

Characterisation of the IgE-binding immunodominant epitopes on Ara h1

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Peanut allergy is one of the most severe food allergies due to its persistency and life-threatening character. Serum IgE from patients with documented peanut hypersensitivity reactions and synthetic peptides were used to screen the linear IgE-binding epitopes on the major peanut allergen, Ara h 1. Five major epitopes that bound peanut-specific serum IgE from more than 60% of patients tested were identified. Mutational analysis of the immunodominant epitope showed that single amino acid changes had dramatic effects on IgE-binding characteristics. Mapping and characterisation of the IgE-binding epitopes on Ara h1 could be used in future immunotherapeutic approaches for peanut allergy disease.

Keywords: peanut allergy; Ara h1; IgE-binding epitope.

Introduction

Ara h1 has been identified as a major peanut allergen of 63.5kDa (Burks, Cockrell, Stanley, Helm, & Bannon, 1995; Burks et al., 1991; De Jong et al., 1998) and has gained considerable attention in recent years (Burks et al., 1995; Koppelman, Bruijnzeel-Koomen, Hessing, & de Jongh, 1999; Shin et al., 1998). The Ara h 1 allergen belongs to the vicilin family of seed storage proteins (Burks et al., 1995). It is resistant to heat (Maleki, Chung, Champagne, & Raufman, 2000), stable to digestion, and highly structured at the secondary level (Koppelman et al., 1999). Two cDNA clones have been identified as isoforms of Ara h 1 (GenBank accession nos. L34402, L38853). The majority of serum IgE recognition of the Ara h1 allergen appears to be due to epitopes within this protein that are linear amino acid sequences that do not contain significant amounts of carbohydrate (Burks et al., 1991; Burks et al., 1995; De Jong et al., 1998). The elucidation of the major IgE-binding epitopes on Ara h 1 may help to better understand the immunopathogenic mechanisms involved in peanut hypersensitivity.

Information about the epitopes of a protein can be obtained by many different methods. The classical way is to test the antigenic properties of peptides resulting from enzymatic or chemical fragmentation of the protein. Another means of epitope mapping is the immunologic testing of peptides synthesised according to the protein sequence. This epitope scanning method allows us to screen high numbers of small peptides and to

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identify epitopes with the resolution of a single amino acid. Potential epitopes were identified from these studies.

Although the epitopes mapping have been performed on the Ara h 1 allergen, immunodominant epitopes maybe different since the serology of peanut allergies are different from country to country (Burks et al., 1995; Burks et al., 1997). In the present study, synthetic peptides were used to map the major IgE-binding epitopes of Ara h 1. The identification of the immunodominant epitopes of Ara h 1 may lead to a better understanding of the mechanisms responsible for sensitisation to peanut. In addition, mutational analysis of the IgE-binding immunodominant epitopes on Ara h 1 was performed.

Methods and material

Peptide synthesis

Individual peptides were synthesised using Fmoc protected amino acids according to the manufacturer's instructions (Genosys Biotechnologies). Synthesis of each peptide was started by esterification of an Fmoc protected amino acid. Peptides were synthesised by sequential addition of the appropriate Fmoc protected amino acid, one at a time. The N-terminal amino group of the peptide was acetylated before cleavage with trifluoroacetic acid. All peptides were used test for immunopathogenicity using human sera from patients with peanut allergy and from non-allergic subjects (Burks et al., 1997).

Purification and MS identification

After synthesis the raw peptides were purified on a Nucleosil C-18RP-18 preparation column (Macherey and Nagel, Düren, Germany; 21.5x250 mm, 5 µm, 30 nm), at a flow rate of 12.0 ml/min and a gradient of 1% v/v acetonitrile per min. Acetonitrile/water with 0.1% v/v trifluoroacetic acid was used as the eluting solvent, and more than 50 mg of these raw peptides were loaded. Then purity analysis and MS identification were carried out on a LCQ-MS system (FinniganMAT, Bremen, Germany) coupled with an analytical HPLC column (Nucleosil 100-C18, 4.6 × 250 mm, 5 µm, 10 nm). MS was operated in positive electrospray ionisation (ESI) mode.

