Radio-Immunoassay of Gastrin Using Antiserum to Porcine Gastrin[1] (34796)

BIANCA S. S. CHAN YIP AND PAUL H. JORDAN, JR.

Surgical Services, Veterans Administration Hospital and the Cora and Webb Mading Department of Surgery, Baylor College of Medicine, Houston, Texas 77025

Gastrin I and II were isolated from hog antral mucosa by Gregory and Tracy (1) and their structure determined by Gregory *et al.* (2). Subsequently, Anderson *et al.* (3) reported gastrin synthesis. Gastrins from the various species studied differ from each other only by the substitution of 1 or 2 of the 17 amino acid residues. The same C-terminal tetrapeptide amide is common to gastrin from every species studied and is the portion of the molecule responsible for all the physiological effects attributed to gastrin (4).

After these developments, Schneider *et* (5) produced rabbit antibodies to unconjugated partially purified porcine gastrin. McGuigan (6–8) produced rabbit and guinea pig antibodies to C-terminal tetrapeptide amide of gastrin conjugated with bovine gamma globulin and rabbit antibodies to synthetic human gastrin I (SHG-I) 2-17 conjugated to bovine serum albumin (9, 10). Stremple and Meade (11) reported antibodies against gastrin conjugated with polyacrylate particles.

The C-terminal portion of gastrin is an important antigenic determinant of the molecule (8, 12). The recognition by Mutt and Jorpes (13) that the C-terminal pentapeptide amide of gastrin is identical with that of pancreozymin-cholecystokinin complicates the production of a specific antiserum for the radio-immunoassay of gastrin. McGuigan (7) reported that pancreozymin-cholecystokinin inhibited competitively the binding of C-terminal tetrapeptide amide to antibodies evoked in response to immunization with gastrin C-terminal tetrapeptide amide. He also reported that antiserum to coupled SHG-I cross-reacted with pentagastrin and would thus be expected to cross-react with pancreozymin (9). Stremple (11) found a similar cross-reaction between pancreozymin-cholecystokinin, SHG-I, gastrin pentapeptide, and antibodies evoked in response to immunization with SHG-I. Odell *et al.* (14) reported that antiserum produced in rabbits immunized with unconjugated gastrin had less sensitivity but greater specificity for gastrin. Jeffcoate (15) demonstrated that antibodies produced in the chicken in response to porcine antral gastrin coated onto positively charged latex particles was found to be specific for an antigenic site in the N-terminal portion of the gastrin molecule. There was no cross-reaction with either pancreozymin-cholecystokinin, or pentagastrin. The very low binding energy of the chicken antiserum limited its effectiveness for the measurement of gastrin in unextracted plasma.

Materials and Methods. Preparation of Antigen. Porcine gastrin (a partially purified mixture of gastrin I and II, Wilson Laboratories, Chicago, Lot 143150) was conjugated to bovine serum albumin (Sigma) as described by McGuigan (8). Six milligrams of porcine gastrin were dissolved in 0.4 ml of *N*, *N*-dimethylformamide. Sequentially 0.6 ml of 0.05 *M* potassium phosphate, pH 7.4, 12 mg of bovine serum albumin (BSA) in 0.4 ml of 0.05 *M* potassium phosphate, pH 7.4, and 50 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 0.2 ml of 0.05 *M* potassium phosphate, pH 7.4, were added. The reaction mixture was gently stirred with a magnetic mixer for 20 hr at 20° and then dialyzed for 48 hr against 2 μliters of 0.15 *M* NaCl–0.01 *M* potassium phosphate, pH 7.4,

[1] These studies were supported by Public Health Service Grant AM-09399 and by the U.S. Veterans Administration.

at 4°. The final volume of the gastrin–BSA conjugate was brought up to 3 ml with 0.15 *M* NaCl–0.01 *M* potassium phosphate and emulsified with an equal volume of complete Freund's adjuvant (1 mg gastrin/ml).

Immunization of rabbits. After control serum was obtained, 2 ml of emulsion were injected into young, adult, male New Zealand white rabbits using either the foot pad or the subcutaneous route. Four weeks later each rabbit received an injection similar to the first. Twenty-eight days after the second immunization, blood was obtained by cardiac puncture and allowed to clot at 4°. The sera were removed and individually stored at −20° for immunological evaluation.

