Immunoradiometric Assay of Human Proinsulin and Partially Processed Proinsulin with use of Monoclonal Antibody and Streptavidin-biotin Labeling

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The sensitive and specific immunoradiometric assay is described for human proinsulin and its intermediate peptides (65-66 split and 32-33 split proinsulin). We developed a monoclonal antibody-based two-site immunoradiometric assay with use of streptavidin-biotin labeling. The detection limits of the assays lie in the range of 0.5-2.0 pM. In the proinsulin assay proinsulin cross-reacted 66% with 65-66 split proinsulin but not with insulin or 32-33 split proinsulin. In the assay of 65-66 split proinsulin it does not cross-react with insulin, proinsulin or 32-33 split proinsulin. In the 32-33 split proinsulin assay it cross-reacted 84% with proinsulin and 60% with 65-66 split proinsulin. The precision (C.V.) of the assays was less than 15% over the various concentration.

The mean concentrations of insulin, proinsulin, 65-66 split proinsulin and 32-33 proinsulin in eight young male subjects in the fasting state were (pM±S.E.M.) 20±3.6, 2.3±0.3, undetectable (< 1.0) and 2.1±0.7 and at the maximum reached during an oral glucose tolerance test, 150±26, 9.9±1.4, 3.8±0.6 and 19.7±6.0 respectively.

Key Words: Immunoradiometric assay, monoclonal antibodies, streptavidin-biotin, proinsulin, diabetes.

INTRODUCTION

The discovery of proinsulin (Steiner, 1967), the demonstration that proinsulin-like molecules exist in human plasma (Roth et al., 1986) and that human proinsulin reacts like human insulin in many human

insulin radioimmunoassay methods (Heding, 1975) has put in question the specificity of human plasma insulin radioimmunoassays. Since proinsulin-like molecules are present in higher concentration in plasma from diabetic subjects (Given et al., 1985) and their biological, insulin-like activity is very much lower than that of insulin (Peavy et al., 1985), their measurement as 'insulin' may lead to incorrect conclusions concerning the availability of biologically active insulin in diabetes.

Beck and Hales (1975) devised the indirect two-site immunoradiometric assay which gives greatly enhanced assay specificity with use of lesser amount of pure antibody. This approach was used to establish an assay for human proinsulin (Rainbow et al., 1979) but it detected partially processed proinsulins

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#This work was supported by Free Competition Research Grant of Korea Research Foundation (1988), Clinical Research Grant of Seoul National University Hospital (1988) and Research Grant of British Council in Seoul (1986).

#This study was presented at the annual meeting of the Korean Society of Molecular Biology, October 21, 1989

and did not recognize intact proinsulin (Gray *et al.*, 1984). Therefore, the assay systems are needed that can distinguish specifically the individual insulin-like molecules in plasma-insulin, intact proinsulin, and partially processed proinsulin. The processing of human proinsulin is believed to occur by endoproteolysis at the junctions of amino acids 32 and 33 and 65 and 66 to yield respectively 32-33 and 65-66 split proinsulin. Removal of the pairs of basic amino acids thereby exposed at the new C-terminal positions is thought to be carried out by a carboxypeptidase and produces respectively des-31-32 and des-64-65-proinsulin.

We attempted to produce a panel of monoclonal antibodies with different binding specificities for these immunologically insulin-like molecules, such that their use in different combinations would allow us to determine specifically the individual concentrations of these molecules. We biotinylated these monoclonal antibodies and 125 I-labeled streptavidin was used for detection.

The resulting immunoradiometric assay has the following advantages: improved linearity and precision, absence of a high-dose "hook" effect, no damage of monoclonal antibodies by oxidation during labeling, small amounts of radioactive waste. We report the assay procedure together with the concentrations of insulin, intact, and partially processed proinsulins in plasma from normal male subjects.

