

IgG and IgE avidity characteristics of peanut allergic individuals

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The role of antibody avidity in allergy is poorly understood and there is no existing literature describing antibody avidity in food allergy. The main aim of this study was to investigate IgE and IgG avidity to a total peanut protein extract (TPPE) and purified Ara h 2 in a group of well-characterized peanut allergic individuals. Forty peanut allergic patients underwent a double-blind placebo-controlled low-dose peanut challenge, during which the severity of the patients' peanut allergy was scored. Serum peanut-specific IgE (psIgE) and IgG (psIgG) concentrations were measured for 37 individuals and the avidities of the same antibodies to a TPPE and purified Ara h 2 were determined using a thiocyanate ELISA method. Both IgE and IgG avidity to Ara h 2 showed weak positive correlations with challenge score [$r = 0.459$ ($p = 0.012$) and $r = 0.486$ ($p = 0.003$), respectively]. IgE avidity to TPPE showed a weak positive correlation with skin prick test results (SPT), $r = 0.467$ ($p = 0.004$) and there was an inverse relationship between the ratio of total IgE:psIgE and challenge score $r = -0.561$ ($p < 0.001$). No significant relationship was found between the ratios of IgE avidity:IgG avidity and challenge score or SPT. This is the first description of IgE and IgG avidity in peanut allergy, and it appears that the avidities of IgE and IgG antibodies to purified Ara h 2 are weakly related to the severity of peanut allergy (as measured by a challenge score).

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The double-blind, placebo-controlled food challenge (DBPCFC) is considered the gold standard for diagnosing food allergies, yet it is not without disadvantages, e.g. it is time-consuming, expensive and stressful to the patient (1). In terms of clinical outcome and the effect on patients' quality of life, the difficulties associated with accurate diagnosis and a lack of effective therapy, it is clear that further research into different aspects of food allergy is required.

In the case of peanut allergy, peanut-specific IgE (psIgE) concentrations in the serum and SPT wheal diameters have been correlated with patient histories and the outcome of peanut challenges to produce probability curves that predict the likelihood of patients with certain psIgE levels reacting to peanut (2–6). More recently a relationship between psIgE serum concentration and symptom severity (as meas-

ured by a challenge score) has been reported (7, 8) and between reaction severity and epitope diversity (9). However, there is still a lack of information concerning how specific antibodies change over time and how antibody characteristics relate to peanut allergy.

Antibody affinity is the biological measure of tightness of the molecular fit between antibody and antigen, and its measurement is well established in many areas of immunology. Antibody affinity is the strength of the binding between a single antigenic determinant and a single combining site on the antibody, but it is difficult and time-consuming to measure, due to the requirement for pure antibodies and epitopes. In most practical and clinical circumstances the interaction of an antiserum and multivalent antigen could be argued to be of more concern and therefore measurement of antibody avidity (the

overall strength of binding of an antigen with many antigenic determinants and multivalent antibodies) more relevant. Antibody avidity is more than the sum of the individual affinities, taking into account the heterogeneity of antibodies in a given serum and the heterogeneity of epitopes involved.

It is thought that persistent exposure to dietary antigens during infancy drives avidity maturation (IgG) and in healthy children this may be a beneficial physiological process enabling the immune system to eliminate harmful antigens (10). In contrast, animal models have shown that persistent exposure to dietary antigens may result in oral tolerance, which manifests in decreasing antibody levels and a paralysis of the T-helper cell response to the specific antigen. It remains unknown whether such tolerance results only in lower antibody levels, with persistent or increased avidity, or if avidity will eventually fall following decreasing antibody titres (10).

The binding kinetics of the IgE–antigen interaction appear to strongly affect intracellular signalling processes and may dictate the level of activation of the mast cell (11). Although the importance of high affinity for IgE function remains controversial, Mita et al. have reported that sensitivity of histamine release, as determined by basophil histamine release, is closely related to the affinity of IgE antibody to its antigen (12).

