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ADDENDUM

Figure 3.2 (following p. 68)

Legend: sentence commencing line 4 is amended to read "The absorbance in control wells containing no antigen (mean for all patients = 0.290 ± 0.059) was subtracted from antigen coated wells".

Immunological and Molecular Characterisation of Major Peanut Allergens and their Cross-Reactive Components in Tree Nuts

Maria P. de Leon

B.Sc. (Hons)

Department of Pathology and Immunology

Monash University

Melbourne, Victoria, Australia

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SUMMARY

Peanut and tree nuts account for the majority of fatal food-related anaphylactic reactions in children and adolescents. The prevalence of this type of food allergy is increasing and at present, the only available form of treatment is allergen avoidance which is often difficult due to the frequent use of peanuts and tree nuts in many different foods. Active avoidance of the offending food is sometimes insufficient as some allergic reactions occur through accidental exposure to peanut and tree nut allergens which can be attributed to the inadequate labelling and contamination of food products during the manufacturing process. Although allergy to peanuts is a more frequent presentation, sensitisation to both peanut and tree nuts is a common clinical observation. It is not known whether this is due to cross-reactive peanut and tree nut allergens.

This thesis investigated the molecular and immunological basis of IgE cross-reactivity between peanut and the tree nuts almond, Brazil nut, cashew and hazelnut. The initial assessment of IgE reactivity to peanut, almond, Brazil nut, cashew and hazelnut extracts in a population of 22 peanut allergic subjects included in this study confirmed that the majority of these subjects had specific IgE antibodies to at least one tree nut type. Subsequent inhibition studies using sera from a sub-population of peanut allergic subjects indicated that peanut-specific IgE antibodies cross-reacted with almond, Brazil nut and hazelnut allergens. The highest level of cross-reactivity was observed WITH almond, followed by Brazil nut and hazelnut. In contrast, negligible IgE cross-reactivity was observed between peanut and cashew. This pattern of cross-reactivity did not reflect the taxonomic classification of peanut and the tree nuts tested.

These results led to further investigations of IgE cross-reactivity between peanuts and tree nuts at the individual peanut allergen level. The major peanut allergens, Ara h 1 and Ara h 2, and the minor peanut allergen, Ara h 3 were cloned, expressed and purified. These recombinant allergen preparations were shown to be IgE reactive and to have biological activity through the activation of basophils from a panel of peanut allergic subjects. Subsequent inhibition studies using sera from peanut allergic subjects previously shown to have specific IgE to rAra h 1, rAra h 2 and rAra h 3, demonstrated that these peanut allergens contribute to the observed IgE cross-reactivity between peanut, almond, Brazil nut and hazelnut. rAra h 1 and rAra h 3 demonstrated low-level cross-reactivity with almond and hazelnut allergens, respectively, while rAra h 2 showed a higher level of cross-reactivity with almond and Brazil nut. Western immunoblotting studies using affinity-purified antibodies specific for rAra h 1, rAra h 2 and rAra h 3 confirmed the presence of cross-reactive allergens and demonstrated the presence of potential allergen homologues in almond, Brazil nut and hazelnut.

Confirmation of the presence of tree nut cross-reactive peanut-specific IgE antibodies in peanut allergic subject serum led to the investigation of the biological activity of these antibodies. An *in vitro* assay was established whereby basophils stripped of surface IgE were resensitised with affinity-purified peanut-specific antibodies. Basophil activation upon exposure to tree nut allergens was subsequently examined as a measure of biological activity. Using this assay, it was demonstrated that basophils resensitised with peanut-specific and rAra h 2-specific IgE antibodies became activated following stimulation with almond and Brazil nut allergens. This correlated with the high level of cross-reactivity observed in the previous inhibition studies. In contrast, peanut-specific and rAra h 1-specific IgE antibodies involved in low-level cross-reactivity between hazelnut and almond allergens, respectively, did not induce basophil activation upon

stimulation with the cross-reactive allergen. Thus it appears that the ability of cross-reactive IgE antibodies to induce effector cell activation is dependent on the degree of cross-reactivity between the allergen sources.

In conclusion, the information presented in this thesis provides further insight into the immunological mechanisms involved in the co-sensitisation to peanut and tree nuts in allergic individuals. Establishing the presence of cross-reactive allergens in peanuts and tree nuts is important for patient management and may lead to simplified diagnosis. It may also provide avenues for the development of an effective treatment for multiple nut allergy.

DECLARATION

The work embodied in this thesis was conducted in the Department of Pathology and Immunology, Monash University, during 2000 to 2004. It contains no material which has been accepted for the award of any other degree or diploma in any other university or institution. To the best of my knowledge this thesis contains no material previously published or written by another person, except where specific reference has been made in the text.

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Maria P. de Leon (23rd January 2004)

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PUBLICATIONS

The results reported in this thesis have been published and presented at scientific meetings as follows:

REFEREED JOURNAL ARTICLE

1. **de Leon, M. P.**, Glaspole, I. N., Drew, A. C., Rolland, J. M., O'Hehir, R. E., Suphioglu C. (2003). Immunological analysis of allergenic cross-reactivity between peanut and tree nuts. Clin Exp Allergy 33, 1273-1280.

CONFERENCE PRESENTATIONS

1. **de Leon, M. P.**, Glaspole, I. N., Drew, A. C., Rolland, J. M., O'Hehir, R. E., Suphioglu C. Immunological analysis of cross-reactivity between the major peanut allergen Ara h 2 and tree nuts. Australian Society for Immunology, 32nd Annual, Brisbane, Australia, 2002.

2. **de Leon, M. P.**, Glaspole, I. N., Drew, A. C., Rolland, J. M., O'Hehir, R. E., Suphioglu C. Allergenic cross-reactivity between peanut and tree nuts: immunological and taxonomic comparisons. Alfred Science Symposium, Melbourne, Australia, 2002.

3. **de Leon, M. P.**, Glaspole, I. N., Drew, A. C., Rolland, J. M., O'Hehir, R. E., Suphioglu C. Allergenic cross-reactivity between peanut and tree nuts: immunological and taxonomic comparisons. Australian Socieity for Immunology, 31st Annual Scientific Meeting, Canberra, Australia, 2001.

ABBREVIATIONS

7AAD	7-amino-actinomycin D
AA	amino acid
AMP	almond major protein
APCs	antigen presenting cells
BcR	B cell receptor
BGP	Bermuda grass pollen
BHRA	basophil histamine release assay
bp	base pair
BSA	bovine serum albumin
BSGG	bovine serum gammaglobulin
°C	degrees Celsius
CBB	Coomassie brilliant blue
CCD	cross-reactive carbohydrate determinant
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
cm	centimetre
DBPCFC	double-blind placebo controlled food challenge
DNA	deoxyribonucleic acid
EAST	enzyme allergosorbent test
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay

FACS	fluorescence-activated cell sorter
FITC	fluoroscein isothiocyanate
fMLP	*N*-formyl-Met-Leu-Phe
g	grams
GE	latex-glove extract
H_2O	Milli Q water
HDM	house dust mite
HRP	horseradish peroxidase
Ig	immunoglobulin
IgE	immunoglobulin E
IL	interleukin
IFN-γ	interferon-γ
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodaltons
L	litre
LTP	lipid transfer protein
MBP	major basic protein
M	molar
mg	milligram
ml	millilitre
µl	microlitre
mm	millimetre

mM	millimolar
M_r	relative molecular mass
Ni-NTA	nickel-nitrilotriacetic acid
nm	nanometre
OD	optical density (absorbance)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
r	recombinant
RAST	radioallergosorbent test
RGP	rye grass pollen
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor
SDS-PAGE	sodium dodecyl sulphate gel electrophoresis
SGF	simulated gastric fluid
SPTs	skin prick tests
Th	T helper
TNF	tumour necrosis factor
UV	ultraviolet
V	volts

CHAPTER 1

LITERATURE REVIEW

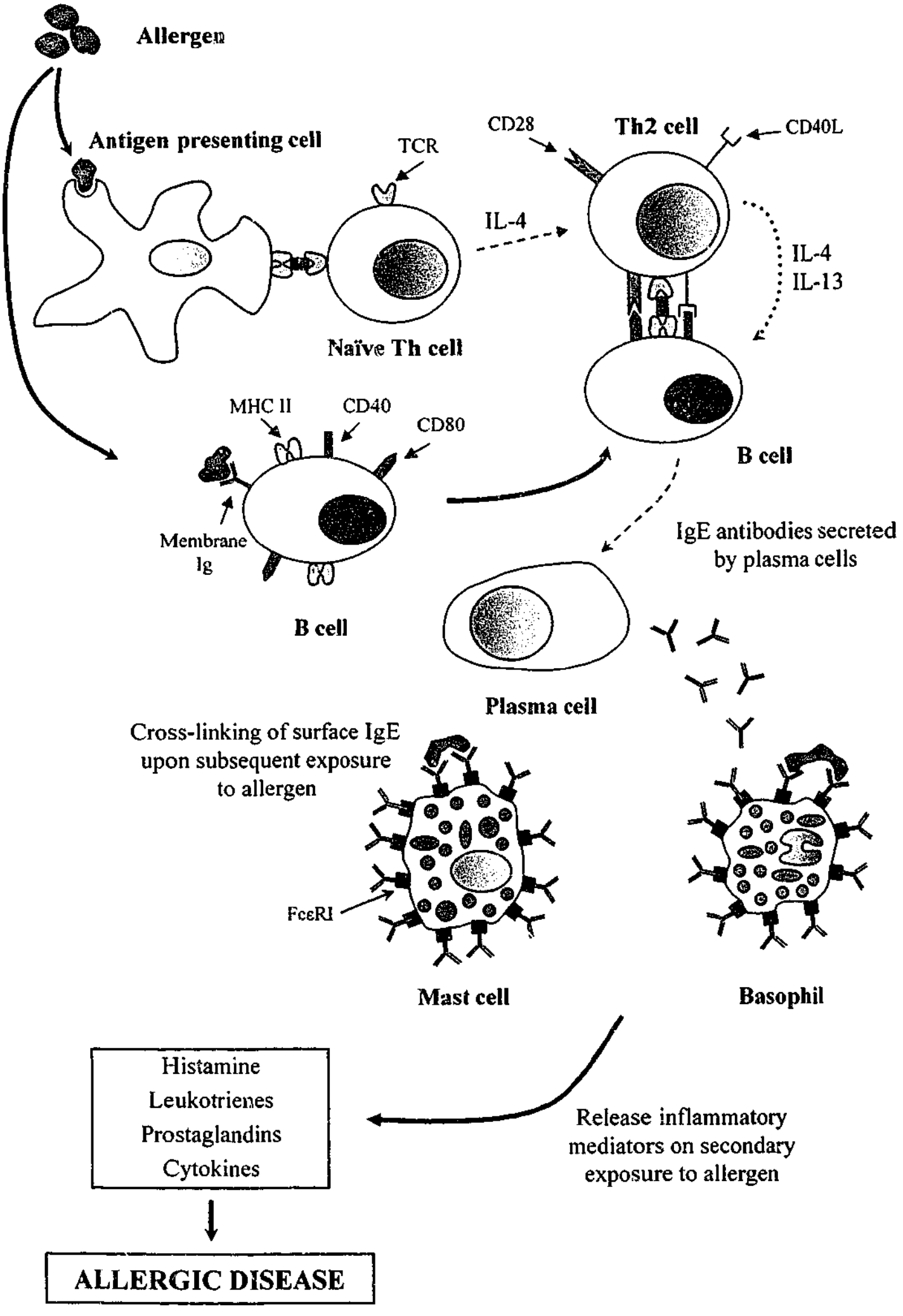
1.1 THE IMMUNE RESPONSE TO ALLERGENS

The exposure to common environmental allergens such as house dust mite, grass and tree pollen, animal dander and fungi can elicit potentially pathogenic inflammatory responses in genetically pre-disposed individuals. This immune response, termed a type I hypersensitivity or allergic reaction, can affect up to 20-30% of the population (Bell and O'Hehir, 1996) and largely defines atopy or the atopic state which is the ability to produce specific immunoglobulin E (IgE) antibodies to common environmental allergens. This production of allergen-specific IgE antibodies triggers a cascade of immunological events, leading to clinical symptoms associated with allergic disease.

The initial exposure of an individual's immune system to an allergen occurs at the mucosal surfaces. This first encounter with an allergen, the sensitisation phase, results in the uptake of the allergen by antigen presenting cells (APCs) such as dendritic cells and macrophages as shown in Figure 1.1 (von Bubnoff *et al.*, 2001). The APCs migrate to the regional lymph node where the allergen is processed into peptide fragments and presented on the cell surface in the context of the class II Major Histocompatibility Complex (MHC) molecules (Figure 1.1). These peptides are presented to naïve T helper (Th) cells via MHC-T cell receptor (TCR) interaction, resulting in priming and

Figure 1.1 The major cellular interactions during the allergic immune response

APCs such as dendritic cells (DCs) encounter allergens at epithelial surfaces such as the gastric mucosa and migrate to the regional lymphoid tissue where DC maturation occurs. Mature DCs subsequently present allergen peptide-MHC complexes to naïve T cells together with the co-stimulatory signals required for activation. Activated Th2 cells recognise allergen peptide-MHC complexes on the surface of B cells, inducing immunoglobulin isotype switching and subsequent production of IgE antibodies by plasma cells. Secreted IgE antibodies bind to FcεRI receptors on effector cells such as mast cells and basophils which become sensitised. Upon secondary encounter with the same allergen, mast cells and basophils release inflammatory mediators which induce the symptoms associated with allergic disease. Image adapted from Burton (2000).


Allergen
Antigen presenting cell
TCR
CD28
Th2 cell
CD40L
IL-4
IL-4
IL-13
Naïve Th cell
MHC II
CD40
CD80
B cell
Membrane Ig
B cell
IgE antibodies secreted by plasma cells
Plasma cell
Cross-linking of surface IgE upon subsequent exposure to allergen
FcεRI
Mast cell
Basophil
Histamine
Leukotrienes
Prostaglandins
Cytokines
Release inflammatory mediators on secondary exposure to allergen
ALLERGIC DISEASE

activation, triggering the humoral and cellular events associated with allergic inflammation.

In allergic individuals, the activation of Th cells results in the secretion of cytokines that stimulate B cells to synthesise IgE antibodies specific to the allergen. Th cells are classified into two functionally distinct subgroups: Th1 and Th2 cells. These cells are derived from a common precursor cell but are defined by the pattern of cytokine secretion, as shown in Figure 1.2. Among a number of cytokines, Th1 cells mainly secrete interleukin-2 (IL-2), interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and lymphotoxin (LT) which promote the production of IgG2a antibodies in mice or IgG3 antibodies in humans. In contrast, Th2 cells secrete interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-9 (IL-9) and interleukin-13 (IL-13). Th2 cells play a pivotal role in the allergic response as the activation of these cells leads to the secretion of IL-4 and IL-13 which drive B cell differentiation into IgE-secreting plasma cells.

Induction of IgE synthesis by B cells also requires the B cell to internalise, process and present the allergen to Th cells. Allergen uptake occurs via its membrane bound immunoglobulin antigen receptor (BcR) and antigen presentation as peptides to Th cells also occurs in the context of MHC Class II complex. This MHC class II-TCR interaction between B cell and T cell results in the engagement of cell surface accessory molecules such as CD80/CD86 on the surface of the B cell with CD28 on the T cell or CD40 on the B cell and the T cell surface molecule, CD154, stabilising the cell-to-cell interaction as well as further augmenting the IgE-mediated immune response (Figure 1.1).

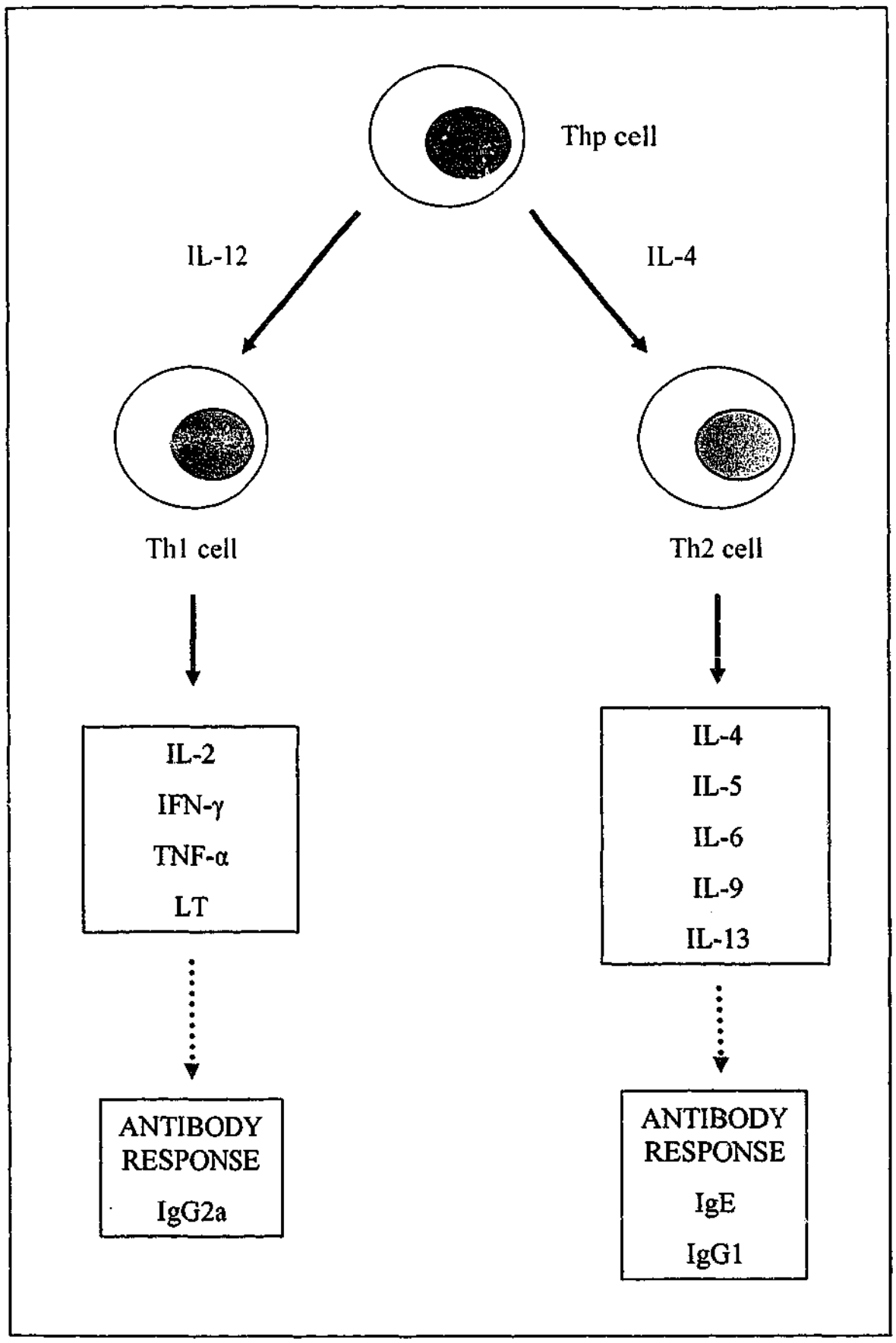


Figure 1.2 Th1 and Th2 cell cytokine secretion

Precursor cells (Thp) differentiate into two distinct subgroups: Th1 and Th2. These two cell types are defined by the pattern of cytokine secretion which, in turn, determines the antibody response. During an allergic response, Th2 cells become activated and secrete cytokines, in particular IL-4 and IL-13, which drive B cells to differentiate into IgE-secreting plasma cells. (Adapted from Corry and Kheradmand, 1999)

Following secretion by B cells, IgE antibodies are bound by high affinity surface IgE receptors (FcεR^1) present on inflammatory cells such as mast cells and basophils, thus sensitising these cells (Figure 1.1). IgE antibodies can also be bound by a second major IgE receptor, CD23 or FcεRII, which is present on B cells, monocytes, platelets and eosinophils although this interaction is of a lower affinity compared with FcεRI. However, during the intermediate phase of the allergic immune response when IgE levels are high, IgE antibodies can bind significantly to CD23 on APCs and subsequently present allergens to Th cells as well as stimulate the production of IgE antibodies by B cells thus further promoting the allergic response.

