

Pretreatment with glycomacropeptide reduces allergen sensitization, alleviates immediate cutaneous hypersensitivity and protects from anaphylaxis

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Summary

Allergic disorders are characterized by the involvement of allergen-specific immunoglobulin (Ig)E antibodies and T helper type 2 (Th2) cells. The search for new therapies for allergic diseases has been the primary focus of interest for many investigators in recent years. Glycomacropeptide (GMP) is a biologically active component of milk that exhibits a range of immunomodulatory functions. We examined whether oral administration of GMP could affect the development of allergic sensitization and the severity of immediate cutaneous hypersensitivity reactions and of anaphylaxis. Rats treated with or without GMP were ovalbumin (OVA)-sensitized and several indicators of allergy were evaluated. Pretreatment with GMP resulted in reduction of antigen-specific IgE titre in rats when sensitized with OVA. GMP administration also markedly suppressed the proliferative response of splenocytes to antigen and the production of interleukin (IL)-13 by splenocytes of sensitized animals. In addition, GMP pretreatment attenuated the intensity of the immediate cutaneous reaction induced by antigen and protected the sensitized rats from severe anaphylaxis. These data demonstrate, for the first time, that the administration of GMP prevents allergen sensitization and reduces the severity of the early-phase reaction induced by antigen in cutaneous hypersensitivity and in anaphylaxis. GMP may be used as a novel prophylactic agent for the control of allergic diseases.

Keywords: allergy, anaphylaxis, glycomacropeptide, IgE, immediate cutaneous hypersensitivity

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Introduction

The prevalence of allergic diseases is increasing globally, and approximately 30–40% of the world's population is now affected by one or more allergic conditions [1]. Allergic disorders, such as anaphylaxis, allergic rhinitis (hay fever), some food allergy and allergic asthma, are characterized by the involvement of allergen-specific immunoglobulin (Ig)E antibodies and T helper type 2 (Th2) cells that recognize allergen-derived antigens [2]. IgE antibodies are bound immediately to high-affinity receptors on tissue mast cells and the individual becomes sensitized to the relevant allergen. Subsequently, antigens cross-link receptors on the surface of mast cells and induce mast cell degranulation, which results in the release of preformed mediators, such as histamine, and the synthesis and release of compounds derived from the metabolism of arachidonic acid and an array of inflammatory cytokines [3]. These mediators cause acute signs and symptoms associated with the early phase of

immediate-type allergic reactions, such as vasodilation, increased vascular permeability with oedema and functional changes in affected organs [contraction of bronchial smooth muscle, increased secretion of mucus, urticaria (hives), vomiting and diarrhoea] [2].

While the exact aetiology of allergic diseases is still unclear, important risk factors are genetic predisposition and the environment. In recent years, attention has been given to new findings in allergy research that link exposure to microbes with the development of allergic illness. This theory is called the hygiene hypothesis, which proposes that infections and unhygienic contact might protect against development of allergic illness [4]. One mechanism, which is cited frequently in relation to this hypothesis, is the skewing of the Th1/Th2 balance towards Th1 cells and away from allergy-promoting Th2 cells, under poor hygienic conditions. Microbial contact, and in particular microbial flora, play a crucial role in the maturation of the host's immune system during the first years of life, favouring the

development of Th1 cell responses [5]. Prebiotics also play an important role in this balance, as they promote selectively the growth of microbiota that is beneficial to health [6].

Glycomacropeptide (GMP), or caseinomacropeptide, is a peptide of 64 amino acid residues obtained from κ -casein hydrolysis by the action of chymosin (rennet) during the process of cheese-making [7–9]. Due to its nutritional value, GMP is currently added to infant formulas [10]. As it has no phenylalanine in its amino acid composition, it is an excellent additive for products to be consumed by individual with phenylketonuria [11]. GMP is also considered to be one of the biologically active components of the milk [12,13]. It is included in toothpastes because of its anti-cariogenic properties [14]. Prebiotic effects have been attributed to GMP, as it can promote the growth of bifidobacteria and lactobacilli *in vitro* [15,16]. This peptide also has immunoregulatory functions, as some *in vitro* studies have described that GMP inhibits mouse splenocyte proliferation induced by lipopolysaccharide and phytohaemagglutinin [17], suppresses expression of interleukin (IL)-2 receptor on mouse CD4⁺ T cells [18], induces expression of an IL-1 receptor antagonist-like component in mouse spleen cells [19] and inhibits serum IgG antibody production by mouse lymphocytes [20]. In models of colitis and ileitis induced with trinitrobenzene sulphonic acid in rats, GMP was reported to have an anti-inflammatory effect [21,22], acting at least in part on lymphocytes [10].

The aim of this study was to investigate whether oral pretreatment with GMP can influence the development of allergic disease. We further examined the effect of GMP on the severity of immediate cutaneous hypersensitivity reactions and of anaphylaxis.

Materials and methods

Animals

Male Wistar rats (150–180 g) obtained from the Laboratory Animal Service of the Autonomous University of Aguascalientes were used throughout the study. Rats were housed under controlled conditions of temperature (22–24°C) and illumination (12 h light cycle), and maintained with Rodent Laboratory Chow 5001 and tap water *ad libitum*. All experiments were carried out with strict adherence to ethical guidelines approved by the Institutional Normative Welfare Standards.

