Proinsulin-Specific Antibodies in Human Sera

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SUMMARY

Sera from nine diabetic patients treated with commercial insulin contained antibodies which reacted to I-125-bovine proinsulin. These antibodies had two distinct components: anti-insulin antibodies which crossreacted with proinsulin, and antiproinsulin antibodies, which were specific to proinsulin and did not react with insulin (purified, single component). Proinsulin-specific antibodies were detectable by their binding to I-125-bovine proinsulin in sera adsorbed with insulin-phe-sepharose or in sera preincubated with excess insulin.

I-125-bovine proinsulin-reactive antibodies showed species specificity. They reacted weakly with porcine proinsulin and appeared to have no crossreactivity with porcine-C-peptide.

The data suggest that proinsulin-specific antibodies (a) may be induced by the contaminants present in commercial insulin; (b) do not inactivate exogenous insulin and therefore, would not influence directly the daily insulin requirements of diabetic individuals. DIABETES *22*:361-66, May, 1973.

Since proinsulin crystallizes together with insulin during the manufacturing process,[4] commercial insulin preparations contain variable amounts of proinsulin and related proteins. These contaminants are antigenic and induce antibody formation in experimental animals.[3,4,8,14-16] Recent investigations[9,11] indicate that proinsulin-reactive antibodies are detectable in patients treated with commercial insulin. Lack of information in regard to the specificity of these antibodies in human sera, however, precludes the determination of their clinical significance. Therefore, we decided to investigate the specificity of proinsulin-reactive antibodies in diabetic patients.

MATERIALS AND METHODS

Antisera. Fasting serum samples were obtained from nine *insulin-treated* diabetic patients. In the absence of either infection or ketoacidosis, these patients had required large doses of insulin and were clinically classed as mildly to severely insulin-resistant.[1] Their current therapy at the time of sampling is given in table 1. Sera from ten *diet-controlled* diabetic patients, mean age 57.5 ± 2.8 (S.E.M.) years and mean known duration of diabetes 3.5 ± 0.6 years, served as control.

Radioiodinated proinsulin and insulin. I-125-bovine insulin (lot no. 98, specific activity 50 μc./μg., Amersham-Searle, Arlington Heights) and I-125-bovine proinsulin (specific activity 250 μc./μg., a gift of Dr. A. Rubenstein, Fisher Endocrinology Laboratories, Chicago) were purified by gel filtration (Sephadex G-50, 0.5 x 20 in. column, borate buffer pH 7.4). These were standardized against nonspecific anti-insulin guinea pig serum of known insulin and proinsulin binding capacities, and diluted to 1 ng./ml. The same preparation of labeled proteins was used for all tests.

Unlabeled proteins. Bovine (lot no. 615-1070B-212-2) and porcine (lot no. 615-1070B-277-2) proinsulins, bovine (lot no. 615-1082B-244) and porcine (lot no. 615-1082B-247) single component insulins (single band

TABLE 1

Clinical data of insulin-treated subjects tested

Patient	Age (yr.)	Duration of diabetes (yr.)	Present therapy	Maximum daily insulin requirements during past year
A.P.	56	34	tolazamide* 1 gm./day	2,000
I.H.	60	16	NPH 110	190
I.T.	38	23	NPH 160	200
J.A.	50	2	Lente 25	600
J.R.	39	15	NPH 30	1,000
J.W.	54	3	NPH 55	90
L.S.	64	20	NPH 40	110
O.G.	41	6	Lente 70	160
P.H.	32	14	NPH 50	300

* Poorly controlled with tolazamide for past six months.

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by electrophoresis), and porcine connecting or C-peptide (lot no. 615-1082B-66-A) were supplied by Dr. R. Chance of the Lilly Research Laboratories, Indianapolis. These were dissolved in borate buffer, pH 7.4, containing 1 per cent bovine serum albumin (Mann Laboratories, N. Y.), and stored at 4° C.

