

Isoforms of the Major Peanut Allergen Ara h 2: IgE Binding in Children with Peanut Allergy

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Key Words

Peanut · Allergen · IgE

Abstract

Background: The major peanut allergen Ara h 2 consists of two isoforms, namely Ara h 2.0101 and Ara h 2.0201. The recently identified Ara h 2.0201 isoform contains an extra 12 amino acids including an extra copy of the reported immunodominant epitope DPYSPS. This study aimed to evaluate the IgE binding of the two Ara h 2 isoforms. **Methods:** Ten clones of Ara h 2 were sequenced to assess the relative frequency of the Ara h 2 isoforms and to identify whether there was further variation in the Ara h 2 sequence. IgE binding to Ara h 2.0101 and Ara h 2.0201 was measured for 70 peanut-allergic children using an IgE DELFIA™ assay to quantitate specific IgE binding. A competition assay was used to measure whether Ara h 2.0201 contained IgE epitopes other than those found for Ara h 2.0101. **Results:** The original Ara h 2.0101 sequence was found for 6/10 clones and Ara h 2.0201 was found for 2/10 clones. Ara h 2.0201 had the expected insertion of 12 amino acids as well as substitutions at positions 40 (40G) and 142 (142E). Two new isoforms were identified as different polymorphisms of position 142. One Ara h 2.01 clone (Ara h 2.0102) con-

tained 142E and one Ara h 2.02 clone (Ara h 2.0202) contained 142D. A polymorphism that was previously identified by other investigators at position 77 (77Q or 77R) was not found for any of the 10 sequences. Although the level of IgE binding to Ara h 2.0201 of individual patients was frequently higher than the binding to Ara h 2.0101 ($p < 0.01$), there was a strong correlation in binding to both isoforms ($r = 0.987$, $p < 0.0001$) and when analyzed as a group the means were similar. Ara h 2.0101 was not as efficient at blocking reactivity to Ara h 2.0201 indicating there is an additional IgE specificity for the Ara h 2.0201 isoform. **Conclusions:** Ara h 2.0201 has similar but higher IgE binding than the originally sequenced Ara h 2.0101 isoform and contains other IgE specificities.

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Introduction

Seven peanut allergens have been characterized by cDNA cloning (Ara h 1–7) and their relative importance determined by IgE binding studies [1–5]. Ara h 1 and Ara h 2 are classified as major peanut allergens as they frequently bind IgE (65–90%) in peanut-allergic sera [6, 7], although IgE binding to Ara h 1 is lower (35%) in some populations [2]. The major group 1 and 2 allergens also

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contribute up to 16 and 9% of the total protein content in peanut extracts [8]. Ara h 1 is a large (66–68 kD) vicilin-like protein [1]. Ara h 2, 6 and 7 are smaller proteins (17.3, 14.5 and 15.8 kD) with strong amino acid sequence similarity with proteins from the conglutin family of seed storage proteins [4]. The amino acid sequence identity to Ara h 2 was higher for Ara h 6 (59%) compared with Ara h 7 (35%), but the frequency of IgE binding to Ara h 6 and 7 was similar (approximately 40%). Ara h 3 (57-kD protein) and Ara h 4 (61-kD protein) were found to share 91.3% amino acid sequence identity and are glycinin-like proteins with moderate IgE binding to peanut-allergic sera (44% for Ara h 3 and 53% for Ara h 4) [3, 4]. Ara h 5 bound IgE in only 13% of peanut-allergic sera and was identified as another small protein (14 kD) with sequence homology with plant profilin [4].

Burks et al. [6] initially identified Ara h 2 (termed fraction 4) as two protein bands of approximately 17 kD. The presence of more than one band was also noted by other investigators [2, 9] and recently the Ara h 2.0201 isoform was cloned and sequenced [10]. The isoform contained an extra copy of the reported immunodominant epitope DPYSPS [11, 10], so it was speculated that Ara h 2.0201 could be a more potent allergen than the original Ara h 2.0101. An important feature of the Ara h 2 immunodominant IgE epitopes is that they are resistant to trypsin digestion and are more likely to remain intact in the gastrointestinal tract [9]. The objectives of this study were to measure IgE binding to both isoforms of Ara h 2 to determine whether the recently identified Ara h 2.0201 was a more potent peanut allergen.

