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Systemic Release of Mucosal Mast-cell Protease in Primed Brown Norway Rats after Feeding with β-Lactoglobulin

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The plasma level of mucosal mast-cell protease was examined to find whether such measurements could be an indicator of allergic response to β-lactoglobulin (β-LG) challenged orally by rats. Brown Norway rats, which had been raised on a bovine milk-free diet, were systemically sensitized on day 0 with a low dose of β-LG, and then by an oral administration of β-LG for 3 h on day 14. The oral challenge with β-LG in saline, when compared to saline alone, resulted in a systemic elevation of rat mast-cell protease II (RMCPII), one of the specific markers for gut mucosal mast-cell secretion. The challenge with β-LG in a fat emulsion further increased the level of plasma RMCPII. This manipulation, however, was not successful for detecting any significant difference in mucosal leucotriene C_4, another allergic mediator. An oral challenge with polymerized β-LG did not induce any elevation of the protease, but resulted in a lower plasma level of β-LG-specific IgG. This animal model is thus relevant to investigate the events regulating the mucosal hypersensitivity and humoral immunity to food proteins.

The initial target for an immediate reaction to food is generally the mast cells within the gastrointestinal mucosa, and such mast cells are sensitized *in vivo* by food-specific IgE. Degranulation of these cells facilitates entry of an antigenic epitope into the lymphatic system and blood stream, thereby causing further degranulation of the mast cells and basophils throughout the body. One difficulty in studying food allergies has been the lack of a reasonable animal model in which reactions could be induced by orally administrating foods. Turner *et al.*[1] have recently shown that rats initially sensitized systemically with a low dose of ovalbumin and subsequently fed with protein by mouth released rat mast-cell protease II (RMCPII) into the circulation system, the synthesis of which is restricted to the gut in rats.[2] Although this model offers the possibility for exploring the pathogenesis of food-induced reactions and of desensitizing animals sensitized with specific food proteins, the validity of this model appears not to have been evaluated for food antigens other than ovalbumin. In addition to controlled comparative studies of various pharmacological agents, the measurement of other newly synthesized mediators of the cyclo-oxygenase pathway, which are known to be accumulated in the intestinal mucosa of rats parasitized with *Trichinella spiralis* (*T. spiralis*),[3] would also be of interest in relation to food protein hypersensitivity.

In the present study, we have examined the levels of systemic RMCPII and gut leucotriene C_4 (LTC_4) in order to evaluate the allergenicity of β-lactoglobulin (β-LG), which is one of the major allergens in cow's milk proteins and is absorbed through the gut epithelial cells into the circulation system.[3] The allergenicity of orally administered and chemically modified β-LG was also examined, as chemically modified allergens have been reported to be able to induce unresponsiveness when systemically challenged in experimental animals.[4–6]

Materials and Methods

Animals. Brown Norway (BN) rats (4 wk old), weaned either to a diet containing bovine milk (CRF-1, Oriental Yeast Co., Tokyo, Japan) or to a diet not containing this milk (MF, Oriental Yeast Co.), were obtained from Seiwa Experimental Animals Co. (Fukuoka, Japan). They were maintained at our animal facility in a controlled temperature at 23±1 °C and given a milk-free diet (NMF, Oriental Yeast Co.) until the age of 6 wk. Subsequently, the rats were maintained on a semi-purified diet free of milk proteins as shown in Table.

Experimental protocol. The protocol for sensitizing the rats and then challenging them with β-LG was essentially similar to that described by Turner *et al.*[1] Six-wk-old rats were sensitized on day 0 with an intraperitoneal injection of 100 μg of β-LG (Sigma Chemicals Co., St. Louis, MO, U.S.A.; 3-times crystallized) adsorbed to 0.5 ml of 3% (w/v) $Al(OH)_3$. On day 14, these rats received a gavage feed (2 ml) containing either 100 mg of β-LG or saline alone. In another trial, glutaraldehyde-polymerized β-LG (β-LG-POL) in physiological saline was also given by gavage. The gavage foods were introduced into the stomach by a stomach

Table Composition of the Diet

Ingredient	g/100 g of diet
Safflower oil[1]	10.0
Soybean protein[2]	20.0
AIN-76 vitamin mixture[3]	1.0
AIN-76 mineral mixture[4]	3.5
Choline bitartrate[5]	0.2
DL-Methionine[3]	0.3
α-Corn starch[6]	15.0
Cellulose[7]	5.0
Sucrose[8]	45.0

[1] Linol-Yusi, Tokyo.
[2] Fujipro R, Fuji Oil Co., Osaka.
[3] Nacalai Tesque, Kyoto.
[4] Oriental Yeast Co., Tokyo.
[5] Wako Pure Chemicals Co., Osaka.
[6] Nihon Shokuhin Kakou Co., Aichi.
[7] Nitchiku Medical Industries, Kanagawa.
[8] Nishinihon Sugar Manufacturing Co., Fukuoka.