In allergic individuals, subsequent exposure to the allergen induces inflammatory reactions largely governed by mast cells, basophils and eosinophils. Mast cells are located in connective tissues surrounding blood and lymphatic vessels, nerves and under epithelial membranes where maturation occurs under the influence of stem cell factor (SCF) and Th2 cytokines such as IL-4 and IL-5 (Prussin and Metcalfe, 2003). Basophils and eosinophils originate from the bone marrow where they undergo differentiation and maturation which are predominantly driven by IL-3 and IL-5, respectively (Kepley *et al.*, 1998, Gleich, 2000). Following secondary allergen exposure, adjacent IgE antibodies bound by FcεRI on the surface of mast cells and basophils are cross-linked by the allergen, resulting in an increase in intracellular levels of Ca^{2-} ions (Figure 1.1). These cells degranulate and release inflammatory mediators such as histamine, prostaglandin, leukotrienes, heparin and platelet-activating factor which increase vascular permeability, bronchodilation and mucus secretion. Further cytokine production also occurs during this phase, most notably IL-4 and IL-13 by mast cells and basophils, further augmenting Th2 cell differentiation and IgE synthesis. Mast cells also produce TNF-α, IL-5 and chemokines which result in the activation and

recruitment of eosinophils to the site of inflammation. Eosinophils release mediators such as major basic protein (MBP), eosinophil peroxidase, eosinophil cationic protein and eosinophil-derived neurotoxin which cause mucosal inflammation and subsequent bronchial hyperresponsiveness (Gleich, 2000). These mechanisms ultimately lead to the manifestation of clinical symptoms commonly associated with allergy, some of which include urticaria, asthma, pruritus and, in severe cases, anaphylaxis.

1.2 PEANUT AND TREE NUT ALLERGY

Food allergies are a common cause of allergen-induced anaphylaxis. Peanuts and tree nuts account for the majority of food-related anaphylaxis in children and adolescents (Sampson *et al.*, 1992). Some tree nuts that have been shown to be allergenic include almond, Brazil nut, cashew, hazelnut, macadamia, walnut, and pine nuts and the taxonomic classification of these nut types as well as peanut is outlined in Table 1.1. Peanut allergy is more prevalent than tree nut allergy although co-sensitisation to both is common. The reported prevalence of this type of food allergy within the general population varies widely. In a randomly selected population, it has been estimated that approximately 1.1% of the general population is affected by peanut and/or tree nut allergy (Sicherer *et al.*, 1999). Tariq and colleagues (1996) reported a sensitisation rate of 1.2%, with 0.5% of these subjects experiencing an allergic reaction upon subsequent ingestion of peanut and tree nuts (Tariq *et al.*, 1996). However, studies analysing patients from specialist allergy clinics have reported a prevalence rate as high as 34-40% (Ewan, 1996, Sicherer *et al.*, 1998). It is apparent that increasing consumption of

Table 1.1 Taxonomic classification of peanut and tree nut plants (USDA, NRCS. 2001. The PLANTS Database, Version 3.1. National Plant Data Centre).

KINGDOM: Plantae (Plants)	SUB-KINGDOM: Tracheobionta (Vascular plants)	SUPERDIVISION: Spermatophyta (Seed plants)	DIVISION: Magnoliophyta (Flowering plants)	CLASS: Magnoliopsida (Dicotyledons)	SUB-CLASS	ORDER	FAMILY	GENUS, SPECIES (COMMON NAME)
					Rosidae	Fabales	Fabaceae (Pea family)	*Arachis hypogaea* L. **(Peanut)**
						Proteales	Proteaceae (Protea family)	*Macadamia integrifolia* Maiden & Betche. **(Macadamia nut)**
						Rosales	Rosaceae (Rose family)	*Ivesia pityocharis* Ertter **(Pine nut)** *Prunus dulcis* (P.Mill) D.A. Webber **(Almond)**
						Sapindales	Anacardiaceae (Sumac family)	*Anacardium occidentale* L. **(Cashew)** *Pistacia vera* L. **(Pistachio nut)**
					Hamamelidae	Fagales	Betulaceae (Birch family)	*Corylus avellana* L. **(Hazelnut)**
							Fagaceae (Beech family)	*Castanea sativa* P. Mill. **(European chestnut)** *Quercus ilex* L. **(Acorn nut)**
						Juglandales	Juglandaceae (Walnut family)	*Carya illinoinensis* (Wagenh.) K. Koch **(Pecan)**
								Juglans regia L. **(English walnut)**
					Dilleniidae	Lecythidales	Lecythidaceae (Brazil nut family)	*Bertholletia excelsa* Humb. & Bonpl. **(Brazil nut)**

peanuts over the years has led to an increase in the prevalence of peanut allergy in the general population (Grundy *et al.*, 2002).

1.2.1 Clinical features and diagnosis of peanut and tree nut allergy

Peanut and tree nut allergy generally develop early in life and are commonly associated with other atopic disorders such as asthma, eczema and rhinitis. Sensitisation usually occurs through the consumption of foods such as peanut butter, although exposure can also occur *in utero* (Kaufman, 1971) or through breastfeeding as peanut allergens have been detected in the breast milk of lactating mothers (Vadas *et al.*, 2001). Allergic symptoms following the ingestion of peanut and tree nuts can occur from within minutes to a few hours and these can manifest as oral pruritus, nausea, vomiting, urticaria, angioedema and tightening of the airways (Sampson, 2002). Anaphylaxis can occur in severe cases which can prove fatal without the administration of adrenaline. A characteristic of peanut and tree nut allergy is also its lifelong persistence in allergic individuals, unlike other food allergies which tend to resolve during childhood (Bock and Atkins, 1989). Thus, lifelong vigilance is required for most peanut and tree nut allergic patients.

The diagnosis of peanut and tree nut allergy is usually based on the patient's clinical history. Laboratory tests such as the radioallergosorbent test (RAST) can be utilised to detect and measure specific IgE to peanut and tree nut extracts. Together, positive clinical history and RAST scores provide sufficient information to diagnose this type of food allergy. Oral food challenge is considered the 'gold standard' for diagnosing immediate food hypersensitivity however this is not without risk since such challenges can lead to severe anaphylactic reactions. Skin prick tests (SPTs) also carry the same

risks although along with food challenges can be used as confirmatory tests in circumstances where patient history and RAST scores are both negative (Hourihane, 1997).

1.2.2 Treatment and management of peanut and tree nut allergy

At present, there is no available form of treatment for peanut and tree nut allergy. This type of food allergy can only be managed through avoidance of the offending food. This can be difficult for most patients as peanuts and tree nuts are increasingly being used as additives in different foods and the risks are further enhanced by the inadequate labelling of some foods. Contamination of foods with peanut and tree nut proteins can also occur inadvertently during the manufacturing process, posing the threat of 'hidden allergens' within these foods. Foods cooked using 'gourmet' oils such as peanut, walnut, Brazil, hazelnut, almond and sesame seed oil can also elicit an allergic reaction in sensitive individuals. Sesame seed oil has long been known to induce systemic reactions in sesame seed allergic patients as it contains allergenic proteins (Chiu and Haydik, 1991). Similarly, crude peanut oil has been shown to contain detectable amounts of peanut proteins sufficient to bind IgE from peanut allergic patients (Hoffmann and Collins-Williams, 1994, Hourihane *et al.*, 1997) although refined peanut oil has been proven to be non-allergenic (Taylor *et al.*, 1981). Given the risks associated with peanut and tree nut allergy, it is not surprising that accidental exposures are quite common for many patients, further emphasising the need to maintain vigilance.

Allergen-specific immunotherapy or desensitisation, commonly used in house dust mite and grass pollen allergy, is not available for food allergy. This form of therapy involves

injecting increasing doses of the allergen extract into the patient, resulting in clinical 'tolerance' upon subsequent exposure to the same allergen partly due to a change in cytokine profile of T cells whereby there is a change from Th2 type polarisation to Th1 type polarisation (Rolland *et al.*, 2000). Attempts have been made to desensitise peanut allergic patients and one particular study by Oppenheimer *et al.* (1992) utilised a rush immunotherapy protocol where frequent subcutaneous injections of peanut extract were administered to patients followed by weekly maintenance doses. Three patients recorded a decrease in symptoms following a double-blind, placebo controlled food challenge (DBPCFC) and were successfully desensitised (Oppenheimer *et al.*, 1992). However, some patients experienced systemic reactions following the injections and the study was prematurely terminated after one participant suffered a fatal anaphylactic reaction.

One therapeutic strategy that has been increasingly put forward as a possible treatment for peanut and tree nut allergy is the use of recombinant allergen-based vaccines (Kraft *et al.*, 1998, Valenta *et al.*, 1998). The isolation and cloning of the cDNA of peanut and tree nut allergens and subsequent expression of the recombinant protein has allowed researchers to determine the role each plays in the manifestation of peanut and tree nut allergy. Recombinant techniques can also be used to generate recombinant allergens with a reduced allergenic potential that can subsequently be used for immunotherapy without the IgE-mediated side effects. At present, several peanut and tree nut allergens have been identified and characterised and a number of these have also been cloned. The following discussion will examine these allergens in detail, including aspects that will determine the involvement of these allergens in the manifestation of peanut and tree nut allergic disease.

1.3 ALLERGEN NOMENCLATURE

Allergens are designated according to the accepted taxonomic name of the source in the following format: the first three letters of the genus, followed by the first letter of the species and an Arabic number which is assigned in the order of identification (King *et al.*, 1994). The use of this system requires investigators to have obtained the complete or partial cDNA sequence of the identified allergen and establish the frequency of reactivity in a reasonable sized population to determine if it is a major (>50% IgE reactivity in patients) or minor allergen (<50% IgE reactivity in patients).

1.4 IDENTIFICATION AND CHARACTERISATION OF PEANUT ALLERGENS

Early studies on the identification and characterisation of peanut (*Arachis hypogaea*) allergens focused on the fractionation of crude peanut extract and identification of IgE-reactive fractions. One of these studies was conducted by Sachs *et al.* (1981) and identified an IgE-reactive acidic glycoprotein, Peanut-I, following fractionation of crude peanut extract by anion exchange chromatography. The allergenicity of Peanut-I was confirmed by *in vivo* tests such as skin prick testing and leukocyte histamine release, with positive responses obtained in all three tested peanut-sensitive individuals (Sachs *et al.*, 1981). RAST inhibition studies demonstrated that this protein did not account for all of the allergenic activity of peanut extract. This was confirmed in a study conducted by Barnett *et al.* (1983) where it was found raw peanut extract contained 16 IgE-binding

proteins whereas roasted peanut extract contained 7 IgE-binding proteins (Barnett *et al.*, 1983). Subsequent studies sought to identify and characterise these allergenic proteins.

1.4.1 Ara h 1

Using pooled serum from 9 patients with atopic dermatitis, positive skin prick test to peanut and either a positive DBPCFC to peanut or positive history of peanut anaphylaxis, Burks *et al.* (1991) identified a major peanut allergen, following fractionation of crude peanut extract by anion-exchange chromatography. This allergen, designated Ara h 1, is a glycoprotein and its molecular properties are outlined in Table 1.2. Ara h 1 showed 100% IgE reactivity in a subgroup of 6 peanut allergic patients by ELISA (Burks *et al.*, 1991). The gene encoding this protein was amplified from a peanut cDNA library using primers based on the amino acid sequence of one of three tryptic peptides derived from purified natural Ara h 1 protein (Burks *et al.*, 1995). The resultant clone encoded a protein with a molecular weight of ~68 kDa with amino acid sequence comparisons showing significant sequence similarity between Ara h 1 and a class of seed storage proteins known as vicilins (Table 1.2). More specifically, there was 48% identity between Ara h 1 and pea vicilins.

The expression of recombinant Ara h 1 (rAra h 1) and subsequent comparison of IgE reactivity with natural Ara h 1 was conducted by Burks *et al.* (1995) using IgE immunoblotting experiments. IgE binding to natural Ara h 1 was demonstrated in 17 out of 18 peanut allergic patients (94%) with these patients also showing some level of IgE binding to the recombinant form. Kleber-Janke *et al.* (1999) similarly demonstrated IgE binding to rAra h 1 in 10/14 (65%) peanut allergic patients. However, in a study by de Jong *et al.* (1998), only 5/14 (35%) of peanut allergic subjects (selected on the basis

Table 1.2 Molecular characteristics and allergenicity of cloned peanut allergens

	Peanut allergens							
	Ara h 1	**Ara h 2**	**Ara h 3**	**Ara h 4**	**Ara h 5**	**Ara h 6**	**Ara h 7**	**Peanut oleosin**
Length (bp)	1972[b]	741[f]	1530[g]	1170[d]	743[d]	627[d]	637[d]	531[h]
AA residues	626[b]	157[f]	510[g]	315[d]	131[d]	124[d]	135[d]	176[h]
M_r (kDa)	63.5[a], 68[b]	17[c], 17.5[f], 17.3[d]	57[g]	35.9[d]	14[d]	14.5[d]	15.8[d]	17-18[h]
AA identity (%)	48[b]	40[f]	62-72[g]	56[d]	83[d]	39[d]	39[d]	41
Protein family	Vicilin[b]	Conglutin[f]	Glycinin[g] Legumin[g]	Glycinin[d]	Profilin[d]	Conglutin[d]	Conglutin[d]	Oleosin[h]
Allergenicity	100%[a], 94%[b], 65%[d], 35%[i]	100%[e], 85%[d]	44%[g]	53%[d]	13%[d]	38%[d]	43%[d]	21%[h]
Genbank Accession no.	L34402	L77197	AF093541	AF086821	AF059616	AF092846	AF091737	AF325917 AF325918

a) Burks *et al.* (1991); b) Burks *et al.* (1995); c) Burks *et al.* (1997); d) Kleber-Janke *et al.* (1999); e) Burks *et al.* (1992); f) Stanley *et al.* (1997); g) Rabjohn *et al.* (1999); h) Pons *et al.* (2002); i) de Jong *et al.* (1998)

of positive DBPCFC, SPT and RAST to peanut) had specific IgE antibodies to natural Ara h 1 as demonstrated by immunoblotting of crude peanut extract (de Jong *et al.*, 1998). Consequently, there is some contention on whether Ara h 1 should be classified as a major peanut allergen. Nevertheless, using synthetic peptides based on the deduced amino acid sequence of Ara h 1, Burks *et al.* (1997) attempted to identify some of the IgE binding sites on the Ara h 1 protein. At least 23 linear IgE-binding epitopes distributed throughout the protein were identified using serum IgE from peanut allergic patients; however, only 4 were classified as immunodominant. Mutational analysis of the immunodominant epitopes revealed that each epitope can be rendered non-IgE reactive by the substitution of an alanine for a single amino acid residue (Burks *et al.*, 1997). It is, however, unclear whether any of the above identified linear IgE-binding epitopes occur within the native conformation of Ara h 1 which is essential given that antigen-antibody interactions usually involve conformational epitopes (Janeway and Travers, 1997).

Structural studies have allowed the identification of the position of the Ara h 1 IgE binding epitopes within its native conformation. This was conducted using homology-based modelling whereby the tertiary structure of Ara h 1 was generated using the X-ray crystal structure of phaseolin which is highly homologous to Ara h 1 at the primary amino acid sequence level (Shin *et al.*, 1998). Of the 35 amino acid residues identified as critical for IgE binding in the above study, 25 were found to be evenly distributed on the surface of the molecule, clustered in two regions, as depicted in Figure 1.3 (Shin *et al.*, 1998, Bannon *et al.*, 1999). The presentation of clustered epitopes to mast cells and basophils may result in a more efficient release of mediators which may be responsible for the severe clinical symptoms associated with peanut allergy. Ara h 1 was also found to be capable of higher order aggregation, forming a stable trimeric complex through

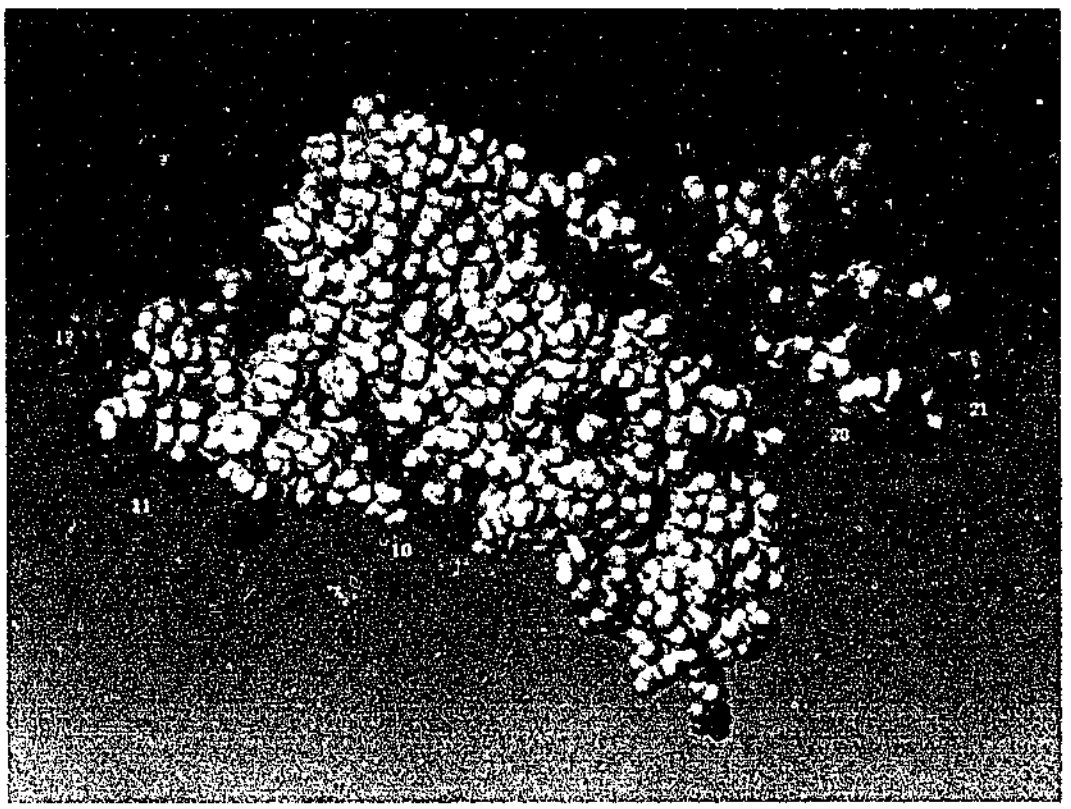


Figure 1.3 IgE-binding epitopes of Ara h 1

A space-filled model of the Ara h 1 monomer shows the location of the IgE-binding epitopes (red areas) including the residues which were determined to be critical for IgE binding (yellow atoms). Image from Shin *et al.* (1998).

interactions between hydrophobic amino acid residues (Bannon *et al.*, 1999, Maleki *et al.*, 2000b). Structural analysis of the Ara h 1 monomer showed that each end of the Ara h 1 protein contains hydrophobic regions which act as contact points for trimer formation with most of the IgE binding epitopes clustered around these contact points (Figure 1.4).

Given the structural stability and compact nature of the Ara h 1 molecule, some studies have focused on the effects of heating and digestion on the overall structure and allergenicity of this protein. Koppelman *et al.* (1999) demonstrated using SDS-PAGE analysis of heated Ara h 1 that this protein was still capable of forming stable dimers, trimers and larger complexes upon heating. It was shown that IgE antibodies from peanut allergic individuals bind with high affinity to natural Ara h 1 and this interaction is not significantly affected when Ara h 1 is isolated from peanuts that have been heated at various temperatures (Koppelman *et al.*, 1999). Experiments involving the digestion of Ara h 1 showed that treatment of this protein with gastrointestinal enzymes such as pepsin, trypsin and chymotrypsin produced large proteolytic fragments which were still recognised by serum IgE from peanut allergic individuals. This indicates that large, proteolytic fragments of Ara h 1 contain multiple-IgE binding epitopes that can survive the digestive processes (Maleki *et al.*, 2000b). The resistance of Ara h 1 to degradation following heating and treatment with digestive enzymes may be related to its stable, homotrimeric structure. This monomer-monomer interaction reduces the accessibility of catalytic sites within the protein, allowing Ara h 1 to survive as an intact protein during food processing or passage along the digestive tract, thus contributing to its potency as an allergen.

