

Original article

Allergenicity of major cow's milk and peanut proteins determined by IgE and IgG immunoblotting

Background: Specific IgG antibodies are frequently observed in food-allergic patients. However, the allergen-fraction specificity of IgG antibodies in relation to IgE antibodies is not well defined. Our aim was to determine the IgE and IgG antibody profile to major cow's milk and peanut-antigen fractions in food-allergic patients and tolerant individuals.

Methods: Sera were collected from 10 patients allergic to cow's milk and 10 patients allergic to peanuts, as well as from 20 control subjects. Cow's milk and peanut proteins were migrated on SDS-PAGE and immunoblotted for IgE, IgG, and IgG4 antibodies. Food-specific IgE concentrations were measured by CAP System FEIA[®], and IgG and IgG4 concentrations by ELISA.

Results: In food-allergic children, similar fraction-specific IgE, IgG, and IgG4 antibody-binding profiles to the major cow's milk or peanut antigens were found. In nonallergics, the presence of fraction-specific IgG antibodies was mostly dependent on regular ingestion of the food. The presence of specific antibody on immunoblots correlated with their quantitative measurement. The mean value for specific IgE in cow's milk-allergic patients was 450 ± 1326 IU/ml, and 337 ± 423 IU/ml in peanut-allergic patients. Specific IgG antibody values in milk-allergic patients were not different (median OD 1.5, range 0.3–2.3) from controls (median OD 1, range 0.2–1.8). However, in peanut-allergic patients, IgG concentrations were significantly higher than in controls (OD 1.2 [0.5–1.3] vs 0.5 [0.3–0.7]; $P < 0.01$).

Conclusions: Similar fraction-specific IgE and IgG antibody profiles in allergic individuals suggest a common switching trigger involving both isotypes. Intrinsic allergenicity might explain identical IgG antibody fraction specificity in nonallergics and in allergics. The presence of IgG antibodies in nonallergics was related to regular ingestion of the food.

I. Szabó

Department of Pediatrics, University of Geneva School of Medicine, Geneva

P. A. Eigenmann

Department of Pediatrics and Department of Medicine, Division of Immunology and Allergy, University of Geneva School of Medicine, Geneva, Switzerland

Key words: food hypersensitivity; IgE; IgG; immunoblotting; milk; peanuts.

Philippe A. Eigenmann, MD
Allergy Unit
University Hospital of Geneva
24 rue Micheli-du-Crest
1211 Geneva 14
Switzerland

Accepted for publication 5 August 1999

Cow's milk and peanut are two of the most prevalent sources of allergens in IgE-mediated food allergy. The major antigens of cow's milk, caseins, β -lactoglobulin (BLG), and α -lactalbumin (ALA), have been well characterized (1), and their implication in clinical reactions has been established (2). Similarly, Ara h 1, Ara h 2, and Ara h 3 were found to be the major peanut allergens (3–5). Initial identification of these allergens was obtained by IgE antibody (Ab) immunoblotting, but only a few studies have examined IgG Abs in relation to fraction-specific IgE Abs. de Jong et al. found similar IgE and IgG Ab-binding patterns on peanut protein immunoblots with pooled sera from allergic patients (6). In a study on soy allergy, Burks et al. identified in eight patients a similar binding pattern of IgE and IgG Abs to two major antigens (7). Other investigators found similar results in wheat allergy (8). It has been speculated that IgG Abs may

play a role in the pathogenesis of food allergy (9, 10). However, the presence of specific IgG Abs to a food regularly ingested may represent a normal response of the immune system (11–13).

Extensive knowledge has been acquired recently on the mechanisms involved in Ab production. Immunoglobulin isotype switching can result in different types of Abs, but with a similar antigen specificity. In mice, IL-4 has been found to promote predominantly IgG1 and IgE, while interferon- γ induces IgG2a Ab switching. In man, prolonged exposure to an antigen favors an IgG4 response (14). Other studies suggest a sequential isotype switching for antigen-specific Abs in experimental models (15–18). However, little is known about the presence of food-specific IgE and IgG Abs in man in relation to clinical reactivity and to food consumption. In this study, we investigated the antigen-fraction specificity of IgG Abs in relation to IgE Abs in

milk- or peanut-allergic patients. Antibody-fraction specificity was also determined in nonallergic subjects, and food-specific IgG and IgE Ab values were compared in both groups.

Material and methods

Patients

Serum was obtained from 10 patients diagnosed with IgE-mediated cow's milk allergy, and 10 patients diagnosed with IgE-mediated peanut allergy after standardized food challenges and/or by a convincing history of an anaphylactic reaction, elevated antigen-specific IgE, and positive prick skin test according to diagnostic criteria previously defined (19). The characteristics of the patients are summarized in Table 1. Four additional patients suffered from milk-induced enterocolitis syndrome. They were diagnosed by criteria published previously (20). Twenty age-matched subjects provided serum for control blots. None had food allergy, and all were ingesting cow's milk and peanuts without symptoms. Cow's milk and dairy products were ingested daily by all children. Subject 31 was daily consuming peanuts, and subject 36 ate them several times a week, while all other nonallergics (nos. 33–40) only occasionally ate peanut-containing foods. Although the quantity of proteins ingested through

peanut-contaminated foods cannot be quantified, we estimate it to be a minimal amount.

