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Immunogenicity and safety of a novel tetanus toxoid-conjugated anti-gastrin vaccine in BALB/c mice

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ABSTRACT

The objective of this study is to determine the immunogenicity and safety of our novel anti-gastrin vaccine that is composed of the common amino-terminal portions of human carboxy-amidated gastrin-17 (G17) and glycine-extended gastrin-17 (gly-G17) as well as the common carboxy-terminal portion of the gastrin precursor progastrin (in a 50:50 mixture) all covalently linked to tetanus toxoid (TT) via peptide spacers.

The vaccine, or immunogen, was injected intramuscularly into the legs of BALB/c mice, which produced high serum titres of specific IgG antibodies and IFN-γ in their spleen cells, identifiable by enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot assay (ELISPOT), respectively. TT as the protein carrier effectively enhanced the antigenic epitopes' humoural and cellular immune responses, unlike the antigenic epitopes alone or the immunogen's adjuvant emulsion system (AES), all of which failed to provoke any obvious immune response. Notably, the animals' body weights increased significantly after immunization ($P < .01$), while their haematology and serum biochemistry were all generally normal, and the gross anatomy of their main organs (e.g., heart, liver, spleen, lung, kidney) showed no obvious histopathological changes.

1. Introduction

The gastrins, including amidated gastrin-34, glycine-extended gastrin-34, amidated gastrin-17, glycine-extended gastrin-17 and the precursor progastrin are all known gastrointestinal hormones [1,2], which can promote gastric acid secretion and gastrointestinal mucosa growth by binding to their corresponding receptors [3,4]; however, considerable evidence shows that excessive production of gastrin-17 and progastrin plays a role in the genesis and development of gastrointestinal (GI) tumours (including gastric [5–7], colon [8–10], pancreatic [11,12] and oesophageal cancers [13]) via autocrine, paracrine or endocrine mechanisms. Additionally, for these GI malignancies, existing chemotherapeutics have low specificity, a significant number of toxic side effects and poor therapeutic outcomes (e.g., short survival times, generally poor quality of life, and high mortalities). Thus, there is a great deal of clinical interest in developing novel therapeutic products for these GI cancers that would have good specificity, less toxic side effects and potent anti-tumour efficacy.

The signal transduction pathways posited for the promotion of tumours by gastrin-17 and progastrin are both numerous and complex [14–16], but targeting them has become an investigative strategy for the treatment of related GI tumours by anti-gastrin therapies, which specifically include endocrine-secretion inhibitors, receptor antagonism, and anti-gastrin antibody methods.

Endocrine-secretion inhibition (e.g., by somatostatin [17] and antisense oligonucleotides [18]) and receptor antagonism (e.g., by cholecystokinin receptor antagonists [19–21]) are methods that have often had low anti-gastrin specificity, weak anti-tumour effects, and necessarily large dosages, with a significant number of toxic side effects. Anti-gastrin antibody methods include both passive and active immunizations. Passive immunizations, e.g., anti-gastrin polyclonal or monoclonal antibodies [22,23], results in intermittent neutralization, requiring long-term infusions that are quite expensive and are often associated with unwanted immunogenic side-reactions caused by heterogeneous antibodies. The active immunization method has been achieved clinically by the application of vaccine-like immunogens, prepared with the epitope of gastrin 17 [24,25]. The use of targeted vaccines in cancer therapy has become a hot research topic of late, as it requires fewer injections and induces specific neutralizing antibodies. Some targeted vaccines have potent anti-tumour effects and thus have good prospects for clinical application.

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This study employs a new vaccine-like immunogen, which was composed of the common amino-terminal portions of human carboxy-amidated gastrin-17 (G17) and glycine-extended gastrin-17 (gly-G17) as well as the common carboxy-terminal portion of progastrin (proG) all covalently linked to tetanus toxoid (TT) by specific peptide spacers. The aim of this study was to determine if such an anti-gastrin vaccine with the specific capability of neutralizing these multiple gastrins, would have significant potent anti-tumour effects as well as minor toxic side effects. Our research thus mainly includes the assessment and evaluation of the novel vaccine-like immunogen's ability to provoke an immune response as well as its tolerated safety in our animals.

2. Materials and methods

2.1. Mice

Female BALB/c mice that were specific-pathogen-free and 6–8 weeks of age were used. Animals were maintained in aseptic isolation, fed and watered *ad libitum* according to institutional guidelines. All animal use was approved by the Institutional Ethics Committee (IRB) for Animal Use and was conducted in compliance with all state regulations.

2.2. Immunogen

The GAS-TT immunogen was composed of an equal mixture of the common carboxy-terminals of progastrin amino acids and the NH_2-terminal amino acids of the G17 and gly-G17 oligopeptide epitopes, which was collectively defined as GAS, all covalently linked to TT by peptide spacers. AES was the adjuvant emulsion system containing α-tocopherol and squalene in an oil-in-water emulsion.

