Autocrine stimulation of growth of AR4-2J rat pancreatic tumour cells by gastrin

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Summary The control of cell proliferation by gastrin has been investigated in a rat pancreatic tumour cell line, AR4-2J. Exogenous gastrin, 10⁻¹⁰ to 10⁻⁸ M, stimulated cell growth of thymidine-synchronised AR4-2J cells cultured over 48 h in serum-free medium. Cell lysates of AR4-2J cells contained an average of 4.5 and 3.5 pg gastrin per 10⁶ cells, when grown in serum-supplemented or serum-free media, respectively, as revealed by radioimmunoassay. In serum-free medium, AR4-2J secretes 34 ng 1⁻¹ 10⁻⁸ cells of gastrin over 48 h. Addition of an anti-gastrin immunoglobulin preparation, but not control immunoglobulins, caused a maximum 52% reduction in cell growth. These data are consistent with an autocrine role for gastrin in the control of AR4-2J cell growth. These results were supported by studies with gastrin/CCK receptor antagonists. Six non-peptide gastrin/CCK receptor antagonists inhibited AR4-2J cell growth in a concentration-related manner. The concentration required for 50% inhibition (IC₅₀) of cell growth by the amino acid-derived antagonists proglumide (3.5 x 10⁻³ M), benzetrit (1.8 x 10⁻³ M), loxiglumide (1.1 x 10⁻⁴ M) and lorglumide (6.7 x 10⁻² M) were of the same order and significantly correlated with their IC₅₀ for inhibition of ¹²⁵I-gastrin binding to AR4-2J cells. Inhibition of cell growth by these antagonists was partially reversed by the addition of exogenous gastrin. In contrast, the IC₅₀ for inhibition of cell growth with two benzodiazepine-derived antagonists, the CCK-B receptor antagonist L-365,260 (4.6 x 10⁻⁸ M) and the CCK-A receptor antagonist devazepide (1.7 x 10⁻³ M) were two-three orders of magnitude greater than those required to inhibit gastrin binding (10⁻⁵ - 10⁻⁷ M). The growth inhibitory effects of L-365,260 and devazepide were not reversed by exogenous gastrin suggesting these benzodiazepine-derived antagonists do not inhibit cell growth by interaction with gastrin receptors. The results are consistent with gastrin being an autocrine growth factor in AR4-2J cells, and that stimulation of cell growth is due to stimulation of the gastrin, rather than CCK-B, receptor sub-type. This study highlights that gastrin receptor antagonists warrant further investigation as agents to control growth of tumours, such as those from the gastrointestinal tract, which express gastrin receptors.

Gastrin, amongst its range of physiological actions, is a trophic factor for the normal gastrointestinal mucosa (Enochs & Johnson, 1977; Balas et al., 1985). There is increasing evidence that gastrin is also a growth factor for gastric and colonic cancers in vivo. Gastrin increases growth of carcinogen-induced tumours of the rat colon and stomach (McGregor et al., 1982; Yasui & Tahara, 1985). Long-term hypergastrinaemia in man and rat is associated with enterochromaffin cell-like cell hyperplasia and gastric carcinoids (Creutzfeldt, 1988). The trophic effects of gastrin on a transplantable murine colonic adenocarcinoma in vivo and on human gastric and colonic cancer xenografts in nude mice are well recognised (Singh et al., 1986; Winsett et al., 1986; Smith & Solomon, 1988; Watson et al., 1989). Similarly, the structurally-related cholecystokinin (CCK) has been reported to enhance pancreatic tumour formation in carcinogen-treat ed animals and growth of human pancreatic cancer xenografts in nude mice (Howatson & Carter, 1985; Smith et al., 1990b). Gastrin receptors are found on a large proportion of tumours from patients with colon cancers (Upp et al., 1989). Studies linking tumour growth to gastrin have been supported by the use of gastrin antagonists. Proglumide, a non-specific gastrin/CCK receptor antagonist, inhibited the growth stimulatory effects of gastrin on mouse colon cancer in vivo and prolonged the survival of tumour-bearing mice (Singh et al., 1987; Beauchamp et al., 1985). Direct trophic effects of gastrin and CCK on gastrointestinal and pancreatic tumour cells in vitro are widely reported (Sirinek et al., 1985; Kusyk et al., 1986; Watson et al., 1988, 1989; Imdahl et al., 1989; Guo et al., 1990; Smith et al., 1991). Inhibition of gastrointestinal and pancreatic tumour cell growth by gastrin/CCK receptor antagonists has also been shown by several workers (Hoosin et al., 1988; Imdahl et al., 1989; Guo et al., 1990; Smith et al., 1990a). These data provide strong evidence for a role for gastrin in gastrointestinal and pancreatic tumour cell growth, and suggest that gastrin antagonists may provide a novel pharmacological approach to anti-cancer therapy.