This study has been reviewed and approved by the ethics committee of the Department of Pediatrics.

Glycine-SDS polyacrylamide gels and immunoblotting

Cow's milk (2 mg/ml, manufactured by Säntis Milchpulver AG, Sulgen, Switzerland), or peanut antigen (1 mg/ml, kindly provided by Dr A. W. Burks, Children's Hospital, Little Rock, AR, USA) was mixed with an equal volume of SDS sample buffer (50 mM Tris HCl (pH 6.8) containing 4% SDS, 2% B-mercaptoethanol, 12% glycerol bromophenol blue, and pylonin Y). Optimal resolution was found with boiled peanut and unboiled milk antigens. Proteins were migrated on glycine-SDS polyacrylamide gels, modified from the method described by Laemmli (21). The running gel was prepared from a stock of 33.5% acrylamide (Sigma, Buchs, Switzerland) and 0.3% bis-acrylamide (Bio-Rad, Richmond, CA, USA) in 1 M Tris/HCl (pH 9.1) with 10% SDS gel buffer solution, and glycerol. The stacking gel was prepared from a solution of acrylamide-bisacrylamide 30%:0.44% in Tris-HCl 0.5 M (pH 6.8) and SDS 10% with a final concentration of 4% acrylamide and 1% SDS. Both gels were polymerized with 10% ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine. The gels were migrated on a Bio-Rad Mini-Protein II (Bio-Rad,

Table 1. Clinical characteristics of patients

Patient no.	Sex	Age at serum sampling	Symptoms‡	Age at diagnosis	Other food allergies
<i>Cow's milk allergies</i>					
1	M	8 months	AD, W	8 months	Egg
2	M	2 years 1 month	AD, U, W	2 months	Wheat, egg, peanut, fish, oat, barley, pork, tomato, lamb
3	F	1 year	U, W	6 months	No
4	M	1 year	AD	3 months	Peanut, soy, egg, fish, wheat
5	M	9 months	AD, W	3 months	Egg
6	M	8 years 7 months	AD	2 months	Fish, peanut, soy, barley
7	F	7 months	AD, A	3 months	No
8	F	5 years 1 month	U, GI	7 months	No
9	F	5 years 8 months			
10	F	5 years 6 months	AD, W	5 years 6 months	Egg, fish, peanut, nuts
<i>Peanut allergies</i>					
21	F	2 years 3 months	n.a.	2 years 3 months	Egg, sesame
22	M	4 years 4 months	U	1 year	Milk, soy, fish
23	M	8 years 6 months	U, W, AD	1 year	Hazelnut
24	M	1 year	AD	3 months	Milk, soy, fish, egg, wheat
25	M	13 years 4 months	U	13 years	Apple, kiwi, carrot
26	F	2 years 7 months	U, W, AD	2 years 6 months	No
27	M	8 years 7 months	AD	7 years 9 months	Fish, milk, soy, barley
28	M	25 years	A	20 years	No
29	M	19 years 9 months	A	n.a.	Hazelnut, carrot, celery
30	M	16 years 5 months	A	16 years 5 months	No

‡AD: atopic dermatitis; A: anaphylaxis; GI: gastrointestinal symptoms; U: urticaria; W: wheezing.

Richmond, CA, USA) at 80 V through the stacking gel, and at 160 V through the running gel.

The migrated milk and peanut proteins were electrotransferred from the polyacrylamide gel to nitrocellulose paper at 100 V for 1 h in 50 mmol/l of Tris-glycine buffer (pH 9.1) containing 20% methanol. After transfer, the nitrocellulose blots were blocked for 1 h in PBS plus 0.05% Tween (PBS-T) with 0.5% gelatin (Fluka, Buchs, Switzerland). Protein staining with 0.1% amido black was obtained for each procedure to confirm proper electrophoresis and protein transfer on Protran[™] 0.2 µm nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Molecular weight markers (SDS-7) were purchased from Sigma, Buchs, Switzerland. The blots were incubated with serum and PBS-T (1:10 v/v dilution for IgE, 1:200 for IgG, and 1:20 for IgG4) for 2 h at room temperature on a rocking platform. Antibodies were bound by a goat anti-human IgE Ab used at 0.5 µg/ml (Kirkegaard and Perry, Inc., Gaithersburg, MD, USA), or by a sheep antihuman IgG (0.5 µg/ml) or a mouse antihuman IgG4 (1:1000) (both Abs from Serotec Ltd, Oxford, UK). Five washes (for 5 min) with PBS-T were done between each step. The blots were developed with DAB[™] (Sigma, Buchs, Switzerland).