Material and Methods

Study Population

Plasma from 70 peanut-allergic children (age range 0.9–15.2 years) was harvested within 2 h of collection and stored at –80°C. Peanut allergy was defined by clinical symptoms following peanut ingestion and by peanut-specific IgE measured using the CAP-RIA system (Pharmacia Diagnostics, Sweden). The diagnosis and CAP-RIA analysis were performed by the Immunology Department at Princess Margaret Hospital (Subiaco, Western Australia). Plasma samples from 10 children with IgE to inhalant allergens were included as negative controls. The Princess Margaret Hospital Human Ethics Committee approved the procedures in the study.

RNA Isolation and Polymerase Chain Reaction Amplification of Ara h 2.0101 and Ara h 2.0201

Seven different types of peanut from three commercially relevant varieties were obtained from the Peanut Company of Australia (Kingsgrove, Australia). Total RNA was extracted from the peanuts using TRIzol (Invitrogen-Life Technologies, Carlsbad, Calif., USA) and

the aqueous phase was mixed with 70% ethanol and purified over RNeasy columns (Qiagen, Valencia, Calif., USA). RNA from the different peanut varieties was pooled and reverse transcribed into cDNA with Multiscribe RT and random hexamers (Applied Biosystems, USA). Ara h 2.0101 and Ara h 2.0201 were amplified by polymerase chain reaction (PCR) from the cDNA with high-fidelity *Pfu* polymerase (Promega, USA). The PCR products encoding the mature allergens (without the leader sequence) were digested with *Sal*I and cloned in frame into pQE.81L (Qiagen) to incorporate an N-terminal hexahistidine tag. The PCR primers (5'–3') used for cloning, with the *Sal*I site underlined and the stop codon in italics, were GGTCTGACAGGCAGCAGTGGGAAC (forward) and TGTCTGACCTTAGTATCTGTCTCTGCCGC (reverse). The resultant construct encodes MRGS-H6-TDPHASSVPRVD followed by the mature Ara h 2 protein sequence (RQQ...). Ten clones of Ara h 2 were sequenced.

DNA Sequencing and Analysis

Sequencing analysis was performed by a DNA sequencer (ABI373 sequencer, PerkinElmer, USA) at the Royal Perth Hospital (Perth, Australia). The amino acid sequence comparison between Ara h 2.0101 and Ara h 2.0201 was performed at the NCBI using the BLAST network service.

Expression of the Recombinant Allergens

The cDNA from Ara h 2.0101 and Ara h 2.0201 were cloned into pQE.81L (Qiagen) and the cDNA of Der p 2.0101 was cloned into pET19b (Novagen) with an N-terminal six-histidine tag fused with the natural amino acid terminus. The Ara h 2 allergens were produced from plasmids transformed into the *Escherichia coli* strain BL21 (DE3) Codon plus (Novagen) and expressed as soluble protein. The Der p 2 allergen was produced from plasmids transformed into the *E. coli* strain BL21-pLysSE (Novagen) and solubilized from inclusion bodies. The allergens were purified by Ni-NTA (Qiagen), by size exclusion chromatography to remove aggregates according to the method of Mueller et al. [12] and the Ara h 2 isoforms were further purified by anion-exchange using MacroPrep High Q support chromatography (Biorad, Hercules, Calif., USA). Finally the allergens were sterilized and endotoxin removed using 0.2 µM Mustang E filters (Pall Life Science, Portsmouth, UK). The purity of the allergens was checked on a 12.5% SDS-PAGE by the method of Laemmli [13] and the concentrations were determined by Folin assay [14].

IgE Antibody Assays

An IgE DELFIA™ (dissociation-enhanced lanthanide fluoroimmunoassay, Wallace, Turku, Finland) assay, also known as the time-resolved fluorescence immunoassay, was used to quantitate specific IgE binding to allergens. The quantity of IgE was interpolated from a standard curve created with a human/mouse anti-Der p 2 chimeric antibody (Indoor Biotechnology, Cardiff, UK) by the procedures of Schuurman et al. [15]. The standard chimeric antibody contained 6.8 µg/ml of IgE compared with the NIH IgE reference (A-699-001-500).