An autocrine role for gastrin in human colonic tumour cell growth has been suggested, on the basis of studies with anti-gastrin antibodies and gastrin/CCK antagonists (Hoosin et al., 1988; 1990), but not confirmed in mouse colon tumour cells (Guo et al., 1990). However, several groups have suggested that a proportion of the inhibition of tumour cell growth by proglumide, the most commonly used gastrin antagonist for such studies, is unrelated to interaction with gastrin receptors (Singh et al., 1987; Imdahl et al., 1989; Guo et al., 1990). Moreover, a recent report has suggested that the growth inhibitory effects of a potent CCK-A receptor antagonist, devazepide (L-364,718), is also independent of its interaction with gastrin or CCK receptors, while a potent CCK-B receptor antagonist, L-365,260 was unable to inhibit proliferation of several colon cancer lines in vitro (Thumwood et al., 1991).

In the present study we have re-evaluated the role of gastrin in the regulation of growth of a tumour cell line. The AR4-2J rat pancreatic tumour cell line has been advocated for studies of gastrin receptors. AR4-2J cells express both CCK-A, coupled to stimulation of pancreatic enzyme secretion, and gastrin/CCK-B receptors (Scemama et al., 1989; Lambert et al., 1991). A trophic response to gastrin has been shown in these cells which is coupled to the gastrin/CCK-B receptor type (Scemama et al., 1989). Gastrin receptors in AR4-2J cells have been characterised by binding studies (Scemama et al., 1987, 1989; Lambert et al., 1991). The latter point is important as earlier studies describing inhibition of cell growth by gastrin/CCK receptor antagonists have not

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Materials and methods

Cell culture

AR4-2J cells, a rat pancreatic tumour cell line derived from a transplantable tumour of the acinar pancreas (Jessop & Hay, 1980), were obtained from the American Type Culture Collection. Cells were maintained in subconfluent monolayer culture at 37°C in an atmosphere of 5% CO2-air in RPMI 1640 supplemented with 10% FCS, glutamine (2 mM), penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). For hormone binding studies, AR4-2J cells were removed from their flasks by washing with calcium and magnesium free phosphate buffered saline (PBS) and the exposing to 0.02% (w/v) EDTA in PBS for 5 min. For routine passage, cells were harvested with 0.025% (w/v) trypsin in versene.

HT-29 cells, a line derived from a human colonic adenocarcinoma, were obtained from the American Type Culture Collection. Cells were maintained in subconfluent monolayer culture at 37°C in an atmosphere of 5% CO2-air in Dulbecco’s modified Eagle’s media supplemented with 10% FCS, glutamine (2 mM), penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). For all other procedures, HT-29 cells were treated with similar protocols as described for AR4-2J cells.

Measurement of cell proliferation

Cells were seeded into 96-well microtitre cell culture plates at a concentration of 30,000 cells/well in 200 µl RPMI medium with 10% FCS. This density of cells was chosen such that untreated cells were in the exponential phase of growth at the end of the 48 h incubation period. After 24 h, the medium was removed and replaced with 200 µl serum-free RPMI containing 1 mM thymidine to accomplish synchronisation of the cells in the G1/S phase of growth as described by Guo and co-workers (1990). After 24 h, the cells were washed and 200 µl serum-free RPMI containing hG17-1 were added to the cells. Cell number was determined using the tetrazolium-based colorimetric assay (MTT assay) originally described by Mosmann (1983) and confirmed in some experiments by direct cell counting using a haemocytometer (see below).

Following the 48 h incubation, 50 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 1 mg ml⁻¹) solution was added to each well and incubated at 37°C for 4 h. The media were then removed from the wells and the formazan crystals solubilised by adding 75 µl of dimethylsulphoxide (DMSO). Plates were agitated on a plate shaker for 5 min, following which absorbance at 540 nm was immediately determined using a plate reader (Dynatech MR 5000).

