Production of Antisera to des Asn²⁸ Thr²⁹ Homoser²⁷- Glucagon; The Development of Radioimmunoassay for Total Glucagon-like Immunoreactivity in Human Plasma

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Abstract

Antisera having a strong and strictly constant cross-reactivity for gut-GLI were raised in seven rabbits immunized with immunogen, a conjugate of BSA and des-Asn²⁸, Thr²⁹, Homoser²⁷-glucagon (CNBr-glucagon). All of the anti-CNBr-glucagon sera exhibited titer and affinity for glucagon sufficiently high enough to develop a sensitive radioimmunoassay. The relative crossreactivity of the antisera to gut-GLI was comparable to that of antiserum K-4023 which strongly crossreacted with gut-GLI. One of the anti-CNBr-glucagon sera, OAL-196, did not react with the glucagon 19-29 fragment at all. The intra- and inter-assay coefficients of variation were 3.8-5.0 and 6.0-7.3%, respectively, in the radioimmunoassay system for total GLI in human plasma using OAL-196. The fasting plasma total GLI was 374 ± 18 pg/ml. The plasma total GLI during 50 g oral glucose load in normal subjects increased significantly, whereas the plasma IRG level measured with the anti-glucagon 19-29 serum, OAL-123, assay system was lowered. In the gastrectomized subjects, plasma total GLI measured with the present assay system elicited a marked increase following an oral glucose load. These results suggest that the radioimmunoassay using anti-CNBr-glucagon sera will be useful in measuring plasma total GLI.

Because of the lack of antisera specifically reacting with gut-GLI, antisera cross-reacting with gut-GLI as well as pancreatic glucagon has been used for the measurement of total glucagon-like immunoreactivity (total GLI).

Shima *et al.*, (1972a) suggested that the widely divergent results for the plasma total GLI level were able to be attributed to the difference in binding of the antiserum with pancreatic glucagon and gut-GLI. Heding (1969) also showed that different antiglu-

cagon sera reacted to varying degrees with the gut-GLI, resulting in variation in the GLI value.

It has been known that there are at least two immunologic determinants in the pancreatic glucagon molecule (Assan and Slusher, 1972; Faloona and Unger, 1974; Vinik and Hardcastle, 1974; Shima *et al.*, 1975; Heding *et al.*, 1976; Moody, 1978). One, which is essential for binding with antisera specific for pancreatic glucagon, is located in the C-terminal region. The other is located in the N-terminal and/or central portion and is also present in the

molecule of gut-GLI as an immunoreactant. Production of the region-specific anti-glucagon sera is achieved only by chance, in so far as the whole molecule of glucagon is used as the immunogen. Peptide hormone fragments have been used as efficient substrates in the production of region-specific antisera. Use of the N-terminal segment of porcine glucagon, which lacks the C-terminal region, may be useful in producing antisera specific to the N-terminal or central region of the glucagon. In addition, the use of the segment as an immunogen excludes the possible heterogeneity of antisera due to concomitant elicitation of antibodies against the C-terminal portion of glucagon when the whole molecule is used. We have previously reported that all antisera produced against haptenic antigen, glucagon 19-29, were specific for pancreatic glucagon (Imagawa *et al.*, 1979; Yanaihara *et al.*, 1979). The present paper provides the characterization of the antisera produced using des-Asn²⁸, Thr²⁹, Homoser²⁷-glucagon (CNBr-glucagon) as haptenic immunogen, which was prepared by the treatment of porcine glucagon with cyanogen bromide. Development of a radioimmunoassay for total GLI using the antisera is also described.

Materials and Methods

Glucagon related peptides and immunogen

CNBr-glucagon was prepared by a modification of the method of Lin *et al.* (1975). Porcine glucagon (80 mg) was dissolved in 70% formic acid and a 60-fold molar excess (over glucagon) of cyanogen bromide (Wako Pure Chem. Co.) was added. The mixture was stirred for 24 hrs at room temperature and lyophilized. The products were gelfiltered on a BioGel P-2 column. The peak fraction eluting at void volume was pooled and lyophilized. Approximately 45 mg of white powder was obtained. Amino acid analysis of this material showed the absence of methionine, and amino acid composition of the acid hydrolysate of CNBr-glucagon was Lys (1) 0.85, His (1) 0.75, Arg (2) 2.02, Asp (3) 3.25, Thr (2) 2.15, Ser (4) 4.23, Glu (3) 2.87, Gly (1) 1.05,

Ala (1) 1.00, Val (1) 0.95, Leu (2) 2.15, Tyr (2) 2.13, Phe (2) 2.06. High pressure liquid chromatography revealed no detectable peak of pancreatic glucagon in this haptenic preparation.

