Gastrimmune Raises Antibodies That Neutralize Amidated and Glycine-extended Gastrin-17 and Inhibit the Growth of Colon Cancer

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ABSTRACT

The effect of gastrin neutralization was evaluated on the in vivo growth of the rat colon line, DHDK12, which expressed cholecystokinin B/gastrin receptors and secreted glycine-extended gastrin-17 (G17). Gastrin neutralization was achieved by administration of the immunogen, Gastrimmune, which is composed of the amino terminal portion of G17 linked to a diphtheria toxoid. A rat-specific version of Gastrimmune was used to preimmunize rats, with control animals receiving diphtheria toxoid only. The antibodies raised neutralized both carboxy-amidated and glycine-extended G17.

The tumor was implanted into the muscle layer of the abdominal wall, and rats immunized with Gastrimmune had significantly reduced median cross-sectional tumor areas (70.2% reduction; P = 0.005) and weights (56.5% reduction; P = 0.0078) when compared to control rats. Histological analysis revealed that the tumors had an enhanced degree of necrosis, with the area of viable tumor in the Gastrimmune-immunized rat reduced to 40.3% compared to 58.6% in the control rats (P = 0.003).

Immunization with Gastrimmune raised antibodies that inhibited the growth of a rat colon tumor. This could have been mediated by neutralization of both serum G17 and cell-associated precursor gastrin molecules.

INTRODUCTION

The hormone gastrin is now a well-recognized growth factor for human colorectal adenocarcinomas (reviewed in Ref. 1). The 17-amino acid form, G17,2 appears to be particularly implicated in this effect due to a possible increased affinity for gastrin/cholecystokinin B receptors over other gastrin species (2), and such receptors have been found to be expressed in a high affinity form on 56.7% of human primary colorectal tumors (3). The gastrin gene has been shown to be activated in colorectal tumor cells (4—6), and gastrin-like peptides have been identified previously (6—8), which are now confirmed as precursor gastrin species (9, 10). An autocrine growth loop, possibly involving a distinct receptor is involved in the autocrine growth of gastrointestinal tumors. This appears to be mediated by neutralization of other than the CCKB/gastrin receptor (11).

A number of high affinity CCKB/gastrin receptor antagonists have been described, such as L-365,260 (12) and CI-988 (13), which have been shown to effectively neutralize the effects of exogenous gastrin on gastrointestinal tumor growth both in vitro and in vivo (14, 15). However, the antagonists lack specificity as they block the actions of all the potential ligands of the receptor, such as G34 and CCK. In addition, if a distinct receptor is involved in the autocrine growth cascade, then they may be unable to block this mechanism of tumor growth promotion.

The immunogen, Gastrimmune, which consists of the nine NH2-terminal amino acids of G17 linked to DT, raises antibodies that specifically bind both the amidated and glycine-extended forms of G17. They do not bind to or neutralize the effects of either additional gastrin species or CCK (16). The objective of this study was to evaluate the effect of active immunization with rat Gastrimmune on the in vivo growth of the rat colon cancer line, DHDK12.

MATERIALS AND METHODS

Cell Line. DHDK12 is a rat colonic tumor cell line of epithelial morphology (17).

Gastrimmune. The immunogen is composed of the NH2-terminal 9 amino acids of G17 linked to DT and can be made specific for either human or rat G17. Antiserum raised by Gastrimmune immunization is denoted as anti-G17(1-9):DT and was provided by Aphton Corporation.

RIA of Precursor Gastrin Levels. DHDK12 cells were grown to subconfluence in RPMI 1640 culture medium (GIBCO, Irvine, Scotland, United Kingdom) supplemented with 2 mm glutamine (Sigma Chemical Co., Poole, Dorset, United Kingdom) and 10% heat-inactivated FCS (Sigma). The cells were incubated in humidified conditions at 37°C with 5% CO2. Cells were harvested with 0.025% EDTA (15 min at 37°C) and washed by centrifugation; then 2 X 106 cells were seeded into flasks containing serum-free medium [RPMI 1640 in a 1:1 ratio with Ham’s F12 (GIBCO) with 0.5% BSA]. Cells were harvested with 0.025% EDTA, washed, resuspended in 1 ml of sterile distilled water, and heated in a boiling water bath (18). The levels of glycine-extended gastrin were measured by RIA using antibodies 109—21 and L-2 as described (10).