MATERIALS

Reagents

Intact, 65-66 split, des-64-65, 32-33 split and des-31-32 human proinsulins were synthesized, purified by high pressure liquid chromatography by Lilly Research Laboratories (Indianapolis, IN 46285, U.S.A.), (Frank *et al.*, 1981). Human insulin standard (Actrapid) was a gift from Novo Industri A/S and anti-human C-peptide antibody PEP 001 was a gift from Novo Laboratories (Bagsvaerd, Denmark). 125 I-labeled streptavidin (specific activity, 23 Ci/g) was obtained from Amersham, U.K. All chemicals were obtained from Sigma Chemical Co., and tissue culture medium and reagents were purchased from Gibco.

Collection of human plasma

Samples of blood from eight non-diabetic, non-obese healthy male subjects, age range 22-36 years, were collected into heparinized tubes before and during a 75g oral glucose tolerance test and centrifuged at 1800g at room temperature for 5 min. The plasma was stored at -20°C until assayed.

Standards

Standards were made up in 0.05 M-sodium barbitone buffer, pH 8, containing 5% (w/v) bovine serum albumin and stored at -20°C until used.

METHODS AND RESULTS

Preparation and selection of monoclonal antibodies

Mouse monoclonal antibodies reactive with human insulin and intact proinsulin were obtained by fusing mouse spleen cells with NSO mouse myeloma cells (Gray *et al.*, 1987). The monoclonal antibody A6 was obtained from a mouse immunized by subcutaneous injection of intact human proinsulin (20 μg) emulsified in Freund's complete adjuvant. A boost immunization (20 μg) was performed intravenously after 6 weeks and again after 10 weeks from the second immunization. The final boost immunization (20 μg) was carried out by intravenous injection 1 year later (4 days before fusion). This immunization protocol was one of a series utilized in these experiments with various intervals between primary and booster injections. The monoclonal antibody 14B was obtained from a mouse immunized by subcutaneous injection of human insulin (100 μg) emulsified in Freund's complete adjuvant. After 6 weeks, another subcutaneous immunization with insulin (100 μg) emulsified in Freund's incomplete adjuvant was performed. After 18 weeks, a booster of insulin (20 μg) was intraperitoneally administered 4 days before fusion. The monoclonal antibody 3B1 was prepared as previously described (Gray *et al.*, 1987). Serum obtained from mice before fusion and supernatants from hybrid cells were assayed by incubating the serum or supernatant (100 μL) with 125 I-labelled human proinsulin (210 $\mu\text{Ci}/\mu\text{g}$) or 125 I-human insulin (1500-200 $\mu\text{Ci}/\mu\text{g}$, 100 μL , approximately 20000 c.p.m.) for 24h at 4°C , or 3h at room temperature. And then cellulose-bound sheep anti-(mouse IgG) antibody (50 μL of a 5 mg/ml cellulose suspension) was added and incubation was carried for 30 min at room temperature (Soos and Siddle, 1982). Ice-cold 0.025 M-sodium barbitone buffer, pH 8 (1 ml) (0.05 M-sodium barbitone/0.09 M-NaCl/0.0015 M-NaN₃/0.014 M-HCl/0.5% bovine serum albumin, pH 8.0; diluted with distilled water) was added and then centrifuged at 1700g (Fison Coolsin) for 10 min. The supernatant was discarded, the pellet was washed again with 1 ml of ice-cold 0.025 M-sodium barbitone buffer, pH 8, centrifuged and the supernatant again discarded. The pellet was counted for 1 min using a Nuclear Enterprises 1600 Gama Counter (The 125 I counting efficiency was approximately 70%). The monoclonal antibodies A6 and 14B were selected to bind either intact human proinsulin or human insulin respectively to the exclusion of the other. All hybrid cell lines were routinely cloned at least twice before