Experimental design

Rats were assigned randomly to three different groups: sham (SH), sensitized without ovalbumin (OVA) and not GMP-treated; sensitized (S), OVA-sensitized and not GMP-treated; and sensitized with treatment (ST), OVA-sensitized and GMP-treated. Group ST was administered GMP (Lacprodan® CGMP-10; Arla Foods Amba, Viby,

Denmark) orally 500 mg/kg/day dissolved in tap water from 3 days before sensitization to the final of the study, whereas groups SH and S were administered tap water only. An oesophageal catheter was used to deliver GMP solution or water.

Protocol for induction of experimental OVA sensitization

OVA sensitization of rats has been described previously [23]. Briefly, animals from the S and ST groups were sensitized (day 0) with an intramuscular (i.m.) injection of 1 mg of OVA (Grade V; Sigma, St Louis, MO, USA) precipitated in 7.8 mg of aluminium hydroxide gel in 1 ml of saline solution. Simultaneously, and as adjuvant, 0.5 ml of *Bordetella pertussis* vaccine (Zuvirac, Mexico DF, Mexico) containing $10\text{--}15 \times 10^9$ heat-killed bacilli/ml was injected subcutaneously (s.c.). A booster sensitization was given 7 days later. SH rats were injected with aluminium hydroxide gel and the vaccine, but without OVA. Serum was collected from each rat at days 0, 7, 14 and 21 of sensitization and stored at –20°C until used to titrate IgE anti-OVA in the samples.

Passive cutaneous anaphylaxis (PCA) reaction for OVA-specific IgE titre in serum

Sera from SH, S and ST rats were analysed individually by PCA. Male Wistar rats weighing 500 g were anaesthetized with ether and the dorsal skin shaved. Fifty microlitres of each serum diluted 1:256, 1:128, 1:64, 1:32, 1:16, 1:8, 1:4 and 1:2 were injected intradermally (i.d.) in the dorsal skin. Twenty-four hours later the rats were anaesthetized and injected i.d. with 50 μ l of saline solution and histamine (2 μ g) as negative and positive controls, respectively. The rats were then challenged by intravenous (i.v.) (jugular) injection of 2 mg of OVA and Evans blue (34 mg/kg) in 3% saline solution. After 30 min, the animals were killed by anaesthesia overdose. The skin was inverted and the response, in terms of the infiltration of the blue dye rings around the injection sites, was read by measuring the largest and orthogonal diameters of each blue area using a digital vernier. The titre of the anti-OVA IgE antibody was expressed as the highest dilution causing a lesion more than 5 mm in diameter [24].

Spleen cells isolation

Spleens were removed aseptically from rats on day 14 of sensitization. Organs were perfused with cold saline solution and the cell suspension centrifuged at 212 g for 10 min at 10°C. The cell suspension was depleted of erythrocytes by incubation in hypotonic lysis buffer (0.17 M Tris, 0.15 M NH₄Cl, pH 7.2) for 5 min on ice, washed twice in saline solution by centrifugation and the obtained pellet was suspended in RPMI-1640 without phenol red (Sigma-Aldrich),

supplemented with 5% fetal calf serum (Invitrogen, Grand Island, NY, USA) and 1% penicillin/streptomycin (Sigma-Aldrich). Cell viability was quantified in a haemocytometer using the Trypan blue exclusion assay. Only those preparations with a purity $\geq 90\%$ and a viability $\geq 98\%$ were used. Spleen cell suspensions were plated in triplicate into 96-well flat-bottomed plates (Costar, Cambridge, MA, USA) at a concentration of 2×10^5 cells in 100 μl of supplemented RPMI medium/well for cell proliferation and cytokine assays.

Cell proliferation assay

To detect lymphocyte proliferation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) technique was used [25]. Spleen cells were stimulated with 0.1, 0.5 and 2.0 mg/ml of OVA at 37°C under an atmosphere of 5% CO₂. Concanavalin A (ConA, 1 $\mu\text{g}/\text{ml}$) or culture medium was used as positive and negative controls, respectively. All assays were performed in triplicate. After 96 h, 10 μl of MTT (Sigma-Aldrich) solution (5 mg/ml) was added to each well and the cells were incubated further for 4 h. Then, the purple formazan crystals were solubilized with 0.04 N hydrochloride (HCL) in isopropyl alcohol, plates were mixed thoroughly and read at 595 nm on a microplate reader (Bio-Rad, Tokyo, Japan). The stimulation index (SI) was defined as the ratio of optical density of the test sample to that of the control sample.

Cytokine determination

Spleen cell suspensions were cultured in triplicate for 96 h in the presence of OVA at 37°C under an atmosphere of 5% CO₂. ConA (1 $\mu\text{g}/\text{ml}$) or culture medium were used as positive and negative controls, respectively. Supernatants were taken and stored at -80°C until analysed. The levels of IL-10 and IL-13 were measured according to the manufacturer's protocol with commercially available rat enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen Co., Camarillo, CA, USA).

