Pergamon

European Journal of Cancer, Vol. 35, No. 8, pp. 1286–1291, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00115-X

Original Paper

Antibodies Raised by Gastrimmune Inhibit the Spontaneous Metastasis of a Human Colorectal Tumour, AP5LV

S.A. Watson,[1] D. Michaeli,[3] T.M. Morris,[1] P. Clarke,[1] A. Varro,[4] N. Griffin,[2] A. Smith,[1] T. Justin[1] and J.D. Hardcastle[1]

[1]Cancer Studies Unit, Department of Surgery; [2]Pathology Department, University Hospital, Nottingham, NG7 2UH, U.K.; [3]Aphton Corporation, Woodland, California, U.S.A.; and [4]Department of Physiology, University of Liverpool, U.K.

Both precursor forms of gastrin and mature amidated gastrin peptides can enhance proliferation of colorectal tumours and may regulate growth in an autocrine manner. The purpose of this study was to evaluate the effect of neutralisation of precursor and amidated gastrin on primary and secondary *in vivo* growth of a human colorectal tumour. The human colorectal cell line, AP5LV, when injected into the muscle layer of the abdominal wall of severe combined immunodeficient (SCID) mice, grows as a well-vascularised primary tumour and metastasises to the lung. AP5LV expressed the precursor gastrin forms; progastrin and glycine-extended gastrin and gastrin/CCKB receptors, as assessed by immunocytochemistry. Gastrimmune is a gastrin immunogen in which the amino terminus of the gastrin-17 molecule is linked to *diphtheria* toxoid and induces antibodies which neutralise the amidated and glycine-extended forms of gastrin-17. Rabbit antiserum, raised against Gastrimmune, was administered intravenously into SCID mice bearing AP5LV tumours. Control animals were treated with antiserum raised against *diphtheria* toxoid only. Antibodies raised against Gastrimmune significantly limited the growth of primary AP5LV tumours, as assessed by median cross-sectional area (controls = 244 mm^2; antibody-treated = 179 mm^2; $P = 0.033$). In addition Gastrimmune-induced antiserum limited the growth of lung metastasis as assessed by nodule number (controls = 3.5; antibody-treated = 1.0; $P = 0.0001$) and nodule cross-sectional as assessed by image analysis (controls = 11.9 mm^2; antibody-treated = 3.75 mm^2; $P = 0.0064$). In conclusion *in vivo* neutralisation of gastrin forms, which may potentially be fuelling growth by an autocrine pathway, inhibited both primary growth and, to a greater degree, lung metastasis of a human colorectal tumour cell line. Immunisation against tumour-associated gastrin forms may provide an effective therapy for advanced colorectal cancer.

Key words: colorectal cancer, metastasis, gastrin, Gastrimmune
Eur J Cancer, Vol. 35, No. 8, pp. 1286–1291, 1999

INTRODUCTION

THE POSSIBILITY that colorectal tumours are hormonally dependent has been enhanced by the recent finding that, in addition to the fact that mature carboxy-amidated gastrin increases tumour proliferation [1], post-translational precursor molecules such as progastrin and glycine-extended gastrin may exert additional growth-enhancing effects [2–4]. The role of circulating carboxy-amidated gastrin in the incidence of colorectal cancer remains controversial [5]. This was recently clarified by Penman and colleagues [6] who showed that plasma levels of carboxy-amidated gastrin in patients with colorectal cancer are normal and remain the same following tumour resection. However, Ciccotosto and colleagues [7] showed elevated levels of precursor gastrin forms in the serum of colorectal cancer patients when confounding

Correspondence to S.A. Watson, e-mail: sue.watson@nottingham.ac.uk
Received 4 Dec. 1998; revised 18 Mar. 1999; accepted 25 Mar. 1999.

factors, such as *Helicobacter pylori* infection, were taken into account. Serum levels of gastrin-like peptides have also been implicated in predicting the likelihood of hepatic metastasis from a primary colorectal adenocarcinoma [8].