The assay was standardized by using the same concentration of monoclonal antibody to coat the microtitre plates to capture the test allergens. In this case the same anti-His tag monoclonal antibody was used to capture the His-tagged Der p 2 and Ara h 2 allergens. Initially 100 µl of anti-Penta-His mAb (Qiagen) at 1 µg/well in Na₂CO₃/NaHCO₃ (pH 9.6) was absorbed onto the microtitre plate (Maxi SorpH, Nunc, Denmark) and incubated overnight at 4°C. The plates

Fig. 1. Formatted alignments of the mature protein derived from sequencing ten Ara h 2 clones. The insertion of 12 amino acids after position 54 is in bold. The amino acid substitutions at position 40 (glutamic acid to glutamine) and position 142 (aspartic acid to glutamic acid) are in italics. Underlined is the immunodominant IgE binding epitope DPYSPS. The asterisks denote the amino acids are identical. The number out of ten is the number of clones with the reported sequence.

		10	20	30	40
Ara h 2.0101	RQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSYE				
Ara h 2.0102	*****				
Ara h 2.0201	RQQWELQGDRRCQSQLERANLRPCEQHLMQKIQRDEDSYG				
Ara h 2.0202	*****				
		50	60	70	80
Ara h 2.0101	RDPYSPSQDPYSPS-----PYDRRGAGSSQHQE				
Ara h 2.0102	*****				
Ara h 2.0201	RDPYSPSQDPYSPSQDPDRDPYSPSPYDRRGAGSSQHQE				
Ara h 2.0202	*****				
		90	100	110	120
Ara h 2.0101	RCCNELNEFENNQRRCMCEALQQIMENQSDRLQGRQQEQQF				
Ara h 2.0102	*****				
Ara h 2.0201	RCCNELNEFENNQRRCMCEALQQIMENQSDRLQGRQQEQQF				
Ara h 2.0202	*****				
		130	140	150	
Ara h 2.0101	KRELRLNPQQCGLRAPQRCDDLVESSGGRDRY	6/10			
Ara h 2.0102	*****E*****	1/10			
Ara h 2.0201	KRELRLNPQQCGLRAPQRCDDLEVESGGRDRY	2/10			
Ara h 2.0202	*****D*****	1/10			

were washed 5 times with Tris-HCl pH 7, 0.05% Tween 20 buffer prior to blocking for 1 h with 200 µl of 0.5% bovine serum albumin (in Tris-HCl, 0.05% Tween 20 buffer). This washing step was applied after each incubation apart from the final step where the plates were washed 8 times before the addition of enhancement solution. The plates were then incubated with 100 µl of rDer p 2, rAra h 2.0101 or Ara h 2.0201 diluted to 500 ng/ml in DELFIA assay buffer (Wallac) for 1 h at room temperature with shaking. After washing, 2-fold dilutions of the standard anti-Der p 2 chimeric IgE (0.07140 ng/ml) or reference plasma samples for the peanut allergens were added onto the desired wells at 100 µl/well and incubated overnight at 4°C. On the following day, the wells were washed and incubated with 100 µl of biotinylated mouse-antihuman IgE mAb (BD Pharmingen, USA) diluted 1:1,000 in assay buffer (Wallac) and incubated for 1 h at room temperature with shaking. After washing, the plate was incubated with 100 µl of Eu-streptavidin (Wallac) diluted 1:1,000 in assay buffer for 30 min. In the final step, 100 µl of the DELFIA enhancement solution (Wallac) was added and incubated for at least 5 min. The fluorescence signals were measured on the Victor2 multi-label counter (Wallac) using a 405-nm filter and the concentrations of IgE were transformed from the Der p 2 standard curve by the software (WorkOut™, Wallac). The lower limit of detection for the Der p 2 standard curve was approximately 0.1 ng/ml of IgE. The standardized Ara h 2.0101 and Ara h 2.0201 reference plasma were then used to create a standard curve for a DELFIA assay where the respective allergens were coated onto the plate. The absolute value (ng/ml) of IgE antibody to Ara h 2 could be interpolated from the chimeric curve because of the identical nature of the slopes of the DELFIA count to the dilution of plasma ($\times 10^7$) between the chimeric Der p 2 curve ($\beta = 0.44 \pm 0.01$) and the Ara h 2 curve with the peanut-allergic reference plasma ($\beta = 0.45 \pm 0.05$). Then cutoff values were the mean ± 2 SD of the non-peanut-allergic plasma. IgE levels above 0.21 and 0.20 ng/ml were considered positive for Ara h 2.0101 and Ara h 2.0201, respectively.