Optical absorbance readings from test wells were corrected against control wells containing cells plus serum-free medium alone. When direct cell counts were required, media were removed, cells were washed with 100 µl PBS and harvested by a 20 min incubation with 100 µl 0.025% (w/v) trypsin in versene for resuspension in culture medium and counting in a haemocytometer. In experiments using the anti-gastrin Ig preparation half of the wells received dilutions of the control Ig fraction at an identical protein concentration; both Ig preparations had been previously dialysed against PBS. Incubations with Ig preparations were carried out in serum-free media for 4 days prior to measuring proliferation. Measurement of cell growth in the presence of gastrin/CKK receptor antagonists was made in a similar manner, however, incubations were carried out in the presence of 10% FCS over a 4 day period with a lower initial seeding density (10,000 or 3,000 cells/well for AR4-2J and HT-29 cells, respectively). Control wells received media containing DMSO at the same concentration present in the test wells (up to 0.5% v/v). The effect of each reagent was investigated in between four and ten wells on at least three separate occasions.

Ligand binding studies

AR4-2J cells were harvested, washed three times with Eagle’s modified essential medium (EMEM) containing 0.1% (w/v) bovine serum albumin (BSA) and 2 × 10⁶ cells transferred to 1.5 ml polypropylene microcentrifuge tubes. Cells were incubated with 2.5 × 10⁻¹⁰ M of [125I]-labelled ligand in the presence/absence of 2.5 × 10⁻¹⁰ M competing ‘cold’ ligand to determine non-specific/specific binding or with various concentrations of gastrin/CKK receptor antagonists. Incubation was carried out at 30°C for 60 min after which time the cells were washed three times with EMEM plus 0.1% BSA and cell-associated radioactivity was measured with a gamma counter. The IC₅₀ values for the various ligands was investigated from duplicate evaluations at each concentration on at least two separate occasions.

Measurement of gastrin production by AR4-2J cells

AR4-2J culture supernatants and cells lysed in distilled water were assayed for gastrin by radioimmunoassay, using a commercial kit (CIS, London, UK). The detection limit of the assay was 10 pg ml⁻¹ and the anti-serum used recognised hG17-1, hG17-11, hG34 and CCK with equal affinity. Standard curves were constructed in distilled water for the assay of cell lysates or in RPMI, with or without 10% FCS as appropriate, for assay of culture supernatants.

Materials

Media were supplied by Gibco (Life Technologies, Paisley, UK), foetal calf serum (FCS) by Gibopharm Ltd (Surrey, UK) and plastics by Nunc (Life Technologies). Human gastrin 17-1 (hG17-1) was from Bachem Ltd (Essex, UK). A rabbit anti-human gastrin immunoglobulin (Ig) preparation and a control rabbit Ig fraction prepared from sera of non-immunised animals were obtained from DAKO Ltd (High Wycombe, UK). The anti-gastrin Ig preparation reacts equally with hG17-1, hG17-11, hG34 and CCK and cross reacts to more than 50% with CCK-8. [125I]-tyrosyl-iodinated human gastrin (251I-hG17-1, NEN-Dupont, Southampton, UK) with a specific activity of 2,200 Ci mmol⁻¹ and a concentration of 50 Ci ml⁻¹ was used in the gastrin receptor binding assays.

The benzodiazepine-derived CCK-A antagonist devazepide (L364,718, 35 (N)-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-benzotript-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-(3-2h-indole-2-carboxamid)-[3H] hG17-1, L365,260) [3H+(N-(3,2h-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N- (3-methylphenyl)urea) was a gift of Merck Sharp & Dohme Laboratories, West Point, PA, USA. The amino acid derivaives, proglumide (D,L-4-benzamido-N,N-di-n-propylglutamic acid), proglumide-derived longlumide [CR 1409; D, L-4-(3,4-dichlorobenzoylamino)-5-(di-n-pentylamino)-5-oxo-pentanoic acid] and longlumide [CR 1505; D,L-4-(3,4-dichlorobenzoylamino)-5-(N-3-methoxypentylamino)-5-oxo-pentanoic acid], and benzotript (N-p-chlorobenzoyl-L-tryptophan), were a gift from Rotta Research Laboratories, Milan, Italy. Gastrin/CKK antagonists were dissolved in DMSO; controls including DMSO were included in each experiment.

