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Inhibitory effects of the gastrin receptor antagonist CR2093 on basal, gastrin-stimulated and growth factor-stimulated growth of the rat pancreatic cell line AR42J

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AR42J, a rat pancreatic cell line expressing receptors for both gastrin and epidermal growth factor (EGF), has been used to examine the effect of the gastrin receptor antagonist CR2093 on basal, gastrin-17 (G17), EGF and transforming growth factor (TGF)-α stimulated growth *in vitro*. In serum-free conditions, CR2093 reduced the basal growth of AR42J at a concentration known to displace physiological levels of G17 from gastrin receptors and this effect was reversed by G17 at 1×10^{-9} M. Alone, G17 had little effect on growth, but EGF and TGF-α stimulated growth to 181 and 176% of control values, respectively, and marked synergy was observed when G17 was used in combination with both EGF (212%) and TGF-α (259%). When CR2093 was added, the synergistic effects of the G17/EGF and G17/TGF-α combinations were reduced to basal levels. In addition, CR2093 inhibited the growth stimulation induced by EGF and TGF-α alone. When the ability of CR2093 to bind to EGF receptors was assessed in a ligand binding assay, it was found that the antagonist displaced up to 23% of labeled EGF. Thus CR2093 has potent inhibitory effects on the basal growth of AR42J which can be reversed by G17. It can also inhibit type 1 growth factor-stimulated growth, but although this action may in part be related to the antagonist's ability to inhibit binding to the EGF receptor, other mechanisms may be involved.

Key words: Gastrin, gastrin receptor antagonist, pancreatic cancer, synergy, type I growth factors.

Introduction

It is now well established that a number of gastrointestinal cancer cell lines display a trophic response to gastrin,[1] and that a proportion of naturally occurring human colorectal and gastric cancers are likewise sensitive to this hormone.[2] It is also known that the mitogenic effect of gastrin is mediated via cell membrane receptors,[3] and that gastrin-stimulated growth can be abrogated by gastrin receptor antagonists both *in vitro*[3,4] and *in vivo*.[5,6]

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However, gastrin is only one component of a complex array of hormones and growth factors which can exert a trophic effect on gastrointestinal tumors. The type 1 growth factors, epidermal growth factor (EGF) and transforming growth factor (TGF)-α have been shown to stimulate the growth of certain gastrointestinal tumors,[7,8] and the cell-surface receptor for these factors (the EGF receptor) has been demonstrated on human colorectal and gastric carcinomas.[9,10] In a recent study of a human gastric carcinoma cell line, the combination of TGF-α and gastrin was shown to have a synergistic effect on growth,[11] suggesting that type 1 growth factors and gastrin may interact.

One explanation for such synergy involves the intracellular events consequent on exposure to growth factors and gastrin. The type 1 growth factor receptor consists in part of a tyrosine kinase enzyme which is thought to become phosphorylated when the receptor is activated and to phosphorylate tyrosine-containing proteins which then act as growth signals.[12] There is also evidence that gastrin is a substrate for tyrosine kinase[13] and it is therefore possible that activation of the type 1 growth factor receptor is important for the trophic effect of gastrin on tumor cells.

Another factor to be taken into account is the ability of tumors to produce hormones and growth factors. Human colorectal cancer has been shown to produce a gastrin-like immunoreactive substance[14] and to express the gastrin gene.[15] In addition, mRNA for both EGF and TGF-α has been detected in individual human colorectal cancers, and production of TGF-α protein by human colorectal cell lines has been demonstrated.[16] Given also that EGF and TGF-α may stimulate the transcription of gastrin,[17,18] the potential for complex interactions between autocrine and paracrine loops is considerable.

Whatever the precise mechanisms, it is likely that attempts at therapy using anti-gastrin agents will

have to take growth factor effects into account. For this reason, we have carried out a study to investigate the efficacy of a gastrin receptor antagonist, CR2093, on the basal, gastrin-stimulated and growth factor-stimulated growth of AR42J—a pancreatic cancer cell line which is known to express receptors for both gastrin and type 1 growth factors.[19,20]

Materials and methods

Growth modulating agents

EGF and TGF-α were all obtained from Sigma (Poole, Dorset, UK). The growth factors were initially prepared in sterile distilled water at concentrations between 100 and 10 μg/ml and were stored in aliquots at −20°C.