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tube (Becton, Dickinson & Co., Parsippany, NJ, U.S.A.). In some experiments, the rats received a solution of β-LG dissolved in a fat emulsion (Intralipid, Kabi Pharmacia AB, Uppsala, Sweden). Blood samples were collected immediately by aortic puncture before gavage and at various times up to 3 h post gavage under diethyl ether anesthesia. Sera were collected and stored at −40°C until being analyzed. The proximal half of the small intestine was removed intact, and rapidly flushed with 5 ml of phosphate-buffered saline (PBS) at pH 7.2 and then by 10 ml of air to remove all traces of the medium and gut contents.

Preparation of chemically modified β-lactoglobulin. β-LG was treated with glutaraldehyde (Nacalai Tesque, Kyoto, Japan) as previously described.[4] Briefly, 25 mg/ml of β-LG dissolved in a 0.1 M sodium acetate/acetic acid buffer at pH 5.3 was supplemented dropwise with 6% (w/v) glutaraldehyde in 0.15 M NaCl while stirring over a period of several minutes to obtain a final molar ratio of glutaraldehyde : β-LG of 200 : 1. The reaction was allowed to proceed for 5 h and, after extensive dialysis against borate-buffered saline (0.1 M, pH 8.3), the product was freeze-dried. A portion of the product was applied to a Sephadex G-200 (Pharmacia Japan, Tokyo, Japan) gel-filtration column (2.5 × 90 cm).

Preparation of rat mucosal mast-cell protease and the antibody, and immunological determination of the protease. One hundred female retired rats of the Sprague-Dawley strain (Seiwa Experimental Animals Co.) were used to prepare RMCPII by affinity adsorption chromatography as described by Woodbury *et al.*[7] Subunit C of potato chymotrypsin inhibitor I, which had been prepared from 50 kg of potatoes,[8-9] was covalently coupled to Sepharose 4B (Pharmacia Japan) according to the instructions given by the manufactures. The protease preparation exhibited a single band having an apparent molecular weight of 25,000 by SDS-polyacrylamide gel electrophoresis and an amino acid composition comparable to that reported previously (data not shown).[7] In accordance with Knox *et al.*,[10] the isolated intestinal protease actively hydrolyzed a synthetic chymotrypsin substrate, benzyloxy-carbonyl-L-tyrosine-4-nitrophenol ester (CBZ-L-Tyr-NPE, Sigma Chemicals Co.), but not a synthetic trypsin substrate, *N*-benzoyl-L-tyrosine-*p*-nitroanilide (Bz-Tyr-NAN, Sigma Chemicals Co.; data not shown). From these criteria, the protease preparation obtained was considered to be identical to the reported RMCPII.[7,11,12] The antiserum to RMCPII was prepared with a New Zealand white rabbit (Seiwa Experimental Animals Co.) by intradermally injecting 1.5 mg of protein mixed with complete Freund's adjuvant (Wako Pure Chemicals Co., Osaka), boosting twice with 1 mg of protein mixed in incomplete Freund's adjuvant (Wako Pure Chemicals Co.). The antiserum was found to be monospecific to the RMCPII preparation by a double-immunodiffusion analysis (data not shown). The rabbit anti-RMCPII reacted with the 25,000 molecular weight protein in the blood plasma, but appeared to retain slight cross-reactivity with a higher-molecular-weight component of the blood plasma (Fig. 1). RMCPII in the plasma, which was concentrated by freeze-drying prior to the determination, was quantified by rocket immunoelectrophoresis as described previously,[13] and the results are expressed in an arbitrary unit.

