

Sensitization to serum albumins in children allergic to cow's milk and epithelia

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Patients with persistent milk allergy and specific immunoglobulin E (IgE) to bovine serum albumin (BSA) have a greater risk of rhinoconjunctivitis and asthma because of animal dander. To prove the cross-reactivity between serum albumin (SA) of different mammals in milk, meat, and epithelia and determine if heat treatment of meats decrease the allergenicity of albumins. The study was performed using SDS-PAGE and IgE-immunoblotting using sera from eight patients sensitized to milk, BSA, and animal danders. Sera from non-allergic and only animal dander allergic subjects served as a control. With one exception, all patients' sera recognized SA in different meats (beef, lamb, deer, and pork), epithelia (dog, cat, and cow), and cow's milk. Some patients even were only sensitized to SA in meat and epithelia. Danders' allergic only recognized other proteins in epithelia but not SA. No patients reacted to SA from heated meat extracts. Serum albumin is an important allergen involved in milk, meat, and epithelia allergy. The first contact with SA was through cow's milk and patients developed sensitization to epithelia SA even without direct contact with animals. Patients with both BSA and cow's milk allergy must avoid raw meats and furry pets.

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In Spain, cow milk allergy affects 0.36–1.9% of children <1 yr of age. Cow milk allergy is the third more frequent cause of food allergy following egg and fish (1). Milk allergy usually disappears, only 10–15% of children remain allergic after 5 yr old (2). Patients with persistent milk allergy have a greater risk of developing rhinoconjunctivitis, asthma, and sensitization to animal epithelia. More than 70% of these patients also have persistent allergy to bovine serum albumin (BSA; 3).

Serum albumin (SA) is the most abundant protein in plasma with a normal concentration of around 5 g/100 ml. In mammals, albumin is synthesized initially as preproalbumin by the liver. After removal of the signal peptide, the resultant proalbumin is further processed by removal of the six-residue propeptide from the

new N-terminal (4). The molecular weight (MW) of SAs are around 66 kDa. The main functions of albumin are: to maintain osmotic pressure, transport molecules, and buffering pH (5).

Albumin cross-capillary endothelia from plasma to the mammary gland, meat, and epithelia, by transcytosis (vesicular transport of macromolecules; 6, 7). BSA has been identified as allergen in milk, beef meat, and cow dander (8–10). The cross-reactions between cat dander and pork meat (11, 12); beef, sheep, and pig meats (13); and beef meat and hamster epithelium (14) are due to epitopes of albumin. In this sense, Spitzauer et al., described a cross-reaction between dog albumin and mouse, rat, and cat albumins (15).

The aim was to study the existence of cross-reactions between SAs in cow's milk, meats (beef,

pork, deer, and lamb), and danders (cow, dog, and cat) in patients with a history of persistent cow's milk allergy, who suffered from rhinoconjunctivitis and asthma caused by animal dander, all of them sensitized to BSA as demonstrated by CAP and tolerating cooked meat.

Methods

Patients

Sera from one adult and seven children, four male and four female, were included in this study. All of them were persistently milk allergic with an age ≥ 5 yr (after this age milk tolerance is uncommon; 16), and presented rhinoconjunctivitis and asthma due to mammal's epithelia. Any patient had pets in their house, but all of them had eventual indirect exposure to dog and cat allergens. Two patients had direct contact with cows. All patients tolerated meats and showed specific immunoglobulin E (IgE) and positive skin prick test (SPT) to BSA and/or specific IgE to mammal's epithelia (Table 1). Sera from other six patients were used as negative control. Three of them, with specific IgE only to dog and cat dander's (sera pool C1), and the other three negative both to BSA and pet dander's (sera pool C2).

Skin prick test

The SPTs were performed with commercial inhalant allergens (dog, cat, and cow dander) provided by Bial-Aristegui (Vitoria, Spain). The CAP System (Pharmacia Upjohn, Uppsala, Sweden) was used for determination of specific IgE.