Human gastrin-17 (G17) was obtained from Sigma and prepared in sterile distilled water at 1 mg/ml and stored at −20°C. 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.

Cells

AR42J is a rat ductal pancreatic cell line originally described by Jessop and Hay.[21] The cell line grows as an adherent monolayer in RPMI 1640 culture medium (Gibco, Irvine, UK) supplemented with 5 mM glutamine (Gibco) and 10% heat inactivated fetal calf serum (FCS, Sigma). The cells were incubated at 37°C in humidified conditions with 5% CO_2 and re-fed three times weekly.

Proliferation assays

To determine the effect of the growth modulating agents on cell growth, AR42J cells were seeded at a cell concentration of 2×10^6 into 75 cm^3 tissue culture grade flasks (Flow Laboratories, Irvine, UK) in RPMI medium containing 10% FCS. After 72 h, at which stage the cells had reached a confluence level of 75%, they were harvested with 0.025% EDTA (Sigma) and washed with serum free medium [RPMI 1640 medium in a 1:1 combination with Hams F12 medium (Gibco) containing 0.5% bovine serum albumin (BSA, Sigma)]. The cells were then seeded into the inner 60 wells of a 96-well flat-bottomed tissue culture grade microtitre plate (Flow Laboratories) at a concentration of 2×10^4 cells per well in 100 μl volume of serum-free medium. The outer wells were filled with medium alone to prevent artifacts occurring during the assay due to drying effects.

The growth factors were diluted in serum-free medium and added to the wells to give a final concentration of 1.5×10^{-9} M for EGF and TGF-α and 5×10^{-10} M for G17. CR2093 was also diluted in the serum-free medium to give a final concentration in the wells of between 5×10^{-4} and 1×10^{-7} M. Five replicate determinations were performed per growth factor dilution.

After 72 h incubation at 37°C, methyl thiazol tetrazolium (MTT, Sigma) was added to the wells in a 50 μl volume at a concentration of 1 mg/ml. After 4 h incubation, the insoluble formazan crystals were solubilized by the addition of 75 μl dimethyl sulfoxide (DMSO, tissue culture grade; Sigma) and the absorbance measured at 550 nm.

Ligand binding studies

[^{125}I]EGF was obtained from Amersham (Amersham, Buckinghamshire, UK) and had a specific activity of 170 μCi/μg and [^{125}I]G17 was obtained from NEN-Dupont (Stevenage, Hertfordshire, UK) and had a specific activity of 2200 μCi/μg. AR42J cells were grown in RPMI 1640 plus 10% FCS, harvested by 0.025% EDTA, aliquoted into Eppendoff tubes in 100 μl containing 2×10^5 cells and washed in the serum-free medium previously described by centrifugation.

[^{125}I]EGF at a concentration of 1×10^{-9} M was added to the cells in a 30 μl volume in serum-free medium either alone or with 1×10^{-7} M EGF or with CR2093 at concentrations from 5×10^{-4} to 1×10^{-8} M. The cells were then incubated for 1 h at room temperature, washed by centrifugation and associated radioactivity measured by a Gamma counter (counting efficiency 70–80%).

[^{125}I]G17 at a concentration of 5×10^{-10} M was added to the cells in a 30 μl volume in serum-free medium either alone, with 1×10^{-7} M unlabeled G17 or with CR2093 at the concentrations described above. The cells were incubated for 1 h at room temperature, washed by centrifugation and associated radioactivity assessed as for the [^{125}I]EGF studies.

Statistics

The *in vitro* studies were analysed by a one-way analysis of variance and a Tukeys test of multiple comparisons by the SPSS programme for the IBM PC.

Results

In vitro growth response to the growth factors

The *in vitro* growth of AR42J cells was assessed by MTT uptake. This method of assessment of cell proliferation showed a good correlation with direct cell counts of AR42J as shown in Figure 1 (regression coefficient, $r = 0.89$, $p < 0.01$).

The *in vitro* growth response by AR42J to optimal G17, TGF-α and EGF in three separate experiments is shown in Table 1. In serum-free medium, G17 had a minimal effect on the growth of AR42J cells whereas TGF-α and EGF (used at optimal concentrations as assessed in previous dose–response studies) significantly increased the MTT uptake of the cells.

