

Original article

Identification of bovine IgG as a major cross-reactive vertebrate meat allergen

Background: Although beef is a main source of protein in Western diets, very little has been published on allergic reactions to beef or the main allergens implicated in these reactions. The aim was to evaluate the IgE antibody response to beef in suspected meat-allergic subjects and assess cross-reactivity of beef with other vertebrate meats.

Methods: Fifty-seven sera from suspected meat-allergic subjects were tested by grid blot for specific IgE antibodies to vertebrate meats (beef, lamb, pork, venison, and chicken), and the patterns of recognition of meat proteins were assessed by immunoblot studies.

Results: A 160-kDa band, identified as bovine IgG, was detected in raw beef in 83% (10/12) of beef-allergic subjects but in only 24% of the beef-tolerant subjects. IgE reactivity to a band of similar mol. mass was detected also in lamb and venison, but rarely in pork or chicken. Complete inhibition of the IgE reactivity to the bovine IgG was obtained with lamb, venison, and milk. IgE reactivity to this band also completely disappeared when beef or lamb extracts were separated under reducing conditions, indicating conformational epitopes.

Conclusions: Bovine IgG appears to be a major cross-reacting meat allergen that could predict beef allergy. Further studies with oral IgG challenges should be performed to document the conclusion that *in vitro* reactivity correlates with clinical hypersensitivity. The role of bovine IgG in other bovine products such as milk, dander, or hair must also be studied, and the hypothesis that it is a cross-reacting allergen with other mammalian products validated.

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Key words: allergens; beef; cross-reactivity; IgG; meat allergy.

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Accepted for publication 2 November 1999

The foods most frequently implicated in allergic reactions are hen's egg, cow's milk, peanut, soybean, fish, and wheat; this group of foods accounts for approximately 90% of all positive double-blind, placebo-controlled food challenges (DBPCFC) in children with atopic dermatitis in the USA (1). In other countries, the foods most frequently implicated vary according to availability and cultural habits. Among these foods, allergy to cow's milk represents a significant proportion since it accounts for up to 20% of the positive DBPCFC performed (2). When children with a convincing history of milk allergy (anaphylaxis) were included, together with those diagnosed by

DBPCFC, milk allergy was diagnosed in 30% of the 470 food-allergic children studied (2). In contrast to milk, little has been published on allergic reactions to beef or other vertebrate meats.

In vitro sensitization does not necessarily reflect clinical reactivity: in children with atopic dermatitis, sensitization to beef as demonstrated by skin prick test (SPT) varied from 7% to 26% (3, 4); however, clinical reactivity demonstrated by positive DBPCFC with beef was lower than 12% of the SPT-positive cases (4–6).

Since beef is a staple food of the Western diet, more allergic reactions may be expected. Although beef proteins can provoke allergic reactions in milk-sensitized individuals (4, 6), some allergic reactions have been caused by contaminants such as streptomycin (7) or penicillin (8). Several cases of occupational contact urticaria and hand dermatitis due to calf's liver or

Abbreviations. SPT: skin prick test; DBPCFC: double-blind, placebo-controlled food challenge; BSA: bovine serum albumin; BGG: bovine gamma globulin; RAST: radioallergosorbent test; DTE: dithioerythritol.

bovine meat have been reported in patients without milk allergy (9, 10); more frequently, cow's dander or epithelium proteins have been implicated as inhalants in asthmatic allergic reactions (11). This suggests that sensitization to bovine allergens may occur through different routes and exposure to different bovine proteins. Since meat consumption is increasing, yet only limited information exists on the antigenic composition of beef or other meats, the purpose of this study was to characterize the IgE-binding patterns of beef-allergic patients and to assess the cross-reactivity of beef with other vertebrate meats.

