

Is it just lactose intolerance?

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ABSTRACT

Acquired delayed-onset hypolactasia is a common autosomal recessive condition. Cow's milk allergies, conversely, are less common conditions that may manifest with equivalent symptoms and are able to simulate and/or aggravate lactose intolerance. This study was designed to evaluate the contribution of IgE-mediated cow's milk sensitization to the symptomatology of adult patients with lactose-free diet refractory lactose intolerance. Forty-six adult patients with lactose intolerance and persistent symptoms despite a lactose-free diet underwent skin-prick test to investigate cow's milk, goat's milk, and soy protein-specific-IgE. SDS-PAGE immunoblotting was used to investigate the presence of cow's milk protein-specific IgE. The percentage of patients who had skin reactions to whole cow's milk, alpha-lactalbumin, beta-lactoglobulin, caseins, goat's milk, and soy was 69.5, 36.9, 56.5, 56.5%, 54.3, and 50%, respectively. The percentage of patients with immunoblot-detected IgE specific for alpha-lactalbumin, beta-lactoglobulin, caseins, and bovine serum albumin was 21.7, 63, 67.3, and 2.1%, respectively. IgE-mediated sensitization to cow's milk is a frequent comorbidity in subjects with lactose-free diet refractory lactose intolerance and is worth consideration in patients with this condition.

(Allergy Asthma Proc 33:432–436, 2012; doi: 10.2500/aap.2012.33.3584)

Characterizing adverse reactions related to cow's milk is a research point indicated by the Diagnosis and Rationale for Action against Cow's Milk Allergy guidelines.¹ Generally, a simplified dual perspective is proposed to distinguish immunologic (allergic) reactions from nonimmunologic (enzymic or metabolic) reactions.^{2–4} Several excellent reviews have defined the guidelines for differentiating allergy from enzymic intolerance; however, despite the theoretical reasoning of this scientific approach, there are some patients who simply do not "fit" into these categories.⁵ One of the most common intolerance syndromes is lactose intolerance due to hypolactasia.⁶ The gold standard for the diagnosis of lactose absorption syndromes is a deficiency of lactase activity measured directly in enterocytes;⁷ however, this is an invasive test that can be satisfactorily replaced by noninvasive functional

tests, such as the lactose hydrogen breath test,^{8,9} the glucose-monitored lactose tolerance test,¹⁰ and genetic tests.¹¹ Allergic skin tests and specific IgE assays are used to diagnose immunologic sensitization.^{12–14} This clinical diagnosis is not easy to make because even individuals with severe hypolactasia may tolerate the ingestion of a certain amount of milk because of their colonic microbiota and/or individual low gut sensitivity.⁶ Therefore, when diagnosing an adult with adverse reactions to cow's milk, a physician may follow the statistical trends that report a high prevalence of hypolactasia (which may reach 100% of individuals in certain ethnic groups)³ rather than investigating the possibility of a cow's milk allergy.¹⁵ This trend may create a diagnostic bias, with more and more individuals being clinically labeled as lactose intolerant and fewer and fewer individuals being properly investigated for cow's milk allergies. To test this concept, we evaluated lactose-intolerant individuals who were refractory to a lactose-free diet for an IgE-mediated sensitization to cow's milk proteins.

MATERIALS AND METHODS

Study Design and Subjects

This study was submitted to and approved by the Institutional Research Ethics Review Board and was registered in the Brazilian National Ethical Research System (SISNEP 409/2008). Signed consent forms were obtained from all patients in accordance with the Helsinki Declaration. The participants included 46 adults (14 male patients) between the ages of 18 and 70 years (mean, 45.2 years; SD, 12.7) who had convincing clini-

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CE Olivier is funded by a Ph.D. postgraduate fellowship. CR Pavan and SLS Lorena received grant support from Gastrocentro of the State University of Campinas in São Paulo, Brazil. RAPG dos Santos, RP dos Santos Lima, DG Pinto, and MD da Silva received grant support from Instituto Alergoimuno de Americana, São Paulo, Brazil. R de Lima Zollner received grant support from CNPq and FAPESP