In vitro growth response to G17/growth factor combinations

Figure 2(A and B) shows the MTT uptake of AR42J cells in response to G17, EGF and TGF-α and the uptake when G17 is combined with both EGF and TGF-α from a typical experiment which was repeated twice. G17 alone increased the MTT uptake by 109% which was not significantly different from the control. EGF increased the MTT uptake by 181% of control. This was increased to 212% of control when EGF was combined with G17 which was significantly increased compared with EGF alone. The increased stimulation appeared to be greater than an additive effect, indicating synergy had occurred (Figure 2A). All significance was calculated by a one-way analysis of variance with a Tukeys test for multiple comparisons and all were significant to at least the 5% level as shown on the legends.

TGF-α increased the MTT uptake by 177% of control and when TGF-α was combined with G17 the uptake increased to 259% of control. Again, this was significantly different from TGF-α alone, indicative of synergy (Figure 2B).

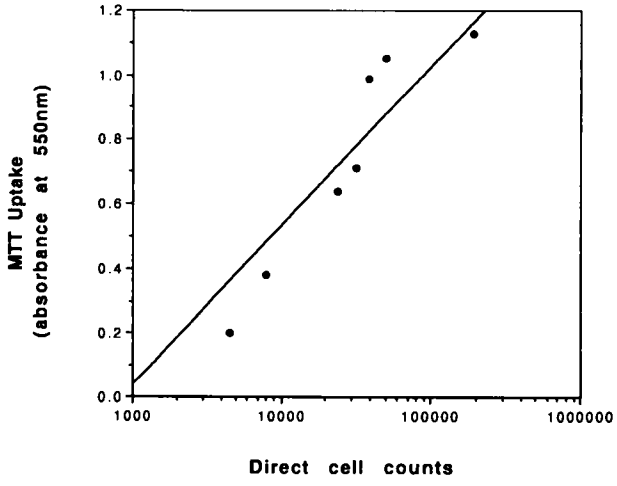

Figure 1. A comparison between MTT uptake and direct cell counts as assessment of the *in vitro* growth of AR42J. Regression coefficient r=0.89, p<0.01.