The latter study was the first to show a link between gastrin peptides and metastatic spread. It is known, however, that gastrin is a growth factor involved in liver regeneration [9]. This fact was recently confirmed when it was shown that there were elevated levels of serum gastrin in patients undergoing partial hepatectomy [10]. Glycine-extended gastrin has been shown to increase the growth of hepatoma cells [11] and thus potentially may also promote the growth of gastrin-sensitive hepatic metastases from a colorectal primary. Gastrin has also been described to influence the growth of small cell lung cancer cells [12], yet the influence of gastrin on pulmonary metastases has not been evaluated.

The aim of this study was to evaluate the effect of neutralising anti-gastrin antibodies on both primary growth and secondary pulmonary spread of the human colorectal tumour cell line, AP5LV.

MATERIALS AND METHODS

Cell lines

AP5LV is a human colorectal tumour originally selected for lung invasion from an intravenous (i.v.) administration route. After selection through the lungs, cells were implanted into the abdominal wall of severe combined immunodeficient (SCID) mice, resulting in a well-vascularised primary tumour and subsequent spontaneous metastasis to the lung [13]. The cell line was maintained *in vitro* in RPMI 1640 growth medium (Gibco Labs, Irvine, U.K.) containing 10% heat-inactivated fetal calf serum (FCS; Sigma, Poole, Dorset, U.K.) and 2 mM L-glutamine (Sigma). The line was routinely passaged with the use of 0.025% EDTA (Sigma) and grown in humidified conditions at 37°C with 5% CO_2.

Immunohistochemical analysis of gastrin species and gastrin/CCKB receptors

Cells were suspended at a concentration of 1×10^6/ml and 200 µl volumes were cytospun onto microscope slides (1200 rpm, 5 min). The cells were fixed with methanol at −20°C (5 min) and permiabilised by treatment with graded alcohols.

Gastrin/CCKB receptors were detected using a rabbit anti-gastrin/CCKB receptor polyclonal antiserum [3]. Human progastrin, glycine-extended and carboxy-amidated gastrin were detected using antisera as previously described [14]. Cells were incubated with a 1:500 dilution overnight at 4°C. Binding was detected using the avidin–biotin technique with immunoperoxidase as the enzyme tracer and diaminobenzidene as the substrate. Tumour sections at the primary site were formalin fixed, embedded in paraffin and 4 µm sections cut. These were stained as described for the cells.

In vivo *growth*

AP5LV cells were injected at a concentration of 5×10^6 cells in 50 µl of sterile 0.8% phosphate buffered saline (PBS, Oxoid, Bucks, U.K.) into the peritoneal wall of severe combined immunodeficient mice (SCID) provided by the Cancer Studies Unit, University of Nottingham. Mice were anaesthetised by a 200 µl subcutaneous injection of Hypnorm (0.315 ng/ml fentanyl nitrate and 10 mg/ml fluanisone; Jannsen, Berrse, Belgium), Hypnovel (5 ng/ml midazolam; Roche, Basel, Switzerland), and sterile distilled water in a 1:1:5 ratio. Mice of both sex were included in groups (each experimental group was composed of $n = 9$ males and 9 females), matched with respect to animal weight, with animals being identified by an electronic tag. Cells were injected randomly between the experimental groups. The clinical condition and weight of the animals were noted once weekly.

Mice were treated with antiserum from rabbits immunised with a gastrin immunogen called Gastrimmune (Aphton

