Application of a Rapid Enzyme-linked Immunosorbent Microassay (ELISA) to Study Human Anti-insulin Antibody

LAURA J. NELL, VALERIE J. VIRTA, AND JAMES W. THOMAS

SUMMARY

A rapid, reproducible enzyme-linked immunosorbent assay (ELISA) for antibody to insulin is characterized and used to study antibodies from insulin-treated diabetic subjects. No radioactivity is involved in the ELISA; rather, peroxidase-conjugated anti-human immunoglobulin is used to detect binding of antibodies to insulin-coated microplates. Color is produced by action of peroxidase on a substrate and an automated reader then measures binding spectrophotometrically. This ELISA was optimized to be at least as sensitive as measurement of antibody by direct ^{125}I-insulin binding. Although detection of IgG directly binding to insulin-coated plates in the ELISA does not reveal species-specific differences, avidity differences are shown in competitive inhibition with insulins from several species. Insulin-binding IgG was purified by affinity chromatography and used to construct a standard curve of the optical density (OD) in the ELISA relative to the concentration of antibody. This assay has been used to quantify and to characterize insulin antibody of sera from a large number of diabetic subjects, ranging from insulin resistant to those who had received only highly purified and human insulins. The assay is shown to be most useful to screen for insulin antibodies in resistant patients. DIABETES 1985; 34:60–66.

The immune response to insulin continues to present a potential complication in the management of diabetes mellitus. Insulin binding immunoglobulins were first demonstrated in the elegant studies of Berson et al.[1] Since then, several modifications of the radioimmunoassay (RIA) have been employed to measure insulin antibodies.[2–4] In the RIA, soluble radiolabeled insulin is used and binding is determined after separation of bound from free ligand. In contrast, the enzyme-linked immunoassay introduced by Engvall and Perlman uses ligand immobilized on a solid phase, usually tubes or microplates.[5] Antibody bound to the ligand is detected by specific anti-immunoglobulin conjugated to an enzyme. This interaction is measured after addition of substrate for the enzyme.The availability of automated readers for substrate color changes has led to wide use of enzyme-linked immunosorbent assays (ELISA) for both antigen and antibody determinations (reviewed in refs. 6 and 7). Since the ELISA is a solid-phase immunoassay, there are some theoretical and practical constraints that differ from fluid-phase immunoassays.[7] Notably, effective antibody affinity is increased due to interaction of the antibody with antigen at the solid-liquid interface. Having multiple antigenic determinants present on a solid phase results in deviation from the law of mass action in that antibody is more likely to interact with another antigen on dissociation than to diffuse away as would occur in solution.[7]

The applicability of a rapid and efficient method to study large numbers of sera without radioisotopes led us to characterize the optimum ELISA for insulin antibody. As used to screen diabetic sera for antibodies, the ELISA requires 20 μl of sample and is completed in less than 3 h. In this study, sera and purified antibodies from representative diabetic subjects were used in the ELISA and in standard ^{125}I-insulin binding assays.[8,9] The ELISA was shown to be efficient, rapid, and reproducible over a period longer than a year. We show that the assay detects 0.5–5.0 μg of insulin-specific IgG antibody and that species-specific antibodies are detected in competitive inhibitions. The ELISA offers a distinct advantage over ^{125}I-insulin binding assays that are influenced by the amount of insulin in serum. The ELISA is especially useful in evaluation of insulin-resistant patients and in studies of hormone metabolism, because pharmacologic levels of insulin attained during intravenous infusion do not interfere with the assay. Although more complex immunochemical analyses, such as Scatchard analysis, require RIA, the specificity, ease, and reproducibility of the ELISA make it extremely powerful in evaluating insulin immunity.

From the Department of Medicine, Rheumatology Section, Baylor College of Medicine, 1200 Moursund Avenue, Houston, Texas 77030.
Address reprint requests to Laura Nell, Ph.D., at the above address.
Received for publication 11 November 1983 and in revised form 25 May 1984.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/1/60/353253/34-1-60.pdf by guest on 26 March 2023

