Highly specific radioimmunoassay for human insulin based on immune exclusion of all insulin precursors

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We describe a rapid and simple insulin RIA in which proinsulin and conversion intermediates do not interfere. Three monoclonal antibodies (S1, S2, and S53) were selected for their specificity (directed, respectively, against the B10 region, the junction between A chain and C-peptide, and the junction between B chain and C-peptide), their affinity constant (~1010 L/mol), and their interactive properties in mixture. S2 and S53 were able to bind simultaneously to the same proinsulin molecule, whereas neither could bind simultaneously with S1. Preincubation of serum samples with an excess of S2 resulted in capture of proinsulin and conversion intermediates modified at the junction between B chain and C-peptide into immune complexes that no longer reacted with S1. Similarly, preincubation with S53 prevented proinsulin and conversion intermediates modified at the junction between A chain and C-peptide from reacting with S1. Preincubation with an excess of both S2 and S53 left insulin as the sole reactant with S1. Thus, separation of insulin precursors from insulin by mutually exclusive antibodies is feasible, and on the basis of this new principle, a highly specific RIA for insulin was designed. The detection limit was 11 pmol/L, and the inter- and intraassay coefficients of variation were 11% and 5%, respectively. The potential of the assay for use in clinical studies was verified by application to serum samples from control subjects and patients with diabetes or insulinoma.

Proinsulin is converted to insulin and C-peptide in beta cell granules by two distinct site-specific endopeptidases (PC1/3 and PC2) and carboxypeptidase H yielding, respectively, split 32,33; split 65,66 proinsulins; and des 31,32; des 64,65 proinsulins as conversion intermediates (1–4). The proinsulin conversion route via des 31,32 proinsulin appears to be the predominant pathway. Indeed, PC1 cleaves intact proinsulin at Arg32, Glu33 more readily than PC2 at Arg65, Gly66; and PC2 has a stronger affinity for des 31,32 proinsulin than for intact proinsulin (5). Moreover, substantial amounts of des 31,32 proinsulin and negligible amounts of des 64,65 proinsulin are found both in the circulation (6, 7) and in human islets (8). In the beta cell and in the circulation, it is generally accepted that the major conversion intermediates found are des 31,32 and des 64,65 proinsulins and not split proinsulins (9), because of the rapid cleavage of the newly exposed C-terminal amino acids by carboxypeptidase H (3).

In many insulin assays, intact proinsulin, des 31,32 proinsulin, and des 64,65 proinsulin cross-react with insulin. In healthy subjects, the contribution of intact proinsulin and conversion intermediates is relatively low, representing at most 10–20% of immunoreactive insulin. However, in patients suffering from type I (10) or type II diabetes (11–13), familial hyperproinsulinemia (14, 15), or insulinoma (16), the insulin concentration can be overestimated by the relatively high concentrations of proinsulin and conversion intermediates.

Because insulin has no unique sequence of amino acids of its own, RIAs or ELISAs based on the use of polyclonal or even monoclonal antibodies (MoAbs)3 are never entirely specific. Specificity can be achieved only if a preliminary step of separation of insulin from proinsulin and conversion intermediates followed by a nonspecific assay.

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Parts of these data were presented in poster form at the 29th meeting of European Association for the Study of Diabetes, Istanbul, Turkey, September 1993.
Received September 23, 1997; revision accepted March 9, 1998.

3 Nonstandard abbreviations: MoAb, monoclonal antibody; RIT, radioimmune titration; RT, room temperature; and IC, immune complex.
MoAbs
Preparation. Hybridomas were produced from spleen lymphocytes of mice immunized with rDNA proinsulin as described in Sodoyez et al. (17). Briefly, BALB/c mice were injected intraperitoneally with 20 μg of proinsulin emulsified in complete Freund’s adjuvant, followed by additional injections of the same amount of proinsulin in incomplete Freund’s adjuvant at 2-week intervals. One or two months after the third injection, the mice received a booster of 40 μg proinsulin without adjuvant, and 4 days later, their spleens were aseptically dissected out. Spleocytes were fused with SP2/O myeloma cells. Fused cells were transferred to 96-well plates containing naïve mouse peritoneal macrophages as feeders in hypoxanthine-aminopterine-thymidine medium. Supernatants of wells containing growing hybridomas were screened for the presence of anti-proinsulin and anti-insulin antibodies by radiobinding assays, and the hybridomas producing an antibody with a high percentage of binding with proinsulin and insulin or proinsulin alone were cloned by limiting dilutions.