Epitope mapping

ELISA was performed as described by Frank and Overwin (1996). Before immunological detection the purified peptides were coupled with bovine serum albumin (BSA). Then the peptides reacted with BSA were combined with 96 well plates. The Ara h 1 peptides were incubated with individual serum or the serum pool from patients with peanut hypersensitivity diluted (1:5) in a solution containing Tris/NaCl (10 mM Tris/HCl, 500 mM NaCl, pH 7.5) and 1% BSA for at least 12 h at 4°C or 2 h at room temperature. The primary antibody was detected using monoclonal mouse-anti-human-IgE (1:2000) conjugated to horseradish peroxidase (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) and subsequently stained for peroxidase activity with 4-chloro-1-naphtol staining, then expressed as A490. Between each step, plates were washed five times with TBS containing 0.1% Tween 20. Non-specific binding was studied using serum from non-allergic individuals and allergic individuals (but not peanut allergic).

Results

The peptides of Ara h 1

The peptide sequences are outlined in Table 1. About 5 mg pure peptide of each was obtained and purification of Ara h 1 peptides were performed with HPLC (see Figure 1), only P4 and P17 were shown, and verified by MS (Figure 2, only P4 and P17 were shown).

IgE-binding epitope mapping of Ara h 1

As shown in Figure 3, the 23 peptides were probed individually with serum IgE from eight different patients with documented peanut hypersensitivity. IgE from patients with peanut allergy displayed significant binding to Ara h 1, and IgE from negative patients did not

Table 1. The peptides of Ara h 1.

The sequence of amino acids	Ara h 1 position
MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQ P1 AA 21–34	1–40
RCLQSCQQEPDDLKQKACESRCTKLEYDPRLVYDPRGHTG P2 AA 43–52 P3 AA 63–72	41–80
TTNQRSPPGERTGRQPGYDDDRRQPRREEGGRWGPAGP P4 AA 89–98 P5 AA 99–108 120	81–120
REREREEDWRQPREDWRRPSHQPRKIRPEGREGEQEWTG P7 AA 122–132 P8 AA 134–144	121–160
PGSHVREETSRRNPPYFPSRRFSTRYGNQNGRIRVLQRFD P9 AA 161–172	161–200
QRSKQFQNLQNHRIQIEAKPNTLVLPKHADADNLIQIQ GQATVTVANGNNRKSFNLDGHALRIPSGFISYILNRHDN P10 AA 253–264	201–240 241–280
QNLRVAKISMPVNTRGQFEDFFPASSRDQSSYLQEFSTRNT P11 AA 306–318	281–320
LEAAFNAEFNETRRVLLEENAGGEQEERGQRRWSTRSEN P12 AA 325–335 P13 AA 342–353	321–360
NEGIVVKVSKEHVEELTKHAKSVSKKGSEEECDITNPINL P14 AA 393–	361–400
REGEPGLSNNFGKLFVVKPDKKNPQLQDLDMMLTCVEIKE 403 P15 AA 418–429	401–440
GALMLPHFNSKAMVIVVVNKGTDGNLELVAVRKEQQQRGR P16 AA 448–458	441–480
EEEEDEDEEEEGSNREVRRTARLKEGDVFIMPAAHPVAI P17 AA 498–507	481–520
NASSELHLLGFGINAENNRIFLAGDKDNVIDQIEKQAKD P18 AA 521–532 P19 AA 536–546 P20 AA 548–557	521–560
LAFPGSGEQVEKLIKQKESHFVSARPSQSQSPSSPEKESPEKE P21 AA 559–570 P22 AA 575–587 P23	561–600
AA DQEEE NQGGKGPLLS ILKAFN 594–605	601–626

Note: P represents peptide.