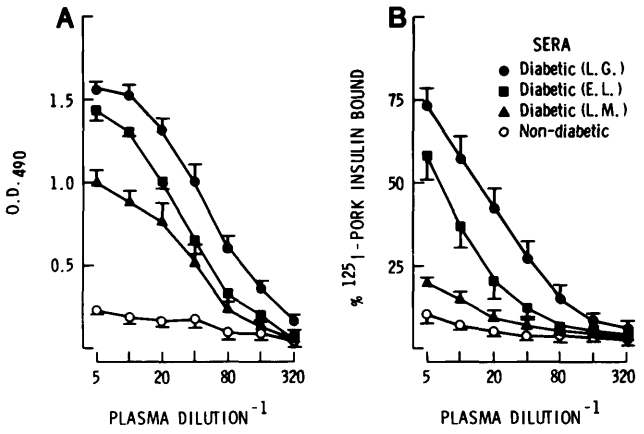

FIGURE 1. Plasma antibody binding to pork insulin. Serial dilutions of plasma from three diabetic subjects and from one nondiabetic control subject were tested for insulin binding in both assays. Panel A: IgG binding to pork insulin-coated plates in the ELISA. After incubation with peroxidase-conjugated, goat anti-human IgG, substrate for the peroxidase was added to the wells. Optical density is plotted versus plasma dilution. Panel B: ^{125}I-pork insulin binding. Plasma from the same subjects was incubated with ^{125}I-pork insulin and immune complexes were precipitated with polyethylene glycol. The percent ^{125}I precipitated is plotted versus the plasma dilution. Bars indicate 1 SD of the mean of at least three replicates.

MATERIALS AND METHODS

Insulins. Crystalline bovine insulin (lot 615-70N-80), porcine insulin (lot 615-O7J-256), and biosynthetic human insulin (lots 615-84S-178-2 and 615-2H2-270-3) were generously provided by Dr. Ronald Chance, Lilly Research Laboratories, Indianapolis, Indiana. Crystalline sheep insulin (lot 71F-0226) and horse insulin (lot 79C-0011) were purchased from Sigma Chemical Company, St. Louis, Missouri. The highly purified insulins contained <1–10 ppm proinsulin and glucagon.

ELISA assay. The assay is carried out in 96-well, flat-bottom microplates (Immulon 2, Dynatech Laboratories, Alexandria, Virginia). Plates are coated with 100 μl/well of 1.0 μg/ml insulin in phosphate-buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.4) for 18 h to 6 days at 4°C. Using ^{125}I-pork insulin tracer, it was determined that 26.8 ± 2.8% of the insulin used to coat each well remains during the assay. On the day of the assay, serial twofold dilutions, beginning at 1/5, of serum, plasma, or purified antibody are made in PBS with 0.05% Tween-20 (Fisher Scientific, Fairlawn, New Jersey), and 0.1% human serum albumin (Calbiochem-Behring Corp, La Jolla, California; PBS-Tween-HSA). The insulin-coated plates are washed 10 times with PBS-Tween and 50 μl of serum dilution is transferred to each well. After 1 h at room temperature, the plates are washed 10 times with PBS-Tween and 50 μl of a 1/250 dilution of peroxidase-conjugated, goat anti-human IgG (lot CG125, Litton Bionetics, Kensington, Maryland) in PBS-Tween-HSA is added to each well. After 1 h at room temperature, the plates are washed 10 times with PBS-Tween. Then, 100 μl of substrate, consisting of 2.2 mM *o*-phenylene-diamine (Sigma) and 0.012% hydrogen peroxide in 0.05 M citric acid, 0.1 M phosphate buffer, pH 5.0, is added to each well. After 10 min at room temperature, the reaction is stopped by addition of 50 μl 4 N H_2SO_4. Optical density (OD) at 490 nm is read in the microwells with an EIA Reader (Model EL307, Bio-Tek Instruments, Inc., Laboratory Division, Burlington, Vermont). For each assay, the background used to adjust the reader to zero (0.000 ± 0.020) is a row of insulin-coated wells that are incubated with serum diluent only, PBS-Tween-HSA, followed by the peroxidase conjugate and the substrate. At least one known diabetic and one nondiabetic control serum are also assayed on every plate. More than 35 individual pools of nondiabetic sera have been used in the ELISA. The mean nondiabetic control binding over 13 mo was an OD of 0.227 at the 1/5 serum dilution (range 0.208–0.241) with the maximum upper limit of nondiabetic serum binding (mean + 2 SD) being 0.249 (CV = 4.8%). Nondiabetic control serum binding is not subtracted from the diabetic binding. Statistical analyses were done using paired-sample *t*-tests to compare OD values at the same serum dilutions.

Competitive inhibition of binding. For inhibition studies, four twofold serum dilutions on the linear portion of the insulin binding curve are prepared in PBS-Tween-HSA. Equal volumes of the insulins at concentrations from 2 to 200 μg/ml

