Current problems in the measurement of gastrin release. A reproducible measure of physiological gastrin release

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Summary

The interpretation of gastrin release is confused because of variation in the technique of the radio-immunoassay of gastrin, the lack of a standard stimulus for the release of gastrin and diversity in the method used to express the results. These problems have been analysed (a) by examining the cross-reactivity of three gastrin antisera and using each of the antisera to measure basal gastrin levels in fasting normal subjects, duodenal ulcer and post-vagotomy patients; (b) by determining a satisfactory stimulus for gastrin release in normal subjects; (c) by examining the results to determine the best method of presenting the data. The different antisera used were found to give different levels of plasma gastrin in the same sample of plasma. This was not related to the cross reactivity of the antisera. An English breakfast was found to be the most satisfactory stimulus for the release of gastrin. The expression of the results of such a stimulus of gastrin release was affected least by assay variation when the incremental integrated gastrin response was used. It is concluded that the incremental integrated gastrin response to an English breakfast is a satisfactory method for exploring variations in gastrin release.

Introduction

The mechanism of the changes in plasma gastrin concentration in response to a food stimulus has been confused by the use of different stimuli for gastrin release, by problems associated with the measurement of gastrin, and by the lack of uniformity in the expression of results. Thus, studies which examine gastrin release in man in the normal subject, in the duodenal ulcer patient, and after gastric surgery are difficult to compare. This situation is reminiscent of the measurement of gastric secretion before the introduction of the augmented histamine test (Kay, 1953). The interpretation of the levels of circulating gastrin has been complicated by the finding that

there are at least four molecular sizes of gastrin: 'big big' gastrin, big gastrin, little gastrin and mini gastrin (Rehfeld, Stadil and Vinkelsoe, 1974; Straus and Yalow, 1974). Each of these gastrin molecules may be present in the sulphated and non-sulphated forms, and they may circulate in different proportions. Thus, antisera with different cross-reactivities will result in different results for the gastrin concentration in a given plasma (Walsh, Debas and Grossman, 1974). A number of stimuli have been used to measure the release of gastrin, such as orange juice and eggs (Walsh, Yalow and Berson, 1971), insulin hypoglycaemia (Stadil, 1972), insulin and bicarbonate as a combined stimulus (Cowley *et al.*, 1973), Oxo (Wyllie *et al.*, 1972) and liquid meals (Becker, Reeder and Thompson, 1973). There are few studies which compare different gastrin stimuli (Korman, Soveny and Hansky, 1971), and none which compares different stimuli in the same individual.

The terms used to express the results are not uniform: peak plasma gastrin concentration is used by some (Wyllie *et al.*, 1972) and integrated gastrin response is used by several others (Ganguli *et al.*, 1974; McGuigan and Trudeau, 1973; Stern and Walsh, 1973). In the latter instance the integrated gastrin response is calculated in a different manner by each group.

This study undertakes to investigate the interpretation of gastrin release first by examining the cross-reactivity of three antisera in detail; second, determining a satisfactory stimulus in healthy volunteers, and third, using the most satisfactory stimulus to determine the difference that the three antisera have on the measurement of the gastrin response to this stimulus.

Methods

(A) Immunoassay of gastrin

Antisera to gastrin were raised in forty New Zealand white rabbits by immunization with synthetic human gastrin I. In twenty-eight rabbits the

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gastrin was conjugated to bovine serum albumin by 1 ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride, and in twelve rabbits by *bis*-diazotized benzidine. The rabbits in the first group received either 1.5 or 3 mg of the gastrin conjugate by subcutaneous injection. In the second group each rabbit received 3 mg of diazo-conjugate in multiple intradermal sites. Booster injections were repeated at three monthly intervals. Three animals produced antisera of high avidity after the second injection: two of the three were in the first group (code name Gas 8 and Gas 9) and the third was from the second group (code name RCS 8).

