

Serological characteristics of peanut allergy

M. C. A. CLARKE, S. A. KILBURN, J. O'B. HOURIHANE*, K. R. DEAN†, J. O. WARNER and T. P. DEAN

University Child Health, Southampton General Hospital, Southampton, *Immunobiology, Institute of Child Health, London and †Department of Biochemistry, University of Southampton, Southampton, UK

Summary

Background Peanut is the most common cause of severe or fatal food-associated anaphylaxis. Studies indicate that peanut extracts contain many allergenic proteins. The identification of major and minor allergenic components is necessary for standardization of experimental and diagnostic extracts.

Objective To identify further major and minor allergenic components of peanut extract using a large population of peanut allergics, and to relate serological findings to clinical parameters.

Methods The crude peanut extract was fractionated by fast protein liquid chromatography and the IgE binding proteins identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis followed by western blotting. Serum from 89 peanut allergics with a positive history of peanut allergy and elevated specific IgE and control serum from four atopic and four non-atopic, non-peanut allergics were used.

Results Nineteen peanut proteins were found to bind IgE from peanut allergic sera. Over 70% of subjects reacted to protein bands of 63 and 17 kDa (consistent with Ara h 1 and Ara h 2, respectively), confirming the importance of these two proteins as major allergens. A high proportion of patient sera also bound proteins at 15, 10, 30, 18 and 51 kDa in decreasing order. The percentage of cases with sensitivity to a 15 kDa protein was found to be higher in patient groups with severe reactions to peanut.

Conclusion This study highlights the diversity of peanut allergens. Diagnostic extracts containing a high proportion of the 15 kDa component may aid in diagnosis.

Keywords: peanut allergy, allergen, immunoglobulin E, CAP system, FPLC, SDS-PAGE, western immuno-blotting, Ara h 1, Ara h 2

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Introduction

Peanut allergic individuals may experience symptoms ranging from mild urticaria, facial swelling, abdominal cramp to hypertension with anaphylactic shock [1–4]. Commonly food allergy is an infantile illness, however, sensitivity to nut tends to persist into adulthood [5]. This together with the fact that a relatively high proportion of the peanut allergic population report severe reactions, leads to peanuts being most frequently associated with fatal and near fatal food related anaphylaxis [6–9].

Identification of allergenic proteins is necessary for the standardization of reliable diagnostic extracts. Understanding the biochemistry of the major allergens may lead to greater understanding of the events leading to the severity of peanut allergy. A source of purified allergens is necessary to investigate the cellular and humoral responses involved, and to establish assays to quantify allergen in food sources.

Several of the proteins in the nut kernel are allergenic. These include the major storage proteins arachin and conarachin, peanut lectin and phospholipase D [10], the acidic glycoprotein, Peanut 1 [11]. In addition there are three major allergens, fully characterized and sequenced and given the nomenclature Ara h 1, 2 and 3 [12–14]. However, sera from relatively few peanut allergics (3–13) have been

Correspondence: S. Kilburn, University Child Health, MP 803, Level G, Centre Block, Southampton General Hospital, Southampton SO16 6YD, UK.

available for allergen identification [10–13]. RAST inhibition studies indicate that individuals have specific serum IgE for more than one component of the peanut [10,11]. The contribution of specific proteins to the potency of this food for provoking severe symptoms is not understood. Identification of both major and IgE proteins for patients with differing severity of disease may shed light on this area.

To this aim we analysed the peanut-specific IgE binding profiles of over 90 peanut allergics. Thus enabling the frequency of reactivity to major and minor allergenic proteins in different severity groups to be determined.