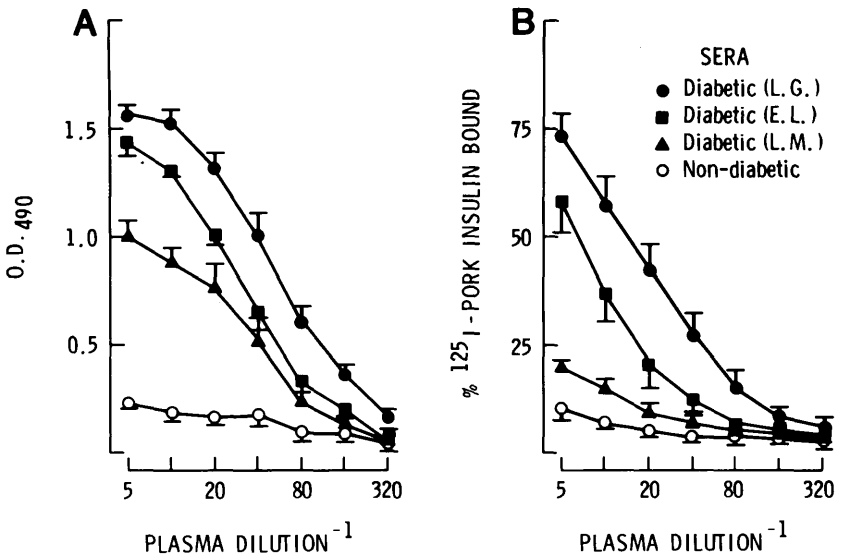

FIGURE 2. *Comparison of direct serum antibody* binding to five species' insulins in the ELISA. Serial dilutions of two diabetic subjects' sera were assayed for IgG binding to the insulins. Panel A is serum from patient E.L., and panel B is serum from patient L.W. Data are the mean binding to each insulin of quadruplicate assays. Bars indicate 1 SD of the mean. In every assay, binding to individual rows of wells coated with each of the five insulins was assayed on a single plate.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/1/60/353253/34-1-60.pdf by guest on 26 March 2023

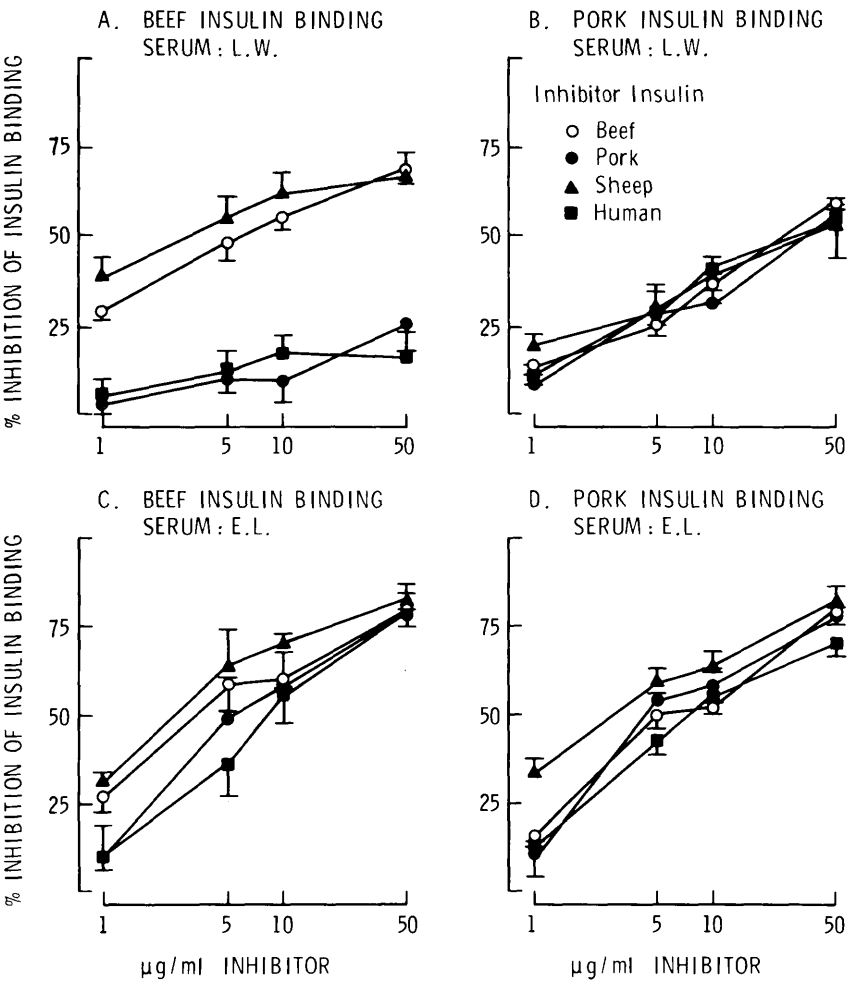

FIGURE 3. Competitive inhibition of antibody binding by insulins in the ELISA. Insulin was added to four serial dilutions of each serum to give final concentrations of 1–50 μg/ml before the diluted sera were transferred to insulin-coated plates. The percentage inhibition of binding by the insulins was calculated as described in the text. Data presented are the mean percent inhibition ± 1 SD of the four serum dilutions calculated for each dose of inhibitor. In panels A and B are inhibitions of the binding of serum from patient L.W., and in panels C and D serum from patient E.L. Panels A and C are inhibition of antibody binding to beef insulin-coated plates and panels B and D are inhibition of the binding to pork insulin-coated plates.

