Pergamon

European Journal of Cancer Vol. 31A, No. 12, pp. 2086–2092, 1995

0959–8049/95 $9.50+0.00

0959-8049(95)00451-3

Original Paper

Gastrin Sensitivity of Primary Human Colorectal Cancer: The Effect of Gastrin Receptor Antagonism

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The purpose of this study was to determine the effect of the gastrin receptor antagonist, CR2093, on basal and gastrin-stimulated growth of primary human colorectal adenocarcinomas and to relate this to gastrin receptor expression. Tumour cells, derived from surgical specimens by enzymatic disaggregation, were grown on matrices of type I collagen and irradiated fibroblasts. Gastrin receptor expression was measured by using a mouse monoclonal antibody directed against the gastrin receptor and an avidin–biotin immunocytochemical method. Increased growth in the presence of gastrin-17 (used at physiological concentrations and as assessed by [^{3}H] thymidine uptake) was shown in 16/34 (47%) tumours. CR2093 significantly reversed this stimulated growth ($P < 0.05$, one way analysis of variance) in 9/16 (56.3%) of the tumours and inhibited the basal growth of 11/34 (32.4%). Basal growth inhibition was reversed by gastrin-17 in 82% (9/11) of tumours. Gastrin receptor expression was widespread, but was not related to the degree of growth response to gastrin, and there was no significant correlation between intensity of receptor expression and inhibition of basal growth by CR2093. In conclusion, both gastrin-stimulated and basal growth of primary human colorectal can be inhibited by gastrin receptor antagonism, but gastrin receptor expression does not predict the sensitivity of tumours to (i) the proliferative effects of gastrin or (ii) the inhibitory effects of a gastrin receptor antagonist on basal growth. Antigastrin agents may have clinical value in the treatment of gastrin-sensitive colorectal tumours, and gastrin receptor expression may be related to endogenous gastrin production by colorectal tumour cells.

Key words: gastrin, gastrin receptors, colorectal cancer
Eur J Cancer, Vol. 31A, No. 12, pp. 2086–2092, 1995

INTRODUCTION

THERE IS now substantial evidence that a variety of gastrointestinal tumour cell lines, derived from both animals and humans, are sensitive to the proliferative effects of the polypeptide gastrointestinal hormone gastrin, and that gastrin receptor antagonism can abrogate growth responses stimulated by this hormone [1]. There is also evidence that human colorectal cancer can be sensitive to gastrin, and, using short term cultures of cells from individual primary human colorectal cancers, our group has previously shown gastrin sensitivity in such tumours [2]. The growth-promoting effect of exogenously applied gastrin is mediated via receptor binding, and high affinity CCKB/gastrin receptors have been reported in primary human colorectal tumours [3]. Gastrin may also be an autocrine growth factor in colorectal tumour growth [4–6], and this autocrine loop has been reported to be mediated via low affinity CCKC/gastrin receptors [7].

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Revised 2 May 1995; accepted 1 Jun. 1995.

There is, however, little evidence relating to the effect of gastrin receptor antagonists in primary human colorectal cancer. Much of the evidence has been generated from the use of established colorectal cell lines. *In vitro* gastrin-17 (G-17) stimulated growth of the human colorectal cancer cell line, HT29, has been shown to be inhibited by the gastrin receptor antagonist, proglumide, and *in vivo* pentagastrin-stimulated growth of two other colorectal cell lines was similarly inhibited by the same compound [8]. It is also of interest that the *in vitro* basal growth of several human colorectal cancer cell lines has been shown to be inhibited by proglumide and an additional gastrin receptor antagonist, benzotript [9, 10]. This is thought to occur via the interaction of these gastrin receptor antagonists with the CCKC/gastrin receptor which has been reported to mediate the autocrine effect of gastrin [7].

