (2, 18, 19), the clinical significance of circulating insulin antibody is still quite debatable. Some workers have suggested that the combination of insulin antibody with insulin results in the formation of circulating immune complexes that accelerate the development of small-vessel complications of diabetes (20). However, as pointed out by Andersen (21), the fact that these late diabetic complications occur in patients who never receive insulin must allow for alternative routes of tissue injury without the presence of insulin antibodies. Since 1956 (2) an assay has been available for the demon-

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**Table 1. Antigenic Specificity of ELISA**

<table>
<thead>
<tr>
<th>Absorbing soln</th>
<th>ELISA results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>4.056</td>
</tr>
<tr>
<td>Insulin</td>
<td></td>
</tr>
<tr>
<td>1 ng/L</td>
<td>2.737</td>
</tr>
<tr>
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<td>1.296</td>
</tr>
<tr>
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<td>100 μg/L</td>
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</tr>
</tbody>
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* Control positive serum with insulin antibodies was reacted with the absorbing solution, then with tubes coated with porcine insulin. * Absorbance at 400 nm after reaction for 100 min.

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**Fig. 5.** Comparison of ELISA and RIA for detection of insulin antibodies

The abscissa shows % binding by RIA.
stration of insulin antibody in human serum. Until recently the methods of detection (22, 23), including assays for human "a component" antibody (24) and proinsulin-specific antibodies (25), have involved variations of radioimmunoassays. We have here explored the use of an ELISA method for rapid, efficient detection of insulin antibodies.

ELISA was originally described by Engvall and Perlmann (15) and was later modified to allow adsorption of antigens onto polystyrene tubes (26).

Telford and Wisdom (27) demonstrated that ELISA can be used to detect anti-bovine insulin antibodies. The optimal concentration of porcine insulin for coating polystyrene tubes was 1 mg/L. Concentrations exceeding 100 mg/L decreased activity. This decrease has been noted before (14, 28) and has been attributed to elution of the coating material during the primary antibody–antigen reaction (26). This eluted antigen can bind antibody in solution, leaving less antibody free to react with the antigen adsorbed to test tube walls.

Binding of the primary and secondary (alkaline phosphate-conjugated) antibodies was complete within 4 h of incubation at room temperature, which is similar to other ELISA systems (14, 28). The rapid completion of the primary and secondary antibody binding allows the assay to be conveniently completed in 24 h.

We found this system to be highly specific. Insulin, 1 mg/L, completely absorbed the antibodies being measured in our system, whereas the same amount of glucagon did not affect the measured antibody (see Table 1). Preincubation with higher doses of the glucagon preparation (100 mg/L) did remove some of the antibody, perhaps because of slight contamination with insulin. As little as 0.1% contamination of the glucagon solution with insulin antigen could account for this difference.

Comparing the ELISA with the RIA revealed a correlation coefficient (r) of +0.84 (statistically significant). We determined the mean ± 2 standard deviations of sera from control individuals. Of the 45 patients tested, one patient had a value that was considered positive for insulin-antibodies by ELISA but negative by RIA, while two patients were considered negative for insulin-antibodies by ELISA but positive by RIA.

The present ELISA system is being used as a screening procedure to measure insulin-antibodies in patients being evaluated for islet-cell antibodies. Because antibodies to insulin and other pancreatic hormones interfere with the immunofluorescence methods used to detect islet-cell antibodies (12, 13), prior screening of samples for insulin antibodies and antibodies to other hormones is essential. This seems to be especially important in methods that use formalin- or Bouin’s-fixed pancreas, as our recent (unpublished) correlation studies with Dr. Bottazzo have shown.

Because the present immunoassay system will measure unbound anti-insulin, it is important to obtain the serum sample just prior to the next scheduled insulin dosage in those patients on insulin therapy.