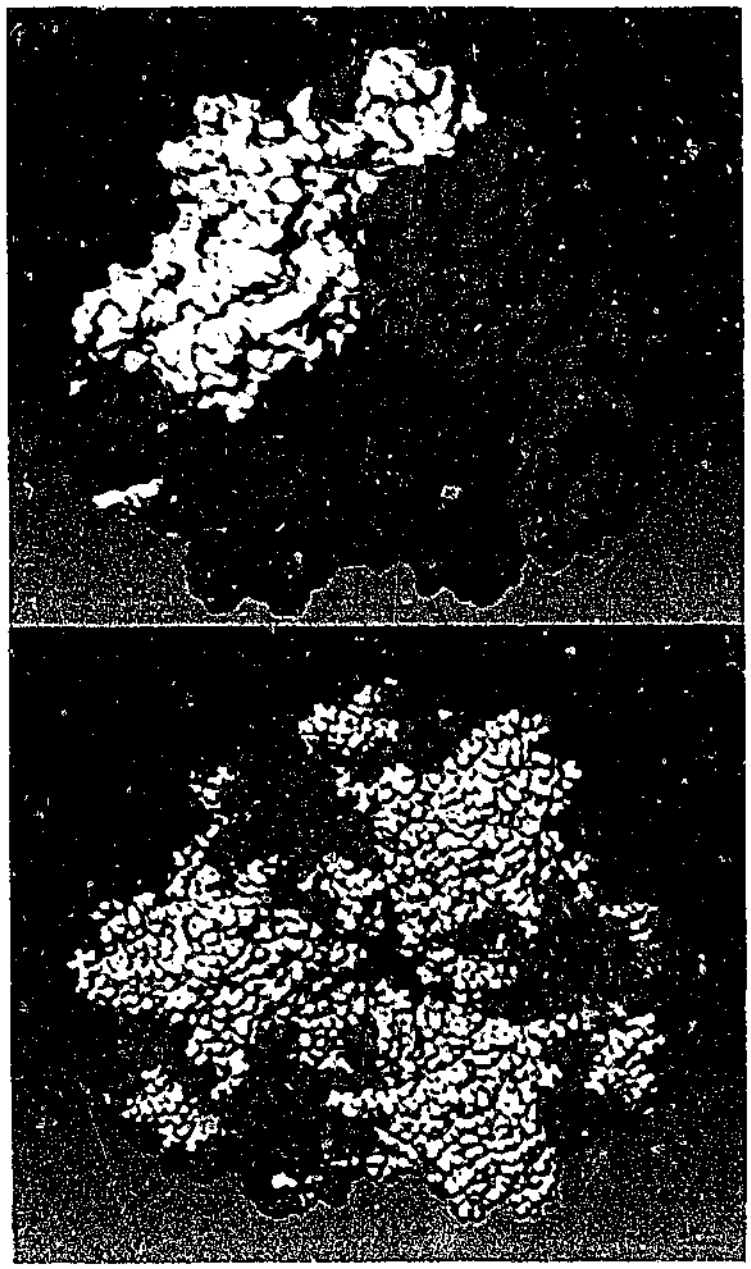


Figure 1.4 Molecular model of the Ara h 1 trimer

Solid Connolly surface depiction of the Ara h 1 trimer shows overlapping α helical bundles at the end of each monomer (top panel). A space-filled model (bottom panel) shows the IgE-binding epitopes (red) which are located in areas close to monomer-monomer contact. Image from Maleki *et al.* (2000).

1.4.2 Ara h 2

A second major peanut allergen has also been identified, using sera from patients with atopic dermatitis and a positive peanut food challenge. Similar to Ara h 1, Ara h 2 was identified following fractionation of crude peanut extract by anion exchange chromatography (Burks *et al.*, 1992). This protein, upon SDS-PAGE and immunoblotting was found to migrate as two IgE-reactive bands with the same *N*-terminal sequence (de Jong *et al.*, 1998) and a mean molecular weight of 17 kDa (Burks *et al.*, 1992). Two dimensional gel electrophoresis of natural Ara h 2 also suggested the presence of isoforms, which was later confirmed by comparisons of recombinant Ara h 2 (rAra h 2) clones (Viquez *et al.*, 2001) and the isolation of cDNA encoding two Ara h 2 isoforms (Chatel *et al.*, 2003). Six peanut allergic patients were used in the above study by Burks *et al.* (1992) and each patient showed IgE binding to this protein by ELISA. Pre-incubation of patient sera with increasing amounts of Ara h 2 inhibited IgE binding to crude peanut extract, suggesting that this allergen contributes significantly to the IgE binding capacity of crude peanut extract (Burks *et al.*, 1992).

Using the *N*-terminal sequence of purified natural Ara h 2, Stanley *et al.* (1997) designed oligonucleotide primers to PCR amplify the gene encoding this allergen from a peanut cDNA library. A 741 bp clone was identified (Table 1.2) which was capable of encoding a 17.5 kDa protein which is in agreement with the molecular weight of the natural form of Ara h 2. From GenBank, Swiss-Prot and EMBL database comparisons, the derived amino acid sequence of Ara h 2 showed significant sequence homology with seed storage proteins from different plant families (Stanley *et al.*, 1997). Ara h 2 was shown to have approximately 40% amino acid sequence identity with conglutin-δ, a sulphur-rich protein from the lupine seed (Gayler *et al.*, 1990). It was concluded that

Ara h 2 belongs to the conglutin family of seed storage proteins (Table 1.2). Other plant proteins such as 2S albumins and mabinlins were also shown to have some sequence homology with Ara h 2. Interestingly, Ara h 2 has 29% homology with α-amylase inhibitors which have been classified as major allergens in individuals who are hypersensitive to wheat proteins (Armentia *et al.*, 1993). Ara h 2 also shows sequence homology with trypsin inhibitors with a recent study by Maleki *et al.* (2003) demonstrating that this allergen acts as a weak trypsin inhibitor with increased activity following roasting, thus protecting itself from trypsin digestion. This inhibitory activity was also found to protect Ara h 1 from trypsin digestion (Maleki *et al.*, 2003). Incubation of Ara h 1 with trypsin in the presence of Ara h 2 purified from roasted peanut extract did not result in any protein degradation after 2 hours. Thus, it appears that the function of Ara h 2 as a trypsin-inhibitor, which becomes enhanced following thermal processing, may contribute to the allergenic properties of peanuts through increased resistance to digestive enzymes.

The use of overlapping synthetic 15-mer peptides allowed the identification of the linear IgE-binding epitopes of Ara h 2. Stanley *et al.* (1997) probed 19 overlapping synthetic peptides based on the deduced amino acid sequence of Ara h 2 with pooled serum from 15 peanut allergic patients. Using immunoblotting, 10 peptides were found to bind IgE with these peptides located on three regions distributed throughout the Ara h 2 amino acid sequence. The immunodominant epitopes of Ara h 2 were subsequently identified by individually probing each of the 10 IgE-binding peptides with serum IgE from 10 different peanut allergic patients. Three peptides (representing amino acid residues 27-36, 57-66 and 65-74 with respect to the Ara h 2 sequence) were found to bind IgE antibodies from all 10 patients. In addition, the majority of Ara h 2-specific IgE antibodies in these patients bound to the same 3 peptides, confirming that the

immunodominant epitopes of Ara h 2 are located within these amino acid sequences (Stanley *et al.*, 1997). However, the importance of these linear epitopes within the context of natural Ara h 2 is not known. Mutational studies have also shown that all of the Ara h 2 IgE-binding peptides can be rendered non-IgE binding by the substitution of an alanine for a single amino acid residue. However, there was no consensus on the type of amino acid that when mutated would lead to the abrogation of IgE reactivity. This information was subsequently used to produce hypoallergenic Ara h 2 mutants by PCR mutagenesis although only 75% efficacy was achieved, with 12 out of 16 peanut allergic patients tested showing diminished IgE reactivity to the modified allergen (Burks *et al.*, 1999). Whether conformational epitopes play a more crucial role in IgE binding to Ara h 2 than linear epitopes is not known although their importance may lie in the fact that only 75% efficacy was achieved when the linear epitopes were rendered non-IgE binding. Consequently, further studies are required to identify any conformational epitopes for Ara h 2 in order to provide avenues for the development of hypoallergenic variants of this major peanut allergen.

1.4.3 Ara h 3

A third peanut allergen has been identified and characterised although it has been classified as a minor allergen. Ara h 3 was initially identified by assessing the IgE reactivity of peanut proteins using sera from peanut allergic patients following adsorption of soy-specific antibodies (Eigenmann *et al.*, 1996). A 14 kDa protein was identified and purified, with primers based on the *N*-terminal sequence of this protein used to isolate the corresponding cDNA from a mature peanut library. The molecular characteristics of the resulting Ara h 3 clone are outlined in Table 1.2, with sequence comparisons indicating that this clone encoded an 11S seed storage protein (Rabjohn *et*

al., 1999). Ara h 3 also showed 62-72% amino acid sequence identity with glycinins and legumins from soybean (*Glycine max*) and pea (*Pisum sativum*), respectively. 11S seed storage proteins are initially synthesised as 60 kDa pre-proglobulins consisting of covalently linked acidic and basic polypeptides and are deposited in storage bodies before being cleaved by asparagine-dependent endopeptidase (Barton *et al.*, 1982, Ereken-Tumer *et al.*, 1982). The protein encoding this Ara h 3 cDNA was subsequently expressed in a bacterial system with an estimated molecular weight of ~57 kDa following SDS-PAGE, much larger than the original 14 kDa protein, suggesting that the smaller protein may be an NH_2-terminal breakdown product. The allergenicity of this recombinant Ara h 3 (rAra h 3) was assessed in a population consisting of 18 peanut allergic patients. Serum from 8 out of 18 patients (44%) were shown to contain IgE antibodies specific to this protein by immunoblotting, thus confirming its status as a minor allergen (Eigenmann *et al.*, 1996, Rabjohn *et al.*, 1999).

The mapping of some of the IgE-binding epitopes of Ara h 3, in particular the linear epitopes, was carried out using 63 overlapping synthetic 15-mer peptides comprising the entire primary amino acid sequence of Ara h 3 (Rabjohn *et al.*, 1999). These peptides were probed with pooled sera from peanut-sensitive patients previously shown to recognise Ara h 3. Four IgE-binding regions were identified throughout the Ara h 3 sequence which consisted of amino acid residues 21-55, 134-154, 231-269 and 271-328. The core epitope for each region was subsequently identified and peptides comprising these epitopes were probed individually with serum from eight peanut allergic individuals known to react with Ara h 3 to determine which, if any, were immunodominant. The results from this study indicate that epitope 3 (279-293), recognised by serum IgE from all 8 patients, was the immunodominant epitope among

this population of Ara h 3 allergic patients. In contrast, epitopes 1, 2 and 4 were only recognised by 25-38 % of the patients tested.

The same study also investigated the effect of mutations on the IgE-binding capacity of the four identified Ara h 3 epitopes. Single amino acid changes were made at each position on the epitopes to determine which amino acids were critical to IgE binding. In general, a reduction in the IgE-binding capacity of each epitope was achieved by substituting the central amino acids with an alanine residue.

In further studies, Rabjohn *et al.* (2002) utilised site-directed mutagenesis as a strategy to develop a hypoallergenic variant of rAra h 3. In this study, the 40 kDa acidic subunit of Ara h 3 containing the four identified Ara h 3 IgE-binding epitopes was expressed using a bacterial system with the critical residues for IgE binding targeted for point mutations by substitution with an alanine residue (Rabjohn *et al.*, 2002). The ability of this modified rAra h 3 protein to bind serum IgE was tested by immunoblotting using serum from 5 patients previously shown to react with the wild-type rAra h 3. All 5 patients showed diminished IgE reactivity to the modified protein in comparison to the wild-type protein. This was confirmed by inhibition immunoblotting whereby higher concentrations of modified rAra h 3 were required to achieve 50% inhibition of IgE binding to wild-type rAra h 3 (Rabjohn *et al.*, 2002).

Using T cell proliferation assays, the same study also investigated whether this rAra h 3 mutant retained the ability to stimulate T cells from peanut-sensitive individuals. Peripheral blood mononuclear cells from the same 5 patients from the previous experiments were stimulated with both wild-type and modified rAra h 3 and T cell proliferation was measured by thymidine incorporation. In general, the modified rAra h

3 was capable of stimulating T cell proliferation to the same degree as the wild-type form in 4 out of 5 patients (Rabjohn *et al.*, 2002). The results from this study suggest that this modified rAra h 3 is a good candidate for use as an immunotherapeutic agent although the retention of some degree of IgE reactivity amongst the patients tested may still pose serious risks for the wider peanut allergic population which most likely has varying sensitivities to this allergen.

1.4.4 Ara h 4, Ara h 5, Ara h 6 and Ara h 7

To date, most of the studies investigating peanut allergens have focused on Ara h 1, Ara h 2 and Ara h 3. However, examination of the IgE-binding profile of crude peanut extract indicates the existence of other IgE-binding proteins (Barnett *et al.*, 1983, de Jong *et al.*, 1998). Kleber-Janke *et al.* (1999) used phage display technology to identify other peanut allergens. Peanut cDNA expression products were displayed on the phage surface and serum from patients sensitised to peanut were used to isolate phages displaying IgE-binding peanut proteins. In total, six IgE-reactive proteins were isolated using this strategy and these included Ara h 1 and Ara h 2 (Kleber-Janke *et al.*, 1999). The four new allergens identified were named Ara h 4, Ara h 5, Ara h 6 and Ara h 7, all of which were previously unknown IgE-reactive peanut proteins and their molecular characteristics are outlined in Table 1.2. Sequence comparisons of Ara h 4 revealed significant amino acid identity (56%) with the glycinin family of seed storage proteins. The Ara h 5 deduced amino acid sequence showed significant identity (83%) with plant profilins, well-known panallergens present in pollen and fruits (Petersen *et al.*, 1996, Reindl *et al.*, 2002). Ara h 6 and 7 have 35% amino acid sequence identity with each other although this was not sufficient to show that they are isoallergens. Both proteins showed amino acid sequence identity with the conglutin family of seed storage proteins

and interestingly, both Ara h 6 and 7 showed 59% and 35% amino acid identity, respectively with the major peanut allergen, Ara h 2, which is also a member of the conglutin seed storage protein family (Kleber-Janke *et al.*, 1999). Alignment of the deduced amino acid sequences of Ara h 2, Ara h 6 and Ara h 7 showed conserved cysteine residues and regions characteristic of the conglutin δ protein.

The immunological relevance of all the recombinant peanut allergens isolated by Kleber-Janke *et al.* (1999) was assessed by examining the frequency of IgE reactivity in a population of 40 peanut allergic patients through Western immunoblotting. This data is summarised in Table 1.2. The status of Ara h 1 and Ara h 2 as major peanut allergens was confirmed with 65% and 85% of peanut allergic subjects showing IgE reactivity to these proteins, respectively. Ara h 4, Ara h 6 and Ara h 7 also showed a high frequency of IgE binding, ranging from 38-53%. In contrast, only 2 out of 40 (13%) patients had serum IgE to profilin-related allergen, Ara h 5, which may indicate that peanut profilin plays a minor role in the sensitisation of peanut allergic individuals.

1.4.5 Peanut oleosin

More recently, a minor peanut allergen has been identified which is involved in the formation of peanut oil bodies. Pons *et al.* (2002) cloned the recombinant form of peanut oleosin from peanut cDNA and its molecular characteristics are summarised in Table 1.2. The encoded protein was expressed and purified and the allergenicity of both the natural and recombinant forms was evaluated. Using IgE radioimmunoassays, only 3 out of 14 peanut allergic patients (21%) were positive for IgE antibodies to peanut oleosin, classifying this protein as a minor peanut allergen (Pons *et al.*, 2002). It was suggested that this allergen may be responsible for IgE-mediated reactions in peanut

allergic individuals exposed to peanut oil, a phenomenon that has been previously reported (Olszewski *et al.*, 1998).

From the preceding discussion, it can be seen that although a number of peanut allergens have been identified, only a few of these have been well characterised. To date, the crystal structure of only one peanut allergen has been resolved, namely Ara h 1, which was achieved through homology-based modelling. Information regarding IgE-binding epitopes is only available for Ara h 1, Ara h 2 and Ara h 3 and the majority of this is limited to linear epitopes, with the exception of Ara h 1. Further structural and epitope mapping studies are required to characterise the other previously identified peanut allergens. Such information may be useful in determining the role each allergen plays in the sensitisation of peanut allergic individuals.

1.5 IDENTIFICATION AND CHARACTERISATION OF TREE NUT ALLERGENS

The increasing prevalence of tree nut allergy and apparent clinical association with peanut allergy has led to the identification and characterisation of various tree nut allergens. Although the occurrence of tree nut allergy in the general population is as frequent as peanut allergy and clinical characteristics are often shared between the two types of allergy, it is only in recent years that information about tree nut allergens has become available.

1.5.1 Brazil nut allergens

One of the most widely studied causes of tree nut allergy is Brazil nut (*Bertholletia excelsa*). Immunoblotting experiments using serum from Brazil nut allergic individuals show the existence of several IgE-reactive proteins (Arshad *et al.*, 1991). Two Brazil nut allergens have so far been cloned, Ber e 1 and Ber e 2. Ber e 2 was cloned and classified as an 11S legumin protein (Table 1.3) but its allergenic properties have not yet been fully characterised. In contrast, Ber e 1 encodes a methionine-rich 2S albumin seed storage protein precursor (Table 1.4) (Ampe *et al.*, 1986, Gander *et al.*, 1991) that was originally examined because of its sulphur-rich amino acid content (3% cysteine and 18% methionine). This protein was subsequently targeted for expression in methionine-deficient foods such as soybeans to improve its nutritional quality (Nordlee *et al.*, 1996, Bartolome *et al.*, 1997).

Because Brazil nut has been previously shown to be an allergenic food, Nordlee *et al.* (1996) investigated the allergenicity of the 2S albumin protein from Brazil nut and in particular, whether expression of this protein in transgenic soybeans resulted in IgE reactivity among individuals with known Brazil nut allergy. Using competitive IgE-binding assays, it was found that transgenic soybean extracts containing 2S albumin competed effectively with Brazil nut proteins bound to a solid phase for serum IgE from Brazil nut allergic individuals, with the degree of inhibition similar to that obtained with raw Brazil nut extract (Nordlee *et al.*, 1996). Immunoblotting studies also showed that serum IgE from 7 out of 9 Brazil nut allergic individuals bound to a protein present in transgenic soybean extract, but not in non-transgenic soybean extract, that had the same molecular weight as 2S albumin (9 kDa). More significantly, three patients with a history of Brazil nut allergy but no history of soybean allergy showed positive results

Table 1.3 Characteristics of tree nut proteins that have been identified as allergenic

	Name	Molecular weight (kDa)	Function	Accession no.	References
Almond	Almond Major Protein (AMP; amandin)	63-65	seed storage protein		Roux *et al.* (1999) Roux *et al.* (2001)
	2S albumin	12	seed storage protein		Poltronieri *et al.* (2002)
	conglutin γ	45	seed storage protein		Poltronieri *et al.* (2002)
Brazil nut	12S globulin	20-30	seed storage protein		Bartolome *et al.* (1997) Pastorello *et al.* (1998)
	11S legumin **(Ber e 2)**	29	seed storage protein	AY221641	Beyer *et al.*, unpublished
Cashew	2S albumin **(Ana o 3)**		seed storage protein	AY081853	Wang *et al.*, unpublished
Hazelnut	profilin **(Cor a 2)**	14		AF327622*	Hirschwehr *et al.* (1992) *Luttkopff *et al.*, unpublished
	lipid transfer protein **(Cor a 8)**	9		AF329829*	*Schocker *et al.*, unpublished Pastorello *et al.* (2002)
	vicilin **(Cor a 11)**	47-48	seed storage protein	AF441864*	*Lauer *et al.*, unpublished Pastorello *et al.* (2002)
	legumin	35	seed storage protein		Pastorello *et al.* (2002)
	2S albumin	32	seed storage protein		Pastorello *et al.* (2002)
Walnut	lipid transfer protein **(Jug r 3)**	9			Pastorello, unpublished
	legumin **(Jug r 4)**		seed storage protein		Teuber *et al.* (2003)

Information extracted, in part, from the International Union of Immunological Societies, Allergen Nomenclature Sub-committee website, http://www.allergen.org/List.htm

Table 1.4 Molecular characteristics and allergenicity of cloned tree nut allergens

	Tree nut allergens						
	Ber e 1	**Jug r 1**	**Jug r 2**	**Ana o 1**	**Ana o 2**	**Cor a 1.04**	**Cor a 9**
Source	Brazil nut	Walnut	Walnut	Cashew	Cashew	Hazelnut	Hazelnut
Length (bp)	1028[b]	660[d]	2057[e]	1781[f]	1375[g]	480[h]	1767[i]
AA residues	154[b]	139[d]	593[e]	540[f]	457[g]	160[h]	650[i]
MW (kDa)	9[c]	15-16[d]	66[e]	65[f]	52[g]	17.4[h]	59[i]
Protein family	2S albumin[a,b]	2S albumin[d]	Vicilin[e]	Vicilin[f]	Legumin[g]	Stress[h]	11S globulin[i]
AA identity (%)	25-33[b]	46[d]	37-51[e]	33-43[f]	50-58[g]	85[h]	36-41[i]
Allergenicity	100%[c]	75%[d]	60%[e]	50%[f]	62%[g]	74-95%[h]	86%[i]
Genbank Accession no.	X54491	U66866	AF066055	AF395893 AF395894	AF453947	AF136945 AF323973 AF323974 AF323975	AF449424

a) Ampe *et al.* (1986); b) Gander *et al.* (1991); c) Pastorello *et al.* (1998); d) Teuber *et al.* (1998); e) Teuber *et al.*(1999); f) Wang *et al.* (2002); g) Wang *et al.* (2003); h) Luttkopf *et al.* (2002); i) Beyer *et al.* (2002)

from skin-prick tests using the transgenic soybean extract. Consequently, this landmark study by Nordlee *et al.* (1996) not only showed that the Brazil nut 2S albumin protein was most likely a major allergen but also demonstrated the risks associated with genetically modified foods whereby potential food allergens may inadvertently be used to improve the nutritional quality of different foods, resulting in the transfer of allergenicity.