The selected antibodies were produced in cell culture devoid of fetal calf serum [culture medium: Hybridoma High Protein (Gibco), 5 × 10⁻⁵ mol/L 2-mercaptoethanol, 2 × 10⁻³ mol/L glutamine, 100 000 units/L penicillin, 100 mg/L streptomycin, 10⁻⁴ mol/L hypoxantine, and 1.6 × 10⁻⁵ mol/L thymidine] and purified on Hi-Trap™ Protein G columns (Pharmacia) and stored at −20 °C.

Characterization. Epitope specificity was tested by radio-immune titration (RIT) or by competitive inhibition. Affinity constants of the selected monoclonal antibodies were measured using the Scatchard method (18). Isotypes, subclasses, and light chains were determined with the Mouse-Hybridoma Subtyping kit (cat. no. 1183117) from Boehringer Mannheim: an enzyme immunoassay for the determination of immunoglobulin class, subclass, and light chain type of mouse monoclonal antibodies.

Materials and Methods
This study was in accordance with the Ethical Committee of the University of Liège.

PETIDES
Human, bovine, and porcine insulins and insulin modified in B10 (histidine replaced by aspartic acid) were generously provided by Novo Research Institute (Copenhagen, Denmark). Human intact proinsulin, des 31,32 proinsulin, and des 64,65 proinsulin were from the Eli Lilly Research Laboratories (Indianapolis, IN).

RADIOPROTEIN ASSAY
A constant amount of ¹²⁵I-Tyr A14 human proinsulin or ¹²⁵I-Tyr A14 human insulin (25 000 cpm in 100 μL) was mixed with 100 μL of culture supernatants and 100 μL of serum pretreated with dextran-coated charcoal (peptide-free serum). After 2 h incubation at 37 °C, 1 mL of 150 g/L polyethylene glycol was added to each tube. After 15 min at room temperature (RT), the tubes were centrifuged, supernatants were discarded, and the radioactivity of the precipitates was measured using a multiwell gamma counter. The percentage of antibody-bound radioligand was calculated as the percentage of the total radioactivity found in the precipitates. Blank values obtained by substituting peptide-free serum for monoclonal antibody solution were subtracted from samples values.

RIT
RIT was performed in duplicate by mixing a constant amount of ¹²⁵I-Tyr A14 human insulin, ¹²⁵I-Tyr A14 B10 (Asp) insulin, ¹²⁵I-Tyr A14 human proinsulin, ¹²⁵I-Tyr A14 des 31,32 proinsulin, or ¹²⁵I-Tyr A14 des 64,65 proinsulin (25 000 cpm in 100 μL) with 100 μL of culture supernatants doubling dilutions, 100 μL of peptide-free serum, and 100 μL of phosphate buffer containing 1 g/L bovine serum albumin. After 90 min at RT, 1 mL of 180 g/L polyethylene glycol was added to each tube. After 15 min at RT, the tubes were centrifuged, the supernatants were discarded, the precipitates were washed with 1 mL of 125 g/L polyethylene glycol, and their radioactivity was measured using a multiwell gamma counter as described for the radiobinding assay.

COMPETITIVE INHIBITION
Epitope specificity was also investigated with ¹²⁵I-labeled proinsulin as tracer and proinsulin, insulin, des 31,32 proinsulin, des 64,65 proinsulin, and C-peptide as competitors. One hundred microliters of radioligand (25 000 cpm in 100 μL), 100 μL of buffer with or without the different competitors at increasing concentration (from 10⁻¹² to 10⁻⁷ mol/L), 100 μL of antibody diluted to bind 50% of the radioligand, and 100 μL of peptide-free serum was incubated 90 min at RT. Bound and free hormone were separated as described for RIT.