Material and methods

Sera

Sera were collected from a total of 57 patients from Spain (Hospital del Niño Jesus and Hospital La Paz, Madrid), USA (Tulane Medical Center, New Orleans, LA; Children's Hospital, Little Rock, AR; Kaiser Permanente Hospital, Hollywood, CA; Plasma-Lab International, Everett, WA; Baton Rouge, LA; Henry Ford Hospital, Detroit, MI; University Suburban Health Center, Cleveland, OH), and Canada (Wellesley Hospital, Toronto, Ontario). All sera donors were suspected meat-allergic patients with at least two of the following criteria: convincing history of allergy to beef, lamb, pork, rabbit, venison, chicken, or turkey; positive skin prick test (SPT); or specific serum IgE by RAST or CAP System (Pharmacia, Uppsala, Sweden) to any of these meats. Twelve had history of allergy to beef, seven to lamb, three to pork, one to venison, one to rabbit, and 23 to poultry. The age of the 29 males and 28 females ranged from 5 months to 48 years; 42 were pediatric patients (mean 6.3 years) and 15 were adults (mean 28.5 years).

In a previous study (12), all 57 sera were tested for IgE antibody reactivities against six raw and cooked meat extracts by grid blot. Forty-one showing strong IgE reactivity to beef extract by grid blot were further tested by immunoblot analysis of the different meats in the present study.

Extracts

Extracts were prepared from raw and cooked beef, lamb, pork, venison, and chicken according to Werfel et al. (4) with slight modifications. Briefly, raw or cooked (20 min in conventional oven, 140°C) meat was homogenized in a blender, extracted for 3 h in PBS (pH 7.2) at 4°C, centrifuged (70 000 g), the fat layer removed and the supernatant concentrated (Amicon, MW cutoff 1 kDa), recentrifuged (100 000 g), aliquoted, and stored at -20°C until use. Protein concentrations of all extracts were determined by the method of Bradford (modified by Pierce Laboratory, Rockford, IL, USA) with BSA as standard. Beef

tropomyosin was obtained from Sigma Laboratories (St Louis, MO, USA), aliquoted (2 mg/ml), and stored at -20°C until use. Nonfat dry milk (Nestle Food Company, Glendale, CA, USA), was dissolved in Millipore water to a final protein concentration of 20 mg/ml just before use.

SDS-PAGE and immunoblot analysis

Minigel (thickness 0.5 mm) SDS-PAGE was performed with the discontinuous buffer system of Laemmli (13). A 1.5-cm-high stacking gel (5% T, 1.5% C) was poured on top of a 6.5-cm-high separating gel (12.5% T, 1.5% C). Of each of the 10 raw and cooked meat extracts, 12.5 µg of protein per cm of gel in nonreducing sample buffer was boiled for 5 min and electrophoresed. Either gels were stained with silver staining (14), or the proteins were transferred onto a CNBr-activated (15) nitrocellulose membrane (0.45 µm, BAS 45, Schneider and Schuell, Germany) at 0.8 mA/cm² for 30 min by semidry blotting (16). The blots were blocked in TBS-Tween for 30 min, dried, and stored between filter paper until use. A volume of 75 µl of sera diluted 1:2 in TBS-Tween was placed per channel and incubated for 2 h. Only 41/57 sera which showed IgE reactivity to beef by grid blot were tested by immunoblot. Of them, 12 reported a convincing history of beef allergy, and the rest presented with specific serum IgE or positive SPT to beef.

For reducing SDS-PAGE, samples were diluted 1:2 in sample buffer (1% DTE; pH 8.2), and boiled for 5 min; next, 10 µl of alkylating reagent (iodoacetamide 15% w/v in H₂O) per 100 µl of sample was added, and the mixture was incubated for 60 min at room temperature in the dark, and then applied to the gel. A pool of five beef-reactive sera was used to test reduced and unreduced beef and lamb extracts.

For the immunoblot inhibition, 75 µl of the pool positive for raw beef was preincubated with 75 µl of raw beef, lamb, pork, venison, or chicken extract and nonfat dry milk with a final concentration of 4 mg/ml of protein and also with 75 µl of beef tropomyosin (80 µg/ml final concentration).

To visualize the IgE-binding proteins, the blot was incubated for 2 h with alkaline phosphatase-conjugated, monoclonal mouse antihuman IgE (Southern Biotechnology Associates, Birmingham, AL, USA) diluted 1:1000 in TBS-Tween, and washed three times for 10 min in TBS-Tween. To identify bovine IgG by immunoblotting, the analysis was performed with raw beef, lamb, and pork extract blots, and an alkaline phosphatase-conjugated rabbit anti-bovine IgG (whole molecule) 1:20 000 (Sigma) as detection antibody.