Intradermal skin test

Rats were tested for immediate cutaneous hypersensitivity by the i.d. skin test. Reactions were elicited in the shaved dorsal skin of rats at day 14 of sensitization. Animals were injected i.d. in triplicate with different OVA amounts dissolved in 0.05 ml of sterile saline solution. A similar volume of saline and histamine (2 μg) solutions were injected as negative and positive controls, respectively. Immediately, an i.v. injection of Evans Blue (34 mg/kg) in 3% saline was performed. Thirty min after OVA challenge, rats were killed and equal areas from the injected sites (blue spots) were removed with a hollow punch and the amount of dye in the tissue samples was determined colorimetrically after dye extraction with

1 ml of 1.0 N KOH and 9 ml of a mixture of acetone and 0.6 N phosphoric acid (5:13). The amount of extravasated dye was determined by measuring absorbance at 620 nm and using a standard curve (0.1 to 5 $\mu\text{g}/\text{ml}$ Evans blue). Negative control value was subtracted from each experimental value, obtained from the same animal, to normalize data.

Systemic anaphylaxis test

Rats were tested for anaphylactic shock by i.v. OVA (1 mg) injection at day 14 of sensitization. Allergen was injected through a catheter inserted previously into the jugular vein. Animals were challenged with OVA 2 h after surgery to be completely recovered. Shock was scored by the following scoring system [26]: 0, no sign of shock; 1, scratching and rubbing around the nose and head; 2, less activity or standing still with an increasing respiratory rate or puffing around the eyes; 3, asthmoid respiration, cyanosis around the mouth and the tail; 4, slight or no activity after prodding or tremor and convulsion; and 5, death. Mortality was monitored for 1 h after induction of anaphylactic shock. At the same time that signs were scored, rectal temperature was measured with a digital thermometer (model CT-513W; Citizen Systems, Nishi-Tokyo-Ski, Tokyo, Japan) just before challenge and every 10 min for 1 h. Changes in the temperature were expressed as time-course changes.

Statistical analysis

The data are presented as mean values $\pm$ standard error of the mean. Statistical analysis was performed by Student's *t*-test. Survival rate data were analysed by χ^2 test. Differences were considered statistically significant when $P < 0.05$.

Results

Reduction of allergen-specific IgE synthesis by GMP administration

To investigate the question of whether oral intake of GMP can modify the development of an allergic response, rats were sensitized by a first OVA/alum i.m. injection in combination with *B. pertussis* s.c and a second OVA/alum i.m. injection without *B. pertussis* (Fig. 1a). Blood samples were taken before and after GMP administration and OVA-specific IgE antibody levels were measured in sera by PCA reaction (Fig. 1b). Sera from all animals before sensitization were negative to PCA, indicating that animals were not sensitized previously to OVA and had no IgE antibodies cross-reacting to OVA that could generate false-positive reactions. OVA-specific IgE titres of sensitized animals exhibited a slight rise at day 7, increased rapidly to maximum level at day 14 and declined rapidly at day 21 (Fig. 1c). The titres of OVA-specific IgE in GMP-treated rats showed a slight rise at

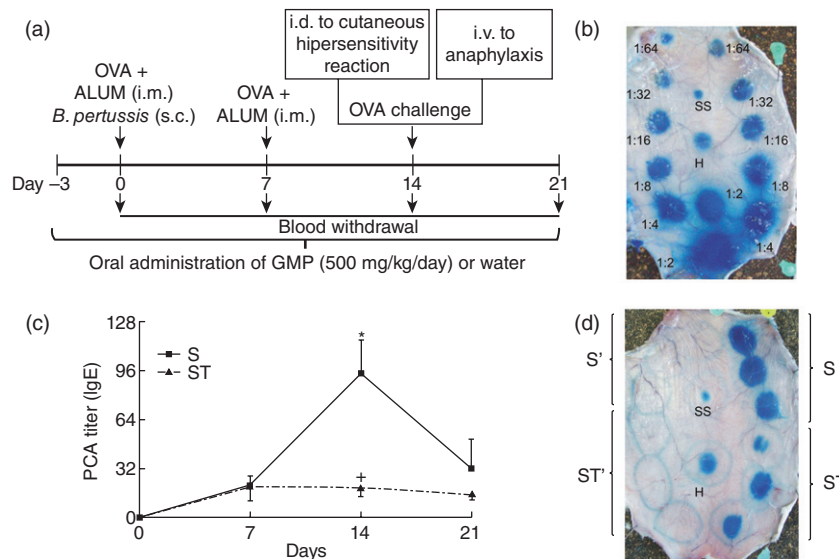


Fig. 1. Treatment with glycomacropeptide (GMP) before sensitization reduced immunoglobulin (Ig)E titres in sera from ovalbumin (OVA)-sensitized rats. (a) Experimental protocol. A rat model of OVA sensitization was used to test the effect of GMP administration in the development and manifestation of allergy. Rats were sensitized on day 0 with an intramuscular (i.m.) injection of 1 mg of OVA precipitated in 7.8 mg of aluminium hydroxide (ALUM) gel in 1 ml of saline solution. Simultaneously, 0.5 ml of *Bordetella pertussis* vaccine was injected subcutaneously (s.c.). A booster sensitization of OVA/ALUM was injected 7 days later. Rats were challenged with OVA on day 14 to develop cutaneous hypersensitivity reactions or anaphylaxis. (b) Photograph of dorsal inner skin reactions generated by increasing twofold dilutions of two sera from OVA-sensitized rats titrated by passive cutaneous anaphylaxis (PCA) reaction. Saline (SS) and histamine (H) solutions were used as negative and positive controls of PCA reaction. (c) Titres of OVA-specific serum IgE before, during and after OVA sensitization. PCA titres represent the reciprocal of the highest dilution of serum which gave a lesion >5 mm in diameter. Values represent the mean $\pm$ standard error of the mean, $n = 10$ in S and $n = 13$ in ST. * $P < 0.001$ compared to days 7 and 21 in S and * $P < 0.01$ compared to day 14 in S. (d) Inactivation of immune sera from sensitized animals. Sera were heated 56°C for 1 h to inactivate IgE. PCA reactions were developed with samples of serum (diluted 1 : 2 in saline solution) from three OVA-sensitized animals (S), three OVA-sensitized and treated animals (ST) and from the same animals but previously inactivated (S', ST'). Saline (SS) and histamine (H) solutions were used as negative and positive controls of PCA test.