IgE Competition Assays

The competition assays were similar to the IgE binding assays. Wells were coated by overnight incubation with 500 ng/well of either the recombinant Ara h 2.0101 or Ara h 2.0201 isoforms diluted in carbonate buffer. After blocking, 100 µl of plasma with or without excess allergen (100 µg) were added to the wells and incubated overnight at 4°C. The assay was then developed as per the IgE binding assay using DELFIA reagents.

Statistical Analysis

Paired data were analyzed by χ^2 and t tests and a correlation was performed with Spearman's correlation. A two-tailed p value of <0.05 was considered significant. Analyses were performed using Prism statistical and graphing package (GraphPad Software, San Diego, Calif., USA).

Results

Protein Sequence and Expression

The predicted amino acid sequences of the mature proteins of Ara h 2.0101 and Ara h 2.0201 found for the cDNA clones are listed in figure 1. The majority were identical to those of Chatel et al. [10]. The original Ara h 2.0101 isoform was found for 6/10 clones and Ara h 2.0201 was found for 2/10 clones. Ara h 2.0201 had the expected insertion of 12 amino acids as well as substitutions of glycine (G) for glutamic acid (E) at position 40 and glutamic acid (E) for aspartic acid (D) at position 142. Interestingly, because the sequence of Ara h 2 is repetitive

Insertion after residue 48			
	50	60	70
Ara h 2.0101	RDPYSPSQ-----DPYSPSPYDR		
Ara h 2.0201	RDPYSPSQDPYSPSQDPDRRD <u>DPYSPSPYDR</u>		
Insertion after residue 49			
	50	60	70
Ara h 2.0101	RDPYSPSQD-----PYSPSPYDR		
Ara h 2.0201	RDPYSPSQDPYSPSQDPDRRD <u>DPYSPSPYDR</u>		
Insertion after residue 50			
	50	60	70
Ara h 2.0101	RDPYSPSQDP-----YSPSPYDR		
Ara h 2.0201	RDPYSPSQDPYSPSQDPDRRD <u>DPYSPSPYDR</u>		
Insertion after residue 51			
	50	60	70
Ara h 2.0101	RDPYSPSQDPY-----SPSPYDR		
Ara h 2.0201	RDPYSPSQDPYSPSQDPDRRD <u>DPYSPSPYDR</u>		
Insertion after residue 52			
	50	60	70
Ara h 2.0101	RDPYSPSQDPYS-----PSPYDR		
Ara h 2.0201	RDPYSPSQDPYSPSQDPDRRD <u>DPYSPSPYDR</u>		
Insertion after residue 53			
	50	60	70
Ara h 2.0101	RDPYSPSQDPYSP-----SPYDR		
Ara h 2.0202	RDPYSPSQDPYSPSQDPDRRD <u>DPYSPSPYDR</u>		
Insertion after residue 54			
	50	60	70
Ara h 2.0101	RDPYSPSQDPYSPS-----PYDR		
Ara h 2.0202	RDPYSPSQDPYSPSQDPDRRD <u>DPYSPSPYDR</u>		

Fig. 2. Amino acid sequence alignments of Ara h 2.0101 and Ara h 2.0201 containing the possible insertion/deletion of 12 amino acids (in bold) for Ara h 2.0201 after residues 48–54. The immunodominant IgE binding epitope DPYSPS is underlined.

in the region of the insertion/deletion (the nucleotide and amino acid sequences were an exact match), several equally plausible alignments can be made depending on whether the insertion is placed after residue 48, 49, 50, 51, 52, 53 or 54 (fig. 2). The alignment in figure 1 places the insertion after residue 54 which is the first residue where the isoforms do not match (starting from the N-terminal). The 12-amino acid insert here would be the previously proposed QDPDRRDPYSPS. The Ara h 2.0201 isoform contains an additional copy of the immunodominant IgE epitope DPYSPS compared to the original sequence regardless of where the insert/deletion is positioned. The polymorphism identified by Chatel et al. [10] at position 77 (77Q or 77R) was not found in any of the 10 sequences. A small difference from the previous sequence was found for two of the clones. The residue at position 142 was