The authors have no conflicts of interest to declare pertaining to this article
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cal histories of adverse reactions to cow's milk. Lactose-free diet refractory milk intolerance was identified when the dietetic direction to avoid lactose-containing foods was not sufficient for the remission of symptoms. All of the subjects underwent hydrogen breath tests to prove lactose intolerance. They were further studied for IgE-mediated sensitization to whole cow's milk, isolated cow's milk proteins, goat's milk, and soy proteins by means of immediate skin-prick tests (SPTs). The participants were also studied for IgE-mediated sensitization to cow's milk proteins that were separated by SDS-PAGE. Fifteen patients (32.6%) exhibited intestinal symptoms that were elicited by lactose-free dairy products (matured cheese and/or lactose-free milk). Twenty patients (43.4%) exhibited associated extraintestinal allergic/atopic symptoms such as urticaria, atopic dermatitis, persistent rhinitis, and/or asthma.

A group of 20 subjects with clinical tolerance to cow's milk was used as a control for the immunoblotting assays and skin tests (6 male patients; mean age, 21.9 years; SD, 17.6 years).

Lactose Hydrogen Breath Test

The subjects were asked to eat a carbohydrate-free diet on the day before the test. After a 12-hour overnight fast, the subjects rinsed their mouths with a wash containing 50 mL of a 1% chlorhexidine solution. Then, two baseline end-expiratory breath air samples were collected at 10-minute intervals using a gas sample collection device. After that step, the participants ingested 50 g of lactose diluted in water. Breath air samples were then taken at 10-minute intervals for 90 minutes and then every 15 minutes up to 180 minutes. The hydrogen concentration from each collected sample was measured on a gas chromatograph (model 12 Microlyzer; QuinTron Instrument Co., Milwaukee, WI) in parts per million (ppm). The difference between the greatest breath hydrogen concentration measured after lactose ingestion and the baseline value was calculated for each test and represented the delta hydrogen excretion. A delta hydrogen excretion of >20 ppm was considered significant for a diagnosis of lactose intolerance.^{16,17}

Skin-Prick Test

A mean wheal diameter (MWD) ≥ 3 mm was considered as positive.¹² SPT reactivity was assessed with commercial glycerinate extracts of cow's milk (20% w/v), bovine α -lactalbumin (Bos d 4; 20% w/v), bovine casein (Bos d 8; 20% w/v), goat's milk (20% w/v), and soy (20% w/v), which were purchased from FDA Allergenic, Rio de Janeiro, Brazil. The bovine β -lactoglobulin (Bos d 5; 20% w/v; >95% purity) was provided by Davisco Foods, Inc. (Le Sueur, MN).

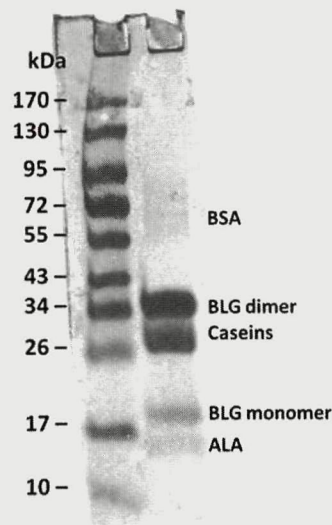


Figure 1. Coomassie blue-stained polyacrylamide gel after the electrophoresis of skimmed cow's milk. BSA, bovine serum albumin; BLG, β -lactoglobulin; ALA, α -lactalbumin.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The samples were analyzed by SDS-PAGE under reducing and denaturing conditions using a Mini Protean Tetra Cell apparatus (Bio-Rad, Hercules, CA) and 5% T/20% C polyacrylamide gel gradients.¹⁸ Lyophilized skimmed cow's milk samples were diluted (4 mg/mL) in a buffer containing 2% SDS and 5% β -mercaptoethanol (without heating). A 5- μ L aliquot of the sample was loaded into each lane. A 10- to 170-kDa prestained protein ladder (PageRuler; Fermentas, Hanovert, MD) was used to identify approximate molecular weights. The gel was run in Tris-glycine running buffer (25 mM of Tris base, 190 mM of glycine, and 0.1% SDS, pH 8.3). The binding analysis was restricted to well-characterized cow's milk proteins: caseins, β -lactoglobulin, α -lactalbumin, and bovine serum albumin (BSA). The gels were stained with Coomassie blue in methanol/acetic acid/distilled water (3:1:6) and destained in methanol/acetic acid/distilled water (3:1:7; Fig. 1).

