

Polyisotypic antipeanut-specific humoral responses in peanut-allergic individuals

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Summary

Background Peanut-containing food products may induce severe clinical reactions in sensitized subjects, and high levels of antipeanut IgE have been reported in the literature. Immunotherapy, proposed for the prevention of severe accidents, is often ill-tolerated and only partly efficient. This could be due to the spontaneous development of polyisotypic antipeanut antibodies.

Objective To appreciate the presence and reactivity of other isotypes other than IgE of peanut-specific antibodies in serum samples from peanut-sensitized subjects.

Methods Serum samples were obtained from 20 non-sensitized subjects and 23 sensitized patients divided in three groups according to their response to peanut oral challenge (no response or response to high or low doses, respectively). Peanut-specific IgG, IgG subclasses, IgA and IgM were assayed using an ELISA, and their reactivity against peanut proteins tested using Western Blot.

Results A large dispersion of antipeanut antibody levels was observed in the three groups of patients, high levels of IgG, IgG1, IgG4 and IgA usually correlating with highly positive radioallergosorbent test (RAST). Such high levels were observed at onset in four patients who underwent peanut immunotherapy who had side effects and poor efficiency. Western blotting demonstrated that the polyisotypic response observed was directed to several peanut antigens, including the major allergens, Ara h1 and Ara h2.

Conclusion Peanut-sensitized patients who spontaneously develop specific IgE, display polyisotypic-specific antibody responses, whatever their response to oral challenge. This might explain the poor efficiency of peanut rush immunotherapy attempts.

Keywords: ELISA, IgA, IgE, IgG subclasses, IgM, immunoglobulin isotypes, immunotherapy, peanut allergy, Western Blot

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Introduction

Food allergies have become a true health problem, as they seem to develop with increasing frequency in young and adult people [1,2]. Among the most common food hypersensitivities, peanut allergy appears especially important not only because of the potential severity of the allergic reaction and the persistence of sensitization, but also because of the

ubiquity of peanut products [3–5]. Peanut allergy causes more severe symptoms than other food allergies and fatal cases of IgE-mediated anaphylaxis have been described [6,7]. Moreover, minimal contact may be sufficient to yield severe reactions [4,8–10]. Epidemiological studies of food allergies have helped to approach the incidence of peanut protein-specific IgE, and of positive cutaneous and/or oral challenges to peanut antigens [4,11,12]. Rush immunotherapy often appears ill-tolerated and satisfactory levels of tolerance are difficult to achieve [13,14].

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Several authors have established peanut-specific T cell clones from sensitized patients. These cells displayed a Th2 cytokine secretion profile potentially involved in favouring antibody and therefore IgE production [15,16]. However, little work has been done to investigate other isotypes of the specific humoral responses to peanut antigens. As immunotherapy is expected to favour the appearance of protective IgG, also called blocking antibodies [17], it would be of interest to have a better knowledge of spontaneous non-IgE responses in sensitized patients.

In the study reported here, we investigated specific isotypic humoral responses in three groups of patients with histories of peanut hypersensitivity. We used an ELISA technique developed with the peanut extract used for skin tests to measure the levels of peanut-specific IgG and IgG subclasses, IgA and IgM. Positive sera were used in a Western Blot technique to define the molecular mass of the proteins involved in these specific humoral responses.

Materials and methods

Subjects

Peanut-specific antibodies were assayed in serum samples from 23 patients with a history of peanut sensitization, enrolled in a prospective study of food allergies, and recruited in a specialized department involved in allergology. Blood samples were collected shortly after the initial clinical manifestations of peanut allergy for diagnostic investigations. The remainder of serum samples was stored at -20°C , and used for this complementary humoral study.

Sensitization to peanut had been later defined according to the proposals of the European Food Allergy Study Group, on the basis of one of the following four criteria: (1) sensitization proven by skin-prick tests and/or (2) specific IgE assays, completed by (3) a standard provocation test, cured by eviction for at least 6 months, or (4) anaphylactic shock reaction to the sensitizing food (provocation tests were not necessary in such cases). Skin-prick tests were performed using a commercial peanut extract (Allerbio, Varennes en Argonne, France). Positive skin-prick tests were considered upon development of a wheal of at least 3 mm diameter. Controls included 9% codeine and saline. Specific IgE were assayed using the radioallergosorbent (RAST) Phadebas CAP System (Pharmacia, Uppsala, Sweden).