Iodination of gastrin was by a trace iodination technique (Bloom, 1974; Jorgensen and Larsen, 1972) using synthetic human gastrin I. To 40 µl of 0.05 M potassium phosphate buffer (pH 7.4) and 1 mCi Na¹²⁵I, 20 µg gastrin I was added. Chloramine T (40 µg) was mixed with the solution for 10 sec, after which time the reaction was stopped by the addition of 40 µg sodium metabisulphite, and 200 µl 0.05 M TRIS buffer containing a trace of potassium iodide. The reaction mixture was then applied to a QAE-Sephadex A 24 ion exchange column, which was precoated with albumin. The column was run overnight at the rate of 10 ml/hr using a gradient elution technique with a low molarity buffer (0.1 M TRIS and 0.5 M NaCl with 1% human serum albumin, pH 8.5) and a high molarity buffer (0.1 M TRIS and 1.0 M NaCl with 1% human serum albumin, pH 8.5). A peak of pure *mono*-iodinated gastrin, free from either damaged or non-iodinated gastrin was eluted. The label was of high specific activity (mean over a 3-month period is 550 mCi/mg gastrin) which, allowing for the isotopic abundance of ¹²⁵I, was close to the theoretical value expected for mono-iodinated gastrin. It maintained full immunological activity for at least 3 months.

The radioimmunoassay was routinely performed in a total volume of 0.8 ml, using a veronal buffer, pH 8.0. Synthetic human gastrin I standards (MRC 68/439) diluted in gastrin free plasma in a volume (0.2 ml) equal to the unknown plasma volume were used in the standard curve. The gastrin free plasma

was obtained from healthy fasting subjects by affinity chromatography (Cuatrecasas, 1970) using antisera Gas 8 attached to cyanogen bromide activated sepharose 4B (Pharmacia). The technique was checked by demonstrating the removal of large quantities of added gastrin and also by the absence of any leaching of gastrin antiserum. The three antisera were used in a final dilution of 1/160,000 for Gas 8, 1/180,000 for Gas 9 and 1/40,000 for RCS 8. Approximately 2.3 pmol of labelled gastrin was added to each tube, and the reaction mixture incubated for 4 days at 4°C. The antibody bound and free labelled gastrin were separated by adsorption of the latter to charcoal, 32 mg/tube (Albano *et al.*, 1972).

The three antisera produced sensitive assays, giving a 10% displacement of binding by 8 pmol/l for Gas 9, 9 pmol/l for Gas 8 and 12 pmol/l for RCS 8. The reproducibility for antiserum Gas 8 has been studied extensively; the mean coefficient of variation between assays was 20% and the within assay variation was 10%, thus changes of approximately 2 pmol/l can be distinguished with 95% confidence.

Each of the three antisera was tested against big gastrin, pentagastrin, cholecystokinin, little gastrin and mini gastrin and the following fragments of the gastrin molecule, 3-17, 11-17, 13-17, 1-13, 3-13, 6-13 and 9-13. Each antiserum was then used to determine the basal gastrin level in ten normal, twenty-five duodenal ulcer patients and eleven post-vagotomy subjects.

(B) Stimulation of gastrin release

Seven healthy volunteers (mean age 28.5 years) agreed to ingest seven types of stimulus, shown in Table 1. The term 'English breakfast' is used because it is recognized by catering departments as corn-flakes (60 mg) with milk, fried bacon and one egg, two slices of toast, butter and marmalade, tea or coffee and sugar to taste. After an overnight fast an indwelling needle was inserted into a forearm vein, and three basal blood samples were taken. The stimulus was ingested and blood samples were taken every 15 min for 3 hr. The blood specimens were

TABLE 1. The substances tested to determine an adequate stimulus for gastrin release. The approximate constituents of the substance are derived either from the manufacturer's list or from a dietician's assessment