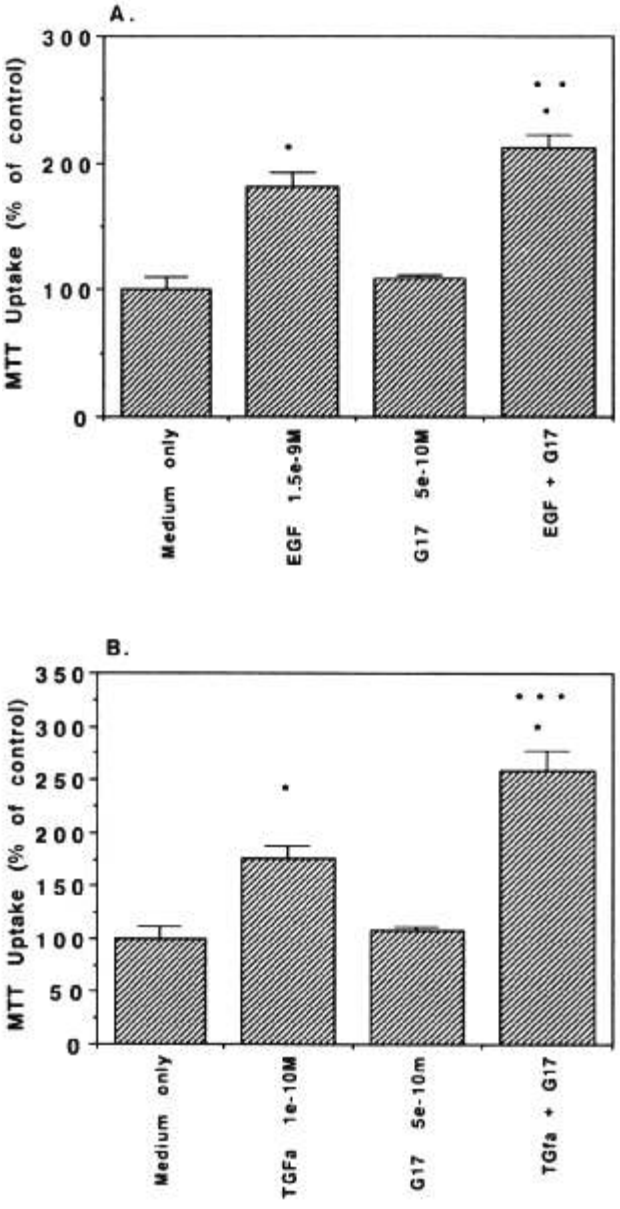

Figure 2. The effect of (A) EGF, G17 and a combination, and (B) TGF-α, G17 and a combination on the *in vitro* growth of AR42J as assessed by the MTT uptake assay. Means ± SD are shown. $^{*}p < 0.05$ from untreated control, $^{**}p < 0.05$ from EGF and G17 only, $^{***}p < 0.05$ from TGF-α and G17 only as assessed by a one-way analysis of variance.

Table 1. Effect of G17, EGF and TGF-α on the *in vitro* growth of AR42J as assessed by MTT uptake

Growth factor	MTT uptake as a % of medium only control mean (± SD)		
	experiment 1	experiment 2	experiment 3
Medium only	100 (8.7)	100 (9.9)	100 (12.2)
G17 5×10^{-5} M	113 (4.8)	112 (1.4)	117 (7.7)
EGF 1.5×10^{-9} M	167 (10.0)	196 (7.3)	180 (11.0)
TGF-α 1.5×10^{-9} M	201 (15.0)	177 (7.0)	163 (13.0)

Effect of CR2093 on the basal growth of AR42J and on the displacement of G17 from gastrin receptors on the cell line

Figure 3(A) shows the effect of CR2093 on the basal MTT uptake of AR42J cells in serum-free medium (results are from four separate experiments). CR2093 at a concentration of 5×10^{-4} M reduced the basal growth of AR42J to 53% of the medium only control. This inhibitory effect titrated out as the CR2093 was further diluted. Microscopical examination of the cells revealed no direct cytotoxic effect. This was confirmed by Trypan blue exclusion of the tumor cells following treatment with CR2093, as greater than 85% of the cells remained viable.

Figure 3(B) shows the ability of CR2093 to displace [^{125}I]G17 from the gastrin receptors on AR42J cells. The maximal displacement occurred at concentrations between 5×10^{-4} and 1×10^{-4} M with the displacement reducing greatly with higher concentrations of CR2093 (1×10^{-4} M).

Effect of CR2093 on G17-, EGF- and TGF-α-stimulated growth of AR42J and growth enhanced by G17/EGF and G17/TGF-α combinations

The effect of CR2093 on the MTT uptake induced by EGF and G17/EGF combinations is shown in Figure 4(A and B). CR2093 reduced basal MTT uptake to 65% of control. EGF-stimulated MTT uptake of 181% of control was reduced to 88% of control when co-incubated with 5×10^{-4} M CR2093 (Figure 4A). The MTT uptake stimulated by G17 and EGF combined of 212% of control was reduced to 97% of control when co-incubated with CR2093 (Figure 4B).

The effect of CR2093 on the MTT uptake induced by TGF-α and TGF-α/G17 combinations is shown in Figure 5(A and B). TGF-α-stimulated MTT uptake of 176% of control was reduced to 82% of control by co-incubation with CR2093 (Figure 5A) and the

