DIRECT MEASUREMENT OF HUMAN GLICENTIN IN PLASMA BY TWO-SITE IMMUNORADIOMETRIC ASSAY

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ABSTRACT

A sensitive and specific two-site immunoradiometric assay (IRMA) for human glicentin was developed using human glicentin as standard, and anti-human glicentin monoclonal antibody 3D5A as a solid phase antibody, and rabbit anti-human glicentin(1–32) antibody NR2 as ¹²⁵I-labeled antibody. The minimal detection limit of the present IRMA for glicentin was as low as 10 fmol/ml. The ranges for intra- and inter-assay coefficients of variance were 1.2–6.2% and 9.4–13.3%, respectively, indicating that the present IRMA was highly sensitive and accurate for the measurement of human glicentin. Gel filtration analysis of human ileal extracts revealed the presence of a single immunoreactive component which co-eluted with human glicentin. Immunoreactive glicentin in plasma of fasting normal subjects was found to be 57.3 ± 9.0 fmol/ml. The present IRMA proved to be extremely useful for the measurement of human glicentin in plasma and tissues.

Glicentin, which was originally isolated from porcine intestine (19), is a 69 residue polypeptide and the C-terminal portion of the peptide comprises the sequence of pancreatic glucagon extended at its C-terminus with an octapeptide (20). The N-terminus of the glucagon sequence in the glicentin molecule was connected to a peptide comprising 32 amino acid residues. In 1983, Bell *et al.* (3) have deduced the amino acid sequence of human glicentin from the genomic sequence of human preproglucagon. The N-terminally-extended portion from the glucagon sequence in the glicentin molecule shows several substitutions in the primary structures between the porcine and human sequences (approximately a 20% difference). It is suggested that glicentin, a major form of gut glucagon-like immunoreactants (gut GLIs) (7, 15), is produced in the intestinal L-cells and glucagon in A-cells in the pancreas (5). In the small intestine in mammals, proglucagon has been known to be processed into glicentin, oxyntomodulin (2) and glucagon-like peptides (GLP) such as GLP-1(7–36)amide (8, 10) and GLP-1(7–37) (14).

These peptides are derived from a common precursor by two different tissue-specific processing pathways (13, 17).

It has generally been accepted that major antibody-recognition sites in glucagon molecule reside in the 11–15 and 24–29 sequence, respectively (1, 6). Antisera elicited against the 24–29 sequence of glucagon are referred to as C-terminal-specific antisera and have proved to recognize specifically pancreatic glucagon, while those against the 11–15 sequence are designated as the N-terminal-specific antisera, which recognize gut GLIs as well as pancreatic glucagon.

Currently, a measurement of gut GLIs is, therefore, deduced from the difference between glucagon immunoreactivities estimated by two glucagon radioimmunoassays (RIA) using the N- and C-terminal specific antisera, respectively (5). Yanaihara *et al.* (22) have previously developed glicentin C-terminal-specific RIA using anti-glicentin(49–69) serum R4804 and ¹²⁵I-glicentin(43–69) as tracer, and demonstrated the practical usefulness of the assay system for measurement of

gut GLIs as well as peptides related to glicentin C-terminal fragments with the C-terminal octapeptide extension, including oxyntomodulin.

On the other hand, the N-terminal-specific glicentin antisera such as R64 by Moody *et al.* (4) have been produced to discriminate glicentin molecule from pancreatic glucagon. However, these C- and N-terminal-specific glicentin RIAs may recognize more or less not only glicentin but also its related peptides.

The availability of human glicentin in a highly purified form (23) led us to develop a sensitive two-site immunoradiometric assay (IRMA) specific for human glicentin. Using this assay system, plasma glicentin levels in normal subjects were examined.

MATERIALS AND METHODS

Peptides

All of the peptides used were prepared in our laboratory (12). Human glicentin produced by the gene technology (23) was used for the production of monoclonal antibody.

Production of Polyclonal Antibody

Human glicentin(1–32)-Cys, which was synthesized by a solid phase methodology, was allowed to react with keyhole limpet hemocyanin (Pierce, Rockford, IL, U.S.A.) using succinimidy1-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Pierce, Rockford, IL, U.S.A.) in the manner described (21). The resulting conjugate was used as immunogen. A solution of the conjugate (2.1 mg of conjugate in 1.2 ml of saline) was emulsified with complete Freund's adjuvant (Calbiochem-Behring, La Jolla, CA, U.S.A.; 1.0 ml) and the emulsion was injected intracutaneously at multiple sites into two Japanese white female rabbits (approximately 2 kg). Immunization was repeated every three weeks using a half of the amount of the conjugate used for the initial injection. Immunization was repeated until sufficient titers were detected, and blood was taken 10 days after the final immunization. Antiserum NR2 thus obtained was used in the present study.