Antihuman immunoglobulin rabbit serum (AHI-RS) was prepared by hyperimmunizing rabbits with purified plasma Cohn fraction II. This serum reacted strongly with both the antigenic types K and λ light chains and was therefore a polyvalent anti-immunoglobulin serum.[7] A sevenfold concentration of this serum was required for the maximal precipitation of immunoglobulins.

Insulin-phe-sepharose immunoabsorbent. Single component porcine insulin was coupled to activated sepharose at pH 5, according to the methods described by Cuatrecasas.[5]

Adsorption of insulin antibodies. This was performed by two different methods: (a) diluted serum was preincubated with excess of bovine insulin (40 μg./ml.), and (b) diluted serum was adsorbed with insulin-phe-sepharose, at 4° C. for twenty hours.

Determination of proinsulin-binding titers. Volumes (0.1 ml.) of the serial dilutions of human sera were incubated with 0.1 ml. of I-125-bovine proinsulin (1 ng./ml.) at 4° C. After forty hours, 1 ml. of diluted *AHI-RS* was added to precipitate the immune complexes. Two days later the precipitate was separated by centrifuging at 1,000 x g for thirty minutes. The radioactivity in the precipitate and supernatant was counted and the percentage of radioactivity in the precipitate was calculated. The serum dilution which bound 33 per cent of the radioactivity is referred to as the binding titer.

The binding titers of the unadsorbed and adsorbed sera are designated as proinsulin-binding titer (PBT) and specific-proinsulin-binding titer (SPBT) respectively.

Competitive inhibition assay. Diluted serum (0.1 ml.) was incubated with a mixture of 0.1 ml. radioiodinated material (1 ng./ml.) and 0.1 ml. of the variable concentrations (0 to 40 μg./ml.) of unlabeled proteins. The percentage of radioactivity bound was determined as described. To obtain a rough comparison of the effectiveness of various inhibitors (unlabeled proteins), the per cent inhibition* was plotted versus logarithm of inhibitor concentration.[2]

Characterization of proinsulin-binding proteins was performed by radioimmunodiffusion[6] using specific anti-immunoglobulin-IgG, IgM, IgA, IgD and IgE sera (Hyland Laboratories, Los Angeles).

RESULTS

Proinsulin-binding titers (PBT). In the diet-controlled group, the undiluted sera bound negligible amounts (less than 3 per cent) of I-125-bovine proinsulin. The values of PBT in insulin-treated patients are given in table 2. Sera preincubated with insulin excess or adsorbed with insulin-phe-sepharose bound negligible amounts of I-125-insulin, although significant binding of I-125-proinsulin persisted. The SPBT ranged from 6 to 780 (table 2). In six cases, the values of SPBT obtained after adsorption with insulin-phe-sepharose were generally lower than the values observed after preincubation with insulin. SPBT was less than PBT in each serum.

There was no correlation between SPBT and the daily insulin requirements in individual patients.

TABLE 2

Proinsulin-binding titers* of human sera

Patients	Proinsulin-binding titer	Specific-proinsulin-binding titer	
		After preincubation with insulin excess	After adsorption with insulin-phe-sepharose
Control†	0		
O.G.	90	6	10
J.R.	600	57	—
I.H.	195	63	45
J.A.	225	66	—
I.T.	654	201	144
J.W.	321	264	200
A.P.	708	306	260
P.H.	4,800	654	—
L.S.	1,191	780	660

* Represents the dilution of serum which bound 33 per cent of I-125-bovine proinsulin (1 ng./ml.).

† Diet-controlled diabetic patients (n = 10).

I-125-bovine insulin-binding antibodies. Sera of insulin-treated patients bound I-125-bovine insulin, and addition of unlabeled bovine insulin or proinsulin inhibited the binding in all cases (one of the representative curves is shown in figure 1). Bovine proinsulin competed less effectively as it required a higher concentration of proinsulin to attain the same degree of inhibition.

$$*100\left[1-\frac{\%\text{ radioactive tracer bound in presence of inhibitor protein}}{\%\text{ radioactive tracer bound in absence of inhibitor protein}}\right]$$