Immunologic procedures for antibody characterization. Ouchterlony agar gel-diffusion system was prepared as described by Campell and associates (16). The rabbit antigastrin serum or control serum was placed in the center well and saline, normal human serum, ovalbumin, bovine serum albumin, bovine serum albumin treated with carbodiimide, SHG-I in sodium phosphate buffer and SHG-I-BSA conjugate at a concentration near its estimated equivalence point were added to the peripheral wells. Another agar-gel plate was prepared with 100 μg SHG-I in the agar. Rabbit antigastrin serum was added to the center well, and SHG-I-BSA and porcine gastrin-BSA conjugates were added to the peripheral wells. The agar plates were stored at room temperature for 5 days.

Radio-iodination of SHG-I. This was done by modifications of the methods of Hunter and Greenwood (17) and McGuigan (9). Four micrograms of SHG-I were dissolved in 100 μliters of 0.5 *M* potassium phosphate, pH 7.6. One millicurie of carrier-free ^{131}I (Isoserve, Cambridge Nuclear Corporation) in 5 μliters was added with mixing. Chloramine T, 35 μg in 10 μliters of 0.5 *M* potassium phosphate, pH 7.6, was added. One minute later 250 μg of sodium metabisulfite in 100 μliters of 0.1 *M* potassium phosphate, pH 7.6, were added. The reaction mixture was immediately applied to a Sephadex G-10 column (1.2 × 30.0 cm) and eluted 4° with 0.1 *M* potassium phosphate, pH 7.6 at a flow rate of 0.4 ml per minute. Under these conditions, iodide, chloramide T, and bisulfite were separated from the ^{131}I-SHG-I which was eluted from the Sephadex column in the void volume. The ^{131}I-SHG-I obtained had a specific activity of from 200–225 μCi/μg of SHG-I.

Radio-immunotitration of antisera. The antibody activity preimmunization and post-immunization rabbit sera was evaluated by the following procedure. Varying dilutions of serum (0.1 ml) were incubated with ^{131}I-SHG-I (0.1 ml of 2 μCi/8 mμg/ml) 0.1 ml of 0.01% rabbit γ-globulin (Cohn fraction II Pentex) in 0.1 ml of 0.25% ovalbumin were added. All dilutions of serum and rabbit γ-globulin were made with 0.25% ovalbumin in 0.15 *M* NaCl–0.01 *M* potassium phosphate, pH 7.4. All reaction mixtures were performed in duplicate in 12 × 75-mm glass tubes. After 24 hr of incubation at 4°, 0.2 ml of goat anti-rabbit globulin serum (Sycco) was added to each tube. After a second 24-hr incubation at 4° the tubes were centrifuged at 2000 rpm for 20 min at 4°. The supernatant fluid and the precipitate were counted separately in a Packard automatic γ spectrometer.

Radio-immunoassay. All dilutions were made with 0.25% ovalbumin in 0.15 *M* NaCl–0.01 *M* potassium phosphate, pH 7.4. To each tube 0.1 ml of standard ranging from 10 to 10,000 μμg SHG-1 or sample was incubated in duplicate with 0.1 ml of ^{131}I-SHG-I (2 μCi/8 mμg/ml) and 0.1 ml of rabbit anti-gastrin serum (1:1000 dilution) and 0.1 ml of 0.01% rabbit γ-globulin for 24 hr at 4°. Then 0.2 ml of goat anti-rabbit globulin serum was added to each tube and incubated an additional 24 hr at 4°. After the second incubation, tubes were centrifuged at 2000 rpm at 4° for 20 min. The supernatant fluid and the precipitate of each tube were counted separately in a Packard automatic γ spectrometer.

The specificity of the anti-gastrin serum was evaluated by measuring the capacity of: (1) porcine antral gastrin (Wilson Laboratories, Chicago), (2) Pentagastrin (Ayerst Laboratories Incorporation, New York), (3) Pancreozymin (Sigma), (4) C-terminal tetrapeptide amide of gastrin (Cyclo Chemical

Company, Los Angeles, Calif.) to inhibit competitively the binding of ^{131}I-SHG-I and antibodies evoked in response to immunization with porcine antral gastrin.