Immunoblotting Assays

After electrophoresis, the proteins were transferred onto 0.45- μ m pore-size nitrocellulose membranes (Bio-Rad) using a Mini Trans-Blot Cell apparatus (Bio-Rad). The membranes were incubated in 3 mL of phosphate-buffered saline (PBS), pH 7.0, that contained 5 μ L of each individual patient's serum overnight at 4°C with gentle agitation and washed three times in PBST (PBS containing 0.05% Tween-20). The membranes were then incubated with polyclonal goat IgG anti-human IgE (15 μ g in 3 mL of PBS) with gentle agitation for 2 hours, washed three times with PBST, and incubated

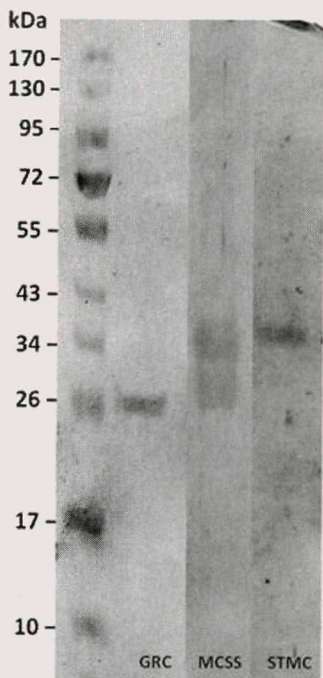


Figure 2. An image of the electroblotted 0.45- μ m nitrocellulose membranes after incubation with the sera from three patients (GRC, MCSS, and STMC), primary antibody (goat IgG anti-human IgE), and secondary antibody (horseradish peroxidase [HRP]–conjugated rabbit anti-goat IgG) and after development with DAB. The blots show three different patterns of sensitization: sensitization to caseins only (19–25 kDa), sensitization to both caseins and β -lactoglobulin dimers, and sensitization to β -lactoglobulin dimers only (36 kDa).

cation of symptoms. The reaction was registered as positive when the ingestion of low lactose-content mature hard cheese was clearly followed by gastrointestinal symptoms, particularly diarrhea.

Statistical Analyses

The results are presented as the mean followed by the 95% confidence interval (CI). All of the statistical analyses were performed with GraphPad Prism software (Version 5.0; GraphPad Software, Inc., San Diego, CA).

RESULTS

All of the 46 subjects included in the milk-intolerant group were proven to have lactose intolerance by the lactose tolerance tests that used H_2 -breath tests (mean delta H_2 , 114.5 ppm H_2 ; 95% CI, 99.6–129.4).

Skin-Prick Tests

Intolerant Group. Histamine showed a mean MWD of 6.7 mm (95% CI, 6.2–7.2). None of the patients reacted to the glycerosaline solution. Thirty-two patients (69.5%) had a skin reaction to whole cow's milk (mean MWD, 5.0 mm; 95% CI, 4.53–5.4), 17 (36.9%) patients had a skin reaction to bovine α -lactalbumin (mean MWD, 3.9 mm; 95% CI, 3.3–4.5), and 26 (56.5%) patients had a skin reaction to bovine β -lactoglobulin (mean MWD, 4.5 mm; 95% CI, 4.0–5.1). There were 26 (56.5%) patients who had a skin reaction to bovine casein (mean MWD, 3.4 mm; 95% CI, 3.4–4) and 25 (54.3%) patients who had a skin reaction to the goat's milk extract (mean, 4.7 mm; 95% CI, 4.0–5.3). Twenty-three (50%) patients had a skin reaction to soy extract (mean MWD, 4.6 mm; 95% CI, 4.0–5.2). Of the 23 patients who exhibited cutaneous reactions to soy, 18 (78.2%) also reacted to casein. Eight (34.7%) of the 23 patients who did not exhibit cutaneous reactions to soy also reacted to casein. The difference between groups was determined to be significant using the chi-squared test ($p = 0.0067$).

