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).

Neutralization was achieved by administration of 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

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/CCK 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 molecules (9). The hormone gastrin is now a well-recognized growth factor for human colorectal adenocarcinomas (9, 10). An autocrine growth loop, possibly involving the hormone gastrin, has been described, such as L-365,260 (12) and CI-988 (13), which have been shown to be expressed in a high affinity form on 56.7% of human primary colorectal tumors (3). The 17-amino acid form, G17, appears to be particularly implicated in this effect due to a possible increased affinity for gastrin/CCK 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 molecules (9, 10). An autocrine growth loop, possibly involving gastrin precursors, has been postulated to be involved in the proliferation of gastrointestinal tumors. This appears to be mediated by a receptor 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.

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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 tail bled at various time points, and an ELISA technique was used to determine the antirat G17:DT antibody titers. A rat G17-BSA conjugate (Aphoton 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% Na₂O₃ (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 antisera 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, 50 μl/well, and incubated for 60 min in the dark at room temperature. The plates were washed with saline buffer, and avidin alkaline phosphatase (Zymed) was added to wells at a 1:100 dilution, 50 μl/well, and incubated for 60 min in the dark at room temperature. After washing with saline buffer, pNPP substrate (Sigma) was added to the wells at 50 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 unknown samples containing free gastrin, bound G17, and free and bound anti-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.

The pellet was washed five times in 0.002 M veronal buffer (pH 8.4) containing 0.5% BSA and solubilized by boiling in 250 μl of water. This was classified as containing bound gastrin. Triplicate aliquots of each of the samples were added to the labeled G17, and the level of inhibition was determined. Gastrin levels in the rat sera were then calculated from the standard curve.

Histological Evaluation of the Rat Tumors. At the termination of therapy, the DHDK12 tumors were fixed in 10% formal calcium and embedded in paraffin. Five-μm sections were cut on a cryostat and stained with hematoxylin and eosin; then the pathological parameters of the tumors were assessed independently by a pathologist. Image analysis was performed on the tumor sections using a Seescan image analyzer in a blind manner to assess the area of viable tumor tissue.

Gastrin receptors were detected using a rabbit anti-CKCB/gastrin receptor polyclonal antiserum (Aphoton Corp.). Sections 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 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 cytospun 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-CKCB/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 Antisera Raised by Gastrimmune Immunization of Rats. Fig. 1 shows the displacement of [125I]G17 from rabbit antihuman G17(1-9):DT antiserum raised by immunization with Gastrimmune (NH₂-terminal specific; Fig. 1A and Fig. 2) and rabbit anti-G17 (C-terminal specific; Fig. 1B). G17

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Table 1  
Precursor gastrin levels associated with DHDK12 cells  

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glycine-extended Glu17 conc* (fmol 10^10 cells^-1)</th>
<th>Amidated Glu17 conc* (fmol 10^10 cells^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>31.2</td>
<td>ND</td>
</tr>
<tr>
<td>(1.0 × 10^7 cells/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td>80.0</td>
<td>ND</td>
</tr>
<tr>
<td>(1.27 × 10^7 cells/ml)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* conc, concentration; ND, not detected.

displaced radiolabeled Glu17 from both antisera preparations with an IC50 of 3500 pg/ml for the rabbit antihuman Glu17(1-9):DT and 800 pg/ml for the rabbit anti-Glu17 (COOH-terminal). Glycine-extended Glu17 did not displace radiolabeled Glu17 from the COOH-terminal-specific antiserum but did from the NH2-terminal-specific antiserum (IC25, 12,000 pg/ml). G34 displaced radiolabeled Glu17 from the specific antiserum but did from the NH2-terminal-specific antiserum.

Table 2  
Precursor gastrin levels of DHDK12 cells after in vitro treatment with rabbit anti-Glu17(1-9):DT antiserum  

DHDK12 cells were grown in serum-free medium (RPMI 1640 in a 1:1 ratio with Ham's F12 with 0.5% BSA. Affinity-purified rabbit anti-rat Glu17(1-9):DT and rabbit anti-DT were then added to the flask at a protein concentration of 3 ng/ml and incubated for 4 days. Cell extracts were recovered by centrifugation, and progastrin, glycine-extended gastrin, and amidated Glu17 levels were measured using antibodies 109-21 and L-2, respectively, as described previously (10). Glucine-extended gastrin-17

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<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glycine-extended Glu17 conc* (fmol 10^10 cells^-1)</th>
<th>Amidated Glu17 conc* (fmol 10^10 cells^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-Glu17(1-9):DT antiserum</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rabbit anti-DT antiserum</td>
<td>67.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

* conc, concentration; ND, not detected.

Levels of Gastrin Precursors Associated with DHDK12 Cells. DHDK12 cells were shown to contain glycine-extended gastrin in two separate experiments, as shown in Table 1.

Effect of in Vitro Treatment with Rabbit Antirat Glu17:DT Antiserum on Precursor Gastrin Levels Associated with DHDK12 Cells. The effect of in vitro treatment with affinity-purified rabbit antirat Glu17(1-9):DT and rabbit anti-DT antiserum on the precursor gastrin levels associated with DHDK12 cells in serum-free medium is shown in Table 2. Rabbit anti-rat Glu17:DT antiserum reduced the levels of glycine-extended Glu17 from 67 pg/ml to an undetectable level.

CCKB/Gastrin Receptor Expression of DHDK12 Cells. CCKB/gastrin receptor expression of DHDK12 cells was evaluated with antisera raised against peptide sequences derived from the human CCKB/gastrin receptor. DHDK12 cells showed a strong and specific membrane-associated immunoreactivity, indicative of a high level of gastrin receptor expression. Cells treated with a control rabbit antisera showed no specific immunoreactivity (data not shown).

Effect of Rat Gastrimmune Immunization on the in Vitro Growth of DHDK12 Tumors. Fig. 3 shows the effect of immunization with rat Gastrimmune (5 immunizations prior to injection of cells) on the final cross-sectional areas and weights, respectively, of DHDK12 tumors. The tumors had significantly reduced cross-sectional areas in rats immunized with Gastrimmune (Fig. 3A; median, 399 to 452 g (13%) 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 Glu17(1-9):DT antibody levels (those in excess of the antibodies required to bind serum-associated Glu17) were measured and are 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 absorbance 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 analysis (P = 0.14; data not shown). The antibodies raised in the immunized 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 antiserum directed against the carboxyl terminus of Glu17) 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% reduction 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 evaluation 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 compared 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-NH2

Gastrimmune

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

Peptide spacer  Diphtheria toxoid
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 quantified by image analysis, and the mean percentage of viable cell area in tumors from Gastrimmune-treated rats was 40.3% (SD, 9.1) compared to 58.6% (SD, 10.4) for the DT-immunized rats ($P = 0.003$, Student’s $t$ test).
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 downregulation 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 isotypic. 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 Gll; 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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