Results. Antibody characterization. In the Ouchterlony agar gel-diffusion system without gastrin in the agar gel, rabbit anti-gastrin serum formed in 12 hr a distinct precipitin band between SHG-I-BSA conjugate, porcine gastrin-BSA conjugate and BSA treated with carbodiimide. At 5 days a precipitin band was found between rabbit anti-gastrin serum and SHG-I in sodium phosphate buffer. No precipitin band was found between rabbit anti-gastrin serum and SHG-I-BSA conjugate or porcine gastrin-BSA conjugate when the agar plate was impregnated with 100 μg SHG-I. No precipitin bands were observed when rabbit anti-gastrin serum was diffused against normal human serum, ovalbumin, bovine serum albumin, or saline. No precipitin bands were observed when preimmunization sera were diffused against SHG-I in sodium phosphate buffer, SHG-I-BSA conjugate, BSA, BSA treated with carbodiimide, normal human serum, ovalbumin, and saline. A summary of the immunological studies performed using the Ouchterlony gel-filtration technique is seen in Table I.

Representative radio-immunotitration curves using pre- and postimmunization sera are shown in Fig. 1. The preimmunization rabbit sera showed minimal precipitation (10–13%) of ^{131}I-SHG-I. The titration curves for anti-gastrin sera obtained 28 days after the second injections were nearly identical whether the injections were made subcutaneously or into the foot pad. Increasing dilutions of anti-serum were incubated with constant amounts of ^{131}I-SHG-I. The useful dilutions for radio-immunoassay ranged from 1:1000 to 1:5000.

TABLE I. Immunological Response of Rabbit Serum Before and After Immunization with Porcine Gastrin-BSA Conjugate.

Response of serum to:	Preimmunization serum	Postimmunization serum	
	Plain agar	Plain agar	SHG-I impregnated agar
1 Saline	0	0	—
2 BSA	0	0	—
3 Human serum	0	0	—
4 Ovalbumin	0	0	—
5 SHG-I phosphate buffer	0	+ 5 days	—
6 SHG-I BSA	0	+ 12 hr	0
7 BSA-carbodiimide	0	+ 12 hr	—
8 Porcine-gastrin-BSA	—	+ 12 hr	0

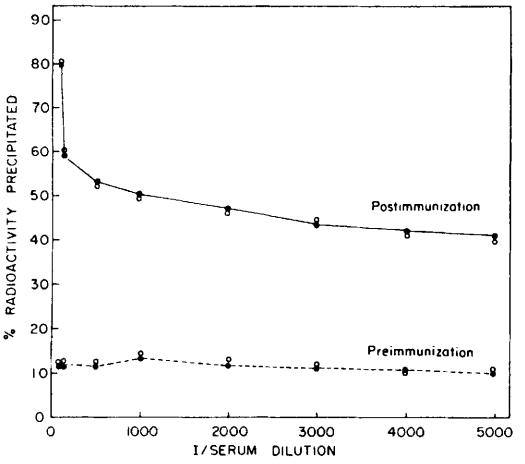

FIG. 1. Titration curves showing the percentage of total radioactivity precipitated by goat anti-rabbit globulin serum when varying dilutions of pre- and postimmunization sera were allowed to react with a constant amount of ^{131}I-SHG-I. Immunization by foot pad injection (●) and subcutaneous injection (○).

Immunoassay. Addition of increasing amounts (10, 50, 100, 500, 1000, 5000, and 10,000 $\mu\mu$g) of unlabeled SHG-I to the reaction tubes which contained constant amounts of anti-gastrin serum and ^{131}I-SHG-I resulted in a progressive reduction of ^{131}I-SHG-I in the precipitates. A straight-line graph was obtained when the percentage of radioactivity precipitated was plotted against the logarithm of the amount of unlabeled SHG-I present (Fig. 2).

To test the specificity of anti-porcine gastrin antibodies, the capacity of pentagastrin, pancreozymin-cholecystokinin, C-terminal tetrapeptide amide of gastrin and porcine gastrin to competitively inhibit the precipitation of ^{131}I-SHG-I in the presence of anti-