Quantitative measurement of specific Abs

Cow's milk- and peanut-specific IgE concentrations were measured by the CAP System FEIA[™] (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. Antigen-specific IgG and IgG4 Ab concentrations were determined by ELISA. Microtiter plates (Nunc-Immuno Plate Maxisorp, Nalge Nunc Int., Denmark) were filled with 50 µl/well of a 10 µg/ml solution of cow's milk, or peanut antigen in coating buffer (0.1 M sodium bicarbonate, pH 9.6), and incubated overnight at 4°C. Fifty microliters of sera (at 1:100 for IgG, and 1:20 for IgG4) in Ab buffer (PBS-T plus 2% human serum albumin) were incubated in duplicates for 2 h at 37°C. Second-step Abs were horseradish peroxidase-conjugated sheep antihuman IgG (0.5 µg/ml) or horseradish peroxidase-conjugated mouse antihuman IgG4 (1:1000) (both Abs from Serotec Ltd, Oxford, UK). Wells were washed thrice between each step with PBS-T. The plates were developed with *o*-phenylenediamine (Sigma, Buchs, Switzerland), and optical densities (OD) were measured at 490 nm with an automated ELISA plate reader (Molecular Device Corporation, Menlo Park, CA, USA).

Statistical analysis

Antigen-specific Ab values obtained by ELISA were analyzed by a nonparametric test for unpaired samples (Wilcoxon rank-sum test).

Results

Fraction-specific Abs by immunoblotting

Whole cow's milk or peanut proteins were migrated on SDS gels and immunoblotted for IgE, IgG, and IgG4 Abs. Fig. 1 shows milk and peanut fractions and molecular weight markers stained with Coomassie brilliant blue. Each set of immunoblots was controlled for nonspecific binding by a blot without serum. Analysis of binding profiles was restricted to well-characterized cow's milk (caseins, BLG, and ALA) and peanut (Ara h 1, Ara h 2, and Ara h 3) major allergenic fractions. On immunoblots to cow's milk, IgE Abs to

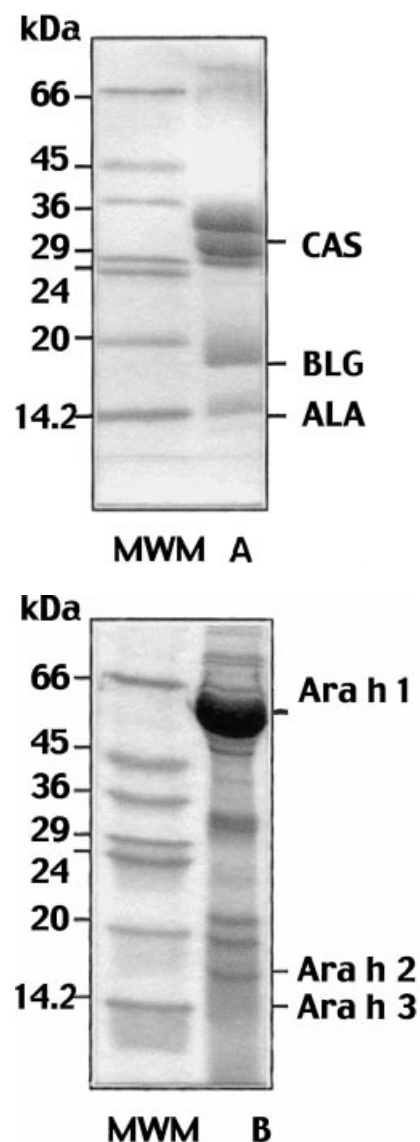


Figure 1. Milk (lane A), and peanut extract (lane B) SDS-PAGE stained with Coomassie brilliant blue. Molecular weight markers (MWM; SDS-7, Sigma, Buchs, Switzerland) are at 66, 45, 36, 29, 24, 20, and 14.2 kDa. Major antigens seen on lane A (cow's milk) are casein (CAS), β -lactoglobulin (BLG), and α -lactalbumin (ALA); on lane B (peanut), they are Ara h 1, Ara h 2, and Ara h 3.

