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A direct two-site peroxidase-based enzyme-linked immunosorbent assay for proinsulin

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Abstract: A non-competitive sandwich assay using an anti-C-peptide IgG and an anti-insulin IgG was developed to measure fasting levels of proinsulin in human serum. The former antibody provided the lower layer in a sandwich immunoassay, the upper layer being composed of an anti-insulin IgG-horse radish peroxidase conjugate. The assay showed negligible cross reactivity at supraphysiological levels of insulin and C-peptide. The method enabled the estimation of proinsulin in fasting non-diabetic control subjects [13.7 ± 1.6(4) pM] and in type 2 non-insulin-dependent diabetic patients [23.2 ± 1.1(8) pM].

Keywords: *Proinsulin (fasting); peroxidase; anti-C-peptide IgG; anti-insulin IgG; immunoassay.*

Introduction

Proinsulin, the precursor of insulin, is synthesized in the β cells of the islets of Langerhans [1]. It is converted by specific enzymes in the β cell secretory granules into insulin via several intermediate forms [2]. Elevated amounts of proinsulin immunoreactive material have been reported in serum in various conditions and diseases and could be an indication of altered β cell function [3, 4]. In recent years monoclonal antibody-based two-site immunoradiometric assays have been established for the specific measurement of insulin, proinsulin and split proinsulin forms in plasma from diabetic patients [5]. These assays have provided new information concerning insulin deficiency in non-insulin dependent diabetes [6]. However, a method for the routine measurement of proinsulin in plasma from fasting normal subjects has been more difficult to develop largely because of the insensitivity of existing two-site immunoradiometric assays and the requirement for frequent iodinations. An amplified immunoenzymometric assay was recently reported to be capable of achieving the desired sensitivity and degree of automation [7]. Here we describe an alternative immunoassay using polyclonal antibodies against human C-peptide and insulin IgG which was sensitive for the measurement of fasting proinsulin levels and which would be suitable for proinsulin determinations in large numbers of sera samples from both diabetic and non-diabetic subjects.

Materials and Methods

Materials

Human biosynthetic proinsulin was obtained from the Lilly Research Centre Ltd (Surrey, UK). Anti-Human C-peptide guinea pig serum M1221 was donated by NovoNordisk. Human C-peptide, anti-guinea pig IgG-peroxidase and antiserum to pig insulin were purchased from The Sigma Chemical Company Ltd (Poole, Dorset, UK). All other reagents were of analytical reagent grade.

Characterization of antibodies

Anti-insulin IgG was conjugated to horse radish peroxidase using the periodate method [8]. Working dilutions and anti-C-peptide IgG and anti-insulin IgG-peroxidase were determined by carrying out checkerboard titrations wherein serial dilutions of proinsulin (0.025–2.5 μg ml^{-1}) were immobilized on a plate and serial dilutions of the antibodies were reacted against the immobilized antigen. Plates were incubated for 2 h at 37°C, washed and incubated with either 100 μl of anti-guinea pig IgG-peroxidase conjugate (for anti C-peptide IgG) or 100 μl of anti-insulin IgG-peroxidase conjugate (for anti-insulin IgG-peroxidase)

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prior to addition of substrate (*o*-phenylenediamine). The reaction was stopped by addition of 50 µl 2 M H_2SO_4 and absorbance measured at 490 nm. Time courses for binding of anti-C-peptide IgG to microtitre plates and for binding of antibodies to proinsulin were determined as described below.

Immunoassay procedure

One hundred µl of anti-C-peptide IgG diluted to its working concentration (10 µg ml^{-1}) in 50 mM sodium carbonate buffer pH 9.6 was added to wells of a microtitre plate. After incubation at 37°C for 2 h, the wells were washed three times with 0.05% (w/v) Tween 20 in phosphate buffered saline containing 12 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 150 mM NaCl, pH 7.4 and blocked with 1% (w/v) bovine serum albumen in carbonate buffer.

