

Allergenicity of Bos d 5 in Children with Cow's Milk Allergy is Reduced by Transglutaminase Polymerization

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Background: Cow's milk allergy in pediatric patients is an unresolved issue. Among the proteins in milk, bovine whey beta-lactoglobulin (Bos d 5) is the most commonly allergenic. Allergenicity to native cow's milk proteins in humans is a well-studied issue, but very little is known about the allergenicity of cross-linked proteins found in bioprocessed dairy products.

Objective: The objective of this study was to evaluate the allergenicity of polymerized bovine whey beta-lactoglobulin in symptomatic children diagnosed with IgE-mediated Bos d 5 hypersensitivity.

Methods: Side-by-side skin prick tests with native and polymerized bovine whey beta-lactoglobulin were performed in 22 symptomatic children allergic to cow's milk with detectable specific IgE to Bos d 5 by CAP Systems Pharmacia. A matched control group tolerant to cow's milk and undetectable specific IgE to Bos d 5 was established for comparison. Wheal mean diameter was compared between the native and polymerized groups by paired *t*-tests.

Results: The mean difference in wheal mean diameter observed between native versus polymerized bovine whey beta-lactoglobulin in the paired skin prick test of the allergic group was 2.27 mm ($p=0.02$; 95% CI 0.38–4.16).

Conclusions: The skin prick test showed a significant reduction in the allergenicity of polymerized compared with native bovine whey beta-lactoglobulin in children with IgE-mediated Bos d 5 hypersensitivity.

Introduction

COW'S MILK ALLERGY in pediatric patients is an unresolved issue of worldwide importance. Among bovine whey proteins, beta-lactoglobulin (BLG; Bos d 5) is one of the most prevalent allergens as evidenced by oral challenges, skin scratch tests, and skin prick tests (SPT).¹ The allergenicity of native cow's milk proteins in humans is a well-studied issue, but little is known about the allergenicity of cross-linked proteins found in bioprocessed dairy products.² An *ex vivo* reduction of polymerized whey immunoreactivity in humans has been demonstrated by immunoblot, CAP, and ELISA inhibition assay.³ Because polymerization is a food engineering process used for some dairy products such as cheese and yoghurts, there is a reason to study the effect of polymerized whey proteins on allergic people. One of the most common techniques employed by food engineers is microbial transglutaminase polymerization in the presence of cysteine.⁴ Microbial transglutaminase (TG) catalyzes acyl-transfer reactions between gamma-carboxamide groups of glutamine residues and the epsilon-amino group of lysine in

proteins, leading to inter- or intramolecular cross-linking. Previous studies in mice have shown that polymerization by TG in the presence of cysteine may reduce beta-lactoglobulin immunogenicity.⁵ This phenomenon has not been demonstrated in humans by *in vivo* methods. The objective of this study was to examine the allergenicity of bovine beta-lactoglobulin polymerized by transglutaminase in the presence of cysteine (TgPol-BLG) in children with a clinical diagnosis of IgE-mediated allergy to Bos d 5 (BLG).

Material and Methods

Study design and subjects

This study was submitted and approved by the Institutional Research Ethics Board Review and registered in the Brazilian National Ethic Research System (SISNEP 409/2008). Signed consent forms were obtained from all parents. There were two groups. The participants allergic to cow's milk (allergic group) included 22 children (14 male) aged between 6 and 95 months (mean 30.6; SD 27.1; 95% CI

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18.5–42.6) with a convincing clinical history of adverse reactions to cow's milk and evidence of serum IgE antibodies specific to beta-lactoglobulin quantified by CAP Systems Pharmacia. The participants tolerant to cow's milk (matched control group) included 22 children (13 male) aged between 6 and 93 months (mean 30.8; SD 26.8; 95% CI 18.9–42.7) selected by their unequivocal tolerance to cow's milk and undetectable specific IgE to beta-lactoglobulin (by CAP Systems Pharmacia) who were asymptomatic (five children) or presenting with minor upper respiratory symptoms (rhinitis).

Allergens and reagents

BLG (>95% purity) was provided by Davisco Foods Inc. (Le Sueur, MN). A commercial preparation of transglutaminase from *Streptococcus mobaraense* (Activa®), a mixture containing 99% maltodextrin and 1% transglutaminase (TG) with 100 U g⁻¹ of declared activity, was donated by Ajinomoto Co. (Tokyo, Japan). Cysteine was obtained from Sigma-Aldrich Chemical Co. (Steinheim, Germany).

BLG polymerization

BLG 7% (w/v) was added to cysteine solution (0.25 mol L⁻¹), the pH was adjusted to 8.0, and TG was then added (25 U g⁻¹ substrate). The polymerization reaction was conducted at 50°C for 180 min, stopped by cooling to 4°C in a water/ice bath, frozen, and then lyophilized before the analyses. The final lyophilized product contained about 1 g of bovine whey beta-lactoglobulin polymerized by transglutaminase in presence of cysteine (TgPol-BLG), approximately 0.25 g TG, and 0.428 g cysteine for each 1.678 g of product. Nitrogen was quantified by Kjeldahl's method (AOAC 991.22), which indicated 14.4 g of nitrogen per g of lyophilized BLG and 11.1 g of nitrogen per g of lyophilized TgPol-BLG.