Table 1. IgE binding of peanut-allergic individuals to Ara h 2.0101 and Ara h 2.0201

	Ara h 2.0101	Ara h 2.0201
Frequency of reactivity, %	77.1 (54/70) ^a	81.4 (57/70)
Specific IgE (mean ± SEM), ng/ml	35.8 ± 11.9	35.5 ± 12.4
Highest IgE binding isoform, %	39.7	60.3 ^b

^a IgE levels above 0.21 and 0.20 ng/ml were considered positive for Ara h 2.0101 and Ara h 2.0201, respectively (> mean + 2 SD of non-allergic sera).
^b Significant at p < 0.01 (χ^2 test).

142E for one Ara h 2.01 clone (Ara h 2.0102) and 142D for one Ara h 2.02 clone (Ara h 2.0202). In addition to the minor amino acid polymorphisms, the nucleotide sequences identified a number of silent changes between Ara 2.0101 and Ara h 2.0201 (data not shown).

Comparative IgE Binding to Ara h 2.0101 and Ara h 2.0201

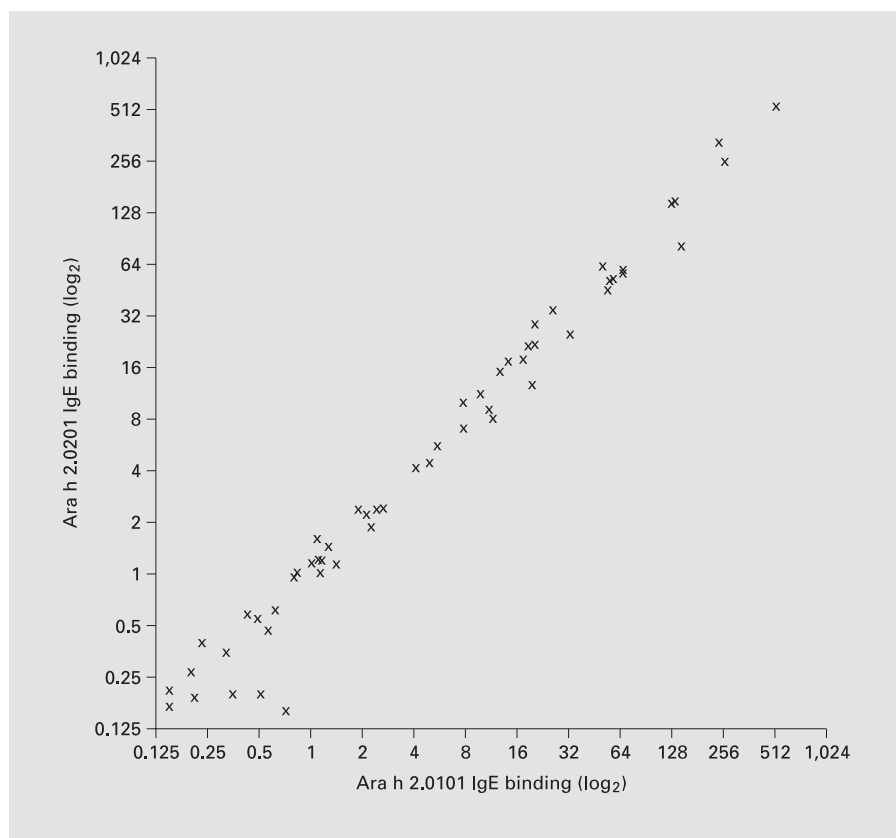
The IgE binding of Ara h 2.0101 and Ara h 2.0201 was examined using plasma from 70 peanut-allergic children (table 1). The frequency of IgE reactivity was 77.1% (54/70) for Ara h 2.0101 and 81.4% (57/70) for Ara h 2.0201 and the means were similar. More plasma, however, had its highest binding to Ara h 2.0201 (60%, χ^2 p < 0.01). The correlation between Ara h 2.0101 and Ara h 2.0201 was extremely strong (r = 0.987, p < 0.0001) (fig. 3). IgE reactivity to only one of the Ara h 2 isoforms was found for 5 patients, but the levels were below 1 ng/ml.