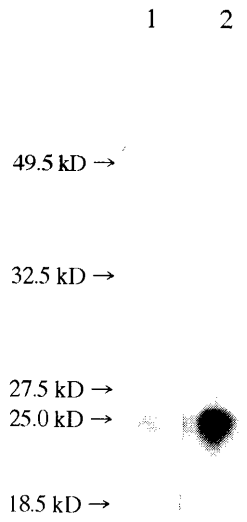

Fig. 1. Western Blot of RMCPII (1) and Blood Plasma Proteins (2) after Incubating with Rabbit Anti-RMCPII and then by Goat Anti-Rat IgG.

The proteins were electrophoretically transferred from the SDS-polyacrylamide gel to a nitrocellulose membrane. The nitrocellulose was incubated with rabbit anti-RMCPII and then by alkaline phosphatase-conjugated goat anti-rabbit IgG.[20]

Quantification of leukotriene C_4. The intestine was slit open and scraped gently with the edge of a glass slide; the mucosal tissue was collected on ice and weighed. The intestinal mucosa was incubated on ice for 1 h in 10 ml of 80% ethanol containing eicosatetraynoic acid (0.1 mM ETYA, Cascade Biochem., England), a lipoxygenase inhibitor. The sample was centrifuged at 3000 rpm and 4°C for 20 min, and the supernatant was collected. A 2.5-ml aliquot of the supernatant mixed throughly with 8 ml of a 0.1 M phosphate buffer at pH 4.0 was passed through a Sep-Pak C-18 reverse-phase cartridge (Water Chromatography Division, Millipore Corporation, Milford, MA, U.S.A.), which had been activated prior to use by rinsing with 5 ml each of ethanol and water. After the cartridge had been subsequently rinsed with 5 ml each of water and hexane, LTC_4 was eluted with a 5-ml mixed solution of ethanol : water = 9 : 10. The eluate was evaporated under a stream of dry nitrogen and stored at −80°C until being used for an enzyme immunoassay according to the manufacturer's instructions (LTC_4 EIA Kit, Cayman Chemical Co., Ann Arbor, MI, U.S.A.).

Determination of IgG specific to β-lactoglobulin. IgG specific to β-LG was determined by an enzyme-linked immuno-adsorbent assay (ELISA). The wells of a microtitration plate (A/S Nunc, Nippon InterMed Co., Tokyo, Japan) were incubated with 150 μl of a β-LG solution (0.5 μg/ml in a 0.05 M carbonate–bicarbonate buffer at pH 9.6) for 1 h at 37°C and then washed three times with PBS–0.05% Tween 20 (TPBS). The free sites were then saturated by incubating at 37°C for 1 h with 300 μl fish gelatin (Sigma Chemicals Co.) in TPBS. After washing with TPBS, the wells were incubated at 37°C for 1 h with 50 μl of rat serum diluted appropriately with TPBS, and then filled with 100 μl of goat anti-rat IgG antibody conjugated with peroxidase (Cappel, West Chester, PA, U.S.A.). Finally, the wells were incubated with 100 μl of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Wako Pure Chemicals Co.) for 15 min at 37°C, and the reaction was terminated by adding 100 μl of 1.5% oxalic acid. The absorbance was recorded at 415 nm with a microplate reader (Nippon InterMed Co., Tokyo, Japan).

Determination of the reaginic activity and IgE. A passive cutaneous anaphylaxis (PCA) reaction was carried out with rat skin as described previously.[14] Briefly, 6-wk-old male Sprague-Dawley rats (Seiwa Experimental Animals Co.) were intradermally sensitized with appropriately diluted rat serum and challenged 48 h later by intravenously injecting 1 ml of 1 mg of β-LG in 0.5%. Evans blue solution (Wako Pure Chemicals Co.). The rats were sacrificed 30 min later under diethyl ether anesthesia, and the diameter of the lesions was measured by using NIH Image (Apple Computer Japan, Tokyo, Japan). Reaginic activity was confirmed by the loss of activity of the immune serum after heating at 56°C for 60 min.