Provocation tests had been performed for all patients according to one of two protocols: labial or oral. Labial tests were graded 15 min after the deposition of a single drop of the food in liquid solution on the outer edge of the lower lip as reported elsewhere [18]. The grades were as follows:

- labial oedema at the site of application;
- erythema or urticaria extending to the chin;

- extension of urticaria on one side of the face, with conjunctivitis or rhinorrhea;
- remote eruptions, i.e. in flexion folds in patients with atopic dermatitis.

If labial provocation was negative, a double-blind, placebo-controlled food challenge (DBPCFC) was performed, according to previously published data [19,20], using original food and a placebo. The patient was monitored during the following afternoon and night. Observation of a clinical reaction led to the i.m. injection of methylprednisolone with antihistaminics. Patients with known asthma who began to react received salbutamol in the form of an aerosol. Positivity of DBPCFC relied on the observation of clinical signs of nausea, abdominal pain, urticaria, conjunctivitis and/or respiratory distress.

According to the clinical history of the patients and to their reaction to a labial test or DBPCFC performed at diagnosis, the sensitized patients were divided into three groups (Table 1). Group I consisted of six patients with a history of clinical reaction after peanut ingestion but negative labial challenge or DBPCFC. Group II included nine subjects with moderate clinical signs at onset and positive reactions to labial challenge or DBPCFC at high antigen cumulative doses (≥ 965 mg). Group III comprised eight allergic subjects who had reported severe clinical reactions and had positive labial challenges or DBPCFC at low antigen cumulative doses (< 965 mg).

Prior to this study on stored serum samples, four of the patients (14, 15, 22 and 23) had agreed to receive peanut immunotherapy. This treatment was accompanied by severe clinical reactions (dyspnea, asthma, nausea), which led to therapy interruption for three of them.

Antibody levels were compared to those observed in serum samples from a control group of 20 males not sensitized to peanut and with no history of atopy. These subjects were recruited outside the department of clinical allergy, in a salt factory. Participation of these subjects was approved by a local ethical committee and all individuals tested freely agreed to participate. They were healthy workers involved in a health care survey who answered an extensive questionnaire including specific items regarding food sensitization. None of them were atopic or reported allergies. These subjects are therefore representative of the French population in terms of peanut consumption.

Peanut extract

A commercial peanut extract prepared for skin testing and immunotherapy was also used in the ELISA test. Briefly, this peanut extract had been obtained from peanut delipidated seeds, by an ammonium bicarbonate (4 g/L) extraction method for 16 h at 4°C . Purification was obtained by

Table 1. Characteristics of the patients studied: clinical manifestations at onset and results of labial and oral challenges (Ara = peanut; nd = not done; Neg = negative; Pos = positive).