in PBS-Tween-HSA are added to the diluted sera before 50 μl of the mixture is transferred to the insulin-coated plate. After 1 h at room temperature, the plates are washed and peroxidase conjugate is added to the wells. The rest of the assay is described above. The percent inhibition of binding by the different insulins is calculated for each dilution of plasma and concentration of inhibitor by the following: % inhibition of binding = $(U - I)/U \times 100$, where U = OD in absence of inhibitor (uninhibited binding) and I = OD in presence of insulin inhibitor (inhibited binding). The mean percent inhibition of binding for the four serum dilutions is then calculated for each concentration of insulin inhibitor.

Radioligand binding assays. ^{125}I-labeled insulin is prepared by the chloramine-T method and purified over cellulose powder.[10] For the assay, serial dilutions of serum in PBS-Tween are incubated with ^{125}I-insulin in 96-well "V"-bottom microplates (PVC, Dynatech) for 1 h at 25°C. Immune complexes are precipitated with 9% polyethylene glycol (PEG) containing 80 μl/ml pooled normal human serum by centrifugation at 1000 × *g* for 45 min as previously described.[8] Precipitates are washed in PEG, and ^{125}I in the precipitates and supernatants is determined in a gamma counter (Model 1270, Rackgamma II, LKB, Turku, Finland). Results are expressed as percent radiolabeled insulin bound at each serum dilution. For experiments in which endogenous and therapeutic insulins were removed from sera, the sera were acidified and insulin was extracted with dextran-charcoal according to the method of Dixon.[2] The radioligand binding assay was done as described by Palmer et al.,[9] with the exception that human gamma globulin (Cohn fraction II, Sigma) was used instead of bovine gamma globulin. Nondiabetic control sera were tested in each binding assay and, as in the ELISA, that background binding was not subtracted from the patients' binding.

Purification of anti-insulin antibodies. IgG was initially purified from serum by precipitation with ammonium sulfate and by ion-exchange chromatography on DEAE cellulose (DE-52, Whatman Chemical Separation Inc., Clifton, New Jersey) as described.[11] Insulin columns are prepared by coupling pork or beef insulin to lysine-Sepharose-4B (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey) with the bifunctional coupling reagents used to couple insulin to erythrocytes.[12] The IgG is incubated on the column for 45 min and the column is washed extensively with PBS. Anti-insulin IgG is eluted from the column with 4 M guanidine HCl (BRL, Rockville, Maryland), extensively dialyzed against PBS, and the protein concentration is determined spectrophotometrically (E_{280} IgG = 1.4).

RESULTS

To use the ELISA as a semiautomated screening assay for multiple serum samples, conditions were optimized for a

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/1/60/353253/34-1-60.pdf by guest on 26 March 2023

reproducible assay with maximal sensitivity at the antibody levels found in most diabetic patients' sera. Several types of microtiter plates were examined for efficiency of coating and consistency of results. Flat-bottom polystyrene plates (Immulon 2) were found to give consistent results with the best discrimination between antibody-containing and nondiabetic control sera. Kinetic studies of 6 diabetic sera demonstrated no statistically significant difference in binding and sensitivity of the ELISA between the standard (1 h) and 24- or 48-h incubations each with sera or peroxidase conjugate. Likewise, in competitive inhibition experiments, preincubation of sera with inhibitor insulins did not change the percent inhibition of binding, calculated as described above ($P > 0.2$). It was also found that 0.1% HSA provided a consistent protein concentration in the serum diluent, blocking nonspecific immunoglobulin sticking to plates.

Using conditions determined to be optimal, the insulin antibody from a large number of randomly selected diabetic subjects has been studied in the ELISA. In Figure 1, data from three insulin-treated diabetic subjects and one nondiabetic control subject are shown. These are representative of the large amounts of antibody occasionally seen (maximum OD 1.0–1.5) as well as of the moderate levels of antibody routinely found (maximum OD 0.5–1.0). Panel A shows IgG binding to insulin in the ELISA. Optical density is plotted for each serum dilution. In panel B are results of a ^{125}I-insulin binding assay done in parallel with the same sera. Both panels show high levels of insulin binding of sera from patients L.G. and E.L. However, as shown with serum from a patient who had taken only purified pork regular insulin for the previous 3 yr (L.M.), the ELISA readily distinguishes between sera containing low levels of insulin-binding IgG and control sera (S.O.). That distinction is less clear in our RIA. The flexibility of the assay system permitted optimizing the ELISA so that a major portion of patients have antibody levels that give an OD from 0.7 to 1.2 at the 1/5 serum dilution. Under these assay conditions, sera with high levels of insulin antibody (L.G.) have a plateau OD near 1.5 at the initial serum dilutions, then have a linear drop in OD with dilution. For antibody screening assays, the same positive and negative control sera (L.G. and S.O.) are used on every plate. Insulin antibody binding in the ELISA is very reproducible over extended periods of time. In 20 assays done in 12 mo, the coefficient of variation (CV) for the binding of the 1/5 dilution of L.G. serum was 3.6% with an interassay CV of 5.2% for binding on 5 plates. Thus, we have optimized the ELISA as a reproducible method for assay of anti-insulin antibody levels found in samples from patients treated with the current insulins.

