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Clinical or Laboratory Pearl

Meat Allergy: III-Proteins Involved and Cross-Reactivity Between Different Animal Species

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Background: Although relatively infrequent, meat allergy represents a serious problem for children both because it is generally associated with intolerance to other protein sources and because of the suggested role of meat in stimulating the gastrointestinal development during weaning.

Objective: With these considerations, the aim of our work was to improve biological-biochemical knowledge of meat allergy.

Methods: This study was performed using *in vivo* skin prick test (SPT) and *in vitro* (electrophoresis associated with the immunoblotting technique) tests.

Results: Bovine serum albumin (BSA) and actin were the proteins most frequently involved in binding with the circulating IgE. BSA involvement was confirmed by SPT, the high number of positive responses observed with actin in immunoblotting was not confirmed by SPT data. Cross-reactivity between serum albumins from different animal species was demonstrated. Our studies show that in this group of children, the correspondence between the percentage of sequence identity (phylogenetic similarity) and the number of positive responses was surprisingly high.

Conclusions: Although further studies are necessary, the data reported here provide new biochemical data on meat allergy.

INTRODUCTION

Meat allergy has long been considered a rare pathology, occurring mainly in children. Sampson reported a 15.9% incidence of meat allergy in 18 out of 113 atopic children tested by skin prick test (SPT), but this percentage decreased to only 1.8 after a double-blind placebo controlled food challenge (DBPCFC) [1]. More recent studies [2-3] performed by our group showed that beef allergy occurs more frequently than previously believed. A prevalence of 8.96% was observed in a group of atopic children.

Meat is an important food for children when weaning starts; it is rich in essential nutrients and, due to its high content of polyamines, it could be involved in the development of children's gastrointestinal mucosa [4]. Consequently, the identification of an alternative protein source able to support fast body growth and development becomes crucial for allergic subjects.

Lamb meat has often been proposed as an alternative for atopic children who present a polyallergy to different foods including cow's milk, other animal protein sources and soy [5-6]. However, this dietary choice came from clinical experiences rather than described biological evidence.

The antigenic activity of serum albumin is well known and the involvement of this protein in milk [7] and bird egg allergy [8] has already been reported. In our previous studies, bovine serum albumin (BSA) has been identified as one of the most important allergens in bovine meat. Moreover, cross-reactivity between bovine and ovine serum albumin has been shown by SPT and DBPCFC when purified proteins are used [2-3]. Different biological implications in the modulation of the allergic reactions to serum albumins from different animal species have also been considered and a role of peptic digestion has been demonstrated in the antigenicity reduction of ovine compared to BSA [3]. This fact could justify the higher clinical tolerance observed for meat

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from calves in atopic children [5-6].

Although the significant role of serum albumin in bovine meat allergy has previously been reported by our group, it seems important to evaluate the implication of other main meat proteins in this pathology. Finally, data on cross-reactivity between meats of different animal species are useful to improve our basic knowledge on meat allergy and could contribute to solving the dietetic and clinical problems of atopic children.

MATERIALS AND METHODS

Clinical Data

Serum samples were taken from 12 children: eight males and four females ranging in age from 1 to 4.33 years (2.21 ± 1.05 , SD). All children presented clinical reactions after ingestion of beef [2]. All children showed SPT positive response to diagnostic reagents (Bayer, Garbagnate) for cow's milk and bovine meat, and to purified BSA (1 mg/mL in 50% glycerol; Sigma Chemical Co.). As a negative control, sera of five children (three males and two females, 1.5 to 5.6 years of age (2.7 ± 1.6 , SD) allergic to foods other than meat or milk and four nonallergic adult subjects were used. Some subjects with high levels of IgE (as obtained in Radio Allergosorbent Test [RAST]) but with no clinical symptoms for bovine meat were also included. These included five males and one female ranging in age from 2.1 to 7.4 years (3.65 ± 1.85 , SD). Informed consent was obtained from the parents of all children participating in this study.

Skin Prick Test

Skin prick test was carried out according to Bock [9]. Wheal diameters 3 mm greater than the negative control were considered a positive response. Since the wheal diameters of negative controls were always zero, a value ≥ 3 mm was considered positive.