injection into mice to produce ascitic tumors. Antibodies for cellulose immunoabsorbent preparation were partially purified from ascites fluids by precipitation with 40% (v/v) saturated $(\text{NH}_4)_2\text{SO}_4$. Antibodies for iodination were further purified on a DEAE-Affigel Blue (BioRad) column and eluted with an NaCl gradient from 0 to 500 mM (Bruck et al., 1982). The antibodies were shown to be homogeneous by electrophoresis on a 10% SDS-polyacrylamide gel. The affinity of the monoclonal antibodies used in these assays was investigated using Scatchard analysis (Scatchard, 1949) essentially as described by Soos and Siddle (1982). The association constants of the monoclonal antibodies used were: A6, $4 \times 10^9 \text{M}^{-1}$ for proinsulin; 3B1, $8.0 \times 10^9 \text{M}^{-1}$ for proinsulin and $1.4 \times 10^{10} \text{M}^{-1}$ for insulin; 14B, $1.1 \times 10^8 \text{M}^{-1}$ for insulin. A6 did not react with 125 I-labelled human insulin, and 14B did not react with 125 I-labelled human intact proinsulin. The isotype of all three monoclonal antibodies was IgG1.

Biotinylation of monoclonal antibody

We used 200 μg of monoclonal antibody in 200 μL of phosphate buffer (60 mmol/L pH 7.4) and 4.8 μg of biotin-N-hydroxysuccinimide (biotin-NHS) (13.3 nmol) in dimethylformamide (DMF). Biotin-NHS was dissolved in 10 μL of DMF and added this to the diluted monoclonal antibody. These were mixed by use of pipette and incubated overnight in the dark at room temperature. We filtered the mixtures three times through a Centricon C30 ultrafilter (Amicon) with a 30000-Da cutoff. We used phosphate buffer (pH 7.4, 60 mmol/L) for washing. The final volume of concentrate was 50 μL . The biotinylated monoclonal antibody was finally diluted to 1 mL with the phosphate buffer. Stored at -20°C and it served as the stock solution.

Preparation of cellulose immunoabsorbents.

Partially purified monoclonal antibodies were coupled to finely divided aminocellulose by diazotization (Hales and Wodhead, 1980), reacting 100 mg of protein with 100 mg of cellulose. The resulting immunoabsorbents contained approximately 200 μg of protein per mg of cellulose and had the following binding capacities: cellulose-linked 3B1 bound approximately 4 μg of insulin per mg of cellulose, and cellulose-linked A6 and 14B bound approximately 2.5 μg of proinsulin and insulin respectively per mg of cellulose. The immunoabsorbents were stored in a suspension of 5 mg of cellulose/mL in 0.05 M-sodium barbitone buffer immediately before use, in order to remove any unbound antibody, were then diluted as required for a particular assay.

Assay optimization

The following points were established (results not shown): assay sensitivity was optimal when the incubation and washing were carried out at 4°C but the degree of improvement with room temperature varied

with the assay; 200 μL samples of standard or unknown gave greater sensitivity than 100 or 50 μL .

Recovery and dilution

Recovery from plasma was assessed by the addition of known quantities of the appropriate hormones to plasma samples. Recoveries of human intact, 65-66 split and 32-33 split proinsulin at 1:4 dilution of normal plasma samples were 77% (60-91), 80% (68-99) and 75% (70-80) respectively.

Assay precision

The intra-assay precision of the assays as determined by replicate analysis of samples gave coefficients of variation of $< 10\%$ over the concentration ranges 20-800 pM, 10-150 pM and 8-250 pM for intact proinsulin, 65-66 and 32-33 split proinsulin respectively. The inter-assay precision (C.V.) determined from the mean of duplicate assays in different assays was $< 15\%$ over the concentration ranges 0.5-50 pM for intact proinsulin, 3-15 pM for 65-66 split and 10-30 pM for 32-33 split proinsulin (Table 1).

Table 1. Precision of assays for intact proinsulin and partially processed proinsulin.