Table 2. Number of patients with positive IgE, IgG, and/or IgG4 antibodies to major cow's milk or peanut proteins by immunoblotting

	IgE	IgG	IgG4	IgE + IgG	IgE + IgG + IgG4
Milk allergics (n=10)					
Casein	9	9	8	8	7
β -Lactoglobulin	5	7	8	4	4
α -Lactalbumin	2	6	1	2	0
Peanut allergics (n=10)					
Ara h 1	6	8	5	6	5
Ara h 2	8	9	8	7	7
Ara h 3	7	9	8	6	6

the casein fractions were found in nine milk allergics, while five patients also had IgE Abs to BLG and two to ALA (Fig. 2A; summarized in Table 2). Absence of significant IgE Ab binding to milk protein was seen in nonallergics (not shown). Most cow's milk-allergic patients had positive bands to casein and BLG for IgG (Fig. 2B), while only one allergic had a band to ALA with IgG4 (Fig. 2C). Of the nine allergics who had IgE Abs to casein, eight had also IgG, and seven had IgE, IgG, and IgG4 Abs. Similarly, 4/5 patients with IgE to BLG had also IgG and IgG4 Abs, and 2/2 patients with IgE to ALA had also IgG, but no IgG4, Abs (Table 2), thus showing a similar fraction binding by all isotypes.

IgG Ab to the casein and BLG fractions were seen in five nonallergics (Fig. 3A). Cow's milk fractions with

IgG4 Ab binding were similar to IgG Ab binding in three subjects (nos. 14, 15, and 19). To confirm the role of a regular exposure to a food antigen on IgG Ab production, we tested sera from four patients with cow's milk-induced enterocolitis syndrome, who were on a milk-free diet. In the absence of Ab-mediated symptoms, a cellular mechanism is suspected in this syndrome. No IgG or IgG4 Abs binding to either major antigen was seen on the blots (not shown).

On immunoblots to peanut proteins, a signal corresponding to a low degree of nonspecific IgE Ab binding to Ara h 1 was seen in nonallergics in the absence of specific IgE Abs (not shown). IgE Ab binding to Ara h 2 and Ara h 3 was seen only in allergic patients (Ara h 2: in eight patients *vs* no controls; Ara h 3: 7 *vs* 0) (Fig. 4A). Most allergics had IgG Abs to Ara h 2 (9/10), and Ara h 3 (9/10) (Fig. 4B and C). Similar binding patterns were seen for IgG4 Abs. Again, most allergics with IgE Abs to Ara h 1, Ara h 2, or Ara h 3 also had antigen-specific IgG and IgG4 Abs to these proteins (Table 2). In nonallergics, a weak signal with Ara h 2 was seen for IgG in only one subject (no. 35), who was not eating peanuts on a regular basis (Fig. 5A). Two other nonallergics consuming peanuts more frequently (nos. 31 and 36) had some IgG Abs. IgG4 Ab to Ara h 1 was seen in only one nonallergic (no. 37) (Fig. 5B).

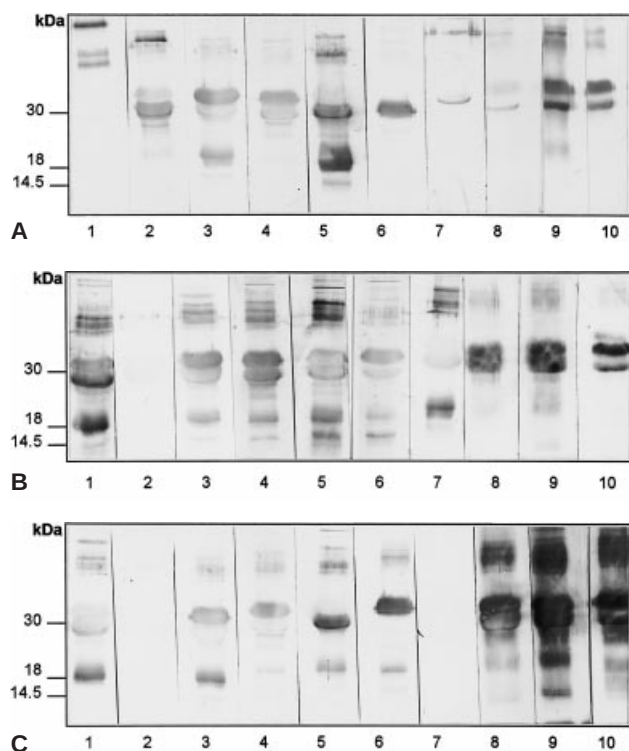


Figure 2. Ab binding to cow's milk fractions isolated on SDS-PAGE immunoblots for 10 cow's milk-allergic patients (lanes 1–10). A) IgE Ab immunoblot, B) IgG Ab immunoblot, C) IgG4 Ab immunoblot.