A recent study by Murtagh and colleagues (2003) examined the physicochemical properties of Ber e 1. It was demonstrated that both the natural and recombinant forms of Ber e 1 (expressed in *Pichia pastoris*) were resistant to digestion following exposure to simulated gastric fluid (SGF) for 15-30 minutes. In contrast, the 7S and 11S globulin fractions of Brazil nut were found to be highly susceptible to digestion upon exposure to SGF for 5 seconds (Murtagh *et al.*, 2003). These data suggest that the 2S albumin is most likely to be the only protein in Brazil nut that is able to survive intact in the gastrointestinal system. It was similarly demonstrated that both natural and recombinant Ber e 1 (rBer e 1) maintain stable secondary structures at acidic pH and at high temperatures (95°C) and are therefore likely to survive the thermal processing of foods. Resistance to proteolysis and denaturation, properties which are suggested to be inherent of food allergens (Huby *et al.*, 2000), may contribute to the overall allergenicity of Ber e 1.

The allergenic nature of Brazil nut is not only due to the 2S albumin fraction but also to the 12S globulin, another class of seed storage proteins, although this protein has not yet been cloned (Table 1.3). The 12S globulin proteins are composed of subunits consisting of a heavy α-chain (~30 kDa) and a light β-chain (~20 kDa) which are linked by

disulphide bonds. IgE reactivity to this Brazil nut protein fraction was first reported by Bartolome *et al.* (1997). Aside from demonstrating IgE-binding to the Brazil nut 2S albumin, immunoblotting studies also showed that the 33.5 kDa and 32 kDa α-subunits and 21 kDa β-subunits of the Brazil nut 12S globulin bound serum IgE from an individual exhibiting clinical symptoms of Brazil nut allergy. However, in a later study by Pastorello *et al.* (1998), IgE-reactivity to these proteins was also observed when sera from asymptomatic patients were used for immunoblotting studies. These data suggest that the 12S globulin subunits identified by Bartolome *et al.* (1997) as allergens may not be involved in eliciting the clinical symptoms observed in Brazil nut allergic patients (Pastorello *et al.*, 1998).

1.5.2 Walnut allergens

2S albumin seed storage proteins from other tree nuts have also been characterised as food allergens. Jug r 1 is a 2S albumin protein that was classified by Teuber *et al.* (1998) as a major walnut (*Juglans regia*) allergen. This allergen was identified as an IgE-reactive clone following immunoscreening of a walnut cDNA library using serum from a walnut allergic patient and its molecular characteristics are outlined in Table 1.4. Amino acid sequence comparisons revealed that Jug r 1 encodes a 2S albumin seed storage protein precursor, which is cleaved into a large subunit and small subunit linked together by disulphide bonds (Robotham *et al.*, 2002). Sequence comparisons showed that this walnut allergen has significant sequence homology (46%) to the Brazil nut allergen, Ber e 1, also classified as a 2S albumin.

The frequency of IgE reactivity of Jug r 1 was assessed by immunoblotting using sera from 16 patients with walnut allergy where it was subsequently shown that this protein

bound IgE in 12 out of 16 patients (75%), indicating that Jug r 1 is a major allergen (Teuber *et al.*, 1998). Inhibition studies were also conducted to assess the relevance of this allergen in terms of the total IgE response to walnut extract. Inhibition of IgE binding to walnut extract was demonstrated when sera from 12 patients previously shown to have specific IgE to Jug r 1 were pre-incubated with purified recombinant Jug r 1 (rJug r 1). The percentage inhibition of IgE binding to walnut extract was 50% or greater in 7 patients, further validating the importance of this allergen.

Similar to the peanut allergens, the linear IgE-binding epitopes of Jug r 1 have been mapped using synthetic peptides. Peptide sequences based on the length of the large and small subunits of Jug r 1 were used in a study by Robotham *et al.* (2002) to identify the IgE-binding epitopes. Three adjacent peptides located in the large subunit were recognised by serum IgE from walnut allergic individuals, with a conserved amino acid sequence of GLRGEEM. In contrast, no IgE-binding peptides were identified from the small subunit. Alanine substitutions identified the core amino acid residues RGEE at positions 36-39 as well as a glutamic acid residue at position 42 as critical for IgE binding (Robotham *et al.*, 2002). However, inhibition studies revealed that this epitope did not account for all of the IgE binding to rJug r 1 as pre-incubation with the above peptide did not abolish IgE binding to rJug r 1. This clearly demonstrates that although synthetic peptides have been useful in mapping the IgE-binding epitopes of peanut allergens, the role of conformational epitopes in the binding of IgE antibodies to allergens must be considered.

A second walnut allergen, Jug r 2, has also been characterised which appears to be a member of the vicilin family of seed storage proteins. Jug r 2 was an IgE-reactive clone isolated from a walnut cDNA library and expressed as a GST fusion protein. The

molecular characteristics of this clone are outlined in Table 1.4. Sequence comparisons indicated that this walnut allergen is related to vicilin-like proteins from cotton, cacao, pea, soybean and most notably peanut (Teuber *et al.*, 1999).

Further characterisation of the expressed recombinant Jug r 2 (rJug r 2) by SDS-PAGE analysis revealed that this protein has a molecular weight of 66 kDa which is in accordance with the deduced molecular weight based on the amino acid sequence. The IgE-reactivity of this allergen was analysed using immunoblotting experiments where it was found that the rJug r 2 fusion protein bound IgE antibodies in 9 out of 15 (60%) walnut allergic patients. Pre-absorption of sera from walnut allergic patients with rJug r 2 abolished IgE binding to several walnut kernel proteins with molecular weights of 52, 48, 44 and 28 kDa with inhibition of IgE binding to the 44 kDa protein observed in all 4 patients. It was suggested from this data that the 44 kDa protein represents the native form of Jug r 2 which may consist of several subunits derived from a single precursor molecule, a characteristic of some members of the vicilin family of seed storage proteins.

Two other walnut allergens have been officially recognised by the Allergen Nomenclature registry (http://www.allergen.org/) but have not yet been fully characterised (Table 1.3). A walnut lipid transfer protein (LTP), designated Jug r 3, has been identified as allergenic (Pastorello, unpublished data). LTPs are clinically relevant panallergens that contribute to immunological cross-reactivity between many botanically unrelated fruits and vegetables (Salcedo *et al.*, 1999, Sanchez-Monge *et al.*, 1999, Asero *et al.*, 2000). Similarly, a legumin-like walnut protein, Jug r 4, has been cloned and expressed as a fusion protein (Teuber *et al.*, 2003). Sera from 15 out of 23 (65%) patients with life-threatening allergic reactions to walnut showed IgE binding to

the fusion protein. Further studies are required to fully characterise these two walnut allergens.

1.5.3 Cashew allergens

Vicilin-like proteins have also been implicated as allergens that are responsible for cashew (*Anacardium occidentale*) allergy. In study by Wang *et al.* (2002), a cashew cDNA library was screened with human sera and rabbit anti-cashew extract antisera, identifying clones which upon sequencing showed homology with the 7S (vicilin) superfamily of plant seed storage proteins and sucrose-binding proteins (Wang *et al.*, 2002). This clone, designated Ana o 1, is 1781 bp in length and was expressed as a maltose-binding fusion protein which upon digestion with thrombin yielded a 65 kDa protein (summarised in Table 1.4). The IgE reactivity of recombinant Ana o 1 (rAna o 1) was assessed among patients with cashew nut allergy using Western immunoblotting which demonstrated IgE binding to this protein in 10 out of 20 patients (50%), establishing it as a major cashew allergen (Wang *et al.*, 2002). Interestingly, 2 out of 8 sera from patients with a history of tree nut allergy but clinically tolerant to cashew nuts also showed IgE binding to rAna o 1.

The identification of native Ana o 1 in cashew extract was also conducted using rAna o 1 in inhibition immunoblotting studies. Rabbit anti-cashew extract antisera and serum from a known cashew-allergic patient were preincubated with 5 μg of purified rAna o 1 and used to probe nitrocellulose strips blotted with cashew extract. It was found that the recombinant protein inhibited IgE binding to a protein with a molecular weight of approximately 50 kDa. Given that the molecular mass of the recombinant form is 65

kDa, it was concluded that the native Ana o 1 undergoes further cleavage which, as stated earlier, is commonly observed among vicilin-like proteins.

Wang *et al.* (2002) subsequently mapped the linear IgE binding epitopes of Ana o 1 by probing overlapping synthetic peptides based on the entire amino acid sequence using rAna o 1 reactive sera from 12 patients. In total, 11 linear IgE-binding epitopes were identified which were located throughout the protein. Comparisons with the linear IgE binding epitopes of the vicilin-like peanut allergen, Ara h 1, showed no significant homology or similarity even though there is 27% identity and 45% similarity between the amino acid sequences of these two allergens. It was observed, however, that 4 of the 11 Ana o 1 epitopes had a significant positional overlap (≥7 amino acids) with previously identified Ara h 1 linear epitopes.

More recently, Wang and colleagues (2003) cloned a second cashew nut allergen, designated Ana o 2, which belongs to the legumin family of seed storage proteins (summarised in Table 1.4). This allergen, expressed as a maltose-binding fusion protein, was classified as a major allergen following IgE immunoblotting which demonstrated reactivity in 13 out of 21 (62%) cashew allergic patients (Wang *et al.*, 2003). Inhibition immunoblotting data using the recombinant form of this allergen revealed that native Ana o 2 exists as two forms in cashew extract – a major band at 33 kDa and a minor band at 53 kDa. Attempts were also made to map the linear IgE binding epitopes of Ana o 2 using 58 overlapping synthetic peptides. Using serum pools, 22 IgE-reactive peptides were identified which were distributed evenly throughout the length of the protein, 7 of which were classified as immunodominant. Two of the IgE-reactive peptides showed significant positional overlap with 4 previously identified Ara h 3 linear epitopes, however there was very little identity or

similarity between the Ara h 3 and Ana o 2 epitopes (Wang *et al.*, 2003). Interestingly, a significant positional overlap and degree of similarity was observed between Ana o 2 IgE-binding peptides and previously identified epitopes of the soybean legumin, G2 glycinin. A third cashew allergen has also been identified, Ana o 3, but this has not yet been fully characterised (see Table 1.3).

1.5.4 Hazelnut allergens

The increasing prevalence of hazelnut (*Corylus avellana*) allergy has led to the identification of hazelnut allergens (Table 1.3), some of which have been cloned and characterised (Table 1.4). Allergy to hazelnuts has often been identified in patients with hazel pollen allergy as these patients commonly present with oral allergy syndrome that is attributed to cross-reactivity between tree pollen allergens and hazelnut allergens. In a study by Hirschwehr *et al.* (1992), it was observed that serum IgE from 25 patients with allergy to tree pollens and hazelnuts bound to Cor a 1, a 17 kDa major allergen of hazel pollen. Serum IgE from the same patients bound to a hazelnut protein of similar molecular mass (18 kDa), suggesting that a Cor a 1 protein homologue may be present in hazelnuts (Hirschwehr *et al.*, 1992). This was further confirmed with inhibition experiments whereby pre-incubation of serum IgE with hazel pollen extract abolished IgE binding to the 18 kDa protein in hazelnut. Pre-incubation of serum IgE with the recombinant form of the major birch pollen allergen Bet v 1 was also able to abolish IgE binding to the major allergens of hazelnut and hazel pollen. Altogether, these data suggest that the 18 kDa major IgE binding protein of hazelnut is similar to the hazel pollen allergen Cor a 1 and also shares IgE binding epitopes with the major allergen of birch pollen, Bet v 1 (Hirschwehr *et al.*, 1992).

The same study also identified a 14 kDa hazel pollen profilin that bound serum IgE from patients with tree pollen and hazelnut allergy. The same patients also displayed IgE binding to a 14 kDa protein in hazelnut extract, suggesting that these two proteins with comparable molecular masses from hazel pollen and hazelnut are related. To confirm this, Hirschwehr *et al.* (1992) used a rabbit anti-celery profilin antibody as a probe in immunoblotting studies using hazel pollen and hazelnut extracts. This antibody was able to bind to the 14 kDa proteins in hazel pollen and hazelnut and subsequent inhibition experiments using serum from patients reactive to these proteins showed that pre-incubation with recombinant Bet v 1 (rBet v 1), a birch pollen profilin, abolished IgE binding to these proteins. Therefore, it appears that the second major IgE-binding protein in hazelnut is a profilin.

Since it has been demonstrated that one of the major allergens in hazelnut is related to the hazel pollen allergen, Cor a 1, attempts have been made to identify and characterise the corresponding homologue in hazelnuts. Luttkopf *et al.* (2002) successfully cloned and expressed four variants of Cor a 1.04, a Bet v 1-related major hazelnut allergen, and compared it with the corresponding homologue in hazel pollen. The molecular characteristics of all four variants were similar (summarised in Table 1.3) and the deduced molecular weight of 17.4 kDa was similar to natural Cor a 1.04. Amino acid sequence comparisons revealed a high degree of identity (85%) with the birch pollen stress protein, Bet v 1-Sc3, as well as known allergens from hazel leaf, hazel pollen, birch pollen, hornbeam pollen, cherry, apple and celery (Luttkopf *et al.*, 2002). The allergenicity of all four Cor a 1.04 variants was subsequently assessed in 43 patients with a positive DBPCFC using enzyme allergosorbent test (EAST). The highest EAST scores were obtained with Cor a 1.0401, with 95% of patients obtaining a score of ≥ 1, followed by Cor a 1.0402 (93%) and Cor a 1.0403 (91%). In contrast, only 74% of

patient sera were positive for IgE binding to Cor a 1.0404. Further studies using inhibition experiments comparing Cor a 1 from hazelnut with the homologue in hazel pollen revealed partial epitope identity as recombinant Cor a 1 (rCor a 1) from hazel pollen was able to inhibit 35% of IgE binding to recombinant Cor a 1.0401 (rCor a 1.0401). These data suggest that the presence of homologues is the most likely contributor to the high incidence of hazelnut allergy in patients sensitive to hazel pollen.

A more comprehensive study conducted by Pastorello *et al.* (2002) examined hazelnut allergens using sera from 65 patients presenting with a positive DBPCFC to hazelnut and these are outlined in Table 1.4. Using IgE immunoblotting to hazelnut extract, 63 out of 65 patients showed IgE binding to an 18 kDa protein (Cor a 1) as well as to proteins with molecular weights of 32, 35 and 47 kDa. Variable IgE binding to the 14 kDa hazelnut profilin was also demonstrated among the same group of patients. In contrast, serum from 7 patients with systemic reactions upon consumption of hazelnuts showed IgE binding to a 9 kDa protein (Pastorello *et al.*, 2002). *N*-terminal sequencing comparisons of the previously unidentified 32, 35 and 47 kDa hazelnut allergens showed homology with different plant proteins. The 47 kDa protein, which appears to have been previously cloned and designated as Cor a 11 (Table 1.3), showed sequence homology with a sucrose-binding protein from soybean that belongs to the vicilin superfamily to which the major peanut allergen, Ara h 1, and the major walnut allergen, Jug r 2, also belong to. Comparisons of the *N*-terminal sequence of the 35 kDa hazelnut allergen revealed that this protein belongs to the legumin family which consists of seed storage proteins from the 11S globulin family which includes allergens from soybean (Burks *et al.*, 1988), coconut (Teuber and Peterson, 1999) and peanut (Rabjohn *et al.*, 1999) . *N*-terminal sequencing of the 32 kDa allergen showed that this protein is a

member of the 2S albumin family which includes the Brazil nut allergen, Ber e 1 (Nordlee *et al.*, 1996), and walnut allergen, Jug r 1 (Teuber *et al.*, 1998).

The same study also conducted inhibition experiments to further characterise these IgE-reactive hazelnut proteins. Pastorello *et al.* (2002) confirmed the high level of homology between birch and hazelnut allergens using inhibition immunoblotting. In this study, IgE binding to both the 14 and 18 kDa hazelnut allergens was completely abolished when serum was pre-incubated with birch pollen extract, confirming the findings reported by Hirschwehr *et al.* (1992). Interestingly, pre-incubation of serum with birch pollen extract did not inhibit IgE binding to the 9 kDa protein using a serum pool of patients with a history of systemic reactions to hazelnut (Pastorello *et al.*, 2002). However, when a serum pool consisting of patients that were previously shown to only react to the 9 kDa protein was pre-incubated with peach extract (50 µg), complete inhibition of IgE binding to the 9 kDa protein was observed. The same serum pool was also pre-incubated with 5 µg of purified peach LTP which similarly resulted in minimal IgE binding to the 9 kDa hazelnut protein. Subsequent *N*-terminal sequencing of this protein showed sequence homology with peach LTP. Altogether, these results suggest that this 9 kDa allergen is a lipid transfer protein in hazelnuts which, as shown in Table 1.3, has been previously cloned and designated as Cor a 8 but not yet fully described. Given that IgE binding to this protein was not abolished by the pre-incubation of serum with birch pollen extract suggests that this hazelnut LTP may be responsible for hazelnut allergy in individuals with no history of pollinosis. This observation is further validated by results obtained in a previous study where hazelnut proteins of approximately the same molecular weight bound IgE antibodies from patients with non-pollen related hazelnut allergy (Schocker *et al.*, 2000). It appears that there are unique

hazelnut allergens also responsible for IgE-mediated reactions in hazelnut allergic individuals.

Given these findings, Beyer *et al.* (2002) sought to further identify other proteins responsible for hazel pollen-independent hazelnut allergy. IgE-binding hazelnut proteins were identified by 2D gel electrophoresis and immunoblotting using serum from 14 patients with hazelnut allergy. Several proteins with a molecular weight of 40 kDa were recognised by sera from 12 of the 14 hazelnut allergic patients. Edman sequencing of the 40 kDa proteins yielded two internal sequences (QGQQQFGQR and HFYLAGNPDDEHQR) which showed homology with 11S globulin seed storage proteins of English oak and almond. Primers based on these sequences were used to identify clones from a hazelnut cDNA library which, upon sequencing, were shown to encode a 59 kDa protein (Beyer *et al.*, 2002). This IgE-reactive hazelnut 11S globulin was designated Cor a 9 (Table 1.3). Sequence comparisons with other 11S globulin proteins from peanut, soybean, sesame, almond and oak revealed that this hazelnut clone encodes a protein composed of acidic and basic subunits which are linked by disulphide bonds. Post-translational modification occurs through the asparaginyl cleavage site which separates Cor a 9 into two subunits with the initial 40 kDa protein representing the acidic subunit. Further sequence alignments with the peanut allergen Ara h 3 (classified as an 11S globulin) revealed 67% identity between one of the 4 known IgE-binding epitopes of Ara h 3 and the corresponding region of Cor a 9, raising the possibility that recognition of this IgE-binding site may contribute to co-sensitisation to peanut and hazelnut.

1.5.5 Almond allergens

Systemic reactions to almonds (*Prunus dulcis*) have also increased in recent years but very little is known about almond allergens. To date, only three almond proteins have been identified and characterised as allergens (Table 1.4) although none of these have been cloned thus far. The first of these is a water soluble storage protein known as amandin or almond major protein (AMP), which accounts for ~65-70% of the total aqueous extractable protein in almond (Wolf and Sathe, 1998). This protein was demonstrated as a major allergen in almond allergic patients (Roux *et al.*, 1999) and migrates as a two major subunits with molecular masses of 63 and 65 kDa. These subunits are composed of two polypeptides with molecular masses of 20-22 and 38-42 kDa, linked together by disulphide bonds (Roux *et al.*, 2001, Sathe *et al.*, 2002).

In a study by Potronieri *et al.* (2002), 2S albumin and conglutin γ proteins in almond seeds were found to be IgE reactive. Almond proteins were initially purified using a process involving globulin separation, ammonium sulphate precipitation and anion exchange chromatography. Analysis of protein fractions revealed two major IgE-binding proteins with molecular weights of 12 and 45 kDa (Poltronieri *et al.*, 2002). *N*-terminal sequencing of the 45 kDa protein followed by sequence comparisons revealed 40% identity and 60% homology with lupine seed conglutin γ (Kolivas and Gayler, 1993). Substantial sequence homology (50%) was also found with a 7S globulin from soybean (Kagawa *et al.*, 1987). In contrast, *N*-terminal sequencing of the 12 kDa protein did not reveal any identity with other plant proteins with the exception of a 2S albumin from English walnut. This protein was further digested with Glu-endoproteinase to obtain a 6 kDa and 2 kDa peptide fraction by gel filtration chromatography. Immunoblot analysis of these fractions revealed that IgE binding to

the intact 12 kDa protein was conserved in the 6 kDa peptide fraction. Attempts to sequence this peptide were unsuccessful due to a blocked *N*-terminus. However, sequencing of the 2 kDa fraction and subsequent database comparisons showed 80% similarity with the *C*-terminal sequence of English walnut and Brazil nut 2S albumins. This suggests that the 12 kDa IgE-reactive protein belongs to the 2S albumin family of seed storage proteins which includes previously identified tree nut allergens from Brazil nut, cashew, hazelnut and walnut (see Tables 1.3 and 1.4)

1.6 PEANUT AND TREE NUT CROSS-REACTIVITY

Clinical studies on peanut and tree nut sensitive patients suggest that it is common for patients to exhibit multiple sensitivities to both peanut and tree nuts. In fact, monoreactivity to peanut or a single tree nut is rare. Given this, it is not known if co-sensitisation to both peanut and tree nuts is due to cross-reactive allergens.