Preparation of solutions for skin tests

For the skin tests, whey proteins and controls were solubilized at pH 7.0 and diluted to their final concentrations with 20% w/v glycerol/saline solution (20% GS). The BLG solution was diluted to a concentration of 0.7 mg mL⁻¹ with 20% GS, and the TgPolBLG solution was diluted to a final concentration of 0.9 mg mL⁻¹ with 20% GS. These concentrations were designed to contain 10,000 PNU mL⁻¹. TG and cysteine skin allergenicities were tested simultaneously. They were diluted in the equivalent concentrations used to prepare TgPolBLG. The TG solution for the skin tests was diluted to 0.2 mg mL⁻¹ with 20% GS, and the cysteine solution for the skin tests was diluted to 0.38 mg mL⁻¹ with 20% GS. A positive control was performed with histamine 1 mg mL⁻¹ in 20% GS, and the negative control was 20% GS alone.

Skin prick test

Skin prick tests (SPT) were performed on the backs of babies or on the volar part of the forearms of cooperative children with a disposable sterile stainless steel lancet with a 1 mm tip (purchased from FDA Allergenic Co., Brazil). The lancet was introduced at a 90° angle perpendicular to the skin, retrieved after 1 sec and discarded. Wheal mean diameter (WMD) was assessed 15 min after the application of the allergen extracts. After subtracting each patient's reaction

to the negative control, a corrected WMD ≥ 3 mm indicated a positive reaction.

Specific IgE measurements

Serum IgE specific to Bos d 5 was measured by CAP Systems Pharmacia, and the results are expressed as kU L⁻¹.

Statistical analyses

All statistical analyses were performed with GraphPad Prism software (v5.0; GraphPad Software, Inc., San Diego, CA). Differences in the means of matched samples were assessed with paired *t*-tests. Box column graphs with 95% CI whiskers were plotted with the means. The data are reported as the arithmetic mean with a 95% CI. For all analyses, a *p*-value of less than 0.05 was considered statistically significant. The D'Agostino & Pearson omnibus *K*² normality test was applied for the skin test results.

Results

Tolerant control group

There were no SPT reactivity with BLG in 20% GS, TgPolBLG in 20% GS, TG in 20% GS, cysteine in 20% GS, or the negative control in the patients of the tolerant matched control group. The mean WMD for histamine (positive control) obtained by SPT was 6.3 mm (95% CI 5.4–7.1; SD 1.8 mm; SE 0.4 mm), which passed on the D'Agostino & Pearson omnibus *K*² normality test ($\alpha=0.05$; *K*²=1.7; *p*=0.4).

Cow's milk allergic group

Clinical features and test results of the allergic group are shown in Table 1. The mean level of IgE specific to BLG was 10.9 kU L⁻¹ (95% CI 5.4–16.4; SD 12.3 kU L⁻¹; SE 2.6 kU L⁻¹). There was no SPT reactivity against TG in 20% GS, cysteine in 20% GS, or the negative control. The mean WMD for histamine (positive control) obtained by SPT was 7.7 mm (95% CI 6.5–8.9; SD 2.67 mm; SE 0.57 mm), which passed on the D'Agostino & Pearson omnibus *K*² normality test ($\alpha=0.05$; *K*²=0.83; *p*=0.65). The mean WMD for BLG obtained by SPT was 4.2 mm (95% CI 2.5–5.9; SD 3.8 mm; SE 0.8 mm), which passed on the D'Agostino & Pearson omnibus *K*² normality test ($\alpha=0.05$; *K*²=4.68; *p*=0.09). The mean WMD for TgPolBLG obtained by SPT was 2.0 mm (95% CI 0.9–3.0; SD 2.4 mm; SE 0.5 mm), which passed on the D'Agostino & Pearson omnibus *K*² normality test ($\alpha=0.05$; *K*²=4.14; *p*=0.12). The mean difference in WMD observed between native versus polymerized bovine whey beta-lactoglobulin in the paired SPT was 2.27 mm (*p*=0.02; 95% CI 0.38–4.16). These data are presented in Figure 1.

Discussion

This study was designed under the direction of the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) 2001 decision tree for analyzing the allergenicity of foods derived from biotechnology, which recommends performing skin allergy tests with the native and bioprocessed proteins in people with a known allergy to the native protein.⁶ Among the proteins in whey, Bos d 5 is the most important due to its relatively high concentration in cow's milk (mean: 3–4 g L⁻¹) and

