A NEW TYPE OF GASTRIN DERIVATIVE AND ITS USE FOR PRODUCTION OF CENTRAL REGION-SPECIFIC ANTI-GASTRIN SERA

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ABSTRACT

Synthetic H-Gly-Lys-[Gln¹]-human gastrin I was used as haptenic immunogen for production of anti-human gastrin sera. The conjugate of the gastrin derivative with human serum albumin prepared by the action of glutaraldehyde was found to be an efficient reagent for production in high probability of anti-human gastrin I sera specific to the central region of the hormone molecule (positions 6-12) where the characteristic structure of gastrin exists. Radioimmunoassay using the antisera discriminated human gastrin I from II as well as from CCK, although the systems detected the big form of human gastrin I to almost the same extent as human gastrin I on molar basis. Immunoreactivities in tissue extracts measured by the present system showed the characteristic distribution of gastrin in Japanese monkey and quail intestines and canine brain.

KEY WORDS synthetic human gastrin derivative / central region-specific anti-gastrin serum / gastrin radioimmunoassay

A number of attempts have been made by a variety of techniques to obtain gastrin specific antisera that could be used for development of radioimmunoassay sufficiently sensitive to measure concentrations of the hormone in biological materials (9, 12). Recent advances in biochemical and immunological studies on gastrin have revealed molecular heterogeneity of gastrin in blood and tissues (4, 6, 7, 10, 13). This in turn has posed problems of specificity of antisera used in gastrin radioimmunoassays. Most of the anti-gastrin sera so far obtained have been shown to be specific to the C-terminal portion of the gastrin molecule where the

hormone in the forms of I and II shares with CCK a common structure. Hauskey *et al.* (5) and Dockray and Walsh (3) reported productions of anti-gastrin sera of specificities for gastrin molecule other than the C-terminal region. Productions of gastrin antisera of these kinds, however, seemed to be encountered by chance.

In this study, we found a easier way to raise anti-human gastrin sera of specificities to the central region of human gastrin I (hG17 I) and successfully produced such antisera in rabbits and guinea pigs in high probability. The immunogen used in this study was a protein conjugate of a new type of synthetic human gastrin derivative, H-Gly-Lys-[Gln¹]-hG17 I (11). The derivative was synthesized by the conventional method for peptide synthesis, which was then coupled with human serum albumin (HSA) in the presence of glutaraldehyde. By

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radioimmunoassay using one of these antisera, immunoreactive gastrin was measured with extracts from various tissues of dog, Japanese monkey and quail.

MATERIALS AND METHODS

Synthetic Peptides

Synthetic peptides used in this study include hG17 I, H-Gly-Lys-[Gln¹]-hG17 I, hG17 I (5-17), hG17 I (8-17), hG17 I (11-17), hG17 (13-17) and pGlu-Gly-Pro-Trp-Leu-Glu-Glu-OMe, corresponding to hG17 (1-7). These peptides were prepared by the conventional method for peptide synthesis as reported previously (11) and purified extensively prior to the use for radioimmunoassay. A new derivative, H-Gly-Lys-[Gln¹]-hG17 I was purified by gel-filtration on Sephadex G-25 followed by droplet counter-current distribution (solvent system: 1-BuOH-0.1% acetic acid-pyridine = 5:11:3) to give homogeneous product: Rf(1-BuOH-pyridine-acetic acid-H₂O = 30:20:6:24) = 0.51, Rf(1-BuOH-acetic acid-H₂O = 4:1:5) = 0.15; $[\alpha]_D^{20} = -48.5^\circ$ (c 0.51, 3M acetic acid); amino acid ratios in acid hydrolysate Lys 1.18 Asp 1.02 Glu 5.89 Pro 1.14 Gly 3.12 Ala 1.02 Met 0.88 Leu 0.93 Tyr 0.96 Phe 0.98.

Natural Peptides

Natural hG17 I and II and human big gastrin I, hG34 I, were prepared by Professor R. A. Gregory, University of Liverpool, which were given us through the courtesy of Professor M. I. Grossman, Veterans Administration Wadsworth Hospital.