Stimulus	Trade name	Quantity	Carbohydrate	Protein	Fat	Calcium
			(wt in g)	(wt in g)		(mg)
Calcium caseinate	Casilan	100 g*	Nil	90	1.8	1130
Composite food	Complan	100 g*	44	31	16	730
Beef extract	Oxo	11.7 g*	1.4	1.1	0.4	21
Groundnut oil emulsion	Prosperol	100 ml*	Nil	Nil	50	Nil
Milk		300 ml	5	3.2	4	120
Calcium carbonate		2 g*	Nil	Nil	Nil	2000
English breakfast			80	25	40	240

* Made up to 300 ml with tap water.

immediately centrifuged and the plasma deep frozen. For each test the mean basal gastrin and the peak gastrin after the stimulus was recorded. The integrated gastrin response was calculated by adding the mean of the adjacent readings multiplied by the time interval between the readings. The incremental integrated gastrin response was calculated in a similar manner, but the mean basal level was subtracted from each result. This term is mathematically only an approximation in that integration is undertaken over finite periods. Moreover, no account is taken of degradation or excretion. Nevertheless, it is acceptable in the present state of our understanding as an indication of the hormonal response to a stimulus. In order to determine the reproducibility of the breakfast stimulus a further seven volunteers were given the meal on four occasions.

Results

(I) Cross-reactivity studies

The pattern of cross-reactivity was different for each of the three antisera when tested against big gastrin, pentagastrin, little gastrin, cholecystokinin and the fragments of the little gastrin molecule (Figs 1–3). None of the three antisera tested reacted significantly with fragments 1–13, 3–13, 6–13, or 9–13, thus suggesting that binding to the C terminal end of the molecule is preferred; nevertheless, by adding massive amounts of fragment 1–13 (10^7 pmol) complete displacement of the label occurred. Pentagastrin, a C terminal analogue of gastrin, also did not show significant cross-reaction, nor did fragment 13–17;

reactivity was found to increase as more of the molecule was present. However, it was apparent that even the most reactive fragment was only one-tenth as active as the whole molecule of little gastrin against which the antibodies had been raised. Although cross-reactivity for cholecystokinin was minimal with all three antisera, there were ten-fold differences between them. These differences were least with RCS 8 and greatest with Gas 8. The cross-reactivity between big and little gastrin was examined in greater detail and was found to be constant throughout the standard curve, being approximately 75% for Gas 8 and RCS 8, and 85% for Gas 9.

(II) The measurement of plasma gastrin using three antisera

The plasma from the ten normal subjects was assayed using the three antisera. The mean plasma gastrin for antiserum Gas 8 was 6.2 pmol/l s.e. mean ± 0.5 pmol/l, for Gas 9 was 6.4 ± 0.4 pmol/l, and for RCS 8 was 12.8 ± 1.5 pmol/l. The level of plasma gastrin was higher in every sample when assayed by antiserum RCS 8, than when it was assayed with the other two. A similar finding was noted with plasma samples from twenty-five duodenal ulcer patients: Gas 8: 6.4 ± 0.9 pmol/l; Gas 9: 6.8 ± 0.9 pmol/l; RCS 8: 11.5 ± 1.2 pmol/l (Fig. 4). This difference between the level determined using RCS 8 and the levels with Gas 8 or 9 was statistically significant (Wilcoxon Rank Sum Test: $P < 0.01$). In the eleven post-vagotomy subjects the mean basal gastrin when estimated with Gas 8 was 10.2 ± 1.6 pmol/l; with

FIG. 1. The cross reactivity of antibody Gas 8 when tested against big gastrin (a), little gastrin (b), pentagastrin (c), cholecystokinin (d) and the fragments of little gastrin (e, 13–17; f, 11–17, g, 8–17; h, 3–17). Fragments 1–13, 3–13, 6–13 and 9–13 are not included for the sake of clarity as no binding occurred until 10^6 pmol were present, and total displacement of the label occurred at 10^7 pmol.