TABLE 1. CLINICAL FEATURES OF PATIENTS ALLERGIC TO COW'S MILK AND TEST RESULTS

Patient	Age (months)	s-IgE kU L^{-1}	WMD histamine	WMD BLG	WMD TGPoBLG	Clinical manifestation
1	6	12.57	3	8	3	Urticaria
2	7	5.10	4	4	0	Urticaria
3	8	41.92	10	8	0	Wheezing
4	8	17.89	10	5	3	IGH
5	8	24.32	10	8	4	Wheezing
6	9	1.88	7	8	0	Urticaria
7	12	1.60	5	3	3	Urticaria
8	12	1.56	6	0	0	Atopic dermatitis
9	13	40.77	9	6	0	Urticaria
10	16	16.07	12	15	5	Urticaria
11	21	8.06	6	5	0	Atopic dermatitis
12	24	17.79	8	3	5	Oral allergy syndrome
13	28	5.90	7	0	7	Atopic dermatitis
14	28	5.52	9	5	3	Urticaria
15	35	1.20	10	0	0	Urticaria
16	37	1.44	7	5	0	Atopic dermatitis
17	39	1.59	7	0	0	Atopic dermatitis
18	47	2.04	9	0	0	Atopic dermatitis
19	52	3.39	7	5	6	Wheezing
20	75	4.68	5	0	5	Atopic dermatitis
21	94	23.53	5	0	0	Wheezing
22	95	1.52	14	6	0	Wheezing

S-IgE: specific IgE against Bos d 5 (CAP Phadia); BLG: bovine whey beta-lactoglobulin (Bos d 5); TGPoBLG: bovine whey beta-lactoglobulin polymerized by transglutaminase in the presence of cysteine; WMD: skin prick test wheal mean diameter; TG: microbial transglutaminase from *Streptococcus thermophilus* (Activa®); IGH: immediate gastrointestinal hypersensitivity.

substantial allergenicity, as human breasts do not produce beta-lactoglobulin. To standardize this study with previous works, we opted to dilute the skin solutions of BLG and TGPoBLG to 10,000 PNU m L^{-1} as used by Goldman.⁷ The patients enrolled in this study (allergic group) were not representative of the general population. They were selected due to their diagnosed clinical hypersensitivity to cow's milk and presence of seric IgE antibodies specific to BLG. Industrial dairy products may be a potential source of immunological sensitization due to the modification of native proteins. Protein polymerization in dairy products by bacterial TG is a common technique to decrease whey syneresis, and improve the gel strength and viscosity of set-type yoghurt and curd cheese.⁸ Because there is evidence that

polymerization may reduce the allergenicity of beta-lactoglobulin⁵ and ovalbumin in mice,⁹ there is a possibility that the polymerization technique could be used to reduce the allergenicity of foods as an "allergoid" desensitization immunotherapy.¹⁰ In a similar way, the use of dietary baked milk had also been proved useful to accelerate the resolution of cow's milk allergy in children.¹¹ The skin test results show a significant reduction of reactivity of TGPoBLG compared with native BLG. Nevertheless, despite average skin reactivity to TGPoBLG being lower, two patients showed no skin reaction to BLG and positive skin reactivities to TGPoBLG. This indicates that transglutaminase polymerization may be deleterious for some patients. To define this better, it would be necessary a challenge protocol to native and polymerized beta-lactoglobulin. Therefore, one must have caution in using general conclusions when dealing with a patient allergic to cow's milk. As a secondary objective, we analyzed skin reactivity to TG and cysteine. Skin tests against TG and cysteine were used to distinguish true skin reactivity against TGPoBLG from a possible reactivity against TG and cysteine. No patient showed a reaction to either TG or cysteine. Gastrointestinal manifestations (oral allergy syndrome and immediate gastrointestinal hypersensitivity) were diagnosed in two patients according to Sampson et al.¹² Oral allergy syndrome was characterized by immediate swelling of the tongue and the lips after contact with cow's milk. Immediate gastrointestinal hypersensitivity was characterized in one patient by gastrointestinal symptoms associated with cow's milk ingestion: vomiting (after some minutes) and diarrhea (after 2 h).

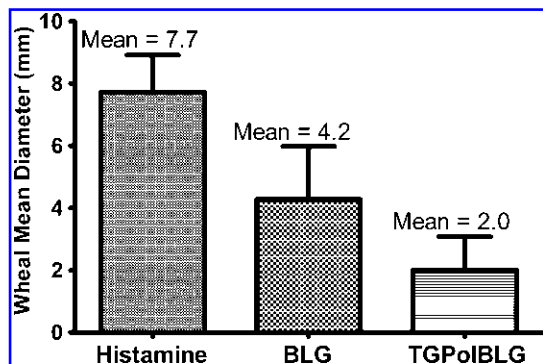


FIG. 1. Box column graphs with 95% CI whiskers from skin prick tests of 22 patients allergic to cow's milk with IgE-mediated Bos d 5 allergy. The positive control (histamine), native beta-lactoglobulin (BLG), and bovine beta-lactoglobulin polymerized by transglutaminase in the presence of cysteine (TGPoBLG) are shown.

Conclusions

Transglutaminase polymerization may be a useful tool for reducing the allergenicity of Bos d 5 in dairy products. This

reduced allergenicity may also be useful to perform allergic oral desensitization as an “allergoid.” Additional studies on the immunogenicity of native and polymerized beta-lactoglobulin must be performed to clarify these issues.

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Author Disclosure Statement

No competing financial interests exist.

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