In summary, the role of avidity in allergy is not well described and literature on the significance of IgE and IgG avidity in peanut allergy does not currently exist. With all this in mind, we sought to investigate the avidity of specific IgE and IgG binding to a total peanut protein extract (TPPE) and purified Ara h 2 (a dominant peanut allergen for the patients studied) in a group of well-characterized peanut allergic individuals.

Methods

Study design

Serum was collected from 40 well-characterized peanut allergic patients, all of whom attended the Wellcome Trust Clinical Research Facility (WTCRF) for clinical evaluation, skin prick testing (SPT), blood tests and a low-dose DBPCFC during which the eliciting dose of peanut protein was determined and the severity of symptoms scored. Details of the DBPCFC and scoring methods have been described elsewhere (7, 8, 13), but in brief challenge-based reactions were scored using a 5 × 5 grid of five grades of increasing clinical severity and five increasing doses. Scores of 1–5 were considered mild reac-

tions, 6–13 were scored as moderate and 14 or more were considered severe reactions. For example, a volunteer who experienced generalized urticaria after mucosal contact with peanut but without ingestion was given a score of 4, whereas a reaction of laryngeal oedema after a dose of peanut between 3 and 99 mg was scored as 15.

All skin test reagents from ALK-Abello (Hungerford, UK). Skin prick tests were performed with single-tine lancets, pricking the skin at an angle of 90°. The weal diameter recorded at 15 min was the mean of two perpendicular diameters.

There was sufficient serum from 37 of these patients to measure the avidity of specific IgE and IgG binding to a TPPE and purified Ara h 2 using the following methods. The Southampton and South West Hants Joint Research Ethics Committee approved the study.

Specific IgE

Serum concentrations of total and psIgE were determined by FEIA (UNICAP System, Pharmacia, Uppsala, Sweden).

Specific IgG

Serum concentrations of psIgG were determined using an indirect ELISA. 96-well, flat-bottomed 1/2-area ELISA plates (Corning Costar, high binding polystyrene #3690, NY, USA) were coated with 100 µg/ml of defatted peanut protein in 0.05 M carbonate-bicarbonate buffer pH 9.6 at 50 µl per well. Defatted peanut protein was prepared from roasted peanut flour (Golden Peanut Company, GA, USA) as previously described (8). The plate was incubated overnight at 4°C, then washed five times with 200 µl per well phosphate-buffered saline (PBS), 0.05% Tween-20 (TPBS) and blocked for 1 h at room temperature with 150 µl per well of 3% bovine serum albumin (BSA) in PBS. The plate was washed again and a pool of sera from five peanut allergic individuals (doubly diluting six times from a starting concentration of one in 200) using 1% BSA in TPBS (dilution buffer) was added to the first two lanes, at 50 µl per well, to produce a standard curve. Serum samples were added to the plate initially diluted 1:300 in dilution buffer, at 50 µl per well, in duplicate. After incubation for 1 h at room temperature, the plate was washed and 50 µl per well of horseradish peroxidase (HRP)-linked antihuman IgG (Dako P0214, Ely, UK) diluted 1:6000 in dilution buffer was added to the plate. After another 1 h incubation and washing, the plate was developed with TMB substrate (100 µl per

well) for 10 min, and stopped with 50 μ l per well H_2SO_4 . The plate was then read at 450 nm. A BSA-coated plate (100 μ g/ml) was also included in each experiment as a control for non-specific binding. Inter-plate and intra-plate CVs for this assay were determined to be <10% and <4%, respectively.

Specific IgE avidity to total peanut protein

A thiocyanate ELISA elution assay based on the method of Goldblatt (14) was used to measure the strength of binding between allergen and antibody. This approach is widely used in vaccine studies and has been described as a useful method for estimating relative avidity of specific IgE antibodies for epitopes of allergenic proteins in allergen extracts (15). Thiocyanate is a chaotropic agent that dissociates antigen-antibody complexes by interfering with non-covalent binding. Under the conditions used in this study, we determined that the thiocyanate is not removing proteins coated onto the ELISA plate [data not shown, but methodology as described by Goldblatt (14)] and therefore the thiocyanate effect is purely acting on the protein-antibody binding.