Variation	n	Intact	65-66 split	32-33 split
Range, pM		20-800	10-150	8-250
Intra-assay (CV, %)	5	8.5	7.9	9.8
Range, pM		0.5-50	3-15	10-30
Inter-assay (CV, %)	10	12.5	13.2	14.7

Assay protocol of intact proinsulin

Biotinylated monoclonal antibody A6 (50 μL) was added to either unknown plasma samples, or standards, mixed and incubated for 24h at 4°C . Cellulose-bound monoclonal antibody 3B1 (50 μL , binding approximately 30ng of insulin/50 μL) was then added, mixed and incubated for a further 24h at 4°C . At the end of this step, 0.5 mL of PBS containing Tween 20 (0.5%, v/v) was added to each tube and mixed. The tubes were centrifuged at 8300g for 3 min at room temperature and the supernatants removed by aspiration. After repeating this washing step again, 50 μL of 125 I-labelled streptavidin was added, mixed and incubated for 1h at room temperature. This mixture was washed two times with 1 mL of PBS containing 0.1% Tween 20 and the pellet was resuspended in 0.025M sodium barbitone buffer, pH 8 containing 0.5% Tween 20. The tubes were spun at 8300g for 2 min and the supernatants were removed by aspiration. The

pellets were counted for 10 min in a Nuclear Enterprises 1600 Gamma Counter. Fig. 1 shows a typical standard curve for the measurement of intact proinsulin together with the cross reaction with 65-66 split human proinsulin ($65 \pm 3\%$). The assay detected down to 1.5 pM intact proinsulin.

Fig. 1. Standard curve of human intact proinsulin and the cross-reactivity of 65-66 split proinsulin assayed with biotinylated monoclonal antibody A6 and cellulose-bound antibody 3B1.

Assay protocol of 65-66 split proinsulin

The cellulose-bound monoclonal antibody 14B ($50 \mu\text{L}$, binding approximately 30 ng of insulin/ $50 \mu\text{L}$) was added to the unknown plasma sample and standard ($200 \mu\text{L}$), mixed and incubated for 24 h at 4°C . Washing was done similarly as in the assay for intact proinsulin. Biotinylated monoclonal antibody A6 was then added to each tube, mixed and incubated for 24 h at 4°C . Then 0.5 mL of ice-cold 0.025 M -sodium barbitone buffer was added to each tube, mixed, centrifuged and the supernatants removed by aspiration. The pellets were washed three times as described in proinsulin assay. A typical standard curve is shown in Fig. 2. The cross-reactivity with intact proinsulin was $6 \pm 1\%$. The assay detected 0.8 pM of 65-66 split human proinsulin (mean ± 3 S.D. of the zero signal).

Fig. 2. Standard curve of human split proinsulin (65-66) and the cross-reactivity of intact proinsulin assayed with biotinylated monoclonal antibody A6 and cellulose-bound antibody 14B.

Assay protocol of 32-33 split proinsulin

The assay was carried out essentially as described for the 65-66 split assay using cellulose-bound antibody 3B1 and iodinated anti-(C peptide) antibody PEP 001, but the cellulose-bound antibody pellets were washed three times before adding the iodinated antibody to remove the potentially large excess of C-peptide. A typical standard curve is shown in Fig. 3. The cross-reactivities of intact proinsulin and 65-66 split proinsulin were $84 \pm 2\%$ and $60 \pm 5\%$ respectively. The assay detected 2.5 pM of 32-33 split proinsulin (mean ± 3 S.D. of the zero signal).

Concentrations of intact proinsulin, 65-66 and 32-33 split proinsulin in human plasma

Concentration of intact proinsulin and glucose in eight normal subjects during an oral glucose tolerance test are shown in Fig. 4. Intact proinsulin rose slowly from a basal concentration of $2.3 \pm 0.3 \text{ pM}$, doubled by 15 min, reached a plateau between 60 and 150 min (4-fold above the fasting concentration) and was still approximately double the fasting concentration 240 min after glucose. The fasting concentration of 65-66 split proinsulin was undetectable ($< 1 \text{ pM}$) in

Fig. 3. Standard curve of human split proinsulin (32-33) and the cross-reactivity of 65-66 split and intact proinsulin assayed with iodinated anti-(C-peptide) and cellulose bound antibody 3B1.