In the present study, our aim was to use short term cultures of individual human colorectal cancers to assess gastrin sensitivity and the effects of the gastrin receptor antagonist, CR2093. This antagonist is a member of the glutaramic acid family of antagonists with a much higher antagonist potency than that of proglumide [11, 12], and in previous studies with gastrin-

sensitive cell lines, we have shown CR2093 to have marked inhibitory effects on tumour cell growth [13, 14]. In this study, we examined specifically the ability of CR2093 to block both the growth-promoting effects of exogenous physiological gastrin concentrations (mediated by CCKB/gastrin receptors) and basal growth (which may be maintained by the autocrine gastrin loop mediated by CCKC/gastrin receptors). In addition, we attempted to assess the significance of gastrin receptor expression.

MATERIALS AND METHODS

Preparation of tumour cells

Tissue from primary colorectal tumours was obtained within 30 min of surgical resection. Malignancy of the tissue was confirmed by immunochemical evaluation of sections taken from adjacent biopsies. Tumour tissue was immediately placed in RPMI 1640 culture medium (Gibco Laboratories, U.K.) containing 10% heat-inactivated fetal calf serum (FCS, Sigma, Poole, U.K.), gentamicin (100 μg/ml, Nicholas, Slough, U.K.) and Amphotericin B (2.5 mg/ml, Flow Labs, Irvine, U.K.), to eliminate surface contamination, and processed as previously described [2].

Swiss 3T3 mouse fibroblasts were grown in 75 cm^3 tissue culture flasks (Costar) in RPMI 1640 plus 10% FCS, and cells were harvested from subconfluent monolayers with 0.025% EDTA (Sigma) for 3–5 min at 37°C. The viability of the cells was assessed by Trypan blue exclusion (0.4% Trypan blue, Sigma), and the cells were suspended at a concentration of 1×10^6/ml in assay medium. The cells were irradiated with a caesium source (9×10^3 rads for 20 min) and then plated out into tissue culture plates containing 96 flat-bottomed wells (Flow Labs) at a concentration of 1×10^4 cells in 25 μl volumes of RPMI 1640 medium containing 1% fetal calf serum (this will be referred to as assay medium). The wells of the tissue culture plates had previously been coated with 25 μl of rat tail type 1 collagen (0.5 mg/ml). The 3T3 cells were then incubated on the collagen coated wells for 2–3 h at 37°C to allow for cell adherence. The tumour cells were plated on to the collagen : fibroblast matrix at a concentration of 1×10^5 cells/well in assay medium.

Test materials

Human G17 (sulphated G17II, Sigma), was stored lyophilised at −20°C and reconstituted to 1 mg/ml in sterile distilled water. For use *in vitro*, G17 was diluted in assay medium and 50 μl aliquots were administered to the tumour cells in the 96-well plates to give final concentrations within the wells of 1×10^{-10} and 5×10^{-10} M.

The gastrin receptor antagonist, CR2093, was a gift of Dr L. Rovati (Rotta Laboratories, Milan, Italy) and was diluted in sterile distilled water at a concentration of 1 mg/ml and stored at 4°C. For use in the assays, CR2093 was diluted in assay medium to give a final concentration within the assay of 5×10^{-4} M. Five replicates were set up per treatment.

Assessment of proliferation

After approximately 5 days incubation (when colony formation was evident within the wells), proliferation was assessed by thymidine uptake. For this, [^{3}H]thymidine (Amersham, Bucks, U.K., specific activity 925 GBq/mmol) was diluted to 37 MBq/ml and 1 ml of this stock was added to 1 ml of cold thymidine (2 mM) and 8 ml phosphate buffered saline (pH 7.3). This mixture was then added to each well in a volume of 10 μl. After 12 h incubation, the cells were washed and harvested on to filter papers. The papers were left to dry before being assessed for associated radioactivity as measured by disintegrations per minute (DPM) on a scintillation counter.

Statistical analysis

The means and their standard deviations of the five replicates were calculated. Wells containing cells but not drugs were used as untreated controls and their thymidine uptake taken to be 100%. All proliferation in the presence of G17 and/or the gastrin receptor antagonist was calculated as a percentage of the untreated control. The results were analysed by a one-way analysis of variance using the SPSS statistical package for the IBM PC.

Data was correlated using a linear regression analysis, from the SPSS Programme.