Assessment of the Effect of Rabbit Anti-ratG17:DT Treatment on the Precursor Gastrin Levels of DHDK12 Cells. Semiconfluent DHDK12 cell monolayers were prepared as described previously in serum-free medium and harvested with 0.025% EDTA. Affinity-purified rabbit anti-G17:DT and rabbit anti-DT were then added to the flasks at equivalent protein concentrations to give an antigen-binding capacity for the former of 3 ng/ml. The cells were incubated for 4 days, after which cell extracts were prepared and assessed for precursor gastrin levels by the RIA described above.

Experimental Animals. Male BDIX rats (The Animal Unit, University of Liverpool) of ages 6—10 weeks weighing 340—430 g were housed in pairs and maintained in a cycle of 12 h light and 12 h dark at 25°C with 50% humidity. The rats were allowed to acclimatize for at least 7 days before use.

Immunization Procedure. Rat G17(1-9) coupled to DT or the DT component alone was dissolved in sterile saline (0.9%), pH 7.3, at 1 mg/ml. The adjuvant nor-muramyl dipeptide (Peninsular Laboratories, Belmont, CA) was added to the conjugate to give a final concentration of 500 µg/ml. The aqueous solution was mixed with oil (Montanide ISA 703; AMS Seppic, Inc., Paris, France) in a 1:2 ratio (v/v) and placed in a glass syringe, which was attached to a second syringe with a three-way stopcock as connector, and the mixture was forced back and forth through the syringes 100 times (the stopcock produced a right angle shear to assist emulsification).

Control animals received an identical emulsion containing the DT peptide only, and all experimental groups were equalized with respect to weight. A 200-µl volume of the emulsion was injected s.c. (right hand flank of the experimental animal). The animals were immunized at 21-day intervals, and the tumor implanted after 5 immunizations.

Initiation of Tumor Growth. DHDK12 cells were suspended in sterile 0.9% saline at a concentration of 2.5 X 106 cells ml−1. Rats were anesthetized by a 1-ml injection of Hypnom (0.315 ng/ml fentanyl citrate and 10 mg/ml fluanisone; Jannsen, Berse, Belgium), Hypnovel (5 mg/ml midazolam; Roche,
Basel, Switzerland), and sterile distilled water in a 1:1.5 ratio. Following a s.c. incision on the right flank, a 200-μl volume of the cell suspension was injected into the muscle layer of the abdominal wall, and the surgical incision was closed with a wound clip. Each experimental group consisted of between 16 and 18 rats. The UK Coordinating Committee for Cancer Research Guidelines were firmly adhered to throughout all animal experimentation.

Antirat G17 Antibody Levels of Rat Gastrimmune-Immunized Rats. To determine the antibody response to the emulsified antirat G17:DT immunogen, rats were bled at various time points, and an ELISA technique was used to determine the antirat G17:DT antibody titers. A rat G17-BSA conjugate (Apthton Corp.) was prepared at a concentration of 2 μg/ml in glycine buffer (0.1 M, pH 9.5), and 25 μl was plated/well into 96-well Immunon U plates (Dynatech Laboratories, Sussex, United Kingdom) and incubated overnight at 4°C. The unabsorbed conjugate was then flicked out, and the wells were washed in buffer that consisted of 0.9% saline (pH 7.3) containing 0.5% Tween 20 (Sigma) and 0.02% NaN3 (Sigma). This buffer was used for both washing and reagent dilutions. The test sera (from animals immunized with the rat gastrin immunogen) were used at a starting dilution of 1:100 and at 10-fold dilutions thereafter. The positive control was rat antirat Gastrimmune antiserum from previously immunized animals, and the negative controls were normal rat serum and sera from rats immunized with DT. These were used at the same dilutions as described for the test sera. The test and control sera were added to the wells in 25-μl volumes, either in the absence or presence of 25 μl/well rat G17-BSA at 100 μg/ml (control wells received 25 μl assay buffer). The plates were then incubated for 60 min at room temperature. The plates were washed with saline buffer; goat antirat immunoglobulin (H+L)-biotin (Zymed, San Francisco, CA) was added to the wells at a 1:500 dilution, 25 μl/well, and after 5 min developing time, the absorbance was read at 405 nm. The difference in absorbance between untreated sera and sera coincubated with rat G17-BSA was calculated as the specific absorbance.

