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Isolation and characterization of soluble insulin–anti-insulin immune complexes formed *in vitro* and *in vivo* in sera from patients with diabetes mellitus

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SUMMARY

The existence of soluble insulin–anti-insulin immune complexes in the serum of patients with diabetes mellitus was investigated. Formation of such immune complexes *in vitro* was studied by adding radioiodinated insulin to the sera of patients with anti-insulin antibodies; immune complexes were formed readily, but apparently differed from patient to patient. Immune complexes formed *in vitro* were precipitated with 5% polyethylene glycol. They eluted in the high-molecular weight fractions when the precipitated material was fractionated by gel filtration, and they remained bound at neutral pH when the high-molecular weight fractions were submitted to affinity chromatography on protein A–Sepharose. When the bound immune complexes were recovered by acid elution and immediately filtered through a Sephadex G-50 column equilibrated with the same acid buffer, free antigen (radiolabelled insulin) and antibody were recovered. This antibody, after neutralization, showed binding capacity when remixed with radioiodinated insulin. When this protocol was applied to a serum that gave positive results in several screening methods for soluble immune complexes, insulin was detected by radioimmunoassay in the high-molecular weight fractions separated from the 5% polyethylene glycol precipitate and in the fractions retained by protein A; however, no free insulin was detected after gel filtration in Sephadex G-50, perhaps due to excessive dilution. The high-molecular weight fraction did have binding capacity for radioiodinated insulin. No insulin-binding protein could be recovered with a similar procedure from a serum negative by all screening tests for soluble immune complexes. These results prove that soluble immune complexes can be formed easily in sera containing anti-insulin antibodies and can be recovered from sera of diabetic patients that show positive results in screening techniques for soluble immune complexes.

INTRODUCTION

Soluble circulating immune complexes (IC) have been suggested to play a role in the pathogenesis of the microangiopathy, which leads to most of the complications seen in diabetic patients (Jayarao *et al.*, 1973). Evidence for the presence of soluble IC in insulin-dependent diabetics has been obtained through the use of the C1q-binding assay, the Raji cell assay, and ultracentrifugation in a sucrose gradient (Kumar & Quismorio, 1978; Irvine *et al.*, 1977; Follig, 1976). Although these results do present a strong case for the existence of IC in insulin-dependent diabetics, this has not been demonstrated directly. There has not been any report of successful isolation of soluble IC and

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demonstration of the existence of insulin and/or anti-insulin antibodies in the isolated material. However, the characterization of isolated IC is essential when their existence has been suggested by screening techniques.

Using the isolation methods first described by Chenais *et al.* (1977), based on polyethylene glycol (PEG) precipitation of soluble IC, gel filtration and further purification by affinity chromatography using staphylococcal protein A as a substrate, we have investigated the *in vitro* formation of IC and the isolation of soluble circulating insulin–anti-insulin IC formed *in vivo* and *in vitro* in the serum of several patients with diabetes mellitus.

MATERIALS AND METHODS

Patients. Sera from four adult diabetics were used in this study. Patient A, 59 years old, was an obese diabetic who had been treated with insulin for the past 15 years, with clinically evident microvasculopathy; patient B, 37 years old, had diabetes associated with chronic pancreatitis and had been treated with insulin for the past 11 years, with a current total daily dosage of 75 units; patient C, 45 years old, had insulin-dependent, ketosis-prone diabetes, recently diagnosed, and the serum was collected at the end of his second week of therapy with regular insulin adjusted according to his blood sugar level (total daily dosages between 20 and 40 units); patient D, 48 years old, had insulin-dependent diabetes of 3 years duration, with no clinical evidence of microangiopathy. His insulin daily dosage at the time of our studies was 45 units.

Sample collection. Blood was drawn by venipuncture and allowed to clot at 37°C. The serum was stored at 4°C if it was to be used within 48 hr or at −70°C if it was to be stored for an extended period of time.

