AN IMPROVED METHOD FOR DETERMINATION OF HUMAN C-PEPTIDE IN SERUM AND URINE

YUKIO IIZUKA¹, EIKO IKEGAYA¹, MAKOTO TASHIRO¹, NOBUHIKO NAKAZAWA¹, TOHRU MOCHIZUKI² and NOBORU YANAIHARA²

¹Daiichi Radioisotope Laboratories, Tokyo 103, and ²University of Shizuoka, School of Pharmaceutical Sciences, Shizuoka 422, Japan

ABSTRACT

Determination of human C-peptide levels (human C-peptide: human CP, human proinsulin 33-63) in serum or urine is a valuable tool in the diagnosis of diabetes mellitus. In order to monitor C-peptide levels more efficiently than with a conventional C-peptide radioimmunoassay kit (CP RIA kit), we have improved kit assay ingredients and modified the assay procedure. The C-peptide used for standard and for label was synthesized by a solid phase method, and a C-peptide antiserum was generated in goats which were immunized with a C-peptide-bovine serum albumin (BSA) conjugate. An anti-goat immunoglobulin G Fc fragment (IgG-Fc) mouse monoclonal antibody (MCA) was used as a second antibody. A solid phase double antibody method in which a second antibody immobilized on beads was used for measurement of human C-peptide levels in serum and urine. Assay results are obtained within 5 h by a one-day procedure and more precise results by a two-day procedure. This human C-peptide radioimmunoassay system can be used to evaluate insulin-dependent diabetes mellitus (IDDM) and non insulin-dependent diabetes mellitus (NIDDM).

The amino acid sequence of human C-peptide was determined by Steiner (9) and Markussen (6) in 1971. Chemical synthesis of human C-peptide by a conventional solution method was reported by Yanaihara (13), Naithani (7, 8) and Geiger (2) using a variety of approaches.

Initially, we developed a human C-peptide radioimmunoassay (RIA) (5) using synthetic human connecting peptide (human proinsulin 31-65) in 1974. Later, human C-peptide was shown to originate as a by-product of the enzymatic cleavage of human proinsulin to human insulin in the pancreatic B-cell, and that human C-peptide and insulin were secreted into the blood in the same molar ratio (3). Hence, we developed a C-peptide RIA kit

using human C-peptide (human proinsulin 33-63) (14) synthesized by a solution technique in 1979. The human C-peptide RIA kits were designed to detect C-peptide in serum by a conventional double antibody method (11). These methods required a long second incubation and separation of bound and free tracer (B/F separation) by centrifugation at 4°C.

In order to improve the separation method, we introduced the use of a solid phase double antibody method, in which the second antibody was made by the hybridoma technique. Anti-goat immunoglobulin G Fc fragment (IgG-Fc) mouse monoclonal antibody-coated beads are used as a second antibody, and the first and second incubations can be done simultaneously. Finally, we developed a C-peptide

Fig. 1 Amino acid sequences of synthetic human C-peptide, N^{α} -tyrosylated human C-peptide and its fragments

RIA kit for human proinsulin C-peptide.

MATERIALS AND METHODS

Peptides

Human C-peptide, C-peptide fragments (CP1-22, CP7-22, CP10-22, CP14-31, CP20-31) and tyrosylated human C-peptide (Fig. 1) were prepared by a solid phase method using an automated peptide synthesizer (type 430A, Applied Biosystems, U.S.A.) (12). Peptides were purified by gel filtration on a Sephadex G-25 column using 1M acetic acid as an eluent and by reverse-phase HPLC using a TSK Gel ODS-120T column with a solvent system of 0.01 N HCl/CH₃CN. Purity of synthetic peptides was confirmed by TLC, analytical reversed phase HPLC with two different solvent systems and amino acid analyses of an acid hydrolysate and amino peptidase-M digestion.

Production of Antiserum against Human C-Peptide (C-Peptide Antiserum)

Human C-peptide (24.8 mg) was conjugated to bovine serum albumin (BSA; 50.3 mg) using ECDI (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl). The C-peptide-BSA conjugate was purified by gel filtration on Sephadex G-10 using 1 M acetic acid as an eluent.

