

Effect of a prebiotic-enriched phytocompound in improving ovalbumin allergenicity

Yussef METUGRIACHUK,^{*,†} Juichiro TSUCHIYA,^{*,†} Francesco MAROTTA,[‡] Sam KAWAKITA,^{*,†} Ruichi OKURA,[†] Olivia KUROI[†] & Emilio MINELLI[‡]

^{*}Tsuchiya Medical Corporation Hospital and [†]Biokenkyusho Research Laboratory, Shizuoka, Japan; and WHO Center for Biotech and Natural Medicine, University of Milan, Italy

OBJECTIVE: The aim of the present study was to test a prebiotic-phytotherapeutic compound in an experimental model of oral allergenicity.

METHODS: Antigen-specific immunoglobulin E (IgE) elevated mice were prepared by injecting them intraperitoneally with 10 µg of ovalbumin. Subsequently, the mice were exposed to ovalbumin solution intranasally and blood samples were obtained on weekly intervals for 4 weeks to measure serum-ovalbumin-specific IgE and total immunoglobulin G. Mice with high titers of ovalbumin-IgE were intragastrically administered with 0.3 mL of phosphate buffered solution containing either 20 mg of ovalbumin, the same

solution with 5 mL of milk, or 20 mg milk added to prebiotic-phytocompound.

RESULTS: Ovalbumin administration caused a significant increase of plasma ovalbumin concentration in sensitized mice while prebiotic-phytocompound-supplemented mice showed a significantly reduced peak value ($P < 0.05$). Prebiotic-phytocompound added to milk exerted a significant effect in lowering the ovalbumin-IgE level and the total immunoglobulin G level as compared to control plain milk ($P < 0.01$).

CONCLUSION: This study provides a rationale basis for a feasible non-pharmacological therapeutic strategy in food allergen hypersensitivity syndromes.

KEY WORDS: hypersensitivity, ovalbumin, prebiotic-phytocompound.

INTRODUCTION

It has been shown that, under normal conditions, the intestinal immune system exists in a state of dynamic homeostasis with the microflora whose evolution is modulated in parallel with that of their human host.¹ This confers to the gut mucosa a leading role in the exclusion and elimination of potentially harmful dietary antigens and microorganisms, while providing the selective absorption of nutrients.² During early stages

of postnatal life, antigen exclusion and elimination and immune regulation mechanisms are known to be only partially developed² and this immature status of the gut barrier represents a predisposing condition to aberrant antigen uptake.³ Nonetheless, later in life environmental factors, when associated with mucosal inflammation, may interfere with the control of this particular phenomenon of systemic hyporesponsiveness, thus triggering a systemic immune response to trivial antigens.⁴ This is likely to perpetuate the damage to the mucosa and, in a vicious circle, further increase the permeability to bystander antigens.⁵ Several intestinal microbiota, being located in juxtamucosal niches in the intestine, are able to significantly modulate specific immune responses in gut-associated lymphoid tissue. Accordingly, it has been shown⁶ that the gut flora are

Correspondence to: Francesco MAROTTA, Via Pisanello, 4, 20146 Milano, Italy. Email: fmarchimede@libero.it

© 2006 The Authors

Journal compilation © 2006 Chinese Medical Association
Shanghai Branch, Chinese Society of Gastroenterology and
Blackwell Publishing Asia Pty Ltd.

involved in the generation of T-helper cells inducing oral tolerance, thus substantiating the use of a probiotic as therapeutic tool in hypersensitive disorders. Since interleukin (IL-4) has been shown to be implicated in atopic patients and to be responsible for the initial sensitization, a number of probiotics are under scrutiny as a potential therapeutic weapon, given the ability of some of them, for example, to decrease the production of IL-4 during casein fermentation.⁷ Further, both lactobacilli and bifidobacteria have been shown to improve the increased gut permeability occurring during exposure to food allergens.⁸ More generally, most of the immuno-stimulating effects of such bacterial strain have been evaluated by measuring in serum and intestinal immunoglobulin A (IgA) and immunoglobulin G (IgG) the increase in certain cytokines and the activation of natural killer cells.^{9,10} Overall, these findings have prompted a number of studies on probiotics engineering as a promising research area in the field of food intolerance and allergy, including intolerance to cows' milk protein and lactose and the improved digestion of these components.¹¹ Therefore, given these premises as well a number of unpublished observational reports on the effectiveness of a prebiotic-enriched phytocompound in oral hypersensitivity syndromes, the aim of the present investigation was to test one such mixed compound in a specific experimental model of oral allergenicity.