IgE specific to β-LG was also determined by ELISA. The wells of a microtitration plate were coated with β-LG (0.5 μg/ml) and then with 0.1% of fish gelatin, as in the determination of IgG. After washing, the wells were incubated for 1 h at 4°C with 50 μl of the serum appropriately diluted with TPBS. Subsequently, the wells were reacted first with biotinylated anti-rat IgE mouse antibody (Zymed, CA, U.S.A.) diluted to 1/2000 in

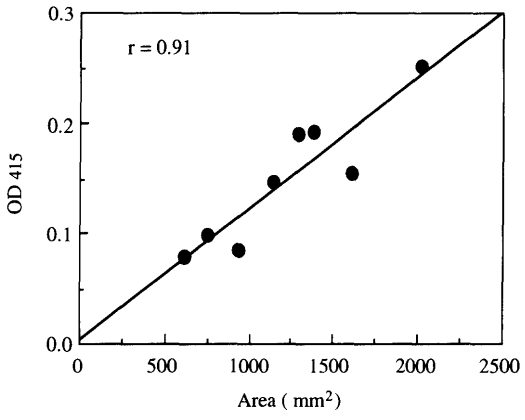

Fig. 2. Correlation between the PCA (Area) and ELISA (OD_{415}) Titers.

TPBS, and then with peroxidase-conjugated avidin (Dakopatts, Denmark) diluted to 1/5000 in TBPS, each at 4 C for 1 h. The procedures followed were the same as those used in the IgG determination already described. As shown in Fig. 2, the plasmic level of IgE specific to β-LG evaluated by PCA was highly correlated with that determined by ELISA.

Statistical Analyses. Statistically significant differences were analyzed by Student's *t* test[15] and by Duncan's multiple range test.[16]

Results

IgE-mediated degranulation and the subsequent secretion of such inflammatory substances as histamine, leucotriene, protease and so on requires the presence of IgE specific to a food antigen before challenging the antigen.[1] In a preliminary experiment, it was found that the milk-free diet during the weaning period resulted in a positive PCA reaction in ten out of eleven BN rats, whereas none of the eighteen rats weaned to the milk-containing diet exhibited a positive PCA reaction. In addition, the concentration of serum IgG specific to β-LG was also lower in the rats weaned to the milk-containing diet than in those weaned to the milk-free diet (Fig. 3). These results indicate that the milk-containing diet during the weaning period induced an oral tolerance to β-LG. BN rats weaned to the milk-free diet were hence used throughout the subsequent experiments.

After intraperitoneal sensitization for 14 days, the rats were orally challenged with saline alone or with β-LG in saline or in the fat emulsion. As shown in Fig. 4, the rats challenged with β-LG in saline, when compared to saline alone, had a higher level of serum RMCPII after the administration. The challenge for 3 h with β-LG in the fat emulsion, when compared to β-LG in saline, further increased the level of plasmic RMCPII. The administration of saline alone also tended to elevate serum RMCPII 3 h after, but without showing a statistical significance.

The concentration of mucosal LTC_4 was measured for the sensitized rats sacrificed just prior to the challenge, and for the rats challenged with saline alone, with β-LG in saline, or with β-LG in the fat emulsion. The challenge with β-LG did not cause any significant change in the concentration of LTC_4 in the gut mucosal homogenates (data not shown).

Figure 5 shows the elution patterns of β-LG and the polymer of β-LG (β-LG-POL) by Sephadex G-200 column chromatography, β-LG-POL having varying size including unpolymerized β-LG. The elevation of plasmic RMCPII due to the challenge with β-LG was also confirmed as shown

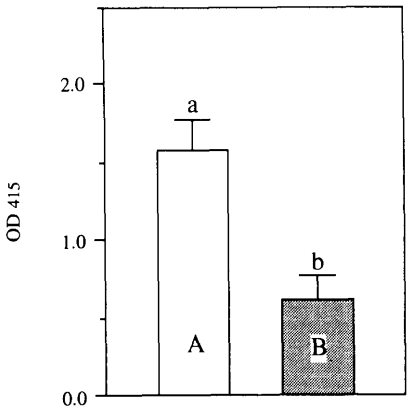

Fig. 3. Effect of Weaning Diet with or without Bovine Milk on the Level of Serum IgG Specific to β-Lactoglobulin in Immunized Rats.

Each bar shows the mean ± SE for 6 rats per group. A and B: Rats weaned to the bovine milk free-diet and bovine milk containing diet, respectively. The serum diluted 100-fold was subjected to ELISA. [ab] Different superscript letters show significant differences at $p < 0.05$.