Immunocytochemical evaluation of the tumours

Test materials. The monoclonal antibody, 2C1, was used to determine gastrin receptor expression. This antibody was originally derived by Mu and associates [15] by immunising mice with canine parietal cell extracts. 2C1 was found to stain parietal cells specifically and displaced gastrin from gastrin receptors [15]. The antibody is believed to recognise a subunit of the gastrin CCK/B receptor [16]. Initial characterisation of the antibody has been performed and it has been shown to stain human parietal cells, inhibit binding of gastrin to gastrin receptors on the rat pancreatic cell line, AR42J, and the level of staining is up- and downregulated by physiological and pharmacological G17 levels, respectively [17–19].

Procedure. Sections were cut from formalin-fixed, paraffin-embedded tumour samples and stained with the 2C1 antibody (1:4000 dilution, 4°C, overnight). Binding of the primary antibody was detected with biotinylated rabbit anti-mouse (1:500 dilution Dakopatts, Bucks, U.K.) incubated at 22°C, for 30 min.

Binding of the secondary antibody was localised using the avidin–biotin complex method with peroxidase as the tracer and diaminobenzidene as the chromagen. Negative controls were included in which the primary antibody was omitted.

The stained sections were assessed blind by an independent observer and graded as follows: 0, 0/+, +, +/++, ++, ++/+++, +++ (intensity levels, 0, 1, 2, 3, 4, 5, 6, respectively).

RESULTS

In vitro *growth response to G17*

Assessment of growth was performed by [^{3}H]thymidine uptake. This method of assessing cell growth has been validated on a series of colorectal cell lines. Significant correlation was shown between direct cell counts and [^{3}H]thymidine uptake ($r = 0.85$, data not shown).

A series of 34 primary human colorectal tumours were evaluated for their *in vitro* growth response to G17 at concentrations of 1×10^{-10} and 5×10^{-10} M, which are known to be close to the K_d of the high affinity receptors reported to be expressed on human colorectal tumour cells [3]. Overall, 16/34 (47%) tumours showed significant growth stimulation (at least to $P < 0.05$, one way analysis of variance) in the presence of G17; 9/34 (26.5%) to 5×10^{-10} M and 14/34 (41.2%) to 1×10^{-10} M G17, ($P < 0.05$). In addition, 2/34 were significantly inhibited; 1/34 by 1×10^{-10} M and 1/34 by 5×10^{-10} M. Figure 1a shows the magnitude of the growth response to G17. The median response at 5×10^{-10} M G17 was 191.1% (range 966.5 to 60%) of control and at 1×10^{-10} M G17, 198% of control (range 317 to 23.7%).