day 7, similar to that reported in sensitized animals, but titres were unchanged thereafter. At day 14, the day with the maximum peak in IgE anti-OVA titre during sensitization, GMP treatment led to a profound and significant decrease of allergen-specific IgE antibodies in rats compared to sensitized animals ($P < 0.01$, ST versus S). To confirm that IgE was responsible for the induction of PCA reaction and to exclude an IgG1-mediated response, sera from sensitized animals inactivated previously by heating 56°C for 1 h were analysed by PCA. As shown in Fig. 1d, inactivation of immune sera from sensitized rats (with or without treatment) eliminated the PCA reaction, indicating that IgE was the reagenic antibody mediating PCA reaction. Sera from sham animals were negative to PCA throughout the study.

OVA-specific proliferative response is inhibited by GMP administration

To determine the effect of GMP on the lymphocyte proliferative response, spleen cells were prepared from sensitized animals at day 14 of sensitization, and cultured with or without antigen or mitogen. As shown in Fig. 2, a marked increase in proliferative response was stimulated by ConA

and OVA in splenocytes isolated from sensitized animals. Oral pretreatment with GMP resulted in a significant inhibition of lymphoproliferative responses to OVA at the three OVA concentrations used. Although spleen cells from GMP-treated rats showed a reduction in levels of proliferation to the mitogen ConA, compared to non-treated animals, the lymphoproliferative response was not abolished totally. After treatment, lymphocyte proliferation to ConA was still significantly greater than that of the control ($P < 0.001$). These results suggest that GMP totally inhibits the lymphocyte proliferation induced by allergen, and only partially when activated by ConA.

GMP administration decreases allergen-induced IL-13 release

Spleen cells obtained from rats at day 14 of sensitization were cultured with OVA or mitogen to detect T cell cytokine responses. As shown in Fig. 3a, high levels of IL-13 and IL-10 were measured in supernatants of OVA-stimulated spleen cell cultures. ConA also had a significant stimulatory effect on IL-13 and IL-10, although production of both cytokines was higher with the antigen. To determine whether GMP

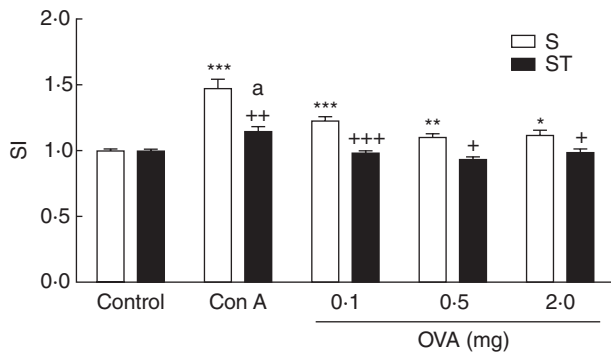


Fig. 2. Glycomacropeptide (GMP) pretreatment inhibited ovalbumin (OVA)-specific lymphocyte proliferative response. Proliferation of spleen cells isolated from sensitized rats in response to OVA (0.1, 0.5 and 2.0 mg/ml) or mitogen concanavalin A (ConA, 1 µg/ml) was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) technique. Proliferative response of splenocytes in medium alone was used as control. Each value represents the mean $\pm$ standard error of the mean, $n = 24$ in each group (eight independent experiments in triplicate). * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$ compared to control S. + $P < 0.05$; ++ $P < 0.005$; +++ $P < 0.0005$ compared to the same condition in S splenocytes. ^a $P < 0.001$ compared to control ST.

administration influences cytokine production by splenocytes *in vitro*, spleen cells isolated from treated animals were stimulated with OVA and mitogen (Fig. 3b) using 2 mg of OVA, because this led to the highest levels of IL-13 and IL-10. Oral pretreatment with GMP resulted in a significant suppression of IL-13 levels in antigen-specific stimulation, without changing IL-13 levels stimulated by mitogen. IL-10 production remained unchanged in GMP-treated animals. The decrease in IL-13 production in GMP-treated rats is in agreement with the finding of reduced levels of serum IgE anti-OVA in these animals.