1.6.1 Cross-reactivity among peanuts and tree nuts

Given the increasing occurrence of multiple peanut and tree nut allergy in individuals, a number of studies have investigated the presence of cross-reactive allergens in peanut and different tree nuts. Although peanut and tree nuts are taxonomically distantly related (Table 1.1), they are defined as 'edible' seeds and are likely to perform similar functions in plant development. Thus, the question arose as to whether the incidence of multiple peanut and tree nut allergy can be attributed to homologous proteins present in peanut and tree nuts that share similar IgE-binding epitopes. One of the earliest studies

to investigate this phenomenon was conducted by Gillespie *et al.* (1976) whereby RAST was evaluated as an *in vitro* measurement of specific IgE antibodies to Brazil nut, almond, walnut, pecan cashew and peanut. Sera from 18 patients with a history of peanut and tree nut allergy were tested for IgE antibodies specific for the abovementioned nut proteins and it was found that 13 out of 18 patients had significantly elevated IgE antibodies to one or more of the nut extracts while 5 patients had specific IgE to at least 2 or more nut types (Gillespie *et al.*, 1976). RAST inhibition was subsequently used to investigate the specificity and cross-reactivity of IgE antibodies to the different peanut and tree nut antigens. No IgE cross-reactivity between Brazil nut and peanut was found in 4 out of 5 patients while IgE antibodies to pecan proteins appear to cross-react with peanut, almond, walnut, cashew and Brazil nut proteins (Gillespie *et al.*, 1976).

Pistachio allergens also appear to share similar IgE-binding epitopes with peanut and other tree nut proteins. Parra and colleagues identified pistachio proteins that elicited Type I hypersensitivity reactions in 3 patients. Four IgE-binding proteins with molecular weights of 34, 41, 52 and 60 kDa were identified by SDS-PAGE and immunoblotting using patient sera (Parra *et al.*, 1993). ImmunoCAP inhibition studies were performed to investigate cross-reactivity between pistachio and almond, cashew, chestnut, peanut, walnut and sunflower seed. The highest degree of cross-reactivity was observed between pistachio and cashew followed by sunflower seed, walnut, peanut, almond and chestnut (Parra *et al.*, 1993). Interestingly, pistachio and cashew both belong to the *Anacardiaceae* family suggesting that highly homologous proteins may be present in these tree nuts. Similar results were obtained by Fernandez *et al.* (1995) where IgE cross-reactivity was established between pistachio and cashew as well as mango seed, also a member of the *Anacardiaceae* family. It is apparent from these

studies that IgE cross-reactivity can occur between members of the same botanical family.

Teuber *et al.* (1999) investigated *in vitro* cross-reactivity between coconut, walnut, almond and peanut after two patients with tree nut allergy reported systemic reactions after ingestion of coconut (*Cocos nucifera*). Absorption of patient serum with walnut extract completely abolished IgE binding to 55, 36.5 and 35 kDa coconut proteins in both patients. Pre-incubation of serum from Patient 1 with almond extract showed complete inhibition of IgE binding to the same proteins while there was minimal IgE binding to the 35 and 55 kDa protein in Patient 2 (Teuber and Peterson, 1999). In contrast, peanut proteins inhibited all of the IgE binding to the above coconut proteins in Patient 2 while some IgE antibodies from Patient 1 were still able to bind to the 55 kDa coconut protein. A previous study has characterised the 35 kDa coconut protein as a subunit of the coconut 11S globulin, which is thought to be similar to other described 11S globulins (legumin group) from different plant families (Carr *et al.*, 1990). These data suggest that legumin proteins may play a role in IgE cross-reactivity between coconut, walnut, almond and peanut.

Allergens present in macadamia nut also appear to share similar IgE-binding epitopes with certain tree nut proteins. Sutherland *et al.* (1999) reported a case of macadamia nut anaphylaxis in a patient with no previous history of peanut or tree nut allergy. Using serum from this patient, a 17.4 kDa IgE-binding macadamia nut protein was identified by SDS-PAGE and immunoblotting. IgE binding to this protein was partially inhibited when patient serum was pre-incubated with hazelnut extract (Sutherland *et al.*, 1999). No inhibition was observed when peanut was used as the inhibitor. Thus, there is

evidence that some macadamia and hazelnut proteins may share cross-reactive IgE-binding epitopes.

Given that vicilin proteins are also present in other plants, Teuber *et al.* (1999) investigated whether cross-reactivity exists between Jug r 2 and vicilins present in peanut and cacao seed extracts. Overall, pre-incubation of serum with peanut extract did not inhibit IgE binding to Jug r 2 in walnut extract even at the highest inhibitor concentration of 200 µg/ml (Teuber *et al.*, 1999). In contrast, pre-absorption of sera with 200 µg/ml of cacao protein extract inhibited IgE binding to Jug r 2, suggesting that there is IgE cross-reactivity between Jug r 2 and proteins present in cacao seeds. However, the concentration of cacao protein extract (200 µg/ml) required to inhibit IgE binding to Jug r 2 was much higher than the concentration of walnut extract required to completely inhibit IgE binding to walnut proteins (1-5 µg/ml), suggesting that this cross-reactivity is of a low affinity or that the cross-reactive allergen is of low abundance.

Similarly, following the identification of almond allergens, Poltronieri and colleagues (2002) examined whether there was any IgE cross-reactivity between these allergens and those present in hazelnut and English walnut extracts, although IgE cross-reactivity was previously demonstrated between almond and pine nut (de las Marinas *et al.*, 1998). Sera from almond allergic patients were pre-incubated with hazelnut and walnut soluble albumin fractions and then used to probe for IgE binding to nitrocellulose membranes blotted with the 12 kDa 2S albumin and 45 kDa conglutin γ allergens from almond extract. IgE binding to the 45 kDa conglutin γ protein was inhibited when sera was pre-incubated with increasing concentrations (250-500 µg) of hazelnut and walnut albumin fractions. No inhibition in IgE binding was observed with the 12 kDa 2S albumin

fraction. This suggests that there is some degree of cross-reactivity between conglutin γ from almond and albumins from hazelnut and walnut.

1.6.2 Peanut and tree nut cross-reactivity with other foods

Hazelnut has been shown to have common allergenic structures with seeds from other plants. Vocks *et al.* (1993) observed that allergy to kiwi, poppy seeds and/or sesame seeds is a common occurrence in patients who are also sensitised to hazelnuts and as a consequence investigated whether or not there were cross-reactive proteins present in these foods. Again using inhibition immunoblotting, it was found that pre-incubation of patient sera with kiwi, sesame seed or poppy seed extracts inhibited IgE binding to hazelnut and rye grain proteins (Vocks *et al.*, 1993).

Cross-reactivity between cashew nut and fruit proteins has also been reported. Rasanen *et al.* (1998) studied a patient who displayed hypersensitivity to cashew nut and pectin, a high molecular weight carbohydrate found in fruit that is frequently used as a gelatinising agent. In RAST inhibition studies, IgE binding to cashew proteins was completely inhibited by pectin extract. This indicates that some allergens present in cashew nut share similar IgE-binding epitopes with pectins (Rasanen *et al.*, 1998).

Corn allergens have been shown to cross-react with peanut proteins. Lehrer *et al.* (1999) initially measured RAST scores for corn, soybean, rice and peanut for 125 patients with a positive history, positive skin test and positive RAST for the above foods. Significant RAST score correlations were found between rice and corn, corn and soybean, rice and soy and peanut and soybean. Using RAST inhibition, inhibition of IgE binding to corn was demonstrated for soybean, rice and peanut with the highest

degree of inhibition observed with rice (Lehrer *et al.*, 1999). This is most likely due to the fact that corn and rice both belong to the *Graminaceae* (grasses) family.

A study by Wensing and colleagues (2003) reported the occurrence of cross-reactive IgE antibodies to vicilin proteins present in peanuts and peas, both of which are classified as legumes. Inhibition immunoblotting and ELISA studies using serum from a patient allergic to both peanuts and peas showed that pre-incubation of serum with increasing concentrations of purified pea vicilin inhibited IgE binding to the vicilin-like peanut allergen, Ara h 1. It was suggested that sensitisation to vicilin induces some degree of allergenic cross-reactivity with other members of the legume family that can be of clinical significance (Wensing *et al.*, 2003).

LTPs also appear to contribute to allergenic cross-reactivity between botanically unrelated foods. LTPs are the major allergens in fruits such as peach, apple and apricot (Lleonart *et al.*, 1992, Pastorello *et al.*, 1999a, Pastorello *et al.*, 1999b, Sanchez-Monge *et al.*, 1999, Pastorello *et al.*, 2000a) all of which belong to the *Rosaceae* family. In a study by Asero and colleagues (2002), IgE reactivity to walnut and peanut was abolished following pre-absorption of sera from LTP-hypersensitive patients with peach LTP. Of the non-*Rosaceae* foods, peanuts and tree nuts were found to be the most common offending foods among this patient population, suggesting that the presence of LTPs in these foods is the contributing factor (Asero *et al.*, 2002).

1.7 CLINICAL AND BIOLOGICAL RELEVANCE OF CROSS-REACTIVE IgE ANTIBODIES

It is clear from the preceding discussion that individuals with peanut and tree nut allergy have IgE antibodies that can cross-react with other food proteins. This may be due to the presence of common IgE-binding epitopes between homologous proteins. This 'cross-sensitisation' has been presented as a possible explanation for the high incidence of co-sensitisation to peanut and tree nut allergens in allergic individuals. It has been suggested that IgE antibodies specific for an allergen from one nut type may cross-react with another allergen from a different nut species. However, this does not necessarily indicate that exposure to the latter will result in a Type I hypersensitivity reaction.

1.7.1 Cross-reactive antibodies to peanut and other legume proteins

Peanut is a member of the legume family which also includes other foods such as soybean, pea, lima bean and green bean. Although these foods are closely related taxonomically, clinical hypersensitivity to more than one legume is rare. In a clinical study by Bernhisel-Broadbent and Sampson (1989), only 2 out of 69 patients with a positive skin prick test to one or more legumes were symptomatic to more than one legume. It was then concluded that clinically relevant cross-reactivity to legumes is very rare and consequently, dietary elimination of all legumes was not warranted (Bernhisel-Broadbent and Sampson, 1989).

These findings were further investigated by analysing the serology of these patients. In a follow-up study, Bernhisel-Broadbent *et al.* (1989) examined the presence of IgE cross-reactivity between peanut, soybean, lima bean, pea, garbanzo bean and green bean

proteins using serum from patients with a positive legume skin prick test. Using immunoblots of legume proteins separated by SDS-PAGE, the authors were able to demonstrate IgE binding to numerous peanut, soybean, lima bean, garbanzo bean and green bean proteins using serum from a patient who was sensitive to only peanut as demonstrated by skin prick tests and food challenges (Bernhisel-Broadbent *et al.*, 1989). Similar results were also obtained from other patients where IgE binding was detected to proteins in legumes to which the patient did not have a positive food challenge. It appears from the results of this study that the IgE cross-reactivity between legume proteins observed *in vitro* does not correlate with clinical hypersensitivity.

1.7.2 Cross-reactive IgE antibodies to carbohydrate epitopes

The possible role of carbohydrate epitopes in the generation of cross-reactive IgE antibodies was investigated by van der Veen and colleagues (1997) following the observation that one-third of patients sensitised to grass pollen had significant serum levels of peanut-specific IgE antibodies but no clinical symptoms. Earlier studies had demonstrated that the *N*-linked carbohydrate groups of glycoprotein allergens induce the production of IgE antibodies which can cross-react with food and grass pollen allergens (Batanero *et al.*, 1996, Petersen *et al.*, 1996). It was hypothesised that these cross-reactive carbohydrate determinants (CCDs) were responsible for the IgE cross-reactivity observed between grass pollen and peanut allergens in patients without clinical sensitivity to peanut. Using RAST inhibition and basophil histamine release assays (BHRAs), van der Veen and colleagues (1997) sought to determine the biological activity of anti-CCD IgE antibodies. In 11 patients with discrepant peanut RAST and SPT results, it was found that pre-incubation of serum with proteinase-k treated grass pollen (CCD source) showed almost complete inhibition of IgE binding to peanut

extract. In contrast, inhibition of IgE binding to peanut extract by CCD was observed in only 1 out of 4 peanut allergic patients, suggesting that these patients have minimal IgE antibodies directed at CCDs.

IgE binding to the major peanut allergens, Ara h 1 and Ara h 2, was also assessed by RAST in patients that showed a false-positive RAST score for peanut but significant levels of anti-CCD IgE. These two allergens are glycoproteins and thus contain carbohydrate moieties. However, minimal IgE binding to Ara h 1 and Ara h 2 was detected in patients with high levels of CCD-specific IgE antibodies compared to peanut allergic patients with low levels of anti-CCD IgE (van der Veen *et al.*, 1997). It appears from these results that not all glycoprotein allergens will induce the production of IgE specific for carbohydrate determinants.

The same study also investigated whether cross-reactive IgE antibodies specific for CCDs have any biological activity. More specifically, histamine release of basophils from patients with a positive peanut RAST score predominantly based on anti-CCD IgE was measured. From these experiments, it was found that higher concentrations of peanut extract were required for histamine release in these patients compared with peanut allergic patients. Similar results were obtained when purified Ara h 1 and Ara h 2 were used to stimulate basophils. Thus, anti-CCD IgE antibodies appear to have very poor biological activity. This finding further validates the assertion that the presence of IgE antibodies directed to CCDs does not necessarily correlate with clinical reactivity.

1.8 SUMMARY AND AIMS

The immune response to allergens in an allergic individual is a cascade of cellular and humoral events driven by the activation of numerous cell types and the release of mediators. The consequence of this is the manifestation of symptoms commonly associated with an allergic reaction which include rhinitis, asthma, urticaria, angioedema and in severe cases, anaphylaxis. Up to 20-30% of the general population suffer from allergies to common environmental allergens such as house dust mite, grass and tree pollen, animal dander and fungi. Although rarely life threatening, it can nevertheless cause discomfort in suffering individuals.

Food allergies are frequently associated with anaphylactic reactions. In particular, allergy to peanut and tree nuts accounts for the majority of fatal food-induced anaphylaxis. Allergy to peanuts is a more frequent presentation although sensitisation to both peanut and tree nuts is common. The prevalence of this type of allergic disease appears to be increasing and currently, treatment is in the form of allergen avoidance and medical prevention of inadvertent exposure. Accidental exposure to peanut and tree nut allergens is unavoidable due to the inadequate labelling of food products and contamination during the cooking or manufacturing process. The situation is further compromised by the observation that peanut and tree nut allergens may share common IgE-binding epitopes.

Much research has focused on identifying the allergens responsible for peanut and tree nut allergy with the intention of providing avenues for the treatment of this type of food allergy. To date, peanut allergens have been the most widely studied with the

identification of a number of allergens, three of which are well characterised, namely Ara h 1, Ara h 2 and Ara h 3. Information about tree nut allergens is more limited, with only a few allergens having been identified for almond, Brazil nut, cashew, hazelnut and walnut, some of which are yet to be cloned. The availability of recombinant allergens provides a useful tool in investigating the association between peanut and tree nut allergy. In particular, purified recombinant allergens can be used to investigate the presence of cross-reactive IgE antibodies in peanut and tree nut sensitive individuals. Such information will be useful in managing allergen avoidance in these patients as well as addressing the observation of co-sensitisation to both peanut and tree nut allergens in allergic individuals. Consequently, this thesis presents a study of IgE cross-reactivity between peanut and tree nut allergens. More specifically, the aims of this project were: 1) to investigate allergenic B cell cross-reactivity between peanut and tree nuts at the crude extract level, 2) to clone and express Ara h 1, Ara h 2 and Ara h 3 as recombinant proteins, 3) to establish whether there is IgE cross-reactivity between these peanut allergens and tree nut proteins and 4) to investigate the biological relevance of cross-reactive IgE antibodies.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Protein gel electrophoresis reagents

Ammonium persulphate	Bio-Rad Laboratories, USA
Benchmark™ Pre-stained Protein Ladder	Invitrogen™ Life Technologies, USA
BIS-Acrylamide (29:1), electrophoresis purity	Bio-Rad Laboratories, USA
Coomassie brilliant blue R-250	Sigma Chemical Company, USA
Gel-Dry™ Drying Solution (1x)	Invitrogen™ Life Technologies, USA
Mini-gel cassettes	Invitrogen™ Life Technologies, USA
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Bio-Rad Laboratories, USA

2.1.2 Immunoblotting reagents and materials

4-chloro-1-naphthol	Sigma Chemical Company, USA
Anti-His_6 mouse monoclonal antibody	Roche Diagnostics, Germany
Unfractionated Bermuda grass pollen (BGP) extract	Kindly provided by Ms. Neeru Eusebius
Goat anti-rabbit IgG (H+L) horseradish peroxidase (HRP) conjugated antibody	Promega Corporation, USA
Hydrogen peroxide (H_2O_2)	BDH Laboratory Supplies, England
Nitrocellulose membrane (BA 0.45 μm)	Schleicher and Schuell

Ponceau S	Sigma Chemical Company, USA
Rabbit anti-human IgE antibody	DAKO Corporation, USA
Sheep anti-mouse immunoglobulin HRP-conjugated antibody	Silenus Labs, Australia

2.1.3 Protein assay reagents and materials

96-well flat bottom microplates	Greiner Bio-One, Germany
BCA Protein Assay Kit	Pierce, USA
BSGG protein standards	Bio-Rad Laboratories, USA

2.1.4 ELISA reagents and materials

Costar® 96-well EIA/RIA Plate, flat bottom	Corning, USA
o-phenylenediamine (OPD) tablets	Sigma Chemical Company, USA
Phosphate citrate buffer with sodium perborate capsules	Sigma Chemical Company, USA
Unfractionated latex glove extract (GE)	Kindly provided by Dr. Alec Drew
Unfractionated house dust mite (HDM) extract	Kindly provided by Ms. Leanne Gardner
Unfractionated rye grass pollen (RGP) extract	Kindly provided by Dr. Cenk Suphioglu
Recombinant Hev b 6.01 (rHev b 6.01)	Kindly provided by Dr. Alec Drew

2.1.5 Protein expression and purification reagents

Econo-Pac® disposable chromatography columns	Bio-Rad Laboratories, USA
Glutathione, oxidised	Calbiochem, USA
Glutathione, reduced	Calbiochem, USA

Imidazole	ICN Biomedicals, USA
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Roche Diagnostics, Germany
Nickel-nitrilotriacetic acid (Ni-NTA) Agarose	QIAGEN, Australia
Sodium phosphate monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)	Sigma Chemical Company, USA
Sodium sulfite (Na_2SO_3)	Sigma Chemical Company, USA
Sodium tetrathionate ($Na_2O_6S_4 \cdot 2H_2O$)	Sigma Chemical Company, USA
Urea	Merck, Australia

2.1.6 Molecular biology reagents

Agarose (molecular biology grade)	Progen Industries, Australia
Ampicillin	Sigma Chemical Company, USA
BL21 *Escherichia coli (E. coli)* cells	Novagen, USA
Dextran Blue	Sigma Chemical Company, USA
DH5α *E. coli* cells	Invitrogen™ Life Technologies, USA
Dimethylformamide	BDH Laboratory Supplies, England
DNA Ligase buffer (10 x)	Promega Corporation, USA
Eco RI restriction enzyme	Promega Corporation, USA
Epicurian Coli® BL21-CodonPlus® (DE3)-RIL Competent Cells	Stratagene, USA
ER1793 *E. coli* cells	New England Biolabs, USA
Ethidium bromide	Sigma Chemical Company, USA
GeneRuler™ DNA Ladder Mix	MBI Fermentas, Lithuania
Maltose	Sigma Chemical Company, USA
Mineral oil	Sigma Chemical Company, USA

pBluescript® II KS+ plasmid vector	Stratagene, USA
PCR and sequencing custom primers	Geneworks, Australia
Platinum PCR Supermix	Invitrogen™ Life Technologies, USA
pPROEX™ HT Prokaryotic Expression System	Invitrogen™ Life Technologies, USA
Pst I restriction enzyme	Promega Corporation, USA
QIAGEN® Plasmid Midi Kit	QIAGEN, Australia
QIAquick™ Gel Extraction Kit	QIAGEN, Australia
Restriction enzyme Buffer D (10 x)	Promega Corporation, USA
Restriction enzyme Buffer H (10 x)	Promega Corporation, USA
Sal I restriction enzyme	Promega Corporation, USA
T_4 DNA Ligase	Promega Corporation, USA
T_4 DNA Ligase Buffer	Promega Corporation, USA
Xba I restriction enzyme	Promega Corporation, USA