The C-peptide-BSA conjugate (1 mg/goat) was dissolved in saline (0.5 ml) and emulsified with Freund's complete adjuvant (0.5 ml). The emulsion was administered to five male goats. After 4 injections at 14-day intervals, goats were bled and the titer was examined by a radioimmunoassay method using 30% polyethylene glycol (PEG) solution for B/F separa-

tion. Immunizations (conjugate 1 mg/goat) and bleedings were repeated every two weeks in order to obtain a high antibody titer. Antisera were purified by precipitation with 40% ammonium sulfate and by an FPLC ion-exchange column (Mono-Q; Pharmacia Fine Chemicals AB, Uppsala, Sweden) with a solvent system of 50 mM Tris-HCl buffer (pH 8.0).

Production of Secondary Antisera

Purified extracts of goat immunoglobulin G (IgG) and goat IgG-Fc were purchased from Cooper Biomedical (Malvern, PA, U.S.A.). Goat IgG-Fc (50 μ g/mice) was prepared in saline (0.1 ml) and emulsified with Freund's complete adjuvant (0.1 ml). The emulsion was initially given intraperitoneally into five BALB/c mice, which were boosted on days 14 and 28 with identical amounts. Mice were then bled and sera titers were examined by a radioimmunoassay using a double antibody method. Three days after the final immunization of goat IgG-Fc 50 μ g in saline, a standard fusion was done (10).

Selected cell lines were cloned by limiting dilution, and the cultures were expanded for ascites production. Ascites titers were monitored by binding of ¹²⁵I-labelled goat IgG and ¹²⁵I-labelled goat IgG-Fc. Two cell lines having a high binding capacity to goat IgG and IgG-Fc were selected. Monoclonal antibodies (MCAs) in ascites were purified by precipitation with 40% ammonium sulfate and by FPLC ion-exchange column chromatography (Mono-Q; Pharmacia). A subclass of anti-goat IgG-Fc mouse MCAs produced by the hybridoma was determined by an ELISA kit (ZYMED Laboratories, San Francisco, CA,

U.S.A.).

Preparation of ^{125}I -Goat IgG, ^{125}I -Goat IgG-Fc and ^{125}I -Anti-Goat IgG-Fc Mouse MCA

Goat IgG, goat IgG-Fc and anti-goat IgG-Fc mouse MCA were iodinated with ^{125}I -Na using a modified procedure of the chloramine T method of Hunter and Greenwood (4). ^{125}I -Goat IgG, ^{125}I -goat IgG-Fc and ^{125}I -anti-goat IgG-Fc mouse MCA were purified by gel filtration on a Sephacryl S-300 column using 0.5 M phosphate buffer (pH 7.4) as an eluent. Peak fractions were used as tracers.

Preparation of ^{125}I -Tyrosylated Human C-Peptide (^{125}I -C-Peptide)

Radioiodination of tyrosylated human C-peptide with ^{125}I -Na was carried out by a modified chloramine T method. Labelled peptide was purified by gel filtration on a Sephadex G-25 column using 1.0 M acetic acid or by reversed phase HPLC on a TSK Gel ODS-120T column (solvent system; 1% AcOH/ CH_3CN). The solvent for HPLC was changed by a linear gradient of 30% CH_3CN to 37.5% CH_3CN for 30 min. A single peak fraction purified by gel filtration and three peak fractions obtained by reversed phase HPLC were examined.

Preparation of Second Antibody Coated Beads

One-fourth inch beads were washed by 5% SCAT 20X-PS (non-phosphoric detergent, Daiichi Kougyou Seiyaku, Kyoto, Japan) and distilled water, and then coated with anti-goat IgG-Fc mouse MCA (second antibody) in 1 mM phosphate buffer (pH 6.4) according to a modified procedure of Catt and Tregear (1). Coated beads were washed with distilled water, protected by 0.5% BSA in 10 mM phosphate buffer (pH 7.4) and dried *in vacuo*. The amount of second antibody coated onto a bead was examined by binding of ^{125}I -anti-goat IgG-Fc mouse MCA. Beads were coated with 5 μg of second antibody.