For detection of bound IgE, the membrane was washed 5 min in 37°C warm TBS-AP (0.1 M Tris-HCl, 0.1 M NaCl, and 5 mM MgCl₂, pH 9.5), and antibody

binding was visualized at 37°C using the substrate/chromogen mixture for alkaline phosphatase (17) containing 450 μ M 5-bromo-4-chloro-indolyl-phosphate disodium salt (BCIP, Sigma) and 400 mM nitroblue tetrazolium chloride (NBT, Sigma) solubilized in TBS-AP. The reaction was stopped with deionized water and the blots were dried.

Results

SDS-PAGE

Under nonreducing conditions on a 12.5% separating gel, the protein patterns of raw and cooked meat extracts differed considerably. Silver staining of raw extracts revealed more than 30 bands of 10–200 kDa with staining intensity highest in the 25–66 kDa range. The pattern was almost identical for all raw mammalian meats tested, while slight differences appeared in the mobility of some bands in the raw chicken extracts. In the cooked meat extracts, the pattern was similar to raw extracts, but fewer bands were visible (data not shown).

The effect of a reducing agent (DTE) on raw beef extract was tested; in general, the same bands found in the nonreduced extract were present in the reduced extract, although better defined. However, one protein of approximately 160 kDa disappeared after reduction.

IgE reactivities to mammalian meats and chicken by immunoblot

Among the 41 sera tested by immunoblotting to raw and cooked beef, 12 patients reported a convincing history of beef allergy. All 41 subjects had specific IgE to beef as demonstrated by grid blot. Twenty-one of the 41 sera with the strongest IgE reactivity to beef by grid blot were also tested with extracts of raw and cooked lamb, pork, venison, and chicken.

Sera with IgE reactivity by grid blot to a particular meat usually also had positive immunoblot to the same extracts, although weaker; three sera previously reactive by grid blot to beef extract did not show any IgE-binding by immunoblotting. This fact suggests that grid blot is a more sensitive technique (probably because it sums reactivity to all proteins); alternatively, the immunoblotting procedure may alter some proteins, rendering them non-IgE-reactive. Nevertheless, sera from two subjects with convincing beef allergy and a negative grid blot to all meat extracts had positive immunoblot to beef, indicating that some false negatives for grid blot may occur.

The reactivities to beef extracts by immunoblot were as follows: 25/41 to raw only, 10/41 to both raw and cooked, and 3/41 to cooked beef only. In raw beef extract, the following bands were identified: 160, 66, 45, 38, and 21 kDa (Fig. 1). In cooked beef extract, only isolated reactivities to proteins of medium to low mol. mass were detected (not shown), suggesting that cooking significantly reduces the allergenicity of beef proteins.

Similar yet more potent IgE reactivities to those found in beef were detected particularly in lamb and venison (not shown). IgE reactivity to cooked meats was generally weaker than to raw meats, supporting the results found in beef. IgE reactivities to pork and chicken meat were in general very weak (data not shown).

Comparison of presence of the 160-kDa band in different meat extracts

The bands most frequently detected by immunoblot were 160 kDa (17/41), 66 kDa (11/41), and 45 kDa (8/41). Almost all bands disappeared when the cooked beef extract was used. The frequencies of detection of