Glucagon fragments 1-12, 13-17 and 19-29 were prepared by trypsin digestion of glucagon in the manner described previously (Imagawa *et al.*, 1979). Highly purified synthetic porcine VIP (Yanaihara *et al.*, 1977, a) and porcine secretin (Yanaihara *et al.*, 1977, b) and natural porcine glicentin (Novo, Denmark) were employed in the present study.

Coupling of the CNBr-glucagon to BSA was carried out in the following manner. The CNBr-glucagon (40 mg) and BSA (60 mg) were dissolved in 20 ml of 0.1 M borate-HCl, pH 9.0, and 0.2 M glutaraldehyde (5 ml) was added dropwise with constant stirring while pH of the reaction mixture was adjusted to approximately 9.0 with 0.1 N NaOH solution. The mixture was stirred at room temperature for 6 h and then dialyzed against saline at 4°C for 48 h.

Production of antisera

CNBr-glucagon-BSA conjugate was emulsified in Freund's complete adjuvant and injected in to seven male New Zealand white rabbits via a subcutaneous route at multiple sites at 2 week intervals for 2 months. Each rabbit received 2 mg of the conjugate (0.8 mg of CNBr-glucagon). All antisera thus obtained were examined for their ability to bind with ¹²⁵I-glucagon, and tested for reactivity with gut-GLI (peak-I, see below) in canine jejunal extract.

For comparison with the antisera produced in this study, antisera raised against glucagon 19-29 fragment as described previously (Yanaihara *et al.*, 1979), especially one of these antisera, OAL-123, and K-4023 (Novo, Denmark) which is the N-terminal specific, were used as references.

Radioimmunoassay procedure

Radioimmunoassay for plasma GLI or glucagon related peptides was carried out by the method described previously (Imagawa *et al.*, 1979). Glucagon was radioiodinated by the chloramine T method (Hunter and Greenwood, 1962) and the labeled compound was purified by ion exchange chromatography on a QAE-Sephadex A-25 column (Jørgensen and Larsen, 1972). The diluent used for the assay and for dilution of standard and reagents was 40 mM phosphate buffer, pH 7.4, containing 0.2% BSA, 7.5 mM EDTA, and 0.0075% NaN₃. Mixed in each incubation tube were properly diluted antisera (200 μ l), labeled glucagon (200 μ l about 10,000 cpm), glucagon standard or unknown sample (200 μ l), and standard diluent containing 1,000 KIU of Trasylol® (200 μ l). The mixture was incubated for 72 h at 4°C. Free and bound ¹²⁵I-glucagon were separated by the dextran-coated charcoal method. Just before

the addition of dextran-coated charcoal, normal sheep serum (200 μ l) was added to standard and standard diluent (200 μ l) to plasma samples. After incubation for 1 h at 4°C, the suspension was centrifuged at 3,000 rpm for 20 min. at 4°C and the supernatant was decanted. Both supernatant and precipitate were counted with a gamma-spectrometer.

Preparation of extracts of canine stomach, ileum and pancreas, and gelfiltration procedure

Gut-GLI was extracted from mucosal scraps of the canine small intestine (ileum) according to the modification of Kenny's procedure (1955). The crude extracts obtained were applied to a BioGel P-10 column (4×100 cm) equilibrated with 50 mM NH_4HCO_3 solution, pH 8.8, and the eluates, the so called "peak-I", located before the insulin marker, were pooled and lyophilized. This material was used for the characterization of antisera produced. The preparation of extracts of canine stomach, ileum and pancreas was carried out in a similar way to that described above. These extracts were gelfiltered with a BioGel P-10 column (1.5×100 cm) equilibrated with 50 mM of phosphate buffer, pH 7.4, containing 0.2% BSA and 0.2 M NaCl. The flow rate was 5 ml/h, and a 2.8 ml fraction was collected. The column was precalibrated with blue dextran, insulin, glucagon and NaCl as molecular size marker.