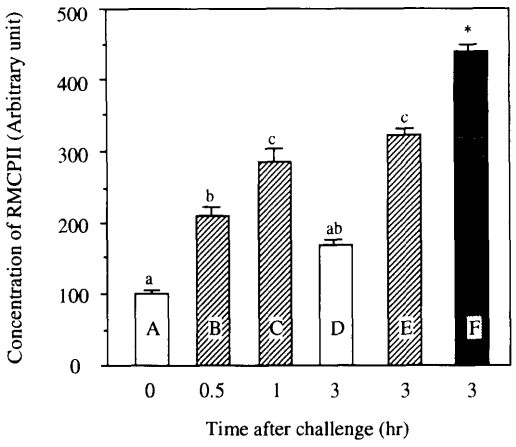

Fig. 4. Levels of Serum RMCPII in Sensitized Rats Challenged with Saline Alone and with β-Lactoglobulin.

Each bar shows the mean ± SE for 6 rats per group. Rats were sensitized with β-LG for 14 days and then gavaged with saline alone (A and D), β-LG in saline (B, C and E) or β-LG in a fat emulsion (F) for 3 hr. [abc] Different superscipt letters show significant differences at $p < 0.05$. *The values for the fat emulsion-fed rats (F) are significantly different from those for the saline-fed rats (E) at $p < 0.05$.

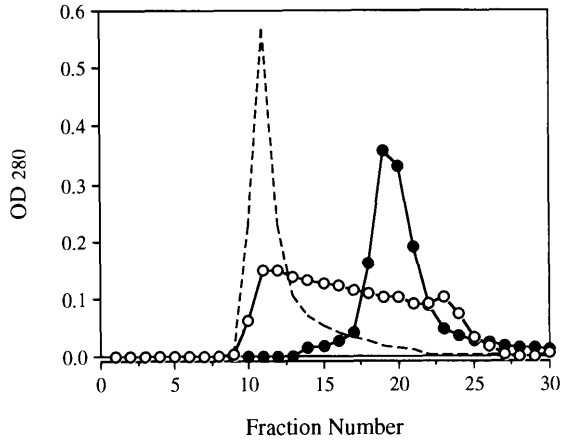

Fig. 5. Elution Profiles of β-Lactoglobulin and Its Polymer by Sephadex G-200 Column Chromatography.

β-LG (5 mg/ml) and β-LG-POL (5 mg/ml) dissolved in borate-buffered saline (0.1 M, pH 8.3) were applied to the column (2.5 × 90 cm), and the eluate of 3 ml per tube was collected. ----- Dextrane, ● β-LG, ○ β-LG-POL.

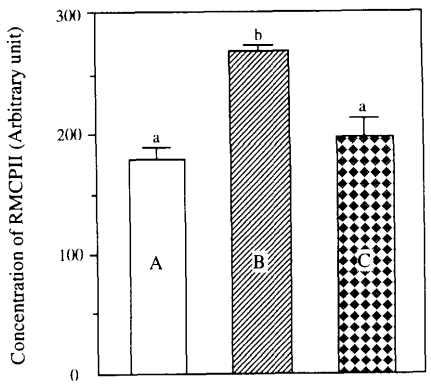

Fig. 6. Levels of Serum RMCPII in Sensitized Rats Challenged with β-Lactoglobulin and Its Polymer (β-LG-POL).

Each bar shows the mean ± SE for 5 rats per group. Sensitized rats were challenged for 3 hr with saline alone (A), β-LG in saline (B), or β-LG-POL in saline (C). [ab] Different superscript letters show significant differences at $p < 0.05$.

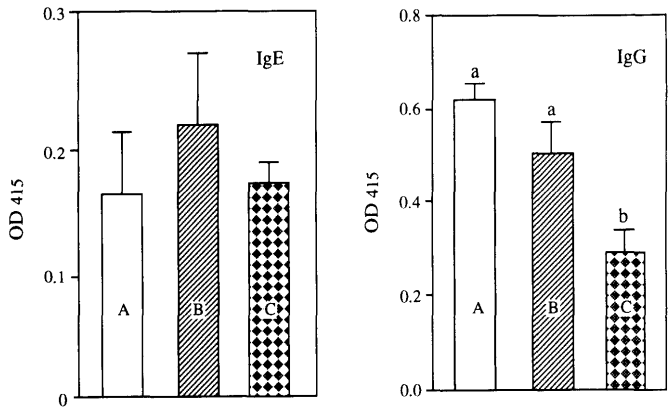

Fig. 7. Level of Serum IgE and IgG Specific to β-Lactoglobulin in Sensitized Rats Challenged with β-Lactoglobulin or Its Polymer (β-LG-POL).