Methods and materials

Study population

Ninety-one individuals with peanut allergy were recruited from the paediatric food allergy clinic at Southampton General Hospital and from a group of patients replying to a press advertisement seeking peanut allergic volunteers. These cases form a subgroup of 632 people recruited to a study in order to investigate the clinical characteristics of peanut allergy [15]. All patients studied had a positive clinical history of symptoms, collected using a questionnaire, to peanut together with positive skin-prick test (SPT) result. The validity of the questionnaire has been described previously [4,15]. The questionnaire has been found to have a false positive rate of only 14% when compared to diagnosis by challenge [4,16]. Seventy children (1–16 years) (male: female = 40:30) and 21 adults (17–38 years) (male: female = 8:13) made up the cohort.

The controls were categorized into two groups: (a) atopic: three adults and one child (aged 5 years) with one or more positive SPT to a battery of common inhalant or food allergen and a history of allergic reactions, and (b) non-atopic: three adults and one child (aged 8 years) who had no history of allergy or positive SPT to a battery of common allergens. Both groups had negative SPT to peanut and no history of adverse reactions despite regular ingestion of peanuts. The age of the adult controls ranged between 24 and 32 years (median = 28.5 years).

Definition of previous reactions to peanut

The symptoms experienced by the study volunteers on two separate occasions were recorded in a formal questionnaire, discussed previously [15]. Reactions were categorized into mild, moderate and severe using the following clinical symptoms. Mild: vomiting, abdominal pain, irritability, pruritus and/or urticaria; moderate: facial oedema and/or breathing difficulty (but not wheeze); severe: cyanosis, wheeze and or collapse/faint.

The survey of 622 individuals [15], showed that a large proportion of individuals' first reported reaction tended to be the same category as for their most recent reaction, 78% for severe, 56% for moderate and 41% for mild. Only 9% of severe reactors reported mild recent reactions but 32% of those who started with mild reactions reported a severe recent reaction.

Blood samples

Venous blood (2–10 mL) was collected from each patient and allowed to clot at room temperature for 1 h. The blood was centrifuged at 1500 *g* for 10 min at 4 °C and the serum was aliquotted and stored at –20 °C.

Total IgE

Serum total IgE concentrations were determined by the CAP System (Pharmacia, Milton Keynes, UK) and carried out according to the manufacturer's instructions. The detection range for this test was between 2 and 2000 kU/L with a 6–10% coefficient of variation within assays and 2–6% between assays.

Specific IgE

Concentrations of specific IgE to raw peanut protein were determined by the CAP System and performed according to manufacturers instructions. The specific IgE was converted to the RAST score 1–6. The detection range for this assay was between 0.35 and 100 kU/L with a 7–10% coefficient of variation of within assays and 6–9% between assays.

FPLC of crude peanut extract

Five gram of crude peanut powder (Sigma, Poole, UK) was freshly homogenized in 10 mL of bis[TRIS(hydroxymethyl)methylamino]-propane (20 mmol/L, pH 7.2) and dialysed against the same buffer for 30 min [12]. Debris was removed by ultracentrifugation (10 min, 27 000 *g*, 4 °C). Mixing and ultracentrifugation was repeated until the lipid layer and debris were sufficiently removed. The peanut extract supernatant was aspirated and used for fast protein liquid chromatography (FPLC) fractionation. The protein content of this extract was quantified using the Bradford reagent assay [17].

Approximately 100 mg of the protein was loaded onto a Mono Q 10/10 column (Pharmacia FPLC System, St Albans, UK), and fractionation was achieved by a stepwise salt gradient of 0–1.5 mol/L NaCl [12].

Fractions resolving around each peak were pooled and lyophilized. A Bradford test was employed to determine the protein concentrations of each pooled fraction. The fractions were reconstituted in sample buffer (0.125 mol/L TRIS, 4%

SDS, 20% glycerol, 0.1 g bromophenol blue) at a concentration of 5 mg/mL. The samples were then stored at -20°C for further analysis.