Isotyping the Antibody Response. A pooled aliquot of rat sera was obtained from rats immunized with rat Gastrimmune. This was coated onto 96-well microtiter plates overnight at a 1:1000 dilution [dilution buffer was 0.9% saline containing 0.5% Tween 20 (Sigma) and 0.02% sodium azide], 50 μl/well. The adsorbed antiserum was then incubated with alkaline phosphatase-conjugated antirat IgG isotypes (The Binding Site, Birmingham, United Kingdom), the binding of which was detected with the substrate pNPP (Sigma).

G17 Levels of the Immunized Rats. Rabbit anti-G17 antiserum (COOH-terminal-specific; Dakopatts, Buckinghamshire, United Kingdom) was coated onto 96-well microtiter plates at a protein concentration of 10 ng/well in PBS. A standard curve was constructed by incubating [125I]G17 at a fixed concentration of 10,000 cpm/well with increasing concentrations of G17. The unabsorbed conjugate was then incubated with alkaline phosphatase-conjugated antirat G17 antibodies were prepared in 250-μl aliquots. A 125-μl aliquot of newborn calf serum (Sigma) and 312.5 μl of 25% polyethylene glycol (Sigma) were added to each sera sample. These were vortexed and spun at 1500 rpm for 30 min. The supernatant was removed and boiled (to ensure that no free antibodies remained) and was classified as the free gastrin sample.

To determine the antibody response to the emulsified antirat G17:DT immunogen, rats were bled at various time points, and an ELISA technique was used to determine the antirat G17:DT antibody titers. A rat G17-BSA conjugate (Apthton Corp.) was prepared at a concentration of 2 μg/ml in glycine buffer (0.1 M, pH 9.5), and 25 μl was plated/well into 96-well Immunon U plates (Dynatech Laboratories, Sussex, United Kingdom) and incubated overnight at 4°C. The unabsorbed conjugate was then flicked out, and the wells were washed in buffer that consisted of 0.9% saline (pH 7.3) containing 0.5% Tween 20 (Sigma) and 0.02% NaN3 (Sigma). This buffer was used for both washing and reagent dilutions. The test sera (from animals immunized with the rat gastrin immunogen) were used at a starting dilution of 1:100 and at 10-fold dilutions thereafter. The positive control was rat antirat Gastrimmune antiserum from previously immunized animals, and the negative controls were normal rat serum and sera from rats immunized with DT. These were used at the same dilutions as described for the test sera. The test and control sera were added to the wells in 25-μl volumes, either in the absence or presence of 25 μl/well rat G17-BSA at 100 μg/ml (control wells received 25 μl assay buffer). The plates were then incubated for 60 min at room temperature. The plates were washed with saline buffer; goat antirat immunoglobulin (H+L)-biotin (Zymed, San Francisco, CA) was added to the wells at a 1:500 dilution, 25 μl/well, and after 5 min developing time, the absorbance was read at 405 nm. The difference in absorbance between untreated sera and sera coincubated with rat G17-BSA was calculated as the specific absorbance.

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G17 Concentration (pg/ml)

A

B

G17 Concentration (pg/ml)

G17 assay 1

G17 assay 2

Gly-G17 assay 1

Gly-G17 assay 2

G34 assay 1

Fig. 1. Displacement of [125I]G17 from rabbit antihuman G17(1-9):DT antiserum by immunization with Gastrimmune (NH2-terminal specific; Fig. 1A and Fig. 2) and rabbit anti-G17 (C-terminal specific; Fig. 1B). G17 dilution overnight at 4°C. Binding was detected using the avidin-biotin technique with immunoperoxidase as the enzyme tracer and diaminobenzidine as the substrate.

Immunocytochemical Evaluation of the CCKB/Gastrin Receptor Expression of DHDK12 Cells. DHDK12 cells were suspended at a concentration of 1 × 106 ml−1, and 200-μl volumes were cytopspun onto microscope slides (1200 rpm for 5 min). The cells were fixed with methanol at —20°C (5 min) and permeabilized by treatment with graded alcohols. The cells were incubated with the rabbit anti-CCKB/gastrin receptor antiserum and stained as described previously.

Statistical Analysis. In vitro results were analyzed by a one-way ANOVA, in vivo results by a Mann Whitney non-parametric test, and a linear regression analysis was used for data correlations by use of the SPSS statistical package for the IBM PC.