Data analyses

Estimates of half-maximal concentrations (IC₅₀) of antagonists required for inhibition of cell growth or gastrin binding were determined by non-linear regression analyses (GraphPad). Scatchard analyses were performed by equilibrium binding data analysis using LIGAND (GnG, Biosoft). Significance of difference between means was investigated by analysis of variance followed by Student’s t-tests. Significance was set at P < 0.05.
Results

Effect of gastrin on AR4-2J proliferation

In preliminary experiments, addition of $5 \times 10^{-10} - 5 \times 10^{-8} \text{M} \text{hG}17-1$ to asynchronous AR4-2J cells in serum-free culture medium had no significant effect on proliferation (data not shown). Subsequent experiments were, therefore, carried out on thymidine-synchronised AR4-2J cells. AR4-2J proliferation in the presence of hG17-1 was assessed by the MTT assay on five separate occasions. These experiments were repeated by direct cell counting on one occasion with similar results (data not shown). A bell-shaped concentration-response curve was obtained (Figure 1). Gastrin concentrations between $10^{-12} \text{M}$ and $10^{-4} \text{M}$ significantly increased AR4-2J proliferation over a 48 h period, with the maximal proliferative response observed between $5 \times 10^{-12} \text{M}$ and $10^{-10} \text{M}$ gastrin. There were no significant growth effects of gastrin when used a concentrations $\leq 10^{-11} \text{M}$ or $\geq 10^{-7} \text{M}$.

Effect of gastrin/CCK receptor antagonists on AR4-2J proliferation

All of the antagonists tested inhibited unsynchronised AR4-2J proliferation over a 4 day period in a concentration-dependent manner. Figure 2 illustrates the results of three separate experiments in which cell number was assessed by the MTT assay; these data were confirmed on one occasion by direct cell counts. Proglumide was the least potent inhibitor of AR4-2J cell growth, while devazepide was the most effective. The rank order for inhibition of AR4-2J proliferation was devazepide $> L-365,260 >$ lorglumide $> loxiglumide >$ benzotript $> proglumide$. The half maximal inhibitory concentration of each antagonist is given in Table 1 and ranged from 3 nM (proglumide) to 17 µM (devazepide).

Figure 3 illustrates the results of experiments in which $5 \times 10^{-7} \text{M}$ gastrin was added to AR4-2J cells 2 h prior to addition of the antagonists. Each antagonist was used at the concentration previously determined to half maximally inhibit AR4-2J growth (Table 1). The inhibition of cell growth caused by proglumide, benzotript, lorglumide and loxiglumide was partially reversed by prior addition of gastrin. In contrast, inhibition of AR4-2J cell growth by L-365,260 and devazepide was not significantly effected by prior addition of gastrin.

We examined the ability of the gastrin receptor antagonists to inhibit the growth of HT-29 cells. In this cell line we were unable to detect specific 121H-GH17-1 binding or endogenous gastrin, and neither exogenous gastrin nor an anti-gastrin Ig preparation effected proliferation. All six gastrin/CCK antagonists inhibited HT-29 cell proliferation with the rank order of potency similar, except for devazepide, to that observed for AR4-2J cells (Table 1). The IC$_{50}$ values for inhibition of HT-29 proliferation by the amino acid derivatives were 2-4 fold greater than in AR4-2J cells. Devazepide did not achieve 50% inhibition of HT-29 proliferation at concentrations up to and including $10^{-4} \text{M}$.

Effect of an anti-gastrin Ig preparation on AR4-2J proliferation

When unsynchronised AR4-2J cells were cultured in serum-free medium in the presence of a dialysed anti-gastrin Ig preparation a significant concentration-related reduction in proliferation occurred compared to wells which received serum-free medium alone (Figure 4). Pre-incubation of the anti-gastrin Ig with $50 \mu\text{g ml}^{-1} \text{hG}17-1$ for 24 h partially reversed the inhibition of proliferation observed on culture with a 1/40 dilution of this reagent (from 45.5% inhibition to 23.0% inhibition with the anti-gastrin Ig alone or with gastrin pre-incubation, respectively). No growth effects of the same dilutions of the control Ig preparation were apparent.