Half-well ELISA plates were coated with 100 μ g/ml of defatted peanut protein or BSA (control plates) in carbonate-bicarbonate buffer and left overnight at 4°C. The plates were then washed with PBS (200 μ l per well) and blocked for 1 h (with shaking) with 3% BSA in PBS (150 μ l per well). The wash step was then repeated and then peanut allergic sera were added to the plates [following the method of Goldblatt (14) different dilutions were used for each patient in order to give an optical density (OD) reading of 1 ± 0.2 when untreated with potassium thiocyanate (KSCN)], with six rows of triplicate wells (50 μ l per well) loaded for each patient. After incubation for 1 h the plates were washed with TPBS (200 μ l per well) and KSCN was added in increasing concentrations [usually 0.5, 1, 2, 3 and 4 M (lower ranges were used for some patients exhibiting very weak antibody-protein binding) diluted in 1% BSA in TPBS, 50 μ l per well] to the rows, with one row loaded with just 1% BSA in TPBS. Immediately after 30 min incubation, without shaking, the plates were washed with 5×200 μ l TPBS per well and incubated for 1 h with biotinylated goat antihuman IgE (Kirkegaard Perry Limited, Gaithersburg, MD, USA) diluted 1:500 in 1% BSA in TPBS, 50 μ l per well. The washing step was then repeated before the final 1 h incubation with HRP-linked extravidin (Sigma, Poole, UK) diluted 1:1000, 50 μ l per well. After a final wash the

plates were developed for 5 min with TMB substrate and stopped with 1 M sulphuric acid, before being read at 450 nm.

The results for the avidity ELISAs are expressed as an avidity index (AI), defined as 'the concentration of KSCN required to elute 50% of allergen antibody', such that the lower the AI or KSCN concentration, the lower the avidity of the allergen-antibody interaction. The AI was calculated as follows:

- 1 the percentage change in absorbance (relative to the samples without thiocyanate treatment) for each serum at different molarities of KSCN was calculated;
- 2 a graph of KSCN concentration was plotted against the percentage reduction in absorbance (Fig. 1 shows a typical avidity plot) and
- 3 a line of best-fit added was fitted to the graph (Fig. 1) and the resulting equation used to calculate the KSCN concentration in molarity (M) required to elute 50% of the allergen-bound antibody (i.e. produce a 50% reduction in absorbance).

Specific IgE avidity to Ara h 2

This method was identical to that for the defatted peanut protein extract, except ELISA plates were coated with 10 μ g/ml of purified Ara h 2. The Ara h 2 used was native protein extracted from *Arachis hypogaea* L., cv. Florunner as previously described (16). In brief, protein was extracted from raw defatted peanut flour, fractionated using ammonium sulphate precipitation and DEAE separation prior to SDS-PAGE with Coomassie blue. The fractions of interest were then pooled, concentrated and buffer exchanged before sterilization by filtration through 0.2 μ m

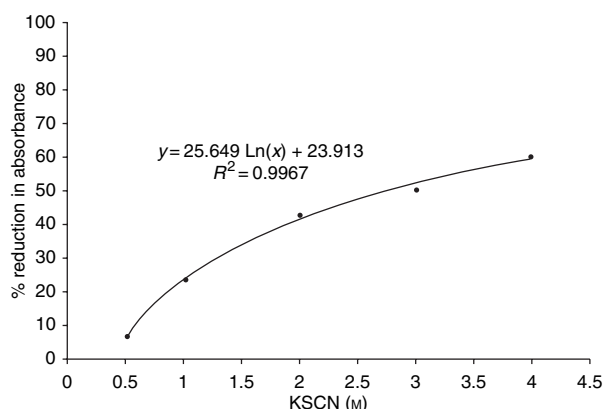


Fig. 1. A typical avidity plot, illustrative of the type of results obtained for both IgE and IgE avidity to TPPE and Ara h 2.

membrane and lyophilization. Typically, when prepared by this method, the Ara h 2 purity is 92–95%. After chromatography separation, special attention was paid when making the pool to prevent contamination with Ara h 1 that was eluted in the latest fractions.

