ANTIBODIES TO PROINSULIN IN DIABETIC PATIENTS TREATED WITH PORCINE INSULIN PREPARATIONS

By

O. Ortved Andersen

ABSTRACT

In most diabetic patients treatment with commercial insulin preparations containing 1 to 2 per cent proinsulin, results primarily in the formation of antibodies reacting with both insulin and proinsulin. Later on the formation of antibodies reacting specifically with proinsulin also seems to occur. In some plasma samples from patients treated with porcine insulin it has been possible to demonstrate the formation of antibodies reacting with proinsulin and intermediate forms, but not with monocomponent insulin, dimer forms or C-peptide from porcine insulin. No reaction of bovine proinsulin with antibodies to porcine proinsulin could be demonstrated.

The observations indicate that proinsulin has considerable significance for antibody formation in diabetic patients treated with the commonly available insulin preparations.

Steiner & Oyer (1967) have established that insulin is synthesized in the islets of Langerhans as a single chain polypeptide, proinsulin. Chance & Ellis (1969) and Melani & Steiner (1970) have found a content of 5 per cent proinsulin in the pancreas. Proinsulin and intermediate forms in amounts of 1 to 2 per cent have been isolated from commercial insulin preparations (Steiner et al. 1968; Chance & Ellis 1969; Schlichtkrull et al. 1969). The purpose of this investigation has been to examine the antigenic properties of proinsulin in commercial insulin preparations given to diabetic patients, and furthermore to examine the specificity of the antibodies formed.
MATERIAL AND METHODS

Dilution media

The dilutions of antisera, plasma and preparations were carried out with 0.04 M phosphate buffer pH 7.4, containing 0.5 % human albumin and 0.9 % NaCl. Human albumin was supplied by Statens Serum Institut, Copenhagen.

Preparations

Recrystallized porcine insulin and bovine insulin with a biological activity as measured by the mouse convulsion method of 23.3 and 25.5 units per mg dry weight respectively, were obtained from Nordisk Insulin Laboratory, Copenhagen.

Porcine proinsulin was kindly supplied by the Lilly Research Laboratories, Indianapolis.

Porcine proinsulin, porcine intermediate forms, porcine C-chain-peptide and porcine dimer forms were isolated, purified and kindly supplied by lic. pharm. E. Pedersen from The Research Laboratory, Nordisk Insulin Laboratory and Steno Memorial Hospital. Lic. pharm. E. Pedersen has also kindly placed Fig. 1 at the author's disposal.

Porcine monocomponent insulin has been kindly supplied by cand. scient. S. Linde, The Research Laboratory, Steno Memorial Hospital.

\[^{125}\text{I}]-preparations

Radioactive labelling of 10 µg porcine insulin and 15 µg porcine proinsulin was carried out according to Hunter & Greenwood (1962) as described by Brunfeldt & Jørgensen (1967). 1 mCi \[^{125}\text{I}\] obtained from The Radiochemical Center, Amersham, was used for labelling. After labelling, the \[^{125}\text{I}\]-porcine insulin and the \[^{125}\text{I}\]-porcine proinsulin were diluted and dispensed in solutions containing approximately 0.5 and 0.75 ng per ml respectively. The solutions, only thawed once, were stored at -20°C until use.

Fig. 1.

Fig. 2.
Competition experiments on plasma from three patients treated with porcine protamine-insulin. The per cent precipitated $^{125}$I-porcine proinsulin and $^{125}$I-porcine insulin in competition with different amounts unlabelled porcine monocomponent insulin and porcine proinsulin are indicated.

**Rabbit anti-human IgA and IgG serum**

The antisera used as precipitating sera were supplied by Brostex A/S, Copenhagen. To obtain maximum precipitation of the human gamma globulins, preliminary antigen-antibody titrations were carried out to estimate the suitable proportion between the dilution degree of human plasma and rabbit anti-human IgA and IgG serum.

**Assay technique**

Blood for antibody determination was collected in tubes containing heparin. Plasma was stored until use at $-20\degree C$.