Tolerant Group. None of the 20 milk-tolerant subjects exhibited a cutaneous reaction to any milk protein. Histamine (positive control) showed a mean MWD of 7 mm (95% CI, 5.7–8.2). None of the patients reacted to the glycerosaline solution (results not shown).

Immunoblot

Intolerant Group. The sera of 10 (21.7%) patients recognized bands at 14 kDa (bovine α -lactalbumin), exhibiting a mean AOD of 13.2 mm and a 95% CI of 8.3–18.1. The sera from 9 (19.5%) patients recognized bands at 18 kDa (bovine β -lactoglobulin monomers) and showed a mean AOD of 21.9 mm and a 95% CI of

with horseradish peroxidase–conjugated rabbit anti-goat IgG (30 μ g in 3 mL PBS) for 2 hours. After three washes with PBST, horseradish peroxidase activity was stimulated by incubating the membranes in 10 mL of PBS containing 0.05% DAB (Sigma, Buchs, Switzerland) and adding 50 μ L of developing solution ($CoCl_2 \cdot 6H_2O$ plus $NiSO_4$) and 20 μ L of hydrogen peroxide (H_2O_2).¹⁹ When the impregnation and color development were complete, the membranes were washed in distilled water and subsequently dried (Fig. 2). The images of the membranes were digitized, and the relative densitometry of the bands was analyzed using ImageJ software (Windows Version 1.44 of NIH ImageJ, Bethesda, MD)²⁰ by measuring the average optical density (OD). After background correction, the adjusted OD (AOD) was calculated by subtracting the average OD of a user-defined area within the image from the average OD of a similar area defined around the measured bands.²¹

Low Lactose-Content Cheese Oral Challenge

The categorical reaction to the ingestion of low lactose-content cheese²² was registered as absent when the patient was able to regularly eat low lactose-content mature hard cheese without the provo-

8.6–35.2. Twenty-eight (60.8%) patients had sera that recognized bands at 36 kDa (bovine β -lactoglobulin dimers), with a mean AOD of 27 mm and a 95% CI of 18.6–35.4. Only one patient's serum recognized the band at 18 kDa and did not recognize the band at 36 kDa. The serum samples from 31 (67.3%) patients identified bands at 19–25 kDa (bovine caseins), exhibiting a mean AOD of 21.9 mm and a 95% CI of 17.5–33.3. One (2.1%) patient's (JVF) serum identified bands at 66 kDa (BSA, result not shown).

Tolerant Group. Only one serum of the 20 milk-tolerant subjects (5%) identified a 36-kDa band (β -lactoglobulin; AOD = 12.5) on the immunoblot (results not shown).

DISCUSSION

In adults, the most common adverse reaction to cow's milk is lactose intolerance due to lactase deficiency. Lactose is a disaccharide (galactose β -1,4 glucose) that can not be absorbed by the intestinal epithelium; therefore, it must be broken down into galactose and glucose by amphiphilic lactase/phlorizin hydrolase from the human small intestine to be incorporated.²³ The ability to produce lactase is genetically down-regulated by humans after infancy (acquired delayed-onset hypolactasia).^{24,25} The symptoms of lactase deficiency include abdominal pain, bloating, excessive flatus, and watery stool and occur in a dose-dependent manner after the ingestion of foods containing lactose.²⁶ These symptoms may also be dose-dependently caused by immediate gastrointestinal hypersensitivity, which is an IgE-mediated condition triggered by cow's milk proteins.²⁷ Because lactose intolerance is highly prevalent, it is reasonable to infer that patients with IgE-mediated gastrointestinal hypersensitivity to milk proteins will also have a high risk of presenting with comorbid lactose intolerance.