IMMUNE EXCLUSION AND COOPERATIVE BINDING
Immune complex (IC) size. Each MoAb S1, S2, and S53 was incubated separately with ¹²⁵I-labeled proinsulin (mol/mol) overnight at 4 °C. In addition, equimolar mixtures of MoAbs S1 and S2, S1 and S53, and S2 and S53 were incubated similarly with ¹²⁵I-labeled proinsulin (2 mol/mol). ICs were submitted to gel filtration on a Superose 6 column connected to a fast protein liquid chromatography system (Pharmacia). The column was equilibrated and run with 120 mmol/L NaH₂PO₄, 1 mmol/L EDTA, and 3 mmol/L NaN₃, pH 7.4. Bovine thyroglobulin (Mr 160 000), mouse IgG (Mr 160 000), bovine serum albumin
(Mₐ 60 000), and ¹²⁵I-proinsulin were used as molecular weight markers to calibrate the column. The samples were eluted at 0.4 mL/min, and fractions of 200 µL were collected. The absorbance at 280 nm was read by an online UV detector, and the radioactivity in each fraction was measured in a well-type counter.

Cooperativity. MoAbs S2 and S53 were diluted to bind 50% of the radioligand and then mixed in different ratios. A constant amount of ¹²⁵I-proinsulin (25 000 cpm in 100 µL) and 100 µL of peptide-free serum were added to 100 µL of mixed antibodies. After 90 min incubation at RT, the ICs were precipitated as described above, and the radioactivity of precipitates was counted. Results were plotted as the percentage of bound radioligand vs the ratio of the two antibodies.

RIA PROCEDURE SPECIFIC FOR INSULIN
An equilibrium RIA was developed to quantify insulin without interference of proinsulin or des 31,32 and des 64,65 proinsulins. Plasma samples (100 µL) were preincubated at 37 °C with an excess of either S2 (2.5 pmol in 50 µL) or S53 (1 pmol in 50 µL) or a mixture of S2 and S53, 100 µL of peptide-free serum, and ¹²⁵I-labeled insulin (25 000 cpm in 100 µL). After 30 min, 100 µL of S1 (diluted to bind 50% of the radioligand) was added, and the tubes were incubated 90 min at RT. Bound hormone was precipitated as described for RIT.

Calibration solutions of insulin calibrated with the 1st International Reference Preparation 66/304 and plasma samples, when needed, were diluted in peptide-free serum.

To validate the assay, insulin, intact proinsulin, des 31,32 proinsulin, and des 64,65 proinsulin were also diluted in human peptide-free serum and calibrated on an insulin calibration curve, using the MoAb directed against an epitope common to all molecules (S1).

Preliminary studies showed that lipids (Intralipid 20%, Pharmacia) and bilirubin (concentrations up to 5 g/L and 500 µmol/L, respectively) did not interfere with insulin measurement in serum. We did not observe an adverse effect of hemolysis. However, in view of its known deleterious effect on insulin (19), it should be avoided.

PATIENTS
Serum samples were collected from 20 fasting healthy subjects [14 women and 6 men; mean age, 30 years (range, 19–65 years); mean body mass index, 21.9 kg/m² (range, 17.5–24.9 kg/m²); mean fasting plasma glucose, 4.2 mmol/L (range, 3.3–5.1 mmol/L)], from 6 patients with non-insulin-dependent diabetes mellitus [5 women and 1 man; mean age, 66 years (range, 40–82 years); mean body mass index, 29 kg/m² (range, 22.8–36.5 kg/m²); mean fasting plasma glucose, 6.3 mmol/L (range, 4.1–8.4 mmol/L)] after fasting and 120 min after a test meal, and from 9 patients suffering from recurrent episodes of hypoglycemia and with surgically proven insulinomas. The patients with non-insulin-dependent diabetes mellitus had not been treated with insulin but were on oral hypoglycemic treatment at the time of the study. Insulin was measured with the specific assay described above. Total insulin was measured with a RIA using the MoAb S1. In patients with insulinomas, the concentrations of des 31,32 and des 64,65 proinsulins were estimated by subtracting the insulin value from the values obtained when sera were preincubated with S53 or with S2, respectively, and the concentration of proinsulin was estimated by subtracting insulin and each conversion intermediate from total insulin.