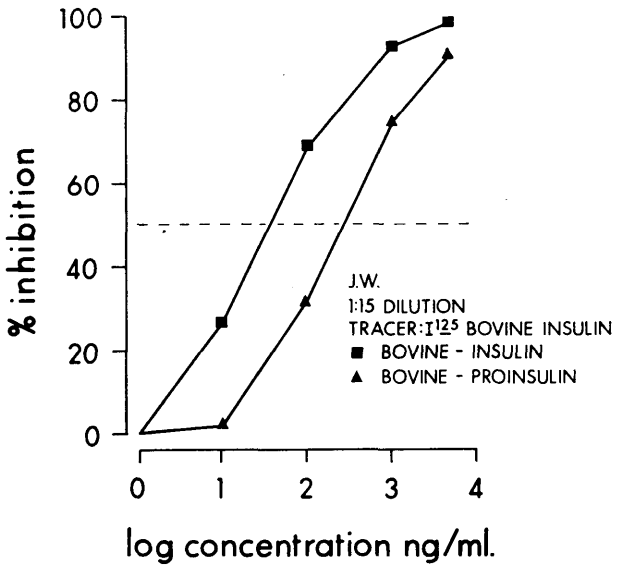

FIG. 1. Using serum (1:15 dilution) of patient J.W., the per cent inhibition of I-125-bovine insulin (1 ng./ml.) binding by the added unlabeled bovine insulin or proinsulin is shown. Plotted on the abscissa are the logarithm concentrations of inhibitor insulin or proinsulin in nanograms per milliliter. Proinsulin competes with tracer, though less avidly. Fifty per cent inhibition of binding is observed at 40 ng./ml. of insulin and 283 ng./ml. of proinsulin.

I-125-bovine proinsulin-binding antibodies. Sera of insulin-treated patients bound I-125-bovine proinsulin, and addition of unlabeled bovine proinsulin effectively inhibited the binding in all cases (figure 2 shows one of the representative curves).

The degree of inhibition by bovine insulin varied in the individual sera and was related to the difference between PBT and SPBT; the maximum inhibition was found in patient P.H. and the minimum in patient J.W. In the latter case, the inhibition curve (figure 2) plateaued at 8 per cent and a further increase in insulin concentration, from 100 ng. to 40 μg. (a four hundred-fold change), did not affect the degree of inhibition. Presumably the insulin antibodies were saturated at 100 ng./ml. leaving behind proinsulin-specific antibodies, which showed no affinity for insulin. Almost identical inhibition curves were obtained with unlabeled porcine insulin.

In unadsorbed sera, porcine proinsulin showed some crossreactivity with I-125-bovine proinsulin (figures 2 and 3). The degree of crossreactivity varied in individual samples, being maximum in patient L.S. and minimum in patient O.G. Eight out of nine adsorbed sera continued to show crossreactivity between bovine and porcine proinsulins, though to a much lower degree (figure 3). In the ninth serum, porcine proinsulin did not crossreact to any measurable extent (figure 4). Thus, the elimination of insulin-reactive antibodies decreased the crossreactivity.