Patients/sex M/F	Age (years)	Clinical features after peanut ingestion	Skin-prick test (mm)	Labial challenge (score)	Oral challenge acc. to DBPCFC
<i>Group I: Patients with negative oral challenge</i>					
1/F	20	Asthma + atopic dermatitis	Ara: 0	nd	Ara: Neg (7110 mg)
2/F	29	Angioedema, urticaria	Ara: 4	Neg	Ara: Neg (11.5 g)
3/F	11	Asthma + atopic dermatitis	Ara: 0	nd	Ara: Neg (10g)
4/M	8	Atopic dermatitis	Ara: 2	Neg	Ara: Neg (965 mg)
5/M	9	Asthma + atopic dermatitis	Ara: 1	nd	Ara: Neg (965 mg)
6/M	8	Asthma	Ara: 5.5		Ara: Neg (7110 mg)
<i>Group II: Patients with positive labial or oral challenge at high doses</i>					
7/F	9	Asthma	Ara: 5	nd	Ara: Pos (2000 mg)
8/M	8	Asthma	Ara: 5	Pos (1)	nd
9/F	12	Asthma	Ara: 10	Pos (3)	Ara: Pos (965 mg)
10/M	9	Asthma	Ara: 15	Neg	Ara: Pos (965 mg)
11/F	7	Angioedema + urticaria	Ara: 5	nd	Ara: Pos (7110 mg)
12/F	2	Angioedema	Ara: 5	Pos (2)	Ara: Pos (7110 mg)
13/F	2.5	Atopic Dermatitis	Ara: 0	nd	Ara: Pos (7110 mg)
14/M	9	Asthma	Ara: 11	nd	Ara: Pos (965 mg)
15/M	17	Shock	Ara: 13	nd	Ara: Pos (965 mg)
<i>Group III: Patients with positive labial or oral challenge at low doses</i>					
16/M	19	Angioedema Generalized pruritis	Ara: 11	Pos (3)	nd
17/M	10	Anaphylactic shock	Ara: 7	nd	Ara: Pos (600 mg)
18/F	6	Asthma Atopic dermatitis	Ara: 8	Pos (4)	Ara: Pos (700 mg)
19/M	11	Asthma Atopic dermatitis	Ara: 7	Pos (2)	Ara: Pos (10 mg)
20/M	10	Recurring angioedema	Ara: 10	Pos (2)	Ara oil: Pos (5 mL)
21/M	13	Asthma Atopic dermatitis	Ara: 8.5	Neg	Ara: Pos (200 mg)
22/M	8	Asthma	Ara: 10	Pos (3)	Ara: Pos (10 mg)
23/M	17	Anaphylactic shock	Ara: 12	nd	Ara: Pos (200 mg)

centrifugation at 1000g, filtration and then sterilizing the filtration before lyophilization (Allerbio, Varennes en Argonne, France). It is devoid of endotoxins.

Specific ELISA

A specially developed ELISA was used to assay the levels of IgG, IgM, IgA, IgG1, IgG2, IgG3 and IgG4 antipeanut antibodies in serum samples from sensitized patients and control samples.

Briefly, lyophilised peanut extract, prepared as described above, was reconstituted in 1 mL of carbonate-bicarbonate

buffer (pH 9.6) yielding a final protein concentration of 5.21 mg/mL.

Ninety-six well microplates (NUNC, Roskilde, Denmark) were coated for 18 h at 4 °C with the reconstituted peanut extract at a concentration of 10 mg/L in carbonate-bicarbonate buffer. Each well received 100 µL of the antigenic suspension. After coating, the plates were emptied and overcoating was performed by a 1-h long incubation period, at 37 °C in a moist chamber, with 200 µL per well of phosphate buffer saline (PBS, pH 7.4), supplemented with 0.5% bovine serum albumin (BSA, Sigma, St Louis, MO, USA) and Tween 20 (Sigma). The plates were then washed three times in PBS.

Aliquots of 100 μ L of serum diluted 1:100 in PBS/BSA were deposited in the wells. After 30 min incubation at 37 °C in a moist chamber, the plates were washed three times in PBS. Each well received 100 μ L of the proper dilution of peroxidase-conjugated antihuman IgG (1:10000, Dako, Glostrup, Denmark), IgA (1:10000, Dako), IgM (1:10000, Dako), IgG1 (1:400, The Binding Site, Birmingham, UK), IgG2 (1:1600, The Binding Site), IgG3 (1:10000, The Binding Site) or IgG4 (1:800, The Binding Site). The plates were further incubated for 30 min at 37 °C in a humid atmosphere, then washed three times in PBS. One-hundred microlitres of citrate buffer (pH 5.6) containing 0.05% of orthophenylene diamin (Sigma) and H₂O₂ were then distributed in each well. The colour was allowed to develop and the enzymatic reaction was stopped with 50 μ L of 2N H₂SO₄. Care was taken to avoid reaching high optical densities (OD) in order to remain in the slope area of quantitative assessment.

Absorbance in each well was measured at 492 nm using a computer driven spectrophotometer (Titertek Multiscan II, Flow Laboratories, Helsinki, Finland). A calibrator of normal pooled human serum was used in each plate, and allowed to express individual results as a ratio of the sample OD/calibrator OD.