RESULTS

Gastrin Specificity of Antiserum Raised by Gastrimmune Immunization of Rabbits. Fig. 1 shows the displacement of [125I]G17 from rabbit antihuman G17(1-9):DT antiserum by immunization with Gastrimmune (NH2-terminal specific; Fig. 1A and Fig. 2) and rabbit anti-G17 (C-terminal specific; Fig. 1B). G17
displaced radiolabeled G17 from both antisera preparations with an
PG/ml for the rabbit anti-Gl7 (COOH-terminal). Glycine-extended
reduced weights in rats immunized with Gastrimmune (Fig. 3B; 
P = 0.005, Mann Whitney). DHDK12 tumors also had significantly
reduced by 70.2% when compared to tumors from the DT controls; 
(P = 0.0078).

The mean animal weight in the Gastrimmune-treated rats rose from 
399 to 452 g (13% increase) over the duration of the experiment and 
in the DT-treated animals from 392 to 447 g (13.8% increase), 
indicating that the growth rate of the animals was not affected by 
administration of the Gastrimmune immunogen.

Antibody Levels of Rat Gastrimmune Immunized Rats. The 
free antirat G17(1-9):DT antibody levels (those in excess of the 
bioassays required to bind serum-associated G17) were measured and 
expressed as the specific absorbance obtained at a 1:100 dilution of 
serum (Fig. 4). After five immunizations, at the time of tumor cell 
injection, the mean antibody titer was 0.243 absorbance units (Fig. 4, 
Group 1). The mean antibody titers had increased, by the termination 
of the study, following two additional immunizations, to 0.66 absorb-
ance units (Fig. 4, Group 2) and were in the range of the positive 
control (Fig. 4, Group 3). Antibody titers from animals immunized 
with DT had a mean absorbance of 0.1 units (Fig. 4, Groups 3 and 4), 
and the negative control (normal rat serum) showed no absorbance 
(Fig. 4, Group 6). There was no apparent correlation between tumor 
weight and antibody levels, as measured by a linear regression anal-
ysis (P = 0.14; data not shown). The antibodies raised in the immu-
nized rats were shown to be predominantly of the IgG1, IgG2a, and 
IgG2b isotypes.

Serum Gastrin Levels of the Gastrimmune Immunized Rats. 
The free serum gastrin level (as measured with an antisera directed 
against the carboxyl terminus of G17) of the DT-immunized rats was 
found to be 114.0 pg/ml (SD, 31) compared to 68.5 pg/ml (SD, 20) in 
the Gastrimmune-immunized group. This corresponds to a 40% re-
duction in total gastrin. Total serum gastrin levels were correlated 
with final tumor weight, and the correlation coefficient was found to be 
statistically significant (P = 0.011, linear regression analysis).

The levels of serum gastrin bound to antibodies were found to be 
zero in the DT-immunized rats and ranged from 30.1 to 253.7 pg/ml 
in the Gastrimmune-immunized rats (median, 53.3 pg/ml).

Histological Evaluation of DHDK12 Tumors. Histological eval-
uation revealed that the tumors from Gastrimmune-immunized rats 
had a smaller rim of viable tumor tissue around the leading edge of the 
tumor and a greater degree of central necrosis (Fig. 5A) when com-
pared to tumors from rats immunized with DT (Fig. 5B). This was

Glycine-extended gastrin-17

Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-Gly

Carboxy-amidated gastrin-17

Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH3

Gastrimmune

Glu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-

Peptide spacer  Diphtheria toxoid

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Fig. 2. Diagrammatical representation of glycine-extended G17, carboxy-amidated G17, and the amino terminal portion of G17 present in the Gastrimmune immunogen construct.
Higher magnification microscopy showed that the tumor cells in the DT-immunized rats grew in a regular trabecular manner (Fig. 6A), whereas the tumor cells from Gastrimmune-immunized rats had a disrupted pattern of growth (Fig. 6B). There was also more connective tissue in the tumors from Gastrimmune-treated rats when compared to tumors from DT-treated rats (connective tissue:tumor ratio, 75:25 and 50:50, respectively). Areas of focal necrosis were present within the viable tumor tissue in the Gastrimmune-treated group and there was also an increased inflammatory infiltrate, which appeared to be composed mostly of lymphocytes. The tumors from rats in both treatment groups were stained with anti-gastrin receptor antiserum, and it was shown that the viable cells remaining in both the DT and the Gastrimmune-treated group had retained their gastrin receptor positivity (data not shown).