One hundred µl aliquots of serum (frozen at −20°C and thawed at 37°C immediately prior to assay) or proinsulin standards (0.1–500 pg ml^{-1}) were added in triplicate to the wells, followed by incubation at 37°C for 2 h. After triplicate washings, 100 µl anti-insulin IgG-peroxidase conjugate (300 µg ml^{-1}) was added and the plate was incubated 24 h overnight at 4°C. After washing five times, 100 µl freshly prepared substrate was added to each well and the plate was incubated for 30 min at 37°C. The reaction was then stopped by addition of 50 µl 2 M H_2SO_4 and absorbance of the oxidized form of *o*-phenylenediamine (yellow) at 490 nm was measured after 5 min at room temperature.

Cross reactivities of insulin and C-peptide in the complete assay were determined by incubating with insulin (1–1000 ng ml^{-1}) and C-peptide (10–20000 ng ml^{-1}).

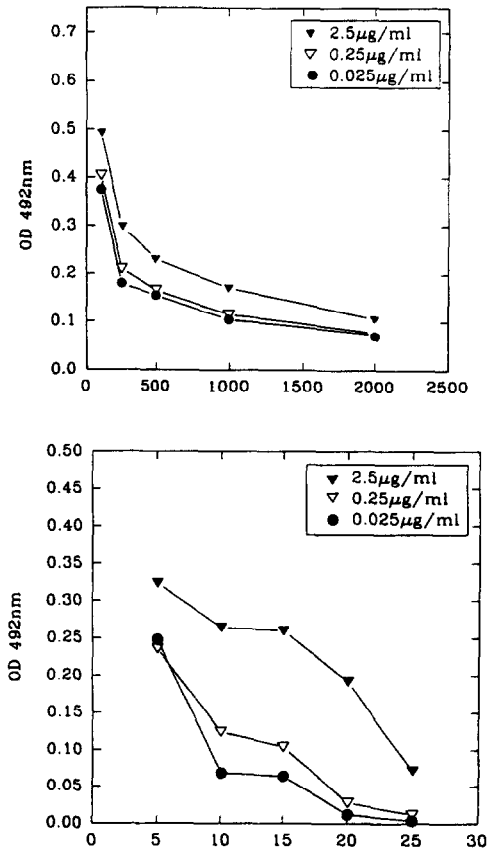

Figure 1
OD at 492 nm as a function of dilution factors for anti-C-peptide IgG (top panel) and anti-insulin IgG conjugate (bottom panel). Titrations of immobilized proinsulin (0.025–2.5 µg ml^{-1}) with serial dilutions of antibodies were carried out as described in the Methods section.

Results

Characterization of antibodies

A 1:10 dilution (300 µg ml^{-1}) of anti-insulin IgG-peroxidase conjugate and 1:500 dilution (10 µg ml^{-1}) of anti-C-peptide were shown to be suitable following reaction of serial dilutions of the antibodies against immobilized proinsulin (Fig. 1). The optimum incubation time for adsorption of the anti-C-peptide IgG to a microtitre plate (Fig. 2) and for its binding to immobilized proinsulin (Fig. 3) was 2 h at 37°C. A much longer incubation time of 8 h at 37°C or 24 h at 4°C was required for the binding of proinsulin to antiinsulin IgG conjugate (Fig. 4).

Assay characteristics

A standard curve (fitted by ruler and pen) for human proinsulin constructed in these conditions is shown in Fig. 5. The data represent the composite standard curve obtained from six separate assays relating $\log_{10}$ proinsulin concentration (pg ml^{-1}) with absorbance at 492 nm. Insulin and C-peptide did not crossreact in this assay at levels below 50 and 10 µg ml^{-1}, respectively. The detection limit of the assay was 1 pg ml^{-1} as assessed by the response representing the mean plus three times the standard deviation of the lowest detectable absorbance. The assay was linear up to 500 pg ml^{-1} which provided a suitable working range for detecting proinsulin in human serum. The robustness of the method was assessed by assaying in triplicate three concentrations of proinsulin (10, 250 and