Quantitative measurement of food-specific Abs

None of the control subjects had measurable specific IgE Abs, while patients allergic to milk had specific IgE Ab concentrations to milk ranging from 8.2 to 4225 IU/ml, with a median of 39.8 IU/ml (Fig. 6A). Patients allergic to peanuts had specific IgE Ab concentrations ranging from 21.1 to 1165 IU/ml, with a median of 134 IU/ml (Fig. 6B). Cow's milk- and peanut-specific IgG

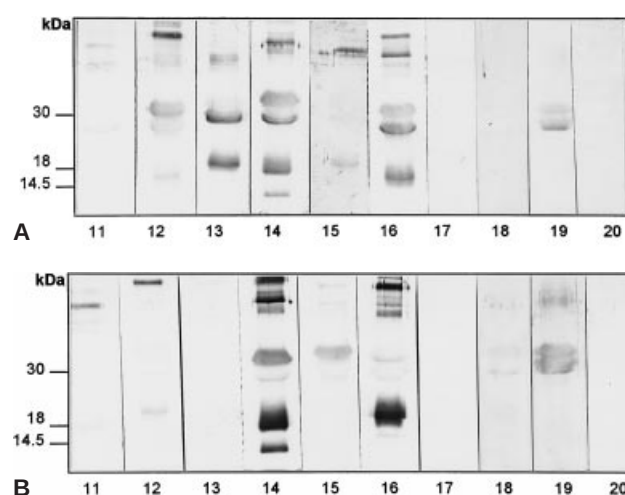


Figure 3. Ab binding to cow's milk fractions isolated on SDS-PAGE immunoblots for 10 nonallergics (lanes 11–20). A) IgG Ab immunoblot, B) IgG4 Ab immunoblot.

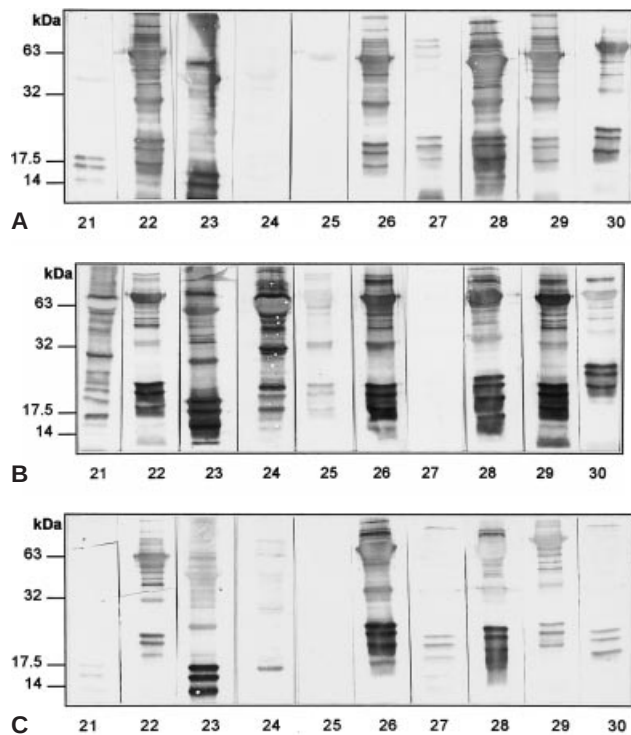


Figure 4. Ab binding to peanut fractions isolated on SDS-PAGE immunoblots for 10 peanut-allergic patients (lanes 21–30). A) IgE Ab immunoblot, B) IgG Ab immunoblot, C) IgG4 Ab immunoblot.

and IgG4 concentrations were measured by ELISA. IgG Ab concentrations (OD values from 0.2 to 1.8, median: 1) in milk-tolerant subjects were overlapping with results from allergies (median: 1.5, range: 0.3–2.3; $P = \text{NS}$) (Fig. 6A). Results for specific IgG4 Ab to milk were similar in both populations (median value in allergies: 0.9 [0.1–2.7], and in controls: 0.7 [0.1–2.4]; $P = \text{NS}$).

In peanut-allergic patients, specific IgG Ab concentrations were found to be elevated in all but one patient (median OD value of 1.2 [0.5–1.3]), while concentrations were all found to be low in nonallergics (0.3–0.7, median 0.5; $P < 0.01$) (Fig. 6B). There were virtually no measurable specific IgG4 Abs in sera from controls (median 0.3, range: 0.2–0.4), while specific IgG4 Abs were found in most allergic patients (median 0.4, range: 0.1–1.5), but without a significant difference between the two groups. Overall, the values for IgG or IgG4 Abs did correspond to the intensity of the signal seen on the immunoblots.

Discussion

We examined the fraction specificity of different Ab isotypes to major peanut and milk allergens. Serum IgE Abs from most allergic patients did bind to the major allergenic fractions already described. Therefore, the study population corresponded to a representative

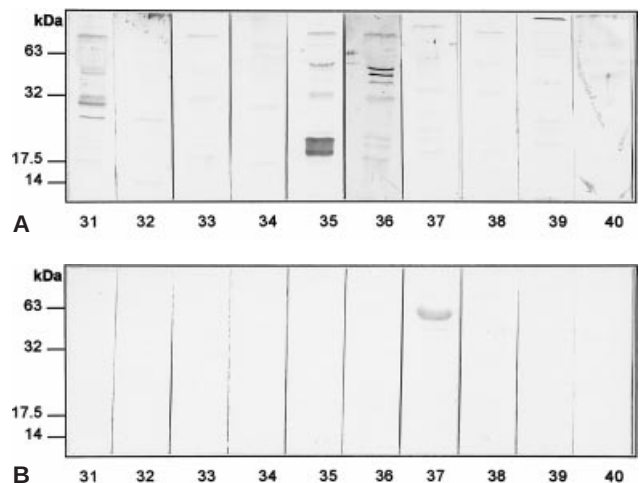


Figure 5. Ab binding to peanut fractions isolated on SDS-PAGE immunoblots for 10 nonallergics (lanes 31–40). A) IgG Ab immunoblot, B) IgG4 Ab immunoblot.