2.1.7 Tissue culture reagents

Ficoll-Paque	Pharmacia, Sweden
RPMI 1640	Invitrogen™ Life Technologies, USA
Foetal calf serum (FCS)	CSL, Australia
Penicillin-streptomycin-glutamate (PSG)	Invitrogen™ Life Technologies, USA
Recombinant human interleukin (IL)-3	R & D Systems, USA
Sodium heparin (preservative free)	David Bull Laboratories, Australia
Trypan Blue	Sigma Chemical Company, USA

2.1.8 Flow cytometry reagents

7-amino-actinomycin D (7AAD)	Sigma Chemical Company, USA
N-formyl-Met-Leu-Phe (fMLP) chemotactic peptides	Sigma-Aldrich, USA
Goat IgG antibody fluoroscein isothiocyanate (FITC)-conjugated isotype control	Caltag Laboratories, USA
Goat anti-human IgE FITC-conjugated antibody	Caltag Laboratories, USA
Goat serum	Sigma-Aldrich, USA
Mouse anti-human CD63 phycoerythrin (PE)-conjugated IgG_1 monoclonal antibody	Caltag Laboratories, USA
Mouse IgG_1, κ monoclonal immunoglobulin PE-conjugated isotype control	Caltag Laboratories, USA
Mouse anti-human CD14 allophycocyanin (APC)-conjugated IgG_{2a} monoclonal antibody	BD Pharmingen, USA
Mouse IgG_{2a}, κ monoclonal immunoglobulin APC-conjugated isotype control	BD Pharmingen, USA
Mouse anti-human CD19 allophycocyanin (APC)-conjugated IgG_1 monoclonal antibody	BD Pharmingen, USA
Mouse IgG_1, κ monoclonal immunoglobulin APC-conjugated isotype control	BD Pharmingen, USA

2.1.9 General reagents

Acetone	BDH Laboratory Supplies, England
Agar	Sigma Chemical Company, USA
Bovine serum albumin (BSA)	Sigma Chemical Company, USA

Bromophenol blue	Sigma Chemical Company, USA
Calcium chloride dihydrate ($CaCl_2$)	Merck, Germany
Complete™ protease inhibitor cocktail tablets	Roche Diagnostics, Germany
Diethyl ether	Merck, Australia
Di-sodium hydrogen orthophosphate (Na_2HPO_4)	BDH Laboratory Supplies, England
DL-Dithiothreitol (DTT)	Sigma Chemical Company, USA
Ethylenediaminetetra-acetic acid (EDTA)	Merck, Australia
Ethanol	Merck, Australia
Glacial acetic acid	BDH Laboratory Supplies, England
Glucose	BDH Laboratory Supplies, England
Glycerol	Merck, Australia
Glycine	Merck, Australia
HEPES	Sigma Chemical Company, USA
Hydrogen chloride (HCl)	Merck, Australia
Keyhole limpet haemocyanin (KLH)	Sigma Chemical Company, USA
Lactic acid	Sigma Chemical Company, USA
Magnesium sulphate ($MgSO_4$)	BDH Laboratory Supplies, England
Methanol	Merck, Australia
Potassium chloride (KCl)	Merck, Australia
di-Potassium hydrogen phosphate (K_2HPO_4)	BDH Laboratory Supplies, England
Raw almonds	Naytura, Australia
Raw Brazil nuts	Naytura, Australia
Raw cashew nuts	Naytura, Australia
Raw hazelnuts	Naytura, Australia
Raw peanuts	Naytura, Australia

Skim milk powder	Diploma, Australia
Sodium acetate	BDH Laboratory Supplies, England
Sodium carbonate (Na_2CO_3)	Merck, Australia
Sodium chloride (NaCl)	Merck, Australia
Sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$)	Merck, Australia
Sodium dodecyl sulphate (SDS)	BDH Laboratory Supplies, England
Sodium hydrogen carbonate ($NaHCO_3$)	Merck, Australia
Sodium hydroxide (NaOH)	Merck, Australia
Tris (hydroxymethyl) aminomethane (Tris)	BDH Laboratory Supplies, England
Trypticase peptone	BD Biosciences, USA
Tween-20 (Polyxyethylenesorbitan monolaurate)	BDH Laboratory Supplies, England
Yeast extract	Sigma Chemical Company, USA

2.2 BUFFERS AND SOLUTIONS

All buffers and solutions were prepared using Milli Q filtered (Millipore, USA) H_2O unless stated otherwise.

10% SDS (stock solution)

10 g of SDS was dissolved in 100 ml H_2O. This was stored at room temperature.

10% glycerol

10 ml of glycerol was dissolved in 90 ml H_2O. This was autoclaved and stored at room temperature.

20% maltose

20 g of maltose was dissolved in 100 ml H_2O. Solution was autoclaved and stored at 4°C.

50% BIS-Acrylamide solution

This solution was prepared by dissolving 300 g BIS-Acrylamide (29:1) in 150 ml H_2O. H_2O was then added for a final volume of 300 ml and solution was stored in the dark at room temperature.

Ampicillin stock (100 mg/ml)

This was prepared by dissolving 100 mg of ampicillin in 1 ml sterile H_2O. Solution was filter-sterilised and stored at -20°C.

1% blocking solution

This was prepared by dissolving 1 g skim milk powder in 100 ml PBS.

5% blocking solution

This was prepared by dissolving 5 g skim milk powder in 100 ml PBS.

10% blocking solution

This was prepared by dissolving 10 g skim milk powder in 100 ml PBS.

0.5% BSA in PBS

This was prepared prior to use by dissolving 0.25 g BSA in 50 ml PBS.

Calcium chloride (500 mM)

This was prepared by dissolving 73.51 g $CaCl_2$ in 1 L H_2O. This was autoclaved and stored at 4°C.

Coomassie destaining solution

This solution consisted of 20% (v/v) methanol, 7% (v/v) acetic acid and 3% (v/v) glycerol in 1 L H_2O. This was stored at room temperature.

Coomassie staining solution

This solution consisted of 50% (v/v) methanol, 7% (v/v) acetic acid and 0.1% (w/v) Coomassie brilliant blue R-250 in 1 L H_2O. This was stored at room temperature.

Denaturing elution buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, pH 4.5)

This buffer was prepared by dissolving 13.8 g sodium phosphate, 1.2 g Tris and 480.5 g urea in 1 L H_2O.

Denaturing elution buffer with imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole and 8 M Urea, pH 8.0)

This buffer was prepared by dissolving 6.9 g sodium phosphate, 17.54 g sodium chloride, 34 g imidazole and 480.5 g urea in 1 L H_2O.

Denaturing lysis buffer (100 mM NaH_2PO_4, 10 mM Tris and 8 M Urea, pH 8.0)

This buffer was prepared by dissolving 13.8 g sodium phosphate, 1.2 g Tris base and 480.5 g urea in 1 L H_2O.

Denaturing wash buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M Urea, pH 6.3)

This buffer was prepared by dissolving 13.8 g sodium phosphate, 1.2 g Tris and 480.5 g urea in 1 L H_2O.

Denaturing wash buffer with imidazole (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole and 8 M Urea, pH 8.0)

This buffer was prepared by dissolving 6.9 g sodium phosphate, 17.54 g sodium chloride, 2.9 g imidazole and 480.5 g urea in 1 L H_2O.

DNA sample loading buffer (6 x)

This buffer consisted of 5 ml glycerol, 100 µl SDS, 0.37 g EDTA and 10 µl bromophenol blue. H_2O was added for a final volume of 10 ml and this was stored at 4°C. This stock solution was added to the DNA sample for a final concentration of 1 x.

ELISA coating buffer (pH 9.6)

This was prepared by dissolving 0.86 g sodium carbonate and 1.72 g sodium hydrogen carbonate in 100 ml H_2O. The solution was stored at 4°C.

Ethanol (70%)

This was prepared by mixing 70 ml ethanol with 30 ml H_2O. Solution was stored at -20°C.

Ethidium bromide stock (10 mg/ml)

20 mg of ethidium bromide was dissolved in 2 ml H_2O. This was wrapped in foil and stored at 4°C.

FACS wash buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, 0.27 mM EDTA, pH 7.3)

This buffer was prepared by adding 2.38 g HEPES, 3.89 g sodium chloride, 0.19 g potassium chloride and 0.05 g EDTA to 500 ml H_2O. This was stored at 4°C.

FACS red cell lysis buffer

This buffer was prepared by dissolving 4.13 g ammonium chloride, 0.5 g potassium bicarbonate and 0.15 g EDTA in 500 ml H_2O.

Glycine buffer (0.2 M) containing 1% BSA (pH 2.6)

This buffer was prepared by dissolving 7.5 g glycine in 500 ml H_2O and stored at 4°C. Prior to use, 0.5 g BSA was added to a 50 ml aliquot of this buffer.

Heparinised RPMI containing penicillin-streptomycin-glutamate (PSG)

This was prepared by adding 5000 units of sterile preservative free sodium heparin and 2 mM/L L-glutamine with 100 IU/ml penicillin and streptomycin to 500 ml RPMI 1640 medium. This was stored at 4°C, protected from light.

HEPES buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 5.5 mM glucose, pH 7.4) containing 5% foetal calf serum (FCS)

This buffer was prepared by dissolving 2.38 g HEPES, 3.86 g sodium chloride, 0.22 g potassium chloride, 0.12 g magnesium sulphate, 0.10 g di-potassium hydrogen phosphate and 0.5 g glucose in 500 ml H_2O. This was stored at 4°C. Prior to use 2.5 ml FCS was added to a 50 ml aliquot of this buffer.

HEPES buffer containing 5% FCS and $CaCl_2$ (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM K_2HPO_4, 5.5 mM glucose, 1 mM $CaCl_2$, pH 7.4)

This buffer was prepared by dissolving 2.38 g HEPES, 3.86 g sodium chloride, 0.22 g potassium chloride, 0.12 g magnesium sulphate, 0.10 g di-potassium hydrogen phosphate, 0.5 g glucose and 0.07 g calcium chloride in 500 ml H_2O. This was stored at 4°C. Prior to use 2.5 ml FCS was added to a 50 ml aliquot of this buffer.

Isopropyl-β-D-thiogalactopyranoside (IPTG) solution (1 M)

A 1 M solution was prepared by dissolving 2.38 g IPTG in 10 ml H_2O. Solution was filter-sterilised and stored in 1 ml aliquots at -20°C.

Lactic acid buffer (13.4 mM lactic acid, 140 mM NaCl, 5 mM KCl, pH 3.9)

This buffer was prepared by dissolving 0.6 g lactic acid, 4.1 g sodium chloride and 0.19 g potassium chloride in 500 ml H_2O. This was stored at 4°C.

Non-reducing sample buffer

This buffer consisted of 2.5 ml stacking gel buffer, 4 ml 10% SDS and 2 ml glycerol. H_2O was added for a final volume of 10 ml and bromophenol blue crystals were added until solution was blue. This was stored at -20°C in aliquots.

Phosphate buffered saline (PBS; pH 7.3)

A 10 x stock solution was prepared by dissolving 85 g sodium chloride, 3.9 g sodium dihydrogen orthophosphate and 10.7 g di-sodium hydrogen orthophosphate in 1 L H_2O. This was stored at room temperature and diluted 10-fold when required.

Phosphate citrate buffer (0.05 M)

This was prepared by dissolving the contents of 1 phosphate citrate buffer with sodium perborate capsule in 100 ml H_2O.

PBS-Tween (0.05%; pH 7.3)

2.5 ml of Tween-20 was dissolved in 5 L of 1 x PBS. This was stored at room temperature.

Ponceau S stain

This solution was prepared by dissolving 1 g Ponceau S and 5 ml acetic acid in 95 ml H_2O. This was stored at room temperature.

Reducing sample buffer

This was prepared by dissolving 78 mg DTT in 2.5 ml stacking gel buffer, 4 ml 10% SDS and 2 ml glycerol. H_2O was added for a final volume of 10 ml and bromophenol blue crystals were added until solution was blue. This was stored at -20°C in aliquots.

Refolding buffer (100 mM $Na_2H_2PO_4$, 10 mM Tris-Cl, 3 M Urea, pH 8.0)

This was prepared by dissolving 13.8 g sodium phosphate, 1.2 g Tris and 180.2 g urea in 1 L H_2O. This was stored at room temperature.

Refolding buffer (100 mM $Na_2H_2PO_4$, 10 mM Tris-Cl, 3 M Urea, 0.2mM oxidised glutathione, 1 mM reduced glutathione, pH 8.0)

This was prepared by dissolving 13.8 g sodium phosphate, 1.2 g Tris, 180.2 g urea, 0.12 g oxidised glutathione and 0.3 g reduced glutathione in 1 L H_2O. This was stored at room temperature.

RPMI 1640 containing 10% FCS

This was prepared by adding 50 ml FCS to 500 ml RPMI 1640 medium. This was stored at 4°C, protected from light.

Running buffer (10 x)

This was prepared by dissolving 29 g of Tris, 144 g of glycine and 10 g of SDS in 1 L H_2O. Buffer was diluted to 1 x with H_2O as required and stored at room temperature.

Sodium acetate (3 M)

This buffer was prepared by dissolving 61.5 g sodium acetate in 250 ml H_2O. This was stored at room temperature.

Separating gel buffer (1 M Tris-HCl, pH 8.8)

30.3 g of Tris was dissolved in 250 ml H_2O and stored at 4°C.

Stacking gel buffer (0.375 M Tris-HCl, pH 6.8)

11.4 g of Tris was added to 250 ml H_2O and stored at 4°C.

Stimulation buffer containing heparin and IL-3 (mM HEPES, 133 mM NaCl, 5 mM KCl, 7 mM $CaCl_2$, 3.5 mM $MgCl_2$, 0.1% BSA, pH 7.4)

This buffer was prepared by dissolving 2.38 g HEPES, 3.89 g sodium chloride, 0.19 g potassium chloride, 0.51 g calcium chloride, 0.36 g magnesium chloride and 0.5 g BSA in 500 ml H_2O. This was stored at 4°C. Prior to use, 200 µl heparin (5000 IU) and 20 µl IL-3 (2 ng/ml) were added to 10 ml of stimulation buffer and this was subsequently used in the basophil activation experiments.

Sulfonation buffer (100 mM Na_2SO_3, 10 mM $Na_2O_6S_4 \cdot 2H_2O$, 100 mM $Na_2H_2PO_4$, 10 mM Tris-Cl, 8 M Urea, pH 8.0)

This was prepared by dissolving 12.6 g sodium sulfite, 3.06 g sodium tetrathionate, 13.8 g sodium phosphate, 1.2 g Tris base and 480.5 g urea in 1 L H_2O. This was stored at room temperature.

TBE buffer (5 x)

This was prepared by dissolving 54 g Tris, 27.5 g boric acid and 20 ml 0.5 M EDTA (pH 8.0) in 1 L H_2O. The buffer was diluted to 1 x prior to use and stored at room temperature.

Transfer buffer (25 x)

A 25 x stock solution was prepared by dissolving 18.125 g of Tris and 90 g of glycine in 500 ml H_2O. Buffer was then diluted to 1 x with H_2O with a 20% final concentration of methanol and this was stored at room temperature.

2.3 MEDIA

All media were prepared using Milli Q filtered (Millipore, USA) H_2O and autoclaved to sterlise.

Luria-Bertani (LB) broth (pH 7.0)

Medium was prepared by adding 5 g trypticase peptone, 2.5 g yeast extract and 2.5 g NaCl to 1L H_2O. Medium was autoclaved and stored at room temperature.

LB-Ampicillin agar plates

Agar plates were prepared by adding 15 g of agar to 1 L LB broth and autoclaved. 1 µl of ampicillin stock was added per ml of sterile molten agar and poured onto plates when required.

LB-Ampicillin-IPTG-X-gal agar plates

These agar plates were prepared by adding 50 µl IPTG (0.1304 g/ml H_2O, filter-sterilised), 40 µl X-gal (50 µg/µl dissolved in dimethylformamide) and 50 µl ampicillin stock to 50 ml of sterile molten LB agar which was poured onto plates when required.

2.4 CLINICAL CHARACTERISTICS OF PATIENTS

Peripheral blood was collected from 22 peanut allergic atopic adults (11 males, 11 females; age range 18-55 years) recruited from the Alfred Hospital Allergy Clinic. All of these subjects had a clinical history of sensitivity to peanut, with the majority also demonstrating sensitivity to tree nuts. Most had specific IgE to peanut and some tree nuts as measured by the Pharmacia ImmunoCAP fluoroenzyme immunoassay (CAP-FEIA) system (Pharmacia Diagnostics, Sweden). Blood was also collected from 17 atopic, non-peanut/tree nut allergic subjects as well as 6 non-atopic subjects (6 females, 17 females; age range 26-60) exhibiting negative skin prick tests to a panel of common environmental allergens. The study was approved by the Alfred Hospital Ethics Committee and informed consent was obtained from all of the subjects before the blood was obtained. The clinical data for all subjects are summarised in Tables 2.1 and 2.2.

Table 2.1 Clinical characteristics of peanut allergic subjects (ND – not done, NA – not available)

Subject no.	Age (yrs)	Sex	Major clinical allergen	Symptoms	Nut allergies	Other food allergies	Peanut CAP-FEIA score	Almond CAP-FEIA score	Brazil CAP-FEIA score	Cashew CAP-FEIA score	Hazelnut CAP-FEIA score
A1	26	F	peanut	facial angioedema	peanut	sesame seed	3	ND	ND	1	ND
A2	29	M	peanut	GIT upset, laryngeal oedema, generalised urticaria, facial angioedema	peanut, Brazil nut, cashew, hazelnut, walnut		2	ND	0	3	ND
A3	28	F	peanut	asthma, laryngeal oedema, generalised urticaria, facial angioedema	peanut, almond		3	0	0	0	0
A4	30	M	peanut	laryngeal oedema	peanut		1	1	1	ND	1
A5	40	F	peanut	asthma, laryngeal oedema, generalised urticaria, facial angioedema	peanut, Brazil nut, hazelnut, cashew		3	0	0	0	0
A6	28	M	peanut	asthma, laryngeal oedema, generalised urticaria, facial angioedema	peanut, almond, hazelnut	avocado, sesame seed	3	ND	ND	ND	3
A7	43	F	peanut	NA	peanut		2	ND	ND	ND	ND
A8	49	F	peanut	GIT upset, asthma, laryngeal oedema, generalised urticaria	peanut, cashew, hazelnut	peas, coconut, sesame seed	5	ND	2	2	1
A9	18	F	peanut	laryngeal oedema, generalised urticaria, facial angioedema	peanut	peas, fish	6	2	2	1	3
A10	27	F	peanut	laryngeal oedema, generalised urticaria, facial angioedema	peanut, hazelnut, walnut	baked beans, sesame seed	2	3	ND	ND	4

Subject no.	Age (yrs)	Sex	Major clinical allergen	Symptoms	Nut allergies	Other food allergies	Peanut CAP-FEIA score	Almond CAP-FEIA score	Brazil CAP-FEIA score	Cashew CAP-FEIA score	Hazelnut CAP-FEIA score
A11	29	M	peanut	GIT upset, laryngeal oedema, generalised urticaria	peanut, cashew, hazelnut, macadamia		6	2	ND	2	2
A12	33	M	peanut	asthma, laryngeal oedema, facial angioedema	peanut, almond, hazelnut, walnut		3	ND	ND	ND	3
A13	25	F	peanut	asthma, laryngeal oedema, facial angioedema	peanut, almond, walnut, cashew	eggs, crustaceans	5	1	1	3	2
A14	35	M	peanut	asthma, generalised urticaria, facial angioedema	peanut, hazelnut	sesame seed	2	2	3	2	3
A15	26	M	peanut	asthma, laryngeal oedema, hypotension	peanut, almond, hazelnut, walnut, cashew, pine nut	milk, sesame seed	2	2	2	3	2
A16	34	M	peanut	GIT upset, asthma, laryngeal oedema, generalised urticaria, facial angioedema	peanut, almond, walnut, pistachio, pecan, pine nut		2	0	0	0	ND
A17	33	M	peanut	asthma, laryngeal oedema, facial angioedema	peanut	chickpea, pea	4	0	0	ND	2
A18	24	F	peanut, pistachio	GIT upset, asthma, laryngeal oedema, facial angioedema	peanut, hazelnut, pistachio	sesame seed	1	2	2	ND	3

Subject no.	Age (yrs)	Sex	Major clinical allergen	Symptoms	Nut allergies	Other food allergies	Peanut CAP-FEIA score	Almond CAP-FEIA score	Brazil CAP-FEIA score	Cashew CAP-FEIA score	Hazelnut CAP-FEIA score
A19	29	M	peanut	GIT upset, laryngeal oedema, facial angioedema	peanut, Brazil nut, almond, hazelnut, walnut, cashew, pine nut	sesame seed	3	0	0	3	2
A20	38	F	peanut	GIT upset, asthma, laryngeal oedema, hypotension	peanut, hazelnut, pine nut	sesame seed	0	0	0	0	0
A21	55	M	peanut	asthma, laryngeal oedema, facial angioedema	peanut, walnut	banana	2	1	0	1	2
A22	35	F	peanut	asthma, laryngeal oedema, generalised urticaria, facial angioedema	peanut	peas, lentils	2	0	0	0	0

CAP-FEIA scores (specific IgE): 0 – < 0.35 kUA/l
1 – 0.35 to < 0.7 kUA/l
2 – 0.7 to < 3.5 kUA/l
3 – 3.5 to < 17.5 kUA/l
4 – 17.5 to < 50 kUA/l
5 – 50 to < 100 kUA/l
6 – ≥ 100 kUA/l

Table 2.2 Clinical characteristics of atopic, non-peanut/tree nut allergic (NA) and non-atopic (NAT) subjects

Subject no.	Sex	Age (yrs)	History of peanut/tree nut allergy	History of atopy	SPT (mm)			
					Mixed pollen	HDM	Aspergillus	Cat
NA1	F	26	N	Y	10	0	0	0
NA2	M	35	N	Y	0	6	0	0
NA3	F	29	N	Y	0	15	0	6
NA4	F	26	N	Y	3	8	0	5
NA5	F	39	N	Y	*NA*	*NA*	*NA*	*NA*
NA6	F	31	N	Y	10	10	0	0
NA7	M	35	N	Y	5	8	0	6
NA8	F	26	N	Y	8	9	0	7
NA9	F	46	N	Y	10	13	0	10
NA10	F	60	N	Y	8	0	0	0
NA11	M	37	N	Y	0	16	0	0
NA12	F	31	N	Y	4	9	2	5
NA13	F	30	N	Y	10	0	0	0
NA14	F	40	N	Y	8	7	2	6
NA15	F	28	N	Y	4	0	0	0
NA16	F	26	N	Y	11	10	0	14
NA17	F	27	N	Y	0	6	0	0
NAT18	M	33	N	N	0	0	0	0
NAT19	F	35	N	N	0	0	0	0
NAT20	F	42	N	N	0	0	0	0
NAT21	M	29	N	N	0	0	0	0
NAT22	F	45	N	N	0	0	0	0
NAT23	M	30	N	N	0	0	0	0

Y – yes; **N** – no; *NA* – not available

2.5 PROTEIN CONCENTRATION DETERMINATION

The BCA Protein Assay Kit was used to determine the concentration of various protein extracts and the assay was performed according to the manufacturer's instructions. Briefly, Reagent A and Reagent B were mixed together at a ratio of 50:1 to make the working reagent. This was dispensed into a 96-well plate (200 µl/well) and 20 µl of bovine serum gammaglobulin (BSGG) standards (at the appropriate concentrations) and protein samples were added to each well. The contents of each well were mixed and the plate was incubated in the dark at 37°C for 30 minutes. The absorbance of each well was then measured at 595 nm using a Bio-Rad Microplate reader (Bio-Rad Laboratories, USA).