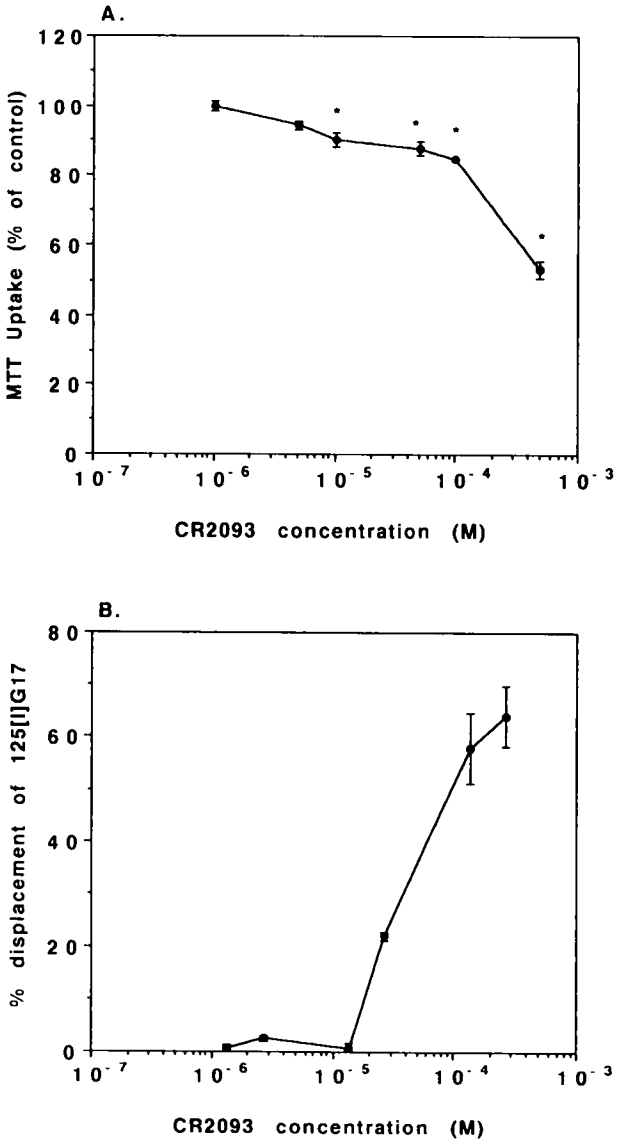

Figure 3. The effect of CR2093 concentrations (A) on the basal growth of AR42J as assessed by MTT uptake, $^*p < 0.05$ from untreated control, and (B) on the displacement of a fixed concentration of [^{125}I]G17 from gastrin receptors on AR42J.

TGF-α/G17-stimulated uptake of 259% of control was reduced to 114% of control (Figure 5B).

Ability of CR2093 to displace EGF from EGF receptors on CR2093 cells

Figure 6 shows the ability of increasing concentrations of CR2093 to displace a fixed concentration (1×10^{-10} M) of [^{125}I]EGF from binding to EGF receptors on AR42J cells. At the highest CR2093 concentration of 5×10^{-4} M, 23% of [^{125}I]EGF was displaced from the EGF receptors.