Insulin antibody determination. Anti-insulin antibodies in serum samples were determined by the dextran-coated charcoal radioimmunoassay (Herbert *et al.*, 1965). The values for this assay were expressed as the ratio (c.p.m. in precipitate − c.p.m. in supernatant/total c.p.m.) × 100. Anti-insulin antibodies in samples other than whole sera were detected by a modified PEG radioimmunoassay (Gerbitz & Kemmler, 1978). Samples to be tested were diluted 1:4 in a 1:10 dilution of normal human serum in 0·1 M phosphate buffer, pH 7·4. Two hundred microlitres of this solution was then incubated with 50 μl of ^{125}I-labelled insulin (Corning Diagnostics) for 1 hr at 37°C and 1 hr at 4°C. This mixture was then mixed with 250 μl 20% PEG in 0·1 M borate buffer, pH 8·0, containing 0·15 M NaCl and incubated for 30 min at 250°C and 1 hr at 4°C. This mixture was then centrifuged at 5,000 ***g*** for 30 min. The amount of antibody present was expressed as a percentage of the counts per minute in the precipitate versus the total counts per minute added.

Screening for immune complexes. Preliminary screening for IC was carried out through the use of the following techniques: direct laser nephelometry (Virella *et al.*, 1979), radiolabelled C1q binding (Zubler & Lambert, 1977) and a modification of the assays for C4 and IgG in PEG precipitates (Digeon *et al.*, 1977).

Our modification of Digeon's procedure included repeated washing of the PEG precipitates (2 × wash with cold 0·1 M borate buffer, pH 8·4, containing 0·15 M NaCl and 3% PEG 6,000), redissolution in 0·4% Tween 20 in the borate buffer at 37°C for 30 min, assay of C4 and IgG by laser nephelometry (Deaton *et al.*, 1976) and the expression of the results as a ratio between the amounts of C4 or IgG found in the resuspended precipitate and in the original serum sample. The limits of normality for both assays have been established by determining the mean and standard deviation (s.d.) values of the results obtained from groups of normal controls. Values more than 1 s.d. below the mean for normal controls were considered negative; values between 1 s.d. above and 2 s.d. below the mean for normals were considered borderline; and values more than 2 s.d. above the mean were considered positive.

Isolation of soluble IC. A modification of the method first described by Chenais *et al.* (1977) was employed for the isolation of soluble IC, either native or formed *in vitro*. One millilitre of 20% PEG 6,000 in borate-buffered saline, pH 8·0, was mixed slowly by stirring with 3 ml of the serum to be examined. The mixture was stirred for 30 min at room temperature and at 4°C for 2 hr. The mixture was centrifuged at 15,000 ***g*** for 30 min and washed with 5% PEG in the same buffer cooled

to 4°C. The precipitate was suspended in 2 ml of 0·1 M phosphate buffer, pH 7·4. This mixture was dialysed for 2 days at 4°C with two changes of 1,000 ml of 0·1 M Tris–HCl, pH 8·0, containing 0·5 M NaCl. The dialysed sample was then subjected to gel filtration using Pharmacia's Sephacryl S-200 superfine equilibrated in 0·1 M Tris–HCl, pH 8·0, containing 0·5 M NaCl. The high-molecular weight fractions were pooled by vacuum dialysis, dialysed against 0·1 M phosphate buffer, pH 7·8, and applied to a 1·5 × 10 cm column of protein A–Sepharose 4B (Pharmacia Fine Chemicals) and allowed to incubate for 2 hr at room temperature. The column was eluted with 0·1 M phosphate buffer and the elution monitored at 280 nm. When the elution had an optical density of less than 0·01, 0·2 M glycine–HCl buffer, pH 3·0, was used to elute the bound IgG-containing complexes. The bound protein eluted with the acid buffer was submitted to a final fractionation on a 1·5 × 30 cm column of Sephadex G-50 fine (Pharmacia Fine Chemicals) equilibrated in 0·2 M glycine–HCl buffer, pH 3·0.