2.6 SDS-POLYACRYLAMIDE GEL STAINING AND WESTERN IMMUNOBLOTTING

2.6.1 SDS-PAGE and gel staining

SDS-polyacrylamide gels were used to analyse unfractionated allergen extracts and recombinant allergens. The separating and stacking gel solutions were prepared as outlined in Table 2.3. The separating gel solution was poured into 1.0 mm mini-gel cassettes (Invitrogen, USA), leaving a 2 cm gap from the top edge. The separating gel was then overlaid with H_2O until the cassette was filled and the gel was allowed to polymerise for approximately 1 hour at room temperature. Following polymerisation, H_2O was removed and a 4% stacking gel was added (Table 2.3). 10 well combs were inserted into the stacking gel which was allowed to polymerise for 1 hour at room temperature. The gels were then stored in running buffer at 4°C until use.

Table 2.3 Composition of separating gel and stacking gel solutions for casting of SDS-PAGE gels

Solution	Separating gel		Stacking gel
	14%	16%	4%
Acrylamide/BIS (ml)	7.0	8.0	1.0
Separating gel buffer (ml)	9.4	9.4	-
Stacking gel buffer (ml)	-	-	4.2
10% SDS (µl)	250	250	125
H_2O (ml)	7.7	6.7	6.3
TEMED (µl)	6.3	6.3	5.0
Ammonium persulphate (µl)	625	625	1000

Protein extracts loaded onto the gel were prepared by diluting samples with reducing sample buffer to obtain the desired protein concentrations. Samples were boiled for 5 minutes and 20-30 µl were loaded into the appropriate wells alongside 8 µl of Benchmark™ pre-stained standards. Proteins were resolved using the Xcell II Mini-Cell apparatus (Invitrogen, USA) at 125 V until dye front was approximately 2-3 mm from the bottom of the separating gel. Separated proteins were visualised by incubating gels in Coomassie brilliant blue stain for 2 hours and subsequently incubating in Coomassie destain overnight at room temperature. After washing in H_2O for 20 minutes, gels were preserved in gel drying solution for another 20 minutes and dried overnight between two sheets of clear cellophane at room temperature.

2.6.2 Serum IgE Western immunoblotting

Unfractionated allergen extracts and recombinant proteins separated on SDS-PAGE gels under reducing conditions (Section 2.6.1) were transferred onto nitrocellulose membranes at 25 V using Xcell II blotting apparatus for 2 hours (Invitrogen, USA). To ensure that the transfer was successful, nitrocellulose membranes were incubated in Ponceau stain to visualise protein bands. Once protein transfer was deemed to be successful, membranes were washed in 0.05% PBS-Tween until stain was removed. The unreacted binding sites on the nitrocellulose membranes were blocked by incubation in 10% blocking solution for at least 1 hour and the membranes were subsequently washed once in 0.05% PBS-Tween and twice in PBS, 5 minutes for each wash. The IgE reactivity of proteins was determined by incubating the membranes in subject and control sera diluted 1/5 with 0.5% BSA in PBS overnight at room temperature. Washing steps were repeated as described above and the membranes were incubated in rabbit polyclonal anti-human IgE (1/500) and HRP-labelled goat anti-rabbit IgG antibodies (1/2000) each for 1 hour with washes conducted in between incubations

as described above. IgE binding was detected by dissolving 0.38 g of the substrate 4-chloro-1-napthol in 10 ml of methanol. This was added to 40 ml of PBS (pre-warmed at 37°C) together with 15 µl of hydrogen peroxide. The solution was poured over the membranes and the reaction was stopped by washing membranes in Milli-Q water (H_2O).

2.7 SERUM IgE ELISA

Allergen extracts and recombinant allergens were diluted to a concentration of 1 µg/ml using ELISA coating buffer, dispensed into 96 well polystyrene plates (50 µl/well), and incubated overnight at 4°C. Plates were washed with 0.05% PBS-Tween and blocked with 5% blocking solution (200 µl/well) for 1 hour at 37°C. After washing 5 times with 0.05% PBS-Tween, 50 µl of subject and control sera, diluted 1/10 with 1% blocking solution, were added to the wells and incubated at 37°C for 2 hours. Plates were washed 5 times with 0.05% PBS-Tween and incubated with rabbit polyclonal anti-human IgE antibody (1/1000; 50 µl/well) for 1 hour at 37°C, followed by HRP-labelled goat anti-rabbit IgG antibody (1/1000; 50 µl/well), incubated similarly for 1 hour at 37°C, with washes conducted in between incubations as described above. IgE binding was detected by dissolving 1 OPD tablet in 10 ml 0.05 M phosphate-citrate buffer and dispensing 50 µl of this solution in each well. The reaction was stopped after 10 minutes with the addition of 4 M hydrochloric acid (50 µl/well) and the absorbance (OD) in each well was measured at 490 nm. The testing of subject sera was performed in triplicates and the absorbance in control wells containing no antigen was subtracted from the absorbance in antigen coated wells to account for non-specific binding.

2.8 ANALYSIS OF BASOPHIL ACTIVATION BY FLOW CYTOMETRY

2.8.1 Activation of basophils in whole blood

Approximately 20 ml of blood was collected from peanut allergic subjects and control subjects using heparinised tubes. 100 µl of whole blood were placed in a FACS tube and 20 µl of stimulation buffer containing IL-3 and heparin were added to each tube and incubated at 37°C for 10 minutes. Allergen challenge was performed with the addition of 100 µl of allergen extract (diluted with stimulation buffer containing heparin and IL-3 to obtain the desired concentration) to each tube followed by incubation at 37°C for 20 minutes. In some experiments, the cells were also stimulated with 100 µl rabbit anti-human IgE antibody (diluted 1/1000 in stimulation buffer containing IL-3 and heparin) and fMLP (diluted 1/200 in stimulation buffer containing IL-3 and heparin) as positive controls. Activation of basophils was stopped by incubating cells on ice for 5 minutes.

2.8.2 Fluorescent labeling of cells

Following allergen challenge, cells were incubated with normal goat serum on ice for 10 minutes to reduce subsequent non-specific binding of labelled antibodies. Cells were stained with PE-conjugated anti-human CD63 and FITC-conjugated anti-human IgE at the previously optimised antibody dilutions and subsequently incubated on ice for 20 minutes, in the dark. B cells and monocytes present in the cell suspension were detected by staining cells with APC-conjugated anti-human CD19 and APC-conjugated anti-human CD14, respectively, at the previously optimised antibody dilutions. Controls for antibody isotype non-specific binding were also included by staining cells with the relevant labelled isotype control antibodies. Red blood cells were lysed by

adding FACS red cell lysis buffer (2 ml/tube) and lysis was allowed to proceed for 10-15 minutes at room temperature. Cells were pelleted by centrifugation at 250 x g for 5 minutes (4°C) and washed once with FACS wash buffer (3 ml/tube) followed by centrifugation as described above. Cell pellets were resuspended in 150 µl of FACS wash buffer per tube and 7AAD was added to the cells to excluded non-viable cells. Flow cytometric analysis was performed using FACScalibur (Becton Dickinson, USA) and Cell Quest software (Becton Dickinson, USA). Approximately 300000 total events were collected per test to obtain sufficient numbers of basophils for analysis. The gating of $CD63^+$ cells was based on the discrimination of the negative control staining (no antigen control) and positive control staining (fMLP and anti-IgE stimulation).

CHAPTER 3

CHARACTERISATION OF PEANUT AND TREE NUT EXTRACTS

3.1 INTRODUCTION

Peanut and tree nuts are a common cause of fatal and near-fatal food-induced anaphylaxis in sensitive individuals. Tree nuts that have been shown to be allergenic include almond, Brazil nut, cashew and hazelnut. Peanut allergy is a more frequent presentation than tree nut allergy but co-sensitisation to both is a common clinical observation. The focus of this project is to determine whether or not co-sensitisation to peanuts and tree nuts is due to cross-reactive allergens.

This chapter details the preparation and characterisation of unfractionated peanut, almond, Brazil nut, cashew and hazelnut extracts which were used in the assays outlined in subsequent chapters. The raw and roasted forms of these extracts were analysed for IgE reactivity with particular focus on the effects of heating on the IgE binding properties of allergenic proteins. The presence of previously identified peanut allergens in the unfractionated peanut extract preparation was also established. Finally, an effector cell-based *in vitro* test, the basophil activation test, was utilised to confirm that these peanut and tree nut allergen preparations were biologically active.

3.2 METHODS

3.2.1 Preparation of unfractionated peanut and tree nut extracts

Ten grams of commercially available almonds, Brazil nuts, cashews, hazelnuts and peanuts (raw or roasted at 180°C for 15 minutes) were crushed and defatted by adding 25 ml of acetone. Contents were mixed and centrifuged at 1000 x g and the pellet was resuspended in 25 ml of diethyl ether. This procedure was repeated 5 times. After the final extraction, the crushed product was separated by vacuum filtration and dried for 10 minutes under vacuum. The dried defatted nut product was then ground to powder using liquid nitrogen and incubated overnight in 30 ml of PBS with Complete™ protease inhibitor cocktail at 4°C, with shaking. The extract was further centrifuged at 1000 x g to pellet debris and at 20000 x g to obtain a clear supernatant. The protein concentration of each extract was determined as outlined in Section 2.5 and extracts were stored in 1 ml aliquots at -20°C.

3.2.2 SDS-PAGE and Western immunoblotting of peanut and tree nut extracts

Raw and roasted peanut and tree nut extracts were analysed by SDS-PAGE using 16% gels. Approximately 30 µg of raw and roasted almond, Brazil nut, cashew, hazelnut and peanut extract were loaded per lane and proteins were resolved and either stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes as outlined in Sections 2.6.1 and 2.6.2. Membranes were incubated with peanut allergic subject sera (diluted 1/5) and IgE binding was detected as described in Section 2.6.2.

3.2.3 Titration of serum IgE against peanut and tree nut extracts by ELISA

Ninety-six well polystyrene plates were coated with raw and roasted almond, Brazil nut, cashew, hazelnut and peanut extracts as outlined in Section 2.7. Patient sera were diluted 1/5, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320 and 1/640 in 1% blocking solution and added to antigen coated wells (50 µl/well). IgE binding to the peanut and tree nut extracts was subsequently measured as outlined in Section 2.7.

3.3 RESULTS

3.3.1 SDS-PAGE analysis and IgE reactivity of unfractionated raw and roasted peanut extracts by Western immunoblotting

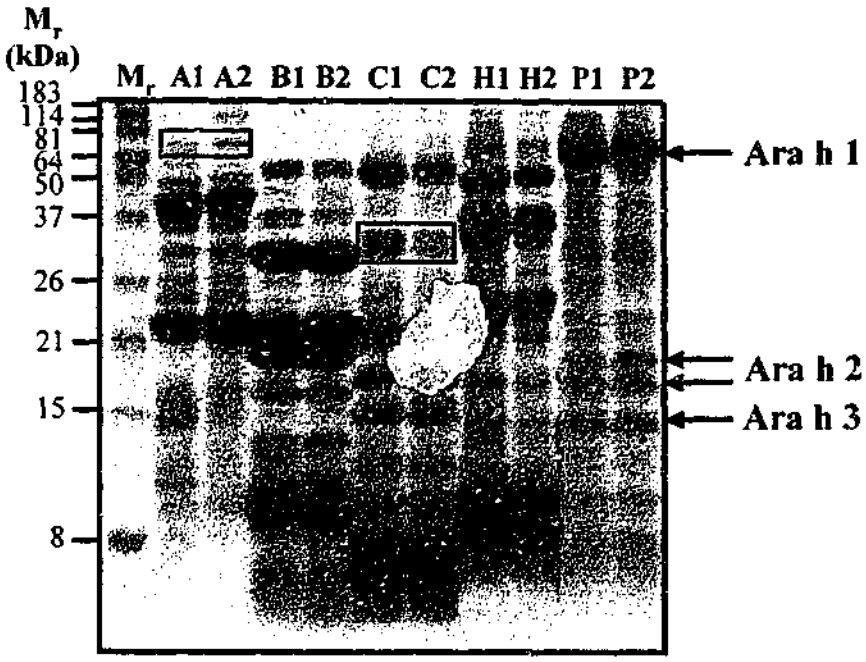
Unfractionated raw and roasted peanut and tree nut extracts were prepared as outlined in Section 3.2.1 and analysed by SDS-PAGE. Figure 3.1a is a Coomassie-stained gel of raw and roasted almond, Brazil nut, cashew, hazelnut and peanut extracts, revealing numerous protein bands for each extract. A comparison of the protein profiles of the raw and roasted peanut and tree nut extracts shows very little difference in content between the two forms. Roasting appears to have minimal effect on the protein profile of the peanut and tree nut extracts, with the exception of a few protein bands (indicated by black boxes).

The IgE reactivity of raw and roasted peanut and tree nut proteins was analysed by Western immunoblotting using sera from 2 subjects demonstrating both peanut and tree nut sensitisation (Figure 3.1b and 3.1c). As indicated by black boxes, some proteins

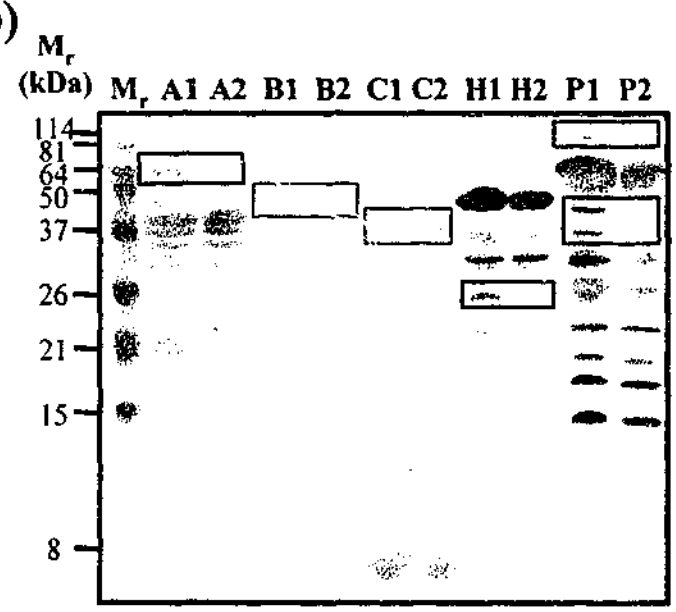
Figure 3.1 SDS-PAGE and Western immunoblot analysis of raw and roasted peanut and tree nut proteins

Following 16% SDS-PAGE separation of unfractionated raw and roasted peanut and tree nut extracts, proteins were electroblotted onto nitrocellulose membranes and subsequently probed with peanut and tree nut allergic subject sera and atopic, non-peanut/tree nut allergic subject serum. **(a)** Coomassie stained gel of raw and roasted almond, Brazil nut, cashew, hazelnut and peanut extracts. IgE binding to raw and roasted peanut and tree nut proteins was demonstrated using sera from 2 peanut and tree nut allergic subjects, A14 **(b)** and A19 **(c)**. Minimal IgE binding was observed when nitrocellulose membrane was incubated in serum from an atopic, non-peanut/tree nut allergic subject (NA1) **(d)**. Membrane was also incubated in 0.5% BSA in PBS which served as the secondary and tertiary detection antibody control **(e)**. M indicates position of molecular mass markers (M_r).

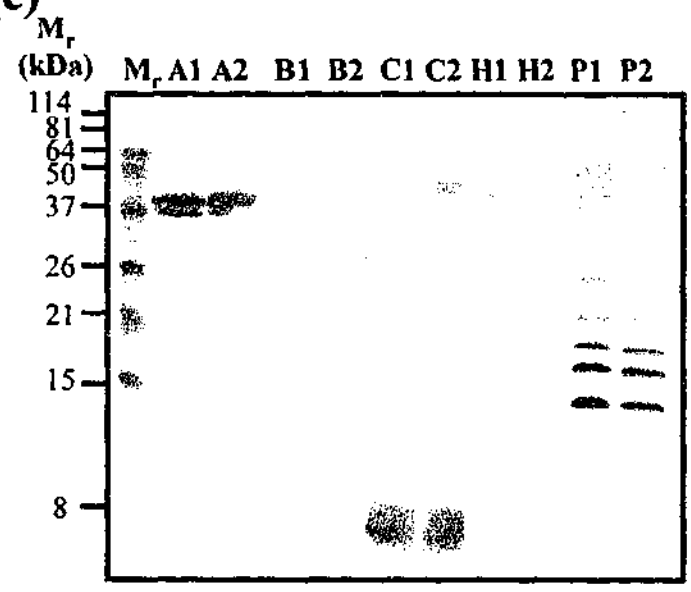
(a)



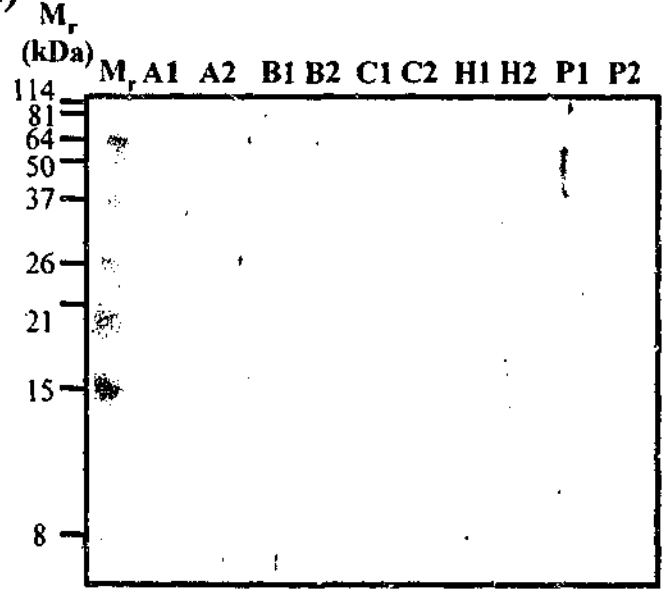
(b)



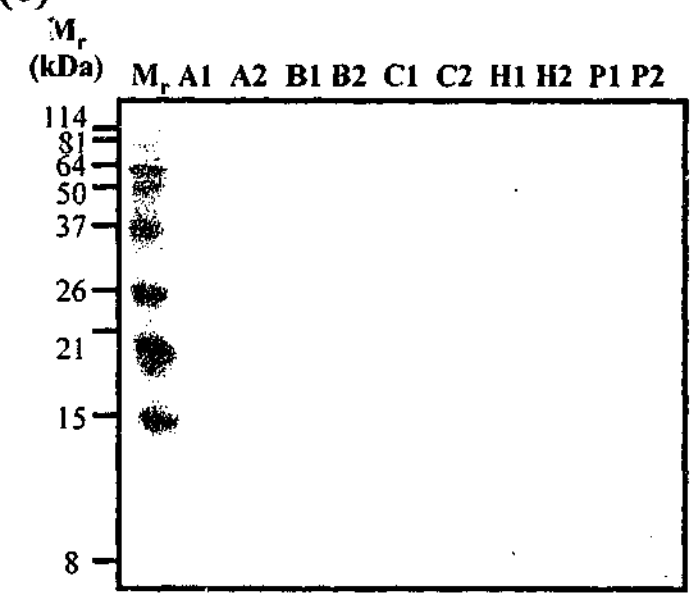
(c)



(d)



(e)



M_r – markers
A – almond
B – Brazil nut
C – cashew
H – hazelnut
P – peanut

1 – raw
2 – roasted

present in almond, hazelnut and peanut appeared to lose IgE reactivity following roasting. With other proteins present in almond and cashew extracts, roasting appeared to enhance allergenicity, as indicated by red boxes. In both cases, the identity of the proteins is not known. It can also be seen that the IgE reactivity of peanut proteins with molecular masses similar to those previously reported for the peanut allergens Ara h 1, Ara h 2 and Ara h 3 are unaffected by roasting. Minimal IgE binding to raw and roasted peanut and tree nut proteins was observed with the atopic, non-peanut/tree nut allergic control subject (Figure 3.1d) and the no serum control (Figure 3.1e). In general, roasting appears to have very little effect on the overall IgE binding to proteins from almond, Brazil nut, cashew, hazelnut and peanut as assessed by Western immunoblotting.