Production of Monoclonal Antibodies

A solution of recombinant human glicentin (120 μ g) in 50% aqueous polyvinylpyrrolidone (Merck, Darmstadt, Germany; 1.0 ml) was stirred for 30

min at room temperature and then the solution was emulsified with complete Freund's adjuvant. The emulsion was injected subcutaneously into four BALB/c female mice (5 weeks old). Subsequently, a half amount of the emulsion was given at three week intervals. Antibodies to human glicentin were detected in three mice after two month immunization. Intravenous immunization (0.02 mg/mouse) was given to mice with the highest titer and three days later, spleens were taken for cell fusion.

Cell fusion was performed in the following manner. Spleen cells (1×10^8) of the immunized mouse were removed aseptically and fused with the mouse myeloma cells P3U1 (1×10^7) in 50% polyethylene glycol. Fusion products were plated in 24-well culture plates in HAT selection medium. All wells containing hybridomas were screened for antibody production. The screening test was performed by glicentin-specific RIA using antiserum NR2 raised in the present study. Positive cells were cloned twice in a limiting dilution and a monoclonal antibody producing cell line 3D5A was established. To produce the ascitic fluid, the cloned hybridomas were inoculated into peritoneal cavities in BALB/c mice treated with pristane(2,6,10,14-tetramethylpentadecane) (Tokyo Kasei Kogyo, Tokyo, Japan). A monoclonal antibody 3D5A thus obtained was used in this study.

Characterization of Monoclonal and Polyclonal Antibodies

Monoclonal and polyclonal antibodies used in this study were characterized by RIA and cross-reactivities of recombinant human glicentin, and glicentin-related peptides such as synthetic human oxyntomodulin [glicentin(33–69)], human glucagon, glicentin(33–61), glicentin(12–69), glicentin(20–69), glicentin(33–51), glicentin(45–61), glicentin(51–69) and glicentin(63–69) were examined in the assay systems. The RIA procedure used was as follows: to each incubation tube, phosphate buffer, pH 7.4 (0.4 ml), antiserum NR2 (0.1 ml) or purified monoclonal antibody 3D5A from ascites (0.1 ml) and recombinant human glicentin or the glicentin fragment (0.1 ml) and 125 I-labeled recombinant human glicentin (0.1 ml; 5,000 cpm) were mixed. The mixture was incubated for 20 h at 4°C. Diluted normal rabbit serum (0.1 ml), goat anti rabbit γ -globulin serum (0.1 ml) and 5% polyethylene glycol (PEG 6,000; 0.5 ml) were added. In the case of monoclonal antibody, diluted mouse serum (0.1 ml) and diluted anti mouse γ -

globulin serum (0.1 ml) were used for the second antibody reaction. After incubation for 2 h at 4°C, the mixture was centrifuged at $2,000 \times g$ for 30 min at 4°C. The supernatant was aspirated and the radioactivity of the precipitate was counted in a gamma counter.

Iodination of Antiserum NR2

Prior to iodination, the antiserum was purified by affinity chromatography using recombinant human glicentin (0.5 mg) coupled to activated CH-Sepharose (Pharmacia, Uppsala, Sweden; 1 g) according to the manufacture's instructions. In brief, antiserum (0.5 ml) was applied to a column (0.8×2.0 cm) equilibrated with 0.01 M phosphate-buffered saline (PBS), pH 7.4. The column was washed with PBS (10 ml) and the bound antibody was eluted with 0.05 M glycine-HCl buffer, pH 2.7 (12 ml). The eluates were immediately neutralized by the addition of 1 M Tris-HCl buffer, pH 8.0. The purified polyclonal antibody (80 μ g) was iodinated by the chloramine-T method (9). The resulting labeled antibody was applied to a Sephadex G-25 column (1.0×25 cm), which was eluted with 0.1 M Tris-HCl buffer, pH 8.5, containing 0.5% (w/v) bovine serum albumin. The specific activity of the iodinated antibody was 25 μ Ci/ μ g. The iodinated polyclonal antibody was stored at 4°C.