Purified Meat Proteins

Purified proteins were purchased from Sigma Chemical Co. (St. Louis, MO) at the highest available purification level and with the following chemical characteristics: bovine meat proteins—actin (lyophilized powder containing approximately 90% protein); myosin (calcium activated in solution 5% glycerol containing 0.6 M potassium chloride and 10 mM potassium phosphate buffer pH = 6.8); myoglobin (lyophilized powder containing 95% to 100% protein); tropomyosin (lyophilized powder); hemoglobin (lyophilized powder); serum albumins—beef, chicken, horse, human, pig, rabbit, sheep, turkey serum albumins (essentially globulin free; lyophilized powder; approximately 99% by agarose electrophoresis). They were suspended in sample buffer (0.25 M

Tris-HCl buffer, pH = 6.8, containing 7.5% glycerol, 2% SDS, and 5% β -mercaptoethanol) at the final concentration of 1 mg/mL and heated for 10 minutes at 100°C. Bovine meat sample was obtained from normal retailers and suspended in sample buffer at the final concentration of 20 mg/mL. Purified protein (3 μ L) and meat (20 μ L) samples were then loaded onto the gel.

When gels were run in native condition, SDS and β -mercaptoethanol were not included in the sample buffer and no heating was applied.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

All chemicals and instruments used in the electrophoretic analyses were purchased from Bio-Rad (Richmond, CA). The composition of electrophoretic gels were:

Gradient running gel: 9-19% acrylamide; 0.08-0.17% bis-acrylamide; 0.36 M Tris (hydroxymethyl) aminomethane (TRIS)-HCl buffer, pH = 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% N,N,N',N'-tetramethylethylenediamine (TEMED). Bromophenol Blue was added to the gel as a run marker.

Stacking gel: 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M Tris-HCl buffer, pH = 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% TEMED.

Running buffer had the following composition: 25 mM TRIS, 0.19 M glycine and 0.1% SDS (w/v), pH = 8.8.

After the electrophoretic run (90 V at room temperature, for approximately six hours), gels were dyed with Coomassie Brilliant Blue G-250 according to the method of Neuhauff et al [10] or used to transfer the proteins onto membranes by immunoblotting.

When gels were run in native condition, SDS was not included in gels and running buffer.

Molecular weight (MW) standard solution contained: rabbit skeletal muscle myosin (MW = 200,000); *E. coli* β -galactosidase (MW = 116,250); rabbit muscle phosphorylase b (MW = 97,400); BSA (MW = 66,200); hen egg white ovalbumin (MW = 42,700); bovine carbonic anhydrase (MW = 31,000); soybean trypsin inhibitor (MW = 21,500); white hen egg lysozyme (MW = 14,400); bovine pancreas aprotinin (MW = 6,500).

Immunoblotting

After SDS-PAGE, proteins were transferred from the gel onto the polyvinylidene difluoride (PVDF, Immobilon P; Millipore, Bedford, MA) membrane by electrophoretic elution (wet transfer). The transfer buffer was Tris 25 mM, glycine 193 mM and methanol 20%. To verify the protein transfer, a solution of 0.2% Ponceau S (in 3% trichloroacetic acid [TCA] and 3% sulfosalicylic acid) was used; protein absence in blotted gel was verified by the usual staining pro-

cedure. After destaining, PVDF membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin (Bio Rad) solution (in 150 mM NaCl, 5mM EDTA, 50 mM Tris, 0.05% TRITON-X) to prevent nonspecific adsorption of the immunological reagents. The membrane was then immersed in 10 mL of 0.25% gelatin solution, containing 0.3 mL of serum from allergic children. Antigen-IgE complex was detected using goat antihuman IgE antibodies labeled with alkaline phosphatase; finally after incubation in the bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) solution, an intense black-purple precipitate developed at the site of enzyme binding. Unless otherwise specified, chemicals were from Merck (Darmstadt, Germany).

When gels were run in native conditions, methanol was not included in transfer buffer and Ponceau staining-destaining procedure was eliminated.

Classes of positive reactions were determined by comparing the precipitate color with a self-prepared scale of black-purple. Only stains of class $\geq$ III were considered significant.

RESULTS

SDS-PAGE of Bovine Meat Proteins

The electrophoretic distribution of bovine meat proteins in 9% to 19% gradient-gel is shown in Fig. 1.