Collection of plasma

Nine healthy volunteers and six gastrectomized subjects were orally given 50 g glucose (OGTT). The gastrectomized group had undergone antrectomy, two with a gastroduodenostomy (Billroth II) and four with a gastroduodenostomy (Billroth I) reconstruction. Blood samples were drawn from the antecubital vein at 0, 15, 30, 60, 90 and 120 min after glucose load, and collected into ice chilled tubes containing 500 KIU Trasylol® and 1.2 mg disodium EDTA per ml of blood. The blood was promptly centrifuged at 4°C, and the plasma was stored at -20°C until the time of assay.

Results

Characterization of anti-CNBr-glucagon sera

Production of antisera was attempted in seven rabbits using CNBr-glucagon-BSA as immunogen. After five injections (over 2 months), production of antibodies was observed in all rabbits given the conjugate. All of the antisera exhibited titer and

affinity for glucagon sufficiently high enough to develop a sensitive radioimmunoassay.

Reactivity of these antisera with "peak-I" fraction in canine gut-GLI was examined and the results were expressed in comparison with those of K-4023 and anti-glucagon 19-29 sera. As shown in fig. 1, the inhibition curves for gut-GLI using all anti-CNBr-glucagon sera was superimposed on those of pancreatic glucagon. Table 1 presents the GLI value in pooled GLI measured with these anti-CNBr-glucagon sera and with anti-glucagon 19-29 sera. The relative crossreactivity of the anti-CNBr-glucagon sera to gut-GLI was $92.9 \pm 9.6\%$ ($n=7$) of that obtained with antiserum K-4023, while the value with anti-glucagon 19-29 sera was $1.7 \pm 0.9\%$ ($n=24$).

Among the antisera elicited in the present study, antiserum OAL-196 having the highest affinity for glucagon was selected for further immunological characterization and for development of a radioimmunoassay for plasma total GLI.

When the elution pattern from a BioGel P-10 column of glucagon-like immunoreactivities in the ileum extract (upper panel in Fig. 2) was monitored with the OAL-196 assay system, there were two peaks eluted; one before insulin and the other between insulin and glucagon markers. The ratio of the values in peak-I estimated with OAL-196 to that with OAL-123 was about 27. In the elution pattern of the immunoreactivity in the stomach extract (middle panel in Fig. 2), five peaks were observed. The largest peak corresponding to the glucagon marker was similar in size to that measured with OAL-123. In the case of the pancreatic extract (lower panel), a single peak eluted in a region similar to that of the glucagon marker was observed with both assay systems.

The displacement curves of the glucagon related peptides are shown in Fig. 3. Antiserum OAL-196 crossreacted with CNBr-

Fig. 1. Crossreactivity of peak-1 material obtained from dog intestinal extract (○) with various antisera (a) K-4023 (1:24,000), (b) anti glucagon 19-29 serum GAL-123 (1:240,000), (c) anti CNBr-glucagon serum OAL-191 (1:200,000), (d) OAL-192 (1:20,000), (e) OAL-193 (1:176,000), (f) OAL-194 (1:352,000), (g) OAL-195 (1:66,000) (h) OAL-196 (1:200,000), and (i) OAL-197 (1:20,000). Tracer: 125 I porcine pancreatic glucagon. Standard: porcine pancreatic glucagon (●). The dilution scale for the extract was fixed in such way that the dose-response curve of the extract was superimposed on that of the standard glucagon in the K-4023 assay system.

Table 1. Crossreactivity of dog intestinal extract with various anti-glucagon sera.

Antiserum	Crossreactivity of dog intestinal extract (ng/ μ g extract, pancreatic glucagon equivalent)*	Relative crossreactivity %
K-4023	3.4	Assumed as 100 %
Anti CNBr-glucagon serum		
OAL-191 ($K_a = 3.1 \times 10^{10} M^{-1}$)**	3.4	100
-192 ($= 4.8 \times 10^9 M^{-1}$)	2.6	76.5
-193 ($= 2.1 \times 10^{10} M^{-1}$)	3.4	100
-194 ($= 8.4 \times 10^{10} M^{-1}$)	3.0	88.2
-195 ($= 4.0 \times 10^{10} M^{-1}$)	3.4	100
-196 ($= 1.1 \times 10^{11} M^{-1}$)	3.4	100
-197 ($= 1.7 \times 10^{10} M^{-1}$)	2.9	85.3
	3.16 ± 0.35	92.9 ± 9.6
Anti glucagon 19-29 sera (n=24)	0.05 ± 0.03	1.7 ± 0.9

* A lyophilized peak-I component equivalent to 3.4 ng standard pancreatic glucagon per μ g extract as determined in the K-4023 system was assayed in each system.