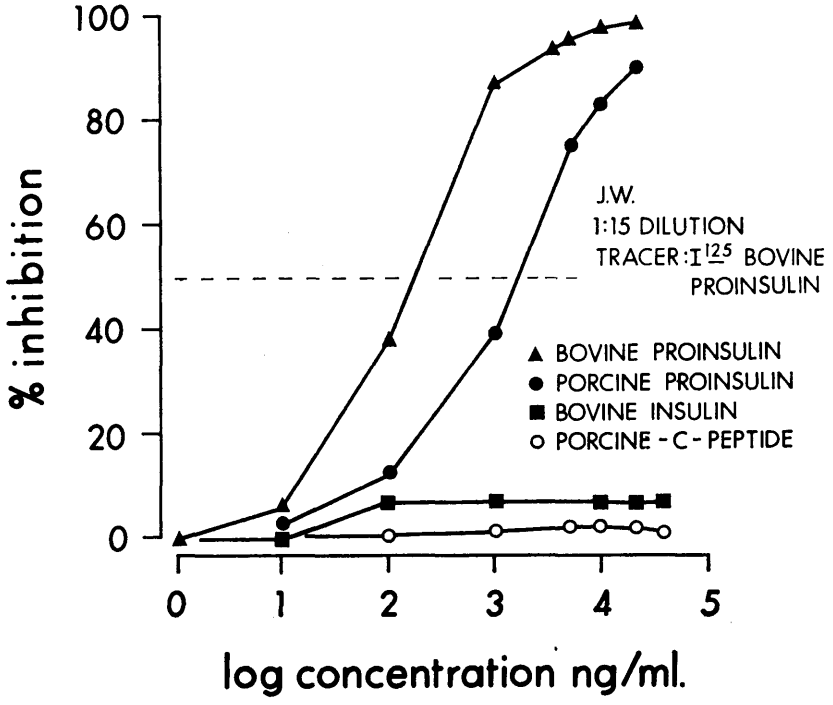

FIGURE 2

Using unadsorbed serum (1:15 dilution) of patient J.W., the per cent inhibition of I-125-bovine proinsulin (1 ng./ml.) binding by the added unlabeled bovine insulin, bovine proinsulin, porcine proinsulin or porcine-C-peptide is shown. Plotted on the abscissa are the logarithm concentrations of inhibitors, unlabeled proteins, in nanograms per milliliter. Porcine proinsulin competes with tracer, though less avidly; porcine-C-peptide does not compete effectively; and bovine insulin produces 8 per cent inhibition.

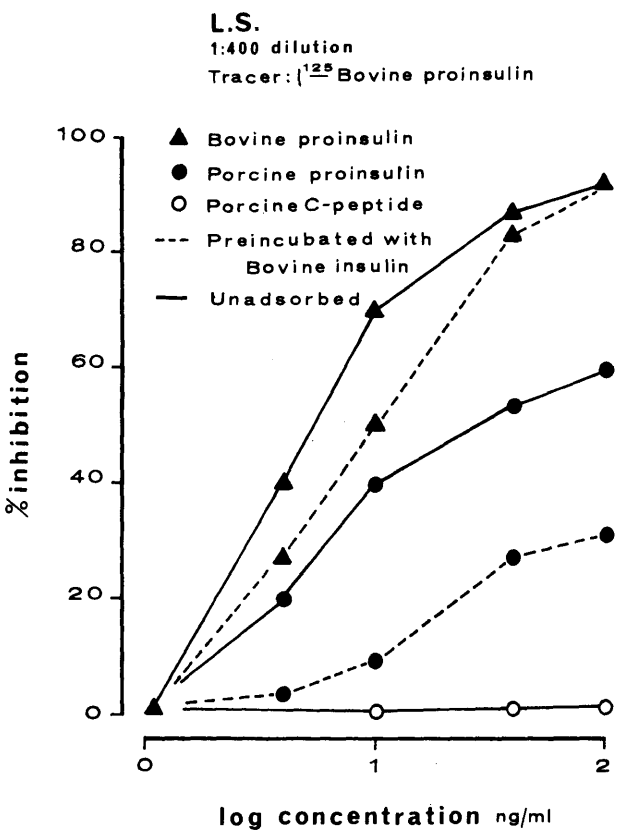

FIG. 3. Curves showing the per cent inhibition of I-125-bovine proinsulin (1 ng./ml.) binding by the added unlabeled bovine proinsulin, porcine-C-peptide and proinsulin in unadsorbed serum and in serum preincubated with excess bovine insulin (40 μg./ml. at 4° C. for twenty hours). Plotted on the abscissa are the logarithm concentrations. The crossreactivity of porcine proinsulin is reduced in the serum preincubated with insulin. Porcine-C-peptide shows no competition.