The different proteins are well separated and their correspondence to meat proteic bands is easily identifiable. Bovine serum albumin shows its band with MW = 66,200 daltons [11] and some other components in trace probably corresponding to the residue of globulins. Myoglobin presents two components: the most abundant having the expected MW (17,000 daltons) [12] and a minor proteic band with approximately MW = 32,000 (a possible dimer of myoglobin). Myosin pattern includes a main band corresponding to

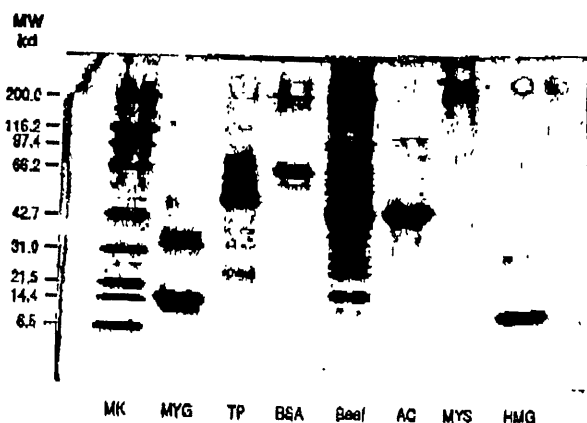


Fig. 1. SDS-PAGE of purified meat proteins. MYG = myoglobin; TP = tropomyosin; BSA = bovine serum albumin; AC = actin; MYS = myosin; HMG = hemoglobin.

the heavy chain of the protein [13]. Tropomyosin presents, apart from a minor component with MW = approximately 28,000 daltons corresponding to the beta chain [14], a diffused area having MW close to 50,000 daltons. Hemoglobin pattern shows only one proteic component, having MW lower than that of the expected alpha and beta chains (15,000 daltons) [15-16]. All proteins described above are also evident in the bovine meat sample, apart from hemoglobin, which was therefore not included in immunoblotting analyses.

Reactivity of Circulating IgE Against Purified Bovine Meat Proteins

Immunoblotting of one of the 12 children included in this study is shown in Fig. 2. This child reacts strongly to BSA

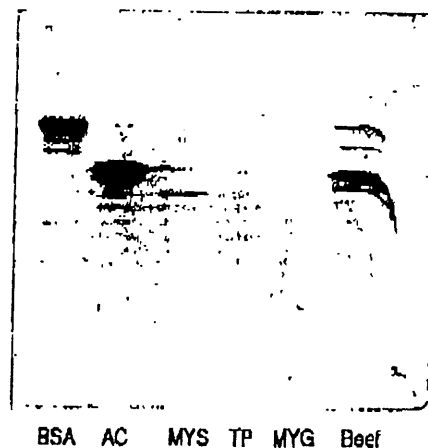


Fig. 2. Immunoblotting of purified meat proteins. The serum used was from a child allergic to bovine meat (abbreviations same as Fig. 1)

and actin; no positive response is observed for other standard bovine meat proteins. In the raw bovine meat sample, the positivity to BSA and actin is clearly visible. To obtain quantitative information, the amount of protein loaded onto the gels was strictly controlled and the absence of proteins in the blotted gel was verified by Coomassie Blue staining procedure. IgE-mediated responses of the 12 children considered in this study are reported in Table 1. Actin and BSA are the proteins more involved in IgE-mediated reactions; in fact, all children reacted to them. BSA showed 10 significant reactions (class equal or higher than III), with six very strong positive responses (class IV and V). Regarding actin, eight subjects presented a reaction considered significant, but no severe response (only class III) was observed. No other important reactions were found; although five children reacted to myoglobin (mainly to the component with MW = 32,000), nobody was included in class $\geq$ III.

Table 1. Specificity of Circulating IgE Against Meat Proteins Detected by Immunoblotting

Meat Protein	Class	Number of Positive Responses
Actin	Class III	8
	Class II	2
	Class I	2
	Total	12
BSA	Class V	4
	Class IV	2
	Class III	4
	Class II	2
	Total	12
Myoglobin	Class II	3
	Class I	2
	Total	5
Myosin	Total	0
Tropomyosin	Total	0

n = 12.

No positive response was registered when the sera of children allergic to foods other than meat were used. Mild reactions to actin (class I and II) were sometimes found with sera of nonallergic adults and of children with high IgE level but with no clinical symptoms after bovine meat ingestion.

SPT with Purified Meat Proteins

All children included in the study presented SPT-positive response to Bayer bovine and ovine meat preparations and to purified BSA and OSA (1 mg/mL) [2]. No significant response (wheal diameter ≥ 3 mm) to actin was obtained in SPT. Because of the low number of positive responses obtained in immunoblotting, other meat proteins were not tested by SPT.