●—● (a); S—S (b); ▲...▲ (c); ◆—◆ (d); X—X (e); △—△ (f); ◆...◆ (g); ■—■ (h).

FIG. 2. The cross-reactivity of antibody Gas 9. Legend as for Fig. 1.

FIG. 3. The cross-reactivity of antibody RCS 8. Legend as for Fig. 1.

FIG. 4. The basal plasma gastrin level in twenty-five duodenal ulcer patients assayed by the three antisera showing the significantly higher level when the plasma samples were assayed with RCS 8 as compared with Gas 8 or Gas 9.

FIG. 5. The incremental gastrin response following an English breakfast in eleven post-vagotomy subjects using the antibodies Gas 8 (●---●), Gas 9 (●....●) and RCS 8 (●—●) to assay the plasma samples.

TABLE 2. The basal gastrin level, the peak gastrin increment and the two hour incremental integrated gastrin response* (IGR) in the seven volunteers after the seven stimuli

Stimulus	Number of tests	Basal gastrin (pmol/l $\pm$ s.d.)	Peak gastrin increment (pmol/l $\pm$ s.d.)	Incremental IGR* (pmol/l $\pm$ s.d.)
Complan	7	8.8 $\pm$ 3.4	5.6 $\pm$ 2.4	346.0 $\pm$ 270.7
Casilan	7	8.1 $\pm$ 3.1	5.9 $\pm$ 2.8	244.1 $\pm$ 186.3
Prosperol	7	8.0 $\pm$ 2.8	7.9 $\pm$ 3.9	463.3 $\pm$ 318.4
Milk	6	6.4 $\pm$ 2.8	4.6 $\pm$ 1.7	270.3 $\pm$ 185.0
Oxo	5	7.9 $\pm$ 3.5	3.8 $\pm$ 2.2	188.2 $\pm$ 190.3
Calcium carbonate	6	6.4 $\pm$ 1.5	4.0 $\pm$ 1.8	204.7 $\pm$ 209.6
Breakfast	7	6.0 $\pm$ 2.0	8.9 $\pm$ 2.3	746.9 $\pm$ 195.7

Gas 9 was 11.1 $\pm$ 1.5 pmol/l; while with RCS 8 the level was 18.6 $\pm$ 3.5 pmol/l; again the level with RCS 8 was in each sample higher than the level with either of the other two antisera.

After the English breakfast test the total integrated gastrin response reflected the difference in the basal levels, being for Gas 8, 3260.7 $\pm$ 517.1 pmol/l; for Gas 9, 3298.8 $\pm$ 422.3 pmol/l; and for RCS 8, 4639.0 $\pm$ 702.7 pmol/l. As the alternative methods of presenting these data were examined it was noted that when the incremental gastrin response was calculated the response after the stimulation was now almost identical when the three antisera were compared: Gas 8, 1296.6 $\pm$ 275.9 pmol/l; for Gas 9, 1159.5 $\pm$ 231.3 pmol/l; and for RCS 8, 1131.9 $\pm$ 264.8 pmol/l (Fig. 5). This method of comparison therefore diminished the variation between different assays.

(III) Stimulation of gastrin release

The basal plasma gastrin level in the seven normal subjects was 7.51 $\pm$ 0.44 pmol/l. The peak gastrin and the total integrated gastrin response for the seven stimuli are shown in Table 2. The stimulus which evoked the highest peak and total integrated gastrin response was the English breakfast. This meal was the stimulus most favoured by the volunteers, and therefore further studies were performed with this meal to test the reproducibility of this method. Seven volunteers had four breakfast tests performed. The total integrated gastrin response and the coefficient of variation for each subject are shown in

TABLE 3. The reproducibility of the breakfast test in seven volunteers tested on four occasions. The pooled estimate of the coefficient of variation was 18.3%

Subject	Number of tests	Mean total IGR (2 hr) (pmol/l $\pm$ s.d.)	Coefficient of variation (%)
C.R.	4	1907 $\pm$ 344	18.0
D.M.	4	1096 $\pm$ 96	8.7
B.P.	4	2918 $\pm$ 447	15.3
B.E.	4	1658 $\pm$ 201	12.1
T.N.	4	1352 $\pm$ 157	11.6
P.F.	4	721 $\pm$ 331	46.0
H.J.	4	2143 $\pm$ 402	18.7

Table 3. The pooled estimate of the coefficient of variation was 18.3%. A one-way analysis of variance of these results showed that of the total variance in the system the 'between individual' variance accounts for 96% and the 'within individual' variance accounts for only 4%.