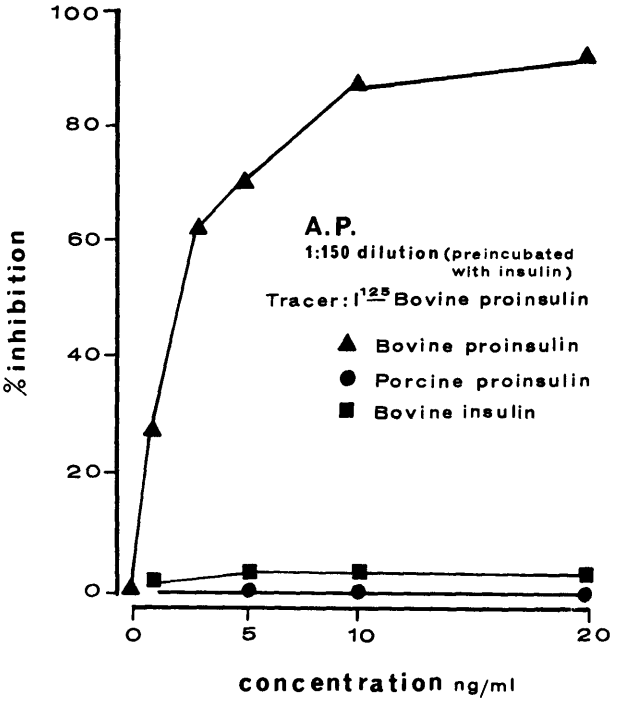

FIG. 4. Curves showing the per cent inhibition of I-125-bovine proinsulin (1 ng./ml.) binding by the added unlabeled bovine proinsulin, insulin and porcine proinsulin, in 1:150 dilution of serum (patient A.P.) preincubated with excess bovine insulin (40 μg./ml. at 4° C. for twenty hours). Porcine proinsulin produces insignificant inhibition.

Competitive inhibition of I-125-bovine proinsulin binding by porcine-C-peptide was studied in the unadsorbed (figure 2) and adsorbed (figure 3) sera. In both situations, there was no detectable competitive inhibition, indicating that porcine-C-peptide did not crossreact with any of the bovine proinsulin binding sites.

Characterization of proinsulin-binding proteins. This was performed in four sera (A.P., J.W., I.T., and P.H.). On the immunodiffusion plates, precipitation lines were seen between the human sera (pretreated with excess bovine insulin) and the anti-immunoglobulin-G, -M and -A; and none were visible aganst anti-IgE or IgD. The autoradiographs revealed the presence of radioactive tracer in the case of anti-IgG only.

DISCUSSION

The avidity and specificity of anti-insulin and anti-proinsulin antibodies have been studied extensively in guinea pig serum.[3,4,8,10,14,15,18,19] In the present study, we investigated the antibodies contained in the sera of diabetic patients. As it was more convenient to work with sera having high antibody titers, samples were obtained from a selected group of patients clinically classed as mildly to severely insulin resistant.[1]

In all nine antisera, bovine proinsulin inhibited the binding of tracer I-125-bovine insulin, indicating that the binding sites for insulin were also reactive and accessible to proinsulin but with a lower affinity for the latter. It has been suggested that the presence of the connecting segment might interfere with the binding of proinsulin to anti-insulin antibodies.[15,18]

It was anticipated that anti-insulin human sera would also bind I-125-bovine proinsulin. However, the partial inhibition of I-125-bovine proinsulin binding by unlabeled insulin in the inhibition assays (figure 2), suggested that there were additional antibodies which re-

acted to proinsulin but not to insulin. This was then demonstrated by the significant proinsulin-binding titers of the adsorbed sera (table 2). Thus, it is evident that the antibodies in these sera consisted of two distinct components, anti-insulin antibodies and antiproinsulin antibodies, the latter being proinsulin-specific. Results of radioimmunodiffusion revealed that antiproinsulin antibodies belong to immunoglobulin class IgG.

C-peptide and proinsulin have common antigenic determinants, located in the C-peptide region.[8] C-peptide and homologous proinsulin compete in specific immunosystems,[3,4,8,12,14,15,17] with significant species specificity; C-peptide and proinsulin molecules of two different species crossreact only weakly.[15,17] This is related to the primary structures, which have species differences.[13] It was not surprising, therefore, that porcine-C-peptide did not produce any noticeable inhibition of I-125-bovine proinsulin binding.