sample of patients with IgE-mediated milk or peanut allergy. Ab-binding patterns were specific to each individual patient, and Ab binding to minor antigens was found in a few patients only. Furthermore, intraindividual analysis of IgG or IgG4 Ab-binding profiles to either milk or peanut fractions was similar to IgE (Table 2).

Several studies have examined IgG and IgE antibody specificity to foods. Spuergin et al. found similar epitope binding of α_{s1} -casein by IgE and IgG Abs (22). Wheat- or soy-specific IgE and IgG Abs recognize the same antigenic fractions (7, 8). de Jong et al. found similar results with peanut-specific IgE and IgG immunoblots (6). However, the use of pooled sera did not allow these authors to analyze individual immunoblot profiles. In our study, the specificity of IgE and IgG Abs to similar fractions in individual peanut- or cow's milk-allergic patients suggests a common antigen-specific secretion trigger in both isotypes. Most of the allergies had avoided the food well before serum was obtained, and had specific IgG Abs. Thus, the presence of IgG in the sera of milk allergies cannot be attributed to an immunologic response due to a constant exposure to a food antigen. As previously shown in experimental models with human cells (15–18), sequential or cyclic isotype switching to IgG and IgE Abs triggered by IL-4 seems to be a more convincing explanation. It should be emphasized that the presence of IgG Abs to major allergenic fractions of peanut or cow's milk does not prove a pathogenic role. However, controversy continues in the literature (9, 10).

Immunoblots with identical IgG Ab profiles were found in tolerant children regularly ingesting cow's milk, while control subjects occasionally ingesting peanuts had low or no detectable specific IgG Abs. These results tend to confirm the hypothesis that the presence of IgG Abs in nonallergics is largely dependent

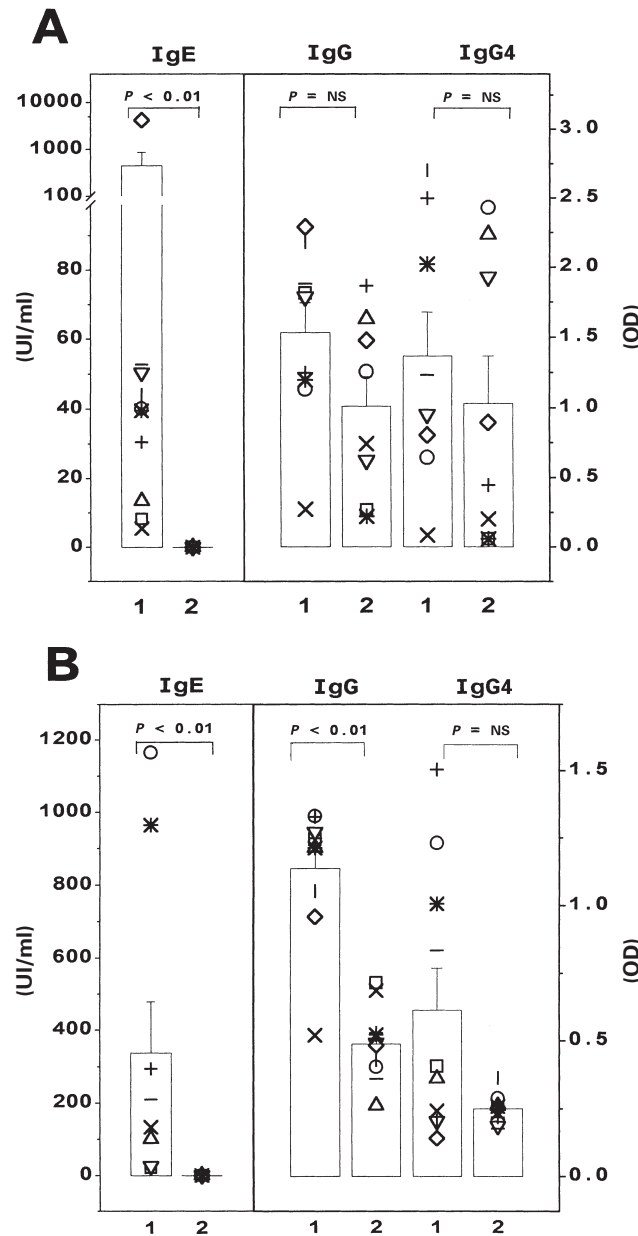


Figure 6. Cow's milk- (A) and peanut-specific (B) IgE Abs (by CAP System), and IgG and IgG4 Abs (by ELISA) measured in sera from allergies (lane 1) and nonallergics (lane 2). ELISA were performed on same plate to allow interindividual comparison. Each symbol represents same serum (either allergic or nonallergic subject) in all test conditions. Columns indicate mean value and SEM.