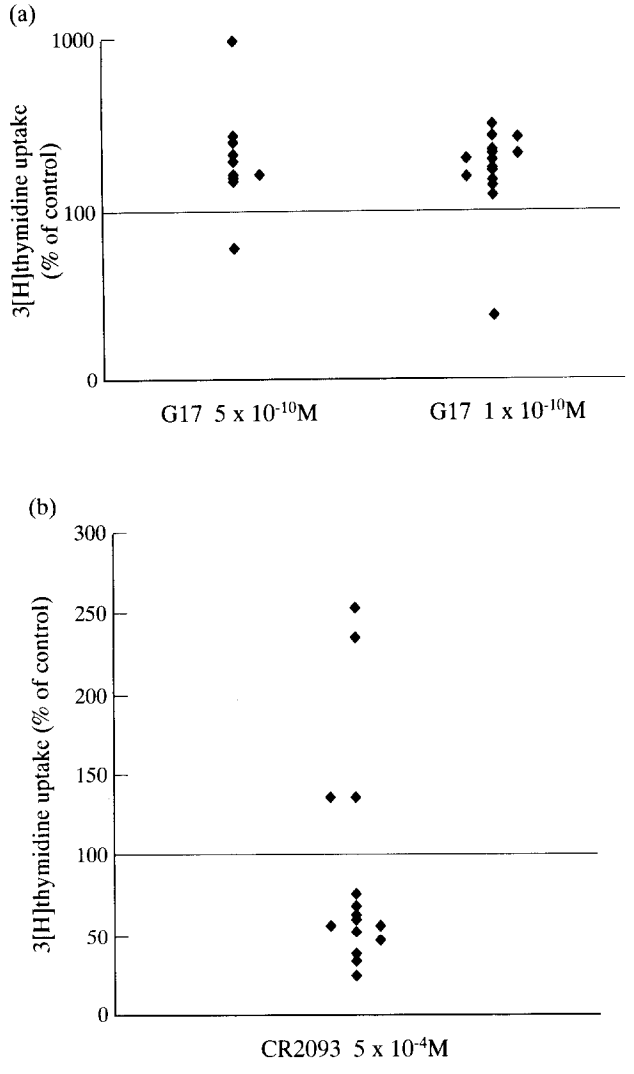

Figure 1. The [³H]thymidine uptake of human colorectal tumours in short-term *in vitro* culture grown (a) in the presence of G17 (5×10^{-10} and 1×10^{-10} M); (b) In the presence of CR2093 (5×10^{-4} M). Each symbol represents an individual tumour.

Effect of the gastrin receptor antagonist, CR2093 on the basal growth of colorectal tumours

The gastrin receptor antagonist, CR2093, was used at a fixed concentration of 5×10^{-4} M in all the assays, as it has been previously shown to displace 70% of G17 from gastrin receptors on the rat pancreatic cell line, AR42J [13].

Figure 1b shows the effect of 5×10^{-4} M CR2093 on the basal growth of the colorectal tumour cells. Of the 34 tumours examined, 11 (32.4%) were significantly inhibited by CR2093 and 4/34 (11.8%) were significantly stimulated (significance assessed by a one way analysis of variance and were significant to at least the 5% level).

Of the 11 tumours in which there was significant growth inhibition by CR2093, significant reversal by G17 was shown in 9/11 (82%).

The effect of CR2093 on tumour growth was correlated to the basal [³H]thymidine uptake of the tumours, but no significant correlation was observed. There was also no significant correlation between the independent effects of CR2093 and G17 on basal growth.

Effect of CR2093 on the growth of the colorectal tumour cells when combined with G17

Table 1 shows the effect of CR2093 on G17-enhanced tumour growth expressed as mean dpm +/− standard deviation. Of the 16 tumours showing a proliferative response to G17, nine (56.3%) had significantly reduced G17-stimulated growth in the presence of CR2093.

Of the three tumours in which CR2093 stimulated basal tumour growth, two had reduced G17-stimulated growth in the presence of CR2093, whereas one did not. Of the two tumours that had inhibited basal growth in the presence of G17, CR2093 reversed the inhibition (data not shown).

Table 1. Effect of CR2093 on the basal and gastrin-stimulated growth of gastrin sensitive primary human colorectal tumours

	[³H] thymidine uptake (dpm +/− S.D.)		
Tumour no.	Untreated	G17	G17 + CR2093
1	2266 (106)	5574* (516)	2609† (114)
2	4612 (248)	5818* (936)	1861*† (495)
3	1980 (432)	4130* (532)	2518† (605)
4	2461 (56)	4789* (1071)	2180† (83)
5	2457 (766)	4069* (1061)	3140 (278)
6	5087 (1067)	7029* (588)	4368† (868)
7	1478 (528)	3111* (921)	3852* (934)
8	889 (52)	2392* (1440)	1418* (341)
9	912 (262)	1284* (137)	1846*† (125)
10	1908 (562)	3395* (1719)	1034*† (379)
11	1760 (119)	4011* (727)	1722† (492)
12	1226 (133)	2424* (231)	985† (238)
13	1147 (252)	1954* (457)	1149† (382)
14	1440 (562)	2271 (352)	2060 (760)
15	2892 (534)	5838* (1520)	3244 (979)
16	465 (36)	1475* (842)	968* (237)

S.D., standard deviation. *Significance at at least the 5% level from the untreated control, †Significance from G17-stimulated growth at at least the 5% level as assessed by a one way analysis of variance.