** Apparent equilibrium constants for pancreatic glucagon were determined by Scatchard plot analysis.

glucagon to nearly the same extent as with pancreatic glucagon and porcine glicentin, whereas the C-terminal specific antiserum OAL-123 showed low crossreactivity with gut-GLI, which was only 2% that of pancreatic glucagon. Antiserum OAL-196 did not react with the C-terminal fragment, glucagon (19-29), synthetic porcine VIP or secretin at all. On the other hand, antiserum OAL-196 crossreacted with the N-terminal fragments, glucagon (1-12) and (13-17), but very weakly.

Development of a radioimmunoassay for plasma total GLI using antiserum OAL-196

A standard curve of the OAL-196 assay system and serial dilution curves of human plasma samples obtained from gastrectomized subjects during OGTT are shown in Fig. 4. The sensitivity of this assay system was 20 pg/ml, which was amount required for a significant decrease in per cent of the labeled antigen bound from that in the absence of unlabeled glucagon. Per cent binding of labeled glucagon with antiserum OAL-196 in the absence of unlabeled glucagon was $42.8 \pm 4.8\%$ (mean $\pm$ SD, n=

10). Serial dilution curves of human plasma were parallel to the standard curve.

Intra-assay variability was determined in the assay with OAL-196 using 20 replicates of three different human plasma samples with different GLI levels. The intra-assay coefficients of variation ranged from 3.4 to 5.0% (Table 2a). For the estimation of inter-assay variability, three different plasma samples were examined on six occasions with OAL-196. The inter-assay coefficient of variation ranged from 6.0 to 7.3% (Table 2b).

Recovery tests were performed by adding glucagon to two different plasma samples containing about 490 and 670 pg/ml of endogenous GLI. When six different amounts of glucagon were added to each sample, the recovery was $104.0 \pm 4.8\%$ (mean $\pm$ SD) and $101.6 \pm 3.8\%$, respectively (Table 3).

Human plasma total GLI levels measured with antiserum OAL-196

The fasting plasma total GLI level in 30 healthy volunteers was 374 ± 18 pg/ml (mean $\pm$ SD). Fig. 5 shows the plasma GLI response during 50 g OGTT in normal and in gastrectomized subjects, measured with

Fig. 2. Elution profiles on BioGel P-10 column of immunoreactive glucagon in dog (a) intestinal, (b) gastric and (c) pancreatic extracts measured with the OAL-196 (●—●) and OAL-123 (○—○) assay systems. Column: 1.4×100 cm. Eluent: 50 mM phosphate buffer (pH 7.4) containing 0.1% BSA, 0.1 M NaCl. Fraction; 2.8 ml each.

Fig. 3. Displacement curves of glucagon related peptides in the (a) OAL-196 and (b) OAL-123 assay systems. Tracer: ¹²⁵I-porcine pancreatic glucagon. ●—● porcine pancreatic glucagon, ■—■ glucagon 19-29, ▲—▲ CNBr-glucagon, △—△ glucagon 1-12, □—□ glucagon 13-17, ○—○ glicentin, ◆—◆ VIP and ◇—◇ secretin.

Fig. 4. A standard curve (●—●) and serial dilution curves of human plasma GLI in the OAL-196 assay system. The plasma samples, sample-1 (▲—▲) and sample-2 (■—■), were obtained from gastrectomized subjects during 50 g OGTT.

Table 2. Reproducibility of radioimmunoassay with antiserum OAL-196

(a) Intra-assay variance (n=20)

	Sample-1	Sample-2	Sample-3
Mean (pg/ml)	279.6	557.9	783.6
S.D.	10.6	19.1	39.0
C.V. (%)	3.8	3.4	5.0

(b) Inter-assay variance (n=6)

	Sample-1	Sample-2	Sample-3
Mean (pg/ml)	272.8	519.1	771.5
S.D.	18.9	31.5	56.3
C.V. (%)	6.9	6.0	7.3

The intra-assay variability of three different human plasma samples was determined in the antiserum OAL-196 system. In the determination of the inter-assay variability, the same plasma samples were examined on six separation occasions.