STATISTICAL ANALYSIS
The data presented in Fig. 5 were compared by ANOVA. Multiple comparisons using Scheffé’s simultaneous confidence intervals were made to compare experimental conditions two by two. The limit of statistical significance was set at P <0.05.

The clinical data were analyzed using the nonparametric test of Wilcoxon for paired or unpaired data.

Results
MoAbs
The characteristics of MoAbs S1, S2, and S53 are outlined in Table 1.

Specificity. The specificity of the different MoAbs (S1, S2, and S53) was investigated by RIT or by competition experiments.

<table>
<thead>
<tr>
<th>Clone name</th>
<th>IgG isotype</th>
<th>Specificity</th>
<th>RI titer*</th>
<th>Affinity as determined by Scatchard analysis, L/mol</th>
<th>r²b</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>IgG1, κ</td>
<td>Hi, Hpi</td>
<td>3200 (Hpi)</td>
<td>7.9 × 10¹⁰</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6400 (Hi)</td>
<td>1.0 × 10¹¹</td>
<td>0.93</td>
</tr>
<tr>
<td>S2</td>
<td>IgG1, κ</td>
<td>Hpi</td>
<td>2000</td>
<td>1.75 × 10¹⁰</td>
<td>0.86</td>
</tr>
<tr>
<td>S53</td>
<td>IgG1, κ</td>
<td>Hpi</td>
<td>1600</td>
<td>1.47 × 10¹⁰</td>
<td>0.96</td>
</tr>
</tbody>
</table>

* RI, radioimmune; Hi, human insulin; and Hpi, human proinsulin.

b Coefficient of correlation for linearity of Scatchard plot.
S1 bound $^{125}$I-human insulin; it also bound $^{125}$I-intact human proinsulin (60% of insulin binding), $^{125}$I-des 31,32 proinsulin (66%), and $^{125}$I-des 64,65 proinsulin (86%). It did not bind $^{125}$I-human insulin modified in B10, thereby suggesting specificity for the B chain portion including B10 (Fig. 1A).

The competition curves obtained with $^{125}$I-labeled proinsulin as tracer and insulin, proinsulin, C-peptide, des 31,32 proinsulin, and des 64,65 proinsulin as competitors indicated that tracer binding to S2 (Fig. 1B) was inhibited equally by proinsulin and des 31,32 proinsulin but not by human insulin, C-peptide, and des 64,65 proinsulin, suggesting that S2 recognizes only the junction between A chain and C-peptide. $^{125}$I-proinsulin binding to S53 (Fig.

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Fig. 1. Radioimmune titration of S1 and competitive inhibition of S2 and S53.

(A) Titration of S1 with insulin (●), proinsulin (■), des 31,32 proinsulin (▲), des 64,65 proinsulin (▼), and insulin modified in B10 (○). Competitive inhibition of binding of $^{125}$I-proinsulin to S2 (B) and S53 (C) with insulin (●), proinsulin (■), C-peptide (▲), des 31,32 proinsulin (▲), and des 64,65 proinsulin (▼). B/Bo, the ratio of radioactivity bound in the presence of unlabeled antigen to the radioactivity bound in the absence of unlabeled antigen.
1C) was inhibited equally by proinsulin and des 64,65 proinsulin, suggesting that the epitope of S53 is the junction between B chain and C-peptide.

**Affinity constant.** The affinity constant of the three MoAbs for proinsulin was determined by Scatchard analysis. Individual values of affinity and the coefficient of correlation for linearity of the Scatchard plots are shown in Table 1. The three MoAbs were characterized by very high affinity constants, and all Scatchard plots were linear.

**MoAb heavy and light chain composition.** MoAb heavy and light chain composition is shown in Table 1. S1, S2, and S53 all belonged to the IgG1 isotype, and all had the same type of light chain (κ).