Preparation of Immunogen

H-Gly-Lys-[Gln¹]-hG17 I (5 mg) and 15 mg of human serum albumin were dissolved in 2 ml of 0.1 M ammonium acetate (pH 7.0) and 1.3 ml of 0.2 M glutaraldehyde was added. The mixture was stirred at room temperature for 15 hr and dialyzed against water at 4°C. The dialysate was lyophilized (yield 19 mg).

Immunization

H-Gly-Lys-[Gln¹]-hG17 I-human HSA (7 mg in 1.8 ml of saline) was emulsified with 2.7 ml of complete Freund's adjuvant. The emulsion was subcutaneously injected to three mixed-bled female rabbits (1.5 mg of conjugate per each) and three guinea pigs (0.75 mg of conju-

gate per each). Using a half amount of the conjugate, the injection was repeated six times at two-week intervals and then three times at one-month intervals. Booster shots were given after the fourth and ninth injections and the animals were bled ten days after each booster shot.

Radioiodination

Radioiodination of hG17 I was carried out by the chloramine T method and the iodinated hG17 I was purified according to the method described by Brown *et al.* (1). Synthetic hG17 I (1 μ g) in 20 μ l of 0.2 M phosphate buffer (pH 7.4) was mixed with 1 mCi of ¹²⁵I Na (New England Nuclear) in 20 μ l of the same buffer, 2 μ l of dimethyl sulfoxide and 10 μ g of chloramine T in 20 μ l of the buffer. The mixture was agitated for 20 sec, when 100 μ g of sodium metabisulfite in 50 μ l of the buffer was added. The reaction mixture was applied to a column of Sephadex G-10 (1.1 $\times$ 15 cm), which was eluted with 0.05 M imidazole buffer (pH 7.5) containing 0.2% BSA. Fractions of 1 ml each were collected. Fraction #7 was then submitted to ion-exchange chromatography on DEAE-Sephadex A-25. The column (1 $\times$ 35 cm) was eluted with a linear gradient of 0-1.2 M NaCl in 0.05 M imidazole buffer (pH 7.5) and fractions were collected in 2 ml each. The fractions were examined in terms of radioactivity and binding ability to anti-gastrin antiserum.

Radioimmunoassay

The standard diluent used was 0.02 M Veronal buffer (pH 8.0) containing 0.025 M EDTA and 1% BSA. To each of incubation tubes containing 0.4 ml of standard diluent were added 0.1 ml of mono-¹²⁵I-hG17 I, 0.1 ml of anti-hG17 I serum and 0.1 ml of standard synthetic hG17 I or unknown sample. The mixture was kept at 4°C for 48 hr, when 0.1 ml of normal serum and 0.1 ml of anti- γ -globulin serum were added. The incubation mixture was further kept at 4°C for 24 hr and then centrifuged at 3,000 rpm for 30 min. The supernatant was removed by aspiration and the precipitate was counted with a gamma counter.

Extraction of Tissue

Each of fresh frozen tissues was homogenized in 5-10 fold excess of water and the homogenate was heated in a boiling-water bath for 5

min. The suspension was centrifuged at 3,000 rpm for 20 min at room temperature and the supernatant was concentrated and lyophilized. The extracts were dissolved in the standard diluent and submitted to radioimmunoassay.

RESULTS

Production of Antisera

Among the six animals received H-Gly-Lys-[Gln¹]-hG17 I-HSA conjugate, three rabbits and two guinea pigs elicited antibodies to hG17 I. Fig. 1 shows binding curves of serial dilutions of the anti-hG17 sera which had been obtained from the five animals after the ninth immunization. These antisera, R-1301-2, R-1302-2, R-1303-2, GP-1301-2 and GP-1302-2, bound 50% of ¹²⁵I-hG17 I added at final concentrations of approximately $\times 5,000$, $\times 1,000$, $\times 50,000$, $\times 15,000$ and $\times 25,000$, respectively. The amount of standard synthetic hG17 I that was required to reduce the ratio of B/B₀ from 100% to 90% (10% fall) in each system is shown in Table 1.