For the assay 50 µl human plasma diluted 1:25 was mixed with 50 µl undiluted rabbit anti-human IgA and IgG serum. After standing for 24 h at $4\degree C$, 100 µl labelled antigen ($^{125}$I-porcine insulin or $^{125}$I-porcine proinsulin), and 100 µl buffer or unlabelled
antigen solution were added. While standing for a further 24 h at 4°C, the tubes were carefully shaken twice in order to suspend the precipitate. Finally the precipitate was filtered off, using an Oxoid membrane filter. The radioactivity remaining on the filter was determined in a well counter. All plasma samples were tested in duplicate.

RESULTS

Fig. 2 shows the binding capacity of plasma from three patients treated with porcine protamine-insulin. The binding capacity was determined, partly to 125I-porcine insulin and partly to 125I-porcine proinsulin in competition with unlabelled porcine monocomponent insulin or porcine proinsulin. Each patient is representative of the patterns of several patients. In the plasma from patient (A) the binding capacity of 125I-proinsulin is seen to be about 100 times that of 125I-insulin. Unlabelled proinsulin is seen to reduce the amounts of 125I-proinsulin precipitated, whereas monocomponent insulin is not capable of competing, showing no cross-reaction with the antibodies binding the 125I-proinsulin. On the other hand, the amounts of precipitated 125I-insulin are seen to be reduced by the addition of even small amounts of monocomponent insulin showing antibodies reacting with the monocomponent insulin. In this experiment, small amounts of proinsulin did not influence the amounts of 125I-insulin precipitated. When greater amounts of proinsulin were added, the amounts of precipitated 125I-insulin were seen to be reduced to the same degree as if monocomponent insulin in the same molar concentration had been added. In the plasma from patient (B), small amounts of monocomponent insulin did not influence the amounts of 125I-proinsulin precipitated. When greater amounts of monocomponent insulin were added, the amounts of 125I-proinsulin precipitated were seen to be reduced to a certain extent. The pattern of competition experiments with 125I-insulin is seen to be very similar to the same experiment on plasma from patient (A). In the plasma from patient (C), unlabelled monocomponent insulin and proinsulin are seen to compete almost equally with 125I-proinsulin and 125I-insulin.

Fig. 3 shows the plasma binding capacity of 125I-proinsulin and 125I-insulin respectively in a patient during treatment with porcine protamine-insulin. The binding capacities with and without competition of unlabelled proinsulin or monocomponent insulin respectively, are shown. After a short period of treatment, the binding capacity of 125I-insulin is seen to be of greater magnitude than that of 125I-proinsulin. Furthermore, it is seen that the binding capacity of 125I-insulin has reached a maximal level before that of 125I-proinsulin. Unlabelled proinsulin and monocomponent insulin are seen to compete equally with 125I-insulin. The same pattern is seen with 125I-proinsulin after a short period of treatment. After treatment for a longer period, monocomponent
The binding capacity of plasma from a patient at different times in the treatment with porcine protamine-insulin. The per cent precipitated $^{125}\text{I}$-porcine proinsulin and $^{125}\text{I}$-porcine insulin without and in competition with unlabelled porcine monocomponent insulin and porcine proinsulin are indicated.

Insulin is seen to compete to a smaller and smaller extent while unlabelled proinsulin is seen to compete with $^{125}\text{I}$-proinsulin to the same extent as to $^{125}\text{I}$-insulin.

Fig. 4 shows a competition experiment between $^{125}\text{I}$-proinsulin and different derivates of insulin preparations. In equimolar concentrations, intermediate forms are seen to compete to a lesser extent than proinsulin. C-peptide, monocomponent insulin and dimer forms shows no detectable cross-reaction to antibodies binding $^{125}\text{I}$-proinsulin.

In the assay, plasma from a patient with an antibody titre to proinsulin about 100 times as large as to that of insulin was used.

Fig. 5 shows a competition experiment between $^{125}\text{I}$-proinsulin, recrystallized porcine insulin, recrystallized bovine insulin and monocomponent porcine
As antibody, a patient plasma with an antibody titre to proinsulin 100 times as large as to that of insulin was used in a concentration binding 30 per cent of the added $^{125}$I-proinsulin. It is seen that neither the addition of monocompoment porcine insulin nor recrystallized bovine insulin could reduce the amounts of $^{125}$I-proinsulin precipitated. On the other hand, recrystallized porcine insulin reduced the amounts of precipitated $^{125}$I-proinsulin to a certain extent, indicating that the recrystallized porcine insulin contained proinsulin (about 1.8 per cent). Furthermore the experiment shows that bovine proinsulin does not react with antibodies to porcine proinsulin.