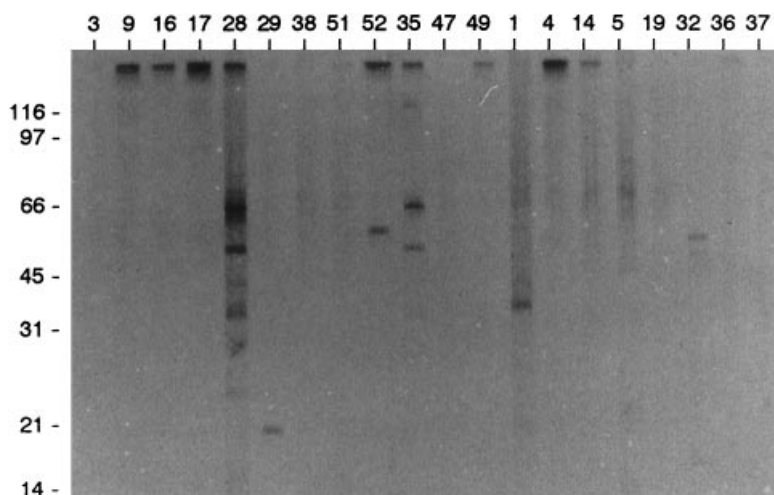


Figure 1. Immunoblot analysis of raw beef extracts. Sera numbers 3, 9, 16, 17, 28, 29, 38, 51, 52, 35, 49, and 4 belong to beef-allergic subjects. Sera 47, 1, 14, 5, 19, 32, 36, and 37 are from beef-tolerant subjects with specific IgE to other vertebrate meats. Serum 14 is from rabbit-allergic subject. Band of 160 kDa was detected by most (10/12) beef-allergic subjects.

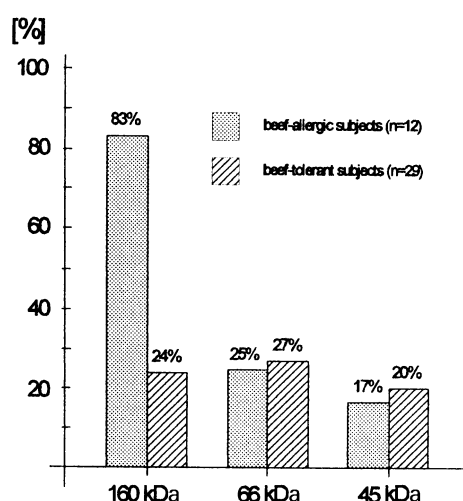


Figure 2. Allergogram of main IgE-binding allergens in raw beef. 160-kDa band was only major allergen detected, binding IgE antibodies from 83% of beef-allergic subjects. Other IgE-binding proteins (45 and 66 kDa) were less frequently detected by beef-allergic subjects.

the main beef reactivities among beef-allergic and beef-tolerant subjects are shown in Fig. 2. The 160-kDa band was present in 83% (10/12) of beef-allergic subjects, while only 24% (7/29) of beef-tolerant subjects reacted to this protein. The 66- and the 45-kDa bands were present in similar proportion in beef-allergic and beef-tolerant subjects. Thus, the 160-kDa band in this study appears to be the only major clinically relevant beef allergen (Fig. 2). Nevertheless, reactivity to this protein was not completely specific to beef-allergic patients, since 24% of beef-tolerant subjects also reacted to it. When reactivity to this band in the different meat extracts was analyzed, 10/12 (83%) of beef-allergic subjects recognized a band with similar mol. mass in raw lamb and venison that was rarely observed in pork and chicken extracts (Table 1).

Since the initial SDS-PAGE had been performed under nonreducing conditions, and IgE-reactive beef

proteins with a mol. mass higher than 100 kDa have been rarely reported to date, we suspected that IgE-reactive fragments smaller than the 160-kDa protein may have been identified by other investigators. Therefore, the effect of reducing agents on IgE binding was evaluated. Extracts of raw beef and lamb were separated by SDS-PAGE under reducing and non-reducing conditions and tested with sera of five beef-allergic subjects who reacted exclusively to the 160-kDa protein in beef. IgE reactivity to this band completely disappeared when beef or lamb extracts were separated under reducing conditions, without any concurrent reactivity to polypeptides with lower mol. mass (Fig. 3). This suggests that the main reactivities to this protein are directed against conformational epitopes.

In order to determine whether the same 160-kDa protein was present in all meat extracts, and whether it was also present in bovine products such as milk, immunoblot inhibition was performed. A pool of five beef-allergic patients with demonstrated sensitivity to the 160-kDa protein was used. Total inhibition of the IgE reactivity to the 160-kDa band in beef was obtained with raw beef, lamb, and venison extracts as well as with nonfat milk. Inhibition was not observed with either raw pork, raw chicken, or beef tropomyosin (Fig. 4).