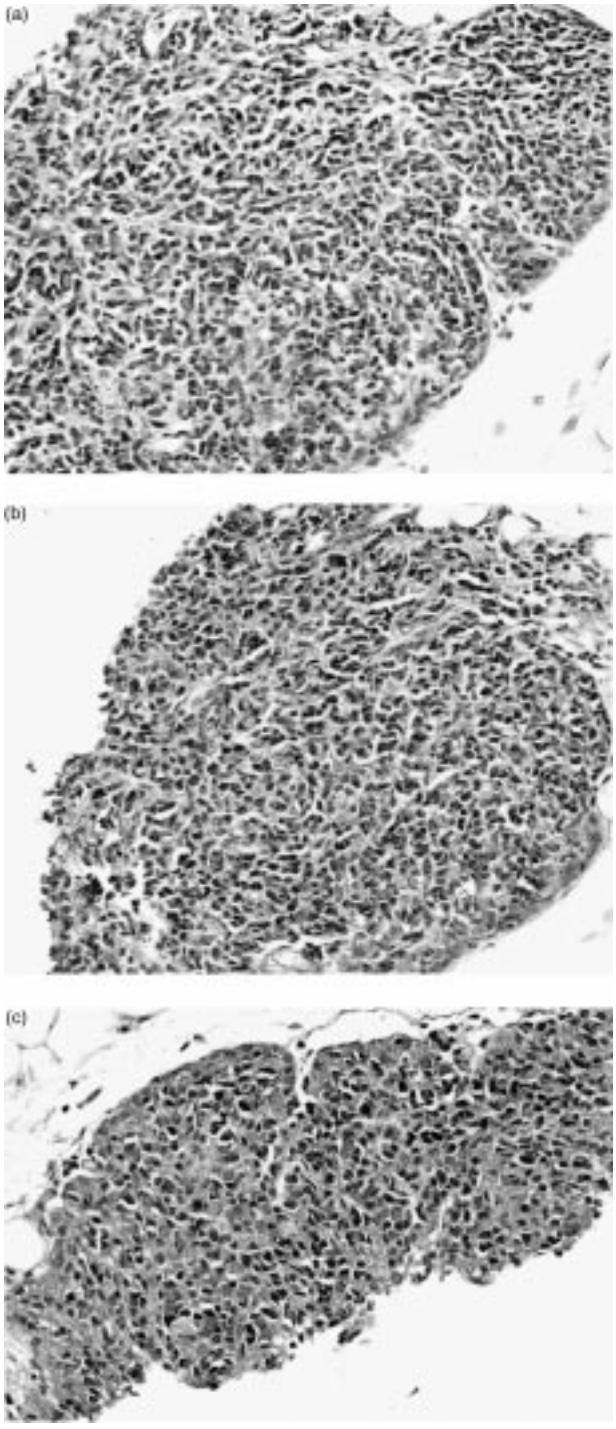

Figure 1. AP5LV lung metastases stained with (a) normal rabbit antiserum, (b) antiserum directed against Glygastrin, (c) antiserum directed against progastrin. Magnification ×250.

Table 1. *Determination of gastrin peptide(s) and gastrin/CCKB receptor expression by AP5LV. Immunochemical detection of gastrin peptides and gastrin/CCKB receptors in AP5LV cells grown* in vitro *(see also Figure* 1*)*

Parameter	Level of expression	% Positive cells
Progastrin	+++	80
Gly-gastrin	++	60
Amidated gastrin	–	–
Gastrin/CCKB receptors	++	60

Corporation, California, U.S.A.). The immunogen is composed of the 9 amino terminal amino acids of human or rat G17 linked to *diphtheria* toxoid (DT) which acts as the carrier [15] and neutralises both the carboxy-amidated and glycine-extended forms of G17 [3]. The antiserum was denoted rabbit anti-G17(1-9):DT. Antiserum against both human Gastrimmune and rat Gastrimmune were combined potentially to neutralise both cell-associated and serum gastrin forms, respectively. Antibodies directed against rat gastrin have previously been shown to cross-react with mouse gastrin as the epitope to which they are raised differs by only one amino acid when comparing rat with mouse. The sera were injected i.v. as previously described which involved a 0.5 ml injection of sera, given daily for 28 days from day 1, resulting in stable serum antigen binding capacity levels of 3.75×10^{-9} M [13]. Control mice received antiserum raised against the DT component only which was denoted rabbit anti-DT.

After 28 days (at a time when nodules are known to develop in the lungs) mice were terminated and macroscopically visible nodules were counted by an independent observer, blind to the treatment groups. In addition, the lungs were formalin fixed, paraffin embedded, and 4 μm sections were cut and stained with haematoxylin and eosin. The area of tumour in cross-sections of the lung was determined using image analysis by two independent observers blind to the treatment groups. The results presented are from a single large study.

Primary tumour growth was monitored by calliper measurement of the two largest perpendicular diameters. In addition the tumours were carefully dissected free of muscle and connective tissue at the end of the experiment and weighed.

All animal experiments were performed according to U.K. co-ordinating committee for Cancer Research (UKCCCR) guidelines.

Statistics

Data were analysed either by a Mann–Whitney or by a one way analysis of variance using the SPSS statistical program for the IBM PC.