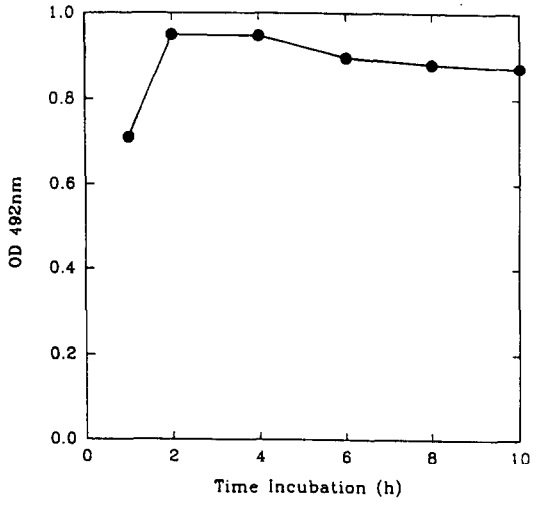

Figure 2
Time course and anti-C-peptide IgG binding to microtitre plate vs OD at 492 nm. One hundred μl anti-C-peptide IgG diluted 1:500 was added to wells of a microtitre plate for varying lengths of time. After blocking, the plate was incubated for 2 h at 37°C with 100 μl of anti guinea pig IgG-peroxidase conjugate and subsequently incubated with substrate as described in the Methods section.

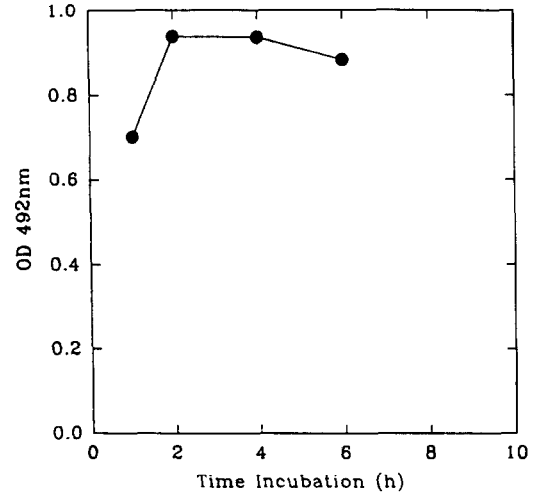

Figure 3
Time course and anti-C-peptide IgG binding to immobilized proinsulin vs OD at 492 nm. One hundred μl of proinsulin (10 ng ml^{-1}) was added to wells of microtitre plates, incubated at 37°C for 2 h, washed and blocked. One hundred μl anti human C-peptide IgG diluted 1:500 was added to each well and incubated for varying lengths of time. After blocking, the plates were incubated for 2 h at 37°C with 100 μl of anti guinea pig IgG-peroxidase conjugate. Plates were subsequently incubated with substrate as described in the Methods section.

500 pg ml^{-1}) over six different days. Interassay RSD was 3.6, 4.1 and 2.6% at proinsulin concentrations of 10, 250 and 500 pg ml^{-1}, respectively. 100 μl aliquots of undiluted serum from eight Type 2 (non-insulin-dependent) diabetic patients and four non-diabetic control subjects were assayed in triplicate. Mean serum proinsulin concentration was

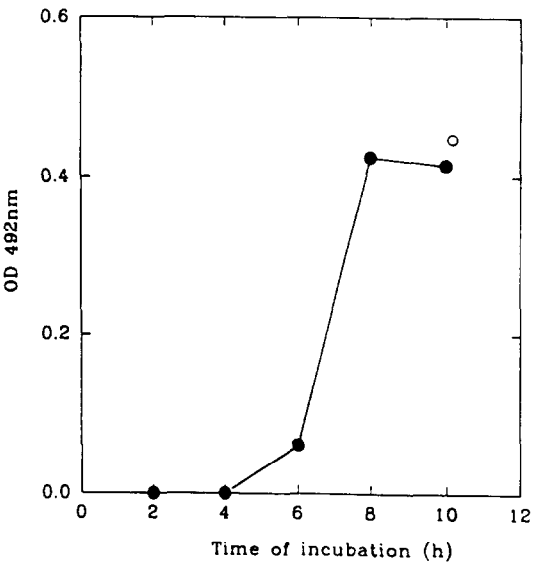

Figure 4
Time course of anti insulin IgG conjugate binding to immobilized proinsulin vs OD at 492 nm. One hundred μl of proinsulin (10 ng ml^{-1}) was added to wells of microtitre plates, incubated at 37°C for 2 h. After washing and blocking, the plates were incubated for varying lengths of time at 37°C with 100 μl of anti insulin IgG-peroxidase conjugate. Plates were subsequently incubated with substrate as described in the Methods section. Key: Closed circles: Time (h) at 37°C, Open circle: 24 h at 4°C.