Measurement of Human C-Peptide in Serum and Urine

For measurement of human C-peptide in

Table 1 *Composition of Human C-Peptide Radioimmunoassay System for 100 Tests*

1. Human C-peptide standards (lyophilized) 0, 0.030, 0.10, 0.30, 1.0, 3.0, 10, 30 ng/ml Reconstitute with distilled water (1.0 ml)	8 vials
2. Goat anti-human C-peptide serum (lyophilized) Reconstitute with distilled water (11 ml)	1 vial
3. ^{125}I -C-peptide (lyophilized) Total activity: ~ 74 kBq (2 μCi) Reconstitute with distilled water (11 ml)	1 vial
4. Second antibody beads (dried) (105 beads)	1 bottle
5. 0.01 M Phosphate buffer (pH 7.4) (50 ml) Containing 0.15 M NaCl and 0.5% BSA	1 bottle
6. Human C-peptide in human serum (lyophilized) Containing approximately 1.2 and 5 ng/ml Reconstitute with distilled water (1.0 ml)	2 vials

serum and urine by radioimmunoassay, the following reagents were prepared for an assay (Table 1). Using this system, two assay procedures were examined under several conditions.

One-day procedure

1. 100 μl of C-peptide standard, patient sample or control serum was pipetted into appropriate test tubes. Previously, patient urine was diluted 10- to 20-fold with 0.01 M phosphate buffer (pH 7.4).
2. 100 μl of ^{125}I -C-peptide was added to each tube.
3. 100 μl of C-peptide antiserum was added to each tube.
4. One bead of secondary antiserum was added to each tube, and tubes were mixed using a RIA shaker.
5. Tubes were incubated for 3 h at room temperature ($20 \pm 5^\circ\text{C}$) with shaking at 200 cycles/min by a reciprocating shaker.
6. Solution in each tube was removed by aspiration, and each bead was washed twice with 1.0 ml of distilled water.
7. Radioactivity on the beads of all incuba-

tion tubes was counted in a scintillation counter.

Two-day procedure (highly sensitive method)

1. 200 μ l of C-peptide standard, patient sample or control serum was pipetted into appropriate test tubes.
2. 100 μ l of 125 I-C-peptide was added to each tube.
3. 50 μ l of C-peptide antiserum was added to each tube.
4. One bead of secondary antiserum was added to each tube, and tubes were mixed with a RIA shaker.
5. Tubes were incubated for 20 h at room temperature ($20 \pm 5^\circ\text{C}$).
6. Solution in each tube was aspirated, and each bead was washed twice with 1.0 ml of distilled water.
7. Radioactivity on the beads for all incubation tubes was counted in a scintillation counter.

Using both procedures for the determination of human C-peptide levels in serum and urine, the characteristics of a C-peptide RIA system, including standard curves (sensitivity of antiserum), intra- and inter-assay variance, correlation test, dilution test, recovery test, cross-reaction test (specificity of antiserum) were examined. Correlation test of this assay system was performed with commercially available C-peptide RIA kit (Daiichi Radioisotope Laboratories, Tokyo, Japan). Commercial C-peptide RIA kit contained the following reagents: human C-peptide standards (lyophilized; 0, 0.3, 1.0, 3.0, 10 and 30 ng/ml), rabbit anti-human C-peptide serum (lyophilized), 125 I-tyrosylated human C-peptide (lyophilized), second antiserum (goat anti-rabbit γ -globulin serum, lyophilized), 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.5% BSA (100 ml), 24% dextran T70 solution in 0.06 M phosphate buffer (pH 7.4, 10 ml) and two kinds of control sera containing human C-peptide (approximately 1.5 and 5 ng/ml).

RESULTS

Anti-Goat IgG-Fc Antisera

After cloning of cell lines, the binding capacity of anti-goat IgG-Fc mouse MCA secreted into culture medium was assayed by a radioimmunoassay using two types of 125 I-

Table 2 *Binding Capacity of Anti-Goat IgG-Fc Mouse MCAs in Culture Media to 125 I-Goat IgG-Fc and 125 I-Goat IgG, and Subclass of MCAs Examined by ELISA*

Clone No.	B/T (%)		Subclass
	125 I-goat IgG-Fc	125 I-goat IgG	
2-1-96-60	5.7	8.2	IgG _{2b}
2-1-96-84	5.7	7.7	IgG _{2b}
2-1-96-88	6.9	10.2	IgG _{2b}
2-1-96-91	6.4	9.9	IgG _{2b}
2-1-96-95	5.4	2.4	IgG _{2b}
2-9-58	0.2	0	IgG _{2b}
2-22-19	15.8	26.2	IgG _{2b}
2-22-49	19.0	32.2	IgG _{2b}
3-23-41	2.6	1.6	IgG ₁
3-23-84	19.7	19.3	IgG ₁
3-23-87-49	17.5	17.6	IgG ₁
3-23-87-50	19.5	19.5	IgG ₁

labelled antigen, 125 I-goat IgG and 125 I-goat IgG-Fc. Five of 12 clones were selected for production of second antibody for a C-peptide radioimmunoassay system (Table 2). In addition, a subclass analysis confirmed that the MCAs generated from 8 clones were IgG_{2b}, and that the MCAs from 4 clones were IgG₁.