Pretreatment with GMP attenuates OVA-induced immediate cutaneous hypersensitivity

To investigate whether GMP modifies the intensity of the immediate cutaneous reaction induced by allergen, OVA was injected *i.d.* in sensitized rats and the magnitude of local hypersensitivity reaction was examined 30 min later. Normalized data of tissue Evans blue amounts indicated the vascular extravasation activated by allergen. It has been demonstrated that intradermal injection of antigens used in sensitization can develop a cutaneous reaction in non-sensitized animals [27]. The dose-response relationships obtained in sham and sensitized rats using twofold dose increments of allergen are shown in Fig. 4a. OVA injection increased vascular permeability in a dose-related manner both in sham and sensitized animals. However, the amount of extravasated Evans blue in sensitized animals was greater than that in sham rats, and this difference was significant at lower OVA concentrations (from 3.9 to 31.2 µg). Thus, active cutaneous

anaphylaxis was developed by intradermal injections of 15.6 and 31.2 µg of OVA in sham, sensitized and GMP-treated sensitized animals (Fig. 4b). High levels of colorant were extravasated in sensitized animals to both OVA stimuli. GMP treatment decreased significantly to half the amount of extravasated Evans blue induced by allergen (Fig. 4c). These data indicate that GMP pretreatment before OVA sensitization inhibited the development of OVA-induced immediate cutaneous hypersensitivity. To study whether GMP blocks mast cell activation, immediate cutaneous reaction was developed in non-sensitized rats treated with or without GMP by intradermal injection of compound 48/80 (C48/80, 50 µg/50 µl of saline solution). Three days before C48/80 injection rats were separated into two groups to be administered orally with GMP or water. GMP did not show any effect on vascular permeability induced by C48/80, as GMP-treated and non-treated rats extravasated an equal amount

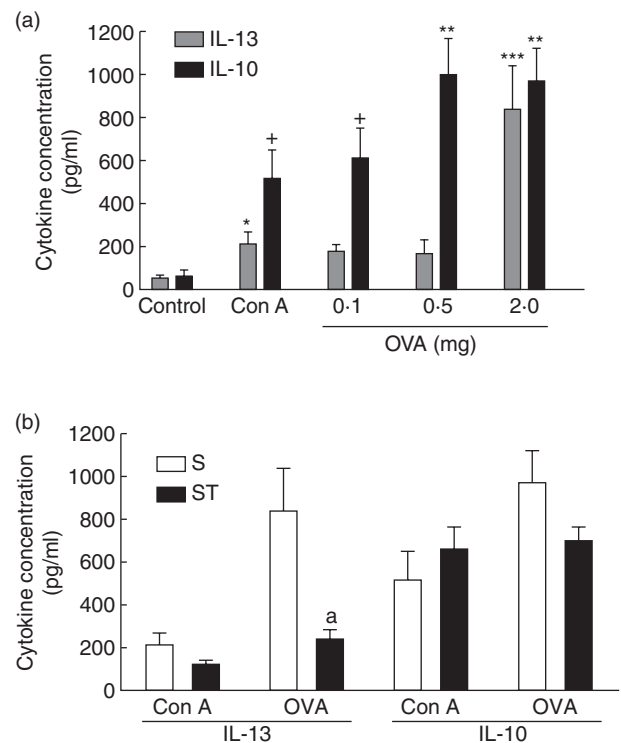


Fig. 3. Glycomacropeptide (GMP) pretreatment suppressed ovalbumin (OVA)-specific interleukin (IL)-13 production in spleen cell cultures from sensitized animals. (a) T cell cytokines (IL-13 and IL-10) production in spleen cell cultures from OVA-sensitized rats. Splenocytes were cultured with OVA (0.1, 0.5 and 2.0 mg/ml) or mitogen concanavalin A (ConA, 1 µg/ml). (b) Comparative production of T cell cytokines in splenocyte cultures from OVA-sensitized rats with and without GMP-treatment. Splenocytes were stimulated with OVA (2.0 mg/ml) or mitogen ConA (1 µg/ml). In all assays cytokine levels were determined in supernatants after 96 h of stimulation by enzyme-linked immunosorbent assay. Each value represents the mean $\pm$ standard error of the mean, $n = 3$ spleens. * $P < 0.05$; + $P < 0.01$; ** $P < 0.005$; *** $P < 0.001$ compared to control of each cytokine. ^a $P < 0.001$ compared to IL-13 in S animals.