MATERIALS AND METHODS

Preparation of fermented milk

Twenty mg of Markalact (100 g: matricariae flos 1.350 g, saccharum lactis 98.5 g, Named srl, Lesmo, Italy) were added to 9% (wt/vol) of skim milk medium containing 0.1% yeast extract and fermented at 37°C for 24 h and the pH and lactic acidity were monitored. The number of cells in the fermented milk was counted after neutralization with 2.5 mol/L sodium hydroxide (NaOH). This fermented sample was used for oral administration to mice.

Preparation of antigen-specific immunoglobulin E-elevated mice

Six-week-old male Swiss mice were housed in plastic cages with sterilized woodchip bedding and bred in animal rooms kept at a temperature of $23 \pm 2^\circ\text{C}$ and a relative humidity of $55 \pm 10\%$ under a 12-h light–dark cycle. They were fed a standard diet while being allowed 1-week acclimatization with free access to water throughout the experimental period. All experimental procedures mentioned below were conducted

in accordance with international guidelines for the care and use of laboratory animals in research. Before their sensitization to antigen, blood samples were obtained from the tail vein of the mice. The mice were injected intraperitoneally with 10 μg of ovalbumin mixed with 2 mg of aluminum hydroxide in a total volume of 0.3 mL saline on days 0 and 4 (the first immunization). On day 10–16, the mice were exposed to ovalbumin solution intranasally (25 mg/mL saline) three times twice a day (second immunization). Blood samples were obtained on weekly intervals for 4 weeks from the tail veins of the mice to measure serum-ovalbumin-specific immunoglobulin E (IgE) and total IgG.

Oral administration to mice

To test the effect of prebiotic-phytocompound on the mouse serum ovalbumin-IgE levels, the mice with high titers of serum ovalbumin-IgE were selected first. After food deprivation for 12 h, mice with high titers of ovalbumin-IgE were intragastrically administered with 0.3 mL of PBS containing 20 mg of ovalbumin. This schedule was applied to mice who had been previously given for 2 weeks 1 mL/day of either non-fermented milk (control) or milk added with prebiotic-phytocompound.

Blood samples

Blood samples from mice after the administration of test samples were obtained from the tail veins before the first sensitization on day 17 and on day 22. Serum samples were prepared by centrifugation of the blood samples at 900 g for 15 min after incubating them on ice for 3 h. Serum samples were stored at -80°C until used. Serum samples with high titers of ovalbumin-IgE were prepared in advance by the procedure described above and used as a positive standard for the quantitative analysis of ovalbumin-IgE in mice.

ELISA of ovalbumin-specific IgE

For measuring the ovalbumin-IgE titers in mice serum, a sheep polyclonal antibody to mouse IgE was diluted with PBS (137 mmol/L sodium chloride, 8.1 mmol/L disodium hydrogen phosphate, 2.7 mmol/L potassium chloride and 1.5 mmol/L potassium dihydrogen phosphate, pH 7.4) to 10 $\mu\text{g}/\text{mL}$. Then 100 μL of the diluted antibody was soaked into each well of a microplate and the microplate was stored overnight at 4°C . The microplate was washed three times with PBS and coated with 0.5% casein-PBS for 3 h at room temperature. After the microplate was washed with PBS, 100 μL portions of diluted mouse serum and standard positive serum were added to each well and the plate was incubated overnight at 4°C . After the plate was washed

with PBS, ovalbumin that had been biotinylated using a specific kit (American Qyulex, CA) was diluted with 0.5%-casein PBS (10 µg/mL), was added to each well. The plate was then incubated for 2 h at room temperature. After washing the plate with PBS, 100 µL of streptavidin-peroxidase diluted to 0.5 µg/mL with 0.5% casein-PBS was added to each well and incubated for 1 h at room temperature. After the plate was washed five times with 0.1%-Tween 20 in PBS, 100 µL of ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid, 0.0006% H₂O₂ in 0.2 mol/L citric acid buffer, pH 4.7) was added to the wells. After incubation for 3 h at 37°C in the dark, absorbance at 405 and 492 nm was measured using a microplate reader. The value of ($OD_{405} - OD_{492}$) was used for the specific absorbance from ABTS. The assay sensitivity was 2.5–250 ng/mL, and the samples were appropriately diluted.