SDS-PAGE and Western Blot

SDS-PAGE separation was performed using a 10%–15% gradient electrophoresis gel, after reduction with Laemmli's buffer. Proteins were allowed to migrate during a 4-h period at a constant voltage of 200 V at 4 °C. After overnight electrophoretic transfer onto nylon membranes (Millipore, Bedford, MA, USA) and overcoating in PBS/BSA/Tween-20, serum samples from eight patients who had the highest levels of anti-peanut antibodies, diluted 1:100 in PBS, were incubated with strips of the transfer membrane. One subject with no detectable anti-peanut antibodies was tested as a control to verify the absence of non-specific binding on peanut lectins. After overnight incubation at room temperature and three washes, each blot was further divided into three strips and incubated with antihuman IgG (Dako), IgG4 (The Binding Site) or IgE (Dako) peroxidase-conjugated goat antiserum, respectively. After a new series of washes, the blots were soaked in a diaminobenzidine-containing revelation buffer with H₂O₂, dipped in distilled water in order to stop the reaction and air dried. The molecular masses of immunoreactive proteins were calculated using a prestained protein standard migrated together with the peanut extract (Kaleidoscope, BioRad, Hercules, CA, USA).

Statistical analysis

Data were expressed as mean $\pm$ SD. Individual data were fed to a computer using Microsoft Excel and Slide Write Plus

software (Advanced Graphics Software, Carlsbad, CA, USA) for statistical comparison between the different groups using ANOVA or Student's *t*-test.

Results

Significant levels of anti-peanut antibodies of the seven isotypes investigated were observed in peanut-sensitized subjects. Statistically significant differences were observed between sensitized (all groups, $n = 23$) and nonsensitized (controls, $n = 20$) subjects for peanut-specific IgG (respectively 3.57 ± 2.88 vs 1.03 ± 0.37 , $P = 0.0003$), IgG1 (5.85 ± 6.53 vs 0.85 ± 0.3 , $P = 0.001$), IgG2 (1.81 ± 0.88 vs 1.08 ± 0.13 , $P = 0.0007$), IgG4 (7.65 ± 7.51 vs 1.19 ± 0.80 , $P = 0.0004$). No differences were observed for the other isotypes between sensitized patients and controls (IgA 2.32 ± 2.69 vs 1.24 ± 0.47 , IgM 1.13 ± 0.50 vs 0.90 ± 0.56 , IgG3 1.21 ± 1.00 vs 1.02 ± 0.19).

In the three groups of sensitized patients, there was a large dispersion of specific antibodies as shown in Fig. 1 and Table 2. The lowest levels were observed in group I patients. Low levels of IgG2 and IgG3 were observed in the three groups, not significantly different from controls. Patients from group I had low levels of specific IgA compared to controls. Higher levels of peanut-specific IgA were observed in patients from groups II and III (Fig. 1).

Patients in groups II and III with high levels of IgG, IgG subclasses and IgA had high levels of IgE detected by RAST, as shown in Table 2. This included the four patients later enrolled in rush immunotherapy.

Western Blots were performed to determine antibody-reactive peanut proteins. A control sample devoid of anti-peanut

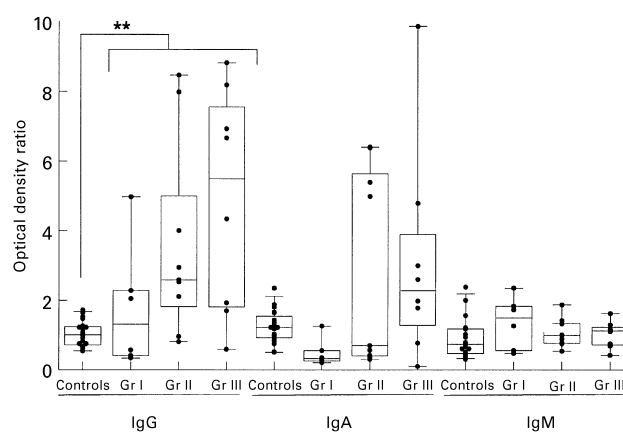


Fig. 1. Partition of specific IgG, IgA and IgM expressed as OD ratio in the controls and the three groups of sensitized patients. OD, optical density. OD ratio = sample OD/calibrator OD. Boxes indicate the 25 and 75 percentiles as well as the median level for each group of patients. Whiskers are for 99 percentile. ** $P = 0.0003$.