SDS-PAGE methodology

Electrophoresis was carried out according to the Laemmli method [18], using the modified protocol described by Dean *et al.* [19]. Sodium dodecyl sulphate (SDS) gradient (10–20%) gels (8×10 cm, 1 mm width) were loaded with $10 \mu\text{L}$ of the peanut FPLC fractions ($50 \mu\text{g}$ protein/well), on a 4% stacking gel (10 wells). Prestained 'Seeblue' molecular weight markers ($10 \mu\text{L}$) (R & D Systems, Abingdon, UK) ranging from 4 to 250 kDa, were run alongside all the samples. Trypsinogen was used as a control protein to test for non-specific binding during the immunolabelling stage. Electrophoresis was performed at 15 mA/gel for 90–110 min on a Hoefer minigel system (Pharmacia, St Albans). Proteins were visualized with Coomassie Blue R-250 (BDH, Poole, UK).

Western blotting

Western blotting was performed according the methods of Towbin *et al.* [20]. The separated proteins were electrophoretically transferred to nitro-cellulose membrane (NM) (Amersham, Little Chalfont, UK), in a TRIS-glycine buffer containing 10% SDS and 40% methanol (pH 8.3), for 90 min at 0.075 A. Proteins were visualized with Ponceau S (Sigma).

The NMs were blocked overnight at 4°C in 1% bovine serum albumin (BSA) in blocking buffer (10 mmol/L TRIS, 0.9% NaCl, pH 7.6), washed in wash buffer (50 mmol/L TRIS, 0.9% NaCl, 0.025% Tween 20, pH 7.4), and incubated with sera diluted (1:1) in antibody buffer (50 mmol/L TRIS, 0.9% NaCl, 0.25% Tween 20, 0.25% BSA, pH 7.4) for 2 h at room temperature. After three washes, bound IgE was labelled with sheep horseradish peroxidase-conjugated antihuman IgE (Serotec, Oxford, UK) in antibody buffer (1:1000) for 1 h at RT, before detection with hydrogen peroxide/Diaminobenzidine (Sigma). The NMs were air dried. The top and bottom of the gels were marked after transfer enabling the distance of migration for each band to be calculated. Standards were transferred on each gel and the distance migrated plotted against molecular weight, so that the molecular weights of all protein bands could be accurately determined. The person analysing the blots was blind to the clinical characterization of the cases.

Results

Total IgE

The total IgE concentration range observed (1–16 years)

was between 3 and 2000 kU/L (median = 554 kU/L) in the children and between 38 and 2000 kU/L (median = 632 kU/L) in adults. Only 16 (17.6%) individuals had concentrations less than 100 kU/L, eight of which were under 5 years of age. Eighteen people (20%) had serum total IgE greater than 1500 kU/L.

Specific IgE to crude peanut extract

Only two individuals had a RAST score of 0, both had total IgE within the normal range and were under 3 years of age. These children were, however, SPT-positive to peanut extract and had a positive clinical history of peanut sensitivity on face-to-face interview with a clinician. The median concentrations of peanut-specific IgE in children and adults were 48.8 kU/L (RAST 4) and 20.3 kU/L (RAST 4), respectively. A high proportion (29%), had a RAST of 6. All control subjects had specific IgE concentrations ≤ 0.35 kU/L (RAST 0) (data not shown).

SDS-PAGE of peanut fractions

The FPLC Fractions I to VI were analysed by SDS-PAGE. In total, 53 protein bands of differing molecular weight were visualized using Coomassie blue (Fig. 1). The resolution of the column was not designed to achieve fractionation of individual proteins, but enabled the visualization of an increased number of proteins without overloading the lanes, so called antigen stretching [21].