ELISA of IgG

To measure the concentrations of total IgG in mice, goat polyclonal antimouse IgG was diluted to 10 µg/mL with PBS. Then, 50 µL of diluted antibody was soaked into each well of a microplate. After overnight incubation at 4°C, the microplate was washed three times with PBS and was blocked with 0.5% casein-PBS for 3 h at room temperature. After the plate was washed with PBS, 50 µL portions of the serum samples were added to each well of the IgG plate, which was washed four times with PBS. Peroxidase-labeled goat antimouse IgG and biotin-labeled rat monoclonal antimouse IgE were diluted 500-fold with 0.5% casein-PBS. Diluted goat antimouse IgG (50 µL) was added to the IgG plate. Each plate was incubated for 2 h at room temperature. After washing, 1.5 µg/mL of streptavidin peroxidase was added followed by incubation at 25°C for 1 h. After washing, tetramethylbenzidine substrate solution was added to produce a color reaction. The reaction was blocked by the addition of 6 N sulfuric acid (H₂SO₄). Optical density was determined at 450 nm using the same methodology as above.

Statistical analysis

The data were subjected to statistical analysis and results were expressed as mean ± S.D. Significance of differences was determined by use of a two-tailed Student's *t*-test. A probability value of less than 0.05 was considered to be statistically significant.

RESULTS

During the study period, the mice showed no adverse effect nor any bowel movement abnormality.

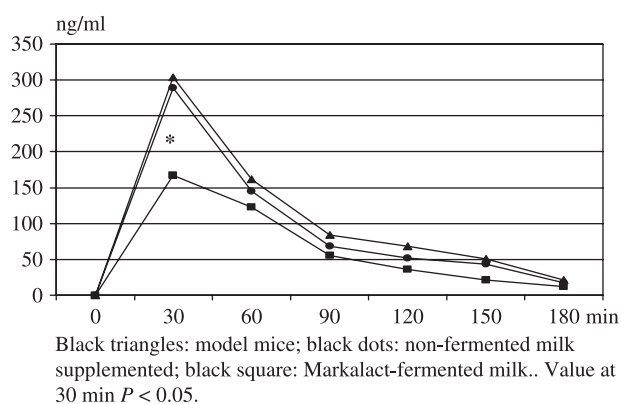


Figure 1. Plasma ovalbumin level after oral administration of ovalbumin in sensitized mice: effect of supplementation with non-fermented milk and with Markalact-fermented milk.

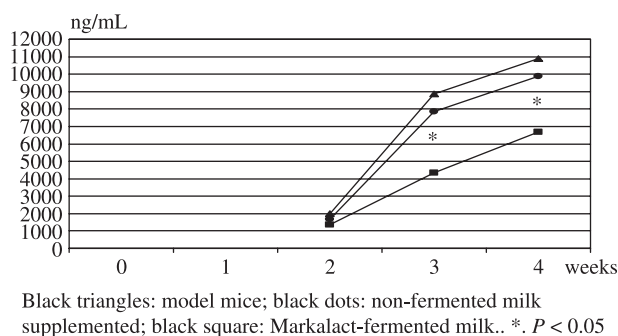


Figure 2. Ovalbumin-specific immunoglobulin E in sensitized mice: effect of supplementation with non-fermented milk and with Markalact-fermented milk.

Effect of markalact on plasma level of ovalbumin

As shown in Figure 1 during the 'acute' study, ovalbumin administration brought about a significant increase of plasma ovalbumin concentration in sensitized mice with a peak at 30-min sampling ($P < 0.01$ vs baseline). While non-fermented milk-supplemented mice did not show any difference from the model mice, the Markalact-supplemented mice showed a significantly reduced peak value ($P < 0.05$).

Effect of Markalact-fermented milk on serum immunoglobulin E and immunoglobulin G levels

Markalact-fermented milk had a significant effect in lowering the ovalbumin-IgE level as compared to control milk (Fig. 2, $P < 0.01$). There was no correlation between the ovalbumin-IgE lowering effect and the pH, acidity or the number of cells in the fermented milk (data not shown). Also total IgG was significantly

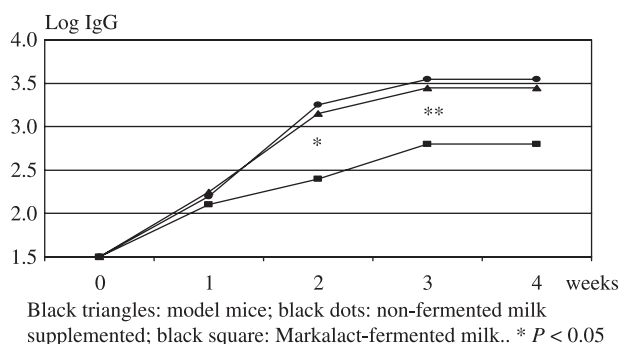


Figure 3. Total immunoglobulin G in ovalbumin sensitized mice: effect of supplementation with non-fermented milk and with Markalact-fermented milk.

raised in ovalbumin-sensitized mice (Fig. 3, $P < 0.001$ vs baseline) and this phenomenon was significantly reduced in the mice given Markalact-fermented milk ($P < 0.05$ vs control model). No benefit appeared when employing only the milk medium.