Table 2. Individual patients peanut-specific IgG, IgG, IgG4 and IgA levels assayed in ELISA, and IgE levels as assayed in RAST.

Patient	Peanut-specific IgG	Peanut-specific IgG1	Peanut-specific IgG4	Peanut-specific IgA	Peanut-specific IgE (KU/L)
<i>Group I: Patients with negative oral challenge</i>					
1	0.34	0.55	00.93	00.21	01.45
2	0.57	0.45	01.80	00.30	12.90
3	0.41	1.20	01.50	00.36	15.90
4	4.97	4.35	01.34	00.27	07.40
5	2.05	1.37	11.91	01.26	10.00
6	2.28	1.70	05.28	00.56	03.80
<i>Group II: Patients with positive labial or oral challenge at high doses</i>					
7	00.96	01.23	01.52	00.31	02.68
8	08.46	14.73	19.96	05.39	> 100
9	07.98	11.15	12.72	04.98	> 100
10	04.00	03.34	04.38	00.57	58.00
11	00.81	01.36	00.97	00.37	00.76
12	02.53	01.77	01.99	00.42	00.70
13	02.95	01.66	02.74	00.70	01.54
14	02.59	08.63	23.77	06.38	> 100
15	02.11	07.95	04.49	06.40	> 100
<i>Group III: Patients with positive labial or oral challenge at low doses</i>					
16	08.82	28.33	13.98	09.87	> 100
17	04.33	03.65	10.19	01.78	> 100
18	01.93	01.80	01.25	00.10	13.80
19	06.66	10.36	05.72	00.77	> 100
20	08.18	12.73	15.29	03.00	> 100
21	06.92	09.15	01.83	01.98	> 100
22	01.70	05.00	24.41	04.78	> 100
23	00.59	02.19	07.90	02.60	32.00

antibodies showed no reactivity in Western Blot, excluding the possible nonspecific binding of human immunoglobulins on peanut lectins. Reactivity patterns varied according to the isotype tested as shown in Table 3. As a whole, seven bands were observed, six bands with molecular masses ranging from 14.5 to 61 kDa were recognized by patients' IgG. Three bands, with strong reactivity (Fig. 2) were shared by most of the samples: 17.6 kDa (8/9 samples), 24 kDa (8/9) and 33 kDa (7/9). The 61 kDa band, more faintly stained, was recognized in 5/9 cases. IgG4 antibodies reacted with six proteins of the extract, with molecular

masses ranging from 17.6 to 102 kDa. Major reactivity was seen for the 24 kDa band, reacting with six samples. Specific IgE antibodies reacted with four protein bands (molecular masses from 17.6 to 61 kDa) in 5/9 samples tested in Western Blot. Major reactivity was seen for the 17.6 kDa and 24 kDa bands.

Discussion

This study reports about specific humoral responses in three groups of patients with graded response patterns to peanut

Table 3. Reactivity of the nine serum samples tested in Western Blot against peanut proteins

	14.5KDa	17.6KDa	24KDa	33KDa	52KDa	61KDa	102KDa
IgG	<i>n</i> = 2	<i>n</i> = 9	<i>n</i> = 9	<i>n</i> = 8	<i>n</i> = 1	<i>n</i> = 5	<i>n</i> = 0
IgG4	<i>n</i> = 0	<i>n</i> = 3	<i>n</i> = 6	<i>n</i> = 4	<i>n</i> = 2	<i>n</i> = 4	<i>n</i> = 1
IgE	<i>n</i> = 0	<i>n</i> = 3	<i>n</i> = 4	<i>n</i> = 1	<i>n</i> = 0	<i>n</i> = 2	<i>n</i> = 0