Table 3. Recovery test in radioimmunoassay with antiserum OAL-196

Sample-1		
Added (pg/ml)	Measured (pg/ml)	%-Recovery (%)
0	490.5	—
31.3	512.6	98.2
62.5	571.7	103.4
125.0	624.7	101.5
250.0	768.1	103.7
500.0	1091.7	110.2
1,000.0	1641.9	110.2
Mean		104.5
S.D.		4.8
C.V. (%)		4.6

Sample-2		
Added (pg/ml)	Measured (pg/ml)	%-Recovery (%)
0	668.9	—
31.3	694.7	99.2
62.5	736.9	100.8
125.0	773.1	97.4
250.0	923.2	100.5
500.0	1212.0	103.7
1,000.0	1805.9	108.2
Mean		101.6
S.D.		3.8
C.V. (%)		3.7

Recovery studies were performed by adding crystalline glucagon to two different samples, containing 490.5 and 668.9 pg/ml of endogenous glucagon. Six different amounts of glucagon were added to each sample.

OAL-196 and OAL-123. In the group of normal subjects, plasma GLI increased significantly at 30 min, whereas the plasma immunoreactive glucagon (IRG) level measured with OAL-123 was lowered. In the gastrectomized subject, plasma GLI measured with OAL-196 elicited a marked increase following an oral glucose load.

Discussion

It was shown in the present study that the cyanogen bromide cleaved glucagon, CNBr-glucagon, was a suitable antigen for the production of antisera possessing immunologic determinants in the N-terminal region of glucagon. All rabbits immunized with CNBr-glucagon elicited antisera sufficient enough to develop a radioimmunoassay for total GLI.

Purity of the CNBr-glucagon used as haptenic antigen was proved by the amino acid analysis, and by high pressure liquid chromatography.

Anti CNBr-glucagon serum, OAL-196, thus obtained was found to react not only with glicentin and CNBr-glucagon nearly equally to pancreatic glucagon, but also with peak-I in gut-GLI of the canine ileum

Fig. 5. Mean plasma GLI levels in human subjects during 50 g OGTT measured with the OAL-196 (●—●) and OAL-123 (○—○) assay systems. (a) Response to 50 g OGTT in 9 healthy subjects and (b) in 6 gastrectomized subjects.

extract. These observations indicate the prerequisite characteristics of the antiserum for measurement of total GLI in blood. The radioimmunoassay system developed using OAL-196 was able to detect as little as 4 pg/tube glucagon and its detection limit was very close to that of the K-4023 assay system. Serial dilution curves of human plasma containing endogenous GLI were parallel to the standard curve. These GLI values fit well with the least square regression lines of measured GLI on expected concentration. The reproducibility of this system was satisfactory as assessed in coefficient variation of intra- and inter-assay, ranging from 3.4 to 7.3%. The recovery of the glucagon added to plasma was also satisfactory.

GLI values in the fasting human plasma measured with the OAL-196 system, 374 ± 18 pg/ml (mean $\pm$ SD, $n=30$) ranging from 232 to 676 pg/ml, were similar to those measured with K-4023, 353 ± 23 pg/ml. OGTT in gastrectomized subjects, resulted in markedly rising plasma GLI levels as re-

ported (Rehfeld *et al.*, 1970; Heding, 1971; Shima *et al.*, 1972b; Marco *et al.*, 1972; Breuer *et al.*, 1975), and the ratio of increment of plasma GLI from the basal level at each time to the basal value was closely related to the corresponding value calculated from the data reported by other, in both normal and gastrectomized subjects.

In conclusion, the des-Asn²⁸, Thr²⁹, Homoser²⁷-glucagon, CNBr-glucagon, was proved to be a useful haptenic immunogen for the production of antisera possessing an immunodeterminant in the N-terminal region of pancreatic glucagon, and having a strong and strictly constant crossreactivity with gut-GLI. The radioimmunoassay using such antisera provided the method suitable for a measurement of plasma total GLI.

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