Specificity in the ELISA. In addition to screening for insulin antibody, we have used the ELISA to examine the species specificity of these antibodies. Species specificity is not apparent in the direct IgG binding to insulin-coated plates. Shown in Figure 2 are ELISA results of direct binding of two patients' sera to insulins from five species. The data show that the antibody binding on plates coated with different mammalian insulins is the same. Increasing the time of incubation does not reveal any species preference in direct binding. However, species preference can be demonstrated by competitive inhibition of binding to insulin-coated plates. Figure 3 shows inhibition profiles when soluble insulins are used to inhibit binding to plates coated with beef (panels A and C) and pork (panels B and D) insulins using the same sera as shown in Figure 2. Figure 3, panel A, demonstrates that serum from L.W. is inhibited more readily by beef and sheep insulin (30–70%) than by pork and human insulin (1–23%). This serum (L.W.) contains high-avidity antibody for the A-chain-loop determinants (A8 and A10), where beef and sheep insulin differ from pork and human insulins. Similar experiments using pork insulin-coated plates are shown in panel B, where the lower avidity pork insulin binding IgG is inhibited to the same extent by the beef, pork, sheep, and human insulins. Further, the high insulin binding capacity of this low-avidity antibody is demonstrated; 50% inhibition of the binding is seen only at the highest dose of inhibitor insulin (50-μg/ml). The antibody from L.W. has 100 times higher affinity for beef than for pork insulin in our ^{125}I-insulin binding assay in which 9 ng beef insulin and 900 ng pork insulin were required to inhibit 50% of the binding to ^{125}I-beef insulin.

In contrast, the data in panels C and D show that the serum from E. L. does not contain antibodies with marked species-specific differences in avidity. The data indicate relatively high avidity for both beef and pork insulins. In panel C, the slightly higher inhibition of the beef binding by the 1.0 μg/ml doses of beef and sheep insulins indicates a subpopulation of antibody that may have a higher avidity for those insulins than for pork and human insulins. However, most antibody has the same high avidity for all the insulins, demonstrated by the >50% inhibition of the binding to the beef-coated plates at the 10- and 50-μg/ml doses and the strong inhibition of the pork binding (panel D). These data demonstrate that antigen specificity and avidity differences can be shown using the ELISA. The differences cannot be seen

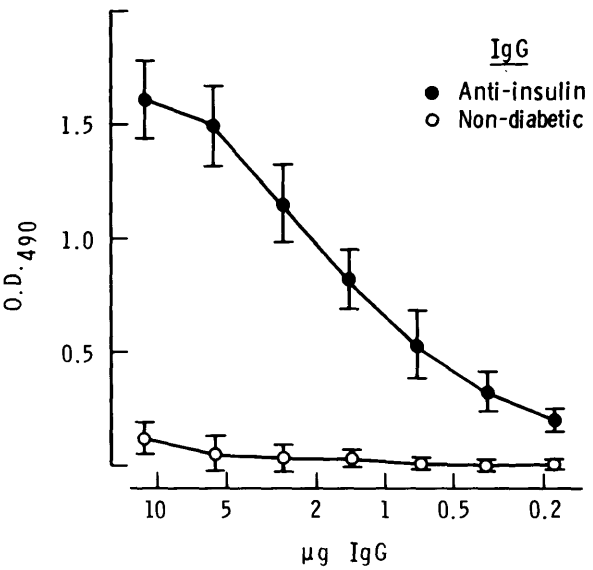

FIGURE 4. Binding of pork insulin affinity purified antibody in the ELISA. Insulin-specific IgG from three patients (R.B., E.L., and L.W.) was assayed for binding to pork insulin-coated plates. Data are plotted as the mean OD_{490} versus the amount (μg) of insulin affinity-purified IgG added per well. The solid circles are the mean binding of IgG preparations from three different diabetic subjects in four different assays, with the open circles being the mean binding of four nondiabetic IgG preparations. Bars indicate the range of binding of all the preparations in all four assays.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/1/60/353253/34-1-60.pdf by guest on 26 March 2023

in the direct binding assay, but are demonstrated by competitive inhibition assays.