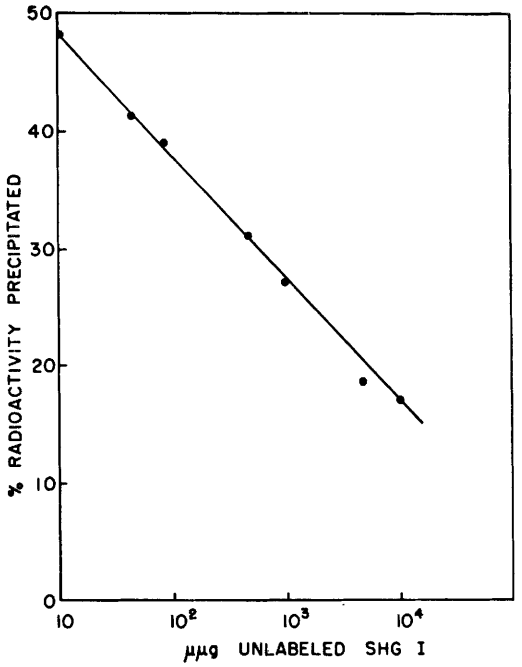

FIG. 2. SHG-I gastrin immunoassay standard curve, indicates percentage of total radioactivity precipitated by goat anti-rabbit globulin serum when a constant amount of porcine gastrin antiserum (0.1 ml of 1:1000) reacted with varying amounts of unlabeled SHG-I and a constant amount of ^{131}I-SHG-I.

porcine gastrin antibodies was tested. The results are shown in Fig. 3. When porcine gastrin was used the reduction in ^{131}I-SHG-I precipitated was similar to that observed in Fig. 2 when unlabeled SHG-I was used. When pentagastrin, pancreozymin, or C-terminal tetrapeptide amide of gastrin was substituted for SHG-I on an equiweight basis, there was virtually no measurable inhibition of immunoprecipitation of ^{131}I-SHG-I indicating an absence of cross-reaction between these different peptides.

Fasting serum from 21 persons without recognized gastrointestinal disease were tested. The gastrin values obtained ranged from 200–630 with a mean of 391 μμg gastrin per milliliter. Serum level of gastrin from 10 normal dogs ranged from 661–3170 with a mean of 1871 μμg per milliliter.

Discussion. The results of this study show that antibodies specific for gastrin are produced in rabbits after immunization with partially purified porcine gastrin conjugated with bovine serum albumin. The partially purified porcine gastrin containing gastrin I and II is conjugated with bovine serum albumin by the use of a water-soluble carbodiimide that couples peptides to proteins by the formation of peptide bonds. Although the N-terminus and the C-terminus of naturally occurring gastrins are blocked, it is likely that amide bonds are formed between reactive glutamyl-carboxyl groups of the peptide and lysle-e-amino groups of bovine serum alubmin to produce an effective antigen for production of anti-gastrin antibodies If the antiserum is specific for a reactive site located somewhere other than the C-terminal tetrapeptide as is suggested by our studies, it is possible that the assay may detect fragments of the gastrin molecule which are not biologically active.

The development of rabbit antibodies to porcine gastrin is demonstrated by the Ouchterlony agar gel-diffusion studies and the radio-immuno precipitation of ^{131}-I-SHG-I. The appearance of precipitant bands between both SHG-I-BSA and porcine gastrin-BSA

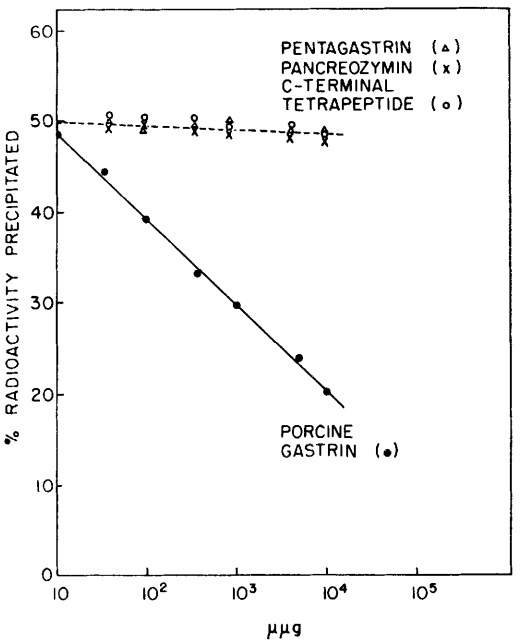

FIG. 3. Comparison of the inhibition of ^{131}I-SHG-I immunoprecipitation by pentagastrin (△), pancreozymin (X), C-terminal tetrapeptide amide of gastrin (○), and porcine gastrin (●).

and the antisera which are absent if the agar-gel is impregnated with gastrin is evidence for anti-gastrin antibodies. The absence of competitive inhibition of ^{131}I-SHG-I precipitation by pancreozymin, pentagastrin, and C-terminal tetrapeptide is indicative of the specificity of the antibodies. In the immunoassay system we use rabbit anti-gastrin serum obtained from rabbits immunized either by foot pad or subcutaneous injections at dilutions between 1:1000 and 1:5000 and are able to measure gastrin levels less than 10 $\mu\mu$g/ml.