Preparation of Monoclonal Antibody Immobilized Polystyrene Beads

Monoclonal antibody 3D5A in ascites was purified on a Protein A-Sepharose column (Pharmacia, Uppsala, Sweden) (0.8×1 cm) and then immobilized on polystyrene beads (Sekisui Kasei Kogyo, Osaka, Japan). In brief, a solution of the monoclonal antibody 3D5A (0.5 mg/5 ml) in 0.1 M carbonate buffer, pH 9.5 (70 ml) was kept with polystyrene beads (100 beads). After treatment for 12 h at 4°C, the buffer was removed and the polystyrene beads were washed with 0.05 M phosphate buffer, pH 7.5 and then soaked with 0.05 M phosphate buffer, pH 7.5, containing 0.1% (w/v) bovine serum albumin and 0.02% (w/v) NaN_3 .

Effects of pH and Buffer on IRMA

Recovery test of human glicentin from human plasma was carried out at varying pH and buffer. Human glicentin (100 fmol/ml) was added to

pooled human plasma. The effects of pH varied from 5.0 to 8.0, and of 0.05 M phosphate buffer or, 0.05 M citrate buffer were examined. Recovery was calculated as the percentage of the theoretical glicentin value (endogenous plus added human glicentin) against the measured value.

The Two-Site IRMA

IRMA was designed to produce an immune complex of ^{125}I -labeled polyclonal antibody NR2, recombinant human glicentin as standard and solid phase mouse monoclonal antibody 3D5A. Standard diluent buffer was 50 mM citrate buffer, pH 5.0, containing 10% (v/v) horse serum, 0.15 M NaCl and 0.02% (w/v) NaN_3 . For dilution of ^{125}I -labeled polyclonal antibody, 50 mM citrate buffer, pH 5.0, containing 10% (v/v) horse serum and 0.02% (w/v) NaN_3 was used. The assay was carried out in polypropylene tube (12×75 mm). In each incubation tube, the standard diluent (0.7 ml), recombinant human glicentin or sample (0.1 ml) and solid phase monoclonal antibody (1 bead) were mixed. After incubation for 20 h at 4°C, the bead was washed twice with H_2O (1.0 ml), and ^{125}I -labeled polyclonal antibody in the citrate buffer (approximately 100,000 cpm/0.2 ml) was added. The mixture was then incubated at 4°C for 20 h. The bead was washed twice with H_2O . Radioactivity of the solid phase was counted in a gamma-counter.

RIA Using OAL196

Gut glucagon-like immunoreactivities were determined by an RIA using the N-terminal-specific glucagon antiserum OAL196 (16).

Collection of Plasma

Blood samples were obtained from overnight fasted 8 healthy male volunteers, ranging from 22 to 37 years old. Blood samples were collected into ice-chilled tubes containing EDTA (2 mg) per 1 ml of blood. Plasma samples were centrifuged immediately and stored at -30°C .

Oral Glucose Load

Glucose (75 g) in water (225 ml) was ingested within 5 min. Blood was collected at 0, 5, 10, 15, 30, 45, 60 and 120 min after the glucose ingestion in the manner described above. Plasma samples were centrifuged and stored at -30°C .

Table 1 *Characterization of Anti-Human Glicentin Antibodies in RIAs Using Polyclonal Anti-Human Glicentin(1–32) Antibody NR2 (a) and Monoclonal Anti-Human Glicentin Antibody 3D5A (b)*

a)		b)	
Peptide	Cross-reactivity	Peptide	Cross-reactivity
Human glicentin	100%	Human glicentin	100%
Human oxyntomodulin	0	Human oxyntomodulin	22.9
Glucagon	0	Glucagon	25.7
GLP-1(7–36)-NH ₂	0	GLP-1(7–36)-NH ₂	0
Human glicentin(12–69)	23.2	Human glicentin(33–51)	0
Human glicentin(20–69)	0	Human glicentin(45–61)	20.4
Human glicentin(1–32)	197.7	Human glicentin(49–69)	12.0
		Human glicentin(51–69)	0
		Human glicentin(63–69)	0

Fig. 1 A typical standard curve of the present IRMA specific to human glicentin. ●, human glicentin; ○, glicentin-related peptides such as oxyntomodulin, glucagon and GLP-1(7–36)NH₂

Tissue Extraction

Normal human tissue specimens obtained after surgical resection were used. Specimens were frozen on dry ice immediately after surgical excision and stored at -30°C . For extraction, the tissues were weighed, minced on ice-cold bath, and homogenized in 2–3-fold excess of 0.1 M acetic acid in an ice-cold bath. Then, the homogenate was heated in a boiling water-bath for 10 min. After cooling, glacial acetic acid was added to a final concentration of 1.0 M. The homogenate was centrifuged at $15,000 \times g$ for 30 min and the precipitate was

washed with 1 M acetic acid. The combined supernatant and washing were lyophilized. The lyophilized extracts were used as samples for IRMA and gel permeation chromatography.