**Cooperativity and immune exclusion**

**Description of IC size.** ICs formed with 125I-proinsulin and a mixture of S2 and S53 yielded an asymmetrical peak of radioactivity preceding the elution volume of S2 alone with 125I-proinsulin and S53 alone with 125I-proinsulin (Fig. 2A). These results indicate that, in the presence of proinsulin, S2 and S53 are able to form ICs of a size greater than one IgG. In the absence of the antigen, the mixture of S2 and S53 eluted in the volume of one IgG, as demonstrated by subsequent incubation of each fast protein liquid chromatography fraction with 125I-proinsulin (data not shown). Moreover, the combination of these two MoAbs in variable proportions did not increase the amount of proinsulin bound (Fig. 3), indicating that these two monoclonals have an additive effect and no positive cooperativity.

ICs formed by 125I-proinsulin and a mixture of S1 and S2 eluted in the same volume as that of S1 alone and labeled proinsulin or S2 alone and labeled proinsulin, thus in the volume of one IgG (Fig. 2B). The same elution profiles were obtained if S2 was replaced by S53 (data similar but not shown). This indicates that, in the presence of proinsulin, S1 and S2 or S53 do not form immune complexes of a size greater than one IgG. This inability of S1 to form complexes with S2 and S53 in the presence of antigen was further demonstrated by the following experiment: an insulin calibration curve performed with S1 was

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**Fig. 2.** Elution profiles of ICs made with 125I-proinsulin.

(A) With S2 alone (1), S53 alone (1), and a mixture of S2 and S53 (2); (B) with S1 alone (1), S2 alone (1), and a mixture of S1 and S2 (1). Calibration was performed using thyroglobulin (1), IgG (2), bovine serum albumin (3), and 125I-proinsulin (4).
displaced to the left when a constant amount of proinsulin was added to each insulin calibrator but resumed its original position if the mixed insulin and proinsulin calibrators were preincubated with S2 (Fig. 4A) or S53 (data similar but not shown). Similar displacements were obtained by addition of des 31,32 proinsulin preincubated or not with S2 (Fig. 4B) or by addition of des 64,65 proinsulin preincubated or not with S53 (Fig. 4C). Thus, MoAbs S1 and S2 or S53 are mutually exclusive.

RIA SPECIFIC FOR INSULIN

The above described characteristics of MoAbs S1, S2, and S53 allowed us to design a new insulin RIA specific for insulin. Indeed, immune exclusion of all insulin precursors by two MoAbs directed against the junction between A chain and C-peptide and the junction between B chain and C-peptide, respectively, leaves insulin as the sole reactant with the MoAb directed against the B chain.

Effects of known amount of proinsulin and conversion intermediates on insulin measurement. Different concentrations of insulin, proinsulin, des 31,32, and des 64,65 proinsulins were measured with S1 (Fig. 5). A known concentration of each proinsulin-like molecule was added to a known concentration of insulin (Fig. 5, column 1) and the concentration of each mixture was measured before immune exclusion (Fig. 5, column 2). If each mixture was preincubated with S2 (Fig. 5, column 3), the values obtained corresponded either to insulin or to the sum of insulin and des 64,65 proinsulin. Indeed, S2 bound to proinsulin and des 31,32 proinsulin and excluded them. If the same mixtures were preincubated with S53 (Fig. 5, column 4), S53 bound to proinsulin and des 64,65 proinsulin and excluded them, and only insulin or insulin with des 31,32 proinsulin were available for S1. The measurements of each mixture after preincubation with S2+S53 (Fig. 5, column 5) were between 141 and 187 pmol/L, corresponding to insulin only.

Assay characteristics. The calibration curve of this human insulin assay is shown in Fig. 6. The assay characteristics were as follows:

1. Limit of detection/limit of quantification.
   The detection limit of this assay, calculated by the mean + 3 SD of 10 zero signals, was 11 pmol/L.
   The working range of this assay was established by calculating the CV of each calibrator in 10 independent calibration curves. CVs were <20% between 10 and 600 pmol/L and <10% between 10 and 300 pmol/L.