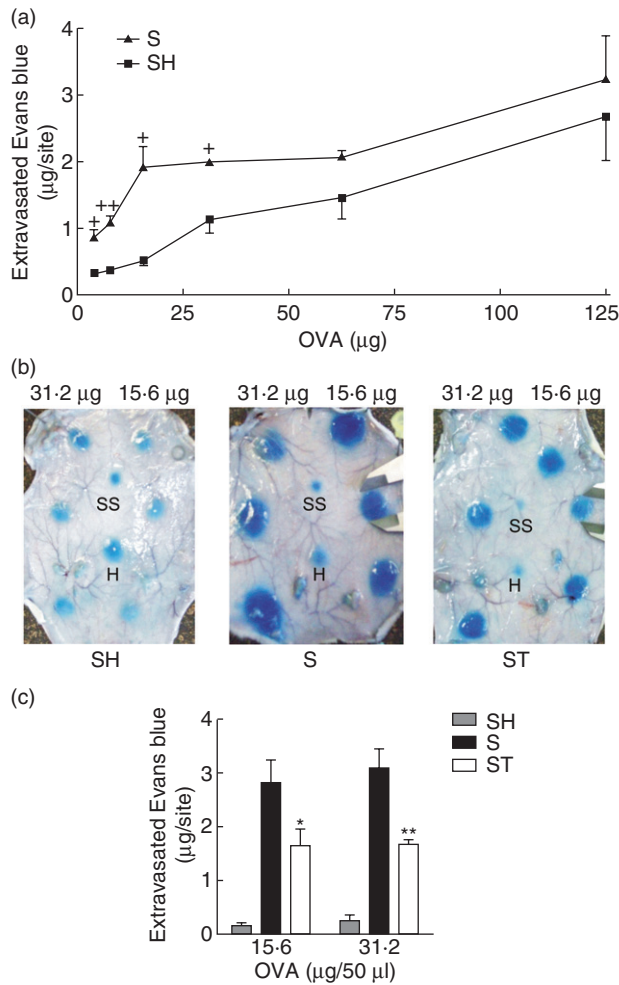


Fig. 4. Pretreatment with glycomacropeptide (GMP) reduced the intensity of immediate cutaneous reaction induced by ovalbumin (OVA) in sensitized animals. (a) Vascular extravasation induced by injection of increasing doses of OVA in sham and sensitized rats. OVA was injected at 3.9, 7.8, 15.6, 31.2, 62.5 and 125.0 µg/50 µl of saline solution. Evans blue extravasated in each cutaneous reaction was calculated colorimetrically. Each value represents the mean $\pm$ standard error of the mean, $n = 4$. * $P < 0.05$; ** $P < 0.01$ compared to the same condition in SH. (b) Photographs of dorsal inner skin reactions generated by intradermal injection of 15.6 µg (three blue spots at the right of SS) and 31.2 µg (three blue spots at the left of SS) of OVA/50 µl of saline solution in sham (SH), OVA-sensitized (S) and OVA-sensitized and GMP-treated (ST) rats. Saline (SS) and histamine (H) solutions were used as negative and positive controls. (c) Evaluation of vascular extravasation generated in immediate cutaneous reactions to OVA. The amount of Evans blue dye extravasated in the skin of SH, S and ST rats was calculated. Each value represents the mean $\pm$ standard error of the mean, $n = 12$ (four independent experiment in triplicate). * $P < 0.01$; ** $P < 0.001$ compared to S.

of colorant (2.27 ± 0.53 and 2.77 ± 0.15 µg/site of Evans blue, respectively, $n = 8$). These results indicate that GMP pretreatment does not modify the cutaneous reaction induced by mast cell activation in rats.

GMP administered orally protects from allergen-induced systemic anaphylaxis

To study the effect of GMP administration on anaphylaxis, the severity (score) of the response, changes in rectal temperature and rate of survival were monitored after intravenous challenge of sensitized rats with OVA. As shown in Fig. 5a, all rats in the sham group exhibited no signs of shock (score 0, 81.81%) or scratching and rubbing around the nose and head (score 1, 18.18%). The mean score was 0.18 (range: 0–1). Sensitized animals showed the highest degrees of severity in their signs, as 100% of the rats died of fatal anaphylaxis (mean score 5) during the 60 min following the challenge. However, GMP pretreatment reduced the mean score of sensitized animals significantly to 4.3 (range: 2–5). In GMP-treated group, animals showed signs such as less activity or standing still with an increasing respiratory rate or puffing around the eyes (score 2, 7.7%), asthmoid respiration, cyanosis around the mouth and the tail (score 3, 23.1%) or

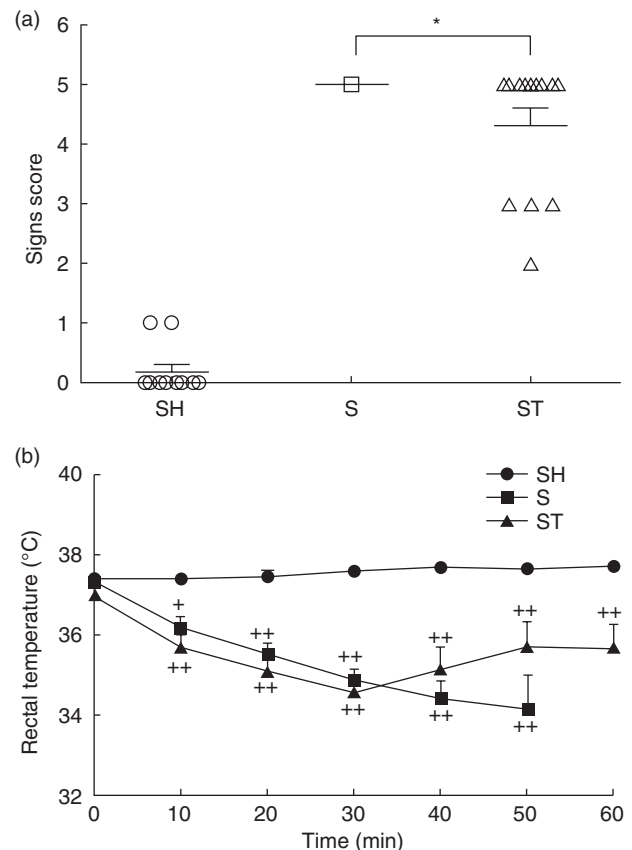


Fig. 5. Inhibitory effect of glycomacropeptide (GMP) pretreatment on allergen-induced systemic anaphylaxis. (a) Anaphylactic sign scores 60 minutes following intravenous ovalbumin (OVA) challenge. Each symbol represents each individual rat. (b) Rectal temperature was measured just before challenge and after every 10 min for 60 min. Values represent the mean $\pm$ standard error of the mean, $n = 11$ in SH and S groups and $n = 13$ in ST group. * $P < 0.05$ compared to S. ** $P < 0.01$; *** $P < 0.001$ compared to SH at the same time.