RESULTS

Immunocytochemical analysis of AP5LV

The cell line stained positively for progastrin and glycine-extended gastrin but not for carboxy-amidated gastrin. The cell line was also shown to express gastrin/CCKB receptor immunoreactivity (Table 1). When grown *in vivo*, the tumour maintained its expression of gastrin precursors (Figure 1). In addition, carboxy-amidated gastrin was also expressed. Figure 1(b) shows the expression of glycine-extended gastrin,

Table 2. *Effect of Gastrimmune–induced antibodies on the spontaneous lung metastasis of AP5LV*

Treatment group	*n* nodules		Cross-sectional area (mm^2)	
	Male	Female	Male	Female
Rabbit anti-DT serum	6	1	215.4	12.2
	4	2	7.6	28.4
	8	4	26.2	9.8
	4	2	27.8	11.6
	6	3	16.4	31.0
	6	2	109.5	0.3
	3	1	6.7	0.0
	4	0	10.1	0.4
	6	1	14.2	
Median IQR	6.0 4.0–6.0	2.0 1.0–2.0	15.4 11.1–89.1	6.4 0.3–24.4
Median (sexes combined) IQR	3.5 1.5–6.0		11.9 7.2–28.1	
Rabbit anti-G17(1-9):DT serum	2	0	3.0	0
	5	0	4.6	0
	2	0	13.1	0
	2	1	3.7	2.4
	1	0	3.8	0
	1	1	4.1	3.6
	3	1	11.1	4.8
	3	1	28.3	2.0
	3	1	6.0	17.0
Median IQR	2.4 1.3–3.0	1.0 0.0–1.0	4.1 3.7–12.6	2.0 0.0–4.5
Median (sexes combined) IQR	1.0 0.5–2.5		3.75 1.5–8.6	

IQR, interquartile range.

and Figure 1(c), progastrin in an AP5LV lung metastasis. There was mainly cytoplasmic immunoreactivity, which in the case of progastrin was expressed in the majority of cells.

Effect of antisera raised against Gastrimmune on the in vivo *primary growth and lung colonisation of AP5LV*

The effect of treatment with antiserum raised against Gastrimmune was evaluated on growth of AP5LV xenografts at both the primary site (muscle layer of the abdominal wall) and the secondary site (lungs). Treatment with rabbit anti-G17(1-9):DT antiserum resulted in primary tumours with a median cross-sectional area of 179 mm^2 (inter-quartile range 114–227) compared with 244 mm^2 (inter-quartile range 138–325) in the group treated with rabbit anti-DT. The 20% smaller tumour size was significantly different ($P = 0.033$, Mann–Whitney). The median tumour weights were not significantly different (data not shown).

The effect of the antisera treatment on the growth of lung metastases was evaluated both by the number of macroscopically visible nodules and by cross-sectional area of the tumours as evaluated by image analysis. The data for individual mice is shown in Table 2 which includes both nodule number per lung and cross-sectional area. Figure 2(a) shows nodule number in all mice in each treatment group. There was a significant difference between the two groups ($P = 0.0001$) as assessed by a Mann–Whitney with median nodule number in the rabbit anti-DT treated control group being 3.5 mm^2 (interquartile range 1.5–6.0) compared with