Each bar shows the mean ± SE for 5 rats per group. Sensitized rats were challenged for 3 h with saline alone (A), β-LG in slaine (B), or β-LG-POL in saline (C). The serum diluted 16- and 100,000-fold for determining IgE and IgG, respectively, was subjected to ELISA. [ab] Different superscript letters show significant differences at $p < 0.05$.

in Fig. 6. In contrast to the challenge with β-LG, the sensitized rats challenged with β-LG-POL did not show any elevated plasmic RMCPII level. Figure 7 shows the level of plasmic IgG and IgE specific to β-LG 3 h after the challenge. The oral administration of β-LG, when compared to the administration of saline alone, did not cause any significant change in the plasmic levels of IgG and IgE, while the β-LG-POL administration resulted in a lower concentration of IgG but not of IgE, than did saline alone or β-LG in saline.

Discussion

In the present experiment, the immune rats showed elevated levels of serum RMCPII after the challenge with β-LG, suggesting that this animal model would be relevant to determine the allergenicity of food proteins and also to examine the principle(s) involved in alleviating food-mediated allergic reactions. The high response of RMCPII following the challenge with food antigens, which activate the mucosal mast cells through the Fc-receptor,[17] indicate that this model would also be relevant to investigate the regulatory events involved in intestinal hypersensitivity to food protein antigens.

It has been reported that there was a significant elevation after 30 min and 1 h in the concentration of LTC_4 in a perfusate and in a mucosal homogenate from the gut of immune rats challenged with *T. spiralis*.[3] In the present study, the immune rats challenged with β-LG in saline or with saline alone did not exhibit any significant effect on the concentration of LTC_4 in the mucosal homogenates. According to Turner *et al.*,[1] who have initially reported the mucosal mast-cell activation in rats after feeding with ovalbumin, the level of RMCPII detected in the circulation system after the protein antigen challenge was in the range of 1–6 μg/ml, and this level was lower than that reported in primed rats challenged intravenously with an extract of the worm, *Nippostronglylus brasilienis*, in which a level up to 400 μg/ml was recorded. These results accordingly suggest that the capacity of the gut mucosa to synthesize LTC_4 is not sufficient to elevate its concentration in a mucosal homogenate with the present β-LG challenge model. It is also plausible that cell types other than mast cells might participate nonspecifically as the source of LTC_4 in the gut mucosa.

In the present study, RMPCII release appears to have occurred in sensitized rats challenged with saline alone, even though it was not statistically significant. This presumably reflects a stress response in animals subjected to oral saline administration, MacQueen *et al.*[18] having shown that the central nervous system functions as an effector of rat mucosal mast cells in the allergic state.

β-LG dissolved in the fat emulsion, when compared to that in saline, caused a significant elevation in the level of plasmic RMCPII. Kvietys *et al.*[19] have shown that, with a histological evaluation of jejunal mucosa, the epithelial lining of the villous tips was damaged during lipid infusion, and that restitution of the lining occurred within 50 min after resuming a saline infusion. They additionally reported that a lipid infusion also resulted in increased ^{51}Cr-EDTA clearance, indicating an increased permeability of gut epithelial cells. The results of the present experiment hence suggest that the challenge with β-LG mixed with the fat emulsion induced an increase in the entry of β-LG to the mucosal epithelial cells.

HayGlass and Stefura[4] have shown that treating mice with glutaraldehyde-polymerized ovalbumin resulted in a long-lived inhibition of the allergen-specific IgE response and an increase in anti-IgG2a when injected systemically. Because of the side effect resulting from the systemic administration of a chemically modified allergen, an alternative administration route is preferable, but such trials as oral immunotherapy have been unsuccessful, probably due to extensive digestion of the allergens. In the present study, sensitized rats challenged with β-LG-POL, when compared to β-LG, showed a lower concentration of serum IgG specific to β-LG and plasmic RMCPII, thereby raising the possibility that orally administered β-LG-POL would modify the immune system. It is, however, not clear at present whether β-LG-POL was directly involved in preventing mast-cell activation or whether the amount of unpolymerized β-LG in the β-LG-POL preparation was insufficient to induce activation of the mast cells.

In summary, the present study shows an altered response of mucosal mast cells, as the gastrointestinal tract is the principal location of RMCPII in rats.[7] This animal model is thus relevant to investigate the regulatory events involved in mucosal hypersensitivity and in humoral immunity to food antigens.

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