Table 1. Pretreatment with glycomacropeptide (GMP) increases the rate of survival after ovalbumin (OVA)-induced anaphylactic shock in sensitized animals.

Groups	Survival (%) (alive/total)			
	30 min	40 min	50 min	60 min
SH	100% (11/11)	100% (11/11)	100% (11/11)	100% (11/11)
S	72.7% (8/11)	23.27% (3/11)	9.1% (1/11)	0% (0/11)
ST	69.23% (9/13)	30.77% (4/13)	30.77% (4/13)	30.77% (4/13)*

* $P < 0.05$ compared to ovalbumin-sensitized (S) at 60 min analysed by χ^2 test. SH: sham; ST: sensitized with treatment.

died of fatal anaphylaxis (score 5, 69.29%). Rectal temperature showed a pattern in agreement with the score of anaphylactic response. After OVA challenge, rectal temperature in sham rats ranged between 37°C and 38°C, whereas in OVA-sensitized rats temperature was 1–4° below normal (Fig. 5b). There were significant differences in mean temperatures between sham and sensitized animals at 10–50 min after allergen challenge. GMP treatment induced an increase in rectal temperature from 40 to 60 min after OVA challenge, indicating that surviving rats in the GMP-treated group were recovering from the fall in temperature. In relation to the rate of survival (Table 1), sham animals reported a survival of 100% to the challenge with OVA. However, in sensitized rats the percentage of survival was time-decreasing, with 0% of survival 60 min after challenge. GMP pretreatment increased the rate of survival of sensitized animals to 30.77% at 40 min after OVA challenge and was unchanged thereafter. The survival rate of GMP-treated rats was increased significantly compared with sensitized animals at 60 min after OVA challenge ($P < 0.05$). These results demonstrate that pretreatment with GMP was effective in protecting rats from allergen-induced anaphylactic reactions.

Discussion

In the present study, we demonstrate that oral pretreatment with GMP induces a significant reduction in the development of experimental systemic sensitization related to allergy. GMP administration prior to OVA-sensitization strongly decreases the levels of serum antigen-specific IgE, inhibits the proliferative response of splenocytes to OVA and decreases the production of IL-13 stimulated by OVA in cultures of spleen cells from sensitized animals. Also, we show that GMP pretreatment impacts upon the clinical manifestations of immediate cutaneous hypersensitivity by largely reducing the intensity of local inflammatory response. GMP also impairs the systemic anaphylactic response of animals by increasing the survival and decreasing the signs of severe anaphylaxis.

IgE production and Th2 differentiation are clearly essential to the development of allergy [28,29]. In our experimental model, high levels of OVA-specific IgE and IL-13 were quantified, respectively, in serum and supernatants of splenocytes obtained from sensitized animals. These two mol-

ecules are related to each other, as it has been reported that IgE expression can be regulated by IL-13 in mice [30], and in allergic asthma patients elevated levels of IL-13 have been found to correlate with IgE [31]. We show that pretreatment with GMP reduces the levels of IgE specific to antigen significantly when synthesis of IgE is induced experimentally in an allergy model. This result is in line with the inhibitory effect of GMP on IL-13 production that we observed. Previous reports in mice show that a diet supplemented with GMP suppresses the level of serum IgG specific to injected antigens [20]. Taken together, these results demonstrate an immunomodulatory function of dietary GMP in the humoral immune response.

IL-10 is also highly secreted by splenocytes of allergic animals in response to antigen [32,33]. We detected high levels of this cytokine in the supernatant of splenocytes of sensitized animals activated by OVA or by mitogen. IL-10 is a regulatory cytokine secreted by numerous cell types, including Th2 cells, which plays a central role in controlling inflammatory processes [34]. A role for IL-10 in regulation of immune responses to allergens has been demonstrated [35–38]. We showed that IL-10 is not apparently a key element in GMP immunoregulation, as no significant changes in IL-10 synthesis in response to antigen or mitogen were observed after GMP treatment. This is consistent with the evidence that GMP does not affect IL-10 production by lipopolysaccharide (LPS)-stimulated dendritic cells [39]. Previous studies show that oral GMP administration increases forkhead box protein 3 (FoxP3) expression in splenocytes, indicating a positive effect on regulatory T cell differentiation [10]. Whether other regulatory mechanisms mediated by regulatory T cells, such as transforming growth factor (TGF)- β secretion, anergy and/or cell–cell contact, are involved in the suppressive effect of GMP on allergy development needs to be investigated.