Bovine serum albumin

Globulin-free, 98% pure, lyophilized BSA was purchased from Serva (Feinbiochemica GmbH & Co., Heidelberg, Germany). BSA was solubilized

at a final concentration of 1 mg/ml in distilled water. About 6 μ l of this final concentration were mixed with 54 μ l of sample buffer (1:10) and studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and IgE-immunoblotting with sera from the patients.

Milk extract

About 500 μ l of commercial non-fat milk were centrifuged at 15,500 *g* for 5 min, the supernatant was then recovered and mixed with sample buffer at 1/50 dilution and studied by SDS-PAGE and IgE-immunoblotting.

Meat extracts

Crude meat extracts from beef, lamb, deer, and pig were prepared as follows: 10 g of each raw meat in 10 ml of phosphate-buffered saline (PBS) were ground in a domestic electric mixer. The mixtures were shaken during 30 min and centrifuged at 4500 *g* for 15 min, and 6 μ l of each supernatant were then mixed with 54 μ l of sample buffer and studied by SDS-PAGE and IgE-immunoblotting.

Epithelia extracts

For *in vitro* studies 300 μ l of the commercial extracts used to perform SPTs were centrifuged at 5000 *g* for 60 min in a Ultrafree®-MC centrifugal filter, 10,000 nominal MW limit (Millipore Corporation, Bedford, MA, USA) to concentrate (4 $\times$) and eliminate phenol and glycerin. Extracts were then mixed with sample buffer at the appropriated dilutions.

Heating procedure

Meat extracts were centrifuged at 15,500 *g* for 5 min and 50 μ l of each supernatant were then mixed with 200 μ l of distilled water and heated for 60, 120, and 180 min in a boiling water bath. The heated samples were then centrifuged at 15,500 *g* for 5 min. The supernatants were recovered and mixed (1:1 v/v) with sample buffer. The pellets (50 μ l) were resuspended in 50 μ l of sample buffer and heated 5 min in the boiling water bath. Both the supernatants and pellets were studied by SDS-PAGE and IgE-immunoblotting with a pool of sera.

SDS-PAGE

About 15 μ l of each sample were loaded in a 16% polyacrylamide gel and electrophoresis was

Table 1. Demographic, clinical, and laboratory data from patients

	1	2	3	4	5	6	7	8
Sex	M	F	F	M	F	M	M	F
Age (yr)	35	8	6	8	6	6	6	6
Milk allergy	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Dander allergy	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Meat allergy	No	No	No	No	No	No	No	No
IgE (CAP) BSA	62.4	0.98	91	3.87	3.61	65	6.71	13.9
SPT dog dander	+	+	—	—	—	+	—	ND
SPT cat dander	+	+	—	—	—	+	—	ND
SPT cow dander	+	+	—	+	—	+	+	+
CAP dog dander	27.4	0.56	27.6	ND	1.83	37.4	ND	ND
CAP cat dander	12.5	0	32.2	ND	1.68	9.13	ND	ND
CAP cow dander	41.4	0.94	6.13	3.87	ND	ND	17.1	ND

SPT, skin prick test; ND, non-determined.

carried out at 150 V. Gels were dyed with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Hercules, CA, USA) according to the method of Neuhoff et al. (17) or used to transfer the proteins onto membranes for immunoblotting.

IgE-immunoblotting

Proteins separated by SDS-PAGE were transferred to two nitrocellulose sheets (supported nitrocellulose membrane 0.45 μm ; Bio-Rad) by diffusion during 24 h. Thereafter, the nitrocellulose membranes were incubated for 30 min in 3% Nonidet P-40 (Amresco, Solon, OH, USA) in PBS. The membranes were then incubated overnight (room temperature and continuous shaking) with 500 μl of sera from patients and controls diluted in 10 ml of incubation buffer (10 mM Tris-saline pH 7.4, 1% Tween 20) without fetal bovine serum (18). Next day, membranes were washed three times with 0.5 M NaCl, 2 mM Tris pH 7.4, 0.1% Tween 20, and incubated again for 3 h with 10 ml of a 1:1000 dilution of a monoclonal anti-IgE antiserum (Ingenasa, Madrid, Spain) in incubation buffer with 5% fetal bovine serum. After new three

washes, the membrane was incubated with 10 ml of a 1:4000 dilution of an alkaline phosphatase-labeled goat antimouse antiserum (Bio Source International, Camarillo, CA, USA) in incubation buffer with 5% FBS. Finally, the membrane was washed and the substrate nitro-blue-tetrazolium/5-bromo-4-chloro-indolyl phosphate (Amresco) added for 30 min (19).