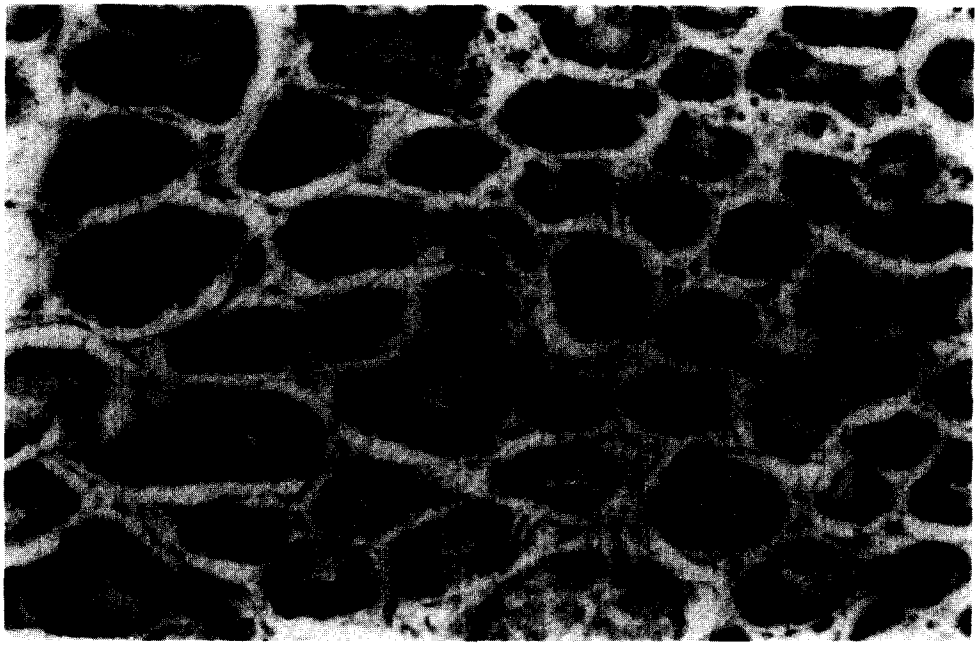

Figure 2(a).

A comparison of G17-stimulated tumour growth with gastrin receptor status

The 2C1 monoclonal antibody was shown to specifically stain parietal cells in human gastric mucosa (Figure 2a). Gastrin receptor expression was examined on 20 of the tumours in the *in vitro* screen using the same monoclonal antibody. Figures 2b and c shows a colorectal tumour staining with low (0/+, intensity level 1) and high intensity (+++, intensity level 6), respectively.

Some degree of gastrin receptor expression as detected by immunocytochemistry was shown in all the tumour specimens examined (see Figure 3 for the distribution of tumours versus intensity of staining) with the majority of tumours showing a + or level 2 staining intensity. Expression was mostly cytoplasmic, although expression on both the plasma membrane and nucleus was evident. There was no relationship between expression and histological grade or Dukes' stage (data not shown).

Figure 4 shows a comparison of gastrin receptor immunoreactivity with the *in vitro* proliferative response to G17 in the 20 tumours examined. There was no correlation between the intensity of cell-associated staining and the level of proliferative response to G17. All tumours responding to G17 were shown to stain with the gastrin receptor antibody, however, eight tumours showing no growth response to G17 also expressed gastrin receptor immunoreactivity. Gastrin receptor immunoreactivity was also compared to the effect of CR2093 on basal growth (Figure 5) and, although a negative correlation between the intensity of gastrin receptor immunoreactivity and the degree of inhibition of growth shown by CR2093 was observed, this failed to reach significance ($P = 0.092$, linear regression analysis).

Figure 2(b).

Figure 2. (a) 2C1 immunoreactivity staining in human gastric fundus, magnification × 1000. (b) Poorly staining human colorectal primary tumour, magnification × 1000. (c) Highly staining human colorectal primary tumour, magnification × 1000.