DISCUSSION

Probiotic lactic acid bacteria (LAB) are able to modulate the host immune system and clinical trials have demonstrated that specific strains have the capacity to reduce allergic symptoms.^{12,13} This is because the intestinal microflora are known to contribute to antigenic exclusion¹⁴ by preventing the adherence of antigens through competition for nutrients, by producing antimicrobial agents and interferons¹⁵ and by increasing the production of specific antibody-secreting cells and mucus. Thus, antigen elimination involves the ability of the mucosa to process antigens and decrease the antigen load to the immune system. Immunoglobulin E is one of the major mediators of the immediate hypersensitivity reactions that underlie atopic conditions such as urticaria, seasonal allergy, asthma and anaphylaxis. Unlike other immunoglobulin isotypes, IgE has the peculiarity to display very low plasma levels and short half-life. Also prebiotics are important regulatory component of gut ecology and have been recently considered as potential targets of therapeutic intervention in allergic diseases.^{16,17} Moreover, Moro and Arslanoglu¹⁸ have suggested how a prebiotic mixture could dose-dependently mimic a bifidogenic effect. Although lactose in dairy products is maldigested by up to 70 to 75 percent of the world's population and is often experienced by people suffering from irritable bowel syndrome, it has also a number of prebiotic characteristics as recently outlined.¹⁹ This may be in agreement with earlier report suggesting the role of fermented milk, as compared to base milk, in modifying

the enzymatic characteristics of lactobacilli flora in the gut.²⁰ Thus, in the present study we used a lactose-phytocompound mixture in an established model of food allergenicity. Interestingly, our first finding was that mice co-treated with the prebiotic-phytocompound-added milk showed a significantly lower plasma level of ovalbumin. Although the antigenicity of macromolecules is characterized by several factors, such as the foreignness to the host, their molecular complexity, solubility and stability, and by their concentration,²¹ we can make a preliminary inference that our treatment decreased the gut absorption of ovalbumin. Accordingly, the rise of plasma concentration of either ovalbumin-specific IgE and the total IgG were significantly mitigated. The proinflammatory cytokines locally secreted by intestinal absorption of antigen can directly disrupt the tight junctional integrity and this can cause further leakage of macromolecules in their intact forms.²² Although we did not perform microbiological examinations, it is likely that the gut flora modification induced by the prebiotic-phytocompound must have played a role. Interestingly, quite recently Matsumoto and Benno,²³ investigating the relationship between probiotic metabolites and host cytokine production, have demonstrated that some intestinal bacterial metabolites produced by a specific yogurt consumption contributed to suppressing the inflammatory cytokine produced by macrophages. Moreover, the (albeit mild) anti-inflammatory properties of matricariae²⁴ contained in the compound is likely to be a contributory factor. Taken together, such preliminary data confirm that oral allergen-related disease may benefit from a prebiotic-supplemented diet while the present lactose-matricariae mixture may be a feasible candidate in such therapeutic regimes.

REFERENCES

- 1 Conway PL. Microbial ecology of the human large intestine. In: Gibson GR, Macfarlane GT, eds. *Human Colonic Bacteria. Role in Nutrition, Physiology and Pathology*. Boca Raton: CRC Press, 1995; 1–18.
- 2 Brandzaeg P. Molecular and cellular aspects of the secretory immunoglobulin system. *APMIS* 1995; 103: 1–19.
- 3 Teichberg S, Isolauri E, Wapnir RA, Roberts B, Lifshitz F. Development of neonatal rat small intestinal barrier to non-specific macromolecular absorption: effect of early weaning to artificial diets. *Pediatr Res* 1990; 28: 31–7.
- 4 Macpherson A, Khoo UY, Forgacs I, Philpott-Howard J, Bjarnason I. Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. *Gut* 1996; 38: 365–75.
- 5 Wyatt J, Vogelsang H, Hub W, Waldhoer T, Lochs H. Intestinal permeability and the prediction of relapse in Crohn's disease. *Lancet* 1993; 341: 1437–9.
- 6 Sudo N, Sawamura S, Tanaka K, Aiba Y, Kubo C, Koga Y. The requirement of intestinal bacterial flora for the development