Gel Filtration Analysis

Crude extracts of human ileal tissue were fractionated by gel filtration on Superdex 75 HR 10/30 column (1.0×30 cm) (Pharmacia, Uppsala, Sweden) using 0.05 M PBS, pH 7.2 as eluent. Elution was performed at a flow rate of 0.5 ml/min and fractions (0.5 ml each) were collected.

RESULTS

Production and Characterization of Antibodies

To produce the N-terminal-specific human glicentin antiserum, a conjugate of synthetic human glicentin(1–32)-Cys with keyhole limpet hemocyanin was used as an immunogen. The polyclonal antiserum NR2 thus raised in a rabbit was tested for cross-reactivities with synthetic human glicentin-related peptides by the RIA.

The results were summarized in Table 1 (a). The polyclonal antibody was found to recognize within the amino-terminal 1–19 sequence of human glicentin, since human glicentin(20–69) did not cross-react in the assay system.

The monoclonal antibody 3D5A produced using a recombinant human glicentin was also characterized by the RIA. Table 1 (b) shows the cross-reactivities of synthetic human glicentin-related peptides in the RIA using antibody 3D5A, indicating that this monoclonal antibody seems to recognize the epitope within the 45–51 sequence of human

Table 2 *Effect of pH on the Recovery and Non-Specific Binding in Human Glicentin IRMA*

pH	Phosphate buffer		Citrate buffer	
	Recovery (%)	NSB (%)	Recovery (%)	NSB (%)
5.0	99.9	2.1	109.3	1.1
6.0	118.5	2.5	58.2	1.8
7.0	67.9	2.3	—	—
8.0	70.9	1.9	—	—

Recovery is expressed as a percentage of the theoretical glicentin value (endogenous plus added glicentin). NSB, Non-specific binding; —, not tested

Fig. 2 A standard curve (●) and dilution curves of human ileal extracts (△) and normal plasma (○) in the present human glicentin IRMA

glicentin.

These results indicate that the antibodies are specific for different regions of human glicentin and they can be used for the development of the IRMA for human glicentin.

Development of IRMA Specific to Human Glicentin

Fig. 1 shows a log-log presentation of a typical standard curve for the IRMA developed using the monoclonal and polyclonal antibodies. The minimal detection limit of the IRMA was as low as 10 fmol/ml, which was 7-fold higher in sensitivity, as compared to that of the RIA using anti-human glicentin(1–32) serum NR2. Table 2 shows the effects of varying pH in the recovery of human glicentin added to pooled human plasma. In the phosphate

Table 3 *Recovery of Human Glicentin Added to the Plasma*

Added human glicentin (fmol/ml)	Measured value (fmol/ml)	Recovery (%)
25	81.8	94
100	176.6	109
300	380	105

The concentration of the IR-glicentin in human plasma used was estimated as 62 fmol/ml.

buffer, pH 5.0, the recovery of the immunoreactive (IR) human glicentin was 99.9% and the non-specific binding was reduced, but slightly. In the citrate buffer, pH 5.0, the recovery of IR-human glicentin was estimated as 109.3% and the non-specific binding was reduced markedly as compared to that in the phosphate buffer. Based on these observations, the citrate buffer, pH 5.0, was used as the standard diluent.

The present IRMA did not recognize human oxyntomodulin corresponding to the 33–69 sequence of human glicentin as well as glucagon (Fig. 1), indicating the high specificity of the present IRMA for human glicentin.

As shown in Fig. 2, dose-response curves of human ileal extracts and plasma sample were parallel to that of standard antigen, indicating that endogenous IR-human glicentin is measurable by the present IRMA.

The results on the recovery test in human plasma were summarized in Table 3, and the ranges for the intra- and inter-assay coefficients of variation were summarized in Table 4. These results indicate strongly the practical usefulness of the present

Table 4 Intra- and Inter-Assay Reproducibility of the Present Human Glicentin IRMA

	IR-glicentin (fmol/ml)		
	Mean	SE	CV (%)
Intra-assay reproducibility (n = 3)			
Sample A	70	18.0	5.3
Sample B	86	7.3	2.0
Sample C	533	18.6	6.0
Sample D	603	21.9	6.2
Sample E	2,077	14.5	1.2
Sample F	2,417	44.1	3.2
Inter-assay reproducibility (n = 3)			
Sample G	78	4.6	10.3
Sample H	594	32.4	9.4
Sample I	2,404	185.0	13.3

SE, standard error; CV, coefficient of variance

IRMA for human glicentin.