Since total inhibition of the IgE reactivity to the 160-kDa band was obtained with defatted milk, the presence of the same or similar protein in milk and beef was hypothesized. Among the milk proteins, a mol. mass of approximately 160 kDa corresponded to immunoglobulins. Since IgE reactivity to IgG antibody in bovine hair and dander has been described, a possible role of IgG as a beef allergen was suspected. Thus, alkaline phosphatase-conjugated rabbit anti-bovine IgG was tested and reacted to the 160-kDa protein in beef, identifying the protein as bovine IgG (data not shown). Cross-reactivity was detected with lamb, but not with pork IgG, indicating similar epitopes in beef and lamb IgG antibodies.

Table 1. IgE reactivity of beef-allergic subjects to 160-kDa protein in beef, lamb, pork, venison, and chicken

Subject no.	Beef	Lamb	Pork	Venison	Chicken
3	No*	No	No	Yes	ND
9	Yes*	Yes*	No*	Yes	No
16	Yes*	Yes*	No*	Yes	No*
17	Yes*	Yes	No	Yes	No
28	Yes*	Yes*	No	Yes	No
29	Yes*	Yes*	No	Yes	ND
38	No*	No	No	Yes*	ND
51	Yes*	Yes	No	No	ND
52	Yes*	Yes*	No	Yes	No
35	Yes*	Yes	ND	Yes	ND
4	Yes*	Yes*	No	Yes	ND
49	Yes*	No	ND	No	No

Yes: IgE-binding to 160-kDa protein in that extract. No: no IgE-binding to 160-kDa protein in that extract. *Clinical symptoms after ingestion of particular meat. ND: not determined.

Discussion

Our results show that suspected beef-allergic subjects have IgE reactivity directed against meat proteins and that this reactivity is mainly directed to a protein of 160 kDa, identified as bovine IgG. Since IgG was bound by IgE of more than 80% of the beef-allergic subjects, it may be a good predictor of beef allergy. Although we were able to identify IgG as a major beef allergen, we cannot exclude the possibility that IgE reactivity to other immunoglobulins of similar mol. mass (such as IgA) may be important in beef-allergic subjects.

Although allergy to mammalian proteins has been widely studied, such studies have mainly focused on IgE reactivity to inhalants such as dander or epithelia from cat, dog, horse, or cattle. In addition, mammalian

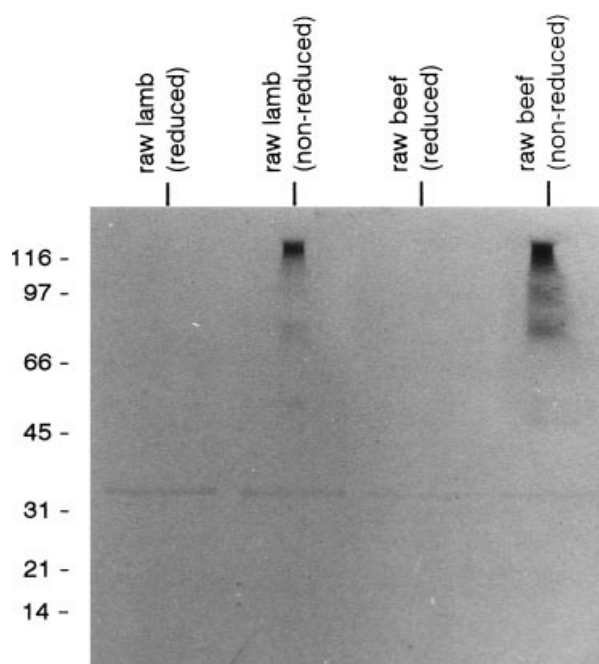


Figure 3. Effect of reducing agent on IgE-binding by 160-kDa protein. Lane 1) reduced lamb; lane 2) nonreduced lamb; lane 3) reduced beef; lane 4) nonreduced beef. IgE reactivity to 160-kDa band in nonreduced extracts was abolished after reducing treatment. No new IgE reactivities of lower mol. mass were detected.