![Figure 1](image1.png)

**Figure 1** Effect of various concentrations of gastrin on the proliferation of synchronised AR4-2J cells. Experiments were carried out as detailed in the Methods with proliferation assessed by the MTT assay. Each point represents the mean of 12 determinations from two separate experiments; error bars represent the standard error of the mean (s.e.m.). Similar results were obtained in a further three experiments with the MTT assay and a single experiment with direct cell counting over a limited range of gastrin concentrations. Proliferation is normalised to control wells which received fresh serum-free medium containing no exogenous gastrin. *P < 0.05 compared with control.

![Figure 2](image2.png)

**Figure 2** Effect of a 4 day incubation of AR4-2J cells with varying concentrations of the gastrin/CCK receptor antagonists proglumide, benzotript, loxiglumide, lorglumide, L-365,260 and devazepide. Experiments were carried out as detailed in the Methods with proliferation assessed by the MTT assay. Each point represents the mean of at least 30 determinations from three separate experiments; error bars represent s.e.m. Proliferation is normalised to control wells which received fresh medium containing the same amount of DMSO used in test wells. 

![Table 1](image3.csv)

| Antagonist  | IC$_{50}$ (M) | IC$_{90}$ (M) | HT-29 growth inhibition |
|------------|---------------|---------------|-------------------------|
| Proglumide | $3.5 \times 10^{-5}$ | $1.3 \times 10^{-3}$ | $1.4 \times 10^{-2}$ |
| Benzotript | $1.8 \times 10^{-3}$ | $2.6 \times 10^{-4}$ | $3.4 \times 10^{-3}$ |
| Loxiglumide | $1.1 \times 10^{-4}$ | $3.0 \times 10^{-5}$ | $4.7 \times 10^{-4}$ |
| Lorglumide | $6.7 \times 10^{-5}$ | $1.5 \times 10^{-5}$ | $1.5 \times 10^{-4}$ |
| L-365,260 | $4.6 \times 10^{-5}$ | $1.0 \times 10^{-8}$ | $2.2 \times 10^{-5}$ |
| Devazepide | $1.7 \times 10^{-3}$ | $1.2 \times 10^{-7}$ | $> 10^{-4}$ |

IC$_{50}$ values for growth inhibition were calculated from three experiments using the MTT assay (Figure 2) and one experiment using direct cell counts. IC$_{90}$ values for growth inhibition of HT-29 were calculated for three experiments using the MTT assay.
Endogenous gastrin in AR4-2J cells

Lysates of AR4-2J cells cultured in serum-supplemented medium contained gastrin-like immunoreactivity which diluted in parallel with standard gastrin. From a total of 24 determinations on three separate occasions, a mean value of 4.5 pg equivalent to hG17-1 per 10^6 AR4-2J cells was estimated. Immunoreactive gastrin in the medium collected from up to 4 x 10^6 AR4-2J cells cultured over a 48 h period in RPMI containing 10% FCS was at the limit of detection of the assay. AR4-2J cells cultured for 48 h in serum-free RPMI also contained gastrin-like immunoreactivity which diluted in parallel with standard gastrin (Figure 5), giving a mean value of 3.5 pg equivalent to hG17-1 per 10^6 cells, which was not significantly different to the value obtained from cells grown in serum-supplemented medium. Gastrin-like immunoreactivity was also observed in the media collected from cells cultured under serum-free conditions (Figure 5). Over a 48 h period, AR4-2J cells secreted 34 ng l^-1 10^-8 cells.

Gastrin receptor analyses

125I-hG17-1 binding to AR4-2J cells was displaced by unlabelled hG17-1 with an IC₅₀ of 1.9 x 10^-9 M. From Scatchard analyses of four separate experiments, the apparent dissociation constant for gastrin was 4.6 ± 0.8 x 10^-9 M, with a maximum binding capacity of 89.3 ± 58.3 fmol 10^-6 cells.

Figure 6 illustrates the displacement of 125I-hG17-1 binding to AR4-2J cells by proglumide, benzotript, loxiglumide, lorglumide, L-365,260 and devazepide. The IC₅₀ values for each antagonist are given in Table I. The two benzodiazepine derivatives (devazepide and L-365,260) were the most potent in displacing 125I-hG17-1 binding, with the CCK-B antagonist L-365,260 the most potent. Lorglumide and loxiglumide showed similar potencies in displacing gastrin binding. Proglumide and benzotript were the least potent antagonists tested with IC₅₀ values of 1.3 mM and 0.26 mM, respectively. Consideration of Table I reveals that the concentrations of proglumide, benzotript, loxiglumide and lorglumide required to half maximally inhibit AR4-2J cell growth were within an order of magnitude of the half-maximal concentrations required to displace 125I-hG17-1 binding and there was a good correlation between these two variables (Figure 7). In contrast, much higher concentrations of L-365,260 and devazepide were required for inhibition of AR4-2J cell growth than those required to displace gastrin binding (Figure 7).