2. CVs.
   The interassay CV determined from the mean of duplicate measurements of three serum samples in 10 independent assays was 8.5%, 11.8%, and 4.2%, respectively, at 71, 169, and 655 pmol/L insulin. The intraassay CV estimated by analysis of 10 duplicates of three serum samples in one assay was 5.4%, 5%, and 4.5%, respectively, at 73, 173, and 655 pmol/L insulin.
   As can be expected in a RIA, the interassay CV of samples is lower than the interassay CV of calibrators because of the inclusion of a calibration curve in each run.

3. Analytical recovery.
   Known amounts of purified insulin (ranging from 20 to 1000 pmol/L) were added in five assays to 10 sera of known insulin contents (ranging from 28 to 245 pmol/L). The recovery of human insulin was between 105.1% and 111.2%.

4. Dilution test.
   Insulin recovery after serial dilutions (2 to 8) of 10 human serum samples (ranging from 88 to 283 pmol/L) varied between 97.5% and 106% (mean, 101.8%), showing a good linearity between the degree of dilution and the measured concentrations.

CLINICAL APPLICATIONS

The data presented correspond to small groups of subjects. They have essentially a value of example and will require validation by larger clinical studies.

In fasting healthy subjects, insulin concentrations (median, 25 pmol/L; range, <11–62 pmol/L) were slightly lower (2a ≤0.05) than total insulin concentrations (median, 36 pmol/L; range, <18–108 pmol/L).

In patients with non-insulin-dependent diabetes mellitus, the median fasting concentrations of insulin and of total
Insulin were higher than in healthy subjects ($2\alpha \leq 0.01$), at 100 pmol/L (range, 43–139 pmol/L) and 111 pmol/L (range, 50–141 pmol/L), respectively; 120 min after a meal, the concentrations of insulin and total insulin were 180 pmol/L (range, 103–267 pmol/L) and 218 pmol/L (range, 127–371 pmol/L), respectively.

In nine patients with insulinoma, insulin values were relatively low (range, 30–248 pmol/L) compared with total insulin (range, 130–709 pmol/L). The insulin/total insulin ratio and the contribution of each proinsulin-like molecule were highly variable. In six patients, insulin values were lower than proinsulin-like molecules values. Proinsulin and des 31,32 proinsulin were usually predominant, and des 64,65 proinsulin was usually undetectable. In one patient, the insulin value represented 67% (248 pmol/L) of the total immunoreactive insulin (367 pmol/L), and the two conversion intermediates were present in almost equal amounts (estimated concentrations of des 31,32 proinsulin, 48 pmol/L; estimated concentration of des 64,65 proinsulin, 42 pmol/L). The concentrations of des 31,32 proinsulin, des 64,65 proinsulin, and proinsulin have not been corrected according to their affinity for S1 (Fig. 1A); the concentration of each insulin precursor is therefore underestimated in these uncorrected data.