on a regular ingestion of the food (11, 12). IgG4 was correlated to regular exposure to a food in a previous study (14). However, the low amount of peanut-specific total IgG found in nonallergics might explain undetectable peanut-specific IgG4. The role of regular exposure to a food in IgG Ab production was confirmed by the absence of cow's milk-specific IgG Abs in patients with cow's milk-induced enterocolitis syndrome, a non-IgE-mediated disease (23), who were on a milk-free diet. Levels of specific IgG Abs might also be influenced by early feedings, as children exposed early in life to cow's milk proteins have higher levels of specific IgG Abs (12, 13). Thus, no food-specific IgE

Abs were detectable in nonallergics, suggesting an inhibiting stage for immunoglobulin between IgG and IgE switching. Several *in vitro* studies with human cells have shown inhibition of IgE switching by IFN- γ (24, 25). Furthermore, it has been shown that immunotherapy with Hymenoptera venom induces a Th1-type switch with increased secretion of IFN- γ (26), associated with a progressive decrease of specific IgE, but an increase of IgG4 levels (27). It remains unknown whether a similar cytokine dysbalance toward Th2 cytokines can explain the pathogenesis of food allergy. Interestingly, the intensity of IgG binding to different protein fractions on the blots from nonallergics did not

correspond to the relative amount of the protein in the food; for example, BLG represents 9% of total cow's milk proteins, but most nonallergics had a strong signal for IgG Abs to the BLG fraction. Whether intrinsic allergenicity of specific fractions plays a role in the intensity of the immune response in nonallergics remains to be determined.

The IgG4 Ab subtype has been the focus of several studies on food allergy. Høst et al. found elevated BLG-specific IgG4 Abs in sera from cow's milk-allergic children (11), and Morgan et al. found elevated specific IgG4 Abs in shrimp-allergic patients (28). James et al. followed IgG4 and IgE Ab titers in patients "growing out" of their sensitivity to cow's milk. They found lower IgE/IgG4 and IgE/IgG1 Ab ratios in patients becoming tolerant (29). In our study, most patients with food-specific IgG Abs also had IgG4 Abs, confirming that the sequential Ab switching mechanism to IgE includes IgG4.

In summary, most food-allergic patients had specific IgG Ab and IgE Abs to a similar fraction. These findings suggest an IgE switching mechanism, including IgG production, in cow's milk- and peanut-allergic individuals. The presence of food-specific IgG Abs in tolerant controls can be correlated with the regular ingestion of a food. In nonallergics, specific IgG Abs are found to the same major allergenic fractions, but in the absence of IgE Abs. Whether food allergy results from a defective regulatory mechanism with decreased IFN- γ levels remains to be determined.

Acknowledgments

This study was supported in part by grants from the Swiss National Research Foundation, the De Reuter Foundation, the Société Académique de Genève, and the Ciba-Geigy Foundation. We thank L. Tropa for technical assistance, and Dr H. Sampson for kindly providing serum samples from cow's milk-induced enterocolitis patients.