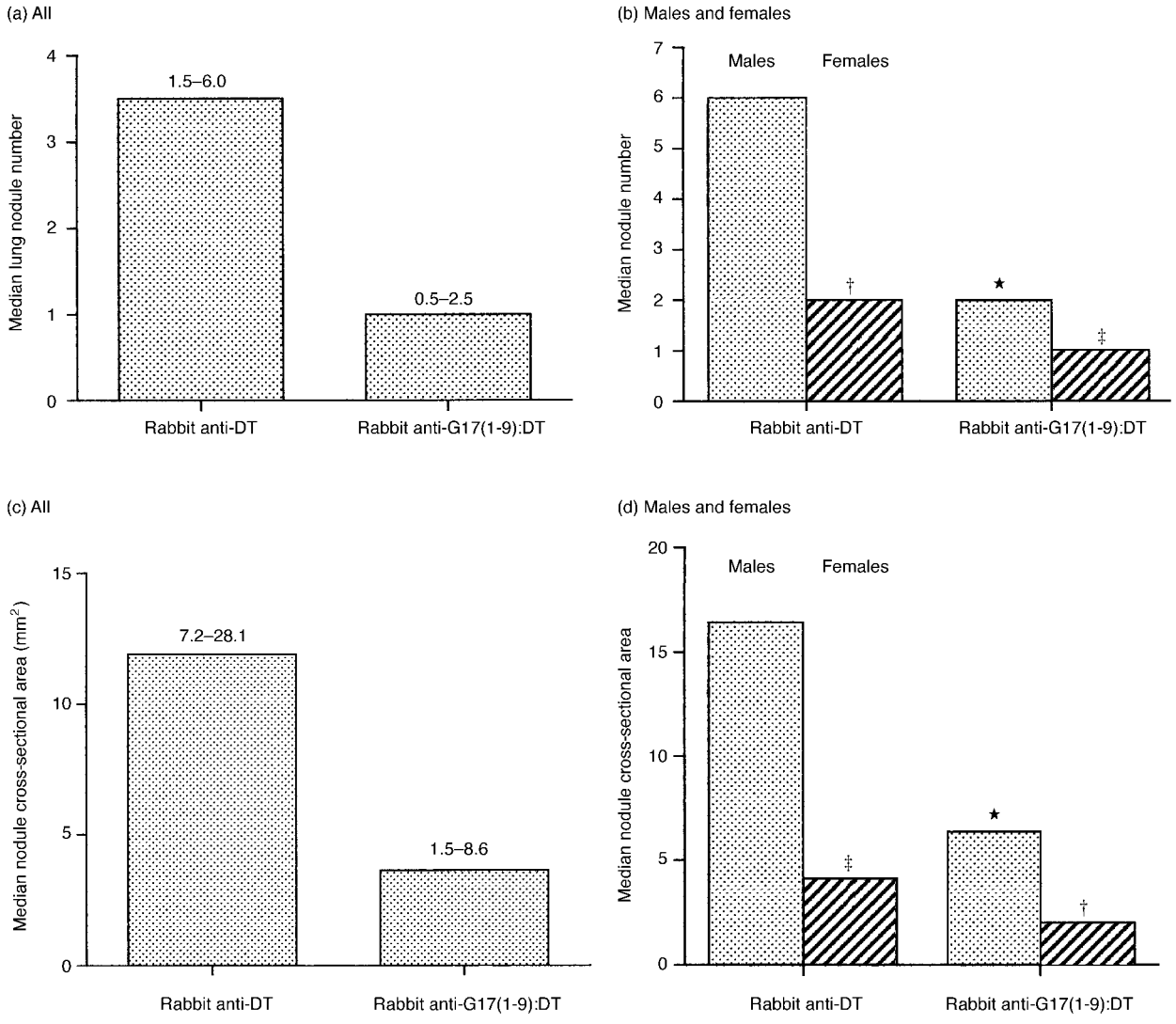

Figure 2. Effect of treatment with antiserum raised against Gastrimmune on the spontaneous lung colonisation of the human colorectal tumour AP5LV when implanted into the muscle layer of the abdominal wall. (a)–(b) Median nodule numbers are shown with the interquartile ranges above the bars. (c)–(d) Median cross-sectional areas of lung nodules are shown with interquartile ranges. (a) Combined sexes ($n = 18$/group) *$P < 0.0001$ when compared with rabbit anti-DT treated group as assessed by a Mann–Whitney non-parametric test. (b) In male and female mice ($n = 18$/group) *$P = 0.0017$ or †$P = 0.0149$ when compared with respective rabbit anti-DT controls. ‡$P = 0.0001$ when compared with mice treated with rabbit anti-DT as assessed by a Mann–Whitney non-parametric test. (c) Combined sexes ($n = 18$/group) *$P = 0.0064$ when compared with rabbit anti-DT group as assessed by a Mann–Whitney non-parametric test. (d) In male and female mice ($n = 18$/group) *$P = 0.0152$ or †NS when compared with the respective rabbit anti-DT control. ‡$P = 0.0002$ when compared with male mice treated with rabbit anti-DT as assessed by a Mann–Whitney non-parametric test.