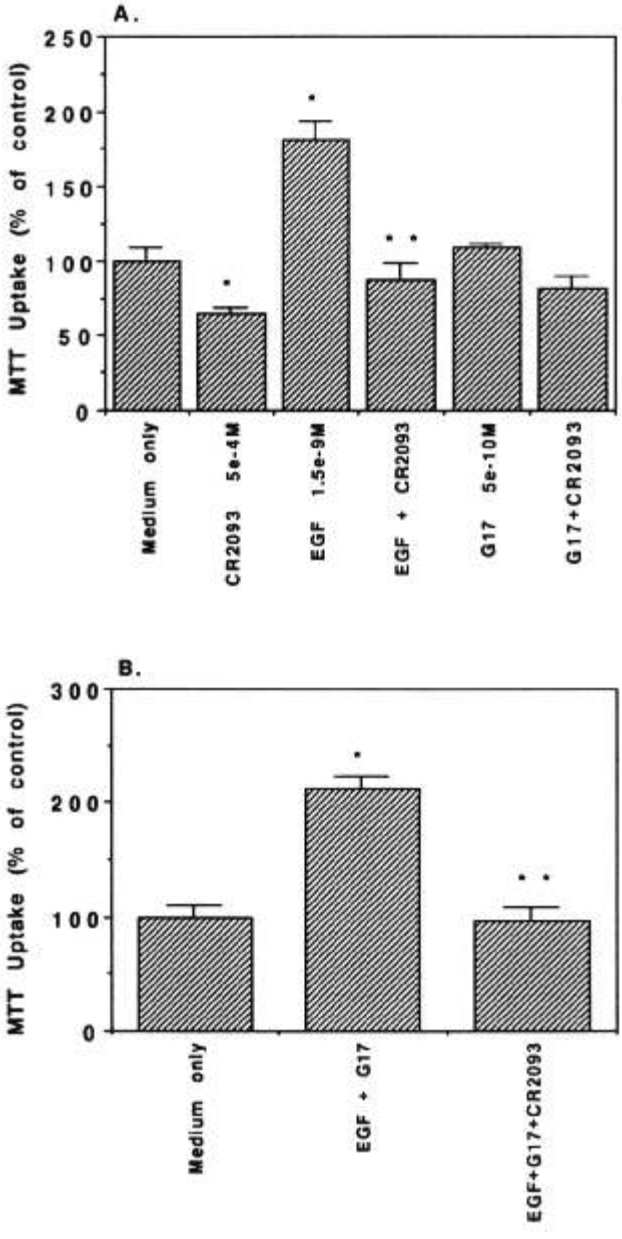

Figure 4. The effect of CR2093 on (A) EGF- and G17-stimulated AR42J cell growth when given alone, $*p < 0.05$ from untreated control, $**p < 0.05$ from EGF alone, and (B) EGF- and G17-stimulated AR42J cell growth when combined as assessed by MTT uptake, $*p < 0.05$ from untreated control, $**p < 0.05$ from EGF and G17.

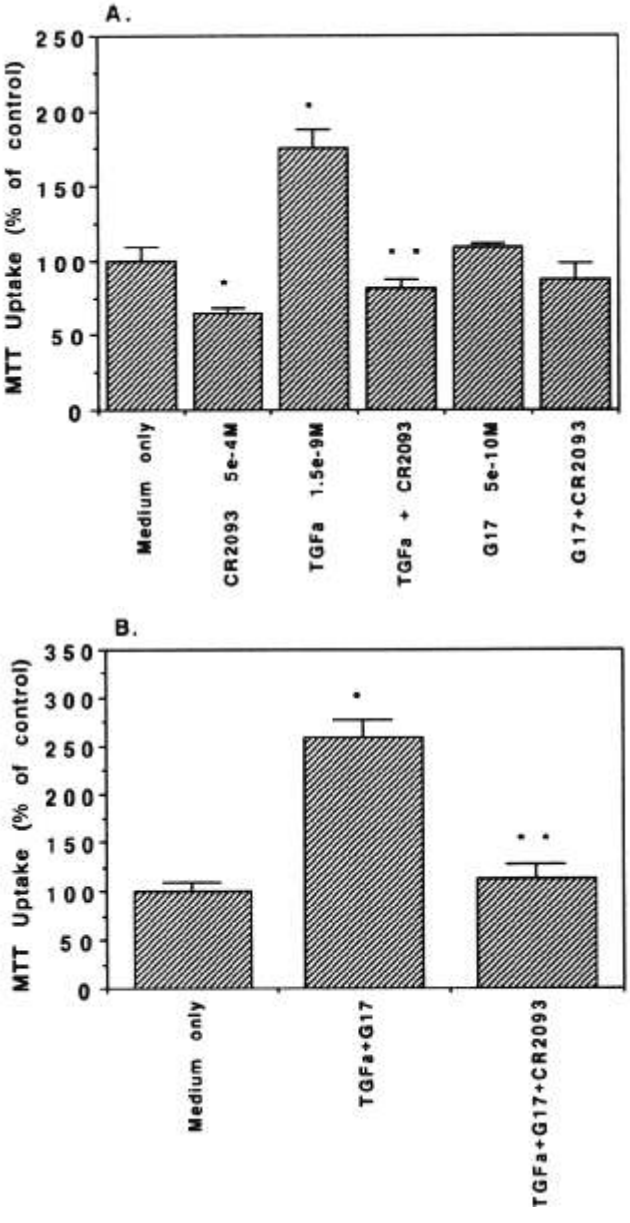

Figure 5. The effect of CR2093 on (A) TGF-α- and G17-stimulated AR42J cell growth when given alone, $*p < 0.05$ from untreated control, $**p < 0.05$ from TGF-α alone, and (B) TGF-α- and G17-stimulated AR42J cell growth when combined as assessed by MTT uptake, $*p < 0.05$ from untreated control, $p < 0.05$ from TGF-α and G17

Discussion

AR42J is a cell line derived from an azaserine-induced rat pancreatic tumor[21] which is known to express receptors for gastrin/CCK-B[22] and type 1 growth factors (EGF receptors).[20] There is also evidence that this cell line may produce gastrin,[23] and it therefore provides a useful tool for the elucidation of the interactions between gastrin and growth factors in promoting tumor cell growth.