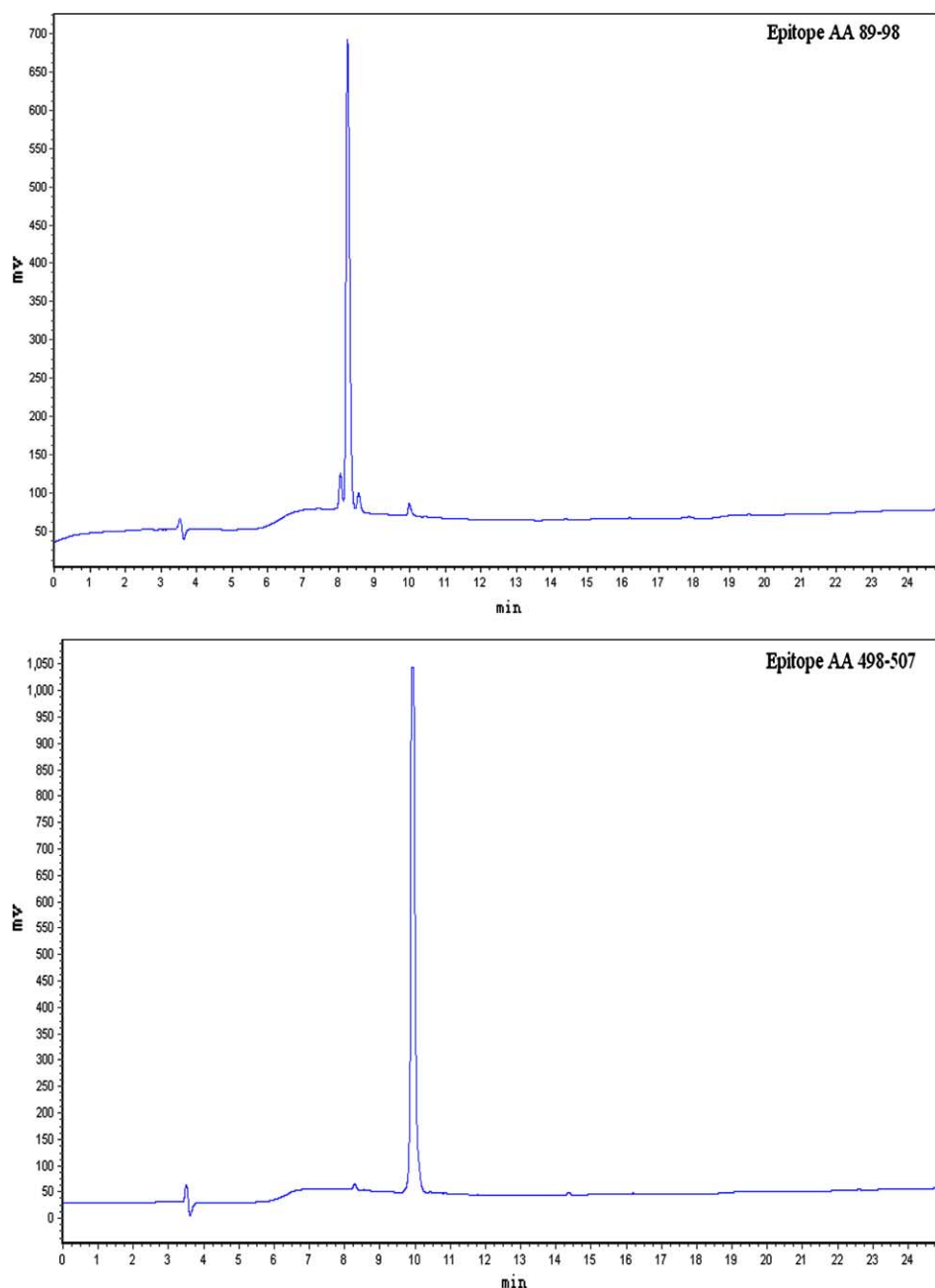


Figure 1. The HPLC profile of synthetic peptides at AA 89–98 and 498–507.

(data not shown). The ELISA results indicated that IgE from each patients interacted with different synthetic peptides.