Fraction I contained the largest number of bands together with smearing, this fraction contained protein components that had not been retained by the column. Fractions II and III contained deeply stained bands at the lower molecular

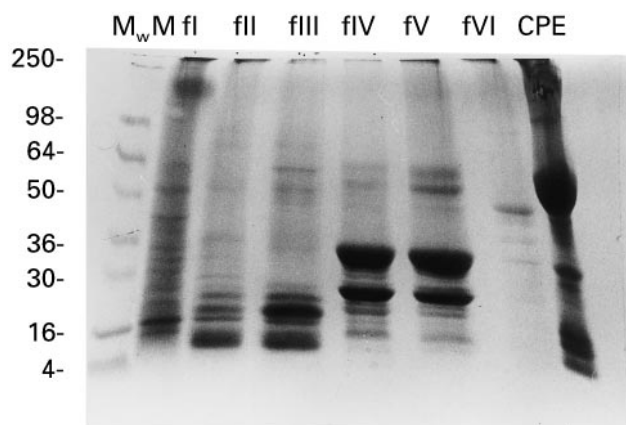


Fig. 1. Protein analysis of the six pooled FPLC fractions, each contained 20, 10.2, 5, 10.4, 7.2 and 15.4 mg of protein, respectively. SDS-PAGE stained with Coomassie brilliant blue. Key: M_w = molecular weight markers; fl to fVI = fractions I–VI, respectively ($50 \mu\text{g}$ /lane); CPE = crude peanut extract ($50 \mu\text{g}$ /lane).

weights, three bands closely migrating at 15, 17 and 18 kDa. Several faint bands were also visible; one at 7 kDa and several between 25 and 80 kDa. Fractions IV and V were almost identical with very large and intense bands at ≈ 28 and 33 kDa. Two other deeply stained bands separated closer to the top of the gel at around the 51 and 63 kDa area. Many other minor bands were visible. Fraction VI contained faint bands at 20, 33, 38 and 96 kDa with a moderately stained band at 44 kDa. This band was also faintly visible in fractions II–V.

Western blotting

IgE binding of sera from the 89 individuals with a positive RAST revealed IgE reactive bands were present in all fractions and allergens had a molecular weight ranging between 7 and 96 kDa.

An example of the IgE binding of one individual with peanut-sensitivity RAST score 5 is shown (Fig. 2). Intensely labelled bands of 15 and 17 kDa were observed in fractions II and III, and 20, 51, 63 and 71 kDa bands in fractions IV and V. Less intensely stained bands were recognized between 10 and 96 kDa in fraction I, a 7 kDa band in fractions II and III, and a 30 kDa band in fractions IV and V.

A summary of the percentage of patients with positive IgE to each band is shown (Fig. 3). Three proteins reacted with over 50% of the peanut allergic groups sera. Two were located in fractions II and III, one of 17 kDa was bound by 71% of patient sera (most probably Ara h 2), while IgE of

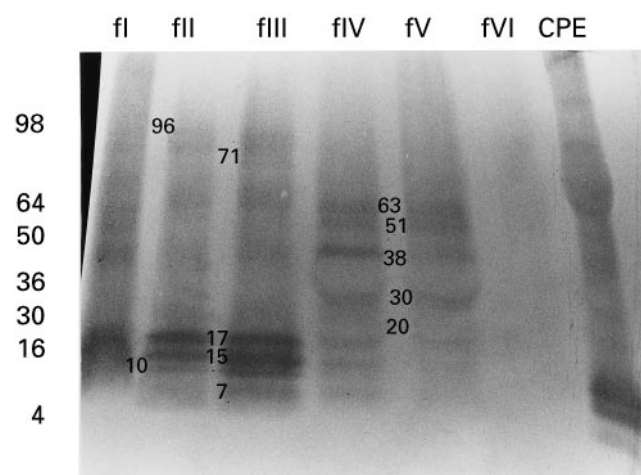


Fig. 2. Detection of allergenic components of the peanut fractions. An example of IgE binding from the serum of a peanut allergic individual (RAST 5). Western blot of FPLC preparation of peanut extract (fl–flVI) and crude peanut extract (CPE) with peanut serum as primary antibody and anti-human IgE peroxidase conjugate as secondary antibody. The molecular weights of some IgE binding proteins are shown.