Unlabeled porcine proinsulin competed with I-125 bovine proinsulin to a variable extent in the individual antisera tested. Since this crossreactivity was partly eliminated with removal of anti-insulin antibodies, it is reasonable to assume that the observed crossreactivity was, in part, due to anti-insulin antibodies. The fact that porcine proinsulin continued to show some crossreactivity with antibovine proinsulin antibodies in eight adsorbed sera, but porcine C-peptide did not crossreact, may suggest that the crossreacting binding sites for heterologous proinsulins were not shared by porcine C-peptide; were not accessible to porcine C-peptide; were somehow related to the configuration of the molecules and were due to some "miss-fit"; or may have been located on antibodies directed toward various intermediate forms of proinsulin.

Rubenstein et al.[15] observed that most of the crossreactivity between heterologous proinsulins was accounted for by anti-insulin antibodies and was removable by adsorbing the sera with insulin. The variation between our results and theirs may be due to the fact that the sera they analyzed were from animals which were immunized with one kind of insulin or proinsulin under controlled conditions, while all our patients had received not only the proteins of both bovine and porcine origins but of different compositions over prolonged periods. It is conceivable that human sera contained several heterogeneous antibodies of different avidity and specificity, which may differ from patient to patient. For example, patient A.P. appeared to have relatively specific antibovine proinsulin antibodies and others did not.

Since proinsulin-specific antibodies were detectable only in the insulin-treated patients and appeared to have significant specificity for bovine proinsulin, it is felt that the contaminants present in therapeutic insulin may have induced their production.

Nonreactivity of antiproinsulin antibodies with insulin suggests that these antibodies do not inactivate exogenous insulin and, therefore, would not influence directly the insulin requirements of diabetic individuals. Indeed, we observed no correlation between SPBT and the insulin needs in nine insulin-resistant patients. This was also the case in a group of twenty-five unselected, insulin-treated diabetic subjects presented elsewhere.[11]

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Dietary Carbohydrate and Postprandial Hyperlipemia

(*Continued from page 353*)

serum triglycerides of approximately 50 mg. per 100 ml. over the fasting value two hours after the meal with sucrose, while at a similar time after the glucose meal the rise was 22 mg. per 100 ml. Even four hours after the meal, although there was some decrease from the two-hour value, the triglycerides were still higher in those instances in which the carbohydrate taken was sucrose. In the younger subjects the serum triglycerides, serum insulin, and blood glucose levels were followed at half hourly intervals after the formula breakfasts. There was no difference in the increment in serum triglycerides after either meal, but whereas after the glucose meal the values started to fall at one and one-half hours, they stayed elevated after the sucrose meal. The peak insulin levels were the same in both groups, but after the sucrose meal the serum insulin fell more rapidly toward the fasting value, so that at one and one and one-half hours there was a significant difference in insulin levels between the two groups. The blood glucose rose to significantly higher levels after the glucose formula was fed. Insulin response was related to the glycemia and there was also a good inverse relationship between the serum insulin response and the lipemic response.

The authors do not comment on the differences in the increments of triglycerides in the two age groups, and give fasting values only for the older men. Denborough (loc. cit.) has shown that the extent of a postprandial rise in serum triglycerides is related to the fasting value, hence one may assume that the younger men had lower fasting levels of triglycerides.

The authors suggest that endogenous triglyceride synthesis is not apparently involved in the elevation of the serum triglycerides seen when an equivalent quantity of glucose or sucrose was given without the sunflower-seed oil. This finding agrees with that of D. C. Swan, P. Davidson, and Albrink (Lancet *1*:60, 1966). Thus the rise in serum triglycerides is apparently dependent upon the dietary fat, and it appears that the elevation in these lipids after sucrose administration is due to a reduced rate of clearance from the plasma. After sucrose feeding the blood glucose does not rise as high as after glucose, the increment in insulin is less, and since lipoprotein lipase is activated by insulin (E. D. Korn, J. Biol. Chem. *215*:1, 1955), there is a reduced rate of triglyceride clearance.

It will be of interest to determine the degree to which insulin response explains the various reported effects of different diets upon the levels of plasma triglycerides.

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