An epitope can be considered as an immunodominant if it is recognised by serum IgE from the majority of patients with peanut hypersensitivity. In general, the peptides at AA 21–34,

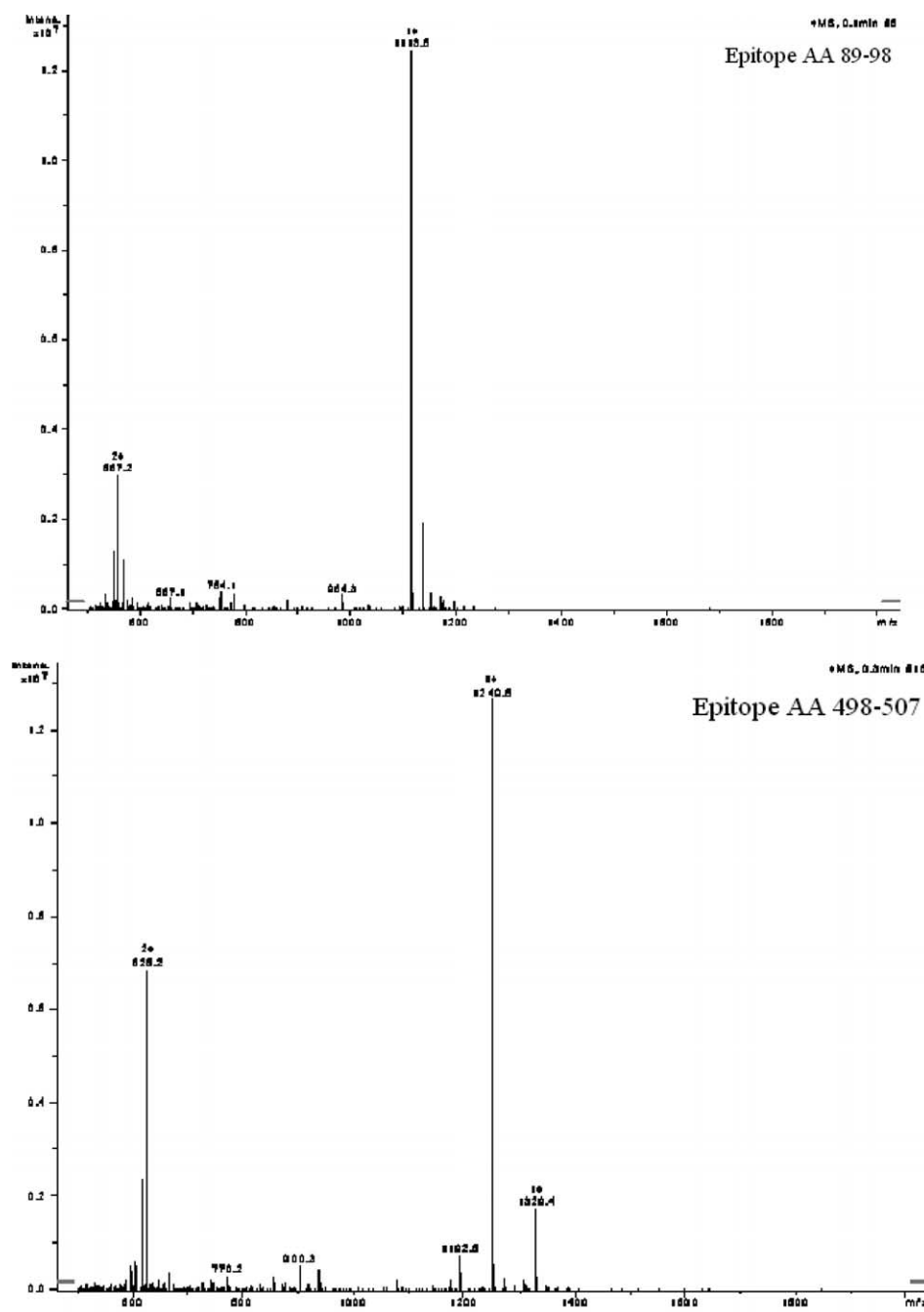


Figure 2. Mass spectrum of the peptides at AA 89–98 and 498–507.

89–98, 393–403, 498–507, and 594–605 were considered as immunodominant epitopes since they were recognised by IgE from at least 60% of the sera tested, moreover, 100% IgE from patients interacted with the epitope at AA 498–507, and 87.5% with the epitope at AA 89–98.