One expects to find four major allergens in a cow's milk immunoblot. The caseins (Bos d 8) constitute a family of related hydrophobic phosphoproteins (α S1, α S2, β , and κ) with a high number of proline peptides (19–25.2 kDa). α -Lactalbumin (Bos d 4) is a whey protein with an MW of 14.2 kDa. BSA (Bos d 6) is a 66.5-kDa serum component that is secreted into milk. Bovine β -lactoglobulin (Bos d 5) is an 18.4-kDa whey protein found as a dimer (36.8 kDa) at neutral pH and a monomer at pH < 3.^{28,29} Because we ran the SDS-PAGE in a running buffer with a pH of 8.3, we observed a higher proportion of β -lactoglobulin dimers (Figs. 1 and 2). Of the 46 patients with lactose-free diet refractory lactose intolerance, 41 (89.1%) had IgE antibodies specific for one or more milk proteins, as detected by SDS-PAGE immunoblotting or skin tests. Only five patients (10.8%) did not show any evidence of IgE-mediated sensitization.

The diagnosis of an associated IgE-mediated sensitization to cow's milk proteins is helpful in prescribing handling guidelines, as well as gaining knowledge of the specific proteins involved. Cow's milk has two phases. Caseins compose the solid phase (cheese). The liquid phase (whey) retains lactose and dozens of proteins, such as β -lactoglobulin, α -lactalbumin, BSA, lactoferrin, and immunoglobulins. Of the proteins found in whey, β -lactoglobulin (Bos d 5) is the most important because of its relatively high concentration in cow's milk (mean, 3 g/L) and greater allergenicity, which results from low digestibility.³⁰ Industrial dairy products may also be additional sources of immunologic sensitization due to the modification of native proteins.^{31,32} To establish dietetic guidelines for a patient with milk intolerance, it is essential to know all of the components to which he/she is sensitive. When performing a clinical diagnosis of "lactose intolerance" based only on statistics, one assumes the risk of overlooking important comorbidities, such as allergies to whey proteins or cheese caseins. The exclusion diet is quite different for each condition. It is very common in clinical practice to find patients who have true cow's milk allergies making use of lactose-free products, feeding the illusion that they are not harmful. The lactose level is reduced in cheese by the removal of whey and fermentation by bacteria.²² A patient with lactose intolerance or an allergy to whey proteins will be able to eat mature whey-free cheese if there is no concomitant allergy to casein. A patient with an allergy to casein must avoid all milk derivatives and sometimes soy products because the S30 component of soy protein may cross-react with cow's milk caseins.³³ This fact explains the significantly greater frequency of concomitant cutaneous reactivity to soy among our patients with cutaneous reactivity to casein. Sometimes, a simple anamnesis is enough to distinguish between these conditions, but cutaneous tests and immunoassays are usually necessary and are very useful in raising the justified suspicion of a primary and/or comorbid immune sensitization. All of the patients from the intolerant group who presented symptoms after the ingestion of hard mature cheese showed a concomitant IgE-mediated sensitization that was verified by immunoblotting and/or SPT. The coexistence of milk sensitization in patients with lactose intolerance is not *per se* an indication of food allergy, but, perhaps, some lactose-intolerant patients who are not responding well to lactose avoidance may be allergic to cow's milk proteins, particularly in patients with comorbid gastrointestinal manifestations. The milk challenge testing with a lactose-free formula is needed for confirmation.

In conclusion, IgE-mediated sensitization to milk proteins is a frequent comorbidity among lactose-intolerant subjects who are refractory to a lactose-free diet. Investigating IgE-mediated sensitization to cow's milk

proteins in lactose-intolerant subjects can lead to more accurate diagnostic and therapeutic decisions, as indicated by the Diagnosis and Rationale for Action against Cow's Milk Allergy guidelines.¹

ACKNOWLEDGMENTS

Celso Eduardo Olivier, Sônia Letícia Silva Lorena, and Ricardo de Lima Zollner conceptualized the clinical trial and the article, outlined the content, and were integrally involved in the writing/rewriting of the final article. Célia Regina Pavan and Sônia Letícia Silva Lorena performed/supervised the lactose tolerance tests and the hydrogen breath tests. Raquel Acácia Pereira Gonçalves dos Santos, Daiana Guedes Pinto, Celso Eduardo Olivier, and Ricardo de Lima Zollner performed/supervised the allergy tests. Regiane Patussi dos Santos Lima, Mariana Dias da Silva, Daiana Guedes Pinto, and Celso Eduardo Olivier performed/supervised the immunoblotting.

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