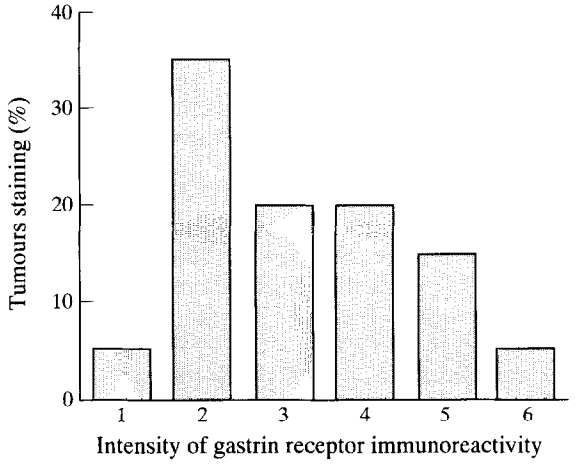

Figure 3. The intensity of gastrin receptor immunoreactivity of a series of colorectal primary tumours.

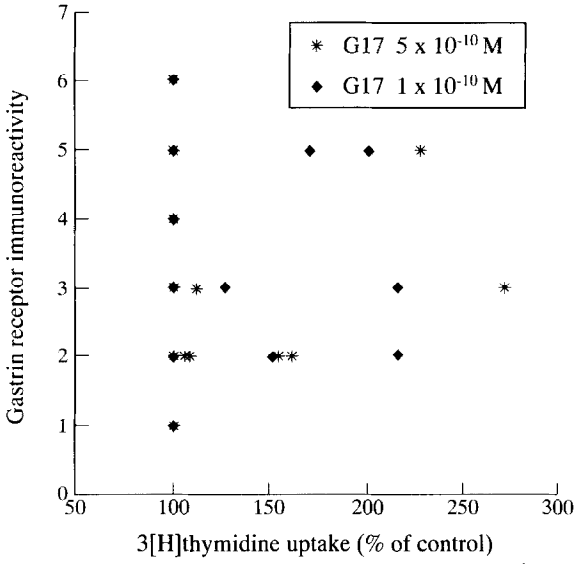

Figure 4. A correlation between gastrin receptor immunoreactivity and [³H]thymidine uptake in the presence of G17 at 5 × 10^{-10} M as denoted. Gastrin receptor immunoreactivity which was scored according to staining intensity with the 2C1 monoconal antibody as follows: 0 0, 1 0/+, 2 +, 3 +/++, 4 ++, 5 ++/+++, 6 +++. Each symbol represents an individual tumour.

DISCUSSION

In this study, we have shown that G17 significantly increased the growth of 47% of human primary colorectal tumours as assessed by [³H]thymidine uptake in short-term *in vitro* culture. This corresponds well with results previously reported by our group [2] and is also in keeping with the percentage of colorectal tumours expressing external CCKB/gastrin receptors as measured by radioligand binding [3]. However, using the 2C1 monoclonal antibody, we found that all tumours expressed some degree of gastrin receptor immunoreactivity, and that there was no correlation between degree of response to exogenous G17 and the intensity of receptor expression. This may be explained by the findings that the 2C1 monoclonal antibody binds to the CCKC/gastrin receptor [7] in addition to binding an epitope of the CCKB/gastrin receptor [16].

The gastrin receptor antagonist, CR2093, inhibited G17-stimulated growth in 9/16 tumours evaluated. In addition, CR2093 inhibited basal tumour growth in 11/34 tumours and this basal growth inhibition was reversed in the presence of G17 in 9/11 tumours. The inhibitory effect of CR2093 on basal tumour growth was not related to the sensitivity of the tumours to exogenous G17, and failed to reach significance when related to intensity of CCKB/CCKC/gastrin receptor expression.