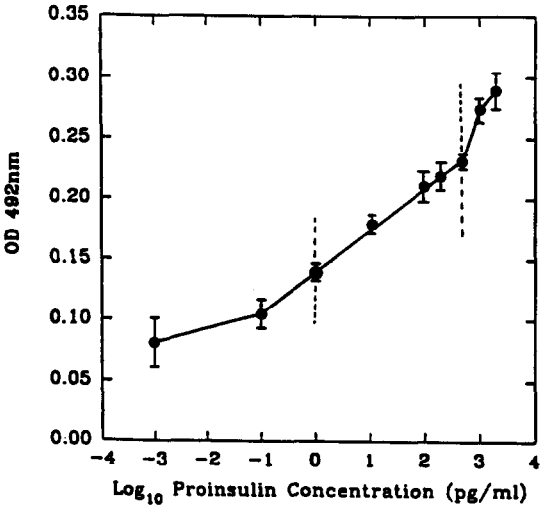

Figure 5
Standard curve of OD 492 nm vs $\log_{10}$ proinsulin concentration (pg ml^{-1}). Data represent mean ±SD for six determinations. Insulin and C-peptide did not react <50 ng ml^{-1} or 10 μg ml^{-1} respectively. The corresponding OD 492 values for Insulin (50 ng ml^{-1}) and C-peptide (10 μg ml^{-1}) were 0.024 and 0.011, respectively. The dotted lines show the lower (1 pg ml^{-1}) and upper (500 pg ml^{-1}) limits of quantification.

significantly higher ($P < 0.05$) in the patient group (23.2 ± 1.1 pM) relative to the control group (13.7 ± 1.6 pM).

Discussion

In the process of establishing the design of this assay with respect to suitable signalling and capturing antibodies, it was necessary to consider the very wide range of concentrations of insulin, its related peptides and C-peptide in serum. A method is described here where proinsulin was conveniently estimated by a two-site technique using anti-C-peptide IgG as capturing antibody that recognized some part of the peptide moiety of proinsulin. The sandwich was completed with an enzyme labelled antibody specific for the insulin moiety of the proinsulin such that the only possibility for the sandwich formation was when the intact proinsulin and/or one or both of its split forms was present. Characterization of the antibodies involved determination of the working dilutions, cross reactivities and optimum incubation times for each step in the assay. Initially the optimum incubation time for anti-insulin IgG-HRP conjugate was shown to be 8 h at 37°C. To overcome possible adverse side effects on conjugate stability that might seriously affect sensitivity we examined the effect of a longer incubation time at 4°C while employing the other previously described optimum assay conditions. Since incubation at 4°C overnight gave similar absorbance readings as those at 37°C for 8 h we chose to carry out all subsequent assays at the lower temperature.

Although the cross reactivities of des- and split proinsulins in this ELISA were not determined, the assay did however exhibit remarkably low cross reactivity with both human insulin and C-peptide. The high binding capacity of the anti-C-peptide IgG coated solid phase as shown in Fig. 2 suggests that the serum concentration of C-peptide would not be so greatly in excess of the proinsulin concentration as to have any marked effect on the proinsulin concentration. By incubating the serum sample in the coated well and then removing it and washing the well before adding the labelled anti-insulin IgG, interference by binding of insulin in the serum sample to the labelled antibody was precluded.

This two-site (non-competitive) ELISA using antisera to C-peptide and insulin represented an improvement in sensitivity for the assay of proinsulin by 10-fold over previous and more lengthy techniques [9] and yet was as sensitive as the recently described amplified enzyme linked immunoassay [10]. The low limit of detection, 1 pg ml^{-1} (0.1 pM) as shown in Fig. 5 combined with a wide range of detection up to 500 pg ml^{-1} (55.5 pM) enabled the estimation of the low concentration of proinsulin expected in fasting normal subjects (13.7 ± 1.6(4) pM) as well as circulating proinsulin levels in Type 2 (non-insulin-dependent) diabetic patients (23.2 ± 1.1(8) pM). Such an assay has potential for providing information on aspects of proinsulin secretion dynamics in both fetal and adult β cells and in the diagnosis of insulinoma.

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