Discussion

Proliferation of AR4-2J rat pancreatic tumour cells in vitro was stimulated by exogenous gastrin, inhibited by anti-gastrin antibodies and by gastrin receptor antagonists. In addition, AR4-2J cells contain and secrete immunoreactive gastrin. These results indicate an autocrine role for gastrin-like peptides in the regulation of growth in this cell line. The lack of correlation of inhibition of AR4-2J proliferation and displacement of gastrin binding observed with devazepide and L-365,260 suggest that the growth inhibitory actions of these two compounds is unlikely to be mediated via gastrin/CCK receptors.

A direct and significant trophic effect of exogenous gastrin was found on AR4-2J cells growth arrested with 1.0 mM...
However, the MTT assay has been shown to provide a reproducible index of cell proliferation in comparison with assays of cell protein in over 30 human and other tumour cell lines (Alley et al., 1988; Rubinstein et al., 1990), and in our study we confirmed the MTT results with direct cell counts. Gastrin at $10^{-7} \text{m}$ increased the proliferation of AR4-2J cells and at this low concentration would not be expected to interact with CCK-A receptors, as these require gastrin to be present in the micromolar range. This conclusion is supported by the rank order of potency of the antagonists for displacement of $^{125}\text{I}-\text{hG17-1}$ binding which is consistent with interaction at gastrin/CCK-B receptors. Previous studies have provided further evidence for growth stimulation coupled to gastrin/CCK-B, and not CCK-A receptors in AR4-2J cells (Scemama et al., 1989). The response in pancreatic AR4-2J tumour cells contrasts with that in the normal mouse pancreatic acinar cells in which the growth response to gastrin is reported to be mediated via the CCK-A receptor (Logsdon, 1987). Human pancreatic cancer cell growth is stimulated by CCK (Smith et al., 1990b; 1991); the receptors mediating this response require evaluating. The sequence of events linking occupation of gastrin/CCK-B receptors to enhanced cell division has yet to be elucidated, but early events may include the activation of Na$^+$/H$^+$ exchange (Bastie et al., 1988) and ornithine decarboxylase (Scemama et al., 1989).

The inhibition of AR4-2J proliferation by the anti-gastrin Ig preparation is evidence for an autocrine role for gastrin-like peptides in the growth regulation of these cells. Similarly, Hoosein and co-workers (1988, 1990) demonstrated that a gastrin antiserum, as well as gastrin/CCK receptor antagonists, inhibited proliferation of six human colonic cancer cell lines and suggested that gastrin-like peptides may function as autocrine growth factors in these cells. In the present study, the reduction in cell growth in the presence of the anti-gastrin reagent was very dramatic with a maximal mean reduction in proliferation of 52%. This would imply that gastrin-like peptides are very important autocrine growth factors for these cells and would explain the relatively small growth response to exogenous gastrin. An autocrine regulatory role for gastrin is supported by the presence of gastrin-like immunoreactivity in AR4-2J cells. The amount of gastrin-like immunoreactivity secreted by the cells was towards the detection limit of the assay, but within the range of concentrations of exogenous gastrin required to stimulate proliferation. Gastrin-like immunoreactivity has recently been reported in several human gastric and colonic tumour cell lines and this is secreted into the medium (Hoosein et al., 1990; Watson et al., 1991). That this reflects endogenous gastrin-like peptides has been confirmed by detection and sequencing of gastrin mRNA in these cells (Hoosein et al., 1990; Baldwin et al., 1990). The nature of the gastrin-like immunoreactivity in AR4-2J cells has yet to be elucidated.