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Simultaneous Determination of 25-Hydroxyvitamin D, 24,25-Dihydroxyvitamin D, and 1,25-Dihydroxyvitamin D in Plasma or Serum

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We describe a simultaneous assay for the principal vitamin D metabolites: 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D, and 1,25-dihydroxyvitamin D. Special attention has been paid to simplification of the extensive extraction and purification procedures used in previously described simultaneous assays. All three metabolites were isolated with a single extraction step, followed by only one gradient liquid-chromatographic procedure. For final quantitation we used competitive protein binding assays, involving readily available binding proteins and commercially purchased tritiated vitamin D metabolites. Concentrations in the plasma of healthy subjects (mean age, 27 years), sampled during December were 51 (SD 17) nmol/L, 4.1 (SD 1.3) nmol/L, and 124 (SD 26) pmol/L for 25-hydroxyvitamin D, 24,25-dihydroxyvitamin D and 1,25-dihydroxyvitamin D, respectively. Intra- and interassay CVs for the three metabolites were 4.4 and 3.9%, 6.7 and 8.0%, and 7.0 and 4.8% respectively.

Additional Keyphrases: liquid gradient chromatography - competitive protein binding - reference interval

In the past decade, knowledge of the metabolism and the mechanism of action of vitamin D has substantially progressed as a result of availability of radiolabeled vitamin D metabolites for metabolism studies and the development of sensitive assays for the biologically active vitamin D metabolites (1–9).

Five reports have described the concurrent determination of these metabolites in blood, including 25(OH)D2, 24,25(OH)2D3, and 1,25(OH)2D3 (4–6). However, these methods are all too laborious for routine use with large numbers of samples. Here we report a relatively fast and reliable procedure for concurrently determining 25(OH)D3, 24,25(OH)2D3, and 1,25(OH)2D3.

Materials and Methods

Vitamin D metabolites. Crystallized 25(OH)D3 and 25(OH)D2 were obtained from Philips-Duphar, Weesp, The Netherlands, and The Upjohn Co., Kalamazoo, MI 49001, respectively. All other vitamin D3 metabolites (24R,25-dihydroxyvitamin D3, 24,25-dihydroxyvitamin D3, and 1,25-dihydroxyvitamin D3) were from Hoffmann-La Roche, Basle, Switzerland. 25-Hydroxy[27, methyl-3H]cholecalciferol (spec. act. 5–15 kCi/mol), 24R,25-dihydroxy[23,24(n)-3H]cholecalciferol (spec. act. 68 kCi/mol), and 1,25-dihydroxy[23,24(n)-3H]cholecalciferol (spec. act. 70–110 kCi/ml) were purchased from the Amersham International Ltd., Amersham, U.K. All vitamin D metabolites were stored in absolute ethanol at –20°C. 1,25(OH)2[3H]D3 for recovery monitoring was purified by HPLC on a Nucleosil 10-No column developed in n-hexane/isopropanol/water (90/10) at a flow rate of 2 ml/min. 25(OH)[3H]D3 and 24,25(OH)2[3H]D3 were used for recovery determination without further purification.

Reagents. All solvents for extraction and HPLC were of analytical grade and were used without further purification. Dextran (Grade C) was obtained from BDH Chemicals, Poole, U.K. Activated charcoal (analytical grade) was from Merck, Amsterdam, The Netherlands. Nucleosil 10-No packing material for HPLC columns was obtained from Macherey-Nagel, Düren, F.R.G. Conical 13 × 55 mm polyethylene tubes for competitive protein binding were from Baker Chemicals, Deventer, The Netherlands. Gelatin was from Brocades-ACF, Maarssen, The Netherlands. Radioactivity in aqueous solutions was measured in Lumagel liquid scintillation fluid (Baker Chemicals), with 36% counting efficiency. The scintillation fluid used in liquid scintillation counting for recovery measurement was Permafluor III (Packard Instrument Benelux S.A., Brussels, Belgium) diluted 10-fold with toluene.

Apparatus. The HPLC system consisted of two Model 110A pumps controlled by a Model 420 microprocessor (Altex Scientific, Berkeley, CA 94710), a 1203 UV III detector operated at 254 nm (LDC, Riviera Beach, FL 33404), and a WISP 710A automatic sample processor (Waters Associates, Milford, MA 01757). HPLC columns were packed by use of a Model 70.00 packing apparatus (Knauer, Berlin, F.R.G.). In turn with the HPLC column, a low-dead-volume three-way valve (Model 7030A, fitted with a Model 7163 solenoid valve; Rheodyne, Inc., Berkeley, CA 94710) was mounted to direct the flow to the fraction collector or to the ultraviolet detector. Fractions were collected with a Redicar 2112 fraction collector (LKB, Bromma, Sweden). Both the fraction collector and the three-way valve were controlled by the Model 420 micropro-