The coating capacity of a one-fourth inch bead was examined by a procedure using 125 I-anti-goat IgG-Fc mouse MCA in the same way as unlabelled MCA. The amount of labelled MCA on a bead was found to be approximately 2.5 μ g. Five μ g of anti-goat IgG-Fc mouse MCA was sufficient for coating beads. Finally, an anti-goat IgG-Fc mouse MCA, coded No. 3-23-87-50, was selected for use as a second antibody and used to separate bound and free tracer. The incubation time was examined using 125 I-labelled antigen and beads coated with No. 3-23-87-50 of MCA. A 3 h incubation time was adequate, with shaking at 200 cycles/min (Fig. 2).

125 I-Tyrosylated Human C-Peptide

The peak fraction of 125 I-tyrosylated human C-peptide, purified by Sephadex G-25 filtration, gave three peaks on reverse-phase HPLC (TSK Gel ODS-120T column). Electrophoresis of the first peak (Fr. No. 6, retention time; 4.5 min), the second peak (Fr. No. 23,

Fig. 2 Binding of goat IgG and IgG-Fc tracers to beads coated by anti-goat IgG-Fc mouse MCA: ●, ^{125}I -goat anti-human C-peptide IgG; ○, ^{125}I -goat IgG (1); ■, ^{125}I -goat IgG (2); □, ^{125}I -goat IgG-Fc

retention time; 17 min) and the third peak (Fr. No. 27, retention time; 20 min) showed that the peaks were, respectively, free ^{125}I , monoiodinated C-peptide and diiodinated C-peptide (Fig. 3). Monoiodinated C-peptide showed a higher binding, higher stability and a lower sensitivity when compared to diiodinated C-peptide.

By gel filtration, the specific activity of the ^{125}I -C-peptide was 11.1–12.95 MBq/ μg (300–350 $\mu\text{Ci}/\mu\text{g}$). The specific activity of monoiodinated C-peptide which was separated by reversed phase HPLC was 27.2 MBq/ μg (735 $\mu\text{Ci}/\mu\text{g}$). This tracer is used for the C-peptide RIA kit.

Anti-Human C-Peptide Serum

^{125}I -Labelled goat IgG, which was purified from anti-human C-peptide antiserum (goat #938), bound well to a bead coated with anti-goat IgG-Fc mouse MCA. Scatchard plot analysis of this goat IgG to beads showed an affinity constant of $1.73 \times 10^9 \text{ L/M}$.

Titer and specificities of anti-human C-peptide antiserum were done by radioimmunoassay as described above. Antiserum (goat #938) was used at a final dilution of 1:9,000 and bound 40–50% of ^{125}I -C-peptide (35,000 cpm). Inhibition of C-peptide tracer with C-peptide fragments (CP1-22, CP7-22, CP10-22, CP14-31, CP20-31) and human proinsulin, showed

Fig. 3 Elution pattern from reverse-phase HPLC using a TSK Gel ODS-120T column (0.7 ml/min, 45 sec/fraction)

Fig. 4 Cross-reactivities of human proinsulin and C-peptide fragments with goat anti-human C-peptide serum: ●, CP1-31; ○, proinsulin; ■, CP1-22; □, CP7-22; ▲, CP10-22; △, CP14-31; ◆, CP20-31

that the determinant of this antiserum was located in the N-terminal portion of C-peptide (CP1-7). Its cross-reactivity to human proinsulin was parallel to that of human C-peptide (Fig. 4). Other C-peptides (duck, porcine, rat-I, rat-II and dog) and other peptides (human insulin, human calcitonin, porcine glucagon, porcine secretin, porcine somatostatin) did not inhibit the binding of C-peptide tracer to this antiserum.