Discussion

This study shows that the pattern of cross-reactivity on an antiserum for the different polypeptides tested is unique to that antiserum. Of particular interest is that two antisera with similar cross-reactivity to big gastrin, namely Gas 8 and RCS 8, gave significantly different basal levels. However, Gas 8 and Gas 9 gave identical basal gastrin levels yet had a slightly different cross-reactivity to big gastrin. The actual pattern of cross-reactivity suggests that each antiserum is not specific for one fragment of the gastrin molecule because all three react with fragments 1-13 and with fragments 13-17, totally displacing the label when 10⁷ pmol of either substance is assayed. Such behaviour of gastrin antisera perhaps makes it unlikely that an antibody will be raised against one of the molecular species of gastrin, to which it is totally specific. Nevertheless, antisera relatively more specific than those described in this study have been reported (Hansky, Soveny and Korman, 1974).

The considerable difference in the basal levels between Gas 8 and RCS 8 antisera assays are difficult to explain. Both big gastrin (Yalow and Wu, 1973) and component 1 (Rehfeld *et al.*, 1974) are known to be present in fasting plasma and differences in the cross-reactivity of the antisera to these molecular forms of gastrin could account for the result. As pure preparations are not yet available, neither of these could be tested in the cross-reactivity studies. Therefore, a possible explanation is that there are high levels of big gastrin in fasting plasma which may be measured more by antiserum RCS 8 than antiserum Gas 8 or 9. An alternative explanation for the higher basal levels with RCS 8 could be attributed to its lower avidity. Nevertheless, the sensitivity of the three assays as shown by the concentration of gastrin required to produce a 10% displacement

of label in the standard curve was really quite similar for each assay. Another explanation is that the difference is connected with non-specific plasma effects in an as yet unknown manner, although in these assays the non-specific effect has been minimized by the use of affinity chromatography to obtain a pure gastrin-free plasma for the standard curve. It is thus probable that even when antisera more specific for each type of gastrin are available the basal levels reported by different workers will still show variation.

The second problem in the quest for reproducible gastrin results is the lack of a satisfactory stimulus for the release of gastrin. Of the seven foods given to the volunteers, the English breakfast was the most natural and therefore the most acceptable, and produced the highest peak gastrin level, as well as the greatest total integrated gastrin response. This stimulus was chosen for further study, and found to be reproducible, the pooled estimate of the coefficient of variation being 18%. Such a coefficient of variation takes into account both assay and individual variation. It is unlikely, therefore, that a system with significantly less variation will be found. The advantage of this test is its simplicity and the avoidance of gastric tubes. The meal was acceptable to the eleven post-vagotomy subjects, which suggests that it would be satisfactory for post-operative studies. Slight variations in the speed of ingestion were found to make little difference to the results.

The expression of gastrin results in the literature varies, yet data are not available to show which method of presentation is more significant. This study suggests that between-assay variation can be reduced by expressing the result in terms of increment, rather than the observed peak or the total integrated gastrin response. The incremental integrated gastrin response may therefore be more useful in the comparison of results between different studies especially when different antisera are used in the gastrin assays.

It can be concluded that at present the results of one study cannot be extrapolated to another unless identical antisera are used. Further, a simple meal such as the English breakfast appears to be an ideal stimulus for gastrin release and the results from such a stimulus are best expressed on an incremental basis.

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