Splenocyte proliferation is intense in the development of adaptive immune response. Inhibition of splenocyte proliferation can be used to demonstrate the suppression of an immune response such as allergic reaction. There are controversial data in relation to the *in vitro* effect of GMP on spleen cell proliferation. GMP has been demonstrated to inhibit mouse splenocyte proliferation induced by mitogens [40,41]. However, recent studies report that GMP enhances ConA-stimulated splenocyte proliferation [10]. Studies *in vivo* have reported that oral administration to mice of a diet

supplemented with GMP enhances the proliferative response of spleen cells to ConA, with no significant changes in LPS and phytohaemagglutinin responses [20]. Our results show that oral administration of GMP inhibits the proliferative response induced by antigen totally, but reduces the response elicited by ConA only partially. These data indicate that suppression induced by GMP on splenocyte proliferation is antigen-specific without altering the general immune function of lymphocytes. Differences in the doses of GMP administered may be the cause of the different results observed *in vivo*, as in the *in vitro* assays [40,41].

Immediate cutaneous reaction is a model for evaluating sensitized skin mast cell activation in response to i.d.-injected specific antigen [42]. We observed that after GMP treatment there is an important decrease in the intensity of the inflammatory reaction induced by antigen in the skin of sensitized rats. Immediate cutaneous reaction involves a process from IgE production to IgE-mediated skin mast cell activation. It is known that concentration of IgE bound to FcεRI can determine the intensity of the reaction developed by mast cell activation [43]. As we observed that GMP does not change the inflammatory response induced by intradermal injection of C48/80, it apparently does not have effects on skin mast cell activation. We propose that the decrease in IgE synthesis caused by GMP reduces the intensity of local inflammatory response in sensitized animals when challenged by antigen.

Experimental systemic anaphylaxis is characterized by hypothermia, scratching, decreased mobility, rapid and potentially reversible hypotension, and finally by death in severe shocks [44]. It is well established that systemic anaphylaxis induced by antigen is mediated mainly by IgE, FcεRI, mast cells and histamine in humans [45]. Recently, it has been suggested that an alternative pathway involving IgG, macrophages, FcγRIII and platelet-activating factor is also developed in mice in response to antigen challenge [44]. The common denominator in all types of systemic anaphylaxis is that mediators released by mast cells, macrophages or other cells in response to antigens are responsible for the development of signs and symptoms of the reaction, although anaphylaxis is also regulated by agents that modify responsiveness to this mediators [44]. The role of IL-13 in the regulation of anaphylaxis reaction has been demonstrated. Data obtained in transgenic mice expressing IL-13 indicate that mice become highly predisposed to anaphylaxis following systemic antigen sensitization [46]. Studies in mice pretreated with IL-13 have demonstrated that these mice develop an anaphylactic shock more severe than non-treated animals due to an increase in sensitivity to vasoactive mediators, predominantly through an effect on vascular endothelium [47]. In our study, GMP pretreatment induces a reduction in the severity of anaphylactic response that becomes evident by the increase in the rate of survival of sensitized animals, the reduction in the mean score of the anaphylactic response and the tendency of animals to

recover from hypothermia. Taken together, these results suggest that one of the main biological actions of GMP to protect from systemic anaphylaxis may be the reduction in IL-13 production. However, although we report that GMP has no effect on mast cell activation, we cannot exclude a possible effect of GMP on other effector cell or mediator that develops or regulates anaphylaxis.

GMP is obtained physiologically in the stomach of neonates and adults by the chymosin digestion of native protein [8,48]. We can also ingest GMP from many commercial foods, such as margarine, yogurt and dietary supplements [49]. However, the intake of GMP by foods is poor. Yogurt provides approximately 0.12–0.15 mg of GMP/ml [49,50], much less than the concentration that we used to obtain an immunomodulatory effect on allergy development. Little is known about the pharmacokinetics of GMP, but two studies have shown previously that after milk or yogurt ingestion it reaches the bloodstream in significant amounts (approximately 1 µg/ml) and the concentration remains relatively stable for at least 8 h. In addition, GMP is detected in the duodenum shortly after milk or yogurt intake [51,52]. Therefore, the protective effect of GMP on allergy development and on the severity of immediate allergic reactions may be due to luminal and/or systemic actions. In addition to the previously mentioned systemic mechanisms, GMP may also protect from allergy by prebiotic effects. GMP has growth-promoting effects on *Bifidobacterium* and *Lactobacillus* strains, as demonstrated by other investigators [15,16,53]. The possible role of probiotics on preventing allergic diseases is still controversial [54]. However, there are strong data indicating a beneficial role of *Bifidobacterium* and *Lactobacillus* strains in atopic diseases by the modulation of Th1/Th2 response to allergens, the activation of tolerogenic dendritic cells or the production of regulatory T cells [54]. In future experiments we plan to assess the hypothesis of prebiotic effects of GMP modulating the allergic response.

In summary, we found that GMP possesses a prophylactic effect in the development of allergy. This effect is associated with down-regulation of the specific IgE levels, T cell proliferative response and IL-13 production. In addition, GMP pretreatment protects from the severity of immediate cutaneous reaction and anaphylaxis induced by antigen. Because type I immediate disorder is a multi-factorial allergic disease that involves both humoral and cellular elements, we speculate that GMP probably possesses many actions that may regulate various immune components involved in allergic response. This study provides the first experimental basis for the therapeutic potential of GMP in the regulation of allergy.

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Disclosure

The authors confirm that there are no conflicts of interest.

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