All of the gastrin/CCK receptor antagonists tested inhibited AR4-2J proliferation over a 4 day period. It has been suggested by some investigators that proglumide-inhibition of AR4-2J proliferation may be a result of non-specific effects rather than its ability to interact with gastrin receptors (Indahl et al., 1989; Singh et al., 1987; Guo et al., 1990). In previous studies, ornithine decarboxylase activity, or $^{3}H$-thymidine or $^{35}S$-selenomethionine incorporation were used as indices of responsiveness of AR4-2J cells to gastrin (Scemama et al., 1989; Seva et al., 1990; Watson et al., 1991a). Interpretation of data obtained by the latter approach is frustrated by the fact that gastrin increases secretion, and presumably synthesis, of proteins such as the enzyme amylase from AR4-2J cells (Lambert et al., 1991). We assessed cell numbers using the MTT assay which relies on the ability of viable cells to reduce tetrazolium to formazan. It is recognised that this assay might be influenced by a variety of factors independent of cell numbers, including chemical interference with the tetrazolium or cellular enzymes, or cellular metabolic conditions (Scudiero et al., 1988).
again consistent with involvement, at least in part, of gastrin receptors in the mechanism of action of these antagonists. In contrast, the two benzodiazepine derivatives (L-365,260 and devazepide) did not appear to inhibit AR4-2J cell growth by interaction with gastrin/CCK-B receptors. Much greater concentrations (several orders of magnitude) of these compounds were required to inhibit AR4-2J cell proliferation as compared with their ability to compete with [125]H-gastrin binding. Inhibition of AR4-2J cell growth by these two agents was not reversible with exogenous gastrin. These results with L-365,260 and devazepide are similar to those recently reported by Thumwood et al. (1991) who found that devazepide in high concentrations inhibited the growth of a variety of cell lines but that this inhibition could not be reversed by addition of gastrin or CCK. These workers failed to observed inhibition of cell growth with the specific gastrin CCK-B receptor antagonist L-365,260, probably a reflection of the concentrations investigated, which were lower than in the present study. Devazepide and L-365,260 have gained in popularity as tools to define gastrin/CCK receptor sub-types since their introduction as specific CCK-A and CCK-B receptor antagonists. The results from the present study would urge caution in their unqualified use for such studies.

To substantiate the results obtained in this study, we chose to test the present study with L365,260 and devazepide would be the existence of two gastrin/CCK-B receptor sub-types on AR4-2J cells; one major sub-type which recognises all six of the antagonists with varying affinity but which is not linked to cell growth processes and a second minor receptor sub-type with low affinity for L-365,260 and devazepide, but similar affinity to the first receptor sub-type for the other antagonists and which is responsible for the growth effects of gastrin on AR4-2J cells. In this hypothesis, it would be postulated that L-365,260 and devazepide decrease AR4-2J proliferation by a receptor-independent mechanism, consistent with the failure of exogenous gastrin to reverse the inhibition. At this time, the existence of such receptor sub-types on AR4-2J cells is speculation. The existence of distinct gastrin and CCK-B, as well as CCK-A, receptors has been argued by others on the basis of agonist studies (Jensen et al., 1989), although previous studies with the benzodiazepine-derived antagonists have suggested that gastrin and CCK-B receptors are equivalent (Freidinger et al., 1989). Functional studies using L-365,260 report it to have a lower affinity than predicted from binding studies in gastric mucosa; estimates of binding affinity may reflect interaction with CCK-B receptors, while the biological response may be a result of interaction with gastrin receptors (Patel & Spraggs, 1992). Three types of gastrin/CCK receptors have been described on guinea-pig smooth muscle cells from protection studies (Grider & Maklouf, 1990). To extrapolate to the present results, on the basis of the studies with antagonists, gastrin receptor binding displacement may be an indication of CCK-B receptor affinity while inhibition of cell proliferation may be a consequence of gastrin receptor antagonism. However, the rank order for displacement of gastrin binding by agonists in AR4-2J cells suggested that gastrin binding is mainly a reflection of gastrin, rather than CCK-B, receptors (Scemama et al., 1989). Obviously, further investigation is required to clarify which receptor sub-types reflect which functions.

In conclusion, we have shown a direct trophic effect of exogenous gastrin on AR4-2J rat pancreatic tumour cells. Evidence was also found for gastrin acting as an autocrine growth regulatory factor in these cells; significant concentrations of gastrin-like immunoreactivity were demonstrated in extracts of AR4-2J cells and their proliferation was inhibited by gastrin/CCK receptor antagonists and by an anti-gastrin Ig preparation. From this study it would appear that gastrin receptor antagonists, or compounds which interfere with gastrin release by tumour cells, warrant further investigation as possible anti-tumour agents for cancers which express gastrin receptors. In this respect gastrin, rather than CCK-B, receptor antagonists would appear to be more promising.

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