Schneider *et al.* (5) also used partially purified porcine antral gastrin preparations to immunize experimental animals and were able to demonstrate, using a passive hemagglutination system, antibody-induced hemagglutination with gastrin I and gastrin-like pentapeptide. The criticism of their method was the inability to characterize the antigen and to determine whether antibodies to porcine mucosal constituents other than gastrin were also produced. The results of our study appear to eliminate these objections to the use of partially purified porcine antral gastrin as the antigen for production of gastrin antibodies.

Mutt and Jorpes (13) reported that the C-terminal pentapeptide amide of gastrin and pancreozymin-cholecystokinin are identical One concern regarding the gastrin immunoassay has been its specificity and whether there is a cross-reaction with other peptides, especially pancreozymin-cholecystokinin. Jeffcoate (15) demonstrated that even though the N-terminus of gastrin is blocked, there is at least another antigenic site in the gastrin molecule. In his opinion antibodies to this antigenic site do not cross-react with the C-terminal group of gastrin. Our studies support this conclusion as well as the opinion that the antibodies do not cross-react with other compounds in which the C-terminal group is similar to gastrin. Comparison of our results with rabbit anti-porcine gastrin serum suggests that the titer and sensitivity are similar but that the specificity of the antibodies may be greater than that reported by others using SHG-I (2-17) as the antigen (9). Our results suggest that antibodies evoked in response to porcine gastrin conjugated with BSA are sufficiently sensitive and specific to permit them to be used practically for the determination of gastrin in biological samples. Using this method we have established that the average gastrin levels in the peripheral blood of man and dog are 391 and 1871 $\mu\mu$g/ml respectively.

Summary. Rabbits immunized with partially purified porcine gastrin conjugated to bovine serum albumin stimulated the production of porcine-gastrin antiserum of high titer. A radio-immunoassay system for gastrin is described utilizing the precipitation of antibody-bound radioiodinated synthetic human gastrin I. The sensitivity of the method permits measurment of less than 10 $\mu\mu$g of gastrin without the possibility of a cross-reaction with molecules having a terminal tetrapeptide similar to gastrin. The technique is capable of measuring human and canine serum gastrin.

1. Gregory, R. A., and Tracy, H. J., J. Physiol. **169**, 18 (1963).

2. Gregory, R. A., Hardy, P. M., Jones, D. S., Kenner, G. W., and Sheppard, R. C., Nature **204**, 931 (1964).

3. Anderson, J. C., Barton, M. A., Gregory, R. A., Hardy, P. M., Kenner, G. W., Macleod, J. K., Preston, J., Sheppard, R. C., and Morley, J. S., Nature (London) **204**, 933 (1964).

4. Gregory, R. A., Gastroenterology **51**, 953 (1966).

5. Schneider, D. R., Endahl, G. L., Dodd, M. C., Jesseph, J. E., Bigley, N. J., and Zollinger, R. M., Science **156**, 391 (1967).

6. McGuigan, J. E., Gastroenterology **53**, 697 (1967).

7. McGuigan, J. E., Gastroenterology **54**, 1012 (1968).

8. McGuigan, J. E., J. Lab. Clin. Med. **71**, 964 (1968).

9. McGuigan, J. E., Gastroenterology **54**, 1005 (1968).

10. McGuigan, J. E., N. Engl. J. Med. **278**, 1308 (1968).

11. Stremple, J. F., and Meade, R. C., Surgery **64**, 165 (1968).

12. Stremple, J. F., Abramoff, P., Van Oss, C. J., Wilson, S. D., and Ellison, E. H., Lancet **2**, 1180 (1967).

13. Mutt, V., and Jorpes, J. E., Biochem. Biophys. Res. Commun. **26**, 392 (1967).

14. Odell, W. D., Charters, A. C., Davidson, W. D., and Tompson, J. D., J. Clin. Endocrinol. **28**, 1840 (1968).

15. Jeffcoate, S. L., Scand. J. Gastroenterol. **4**, 457 (1969).

16. Campbell, D., Garvey, J., Cremer, N., and Sussdorf, D., "Methods in Immunology," p. 83. Benjamin, New York (1963).

17. Hunter, W. M., and Greenwood, F. C., Nature (London) **194**, 495 (1962)

Received Feb. 3, 1970. P.S.E.B.M., 1970, Vol. 134.