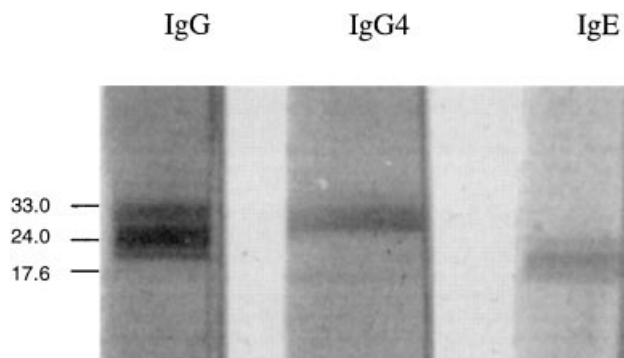


Fig. 2. Representative reactivity in Western Blot towards peanut proteins.

challenge, compared to non-sensitized controls, and demonstrates that whatever the type of clinical reaction to peanut, high IgE levels are associated with a polyisotypic response of also high levels of nonIgE isotypes.

For the determination of specific antibodies, we used an extract efficient for skin testing, suggesting that in this preparation the epitopes of peanut antigens are well preserved. Two major peanut allergens have been defined in the literature: Ara h1 [21] and Ara h2 [22]. Ara h3 was later reported by Bannon *et al.* [23] as a 14-kDa protein. Ara h1 is a 65-kDa glycoprotein, displaying significant homology with the vicilin seed storage protein family. Burks *et al.* [24,25] have identified 33 epitopes recognized by specific anti-peanut IgE although only four of them appear to be immunodominant IgE binding epitopes. Stanley *et al.* [26] have identified the epitopes of Ara h2 using serum samples from 15 sensitized patients. Ara h2 is a glycoprotein of 17.5 kDa, in which 10 IgE binding epitopes have been identified, distributed throughout the length of this protein. Three of these epitopes are recognized by all tested IgE.

Serum samples from patients with high specific antibody levels, tested in Western Blot in our study, showed the presence of IgG, IgG4 and IgE recognizing 17.6- and 61-kDa proteins. The molecular mass of these two proteins is consistent with them being Ara h1 and Ara h2. We found other reactive protein bands of 24 and 33 kDa, recognized, respectively, in 44% and 11% of the cases by IgE, 67 and 44% of the cases by IgG4, and 100 and 89% of the cases by IgG. These proteins do not represent major allergens (defined by IgE specificity) but were evidenced by most of specific IgG. These data are consistent with the polymorphic pattern of antibody response to peanut proteins recently reported by De Jong *et al.* [27].

Antipeanut IgA antibodies were observed at lower levels in some sensitized patients than in controls. However, seven patients from groups II and III had significantly elevated antipeanut serum IgA, together with high specific IgE. These data suggest that these patients may have broken

mucosal protective barriers and developed systemic IgA responses [28–30].

The assessment of specific IgG and IgG subclasses levels of anti-peanut antibodies indicated that high levels of these antibodies could not be predictive of the type of reactivity to expect upon re-exposure. The levels of peanut-specific IgE, measured by RAST, have been described as not correlated with the severity of clinical symptoms [4,31], and the data reported here indicate that this is also the case for other anti-peanut antibodies. This suggests that the putative Th2 response [32,33] leading to specific IgE production also triggers the proliferation of specific B cells having undergone other types of isotype switching. Variations of specific IgG4 levels in allergic patients have been described for other allergens with conflicting results reporting the presence [34] (dust mite *Lepidoglyphus destructor*) or absence [35] (mosquito salivary gland proteins) of specific IgG4 in sensitized patients, or increasing titres after immunotherapy [36] (*L. destructor* and major allergen, Lep d1). These immunoglobulin isotypes could be involved in other pathophysiological mechanisms, for instance, through complement activation.

Such multi-isotypic humoral responses further suggest that the antibodies produced have no protective role. The reported difficulties observed in rush immunotherapy protocols in peanut allergy [13,14], also observed here in four patients with high levels of all isotypes, are consistent with these data, as each injection might increase the production of such non-protective immunoglobulins.

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