Competition Assays

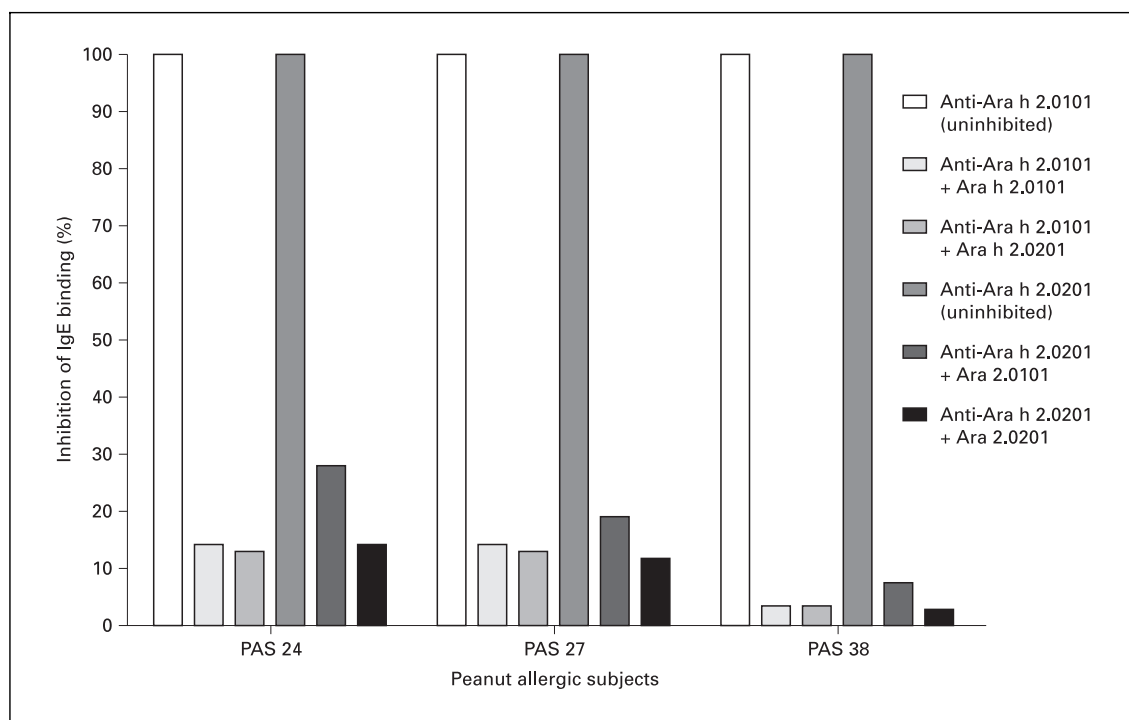
The Ara h 2.0201 isoform contains an additional 12 amino acids that incorporate the reported immunodominant epitope DPYSPS and possibly other IgE specificities. To test whether Ara h 2.0201 may contain IgE epitopes other than those found for Ara h 2.0101, plasma from three children with high IgE reactivity to Ara h 2 (peanut-allergic sera 24, 27 and 38) were selected for competition assays. Ara h 2.0201 was able to inhibit IgE reactivity to both isoforms to a similar degree but although Ara h 2.0101 was as efficient as Ara h 2.0201 in blocking IgE binding to itself, it was less effective at inhibiting IgE binding to Ara h 2.0201 (fig. 4). The difference in the ability of the Ara h 2.0101 and Ara h 2.0201 isoforms to cross-absorb IgE binding was analyzed by comparing the ratio of the percentage of residual IgE binding after each

Fig. 3. IgE binding (ng/ml) to the Ara h 2.0101 and Ara h 2.0201 isoforms for 70 peanut-allergic children. The correlation of IgE binding between the two isoforms was 0.987.

Fig. 4. The ability for either Ara h 2 isoform to compete for IgE binding was tested for three peanut-allergic sera (PAS 24, 27 and 38) in a competition assay. For each patient uninhibited IgE binding to Ara h 2.0101 is shown and binding with Ara h 2.0101 and Ara h 2.0201 as inhibitors. Furthermore, the uninhibited binding to Ara h 2.0201 is shown and binding with Ara h 2.0101 and Ara h 2.0201 as inhibitors. The absolute values of IgE binding were 203, 511 and 105 ng/ml for Ara h 2.0101 and 136, 536 and 148 ng/ml for Ara h 2.0201 for the PAS 24, 27 and 38, respectively.