Electrophoresis in Nondenaturing Conditions (PAGE) of Serum Albumins from Different Animal Species

Serum albumins from seven animal species (normally consumed in Italy) were run in nondenaturing gel in order to maintain the tridimensional structure of the protein; as a negative control, human serum albumin was run in parallel (Fig. 3A). Electrophoretic mobilities of serum albumins are very similar, as expected from their similar MWs. Two more bands, probably the dimer and trimer of serum albumin, are visible in the electrophoretic pattern of every sample.

SPT and Immunoblotting Positive Responses to Native Serum Albumins from Different Animal Species

Six of the 12 children previously included in the study were tested for reactivity against native serum albumins in SPT and immunoblotting. Fig. 3B shows the positive responses obtained in immunoblotting with the serum of a child who reacts against BSA, ovine serum albumin (OSA) and (PSA) pig serum albumins. Human serum albumin was included as a negative control. The positive reactions always included the corresponding dimer and trimer of serum albumin. In Table 2, IgE-mediated positive responses against serum albumins are summarized. All subjects (as expected) presented positive response against BSA; OSA and PSA were highly involved in IgE-antigen complexes (four and three significant positive responses, respectively) but horse serum albumin (HoSA) and rabbit serum albumin (RSA) in only one case (with very slight intensity equal to class II). No positive response was observed in immunoblotting for chicken or turkey.

In SPT, positive responses correlated only partially with immunoblotting results. All children reacted positively to BSA and OSA, as reported in our previous paper [3]. Three subjects reacted to PSA; two to HoSA, TSA and RSA. No wheal diameter was 3 mm larger than that of control when chicken serum albumin (CSA) was tested.

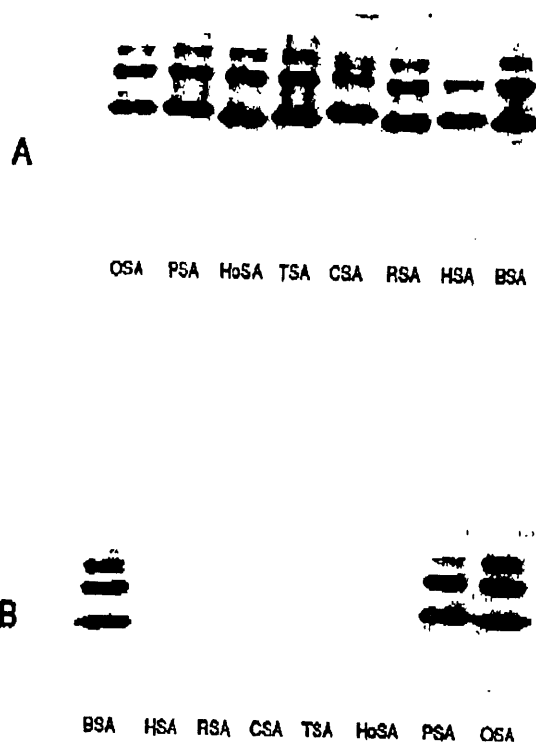


Fig. 3. Native PAGE (A) and immunoblotting (B) of serum albumins from different animal species. OSA = ovine serum albumin; PSA = pig serum albumin; HoSA = horse serum albumin; TSA = turkey serum albumin; CSA = chicken serum albumin; RSA = rabbit serum albumin; HSA = human serum albumin; BSA = bovine serum albumin.

Table 2. SPT and Immunoblotting Positive Responses to Serum Albumins from Different Animal Species

Serum Albumin	Child 1		Child 2		Child 3		Child 4		Child 5		Child 6	
	SPT (mm*)	Imm (class)	SPT (mm*)	Imm (class)	SPT (mm*)	Imm (class)	SPT (mm*)	Imm (class)	SPT (mm*)	Imm (class)	SPT (mm*)	Imm (class)
BSA	8	V	7	III	4	V	4	V	8	III	8	V
OSA	8	IV	4	I	2	I	3	IV	9	III	6	V
PSA	9	I	0	0	0	I	0	IV	6	III	8	IV
HoSA	8	II	0	0	0	0	0	0	2	0	7	0
TSA	0	0	4	0	0	0	0	0	0	0	6	0
CSA	0	0	0	0	0	0	0	0	0	0	0	0
RSA	9	0	0	0	0	0	0	0	0	0	6	II

* wheal diameter

DISCUSSION

Although relatively infrequent, meat allergy can be a serious problem for children, first, because it is often associated with sensitization to other protein sources and secondly, because of the suggested role of meat in stimulating the gastrointestinal development during the weaning period. Previous clinical studies have shown that some kinds of meat are better tolerated than others [5-6], but so far there are no biological data to support clinical evidence. Starting from these considerations, we performed a study with the aim of improving biochemical-biological knowledge on meat allergens.