Characterization of Antisera

In radioimmunoassay systems using the five

Fig. 1 Dilution curves of anti-hG17 sera: R-1301-2 (—■—), R-1302-2 (—○—), R-1303-2 (—●—), GP-1301-2 (—▲—) and GP-1302-2 (—△—). Tracer: ¹²⁵I-synthetic hG17 I

Table 1 Amount of Standard Synthetic hG17 I Required for 10% Fall in B/B₀ (%)

Antiserum used	Synthetic hG17 I (pg/tube)
R-1301-2	14
R-1302-2	14
R-1303-2	22
GP-1301-2	10
GP-1302-2	11

Tracer: ¹²⁵I-synthetic hG17 I

antisera, R-1301-2, R-1302-2, R-1303-2, GP-1301-2 and GP-1302-2, the crossreactivities of the C-terminal pentapeptide amide of hG17, hG17 (13–17), and natural CCK (Karolinska) were remarkably less than that of the standard hG17 I (Table 2) and those of the N-terminal fragment hG17 (1–7) OMe were almost negligible.

Two antisera R-1303-2 and GP-1301-2 were submitted to extensive characterization. Figs. 2a and 2b show the dose-response curves of natural hG17 I and II and natural pG34 I in the two assay systems. The standard synthetic hG17 I and natural hG17 I gave essentially superimposable curves in both systems. In the system using antiserum R-1303-2, the cross-reactivity of natural hG17 II was approximately 9% of that of the gastrin I and the dose-response curve was not completely parallel. Natural pG34 I was less reactive in this system, that is, approximately 38% of that of the gastrin I on an equimolar basis. On the other hand, in the GP-1301-2 system, difference in the crossreactivities of natural hG17 I and II was not so large as that observed in the R-1303-2 system. Natural hG17 II crossreacted to an extent of about 40% of reactivity of hG17

Fig. 2 Dose-response curves of synthetic (—○—) and natural (—●—) hG17 I, natural hG17 II (—■—) and natural pG34 I (—▲—) in R-1303-2 (a) and GP-1301-2 (b) assay systems. Tracer: ¹²⁵I-synthetic hG17 I

Table 2 Relative Crossreactivities of Pentagastrin and CCK (%)

Antiserum used	Synthetic hG17 I	Synthetic pentagastrin	Natural CCK
R-1301-2	100	0.17	0.073
R-1302-2	100	0.01	0.052
R-1303-2	100	<0.001	0.002
GP-1301-2	100	<0.001	0.17
GP-1302-2	100	0.01	0.08

Tracer: ¹²⁵I-synthetic hG17 I

Fig. 3 Dose-response curves of synthetic human gastrin-related peptides in R-1303-2 (a) and GP-1301-2 (b) assay systems. Tracer: ^{125}I -synthetic hG17 I. Peptide: hG17 I ($\bullet$), H-Gly-Lys-[Gln¹]-hG17 I ($\ast$), hG17 I (5-17) ($\circ$), hG17 I (8-17) ($\square$), hG17 I (11-17) ($\blacksquare$), hG17 I (13-17) ($\triangle$), hG17 I (1-7) OMe ($\square$), CCK ($\blacktriangle$) and secretin ($\diamond$).

Table 3 Relative Crossreactivities of Synthetic Human Gastrin-related Peptides (%)

Peptide	Antiserum used	
	R-1303-2	GP-1301-2
hG17 I	100	100
H-Gly-Lys-[Gln ¹]-hG17 I	100	100
hG17 I (5-17)	100	100
hG17 I (8-17)	0.003	0.003
hG17 I (11-17)	0.003	0.005
hG17 I (13-17)	<0.001	<0.001
hG17 I (1-7) OMe	<0.001	<0.001

Tracer: ^{125}I -synthetic hG17 I

I. The reactivity of natural hG34 I was again about 36% of that of hG17 I.

The two antisera were further characterized using various gastrin-related peptides. The results are shown in Figs. 3a and 3b. Table 3 summarized the relative crossreactivities of the peptides in the two systems which were calculated from the amount of each peptide required for 50% inhibition of the antibody-binding of the tracer. In the system with R-1303-2, hG17