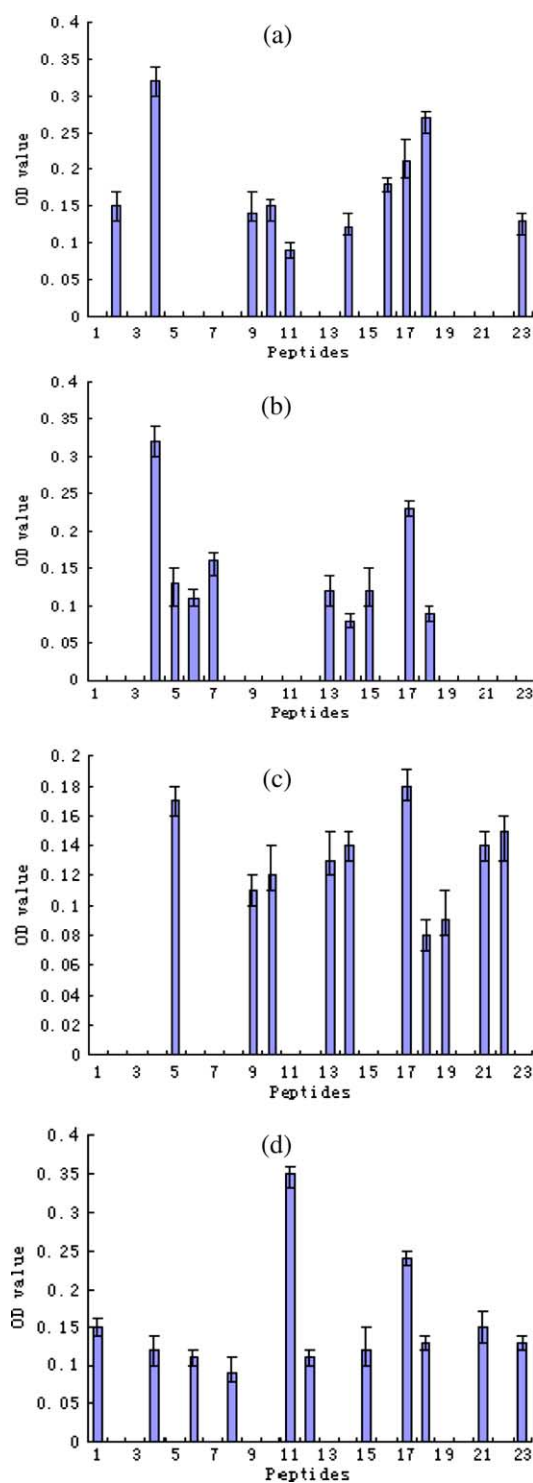


Figure 3. (Continued)

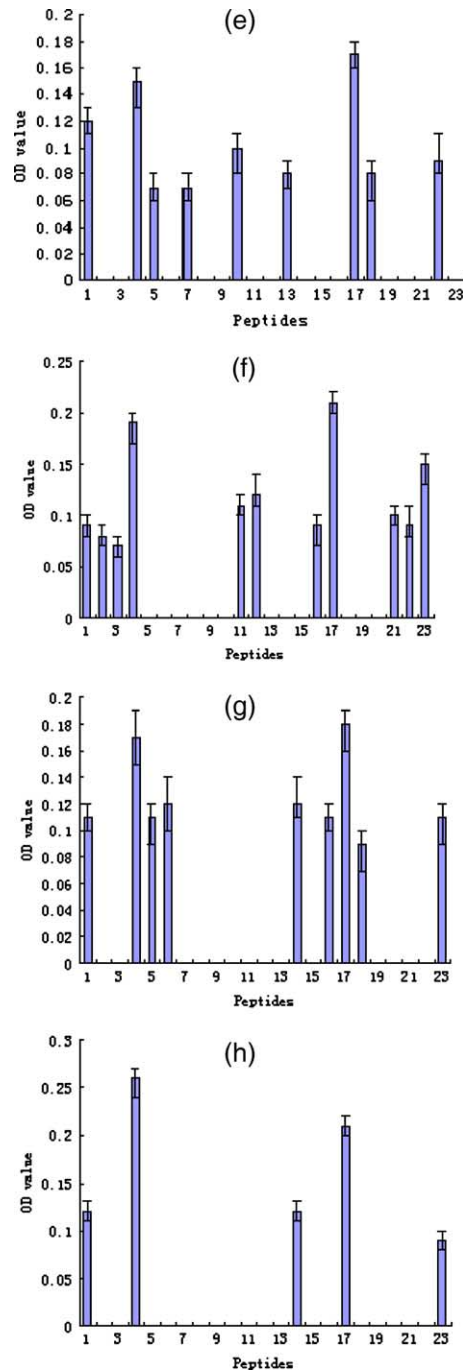


Figure 3. Mapping analysis of the IgE-binding epitopes on Ara h1. The 23 synthetic peptides in Table 1 were probed individually with serum IgE from 8 patients (a,b,c,d,e,f,g,h) with peanut hypersensitivity. All synthetic peptides did not interact with negative sera (data not shown).