Isolation of ^{125}I-insulin–anti-insulin IC. Three millilitres of serum (from either of the patients with evidence of high levels of anti-insulin antibodies) was mixed with 0·5 ml of Corning ^{125}I-labelled insulin and allowed to incubate for 1 hr at 37°C and for 2 hr at 4°C. This mixture was then treated in a manner identical to that of the serum containing native soluble IC. The column elutions were monitored for protein at 280 nm and for radiation with a Beckman 310 gamma counter.

Insulin detection. Insulin was detected using the Phadebas solid-phase radioimmunoassay for insulin (Pharmacia Diagnostics, Rahway, New Jersey).

RESULTS

Studies of IC formed in vitro using ^{125}I-labelled insulin

Four patients' sera were used to test our method for isolation of soluble IC. Table 1 gives the results for our tests for the detection of soluble IC and for the detection of anti-insulin antibodies in the four sera used in the present study. Sera A and B were used in the *in vitro* study.

The PEG precipitate obtained from serum A, after preincubation with radiolabelled insulin, was applied to the Sephacryl S-200 column (Fig. 1). The majority of the radioactivity was detectable in fractions eluted ahead of monomeric IgG indicating that the IC formed *in vitro* were not dissociated. These void volume fractions, pool I corresponding to the ascending limb and pool II to the descending limb, were then submitted to affinity chromatography in Sepharose–protein A. In both cases the only detectable radiation was associated with the protein that remained bound to protein A at neutral pH (Figs 2 and 3). That this radioactivity was associated with intact insulin was confirmed through the detection of insulin by radioimmunoassay in both acid-eluted peaks.

Table 1. Anti-insulin antibody and immune complex levels in the samples studied

Sample	Anti-insulin antibody (%)*†	Immune complexes (%) assayed by: PEG-C4	PEG-IgG	Laser	C1q binding
Patient A	32	0	0·2	—‡	6·1
Patient B	24	29	0·16	1	5·5
Patient C	71	39	0·60	60	n.d.
Patient D	68	2	0·2	1	5·6
Normal controls Mean ± s.d.	76 ± 4	17 ± 8	0·19 ± 0·1	1·5 ± 1·7	6·5 ± 0·98

* By coated charcoal radioimmunoassay.

† For explanation of values in each assay, see Materials and Methods section.

‡ High blank, unreliable result.

n.d. = Not done.

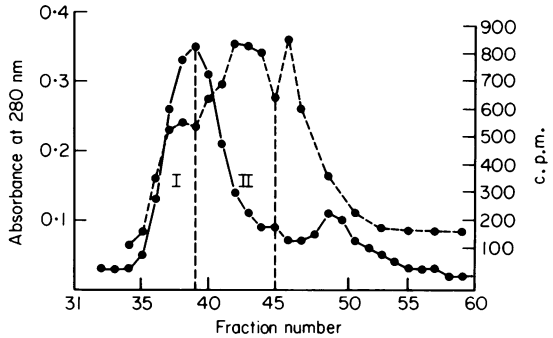

Fig. 1. Elution profile for gel filtration in Sephacryl S-200 of the proteins precipitated with 5% PEG from the serum of patient A after preincubation with radiolabelled insulin. In calibrations run with normal human serum, IgM was eluted in fractions 35 to 41 and IgG in fractions 46 to 56. (●——●) Absorbance, (●– – –●) c.p.m.