Specific IgG avidity to mixed peanut protein and Ara h 2

These protocols were very similar to those used in measuring specific IgE avidity; however, anti-IgG antibody (Dako HRP-linked, 1:6000), was used instead of biotinylated goat antihuman IgE and HRP-linked extravidin.

Statistical analysis

Associations were analysed using the Spearman's rank test. All statistical analyses were performed using SPSS v.11 (Chicago, IL, USA).

Results

Study population

Demographic and DBPCFC details of the 40 challenged volunteers have been described previously (7, 8).

Peanut-specific IgE and peanut-specific IgG serum concentrations

The total IgE, psIgE and psIgG serum concentrations for each patient are detailed in Table 1. The range of serum psIgE concentrations was 0–649 IU/ml (mean 77.6, median 6.7 and s.d. 146.9), with one volunteer having no measurable psIgE despite a history of symptoms, positive SPT and positive DBPCFC. The range of serum psIgG concentrations was 945.1–76334.5 Arbitrary Units (AU; mean 20,730.6, median 14,490.7 and s.d. 21,321.8).

Table 1. Patient serum antibody concentrations and avidity indices

Patient number	IgE avidity to TPPE	IgE avidity to Ara h 2	IgG avidity to TPPE	IgG avidity to Ara h 2	Total IgE (IU/ml)	PsIgE (IU/ml)	PsIgG (AU)
2	1.72	2.16	1.47	2.77	121	101	69,940.9
3	1.60	2.35	1.45	3.64	80	40.5	35,856
4	1.40	1.55	0.58	1.84	103	17.4	5868.1
5	1.97	2.02	1.43	2.18	2398	61.5	5068.4
6	1.63	ND	1.04	2.66	498	5.5	3262.7
7	1.57	3.01	1.26	4.46	88	81.3	47,655.8
8	1.10	1.73	1.38	3.49	36	14.8	55,007.7
9	1.35	ND	1.43	1.48	10	0.9	32,632.6
10	2.05	0.92	0.91	2.98	34	3	14,490.7
12	1.59	3.06	0.91	4.42	169	5.8	1506.8
13	1.80	1.34	1.00	3.04	1609	148	31,897.2
14	0.55	0.78	0.81	1.59	59	4.1	1489
16	1.60	2.44	0.77	2.50	73	81.1	2242.5
17	0.32	0.42	0.89	0.26	62	0	30,027.9
18	1.04	1.09	1.42	1.26	898	1.1	3714.1
19	1.91	2.29	1.39	3.46	214	139	50,740.4
20	1.94	1.56	0.66	1.93	84	7.6	17,607
21	1.69	ND	0.43	1.53	90	1.3	16,316.3
22	1.57	ND	0.57	1.13	103	4	945.1
23	1.79	1.71	1.06	2.14	345	41.7	15,990.7
24	1.51	1.66	1.18	1.59	1122	324	31,027.9
25	2.52	1.12	0.82	2.97	25	3.4	4314.8
26	0.89	0.16	0.36	0.94	78	1.5	1702.8
27	0.50	0.53	1.22	3.72	2	1.4	3166.7
28	1.16	2.59	0.66	4.57	67	45.3	22,586.1
29	0.67	ND	ND	ND	652	0.6	1190.4
30	2.22	2.89	0.70	2.80	2262	4.5	3476
31	1.74	2.52	1.43	2.89	342	55.5	11,758.1
32	ND	ND	0.79	1.25	650	1.8	1307.7
33	1.73	3.03	1.60	5.43	919	171	19,814.9
34	1.64	2.09	1.54	3.57	2298	649	76,334.9
35	0.47	ND	1.53	3.44	783	1.5	3625.2
36	1.57	2.62	1.90	4.61	777	340	56,678.7
37	1.45	0.22	1.32	4.14	1925	502	20,702.3
38	1.84	1.10	1.44	3.12	28	3.6	5121.7
39	1.04	0.25	1.41	0.89	655	1.1	48,586.1
40	1.22	ND	ND	ND	59	6.7	13,376.7

ND, not determined.