2.3. Immunization procedure

GAS-TT (50 μg/mouse), GAS (10 μg/mouse) and TT (40 μg/mouse) were injected i.m. into the left or right leg of BALB/c mice at a final volume of 0.1 ml of AES, which was also used as negative control (n = 8). Mice were initially immunized using three injections at 2-week intervals.

2.4. Anti-human gastrin antibody levels in immunogen-immunized mice [26,27]

Mice were eye bled at time points throughout the experiment. Anti-human gastrin antibody levels were determined by ELISA. A 1 μg/ml solution of human GAS–bovine serum albumin (BSA) conjugate (AoKe Corporation) in a coating buffer (1.5 mg/ml Na_2CO_3, 3 mg/ml $NaHCO_3$, pH = 9.6) was coated into 96-well Immunulon U plates (Corning, USA) with 110 μl per well by an overnight incubation at 4 °C. The positive control, negative control and experimental sera at 3.16-fold serial dilutions, starting at a dilution of 1:100, were prepared in an antibody dilution buffer [phosphate buffer saline tween-20 (PBST), 1% BSA]. Subsequent steps used PBST (8 mg/ml NaCl, 3 mg/ml $Na_2HPO_4{\cdot}12H_2O$, 2.5 mg/ml KCl, 0.2 mg/ml KH_2PO_4, 0.05% tween-20) without BSA for washings. The 96-well plates were washed 4× to free them from non-bound conjugates, then the sera were added (100 μl/well). After a 1.5-h incubation at room temperature (RT), the plates were washed four times and a goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate was added (1:1000 dilution in antibody dilution buffer, 100 μl/well). After another 1.5-h incubation at RT, the plates were washed four times to remove non-bound reagent, and 100 μl/well of pNPP substrate solution (1 mg/ml) was added in substrate buffer (0.01 mg/ml $MgCl_2{\cdot}H_2O$, 10% diethanolamine). After a 5-min incubation in the dark at RT, 100 μl/well of stop buffer (1.0 M NaOH) was added, and the absorbance was measured on a microplate reader at 405 nm (reading wave) and 490 nm (reference wave). The values at 490 nm were subtracted from those at 405 nm, and the antibody titre was calculated by using the ED_{50} (50% effective dose) module of SCANLT software.

2.5. IFN-γ secretion by the spleen cells of the immunogen-immunized mice

To evaluate the IFN-γ secretion by the spleen cells of the immunogen-immunized mice, ELISPOT (BD Biosciences) was employed. Mice were sacrificed, and spleens were aseptically removed at weeks 4, 6, and 8 (n = 4). Single-cell suspensions were made, and erythrocytes were lysed with red blood cell lysis buffer. ELISPOT plates were pre-coated with the anti-IFN-γ antibody at a concentration of 15 μg/ml in 100 μl of sterile phosphate buffer saline (PBS, pH 7.2) at 4 °C overnight, and the plates were then blocked with complete medium RPMI1640 containing 10% foetal bovine serum (FBS) for 2 h at RT (200 μl/well). Cells added at 0.2 5×10^6 cells/well in 200 μl of the complete medium RPMI1640 and were cultured for 48 h at 37 °C in a 5% CO_2 humidified atmosphere in the presence of GAS-TT or ConA (positive control), both at 10 μg/well in 50 μl of RPMI1640 (negative control). After the incubation, the plates were washed 5× with PBS (200 μl/well). Then, 100 μl of biotinylated anti-IFN-γ diluted to 1 μg/ml in PBS containing 0.5% FBS was added, and the plates were incubated for 2 h at RT. After 5 washes with PBS (200 μl/well), 100 μl of 1:500-diluted peroxidase-conjugated streptavidin in PBS containing 0.5% FBS was added, and the plates were incubated for 1 h at RT. After 5 washes with PBS (200 μl/well), the spots were developed with tetramethylbenzidine (TMB) substrate (100 μl/well) for 5–6 min at RT. After 5 washes with PBS (200 μl/well), the plates were air dried and counted with an ELISPOT reader.

2.6. Treatments

Animals were inspected daily and weighed weekly. The mice were treated for a period of 8 weeks before they were sacrificed, their blood was collected, and their organs and injection site muscles were removed for study. The weight of the body and organs were measured by an independent observer who was blind to the treatment groups. Means and standard deviation of the means were calculated for each group (means ± SD).

2.7. Histological evaluation of the organs and injection site muscles

Organ and muscle specimens were fixed with 4% formalin. Specimens were then embedded in paraffin, and 4–5 μm sections were cut by a microtome and stained with haematoxylin and eosin (HE). Assessment of pathologic changes was made by a histopathologist in a blinded manner by image analysis.

2.8. Statistical analysis

All experiments were repeated three times. The data were analysed between groups using Student's *t*-test. P values < .05 were considered statistically significant.

3. Results

3.1. Anti-human gastrin antibody levels of immunogen-immunized mice

Mice were immunized with immunogen and their antibody titres followed for a period of 8 weeks following three immunizations. Fig. 1 shows the IgG antibody levels against gastrins as

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