**Discussion**

After the original insulin RIA described by Yalow and Berson (20), polyclonal antibodies that recognized all insulin-like molecules and that had a relatively weak sensitivity were used for many years. With the development of MoAbs (21), more specific and sensitive assays were developed. An IRMA (7) and an immunoenzymometric assay (22) for insulin, designed with the same two MoAbs, had a sensitivity for insulin of 2.3 pmol/L and 0.8 pmol/L, respectively, but both cross-reacted with intact (5.3%) split 65,66 (62%) and split 32,33 (5%) proinsulins. Andersen et al. (23) described a sensitive ELISA for insulin (5 pmol/L) that cross-reacted with split 65,66 (30%) and des 64,65 (63%) proinsulins. The more recently described assays for insulin were an immunoenzymometric assay (24) and an IRMA (25), both sensitive (6 pmol/L and 1.2 pmol/L, respectively) and rapid but that cross-reacted with des 64,65 proinsulin [77% (26) and 100% (Sanofi Pasteur, personal communication) respectively]. Here we describe a RIA specific of insulin, based on a new principle of separation of insulin precursors from insulin by mutually exclusive antibodies: it requires three antibodies selected for their specificity, affinity, and their interactive properties when mixed. Fast protein liquid
chromatography profiles show that S2 and S53 form with proinsulin ICs of a size greater than one IgG. These ICs are the result of simultaneous binding of S2 and S53 to the same proinsulin molecule and are not the result of linkage between S2 and S53 by their crystallizable fraction, as demonstrated by the elution profile of mixtures of the two MoAbs in the absence of antigen. No positive cooperativity was observed between these two MoAbs. This could be related to the fact that individually they already have a very high affinity for proinsulin. ICs formed by proinsulin and a mixture of S1 and S2 (or S53) elute in the volume of one IgG, indicating an inability to bind to the same proinsulin molecule in spite of a molar ratio between antibodies and antigen selected to favor formation of large complexes (27). This immune exclusion might be due to a modification of the conformation of proinsulin-like molecules when they are already bound by one antibody or to a steric hindrance by portions of IgGs distinct from the binding site. This exclusion between MoAbs is responsible for the absolute specificity of this assay and, in addition, has the advantage of suppressing the step of separation of insulin from proinsulin-like molecules, providing a specific, fast, and easy-to-perform RIA. The sensitivity of this competitive assay (11 pmol/L) is less than the sensitivity achieved by recent immunoenzymometric assays or IRMA assays but, nevertheless, is adequate for most clinical studies.

In fasting healthy subjects, the concentrations of plasma insulin measured with polyclonal antibodies are relatively high, 96 pmol/L (28). Our values are much lower, 25 pmol/L, and close to those measured with other assays using MoAbs: 20 pmol/L (7) and 33 pmol/L (24). The very small difference between total insulin and insulin in fasting healthy subjects is not surprising in view of the very low concentration of proinsulin: 2.7 pmol/L (Houssa, data to be published). Thus, insulin precursors do not seem to account for the large differences in
immunoreactive insulin values obtained with polyclonal vs monoclonal antibodies. The numerous epitopes looked at by polyclonal antibodies could allow recognition of structurally related molecules, degraded insulin, or its breakdown products.

In our small series of type II diabetic patients, basal insulin is higher than in control subjects, in good agreement with Reaven et al. (13), and the ratio of insulin/total insulin is ~90% in fasting conditions and 80% 120 min after a test meal.

At variance in our patients with insulinoma, the insulin/total insulin ratio was usually lower and highly variable; indeed only a few patients had predominantly insulin-secreting tumors. This was also the observation of Hale et al. (29). Monti et al. (24) do not mention individual values of insulin or precursors, but their mean values for insulin (119 pmol/L) and total insulin (349 pmol/L) were very close to ours (116 and 298 pmol/L, respectively). This heterogeneity of insulinomas was recognized years ago, and classifications have been proposed on the basis of the inverse relationship between the degree of beta cells differentiation and the ratio of insulin precursors/insulin in tumors (30) and serum (31).

If an increased proportion of insulin precursors/insulin is suggestive of impaired storage of insulin in tumoral cells (30), the variable proportion of conversion intermediates that we have observed suggests, in addition, anomalies of proinsulin conversion mechanisms, des 31,32 proinsulin not always being the predominant pathway. In this respect, a markedly reduced expression of PC3 has been reported in a human insulinoma (32) and in the insulin-producing cell line INS derived from a rat insulinoma (33). In the latter, no des 31,32 proinsulin was detectable, whereas striking accumulation of des 64,65 proinsulin took place during proinsulin conversion.

In view of the heterogeneity of the insulin-like components released by these tumors, a nonspecific assay of insulin cross-reacting with all precursors probably remains an appropriate screening tool for these patients during a fasting test. The increasing availability and use of highly specific assays will require measurement of each component in patients suspected of insulinoma but will concomitantly improve our knowledge of abnormal proinsulin conversion mechanisms.

This work was supported by grants from the Fonds de la Recherche Scientifique Médicale (Brussels, Belgium). We thank the “Service de Transfusion Sanguine de Liège” for their hospitality. We are grateful to A. Albert for precious advice regarding statistical analysis and to M.C. Requer for technical assistance.

References