1.0 (interquartile range 0.5–2.5) in the rabbit anti-G17(1-9):DT treated group, a reduction of 71%. However, there was also a significant difference between rabbit anti-G17(1-9):DT and rabbit anti-DT treated groups when the two sexes were evaluated independently (Figure 2b). The median nodule number was 6.0 in the rabbit anti-DT treated male group compared with 2.0 in the rabbit anti-G17 treated male group ($P = 0.0017$, 67% reduction) and respective medians of 2.0 and 1.0 in the female group ($P = 0.0149$, 50% reduction). Overall the median nodule number was shown to be significantly greater in male mice (median of 6.0) when compared with female mice (median of 2.0) in the rabbit anti-DT treated group ($P = 0.0001$) but not in the rabbit anti-G17(1-9):DT treated group (Figure 2b).

Median lung tumour cross-sectional areas are also shown in Figure 2 for all animals combined; the cross-sectional area was a median of 11.9 mm^2 (interquartile range 7.2–28.1) in the rabbit anti-DT treated group compared with a median of 3.75 mm^2 (interquartile range 1.5–8.6) in the rabbit anti-G17(1-9):DT treated group ($P = 0.0064$, Mann–Whitney, 68.5% reduction). In male mice, the median cross-sectional area was 16.4 mm^2 in controls compared with 4.1 mm^2 in antibody-treated males ($P = 0.0152$, 75% reduction) and, respectively, 6.4 mm^2 and 2.0 mm^2 in female mice ($P = 0.0082$, 68.8% reduction) for rabbit anti-DT and rabbit anti-G17 treatments, respectively. Cross-sectional areas in male and female mice were significantly different in the rabbit anti-DT treated group ($P = 0.002$) but not in the rabbit anti-G17(1-9):DT treated group.

DISCUSSION

Gastrin has been shown to be associated with the primary growth of bronchogenic carcinomas [12]. Thus, there may be a role for gastrin in the lung colonisation of gastrin-sensitive tumours. Due to the high degree of vascularity of the tissue, tumour cells growing in the lung are likely to be exposed to and, therefore, able to respond to serum-associated gastrin and glycine-extended G17, if in possession of the appropriate receptors. Antibodies raised against Gastrimmune, by virtue of binding to the amino terminus of G17, cross-react with both the carboxy-amidated and glycine-extended forms of G17 [3].

In the present study the effect of gastrin neutralisation, induced by administration of antibodies raised against Gastrimmune, on the primary growth and pulmonary metastases of the human colorectal line, AP5LV, were evaluated. The cell line, when grown *in vitro*, was shown to be associated with levels of autonomous precursor gastrin, expressed the CCKB/gastrin receptor and when grown *in vivo* as a subcutaneous graft is known to respond to G17 [13]. When AP5LV was grown as a xenograft *in vivo*, the expression of cell-associated gastrin peptides was maintained, with the majority of cells within the tumour staining positively, indicating that a functional autocrine/paracrine pathway may be operational in addition to endocrine responsiveness to serum-associated gastrin peptides.

Passive infusion of antiserum, raised in rabbits by Gastrimmune immunisation, inhibited growth at both the primary and secondary site. However, the inhibitory effects at the latter site were greater. This may reflect antibody access to the proliferating tumour cells, as the lung tissue surrounding the tumour will be highly vascularised. It may also be related to the size of the tumours; whereas the primary tumour was initiated by a bulk injection of tumour cells which quickly grow into a moderately vascularised tumour with limited antibody access, the secondary tumours are likely to arise from small clumps of cells present at a well-vascularised location amenable to maximum antibody access. The effect on the primary tumour was significant when assessing cross-sectional areas but not on final tumour weights which could reflect differences in tumour composition. It has previously been shown, for instance, that in rat colon tumours treated with Gastrimmune-induced antibodies, increased fibrosis is evident [3].

There was a sex difference in tumour burden between male and female groups, as has previously been documented, with tumour growth highest in male mice compared with female mice. The sensitivity to gastrin neutralisation was still present in both sexes when evaluating tumour nodule number, but an inhibitory effect of Gastrimmune antibodies on cross-sectional area was non-significant in female mice, possibly due to the lower level of tumour growth. A lower sensitivity to gastrin neutralisation in females may be expected due to a lower level of gastrin/CCKB receptors as shown in rats [16, 17] which may relate to lower serum gastrin levels when compared with male rats [16]. The latter data show the drawbacks of using single sex animals in experiments of this kind.