Gel Filtration of Human Ileum Extracts

Fig. 3 shows the elution profiles of the IR-glicentin in human ileal extracts on a Superdex 75 HR 10/30 column (1.0 × 30 cm). IR-glicentin was co-eluted with a standard human glicentin as a single peak, when measured by the present IRMA, while the conventional non-specific glucagon RIA recognized low molecular weight IR-gut GLIs including oxyntomodulin.

OGTT

Fasting plasma IR-glicentin level in 8 normal subjects was 57.3 ± 9.0 fmol/ml (mean $\pm$ SE, n = 8). Fig. 4 shows the rise of IR-glicentin levels after 75 g oral administration of glucose. Oral glucose ingestion for fasting normal subjects resulted in approximately 2–3-fold elevations in plasma human glicentin immunoreactivity during 30–120 min.

DISCUSSION

In the present study, we have used synthetic human glicentin(1–32)-Cys as an immunogen, in which Cys residue was purposely introduced to the C-terminus of human glicentin(1–32) for the preparation of the conjugate with keyhole limpet hemocyanin. Using the conjugate, the N-terminal-specific glicentin polyclonal antibody was successfully raised in a rabbit. On the other hand, recombinant human

Fig. 3 Elution profile of IR-glicentin of crude human ileal extracts on Superdex 75 HR 10/30 column. (a) IR-glicentin was determined by human glicentin IRMA and (b) gut GLIs were determined by non-specific glucagon RIA using anti-glucagon serum OAL 196. The arrows show the elution position of the authentic peptides. The extracts were fractionated on a Superdex 75 HR 10/30 column (1.0 × 30 cm) eluted with 0.05 M phosphate, 0.15 M NaCl, pH 7.2.

glicentin was used as an immunogen for the production of the C-terminal-specific monoclonal antibody 3D5A. These antibodies to human glicentin were indispensable for the development of the IRMA. Thus, a sensitive IRMA for human glicentin was developed using a solid phase monoclonal antibody 3D5A and a 125 I-labeled polyclonal antibody NR2. The minimal detection limits in the assay system were as low as 10 fmol/ml.

As can be seen in Table 2, citrate buffer at pH 5.0 was used for the standard diluent, since the non-specific binding was reduced markedly with this buffer. In addition, the intra- and inter-assay

Fig. 4 Immunoreactive glicentin concentrations in plasma determined by human glicentin IRMA after oral administration of 75 g glucose. Results are presented as mean $\pm$ SE, $n = 8$, *, $P < 0.05$; **, $P < 0.005$

coefficients of variance (Table 3) were within an acceptable range, with the intra-assay coefficient of variance below 6.2% and the inter-assay coefficient of variance below 13.3%. The recovery of human glicentin added to plasma in amounts ranging from 25–300 fmol/ml was found to be excellent (Table 2), indicating the quantitiveness of this assay. The present IRMA for the measurement of human glicentin in plasma is specific (Fig. 1), since the assay did not detect structurally related peptides such as oxyntomodulin and glucagon. Dilution of normal human plasma and ileal tissue extracts gave displacement curves that were parallel to the standard (Fig. 2). Gel filtration of human ileal tissue extracts revealed clearly that the present IRMA detects human glicentin specifically, but not lower molecule weight gut GLIs including oxyntomodulin (Fig. 3).

The fasting plasma IR-glicentin levels obtained by the use of the present IRMA were 57.3 ± 9.0 fmol/ml, while those of the fasting normal subjects in the conventional RIA for gut GLIs ranged from 24 to 73 fmol/ml (18). After an oral glucose load, IR-glicentin levels were elevated approximately 2.5-fold higher than that of basal value at 30 min and the elevated values lasted at least for 60–120 min. The plasma values obtained in the present IRMA were similar to those measured by the conventional RIA for gut GLIs (5, 11), which required the extraction procedure in order to eliminate the non-specific interference of plasma protein. However, the present IRMA can be used for measurement of IR-glicentin in plasma without the extraction procedure, indicating the practical usefulness of this IRMA for routine measurement of plasma

glicentin levels in humans.

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