Sensitivity of the ELISA. To estimate the range of antibody concentration detectable in the ELISA, affinity-purified, insulin-specific IgG from 3 patients was tested in the assay and used to construct a standard curve. The 3 patients from which the IgG was purified had moderate levels of serum anti-insulin antibody. IgG was obtained from the individual sera by ammonium sulfate precipitation and anion-exchange chromatography. Specific antibody was purified over a pork insulin affinity column. In Figure 4 is shown the mean and range of binding of 4 assays of the 3 anti-insulin IgG preparations and of IgG from 4 nondiabetic controls. The OD is plotted versus the concentration of antibody in the individual wells. The range of greatest sensitivity of the assay is in the linear portion of the graph, from 0.5 to 5.0 μg antibody per well (10–100 μg/ml), although 0.2 μg/well of specific antibody is the limit of detection over nonspecific binding of reagents and similarly prepared nondiabetic IgG. This curve has been used to estimate serum anti-insulin antibody by extrapolation between the linear portions of the serum binding and the affinity-purified antibody binding curves.

Application of the ELISA. The ELISA was developed to screen insulin antibody in conjunction with our studies of lymphocyte responses to insulin.[13] Of more than 200 sera from insulin-treated diabetic subjects, only 2 had no insulin-inhibitable IgG binding in the ELISA. Frequently, when >100 U of insulin is required per day, the role of antibody in the insulin requirement is questioned. In Table 1 are data from 11 patients screened for antibody because of the large doses of insulin that each required. The ELISA data show large amounts of antibody in sera 1–4, while sera 5–11 contain very low levels of antibody. The percent ^{125}I-insulin binding confirms the ELISA results. The high doses of insulin administered therapeutically necessitated acid-charcoal extraction of the sera to dissociate antigen-antibody complexes and to clear free insulin from the sera for the radioligand binding assay. Based on the ELISA screening, and later confirmed by the radioligand binding assay, it was concluded that in sera 1–4 large amounts of insulin antibody were present and potentially contributing to the increased insulin requirements, but in sera 5–11 the low levels of insulin antibody were probably not responsible for the patients' "insulin resistance." In addition, for patients 1, 2, 4, and 11 and nondiabetic controls 13 and 14, pork insulin binding in the ELISA was compared for serum samples that did and did not have endogenous insulin extracted by the acid-charcoal neutralization method.[2] Removal of insulin did not change any of the ELISA values. This is consistent with the supraphysiologic amounts of insulin required to block binding for competitive inhibition of the ELISA (Figure 3).

DISCUSSION

In this study, an enzyme-linked immunosorbent assay (ELISA) is used to examine insulin antibodies in sera of insulin-treated diabetic subjects. Enzyme-conjugated, anti-human immunoglobulin is used to detect binding of antibody to insulin-coated plates. Antigen specificity of the anti-insulin sera is confirmed by the inhibition of binding with soluble insulin and by the lack of binding by control nondiabetic sera. In the randomly selected diabetic population, the amount of insulin antibody is highly variable. Therefore, the assay was optimized to detect the moderate amount of antibody present in sera from insulin-nonallergic diabetic subjects. Practical advantages of the ELISA include speed,

TABLE 1
Antibody levels of insulin resistant patients

		Anti-insulin antibodies				
		ELISA			% ^{125}I-insulin binding‖	
Serum	Insulin dose*	OD†	Titer‡	μg/ml§	Not extracted¶	Charcoal extracted#
1	20/h	1.85**	1/5000	864	92	97
2	120/day	1.87**	1/1280	336	54	66
3	20/h	1.57	1/1280	336	75	88
4	280/day	>2.00**	1/640	136	58	76
5	20/h	0.60	1/80	12	2	1
6	210/day	0.42	1/40	7	7	7
7	35/h	0.27	1/20	4	2	6
8	110/day	0.40	1/40	4	2	1
9	200/day	0.52	1/80	4	6	10
10	120/day	0.37	1/40	4	5	8
11	150/day	0.16**	<1/5	<1	1	2
12	Control††	ND			2	3
13	Control	0.20**	—	<1	1	1
14	Control	0.30**	—	<1	1	1

*Units of insulin patients received in 24 h before sample.
†Optical density (at 490 nm) of 1/5 serum dilution binding to pork insulin in the ELISA.
‡Dilution of serum that had an OD the same as the nondiabetic serum background (at 1/5).
§Serum antibody concentration extrapolated from Figure 4.
‖^{125}I-pork insulin binding.
¶Serum treated with acid and neutralized but not extracted with dextran-charcoal to remove endogenous and therapeutic insulin.
#Serum treated with acid, then insulin was extracted with dextran-charcoal.
**OD, titer, and μg/ml antibody were the same in both charcoal-extracted and unextracted sera samples.
††Nondiabetic control sera. ND: not determined.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/1/60/353253/34-1-60.pdf by guest on 26 March 2023

sensitivity, and reproducibility without the need for radioisotopes. Recent widespread use of ELISA technology[6] has made reagents readily available and inexpensive.