References

1. BLEUMINK E, YOUNG E. Identification of the atopic allergen in cow's milk. *Int Arch Allergy Appl Immunol* 1968;**34**:521–543.
2. GOLDMAN AS, ANDERSON DW, SELLERS WA, SAPERSTEIN A, KNIKER WT, HALPERN SR. Milk allergy. I. Oral challenge with milk and isolated milk proteins in allergic children. *Pediatrics* 1963;**32**:425–443.
3. BURKS AW, COCKRELL G, STANLEY JS, HELM RM, BANNON GA. Recombinant peanut allergen Ara h 1 expression and IgE binding in patients with peanut hypersensitivity. *J Clin Invest* 1995;**96**:1715–1721.
4. BURKS AW, WILLIAMS LW, CONNAUGHTON C, COCKRELL G, O'BRIEN TJ, HELM RM. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J Allergy Clin Immunol* 1992;**90**:962–969.
5. RABJOHN P, HELM EM, STANLEY JS, et al. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* 1999;**103**:535–542.
6. DE JONG EC, VAN ZIJVERDEN M, SPANHAAS S, KOPPELMAN SJ, PELLEGRINI H, PENNINKS AH. Identification and partial characterization of multiple major allergens in peanut proteins. *Clin Exp Allergy* 1998;**28**:743–751.
7. BURKS AW JR, BROOKS JR, SAMPSON HA. Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *J Allergy Clin Immunol* 1988;**81**:1135–1142.
8. THEOBALD K, THIEL H, KALLWEIT C, ULMER W, KÖNIG W. Detection of proteins in wheat flour extracts that bind human IgG, IgE, and mouse monoclonal antibodies. *J Allergy Clin Immunol* 1986;**78**:470–477.
9. HINDOCHA P, WOOD CBS. Histamine release from human leucocytes by IgG4 subclass in the sera of allergic children. *Allergy* 1985;**40**:523–528.
10. VAN TOORENENBERGEN AW, AALBERSE RC. IgG4 and release of histamine from human peripheral blood leukocytes. *Int Arch Allergy Appl Immunol* 1982;**67**:117–122.
11. HØST A, HUSBY S, GJESING B, LARSEN JN, LÖWENSTEIN H. Prospective estimation of IgE, IgG subclass and IgE antibodies to dietary proteins in infants with cow milk allergy. *Allergy* 1992;**47**:218–229.
12. KELLER KM, BURGINS-WOLFF A, LIPPOLD R, WIRTH S, LENTZE MJ. The diagnostic significance of IgG cow's milk protein antibodies re-evaluated. *Eur J Pediatr* 1996;**155**:331–337.
13. JENMALM MC, BJÖRKSTÉN B. Exposure to cow's milk during the first 3 months of life is associated with increased levels of IgG subclass antibodies to beta-lactoglobulin to 8 years. *J Allergy Clin Immunol* 1998;**102**:671–678.
14. AALBERSE RC, VAN DER GAAG R, VAN LEEUWEN J. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J Immunol* 1983;**130**:722–726.
15. ZHANG K, MILLS FC, SAXON A. Switch circles from IL-4-directed epsilon class switching from human B lymphocytes. Evidence for direct, sequential, and multiple step sequential switch from mu to epsilon Ig heavy chain gene. *J Immunol* 1994;**152**:3427–3435.
16. MILLS FC, THYPHRONITIS G, FINKELMAN FD, MAX EE. Ig mu-epsilon isotype switch in IL-4-treated human B lymphoblastoid cells. Evidence for a sequential switch. *J Immunol* 1992;**149**:1075–1085.
17. JABARA HH, LOH R, RAMESH N, VERCELLI D, GEHA RS. Sequential switching from mu to epsilon via gamma 4 in human B cells stimulated with IL-4 and hydrocortisone. *J Immunol* 1993;**151**:4528–4533.
18. BASKIN B, ISLAM KB, EVENGARD B, EMTSTAM L, SMITH CI. Direct and sequential switching from mu to epsilon in patients with *Schistosoma mansoni* infection and atopic dermatitis. *Eur J Immunol* 1997;**27**:130–135.
19. SAMPSON HA, HO DG. Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents. *J Allergy Clin Immunol* 1997;**100**:444–451.
20. POWELL GK. Milk- and soy-induced enterocolitis of infancy. *J Pediatr* 1978;**93**:553–560.

21. LAEMMLI UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;**227**:680–685.
22. SPUERGIN P, MUELLER H, WALTER M, SCHILTZ E, FORSTER J. Allergenic epitopes of bovine α_{s1} -casein recognized by human IgE and IgG. *Allergy* 1996;**51**:306–312.
23. SICHERER SH, EIGENMANN PA, SAMPSON HA. Features of food protein-induced enterocolitis. *J Pediatr* 1998;**133**:214–219.
24. PÈNE J, ROUSSET F, BRIÈRE F, et al. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons gamma and alpha and prostaglandin E₂. *Proc Natl Acad Sci U S A* 1988;**85**:6880–6884.
25. THYPHRONITIS G, BANCHEREAU J, HEUSSER C, TSOKOS GC, LEVINE AD, FINKELMAN FD. Kinetics of interleukin-4 induction and interferon-gamma inhibition of IgE secretion by Epstein-Barr virus-infected human peripheral blood B cells. *Cell Immunol* 1991;**133**:408–419.
26. JUTEL M, PICHLER WJ, SKRBIC D, URWYLER A, DAHINDEN C, MÜLLER UR. Bee venom immunotherapy results in decrease of IL-4 and IL-5 and increase of IFN-gamma secretion in specific allergen-stimulated T cell cultures. *J Immunol* 1995;**154**:4187–4194.
27. MÜLLER U, HELBLING A, BISCHOF M. Predictive value of venom-specific IgE, IgG and IgG subclass antibodies in patients on immunotherapy with honeybee venom. *Allergy* 1989;**44**:412–418.
28. MORGAN JE, DAUL CB, LEHRER SB. The relationships among shrimp-specific IgG subclass antibodies and immediate adverse reactions to shrimp challenge. *J Allergy Clin Immunol* 1990;**86**:387–392.
29. JAMES JN, SAMPSON HA. Immunologic changes associated with the development of tolerance in children with cow milk allergy. *J Pediatr* 1992;**121**:371–377.