IgE-immunoblotting inhibition

About 500 μl of serum were pre-incubated for 2 h with 50 μg of BSA in distilled water. Immunoblotting was then performed as described above.

Results

Fig. 1a shows the result of the SDS-PAGE with BSA and the extracts of meats, epithelia, and milk. BSA showed its band with MW = 66,200 Da (20) and two more bands, the dimer and trimer of the same protein as described by Restani et al. (21). Milk, meats, and epithelia showed a protein of similar MW as BSA. These proteins could correspond to the different albumins: Bos d 6 in cow (22), Ovi a SSA in sheep

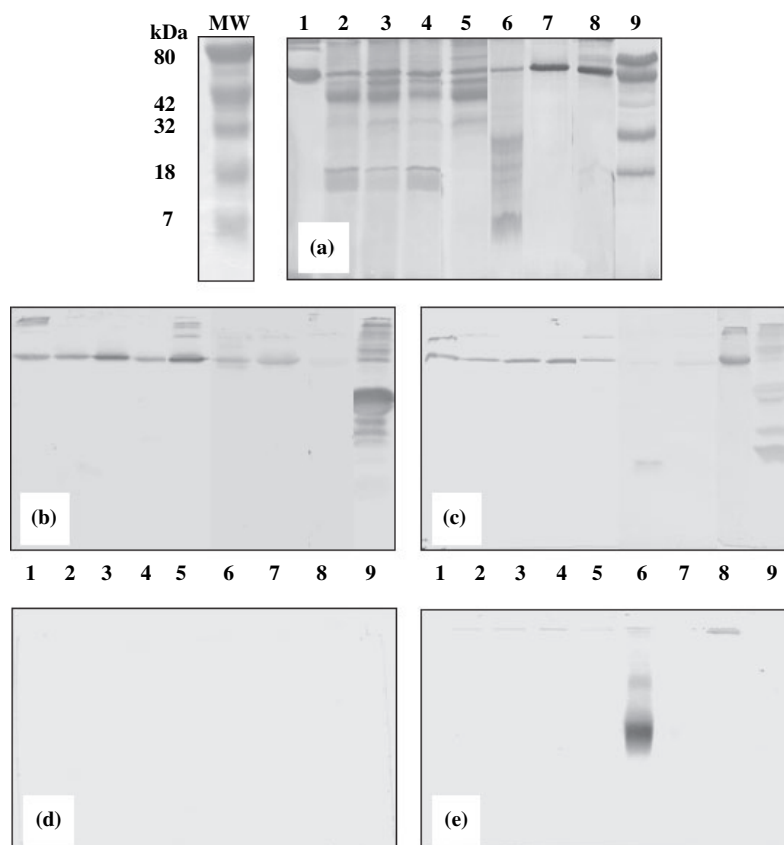


Fig. 1. SDS-PAGE (a) and IgE-immunoblotting patterns of two representative patients (b and c) and controls (d and e). Lanes: 1, BSA; 2, beef meat; 3, lamb meat; 4, deer meat; 5, pork meat; 6, dog epithelium; 7, cat epithelium; 8, cow epithelium; 9, non-fat milk.

(23), Sus s PSA in pork (13), Can f 3 in dog (24), Fel d 2 in cat (25), and deer SA.

Albumins were not detected in the supernatants of heat-treated meats but the pellets obtained after centrifugation of these heated meat extracts contained a protein of identical MW than BSA (data not shown).

IgE-immunoblotting

Circulating IgEs from all patients and controls were tested for reactivity to BSA, meats, epithelia, and cow's milk. Immunoblotting analyses showed that sera of all patients recognized different milk proteins, BSA, and proteins of similar MW than BSA in meats and epithelia (Fig. 1b,c). Three patients recognized only a protein in all the meats and epithelia (Fig. 1b). Five patients also recognized other protein with smaller MW in dog epithelium (Fig. 1c). Any patient showed recognition to proteins of heated meat extracts. Control sera with negative specific IgE to BSA and positive to pet dander recognized proteins different than SA in epithelia (Fig. 1e), and control sera negative both to BSA and pet dander did not recognize any proteins (Fig. 1d). Table 2 summarized the results of IgE-immunoblotting.