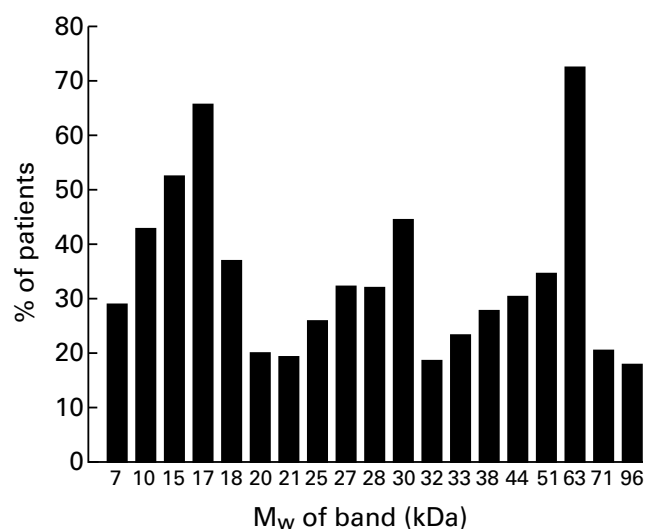


Fig. 3. Histogram showing the percentage of peanut allergic individuals with IgE specificity to the various protein bands in western blotting.

54% recognized a closely migrated band of 15 kDa. The 63 kDa (probably Ara h 1) was located in fractions IV and V, and was bound by 73% of sera. In addition to these major allergens a further four allergenic bands were observed, binding between 33 and 45% of the study groups' sera, and a further 12 bound more than 17% of patient sera. A low molecular weight band of 7 kDa was the smallest protein to be identified in the FPLC fractions. Nonetheless, it was capable of binding specific IgE in nearly 30% of the peanut allergic patients.

The number of protein bands bound by individual sera ranged from 0 to 16. Overall analysis showed 96% of patients with a positive RAST had IgE which bound to two or more of the protein bands. There was a positive correlation (Spearman's Rank: $r = 0.5$, $P < 0.0001$) between the number of bands bound by IgE and RAST grade. Specific IgE to the peanut proteins was not detected in the sera from the control groups.

IgE binding to proteins and clinical severity

No relationship was apparent between the specific IgE and clinical severity. Presence or absence of IgE sensitivity to the major allergenic proteins between the severity groups was compared. There was no difference with the 63 and 17 kDa proteins, all groups showed a high percentage of patients with sensitivity (Table 1). The percentage of patients with sensitivity to the 15 kDa protein however, was significantly different with clinical grouping (Table 1), the patients with severe reactions being most often sensitized. Reactions categorized as severe usually involve respiratory

Table 1. Percentage of patients in the three severity groups with positive serum IgE to individual proteins, determined by SDS-PAGE and Western blotting

Protein M _w	Mild (%)	Moderate (%)	Severe (%)	Significance (χ^2 test)
15	30	36	72	<0.005
17	70	66	70	NS
51	20	29	36	NS
63	80	72	78	NS

NS, not significant.

symptoms. Indeed sensitivity to the 15 kDa protein was significantly associated with wheeze and cyanosis (χ^2 , $P = 0.03$), but not to skin or gut symptoms.

Discussion

The association of IgE sensitization with the presence of clinical peanut allergy is highlighted within this study and elsewhere [15]. The study population consisted of paediatric and adult patients, and using the normal IgE reference values [22] for each age group, 97% of patients had total IgE above the mean value plus one standard deviation. The wide range in the specific IgE values seen here has also been seen in a smaller study of 10 patients [10]. A high proportion of the patients, 82%, had a specific RAST score of 3 or more. The importance, however, of low RAST scores should not be overlooked as over 20% of the patients who had a RAST score of 1 or 2 had reported severe symptoms on their most recent exposure to peanut. This highlights the importance of clearly defined extracts of peanut extracts used for *in vitro* tests in order to detect low levels of specific IgE.