DISCUSSION

Serum-associated G17 has the potential to stimulate the growth of colorectal tumors in an endocrine manner mediated by CCKB/gastrin receptors (1). A potential autocrine loop may also exist due to endogenous production of precursor gastrin peptides by such tumors (9, 10), as it has been shown recently that the precursor gastrin molecule, glycine-extended gastrin, stimulated the growth of a gastrointestinal tumor cell line. This has been shown to be mediated by a receptor other than the CCKB/gastrin receptor (11).

Gastrimmune is an immunogenic form of G17 and, by virtue of its structure, raises specific anti-G17 antibodies directed against the NH2 terminus of both amidated and glycine-extended G17 and can be tailored to be species specific. In the present study, immunization with rat Gastrimmune reduced the in vivo growth of DHDK12 rat colon tumors, as shown by both cross-sectional area and weight measurements. Extrapolation of quantitative assessment of viable tumor tissue by image analysis indicated that the weight of viable tumor tissue may have been reduced by as much as 68%.

DHDK12 rat colon tumor cells growing in vivo, by virtue of their CCKB/gastrin receptors, may have responded to serum G17. In the present study, excess anti-G17 antibodies (i.e., those not bound to serum G17) were measured during the tumor challenge. The total serum gastrin levels were shown to be reduced by 40%, and a
significant positive correlation was shown between tumor weight and serum gastrin levels at the termination of the therapy. In addition, antibody-bound gastrin was also detected in the Gastrimmune but not the DT-immunized rats. Thus, partial neutralization of serum-associated G17 may have contributed to reduced tumor growth.

DHDK12 cells were also shown to express cell-associated, glycine-extended G17 but not amidated gastrin. In vitro treatment of DHDK12 cells with rabbit anti-rat G17(1-9):DT reduced the levels of cell-associated precursor gastrin when compared to cells treated with rabbit anti-DT control antiserum. Thus, potentially antibodies produced by Gastrimmune immunization may interrupt an autocrine growth loop involving such peptides. This may result in a down-regulation of gastrin translation if the autocrine pathway is ineffective at promoting tumor growth.

The antibody titers raised by Gastrimmune were in excess of those required to neutralize serum G17, resulting in high serum levels of uncomplexed antibody. Thus these “free” serum-associated antibodies would have been available to neutralize cell-associated G17 peptides in well-vascularized areas of the tumors.

Gastrimmune immunization resulted in the potential neutralization of two trophic forms of gastrin, G17 and glycine-extended G17, and thus may induce cytostasis within the tumors. The histological observations provide evidence for the theory that tumors from Gastrimmune-treated rats may have a slower growth rate than tumors from control rats because the growth pattern, the degree of fibrosis, and the area of the viable tumor tissue were significantly reduced in the former rats. Interestingly, the viable tumor cells remaining were shown to maintain their expression of gastrin receptors. This indicates that in this tumor model, the hormone-sensitive phenotype may have been expressed by all of the cell clones, and there was no outgrowth of hormone-insensitive clones, leading to escape from Gastrimmune inhibition.

One additional finding was the focal areas of necrosis within the tumor tissue in Gastrimmune-treated rats and the presence of an inflammatory infiltrate in certain areas of the tumor, mainly composed of lymphocytes. One explanation of such findings is that an antibody-directed, cell cytotoxic response was instigated. Antibodies that mediate such a response were shown to be present when the sera from Gastrimmune-treated rats were isotypet. The mechanism of such a response is unknown, and future work is being aimed at evaluating the possibility that such a response may take place.

In conclusion, antibodies raised by Gastrimmune have significant
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antitrophic effects against a rat colon tumor by two potential mechanisms: (a) neutralization of serum GII; and (b) neutralization of cell-associated precursor gastrin molecules. If these mechanisms prove therapeutically important, the agent may be superior to other antigastrin agents, such as gastrin/CCKB receptor antagonists. The latter will not block the newly discovered receptor type that mediates the proliferative effects of precursor gastrin peptides and are nonselective, blocking the effects of all gastrin species and CCK. Gastrimmune is currently being evaluated clinically in patients with advanced colorectal cancer.

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