products such as milk, mainly bovine, have been thoroughly studied and the major milk allergens characterized. Nevertheless, few reports have analyzed allergens in beef, lamb, or pork. Among beef proteins, muscle and serum proteins have been identified as beef allergens. Some reports suggest the importance of BSA (4, 6, 18) and bovine gamma globulin (BGG) (4) as meat allergens. Among the muscle proteins, tropomyosin was found to be a weak meat allergen (12). Other beef proteins that appear to be allergenic are actin and occasionally myoglobin (18). Of the beef allergens, only BSA (18), actin (18), and IgG appear to be major allergens, since in immunoblot studies they bind IgE *in vitro* from more than 50% of the beef-allergic patients. The clinical relevance of sensitization to beef proteins has been demonstrated by DBPCFC only for albumin in some beef-allergic subjects (6). The stronger reactivity observed in our immunoblots, in number and intensity, to the bovine IgG in comparison with reactivity to BSA or any other beef proteins, and the fact that, in some sera, IgE reactivity to IgG was the only positivity detected leave no doubt of the importance of IgG in beef allergy. Nevertheless, further studies are required, including oral challenges, to demonstrate the clinical relevance of sensitization to bovine IgG in beef-allergic subjects.

Hypersensitivity to different bovine products may be due to the existence of cross-reacting allergens; several of the major allergens found in cow hair and dander

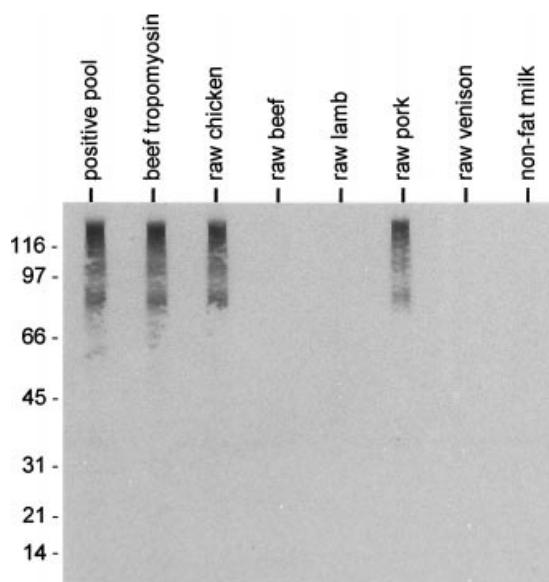


Figure 4. Immunoblot inhibition of beef extract. From left, lane 1) uninhibited IgE reactivity (positive serum pool). Inhibition with beef tropomyosin (lane 2), raw chicken (lane 3), raw beef (lane 4), raw lamb (lane 5), raw pork (lane 6), raw venison (lane 7), and nonfat milk (lane 8). Total inhibition of IgE reactivity to 160-kDa band was obtained with raw beef (homologous inhibition), lamb, venison, and milk, suggesting cross-reacting epitopes. No inhibition was observed with beef tropomyosin or raw chicken and pork.

(Bos d 1, Bos d 2, Bos d 3, and Ag 4) have been identified by crossed-immunoelectrophoresis in other bovine products. Ag 4, with an apparent mol. mass above 200 kDa by gel chromatography (19) and identified as bovine immunoglobulin, was also present in beef as well as in whey, saliva, urine, amniotic fluid, and serum (20). BSA and BGG are milk proteins also present in beef, and BSA is also a bovine hair and dander allergen. Cross-reacting allergens (β -lactoglobulin and casein) have also been demonstrated between cow dander and milk (21). IgE reactivity to bovine IgG in our beef-allergic subjects was completely inhibited by milk, suggesting similar cross-reacting allergens in milk and beef. Our work has identified IgG as a major beef allergen, and it was the only IgE reactivity detected in many of the patients. It is noteworthy, since IgE reactivity to other immunoglobulins (A, G) has been detected previously in bovine dander-allergic patients (20). Therefore, it appears that all bovine products share similar allergens, with IgG playing an important role in the cross-reactivity among bovine products. Thus, sensitization to beef may be secondary to milk allergy (which probably develops since milk is first introduced in the diet), or possibly to hair and dander sensitization (frequently seen in veterinarians); less frequently, sensitization to meat may be primary.