I (5-17) which lacks the N-terminal tetrapeptide of the gastrin molecule crossreacted almost identically with the standard hG17 I, while crossreactivities of hG17 I (8-17) and hG17 I (11-17) were considerably lower, that is, less than 1% of that of the standard. And the dose-response curve of hG17 I (11-17) was not parallel to that of the standard. Pentagastrin, i. e. hG17 (13-17), was much less reactive (<0.001%). In this system, CCK showed 0.002% relative cross-reactivity. In the system using GP-1301-2, cross-reactivities of the peptides examined were almost as same as those in the R-1303-2 system. However, the dose-response curve of hG17 I (11-17) did not show so much discrepancy in parallelism as in the other system. The cross-reactivity of CCK in this system was 0.17% of that of the standard.

Immunoreactivities in Tissue Extracts

Gastrin-like immunoreactivity in tissue extracts was measured with the R-1303-2 assay system. Gastrin-like immunoreactivity in the Japanese monkey stomach was localized in the antrum (39.4 ± 8.6 pg equivalent/mg wet weight of tissue, mean $\pm$ SE, $n=3$) and the intermediate part of the antrum and pylorus (65.3 ± 19.5 pg/mg). The pylorus itself did not contain appreciable amount (<3.4 pg/mg) of the reactivity. Any of three parts, sublingual, submaxilla and parotid, of the salivary gland was also shown to contain little immunoreactive gastrin (<2.6 pg/mg). Among the extracts of various tissues of quail, only the pylorus extract showed exclusively high concentration (357.0 ± 39.8 pg equivalent/mg wet weight tissue, mean $\pm$ SE, $n=3$) of the immunoreactivity. The dose-response curve of the pylorus gastrin immunoreactivity is shown in Fig. 4. Except the duodenal extract (4.3 ± 1.2 pg/mg), the rest of the extracts of the quail tissues examined, i. e. proventriculus, gizzard, proximal jejunum, distal jejunum, ileum, caecum and colo-rectum, did not contain detectable doses of the immunoreactivity. The immunoreactivity found in the quail pancreas extract was variable in quantities and its dose-response curve was poor in parallelism to the standard curve (Fig. 4).

The extracts of the anterior and posterior lobes of the canine pituitary contained fairly high concentrations (152.2 ± 29.1 and 121.3 ± 19.5 pg equivalent/mg wet weight of tissue, mean $\pm$ SE, $n=4$) of the immunoreactivity. The pineal extract also showed high content (135.5 ± 15.6 pg/mg) of immunoreactive gas-

Fig. 4 Dose-response curves of Japanese quail pylorus (Δ) and pancreas ($\blacksquare$) extracts in R-1303-2 assay system. Standard: synthetic hG17 I. Tracer: 125 I-synthetic hG17 I

trin. On the other hand, the extracts of the canine brain tissues such as hypothalamus (0.8 ± 0.1 pg/mg) and cerebral cortex (<0.8 pg/mg) were not notable in content of the immunoreactivity.

DISCUSSION

We obtained five anti-hG17 I sera using H-Gly-Lys-[Gln¹]-hG17 I-HSA conjugate as immunogen and four of them were shown to possess sensitivities satisfactorily high enough to be used for development of radioimmunoassay for gastrin. Crossreactivities of these antisera with the C-terminal pentapeptide amide of hG17 I, i. e. hG17 (13-17), and the N-terminal heptapeptide ester, hG17 (1-7) OMe, were very low, indicating that the major antigenic sites of these antisera seem to locate in the central region of the gastrin molecule.

Various gastrin antibodies have been produced in many laboratories and most of them were reported to possess specificity to the C-terminal portion of gastrin where the hormones, both I and II, share a common structure with CCK. In those laboratories, conjugated of gastrin with macromolecule carriers were preferentially used as immunogens. If such gastrin conjugates are prepared by the action of water soluble carbodiimide which was used in many cases, indeed, the coupling reactions will proceed at the side chain carboxyl groups of the five Glu residues in positions 6-10 and one Asp residue in position 16, since these groups are the only functional moieties in the molecule that can react with the carriers by the action of the car-

bodiimide. This may result in some steric restriction on the characteristic Glu region and reduce the probability that the region participates in the formation of antibodies as a part of the major antigenic determinants. On the contrary, H-Gly-Lys-[Gln¹]-hG17 I presently used as hapten has two amino groups on the N-terminal Gly and Lys residues and conjugation via glutaraldehyde is expected to occur only at these positions, leaving the Glu residues intact. This seems to explain the rather high frequency in eliciting the central region-specific antisera in this study.