Firstly, the involvement of different bovine meat proteins in the allergic phenomenon was considered, using the immunoblotting technique and the sera of subjects with demonstrated allergy to beef. Serum albumin has an important role in meat allergy, as shown in our previous *in vivo* and *in vitro* studies [2-3]; also in immunoblotting, BSA was involved in the formation of antigen-IgE complexes for all subjects included in this study. Six out of 12 sera presented a high specific circulating IgE content, producing class IV and V reactions.

Actin was also involved in 100% of responses, which seems strange since actin is one of the most phylogenetically conserved proteins. In fact, the identity between human and other animal species for alfa actin is close to 100% [17-20]. On the other hand, when the same children were tested in SPT, no positive response to actin was observed. The difference could be justified by the different molecular characteristics of actin when tested in SPT or in immunoblotting. In the former, actin is mainly present in the polymeric form (F-actin), while in immunoblotting, as a consequence of the solubilization in the sample buffer, actin is in monomeric form (G-actin). This important conformational modification could magnify the antigenicity of actin. Since the difference is interesting, we are planning to investigate this aspect further in the future.

In immunoblotting, other muscle proteins (myosin and tropomyosin) appeared to be very weak antigens; myoglobin produced positive responses in five subjects: the intensity

was low (class I and II) and 30,000 Da-component was mainly responsible for binding to IgE. Further studies are necessary to identify other proteic components involved in antigen-IgE complexes.

The second aim of this study was to evaluate cross-reactivity between different animal species whose meat is normally consumed in our country. In order to exclude the possible co-sensitization, we included in this specific study only those children who had never been exposed to meats other than beef [2]. Since BSA was the only bovine meat protein that induced positive responses in all *in vivo* and *in vitro* tests, we decided to consider the cross-reactivity between serum albumins from different animal species. In this study, we used purified proteins and native conditions in electrophoretic runs to maintain the tridimensional conformation, which different papers have indicated as an important parameter in BSA antigenicity [21-22].

In Table 3 we report, in parallel, the percentage of positive responses obtained in SPT and immunoblotting for every serum albumin and the percentage of their sequence identity with BSA. The correspondence between the percentage of sequence identity and the number of positive responses was surprisingly high.

Table 3. Relationship Between Sequence Identity of Different Serum Albumins and Positive Responses Obtained in SPT and Immunoblotting

Species	Reference	% Identity with BSA sequence	% of positive responses (n = 6)	
			SPT	Immunoblotting
Beef	11	100.00	100.00	100.00
Sheep	23	92.26	100.00	66.66
Pig	24	78.84	50.00	50.00
Horse	25	74.14	50.00	0.00
Rabbit	-	not found	33.33	0.00
Turkey	-	not found	33.33	0.00
Chicken	26	44.98	0.00	0.00
Man	27	76.28	nt	0.00

nt = not tested.

Some difference was found between SPT and immunoblotting results; this could be due to the different molecular characteristics of samples used in the tests and/or simply to the differences already reported elsewhere between SPT and the immunoassay data [28].

On the basis of the results of our *in vivo* and *in vitro* tests, BSA seems to be the main or one of the most important antigens of bovine meat; if this is true, the use of some animal species such as rabbit and/or poultry could be suggested in the diet of atopic children. In fact, serum albumins from these animal species were less frequently involved in SPT responses and in the formation of binding with specific circulating IgE. Further studies are now necessary to correlate these results with data of controlled open challenges performed on the same subjects. In fact, the oral challenge is not only the most useful tool to evaluate allergic reactions but it permits a more comprehensive evaluation, including the role of gastrointestinal digestion and/or cooking procedure (heating and technological treatments) in antigenicity reduction.

We have already shown the faster *in vitro* proteolytic attack observed on OSA compared to that on BSA [3] and a study of the effect of digestion and technological treatments on the antigenicity reduction of different serum albumins is now in progress.

Finally, the investigation of the true role of actin (before and after modifications of the polymeric status) in meat allergy and the nature of other proteic components capable of binding specific IgE will be an important task for our group.

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