As with all assays, unique features of the ELISA impose limitations on its use. The assay readily detects insulin binding antibodies. However, direct binding to insulin-coated plates cannot be used to identify species-specific antibody. Shown in Figure 2, the direct serum binding to plates coated with 5 species insulins was the same, despite the species-specific avidity differences demonstrated in Figure 3 in one of the two sera (L.W.). Direct binding to radiolabeled insulins, however, has shown species-specific binding with some antibodies when careful binding titrations are done.[14,15] The high concentration of insulin at the solid-liquid interface is probably responsible for this difference between assays. Therefore, competitive inhibition with soluble insulin is necessary to detect species-specific antibody in the ELISA. The amount of insulin required to block binding in the ELISA to generate inhibition curves (e.g., Figure 3) can be used to estimate relative avidity of antibody for various insulins by expressing the data as the amount of antigen required to inhibit 50% of the binding.[7,16] In the ELISA, 1–200 μg/ml of soluble insulin is necessary to inhibit binding to the solid phase. This exceeds by far (200 times) the amount of insulin required in inhibitions for Scatchard analysis of radioimmunoassays.[15] As a result of this and other constraints of the solid phase, ELISA inhibition does not provide conventional measurements of insulin binding capacity of serum (U/L). The differences in solid- and fluid-phase immunoassays have recently been reviewed in detail.[7]

In the present study, peroxidase-conjugated antiserum specific for the heavy chain of IgG antibodies was used. Anti-insulin antibodies of this heavy-chain isotype are present in more than 99% of the diabetic sera tested in the ELISA. Insulin antibodies of other isotopes, e.g., IgM and IgA, can also be detected by using the appropriate heavy-chain, class-specific, enzyme-conjugated reagents. These isotypes are not frequent among sera from diabetic subjects with established insulin therapy.

The ELISA is used to estimate the amount of insulin-binding IgG in a serum by extrapolation from the OD to the curve of the known affinity-purified IgG binding (Figure 4). It is noteworthy that, when extrapolation is done (Table 1), the OD on the linear portion of the serum binding curve is compared to the linear portion of the standard curve. This is because only within the linear region is the increase in OD directly proportional to the increase in antibody in the wells.[7] Further, the solid-phase ELISA binding is most affected by IgG antibody affinity in the plateau region of the curve,[17] where antibody is in excess and higher affinity antibody competes more effectively for antigen. The ELISA, like other solid-phase immunoassays, does have a "threshold" for antibody detection.[14] Because of the extremely vigorous washing procedures, it was at least a theoretical possibility that low-affinity antibodies might not be detected in this ELISA. It has been shown, using monoclonal IgG antibodies with affinities determined by equilibrium dialysis, that the threshold of the solid-phase ELISA is 14 μg/ml of IgG with affinity greater than 3×10^5 L/mol.[14] This is well below the published affinities of 10^8–10^{10} for the low-affinity–high-capacity insulin-binding antibodies.[3,18]

While the ELISA is sensitive enough to study insulin antibody titers and avidity changes in patients receiving highly purified and synthetic insulins, the assay is not influenced by insulin in patients' sera. In these studies, the amount of insulin required to inhibit binding to insulin-coated plates (1–50 μg/ml) was much greater than circulating insulin levels (Figure 3). More directly, optical densities of sera shown to have increased binding in the radioligand binding assay after charcoal extraction did not change in the ELISA (Table 1). Therefore, it is not necessary that blood be drawn before a scheduled dose (not possible with patients on continuous subcutaneous or intravenous insulin infusion and difficult for those on multiple subcutaneous injections), nor is it necessary to remove insulin from the plasma before assay. As shown in Table 1, we have used the ELISA to test for insulin antibody in "insulin resistant" patients, some of whom were receiving 20 U/h intravenous insulin. In 4 of the 11 patients, the antibody assays were performed and the physicians notified in one-half a working day that large amounts of insulin antibody were present and potentially contributed to the patients' insulin requirements. For the other patients, insulin antibody was ruled out as the direct cause of resistance. This was further supported when other causes for resistance were later identified in patients 5–11. This is consistent with previous reports that insulin antibody contributes to only a portion of insulin resistance.[3,15] Thus, the ELISA is precise and efficient enough for rapid evaluation of antibody-mediated insulin resistance, without interfering with therapy or further compromising patients.