Table 2. IgE-immunoblotting results of patients and sera pool used as controls

	1	2	3	4	5	6	7	8	C1	C2
BSA	+	+	+	+	+	+	+	+	-	-
Milk allergens	+	+	+	+	+	+	+	+	-	-
Dog dander albumin	+	+	+	+	+	+	+	+	-	-
Cat dander albumin	+	+	+	+	+	+	+	+	-	-
Cow dander albumin	+	+	+	+	+	+	+	+	-	-
Beef meat albumin	+	+	+	+	+	+	+	+	-	-
Lamb meat albumin	+	+	+	+	+	+	+	+	-	-
Deer meat albumin	+	+	+	+	+	+	+	+	-	-
Pork meat albumin	+	-	+	+	+	+	+	+	-	-
Other allergen	-	-	+	-	+	+	+	+	-	+

C1, sera pool from non-allergic; C2, sera pool from patients allergic to dog and cat dander; BSA, bovine serum albumin; IgE, immunoglobulin E.

Inhibition assays

Sera of patients were incubated with purified BSA. After incubation, IgE reactivity against BSA in cow's milk, meats, and epithelia was completely removed (Fig. 2b). These sera still weakly recognized SA present in other meats and epithelia (Fig. 2b). The other proteins detected in milk and dog epithelium were not inhibited by BSA.

Discussion

The study by IgE-immunoblotting has shown that the sera of our patients recognized SA in almost every meat and epithelium. This fact suggests that SA is the common allergen present in cow's milk, raw meats, and epithelia of mammals recognized by persistent cow's milk allergic patients. Thus, allergy to BSA in these patients is a risk factor to develop rhinoconjunctivitis and asthma due to sensitization to animal epithelia.

The cross-reactivity between SA of cow, sheep, deer, pork, dog, and cat (meats and/or epithelia) found in all sera from patients was inhibited with pure BSA. Therefore, albumins are implicated as a panallergen in mammals and this protein could be considered as a major allergen for our patients who had developed sensitization to epithelia SA even without direct contact with animals.

No sera recognized SA from extracts of heated meats which agreed with the fact that all patients tolerated well-cooked meat. Most patients were only sensitized to albumin in raw meats and epithelia. Five of them recognized other protein of 20 kDa in dog epithelium that could be Can f 2 (26).

Native SA in epithelia is a relevant allergen in patients with persistent cow's milk allergy who are sensitized to BSA. However, SA present in meats shows a reduction of its antigenicity by heat denaturation (27, 28) being tolerated by patients. In these sense, patients with persistent allergy to BSA must not eat raw meats and have furry pets to avoid allergy and developing respiratory symptoms.

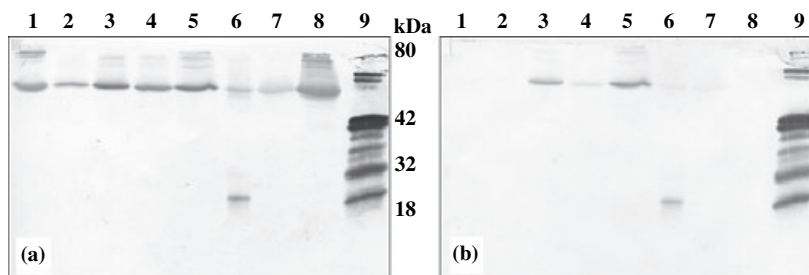


Fig. 2. IgE-immunoblotting inhibition: (a) non-inhibited, (b) inhibited with 50 μ g of pure BSA. Lanes: 1, BSA; 2, beef meat; 3, lamb meat; 4, deer meat; 5, pork meat; 6, dog epithelium; 7, cat epithelium; 8, cow epithelium; 9, non-fat milk.

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