- of an IgE production system fully susceptible to oral tolerance induction. *J Immunol* 1997; **159**: 1739–45.
- 7 Sutas Y, Soppi E, Korhonen H, Syvaoja EL, Saxelin M, Rokka T. Suppression of lymphocyte proliferation *in vitro* by bovine caseins hydrolysed with *Lactobacillus* GG derived enzymes. *J Allergy Clin Immunol* 1996; **98**: 216–24.
 - 8 Rautava S, Kalliomaki M, Isolauri E. New therapeutic strategy for combating the increasing burden of allergic disease: Probiotics-A Nutrition, Allergy, Mucosal Immunology and Intestinal Microbiota (NAMI) Research Group report. *J Allergy Clin Immunol* 2005; **116**: 31–7.
 - 9 Ogawa T, Asai Y, Tamai R *et al.* Natural killer cell activities of synbiotic *Lactobacillus casei* ssp. *casei* in conjunction with dextran. *Clin Exp Immunol* 2006; **143**: 103–9.
 - 10 Roller M, Rechkemmer G, Watzl B. Prebiotic inulin enriched with oligofructose in combination with the probiotics *Lactobacillus rhamnosus* and *Bifidobacterium lactis* modulates intestinal immune functions in rats. *J Nutr* 2004; **134**: 153–6.
 - 11 Sanders MA. Summary of conclusions from a consensus panel of experts on health attributes of lactic cultures; significance to fluid milk products containing cultures. *J Dairy Sci* 1993; **76**: 1819–28.
 - 12 Strobel S, Mowat AM. Oral tolerance and allergic responses to food proteins. *Curr Opin Allergy Clin Immunol* 2006; **6**: 207–13.
 - 13 Ogawa T, Hashikawa S, Asai Y, Sakamoto H, Yasuda K, Makimura Y. A new synbiotic, *Lactobacillus casei* subsp. *casei* together with dextran, reduces murine and human allergic reaction. *FEMS Immunol Med Microbiol* 2006; **46**: 400–9.
 - 14 Bengtmark S, Jeppsson B. Gastrointestinal surface protection and mucosa reconditioning. *J Parent Enteral Nutr* 1995; **19**: 410–5.
 - 15 DeSimone C, Bianchi-Salvadori B, Negri R, Ferrazi M, Baldinelli L, Vesely R. The adjuvant effect of yogurt on production of gamma-interferon by ConA-stimulated human peripheral blood lymphocytes. *Nutr Rep Int* 1986; **33**: 419–33.
 - 16 Noverr MC, Huffnagle GB. The 'microflora hypothesis' of allergic diseases. *Clin Exp Allergy* 2005; **35**: 1511–20.
 - 17 Vos AP, Haarman M, Buco A *et al.* A specific prebiotic oligosaccharide mixture stimulates delayed-type hypersensitivity in a murine influenza vaccination model. *Int Immunopharmacol* 2006; **6**: 1277–86.
 - 18 Moro GE, Arslanoglu S. Reproducing the bifidogenic effect of human milk in formula-fed infants: why and how? *Acta Paediatr Suppl* 2005; **94**: 14–7.
 - 19 Szilagyi A. Redefining lactose as a conditional prebiotic. *Can J Gastroenterol* 2004; **18**: 163–7.
 - 20 Garvie EL, Cole CB, Fuller R, Hewitt D. The effect of yoghurt on some components of the gut microflora and on the metabolism of lactose in the rat. *J Appl Bacteriol* 1984; **56**: 237–45.
 - 21 Thompson PJ, Stewart GA. Allergens. In: Holgate ST, Church MK, eds. *Allergy*. London: Gower Medical Publishing, 1993; 1.1–1.14.
 - 22 Rugtveit J, Nislen EM, Bakka A, Carlsen H, Brantzaeg P, Scott H. Cytokine profiles differ in newly recruited and resident subset of mucosal macrophages from inflammatory bowel disease. *Gastroenterology* 1997; **112**: 1493–505.
 - 23 Matsumoto M, Benno Y. Anti-inflammatory metabolite production in the gut from the consumption of probiotic yogurt containing *bifidobacterium animalis* subsp. *lactis* LKM512. *Biosci Biotechnol Biochem* 2006; **70**: 1287–92.
 - 24 Akihisa T, Yasukawa K, Oinuma H *et al.* Triterpene alcohols from the flowers of compositae and their anti-inflammatory effects. *Phytochemistry* 1996; **43**: 1255–60.