There is now good evidence that human colorectal cancer cells can synthesise their own gastrin. Gastrin mRNA has been demonstrated in a series of colonic carcinoma cell lines [20], and, more recently, in primary human colorectal adenocarcinomas

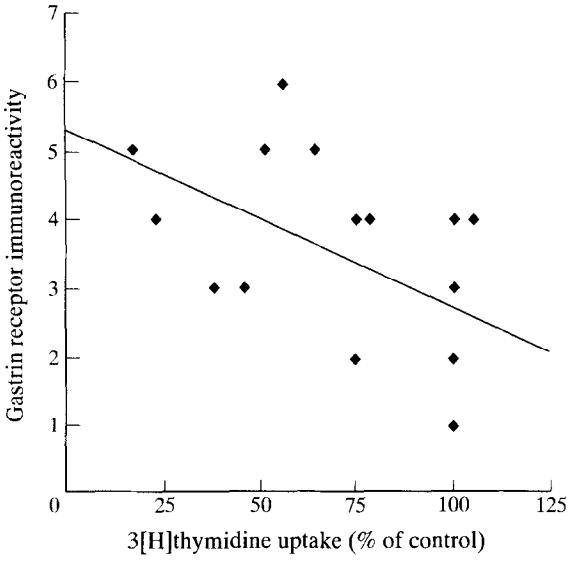

Figure 5. A correlation between gastrin receptor immunoreactivity as measured with the 2C1 monoclonal antibody and [^{3}H]thymidine uptake in the presence of 5×10^{-4} M CR2093.

[6]. Studies have revealed the presence of the precursor gastrin molecules; progastrin and glycine-extended gastrin in both established cell lines [21] and primary tumours [22] but both studies reported the absence of amidated biologically active gastrin. Recently, Seva and associates [23] showed that glycine-extended G17 promoted the growth of the GI tumour cell line, AR42J, and that this was mediated by a receptor other than the CCKB/gastrin receptor.

These studies suggest that endogenous precursor gastrin molecules may have an autocrine role in the growth of colorectal cancer cells, and this would explain some of our findings. First, the *in vitro* response to exogenous G17 may not correlate well with CCKB/CCKC/gastrin receptor expression if the cells are responding in an autocrine manner to their own hormone. This may also explain the inhibitory effect of G17 in some of the tumours; if tumour-associated gastrin is stimulating maximally, additional gastrin may induce receptor downregulation as shown with gastrin concentrations greater than those seen physiologically [24]. Thus, providing exogenous gastrin may push the total cellular gastrin concentration to the far side of a bell-shaped dose–response curve [13].

Gastrin which is stimulating cell growth in an autocrine manner may also induce receptor internalisation which could account for the cytoplasmic expression of receptor observed in the present study. The proportion of tumours shown to express CCKB/CCKC/gastrin receptors by immunocytochemical methods is higher than the levels of CCKB/gastrin receptors shown by ligand binding [3]. The latter method will only detect receptors expressed on the external surface of the plasma membrane and thus will not measure activated receptors. In addition, staining with 2C1 has revealed that the majority of colorectal tumours express either CCKB/gastrin or CCKC/gastrin receptors or possibly both (a discrimination cannot be made by using the existing monoclonal antibody).

Most importantly, however, the concept of autocrine gastrin stimulation is in keeping with the inhibition of the basal growth of the colorectal tumour cells by CR2093 and the reversal of this inhibition by G17. It would also explain why inhibition by CR2093 does not correlate with gastrin sensitivity, as those tumours which are autostimulated may be unlikely to exhibit a marked response to exogenous hormone. These findings are similar to those obtained with recent work carried out by our group on the rat pancreatic cell line, AR42J, which both expresses gastrin receptors and produces its own gastrin [14].

CCKB/CCKC/gastrin receptor expression was not related to inhibition of growth by CR2093. Further research is required to establish the intracellular content of gastrin precursors in human tumours, and this should be related to CCKB/CCKC/gastrin receptor expression, response to exogenous G17 and inhibition by CR2093. This parameter should complete the gastrin profile of colorectal tumours. However, it would appear that a proportion of colorectal cancers are sensitive to hormonal manipulation, and as more powerful means of inhibiting the effects of gastrin are developed, this may have important therapeutic implications.

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Acknowledgements——The work was funded by a grant from the Trent Regional Health Authority. In addition, the authors would like to acknowledge Dr L. Belov and Professor D.L. Morris for provision of the 2C1 antibody and the Pathology Department for their assistance in the immunocytochemical studies. The authors would also like to thank Ms D. Milanowska for typing the manuscript.