Fig. 4. Mean concentration of blood glucose and plasma intact proinsulin in eight non-diabetic, normal weight, young males during an oral (75g) glucose tolerance test.

all eight of the subjects and remained undetectable post-glucose in two of the subjects. The maximum concentration observed after oral glucose was 3.8 pM at 60 min with a mean maximum concentration of 2.2 ± 0.2 pM for the six subjects with detectable levels. The concentration of 32-33 split proinsulin, corrected for the cross-reaction of intact proinsulin in the assay, showed changes intermediate between those of insulin and intact proinsulin (data not shown). The percentage of the total insulin-like molecules accounted for by intact or partially processed proinsulin was 18% in the fasting state and 25%, 27% and 27% at 120, 180 and 240 min after glucose administration respectively.

DISCUSSION

Several radioimmunoassays for proinsulin have been reported and some detailed studies of their specificities have been carried out. But all assays reported thus far either lack sufficient sensitivity to allow the measurement of the individual proinsulin-like molecules in plasma from normal fasting subjects without extraction and concentration (Cohen et al., 1985) or do not have the specificity to discriminate between the individual proinsulin-like molecules present in the circulation. We have previously shown (Gray et al., 1984) that much of the material detected in plasma as 'proinsulin' is partially processed proinsulin.

In the process of establishing the present assays certain interesting technical problems have been encountered. In the first place the sensitivity of the assays for the proinsulin-like molecules is either just adequate (intact proinsulin and 32-33 split proinsulin) or inadequate (65-66 split proinsulin) for the accurate determination of these substances individually in plasma from fasting normal subjects. Further improvement of the sensitivity of these assays is therefore desirable, particularly for studies of the inhibition of the release of these molecules in fasting individuals. Secondly, the very wide range of concentrations of the insulin-related peptides in plasma places considerable constraints on the configuration of the assays. It is often necessary to use a solid-phase antibody preparation with a very high binding capacity to avoid its saturation by potentially cross-reacting materials. In other situations it is necessary to use several washes to eliminate the problem of excess cross-reacting material and this inevitably reduces assay precision. Thirdly, it is not always possible to predict the specificity of monoclonal antibody based two-site immunoradiometric assays from the individual

specificities of the separate monoclonal antibodies as determined in a competitive-binding radioimmunoassay format. It is not clear whether this change of avidity is due to change in the conformation of the antigen induced by antibody binding or to the production of highly stable cyclic complexes (Moyle et al., 1983). Studies with monovalent antibody fragments might discriminate between these possibilities.

The total concentration of proinsulin-like molecules which we have measured in fasting plasma agrees well with that previously determined by less specific assays (Rainbow et al., 1979; Ward et al., 1986). The finding that 32-33 split proinsulin is the major proinsulin-like molecule present in plasma during a glucose tolerance test agrees with our previous observations using an assay which did not detect intact proinsulin (Gray et al., 1984).

The results of the assays carried out in a group of normal relatively young male subjects provide that the mean fasting insulin concentration 20 pM (2.9 munit/L) is somewhat lower than that reported in any previous study. Some reduction of the measured insulin concentration is due to the removal of cross-reactivity with proinsulin. And the inevitable 'noise' seen at the limit of detection of insulin by radioimmunoassays is less significant using the present assay method.

Other advantages of these assays over the assays using iodinated monoclonal antibodies (Sobey et al., 1989) include, firstly, that biotinylation diminishes alterations to the antibody compared with oxidative radiolabeling, so there is no loss of immunoreactivity. Secondly, biotinylated antibody can be stored for at least one year, whereas radiolabeling has to be repeated every month. The decrease in the number of substances that have to be labeled—labeling of streptavidin only—is a great advantage (Peters et al., 1989).

We conclude that the assays we developed represent a significant advance on other methods reported to date. These assays enable the specific assay of proinsulin-like molecules and they will, therefore, be of value in determining the true insulin status of individuals, and in the search for abnormalities of insulin synthesis and processing.

(We thank Mrs. W.J. Sobey, Dr. K. Siddle and Dr. C.A. Carrington, Department of Clinical Biochemistry, University of Cambridge, Cambridge, U.K. and Dr. H.K. Chung, Department of Biochemistry, Seoul National University College of Medicine, Seoul, Korea for their valuable comments and helps for doing this work.)

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