Table 2. Summarized avidity index data

	IgE avidity to TPPE (n = 36)	IgE avidity to Ara h 2 (n = 29)	IgG avidity to TPPE (n = 35)	IgG avidity to Ara h 2 (n = 35)
Mean	1.44	1.73	1.09	2.71
Standard deviation	0.50	0.88	0.38	1.27
Range	0.32–2.52 (2.20)	0.16–3.05 (2.89)	0.36–1.90 (1.54)	0.26–5.65 (5.39)

Table 3. Correlations between avidity indices and patient challenge score and SPT

	Correlation coefficient with challenge score	Correlation coefficient with SPT
IgE avidity to TPPE	$r = 0.09$, $p = 0.603$, $n = 36$	$r = 0.467$, $p = 0.004$, $n = 36$
IgE avidity to Ara h 2	$r = 0.459$, $p = 0.012$, $n = 29$	$r = 0.213$, $p = 0.266$, $n = 29$
IgG avidity to TPPE	$r = 0.244$, $p = 0.158$, $n = 35$	$r = 0.097$, $p = 0.579$, $n = 35$
IgG avidity to Ara h 2	$r = 0.486$, $p = 0.003$, $n = 35$	$r = 0.1976$, $p = 0.260$, $n = 35$

IgE and IgG avidity indices

The IgE and IgG AI calculated for each patient are also detailed in Table 1. For some patients an AI could not be determined, either due to insufficient serum or to a low antibody response, e.g. sometimes there was no measurable response to Ara h 2, suggesting this was not a dominant allergen for these individuals. The ranges, mean values and standard deviations for the AIs for both IgE and IgG to TPPE and Ara h 2 are detailed in Table 2.

Associations

There has been controversy regarding the influence of antibody concentration on the quantitation of affinity/avidity (17), therefore the first correlations that were performed were between the AIs and concentration of sera used (measured as psIgE or psIgG) on the ELISA plates. No correlations were found between the antibody concentration used and AI for any of the measurements made.

IgE AI to TPPE against concentration of psIgE ($r = 0.18$, $p = 0.92$)

IgE AI to Ara h 2 against concentration of psIgE ($r = 0.06$, $p = 0.75$)

IgG AI to TPPE against concentration of psIgG ($r = 0.238$, $p = 0.17$)

IgG AI to Ara h 2 against concentration of psIgG ($r = -0.08$, $p = 0.63$)

As detailed in Table 3, IgE and IgG avidity to Ara h 2 both showed weak positive correlations with challenge score, $r = 0.459$, $p = 0.012$ and $r = 0.486$, $p = 0.003$, respectively. IgE avidity to TPPE also showed a weak positive correlation with SPT, $r = 0.467$, $p = 0.004$. There was no correlation between any AI and patient age. IgE

AI:IgG AI did not correlate with challenge score or SPT. However, the mean ratio for IgE AI:IgG AI for mixed peanut was 1.58 (s.d. 0.87), whereas for Ara h 2 it was 0.75 (s.d. 0.42).

Patient's serum concentrations of psIgE weakly correlated with challenge score, $r = 0.564$, $p < 0.001$; however, serum concentrations of psIgG did not. The ratio of total IgE:psIgE also weakly inversely correlated with challenge score ($r = -0.561$, $p < 0.001$).

Discussion

Understanding the role of antibody–allergen-binding strength in food allergy should contribute to a better understanding of this disorder. Although there has been some research into the affinity/avidity of aeroallergen–antibody interactions, there is almost no published literature on food allergen–antibody interactions. The data presented here are the first to describe the characteristics of IgG and IgE avidity to peanut allergens, using a group of subjects diagnosed using the clinical gold standard test, the DBPCFC.