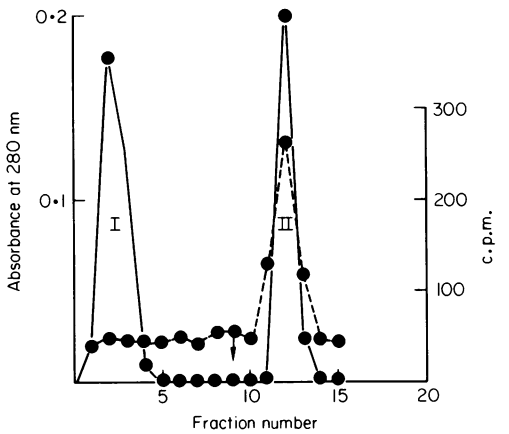

Fig. 2. Affinity chromatography on a Sepharose–protein A column of the proteins separated in pool I of the S-200 separation of serum A (see Fig. 1). The arrow points to the beginning of the elution with 1 M acetic acid. (●– – –●) c.p.m., (●——●) absorbance.

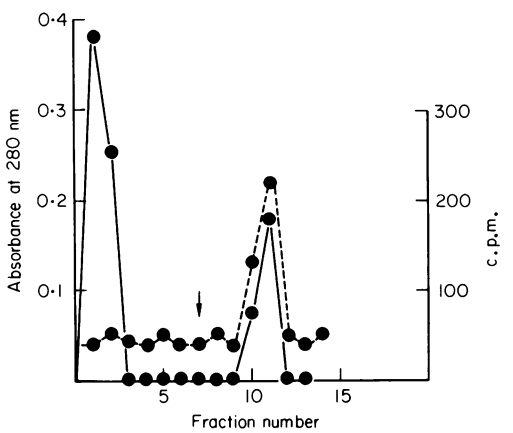

Fig. 3. Affinity chromatography on a Sepharose–protein A column of the proteins separated in pool II of the S-200 separation of serum A (see Fig. 1). The arrow points to the beginning of the elution with 1 M acetic acid. (●– – –●) c.p.m., (●——●) absorbance.

The separation of the PEG precipitate from patient B on a Sephacryl S-200 column produced two distinct peaks of activity, one at the void volume and the second in a position intermediate between the void volume and the IgG peak. After concentration, the first fraction contained only 800 c.p.m./ml, and with this low activity no further studies were possible. The second fraction from the Sephacryl S-200 column, containing 3,000 c.p.m./ml, was submitted to Sepharose–protein A affinity chromatography, and again the majority of the radioactivity was detected in the acid-eluted peak. There was a small amount of radioactivity in the unbound fractions.

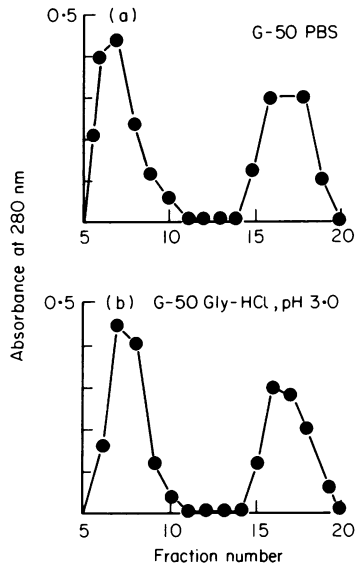

Fig. 4. Elution profile for separation of IgG and insulin on a Sephadex G-50 column equilibrated with PBS (a) or 0·2 M glycine–HCl, pH 3·0 (b).

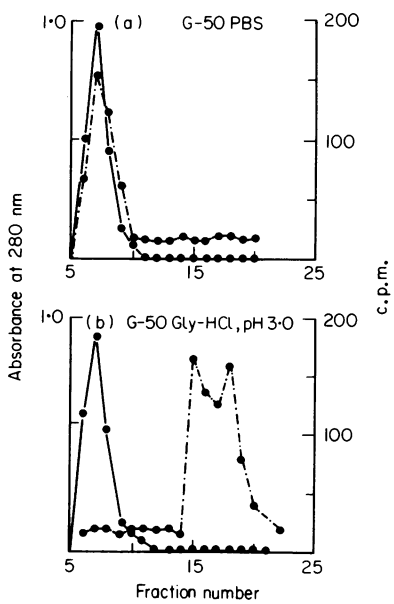

Fig. 5. Elution profile for the 5% PEG precipitate of serum A and radiolabelled insulin on a Sephadex G-50 column equilibrated with PBS (a) or 0·2 M glycine–HCl, pH 3·0 (b).