In conclusion, gastrin appears to play an important role in lung colonisation of the human colorectal tumour, AP5LV. Antiserum raised against the Gastrimmune immunogen has a potent inhibitory effect on spontaneous metastasis and thus may be an important anti-metastatic agent. In this context, Gastrimmune immunisation has recently been shown to enhance survival of patients with advanced colorectal cancer [18].

1. Watson SA, Steele RJC. *Gastrin Receptors in Gastrointestinal Tumours*. Austin, Texas, U.S.A., WG Landes Company, 1993.
2. Baldwin GS. Binding of progastrin fragments to the 78 kDa gastrin-binding protein. *FEBS Lett* 1995, **359**, 97–100.
3. Watson SA, Micheali D, Grimes S, *et al*. Gastrimmune raises antibodies that neutralise amidated and glycine-extended gastrin-17 and inhibit the growth of colon cancer. *Cancer Res* 1996, **56**, 880–885.
4. Stepan MS, Yamada T, Dickinson CJ. Glycine-extended gastrin exerts growth promoting effects on colon cancer cells lines. *DDW Abstract* 1996, **962**, A240.
5. Rehfeld JF. Gastrin and colorectal cancer: a never-ending dispute? *Gastroenterology* 1995, **108**, 1307–1310.
6. Penman ID, El-Omar E, Ardill JES, *et al*. Plasma gastrin concentrations are normal in patients with colorectal neoplasia and unaltered following tumour resection. *Gastroenterology* 1994, **106**, 1263–1270.
7. Ciccotosto GD, McLeish A, Hardy KJ, Shulkes A. Expression, processing and secretion of gastrin in patients with colorectal carcinoma. *Gastroenterology* 1995, **109**, 1142–1153.
8. Kameyama M, Fukuda I, Imaoka S, Nakamori S, Iwanaga T. Level of serum gastrin as a predictor of liver metastasis from colorectal cancer. *Dis Colon Rectum* 1993, **36**, 497–500.
9. Rasmussen TN, Jorgensen PE, Almdal T, *et al*. Effect of gastrin on liver regeneration after partial hepatectomy in rats. *Gut* 1990, **31**, 92–95.
10. Caplin M, Millson C, Michaeli D, *et al*. Serum gastrin levels and identification of CCK-B/gastrin receptor following partial hepatectomy for liver tumours in man. *Gastroenterology* 1996, **110**, A1162.
11. Koh TJ, Nicholls PJ, Wang TC. Glycine-extended gastrin promotes growth of a human hepatoma cell line. *Gastroenterology* 1996, **110**, A1239.
12. Sethi R, Rozengurt E. Gastrin stimulates Ca2+ mobilization and clonal growth in small cell lung cancer cells. *Cancer Res* 1992, **52**, 6031–6035.

13. Watson SA, Michaeli D, Grimes S, *et al*. Anti-gastrin antibodies raised by Gastrimmune inhibit growth of the human colorectal tumour AP5. *Int J Cancer* 1995, **61**, 233–240.
14. Nemeth J, Varro A, Bridson J, *et al*. Increased tissue concentrations of the gastrin precursor in patients treated with omeprazole. *Eur J Cancer* 1992, **22A**, 638–644.
15. Makishimi R, Larkin P, Michaeli D, Gaginella TS. Active immunisation against gastrin-17 with an N-terminal derived immunogen inhibits gastric and duodenal lesions in rats. *Gastroenterology* 1994, **106**, A824.
16. Johnson LR, Peitsch W, Takeuchi K. Mucosal gastrin receptor. VIII. Sex related differences in binding. *Am J Physiol* 1982, **243**, G469–G474.
17. Lichtenberger LM, Lechago M, Johnson LR. Depression of antral and serum gastrin concentration by food deprivation in the rat. *Gastroenterology* 1975, **68**, 1473–1479.
18. Smith AM, Justin T, Watson SA, *et al*. Clinical outcome of advanced colorectal cancer patients treated with the anti-gastrin immunogen, Gastrimmune. *Br J Surg*, 1998, **85** (Abstract).

Acknowledgements—The authors would like to acknowledge Mr David Crosbee and Ms Marian Exton for excellent technical assistance and Miss T. Walker for typing the script.