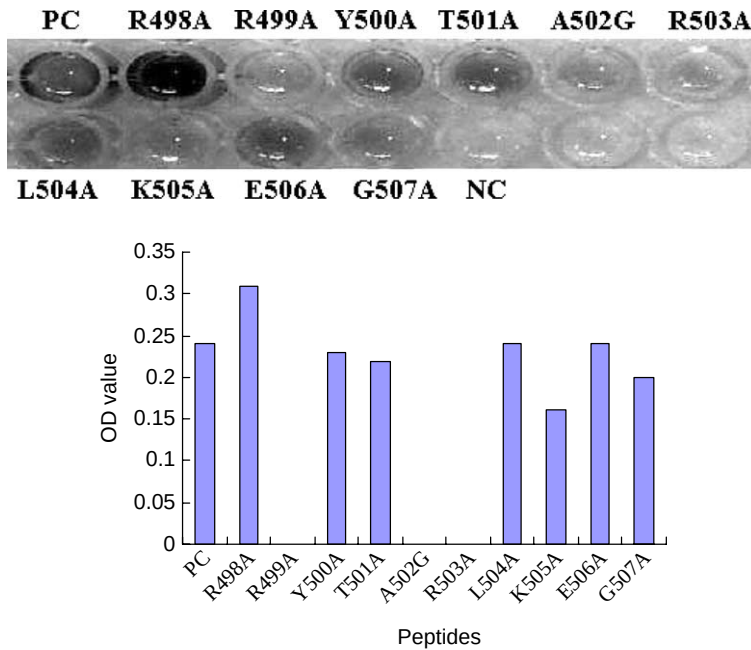


Figure 4. Mutational analysis of the IgE-binding dominant epitope RRYTARLKEG on Ara h1. The epitope at AA RRYTARLKEG was synthesised with an alanine residue substituted for one of the amino acids in this peptide and probed with a pool of serum IgE from eight patients with documented peanut hypersensitivity. The letters across the top of each panel indicate the one-letter amino acid code for the residue normally at that position and the amino acid that was substituted for this residue. The numbers indicate the position of each residue in the Ara h 1 protein. PC indicates the native peptide (no amino acid substitutions). NC indicates negative control (no allergen with ELISA).

Mutational analysis of the IgE-binding epitope on Ara h 1

Pooled sera from eight patients were used to confirm the requirement for each AA in the sequence for epitope-specific recognition by IgE antibodies. In the first phase of experiments, each residue within an epitope was substituted with an alanine (or glycine, when alanine was present) at a time. Figure 4 showed an example of the immunolabelling with pooled sera for peptide 498–507 (RRYTARLKEG). In comparison with the native peptide, single site substituted peptides R499A, A502G, R503A resulted in a total loss of IgE binding. The peptides Y500A, T501A, K505A, and G507A showed diminished but not totally abrogated binding, whereas substitution of the critical AA R498 resulted in an increase of IgE binding.

Discussion

Food hypersensitivity reactions occur shortly after the contact of a specific allergen with its corresponding IgE, when cross-linked by the respective allergen, activates the mast cells to release histamine, heparin, and other mediators responsible for the clinical symptoms observed (Mills, Madsen, Shewry, & Wichers, 2003). Since antigen-specific IgE plays such a critical role in the etiology of allergic disease, determination of allergen-specific, IgE-binding

epitopes is an important first step towards a better understanding of this complex disease process.