Plasma from 8 patients without any detectable antibodies to insulin was tested before treatment and after more than 6 months' treatment with porcine insulin. Proinsulin binding antibodies could not be detected in any of these patients' plasma.

![Graph](image-url)

**Fig. 4.** Competition experiment on plasma from a patient treated with porcine protamine insulin. The per cent precipitated $^{125}$I-porcine proinsulin in competition with unlabelled porcine proinsulin, porcine intermediate forms, porcine C-peptide, porcine dimer forms and porcine monocomponent insulin are indicated.
Competition experiment on plasma from a patient treated with porcine protamine-insulin. The per cent precipitated $^{125}$I-porcine proinsulin in competition with unlabelled porcine monocomponent insulin recrystallized bovine insulin and recrystallized porcine insulin are indicated.

**DISCUSSION**

The content of proinsulin in the islets of Langerhans has been estimated to be approximately 5 per cent (Chance & Ellis 1969; Steiner et al. 1969; Melani & Steiner 1970; **Leading article**: Lancet 1970; Schlichtkrull 1970). Commonly available insulin preparations do not contain C-peptide (Schlichtkrull et al. 1969; Melani & Steiner 1970). Several investigators have demonstrated a content of 1 to 2 per cent proinsulin in the insulin preparations (Steiner & Oyer 1967; Chance & Ellis 1969; Steiner et al. 1969). Schlichtkrull (1970) and **Leading article**: Lancet (1970) have suggested that impurities in the insulin preparations, proinsulin, intermediate forms or dimers could be due to the insulin antibody formation. Contrary to this, Kerp et al. (1970) could not confirm that proinsulin impurities stimulate insulin antibody production.

In the present material, it has been established that treatment of diabetic patients with commercial insulin preparations containing proinsulin leads to the formation of antibodies reacting with insulin and proinsulin. Contrary to the findings of Kerp et al. (1970), several plasma samples exhibited a binding of proinsulin greater than that of insulin, and in some samples the binding of proinsulin was 100 times that of insulin.

In accordance with animal studies by Schlichtkrull et al. (1969) and Heding (1970), antibodies binding porcine proinsulin but not porcine monocomponent insulin (at any rate less than 2 per cent), have been found in patients treated with commercial porcine insulin preparations.

It is generally accepted (Rubenstein et al. 1969; Steiner et al. 1969; Kitabchi 1970; Wright & Makulu 1970), that proinsulin reacts to a lesser extent than
insulin with insulin antibodies. In the present study this view could not be confirmed. In Fig. 2C the results of a competition experiment are shown, where porcine proinsulin and monocomponent insulin are seen to compete equally with \(^{125}\)I-porcine insulin. In Fig. 2A and B small amounts of proinsulin do not compete with \(^{125}\)I-insulin, whereas monocomponent insulin does. This could be explained by a prevailing binding of proinsulin to proinsulin antibodies, present in high titres, and not reacting with \(^{125}\)I-insulin. Contrary to Kitabchi (1970) and Rubenstein et al. (1969) (Fig. 4), but in accordance with Rubenstein et al. (1969) (Fig. 5), it has been found that when proinsulin is added in greater amounts, proinsulin and monocomponent insulin compete equally with \(^{125}\)I-insulin.

The reaction of proinsulin with insulin antibodies seems to be contradictory to the findings that monoclonal insulin did not compete with \(^{125}\)I-proinsulin in Fig. 2A. It is seen that the plasma binding capacity of proinsulin is about 50 to 100 times that of insulin. The most probable explanation of this phenomenon seems to be that the difference in the antibody titres causes about 98 to 99 per cent \(^{125}\)I-proinsulin to be bound to proinsulin antibodies, which do not react with monocomponent insulin, and only 1 to 2 per cent \(^{125}\)I-proinsulin to be bound to insulin antibodies. Monocomponent insulin competes with this 1 to 2 per cent, but the reduction in the amounts of precipitated radioactivity is not detectable in the assay system used.