The protein bands of molecular weights 63 and 17 kDa, tentatively identified as Ara h 1 and Ara h 2, respectively, by virtue of their molecular weight, were confirmed as major allergenic proteins and were bound by over 70% of sera. The 18 kDa protein was bound by fewer sera than the 17 kDa, but showed great agreement ($P = 0.001$), and so may correspond to the isoform of Ara h 2 described [13]. The closely migrating proteins at 10 and 15 kDa showed similar reactivity with individual sera ($P < 0.0001$), but there were differences on comparison of these two proteins with the 17 kDa protein. The 10 and 15 kDa proteins may correspond to the protein described by Bannon *et al.* [14].

The first described allergen of peanut, designated Peanut I [11], occurs as a 180 kDa complex in native state and has been shown in SDS-PAGE electrophoresis to consist of 20

and 30 kDa subunits. The allergenic proteins at 20–21 and 30 kDa described here acting as allergens for over one-third of the patients on the study may correspond to the subunits of Peanut I. The protein migrating at 51 kDa may not have been described previously. This protein was bound by 35% of patients' sera.

Thirty-three per cent of individuals' IgE bound specifically to a protein with a similar molecular weight as peanut agglutinin (27 kDa), a protein described as a minor legume allergen [10,23]. Twenty-eight per cent showed specificity to α -amylase inhibitor-size protein (25 kDa) [24]. Thirty per cent showed positive reactivity to a low molecular weight band of 7 kDa which is similar to the tyrosine- and chymotrypsin inhibitor (7.5 kDa), a protease inhibitor protein found in peanut. Such a small soluble protein could be responsible for rapid allergic responses reported by some patients, as it may elute more rapidly when peanut is in the mouth. In the future research into the quaternary structure of these proteins in aqueous solutions may yield mechanisms for the rapid transport of these proteins across the epithelium.

Symptoms to previous exposures are the most useful criteria used to assess suitable treatment for nut allergics. There are no current *in vitro* tests that can distinguish between patients experiencing mild or severe reactions, or predict the severity of a reaction. Only weak trends of increasing specific IgE or SPT to crude extracts with the increasing severity of a recent reaction have been noted [15]. One of the difficulties in comparing reactions that happen in the community is that the dose and extraneous circumstances are not uniform. This can be demonstrated by comparing the milder symptoms experienced during a controlled challenge procedure compared with accidental exposure [16]. The categories of reaction used here have been shown to be surprisingly consistent for individuals reporting exposure in the community [15].

Using the qualitative analysis, the 15 kDa protein was found to be allergenic for a higher proportion of those patients experiencing severe symptoms than those with reactions classified as moderate or mild. In addition the 51 kDa protein showed a trend for reactivity with a higher proportion of severe reactors. Respiratory symptoms are the major criteria used for discriminating the severe reactions, reactions involving the skin or gut as target organs are classified as mild or moderate. Sensitivity to the 15 kDa protein showed a strong association with respiratory symptoms, and not with skin or gut-associated symptoms.

The population studied here did not highlight a group of peanut-associated eczematous individuals with no immediate symptoms. Food challenges in previous studies [25,26], agree that peanut-associated symptoms tend to be immediate, occurring within 2 h. However, the absence

within this study of delayed onset eczema caused by peanut may reflect the method of sample collection, i.e. self-referral or attendance at allergy clinics. Studies recruiting from dermatology clinics have described patients with atopic dermatitis associated with sensitivity to peanut [27] and cashew nut [28].

It is unlikely that any assay will categorically predict the severity of an individual's reaction or as to whether the symptoms of successive reactions will increase or decrease. However, an assay based on an extract rich in the 15 and 51 kDa or their recombinant forms, may lead to a more meaningful serological assay. Longitudinal studies of specific antibody responses, together with reported clinical reactions would indicate if serological changes precede the increase in symptom severity reported by a subgroup of patients. It is clear that there are many more potentially allergenic proteins in peanut that need to be investigated if we are to understand the relationship between clinical manifestations and the immune response to peanut.

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