A computer algorithm has also been used to predict epitopes of Ara h1. Potential epitopes on Ara h1 were identified from these studies. There are 12 IgE-binding regions along the entire length of the Ara h1 protein recognised by IgE from patients with peanut hypersensitivity, and these IgE-binding regions represent amino acid residues 35–72, 89–112, 121–176, 289–326, 337–350, 361–374, 393–416, 457–471, 489–513, 521–535, 544–583, and 593–607 (Burks et al., 1997).

In this study, we synthesised the 23 peptides for recognition by serum IgE from peanut hypersensitive individuals based on these predicted antigenic regions. Five major IgE-binding epitopes on Ara h1 were identified with individual sera from peanut hypersensitivity because they were recognised by more than 60% of patients' sera tested. This result was contrasted with a previous study. Shin et al. (1998) depicted the 23 IgE-binding epitopes on Ara h1 by peanut hypersensitive individuals, there were no significant differences in the IgE-binding intensity of these epitopes. However, the epitopes at AA 21–34, 89–98, and 498–507 were all recognised by positive sera with peanut hypersensitive patients in this study and Burks et al. (1995, 1997), whereas, the epitopes 393–403 and 594–605 were not observed by Burks et al. (1995). In addition, epitopes 21–34 and 89–98 located in the N-terminal portion of the protein, appear to be unique to this peanut vicilin and do not share any significant sequence similarity with vicilins from other legumes (Gibbs, Strongin, & McPherson, 1989). Moreover, the epitopes 498–507 and 594–605, which were located in the C-terminal end of the protein, were in a region that shares significant sequence similarity with vicilins from other legumes (Gibbs et al., 1989). This may explain the possible cross-reacting antibodies to other legumes that can be found in the sera of patients with a positive double-blind placebo-controlled food challenge to peanuts.

Peptides other than these five epitopes, identified with the absorption technique demonstrated value of IgE binding with only a few sera. These results suggested that these peptides are not recognised as allergic epitopes by all individuals. A possible limitation of this methodology for exploring the epitopes of ingested allergens is the production of IgE antibody to internal epitopes. These buried epitopes may only be revealed to the immune system following gut digestion of the protein and absorption of peptides containing the epitopes. Moreover, it may be that further *in vitro* and *in vivo* techniques utilising the decapeptides will need to be developed before the clinical significance of these peptides can be assessed. We are also in the process of investigating the activity of epitopes recognised in conjugate skin-prick tests.

In the search for critical AAs for IgE binding, alanine and glycine were used for AA substitution because they are relatively small neutral AAs that do not significantly alter the charge or solubility of peptides. This approach has been used successfully for other allergens (Rabjohn et al., 1999; Robotham, Teuber, Sathe, & Roux, 2002; Stanley et al., 1997). Hydrophobic residues appear to be critical for IgE binding in the peanut allergen Ara h1 (Shin et al., 1998), whereas no obvious type of AA was apparent in Ara h2 and Ara h3 (Rabjohn et al., 1999; Stanley et al., 1997). In this study, arginine at 499, 503 and glycine at 502 play an important role for the IgE-binding property of Ara h1. In addition, AAs in the periphery of the epitope appear to be more important for IgE binding in Ara h1 than those found at the centre of the epitope. This was not found for the shrimp allergen Pen a1, the peanut allergen Ara h3, and the walnut allergen Jug r1 (Lehrer, Rosalia, & Reese, 2002; Rabjohn et al., 1999; Robotham et al., 2002). Therefore, the mutational

analysis of the IgE-binding capabilities of core amino acids in epitopes on the Ara h 1 protein will make it possible to address the pathophysiologic and immunologic mechanisms regarding peanut hypersensitivity reactions specifically and food hypersensitivity in general.

In conclusion, we set out to map and mutate the major IgE epitopes of the Ara h 1 allergen because of the prevalence and severity of peanut hypersensitivity reactions and the role B-cell epitopes play in peanut hypersensitivity reactions. Serum IgE from patients with documented peanut hypersensitivity reactions and synthetic peptides were used to identify the IgE-binding epitopes on Ara h 1. Five of the peptides appeared to immunodominant IgE-binding epitopes in that they were identified by serum from more than 60% of the patients tested and bound more IgE than any of the other Ara h 1 peptides. The identification and determination of the immunodominant epitopes showed that single amino acid changes within these epitopes had dramatic effects on IgE-binding characteristics.

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