Dissociation of insulin–anti-insulin IC by Sephadex G-50 gel filtration

Fig. 4a,b shows the separation of purified IgG and insulin, run to calibrate our experiment, at pH 7·4 and 3·0. In both separations, IgG and insulin were eluted at distinct positions, IgG in the void volume and the insulin 3–5 ml after that. Fig. 5a,b shows the dissociation of the IC formed *in vitro*. Equal aliquots from a 5% PEG precipitate of 400 μl of serum from patient A and 100 μl of radiolabelled insulin were submitted to gel filtration using Sephadex G-50 at both neutral (7·4) and acid (3·0) pH. Fig. 5a shows the lack of dissociation of the radioactivity from the void volume at pH 7·4. Fig. 5b shows that, at pH 3·0, the only detectable radioactivity was present in the area corresponding to the free insulin elution volume. Similar dissociation of insulin and anti-insulin antibody was observed when the bound protein peak from the protein A affinity run of serum B was applied directly to an acidic Sephadex G-50 column. The majority of the radioactivity eluted after the major protein peak, and the void volume protein peak, when neutralized and reincubated with 0·01 μCi radiolabelled insulin, showed binding activity. Twenty per cent of the labelled insulin was found in the 5% PEG precipitate obtained thereafter.

Isolation of native IC

One patient (C), positive for soluble IC by all the screening methods used, was chosen for these experiments. A 5% PEG precipitate was obtained and dialysed against the buffer used for the next gel filtration step. Pools I and II from the Sephacryl S-200 gel filtration (ascending and descending limbs of the void volume) were submitted to protein A affinity chromatography, and the bound proteins were further separated on a Sephadex G-50 column under dissociating conditions as shown in Fig. 6. Insulin was detected by radioimmunoassay in pool II from S-200 gel filtration and in the bound, acid-eluted protein peak from protein A affinity chromatography of the same pool.

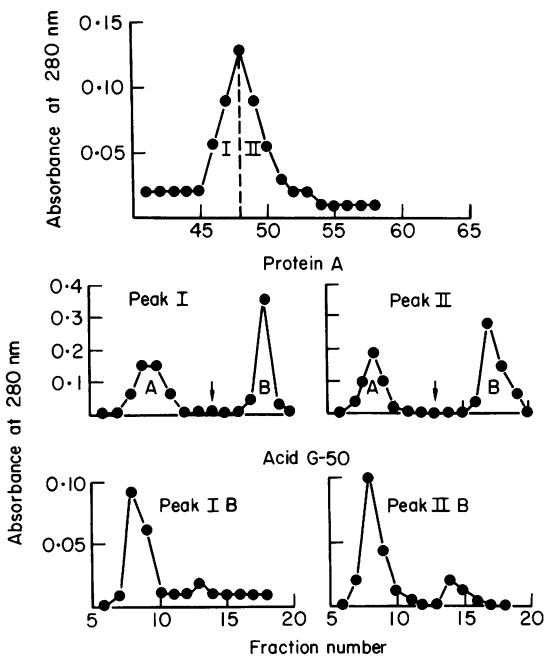

Fig. 6. Elution profiles for the three types of chromatography used for isolation of IC from the 5% PEG precipitate of serum C. The top profile represents gel filtration of the 5% PEG precipitate using Sephacryl S-200; the same column used for the separation shown in Fig. 4 was used for this separation. The middle two profiles correspond to affinity chromatography of pools I and II using Sepharose protein A; the arrows indicate the beginning of elution with 1 M acetic acid. The lower two profiles correspond to gel filtration on a Sephadex G-50 column equilibrated and eluted with 0·2 M glycine–HCl buffer, pH 3·0, of the fractions bound to Sepharose–protein A (IB and IIB).