One of the five antisera obtained, rabbit antiserum R-1303-2, exhibited markedly reduced crossreactivity with natural hG17 II and this suggested the possibility of discriminating gastrin I from II in this R-1303-2 assay system. Natural pG34 I was crossreacted to less extent as compared with gastrin I in the assay system with R-1303-2 or GP-1301-2.

The crossreactivity of hG17 I (5-17) with R-1303-2 which was equal to that of the standard hG17 I distinctly decreased when the N-terminal three amino acid residues of this tridecapeptide were removed. Further three residue-shortening of the decapeptide hG17 I (8-17) chain from the N-terminus resulted in some alteration to parallelism of the dose-response curve. These data suggest crucial role of the 5-7 sequence of the gastrin in antigen-antibody interaction in the R-1303-2 system. In addition, the 8-10 sequence also seemed to participate in the binding to some extent. The marked decrease in crossreactivity resulted by shortening the chain length from hG17 I (11-17) to hG17 (13-17) also indicates the participation of the Ala-Tyr sequence in the binding and this supports the observation that crossreactivity of natural hG17 II was very low in the R-1303-2 system, since hG17 II is sulfated on the Tyr residue in position 12. These findings confirmed that the major antigenic determinants of this antiserum seems to reside within the central part of the gastrin molecule where the characteristic structure of the gastrin exists. Especially, the amino acid residues in positions 6, 7 and 12 were shown to play the most important role in the antigen-antibody binding in the system used. Natural CCK showed 0.002% relative crossreactivity in this system, which can also be explained by the location of the major antigenic sites of this antiserum. It should be, however, noted that significant involvement of the Tyr residue in position 12 in the antigen-antibody interaction requires most careful preparation of the radio-

iodinated antigen used in this system. The major antigenic determinants of antiserum GP-1301-2 seemed to locate in almost the same region as in the case of antiserum R-1303-2, although participation of the residues Ala and Tyr in positions 11 and 12 in the binding was smaller than that in the R-1303-2 system.

We have thus found that the H-Gly-Lys-[Gln⁴]-hG17 I-HSA conjugate is an efficient immunogen which can elicit anti-gastrin antisera specific to the central region of the gastrin where the characteristic structural feature of the hormone exists. Using these antisera, especially one of them, R-1303-2, we developed a sensitive and specific gastrin radioimmunoassay that can discriminate gastrin I from II as well as from CCK.

Localization of gastrin-like immunoreactivity at high concentration has been demonstrated in the antrum and pyloric region of mammalian stomach. Measurement of the immunoreactivity in the extracts of three different parts of the Japanese monkey stomach, i. e. antrum, pylorus and the intermediate region, clarified localization of the reactivity in the antrum and the intermediate region and not in the pylorus itself. The extract of the salivary gland of the Japanese monkey did not contain any detectable amount of the immunoreactivity. In the Japanese quail GI tract, gastrin-like immunoreactivity was found exclusively in the pylorus. The results agreed well with cytochemical observations by Yamada *et al.* (14).

The extracts of the canine pituitary and pineal contained high concentrations of gastrin-like immunoreactivity measurable by the assay system used. There was observed no distinct difference between the concentrations in the anterior and the posterior-intermediate lobes of the pituitary. On the other hand, the hypothalamus contained very little gastrin-immunoreactivity and the concentration in the cerebral cortex was less than the detectable level. These seem to support the observations by Dockray (2) and Rehfeld (8) that gastrin-like immunoreactivity does not exist in brain tissues of rat, porcine, dog or human, and therefore, prove high specificity of this antiserum, R-1303-2, to gastrin.

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