Characteristics of C-Peptide Radioimmunoassay System

1. One-day procedure

Assay conditions The assay conditions of "C-peptide radioimmunoassay kit" were optimized as follows; 100 μl of sample, first incubation time of 3 h at room temperature (15–

25°C) with shaking at 200 cycles/min and washing twice with 1.0 ml of distilled water.

Dose response curves (sensitivity) A dose-response curve was in the range from 0.1 ng/ml to 30 ng/ml (Fig. 5), with a minimum detection dose of 0.05 ng/ml.

Intra- and inter-assay variances Intra-assay variance (coefficient of variation) was 1.9–5.0% with 3 types of sera, and the c.v. was 2.3–6.9% with 3 kinds of diluted urine (dilution ratio, 1:10). Inter-assay variance of 3 kit lots assayed in triplicate was 3.2–10.0% c.v. with 3 batches of sera, and a c.v. of 1.3–5.1% with 3 kinds of diluted urine (dilution ratio, 1:10).

Dilution test Dilution curves of 5 different batches of sera were parallel to the standard curve, and 5 batches of urine were parallel to the standard curve, except when using undiluted and 2-fold diluted urine. These data show that urine samples can be diluted 5-fold for assay. Parallelism between the standard and serum or urine samples confirmed that the synthetic C-peptide was immunologically identical to the endogenous C-peptide.

Recovery test The mean recovery of C-peptide added to 3 batches of sera was 96.3–103.0%, and to 3 batches of diluted urine (dilution ratio, 1:10) was 98.6–103.7%.

Correlation test An acceptable correlation was observed between the commercially available C-peptide RIA kit, serum; Y (this system) = $0.990X$ (commercial RIA kit) – 0.023 ($n = 93$), urine; Y (this system) = $0.991X$ (commercial RIA kit) – 0.357 ($n = 68$).

2. Two-day procedure

Assay conditions The assay conditions of our C-peptide radioimmunoassay system were optimized as follows: 200 μ l of sample; incubated for 20 h at cold room or room temperature (5–25°C).

Dose response curves (sensitivity) A dose-response curve was observed in the range of 0.03 ng/ml to 30 ng/ml (Fig. 5), with a minimum detection dose of 0.01 ng/ml.

Intra- and inter-assay variances The intra-assay variance (coefficient of variation) was 2.1–5.0% for 2 batches of sera, and a c.v. of 2.3–6.9% with 2 batches of diluted urine (dilution ratio, 1:10). The c.v. of 3 kit lots assayed in triplicate was 3.5–5.5% with 2 batches of sera, and 0.4–1.9% for 2 batches of diluted urine (dilution ratio, 1:10).

Fig. 5 Dose-response curves for human C-peptide with a one-day (●) and a two-day (○) procedures

Dilution test Dilution curves of 5 different sera samples were parallel to the standard curve, and 5 urine samples were parallel to the standard curve, except with undiluted and 2-fold diluted urine. These data show that urine samples can be diluted from 5- to 10-fold for assay. Parallelism between the standard and serum or urine samples confirmed that synthetic human C-peptide standard had the same immunologic properties as the endogenous C-peptide.

Recovery test Mean recovery ratio of C-peptide added to 3 kinds of sera was 98.3–102.7%, and with 3 batches of diluted urine (dilution ratio, 1:10) was 99.0–102.1%.

Correlation test Good correlation was observed against a commercially available C-peptide RIA kit, serum; Y (this system) = $1.013X$ (commercial RIA kit) – 0.068 ($n = 93$), urine; Y (this system) = $0.784X$ (commercial RIA kit) – 0.034 ($n = 68$).

DISCUSSION

We have improved a C-peptide radioimmunoassay kit system, which was a solid phase, double antibody method designed for the quantitative measurement of human C-peptide in serum and urine specimens. This assay system is intended for *in vitro* use and as an aid in monitoring pancreatic B-cell activity. This new assay system has two assay procedures, a

one-day procedure using a 3 h incubation, and a two-day procedure using an overnight incubation. It is possible to omit a second incubation step and centrifugation procedure on the B/F separation by using a second antibody-coated beads. This assay system is sensitive, reliable, reproducible, and has a good correlation against our commercial RIA kit. C-peptide levels of patients with insulin-dependent diabetes mellitus (IDDM) are exceptionally low, making it difficult to measure C-peptide concentrations below 0.3 ng/ml using our previous commercial kit. But by using our present two-day procedure, we can detect much lower levels of serum C-peptide (0.01 ng/ml). If C-peptide levels of 0.01 to 0.3 ng/ml are measured precisely, it would indicate whether patients with IDDM have residual B-cell function. Otherwise, C-peptide levels of patients with non insulin-dependent diabetes mellitus (NIDDM), even in the face of treatment with exogenous insulin would differ from those patients with IDDM.