Table 2. Insulin binding by the high-molecular weight fraction obtained after dissociating gel filtration of the acid-eluted peaks from Sepharose–protein A

Sample	Radiolabelled insulin binding (%)*	
	Non-dissociated†	Dissociated‡
Patient C§	14	21
Patient D§	10	9
Control serum¶	9	—

* Percentage of added ^{125}insulin precipitated with 10% PEG.

† Neutralized aliquots of acid-eluted peaks from Sepharose–protein A.

‡ Void volume fractions obtained when the acid-eluted peaks were filtered on Sephadex G-50 under dissociating conditions.

§ See text for details.

¶ Diluted 1:40 in pH 7·8 borate-buffered saline prior to the study of binding capacity.

After dissociation on the G-50 column, free insulin was no longer detectable, but testing of different peaks and a control dilution of normal human serum for binding activity toward radiolabelled insulin showed that the protein peak eluted from the G-50 column had significant binding activity (Table 2).

A fourth patient (D), with negative results in all screening tests for soluble IC, was submitted to the same protocol. Sephacryl S-200 gel filtration of the resuspended 5% PEG precipitate showed practically no protein at the void volume. Two fractions were obtained from the intermediate area between the void volume and the 7S fraction. Both fractions were chromatographed on Sepharose–protein A, and only fraction II (closer to the 7S peak) showed significant binding. The bound material, after acid elution, was dissociated by gel filtration of Sephadex G-50. The protein fraction was shown not to have significant binding capacity when incubated with radiolabelled insulin.

DISCUSSION

These studies had two main objectives: first, to test our isolation procedure for soluble IC, using complexes formed *in vitro* with radiolabelled insulin; and second, to determine whether insulin and anti-insulin antibodies could indeed be identified in the isolates from a serum that appeared to contain soluble IC by our screening methods.

To test our procedure we used two sera shown to contain high levels of anti-insulin antibodies and low levels of soluble IC by non-specific screening methods. Both sera showed binding activity for radiolabelled insulin but formed complexes of different sizes: in serum A the resulting IC were eluted primarily in the void volume fraction of a Sephacryl S-200 column, and in serum B the IC were smaller, eluting after the void volume fractions but well ahead of the monomeric IgG fraction. In both cases, when the IC-containing material was applied to the Sepharose–protein A column we observed that the radiolabelled insulin was eluted together with bound IgG, proving that our basic premise (that IgG-containing IC would bind to protein A) was correct. We also showed that the antigen and antibody could be dissociated, and that measurable antibody activity could be demonstrated in the antibody fraction at the end of this lengthy manipulation.

The results obtained *in vitro* constitute an important achievement, since they provide firm evidence for the soundness of our methodology for isolation of soluble IC. This is not only relevant within the framework of this experiment but also reinforces our earlier contention that this

procedure may be of general use for the isolation of IC. At present, the method has been successfully tested on samples from patients with Sjögren's syndrome, systemic lupus erythematosus and insulin-dependent diabetes.

These results indicate that in any patient with high levels of anti-insulin antibodies, soluble IC are likely to form, probably at antibody excess, when insulin is administered. In addition, our finding that IC could be isolated from a serum that gave consistently positive results with three screening techniques provides unquestionable evidence for the presence of soluble insulin–anti-insulin IC in a diabetic patient. This is the first time that such evidence has been obtained. These observations open a wide range of possibilities for research on the soluble IC of diabetic patients. We have observed (unpublished results) that sera from insulin-dependent diabetics can be divided into three groups: (a) those in which all the screening results are positive for IC; (b) those in which all the screening techniques are negative; and (c) those which give conflicting results. Conflicting results are a general problem in screening for soluble IC, but with diabetic sera we can now try to determine whether factors such as the size or immunoglobulin composition of soluble IC can be correlated with such discrepancies.

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