Although previous work with AR42J has shown that exogenous gastrin can induce ornthine decarboxylase activity,[24] and stimulate both *in vitro*[23] and *in vivo*[6] cell growth, we were not able to demonstrate a significant growth effect with a wide range of G17 concentrations in serum-free conditions. However, basal *in vitro* growth was inhibited

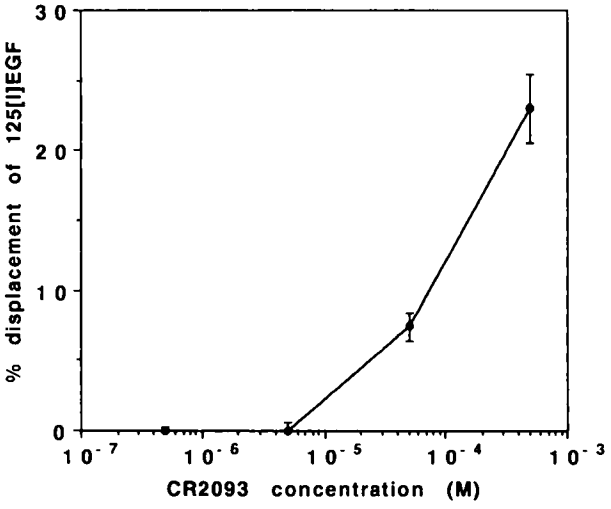

Figure 6. The effect of increasing CR2093 concentrations on the displacement of a fixed concentration of [^{125}I] from EGF receptors on AR42J cells.

by CR2093—a potent gastrin receptor antagonist related to the glutaramic acid derivative proglumide—and this inhibition was almost completely reversed by the addition of G17. This, coupled with the demonstrable ability of CR2093 to displace [^{125}I]G17 from its receptors on AR42J cells, indicates that endogenous gastrin may have an important role in maintaining cell growth. In the present experiments the autocrine pathway may have become more predominant due to growth of the cells in restrictive serum conditions and this effect may have overridden any influence of exogenous hormone. This hypothesis is in keeping with the findings of Blackmore and Hirst,[23] who showed that AR42J cells can secrete gastrin into serum-free medium and that the addition of anti-gastrin antiserum or gastrin receptor antagonist inhibits cell growth.

As might be predicted by the presence of the EGF receptor, both EGF and TGF-α had significant growth effects on AR42J, but the marked synergism exhibited by the G17/EGF and G17/TGF-α combinations is less easy to explain. Previous work with the human gastric carcinoma cell line MKN45 has shown similar synergy with gastrin and TGF-α,[11] and it is possible that type 1 growth factors enhance the effect of gastrin. This may occur by some intracellular interaction involving the phosphorylation of gastrin by the EGF receptor associated tyrosine kinase as suggested by the work of Baldwin.[13] It is possible that as phosphorylated gastrin shows sequence homology with the middle T antigen of the polyoma virus[13] it may, like the polyoma virus, activate the c-*src* oncogene, inducing constitutive kinase activity within the cell, as suggested by Finley *et al.*[15]

Even more intriguing is the observation that the gastrin receptor antagonist CR2093 not only abrogates the synergistic effects of gastrin and growth factors, but also inhibits the growth stimulated by EGF and TGF-α in isolation. It is conceivable that CR2093 acts as an EGF receptor antagonist, but although a weak tendency to displace EGF was demonstrated, this alone is unlikely to account for the observed effect. Another explanation centers on the observed co-operation between G17 and growth factors along with the finding that EGF can stimulate the transcription of the gastrin gene,[17,18] suggesting that a component of EGF-stimulated growth is due to the autocrine-gastrin pathway. Thus it may be that CR2093 inhibits EGF-stimulated growth by blocking the autocrine growth effects induced by endogenous gastrin.

The significance of these observations in human tumors is unclear, but it is known that a proportion of colorectal cancers respond to gastrin[1,25] and express the EGF receptor.[10] In addition, recent work indicates that the majority of human colorectal cancers produce gastrin,[15] and may also produce EGF and TGF-α.[16] The growth promoting effects of gastrin and type 1 growth factors are complex, and seem to interact to a major extent, at least in AR42J cells. It is therefore likely that attempts at anti-gastrin therapy will have to take growth factors into account, and it is encouraging that CR2093 does seem to influence growth stimulation induced by both EGF and TGF-α.

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