We can take another approach to the measurement of C-peptide levels by using both procedures. At first, all patient samples are evaluated by the one-day procedure, which gives results within 5 h of an assay start. Samples with a low C-peptide concentration (0.1 ng/ml) are then repeated by our two-day procedure, which can give results the next day, with a high reliability from 0.1 ng/ml to 0.01 ng/ml. It is possible to determine C-peptide values within 2 days. The present improved C-peptide radioimmunoassay system can be a valuable tool for clinical evaluation of diabetes mellitus.

Received for publication 11 October 1990

REFERENCES

1. CATT K. and TREGGART G. W. (1967) Solid-phase radioimmunoassay in antibody-coated tubes. *Science* **158**, 1570-1572
2. GEIGER R., JÄGER G. and KÖNIG W. (1973) Zur Synthese von Peptiden mit Eigenschaften des Human-Proinsulin-C-Peptids ($_{\text{H}}$ C-Peptid), VI. Darstellung der Gesamtsequenz des Human-Proinsulin-C-Peptids und seines [Glu⁹, Gln¹¹]-Analogon. *Chem. Ber.* **106**, 2347-2352
3. HORWITZ D. L., STARR J. I., MAKO M. E., BLACKARD W. G. and RUBENSTEIN A. H. (1975) Proinsulin, insulin, and C-peptide concentrations in human portal and peripheral blood. *J. Clin. Invest.* **55**, 1278-1283
4. HUNTER W. M. and GREENWOOD F. C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* **194**, 495-496
5. KANEKO T., OKA H., MUNEMURA M., ODA T., YAMASHITA K., SUZUKI S., YANAIHARA N., HASHIMOTO T. and YANAIHARA C. (1974) Radioimmunoassay of human proinsulin C-peptide using synthetic human connecting peptide. *Endocrinol. Japon.* **21**, 141-145
6. KO A. S. C., SMYTH D. G., MARKUSSEN J. and SUNDBY F. (1971) The amino acid sequence of the C-peptide of human proinsulin. *Eur. J. Biochem.* **20**, 190-199
7. NAITHANI V. K. (1973) Studies on polypeptides, IV. The synthesis of C-peptide of human proinsulin. *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 659-672
8. NAITHANI V. K., DECHESNE M., MARKUSSEN J., HEDING L. G. and LARSEN U. D. (1975) Studies on polypeptides, VI. Synthesis, circular dichroism and immunological studies of tyrosyl C-peptide of human proinsulin. *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 1305-1312
9. OYER P. E., CHO S., PETERSON J. D. and STEINER D. F. (1971) Studies on human proinsulin. Isolation and amino acid sequence of the human pancreatic C-peptide. *J. Biol. Chem.* **246**, 1375-1386
10. SHULMAN M., WILDE C. D. and KÖHLER G. (1978) A better cell line for making hybridomas secreting specific antibodies. *Nature* **276**, 269-270
11. SKOM J. H. and TALMAGE D. W. (1958) Nonprecipitating insulin antibodies. *J. Clin. Invest.* **37**, 783-786
12. YANAIHARA C., MOCHIZUKI T., INOUE T., IGUCHI K., HOSHINO M., TOYOSHIGE M. and YANAIHARA N. (1988) Immunochemical demonstration of insulin receptor with use of synthetic peptides and their antibodies. In *Peptide Chemistry 1987* (ed. SHIBA T. and SAKAKIBARA S.) Protein Research Foundation, Osaka, pp. 775-780
13. YANAIHARA N., HASHIMOTO T., YANAIHARA C., SAKAGAMI M., STEINER D. F. and RUBENSTEIN A. H. (1974) Synthesis of human connecting peptide derivatives and their immunological properties. *Biochem. Biophys. Res. Commun.* **59**, 1124-1130
14. YANAIHARA N., YANAIHARA C., SAKAGAMI M., SAKURA N., HASHIMOTO T. and NISHIDA T. (1978) Syntheses of C-peptides and human proinsulin. *Diabetes* **27**, Suppl. 1, 149-160