In addition to screening sera for insulin antibodies, this ELISA has numerous basic and clinical applications. Experiments are currently in progress using the ELISA to monitor anti-insulin antibody levels and avidities in patients being changed from mixed-species insulins to purified single-species insulin preparations. For a number of subjects, competitive inhibitions of antibody binding have been used to determine insulin species preference of the antibodies (Figure 3); these studies may be helpful in recommending therapy with monocomponent or synthetic insulins. Thus, the ELISA is a rapid, reproducible assay for anti-insulin antibody that has proven simple enough to be used for routine screening, as might be done in a clinical laboratory, yet is specific and sophisticated enough to be used for more precise studies.

ACKNOWLEDGMENTS

The authors thank Drs. Howard K. Wilson and Joyce Schroer, and the faculty and staff of the Diabetes Center of the University of Texas Medical School at Houston for cooperation and assistance in obtaining patient samples. We are indebted to Drs. James B. Field and Victor R. Lavis for critically reviewing the manuscript and to Charlene Shackelford for assistance in preparation of the manuscript.

This work was supported in part by the Juvenile Diabetes Foundation, NIH research grant AM-32329, and Lilly Research Laboratories. Dr. Nell was supported by NIH research award 1F32-AI006100.

REFERENCES

1 Berson, S. A., Yalow, R. S., Bauman, A., Rothschild, M. A., and Newerly, K.: Insulin I^{131} metabolism in human subjects. Demonstration of insulin

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/1/60/353253/34-1-60.pdf by guest on 26 March 2023

binding globulin in circulation of insulin treated subjects. J. Clin. Invest. 1956; 35:170–90.

[2] Dixon, K.: Measurement of antibodies to insulin in serum. Clin. Chem. 1974; 20:1275–81.

[3] Goldman, J., Baldwin, D., Pugh, W., and Rubenstein, A. H.: Equilibrium binding assay and kinetic characterization of insulin antibodies. Diabetes 1978; 17:653–60.

[4] Kurtz, A. B., Matthews, J. A., Mustaffa, B. E., Daggett, P. R., and Nabarro, J. D. N.: Decrease of antibodies to insulin, proinsulin, and contaminating hormones after changing treatment from conventional beef to purified pork insulin. Diabetologia 1980; 18:147–50.

[5] Engvall, E., and Perlman, P.: Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen coated tubes. J. Immunol. 1972; 109:129–35.

[6] Maggio, E. T., Ed.: Enzyme Immunoassay. Boca Raton, CRC Press, 1980.

[7] Berzofsky, J. A., and Berkower, I.: Antigen-antibody interaction. *In* Fundamental Immunology. Paul, W. E., Ed. New York, Raven Press, 1984:595–644.

[8] Desbuguois, B., and Aurbach, G. D.: Use of polyethylene glycol to separate free and antibody bound peptide hormones in radioimmunoassays. J. Clin. Endocrinol. Metab. 1971; 33:732–38.

[9] Palmer, J. P., Asplin, C. M., Clemons, P., Lyen, K., Tatpati, O., Raghu, P. K., and Paquette, T. L.: Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 1983; 222:1337–39.

[10] Gavin, J. R., III, Roth, J., Jen, P., and Freychet, P.: Insulin receptors in circulating cells and fibroblasts. Proc. Natl. Acad. Sci. USA 1972; 69:747–51.

[11] Geha, R. S., and Weinberg, R. P.: Anti-idiotypic antisera in man. I. Production and immunochemical characterization of anti-idiotypic antisera to anti-tetanus antibodies. J. Immunol. 1978; 121:1518–23.

[12] Schroer, J. A., Inman, J. K., Thomas, J. W., and Rosenthal, A. S.: H-2-linked Ir gene control of antibody responses to insulin. I. Anti-insulin plaque forming cell primary response. J. Immunol. 1979; 123:670–75.

[13] Nell, L. J., and Thomas, J. W.: The human immune response to insulin. I. Kinetic and cellular aspects of lymphocyte proliferative responses in diabetics. J. Immunol. 1983; 131:701–705.

[14] Kurtz, A. B., Matthews, J. A., and Nabarro, J. D. N.: Insulin-binding antibody: reaction differences with bovine and porcine insulins. Diabetologia 1978; 15:19–22.

[15] Berson, S. A., and Yalow, R. S.: Species-specificity of human anti-beef, pork insulin serum. J. Clin. Invest. 1959; 38:2017–25.

[16] Reed, L. J., and Meunch, H.: A simple method of estimating fifty percent endpoint. Am. J. Hygiene 1938; 27:493–500.

[17] Peterfy, F., Kuusella, P., and Makela, O.: Affinity requirements for antibody assays mapped by monoclonal antibodies. J. Immunol. 1983; 130:1809–13.

[18] Berson, S. A., and Yalow, R. S.: Quantitative aspects of the reaction between insulin and insulin-binding antibody. J. Clin. Invest. 1959; 38:1996–2016.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/34/1/60/353253/34-1-60.pdf by guest on 26 March 2023