3



4

absorption (ratio of Ara h 2.0101:Ara h 2.0201). The amount of residual IgE binding to Ara h 2.0101 was similar after absorption with either isoform (ratio 1.02), whereas the ratio was 2.07 when tested against Ara h 2.0201 ($p < 0.01$). Inhibition of IgE binding to Ara h 2 was negligible using an irrelevant protein Der p 2 (0.7% mean inhibition at 100 $\mu\text{g/ml}$) excluding the possibility of non-specific blocking in the competition assays (data not shown).

Discussion

IgE binding to two isoforms of the major peanut allergen Ara h 2 was examined for 70 peanut-allergic children to assess whether the recently identified Ara h 2.0201 isoform was more allergenic than Ara h 2.0101. Ara h 2.0201 has an insert of 12 amino acids that contains an extra copy of the sequence DPYSPS, which has been described as an immunodominant IgE epitope. There are 2 copies of this motif in Ara h 2.0101 and 3 copies in Ara h 2.0201. Previous studies with a small sample of patients or pooled sera have shown the majority of IgE against Ara h 2 is directed against this region [11, 16]. IgE reactivity in this study was measured using a chimeric mouse/human IgE standard to quantitate specific IgE binding. The levels of IgE binding to Ara h 2.0201 for individual sera were frequently higher than Ara h 2.0101; however, the increase was small at the sample level and there was also a strong correlation in binding to both isoforms and the frequency and means were similar. As expected the frequency of reactivity to Ara h 2.0101 (77%) and Ara h 2.0201 (81%) was high. IgE from a small number of individuals bound to only one of the Ara h 2 isoforms but the level of binding was very low. Therefore, the lack of binding may be due to the sensitivity of the IgE assay rather than a preference for a particular isoform. Both isoforms are well represented in peanut extracts based on the findings of Burks et al. [6], de Jong et al. [2] and Sen et al. [9]. Ara h 2.0101 had moderately stronger Coomassie staining on SDS-PAGE and mass spectroscopy on Ara h 2 purified from peanut protein extract also found slightly more of the Ara h 2.0101 isoform in extracts [10]. The increased number of clones identified for Ara h 2.01 reflects the relative abundance of this isoform in extracts.

The higher level of reactivity to Ara h 2.0201 might not be entirely due to the extra copy of the IgE epitope DPYSPS as indicated by the competition assays. With the high concentration used, both isoforms would be expected to compete for IgE reactivity to a similar extent if their

IgE epitopes were shared. However, Ara h 2.0101 was not as efficient at blocking reactivity to Ara h 2.0201 indicating there is an additional IgE specificity for the Ara h 2.0201 isoform. It is also possible that this region may be important for T cell reactivity. Therefore, although the isoforms are antigenically similar and either would be suitable for diagnosis, an optimal formulation for therapy may need both.

The sequence analysis was similar to that published by Chatel et al. [10], although two additional isoforms were identified. The amino acid polymorphism at position 142 was 142E for one Ara h 2.01 clone (Ara h 2.0102) and 142D for one Ara h 2.02 clone (Ara h 2.0202) showing the conservative amino acid change varied between each isoform. This was not the case for the polymorphism at position 40 which was always glutamic acid for the seven Ara h 2.01 clones and glycine for all three Ara h 2.02 clones.

The sequences of Ara h 2.0101 and Ara h 2.0201 can be aligned by placing the insert after any of the residues 48–54. This is because the sequence is repetitive in this area. It is possible that the tertiary structure when solved could clarify its position. Chatel et al. [10] have nominated QDPDRRDPYSPS as the insert but this is arbitrary. It could be DPYSPSQDPDRR if the insert, was positioned after residue 48 and so on. Figure 1 has equally arbitrarily placed it after residue 54 where the first mismatch occurs counting from the N-terminal. All of the possibilities contain the extra DPYSPS but any studies using peptides to examine the contribution of the insertion need to consider the six possibilities.

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