As serum-derived antibodies consist of populations of antibodies specific for the same antigen but differing in affinity (affinity heterogeneity) and as small populations of low avidity antibodies may be masked by higher avidity antibodies, the AI values used in this study represent an overall average measurement of sera antibody avidity. The range and mean AIs of IgE to TPPE and IgE to Ara h 2 were very similar suggesting similar IgE-binding strengths for TPPE and purified Ara h 2. However, the results were different for IgG. The IgG AIs for TPPE were similar to those of IgE, but IgG AIs to Ara h 2 were much higher and more variable. This suggests that in this patient population, IgG

has a greater binding strength for Ara h 2, compared with IgE binding to both Ara h 2 and TPPE, possibly reflecting a defensive immune response to this major peanut allergen. Recent evidence suggests that there is a preferential variable heavy chain usage in IgE synthesis, and therefore, epitopes responsible for antigen-specific IgE antibody production may differ from those promoting antigen-specific IgG antibodies (18). Different epitope recognition between IgG and IgE may account for the varying avidities of these two antibodies for Ara h 2.

A major objective of this study was to determine if there was any relationship between IgE and IgG avidity to TPPE and/or Ara h 2 and challenge score, a measure of the clinical severity of peanut allergy that takes both symptoms and eliciting dose into account (7). For the patient group studied, the AIs for both IgE and IgG binding to Ara h 2 did correlate weakly with challenge score; however, no significant correlation was found between the AIs to TPPE and challenge score. The correlation with Ara h 2 rather than TPPE could be due to a number of factors, including the composition of TPPE. Ara h 2 only constitutes between 5.9% and 9.3% of total peanut protein content (19) whilst the TPPE may contribute to other non-specific binding, perhaps masking weaker antibody avidities. Ara h 2 is known to be a major allergen in this study population (8) and the challenge score is reflective of reactions to ingested peanut, also the immunodominant IgE-binding epitopes of Ara h 2 are known to be very resistant to enzymatic digestion (16).

We have demonstrated that antibody avidity is weakly positively correlated with challenge score, but found no significant relationship between antibody avidity and age. The age range for this patient group was 7.2–39.2 yr (average age 16.5 yr), so it could be that IgE avidity to peanut had already reached a plateau for these subjects (20).

IgE:IgG avidity ratio to TPPE showed a weak trend of being > 1 , implying that IgE had greater avidity for TPPE than IgG, but the opposite was found for the avidity to Ara h 2. No correlations were found between these ratios and challenge score or SPT. These findings could suggest that IgG is not 'blocking' or interfering with IgE function in these patients. The weak positive correlation between IgG avidity to Ara h 2 and challenge score could be due to IgG playing a role in the pathogenesis of peanut allergy or, as discussed previously, it could indicate a defensive immune strategy. It might also reflect recent exposure to peanut as that was an entry criterion

for the study (7) and it is possible that avidity might decrease with successful avoidance. This phenomenon requires further investigation as a satisfactory explanation has yet to be proposed. No IgG subtypes were investigated in this study. A study investigating the affinity of IgG₁ to Amb 1 (a major ragweed allergen), has shown that non-atopic individuals mount a high affinity IgG₁ response while atopic individuals mounted a high affinity IgE response and their IgG₁ responses were diminished or attenuated (21).

Whilst the method chosen in this study – thiocyanate elution – has the advantages of giving a relatively simple, yet reliable indication of overall antibody avidity with complex patient sera and protein mixtures, we recognize that it is a proxy measure and considered to be rather crude. It is important to build on these initial findings using state of the art techniques such as surface Plasmon resonance to further clarify the kinetics of antibody–allergen interactions in food allergy.

To conclude, this is the first description of IgE and IgG avidity in peanut allergy, indicating that the avidities of IgE and IgG antibodies to purified Ara h 2 are weakly related to the severity of peanut allergy symptoms (as measured by a challenge score) and that other factors also appear to contribute to clinical symptomology such as the ratio of total IgE:psIgE.

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