By the use of plasma with a high titre to proinsulin, it has been possible to demonstrate a reduced immunological reactivity of intermediate forms proportional to proinsulin. Possible as a consequence of the heterogeneity of the preparations tested, the significance of a splitting off at the position near the A-chain or B-chain, cannot be excluded in this connection. Porcine dimers, porcine C-peptide or bovine insulin (containing 1 to 2 per cent proinsulin) did not react with antibodies to porcine proinsulin. As might have been expected, porcine insulin (containing 1 to 2 per cent, calculated as 1.8 per cent proinsulin), did react to a certain extent.

Steiner et al. (1969) showed by amino acid analyses of porcine and bovine proinsulin that their C-peptide sequences differed at 20 out of 33 positions. In agreement with this Root, referred to by Chance & Ellis (1969), in agreement with results in the present study, found that porcine proinsulin antibodies did not cross-react with bovine proinsulin. Rubenstein et al. (1969) and Christiansen & Heding (1970) found a certain reaction of bovine proinsulin with porcine antibodies, but this phenomenon can possibly be explained by cross-reaction with antibodies to the insulin part of the porcine-proinsulin.

In accordance with Rubenstein et al. (1969) and Schlichtkrull et al. (1969), I did not find porcine C-peptide reacting with antibodies to porcine proinsulin, indicating that the antibodies are determined by antigenic sites including both the C-peptide part and the insulin part of proinsulin. The reduced reactivity of
the intermediate forms corroborates the significance of the spatial structure. In contrast Kaneko & Oka and Root & Chance, referred to by Yanaihara et al. (1972) have observed that partially protected peptides consisting of amino acid sequences corresponding to porcine C-peptide reacted with antisera to porcine proinsulin and furthermore the amino acids with positions 49 to 50 should be of decisive significance.

Testing the reactivity with $^{125}$I-proinsulin and $^{125}$I-insulin with and without competition with unlabelled proinsulin or monocomponent insulin in patients treated with commercial porcine insulin preparations containing proinsulin, as a function of time, the binding capacity of $^{125}$I-insulin is seen to be greater than that of $^{125}$I-proinsulin early in the treatment. Later on, after half a year of treatment, the binding capacity of $^{125}$I-insulin is seen to have reached a maximal level, whereas that of $^{125}$I-proinsulin is still rising. Furthermore the observations confirm that unlabelled proinsulin competes equally with mono- component insulin, with $^{125}$I-insulin – whereas the binding of $^{125}$I-proinsulin seems to be more and more specific. The phenomenon could be explained by antibodies to proinsulin becoming more and more specific. More likely seems the assumption of two (or more) antibodies – firstly the formation of antibodies determined by insulin or the insulin part of proinsulin. These antibodies should be able to react with both insulin and proinsulin from several species. Later on would come the formation of antibodies determined by antigenic sites including both the C-peptide part and the insulin part of the proinsulin. The antigen differing so much in primary structure, these antibodies should be rather specific.

The deficiency of demonstrable $^{125}$I-proinsulin binding with proteins in plasma of patients without any demonstrable insulin antibodies, might point to insulin antibodies determined by the insulin component of proinsulin. On the other hand the phenomenon could be caused by divergent immunological reactivity in these patients.

Treatment of patients with porcine monocomponent Lente insulin (Schlichtkrull 1970; Fankhauser & Michl 1971), with porcine monocomponent insulin (Levett & Korp 1971), and porcine insulin free of proinsulin as protamine-insulin, (personal investigation) results in only a very small insulin antibody formation, indicating that proinsulin or proinsulin related products play an important role in the insulin antibody formation. Since most of the investigators mentioned, however, find insulin antibodies in small amounts, proinsulin cannot be the only antigen.

**ADDENDUM**

The porcine C-chain peptide has been isolated according to Sundby & Markussen (1970). The preparation has been tested by Sephadex-gel-filtration, paper electrophoresis in different buffers, qualitative amino acid analyses and N-terminal deter-
mination. Recently the porcine C-chain peptide preparation has been tested in quantitative amino acid analyses, all amino acids but proline were found in mutual concordant proportions. Only fifty per cent of the expected content of proline was found. So the observation of no competitive effect of C-peptide must be taken with some reservation.

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REFERENCES


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