In our study, IgG was not only a major beef allergen, but also a cross-reacting allergen with other mammalian meats. To date, only serum albumin has been

implicated as a cross-reacting meat allergen in beef and lamb (6). *In vitro* cross-reactivity among albumins of different mammalian origin (cattle, horse, dog, cat, etc.) in patients sensitized to animal hair and dander has been described (22, 23). We found that, in general, the IgE-binding pattern of sera from meat-allergic individuals to the various mammalian meats was very similar, especially for beef, lamb, and venison. In contrast, reactivity to pork and chicken was very weak, and no reactivity to IgG was noticed. These results were confirmed by immunoblot inhibition studies. Interestingly, pork and chicken meat did not inhibit IgE reactivity to bovine IgG, probably because of allergenic differences in chicken and pork IgG, rather than the absence of the protein in both meats. These findings are also supported by the cross-reactivity of rabbit antbovine IgG, and bovine and lamb IgG, but not bovine and pork IgG. In view of our results, important *in vitro* cross-reactivity among mammalian meats exists. Pork, a less allergenic meat, could be included in hypoallergenic diets instead of the usually recommended lamb (24). Moreover, since immunoglobulins seem to be important allergens in mammalian dander (19), their role in cross-reactions with other mammalian hair and dander allergens should be further evaluated.

Since beef allergens of more than 100 kDa have been rarely described, the effect of reducing and alkylating agents was studied. The IgE reactivity to IgG after treatment with mercaptoethanol and iodoacetamide completely disappeared without additional IgE reactivities occurring to proteins of lower mol. mass, suggesting conformational epitopes. IgE reactivity to conformational epitopes has been already described for Der p 1. In fact, most of the IgE binds to a flexible loop connecting two domains of the molecule, which appeared on amino-acid residues 81–94 and 101–111 (25). Moreover, B-cell epitope studies of Fel d 1, the major allergen of cat, indicate IgE binding to conformational epitopes; this IgE binding is lost by reduction and alkylation of the protein (26, 27). Therefore, nonreducing techniques may be advisable when identifying new allergens, in view of the risk of overlooking important allergens if only reduced proteins are studied.

In general, IgE binding to raw meat extracts was stronger than that to cooked meat extracts, and other authors have demonstrated similar thermal lability of beef proteins (4). In our studies, no IgE reactivity to

IgG was detected in cooked meat extracts. However, boiling of the raw extract samples for 5 min in denaturing SDS–PAGE sample buffer did not alter the IgE binding, indicating at least some thermal stability of the IgG. Similarly, IgE reactivity to serum albumin was reduced in the cooked meat extracts.

Although beef or mammalian meats are not frequently cited as allergenic foods, some concern about the potential role of IgG in other bovine products such as milk could arise. It appears that the process of heating or pasteurization of the milk before consumption may reduce the immunogenicity of the milk by denaturation of the immunoglobulins (28). The use of SDS–PAGE or other techniques that systematically use denaturing agents when analyzing allergenic extracts may also abolish IgE reactivity to IgG. In fact, although immunoglobulins are not usually considered milk allergens, some data had been published that demonstrate their allergenicity. Most of the children with challenge-proven milk allergy tested had specific IgE antibodies against bovine immunoglobulins (29). The authors did not recommend the administration of bovine immunoglobulin preparations to known milk-allergic children. Interestingly, they show IgE binding specific for 60- and 29-kDa proteins, probably the heavy and light chains of immunoglobulins. This contrasts with our findings, in which IgE-binding capacity was completely lost after reduction and heating, without detection of new IgE reactivities of lower mol mass.

In summary, bovine IgG seems to be a major cross-reacting meat allergen that could predict beef allergy; further studies such as DBPCFC with IgG and other immunoglobulins must be performed to confirm that this *in vitro* IgE reactivity does correlate with clinical hypersensitivity. The role of immunoglobulins, especially IgG, in other bovine products such as milk, dander, or hair should also be studied in more detail, and the implication that they are cross-reacting allergens with other mammalian products clarified.

Acknowledgments

We thank PlasmaLab International, Everett, WA, USA, and Dr A. C. Dalton, Baton Rouge, LA, USA, for their help in obtaining serum samples of meat-allergic subjects. This study was supported by grant 97/5229 from the Instituto de Salud Carlos III, Madrid, Spain, and partly by a grant from Sociedad Española de Alergia e Inmunología Clínica (SEAIC) and the National Fisheries Institute (NFI).

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