3.3.2 IgE reactivity of unfractionated raw and roasted peanut and tree nut extracts by ELISA

IgE binding to raw and roasted almond, Brazil nut, cashew, hazelnut and peanut was assessed quantitatively by ELISA to compare with the results obtained from the Western immunoblotting studies. As shown in Figure 3.2, there were minimal differences in the level of IgE binding between the raw and roasted forms of almond, Brazil nut, cashew, hazelnut and peanut extracts using sera from two subjects demonstrating both peanut and tree nut sensitisation. Again, negligible IgE binding to the peanut and tree nut extracts was observed for the atopic, non-peanut/tree nut allergic control subjects (Figure 3.2c and 3.2d). Titration of sera from the same two peanut and tree nut allergic subjects (A14 and A19) also did not show any differences in IgE binding to the raw and roasted peanut and tree nut extracts (Figure 3.3). These results

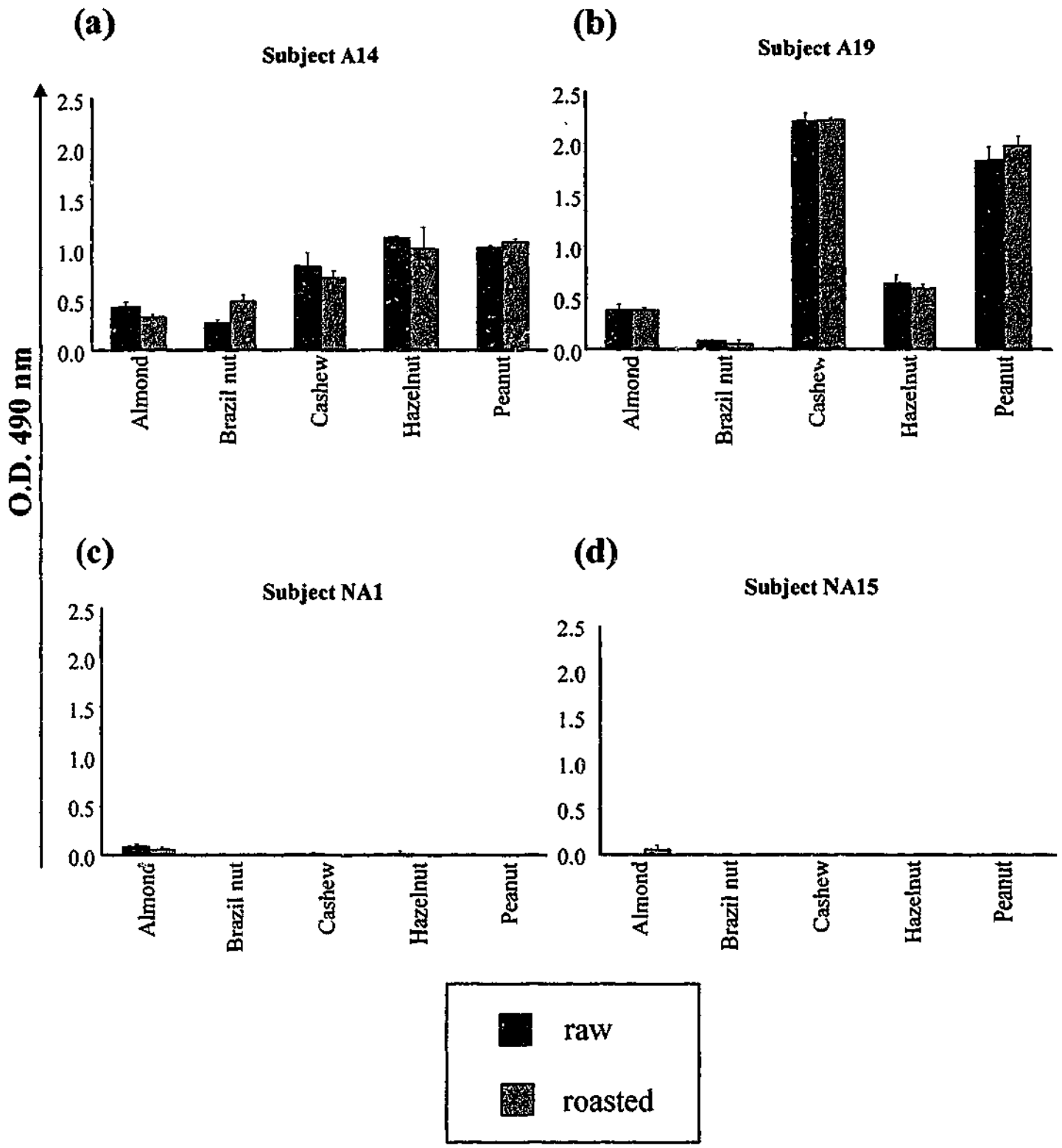
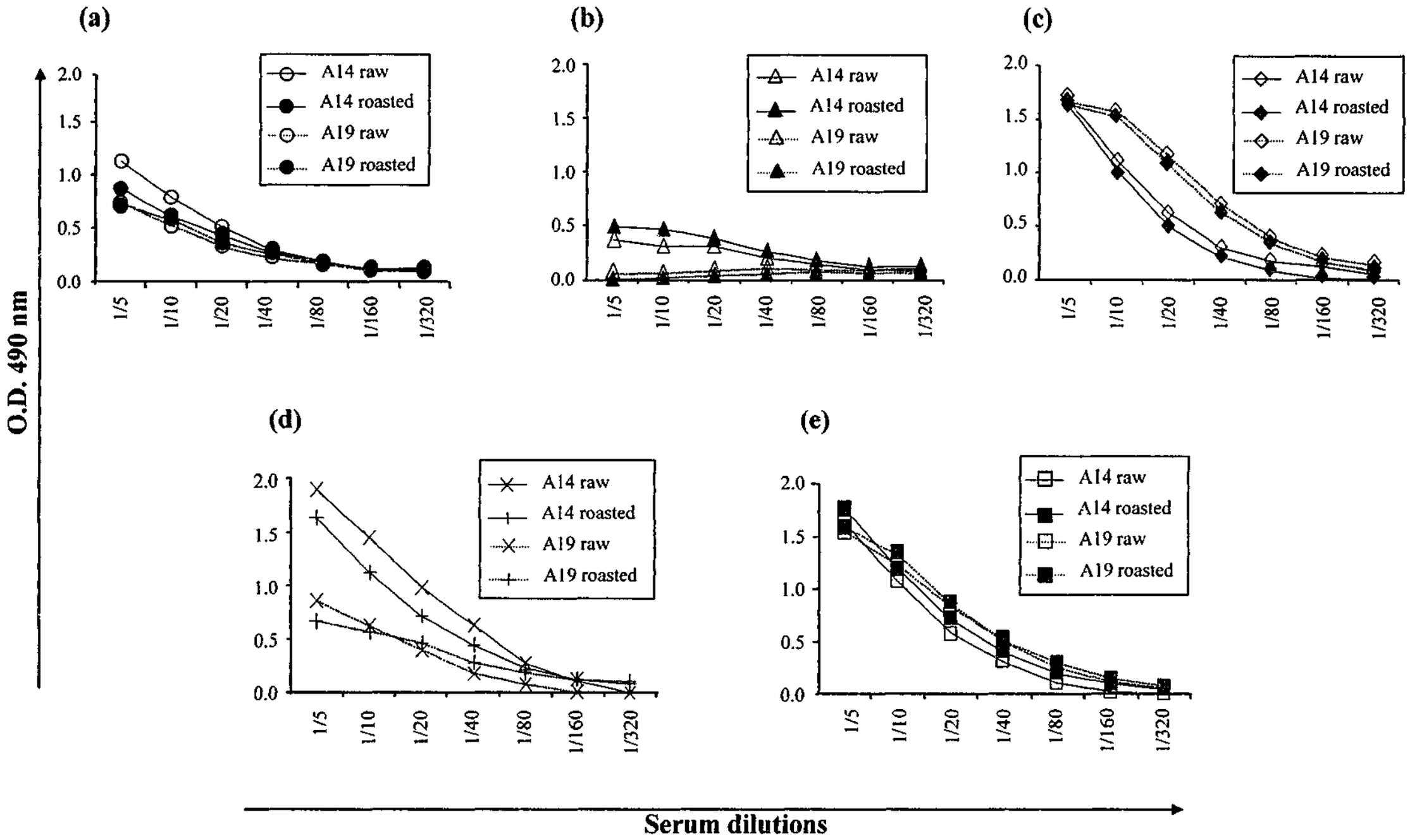


Figure 3.2 IgE binding to raw and roasted peanut and tree nut extracts by ELISA

ELISA plates were coated with 1μg/ml of raw and roasted almond, Brazil nut, cashew, hazelnut and peanut extracts. IgE binding was assessed for 2 peanut and tree nut allergic subjects, **(a)** A14 and **(b)** A19, and 2 atopic, non-peanut/tree nut allergic subjects, **(c)** NA1 and **(d)** NA15. The absorbance in control wells containing no antigen was subtracted from antigen coated wells. Mean values for triplicates are shown and the standard deviation is indicated by error bars.

Figure 3.3 ELISA for titration of serum IgE against raw and roasted nut extracts

ELISA plate was coated with raw and roasted **(a)** almond, **(b)** Brazil nut, **(c)** cashew, **(d)** hazelnut and **(e)** peanut extracts. Sera from subjects A14 and A19 were serially diluted and IgE binding was assessed. Mean values for triplicates are shown. The absorbance in control wells containing no antigen was subtracted from antigen coated wells.


(a)
(b)
(c)
(d)
(e)
A14 raw
A14 roasted
A19 raw
A19 roasted
2.0
1.5
1.0
0.5
0.0
1/5
1/10
1/20
1/40
1/80
1/160
1/320
O.D. 490 nm
Serum dilutions

further confirm that roasting has minimal effect on IgE binding to peanut and tree nut proteins.

3.3.3 Analysis of unfractionated peanut extract by SDS-PAGE and Western immunoblotting

Unfractionated roasted peanut extract was analysed by SDS-PAGE and Western immunoblotting to confirm the presence and IgE reactivity of peanut allergens that have been previously identified by others (Burks *et al.*, 1991, Burks *et al.*, 1992, Eigenmann *et al.*, 1996). The unfractionated peanut extract resolved by SDS-PAGE and visualised with Coomassie brilliant blue staining (Figure 3.1) revealed the existence of numerous proteins, some of which were at positions corresponding to the molecular masses of previously identified peanut allergens. The major peanut allergen, Ara h 1, is the most abundant protein in crude peanut extract and is identified as a large, intensely staining band at approximately 63 kDa which has been previously reported (Burks *et al.*, 1991). The other major peanut allergen, Ara h 2, is present as a double band with a molecular mass ranging from 17-19 kDa, which was first reported by Burks and colleagues (1992). The *N*-terminal breakdown product of Ara h 3 can also be visualised at approximately 14 kDa, as described by Eigenmann *et al.* (1996) and Rabjohn *et al.* (1999). Bands corresponding to other peanut proteins can also be seen but the identity of these has not been established.

The IgE reactivity of the previously described peanut allergens was also analysed by Western immunoblotting. As shown in Figure 3.1b and c, IgE binding to Ara h 1, Ara h 2 and Ara h 3 was demonstrated using sera from two peanut allergic subjects, thus confirming the status of these proteins as allergens. IgE antibodies also bound to other

peanut proteins, indicating the existence of other peanut allergens. The specificity of this IgE binding was confirmed by minimal staining on the atopic, non-peanut/tree nut allergic and the no serum control blots (Figure 3.1d and e).

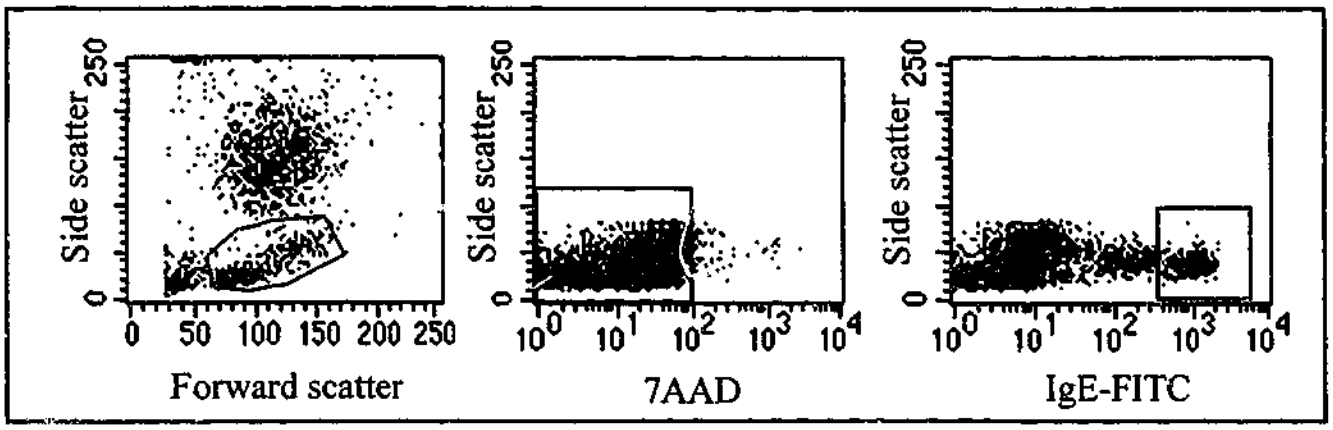
3.3.4 Activation of basophils by peanut and tree nut extracts

The ability of the peanut and tree nut extracts to activate basophils was assessed by flow cytometry. The activation of basophils was detected via CD63 expression following stimulation with allergen extracts. Whole blood from a peanut allergic subject (A14) was challenged with allergen and stained with anti-IgE FITC and anti-CD63 PE. In these experiments, the form (raw or roasted) in which the extracts were used was determined according to that most commonly consumed. Figure 3.4 shows a typical flow cytometric analysis of CD63 expression by basophils. All flow cytometry profiles were based on a 'lymphocyte-monocyte' gate as determined by forward scatter versus side scatter (Figure 3.4a) which was validated as a gate containing basophils in previous back gating studies. Live cells within this gate were selected via 7AAD exclusion (Figure 3.4a) and expression of IgE was analysed (Figure 3.4a). IgE^{hi} cells were selected and activation of basophils was analysed by the expression of CD63. B cells and monocytes were shown to be excluded from the IgE^{hi} cell population by CD19 and CD14 staining, respectively (data not shown). Figure 3.4b is a representative analysis of basophil activation following incubation of whole blood from a peanut, almond, Brazil nut, cashew and hazelnut allergic subject (as demonstrated by positive CAP-FEIA scores) with roasted almond, raw Brazil, roasted cashew, roasted hazelnut and roasted peanut extracts. It can be seen that the incubation of whole blood with 1 μg/ml of peanut and tree nut extract resulted in the activation of between 25-88% of basophils

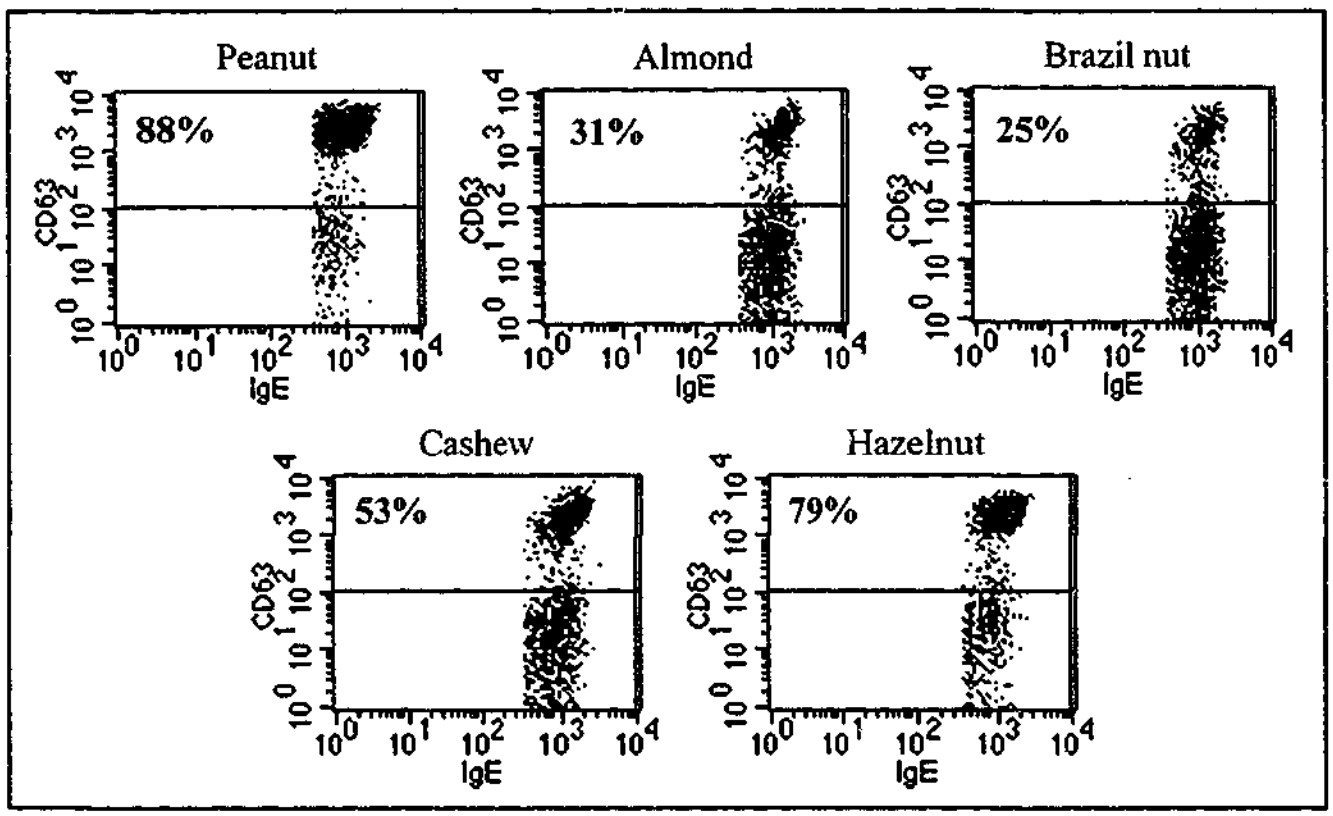
Figure 3.4 Analysis of basophil activation in a peanut, almond, Brazil nut, cashew and hazelnut allergic subject following incubation of whole blood with peanut and tree nut extracts

Whole blood from a peanut, almond, Brazil nut, cashew and hazelnut allergic subject (A14) was incubated with 1 μg/ml of roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut and roasted peanut extract. Activation of basophils as indicated by CD63 expression was analysed as follows: **(a)** Cells were gated based on forward scatter and side scatter. Live cells within this gate were selected via 7AAD exclusion and cells were analysed for high expression of IgE. **(b)** IgE^{hi} cells were analysed for CD63 expression following incubation with peanut and tree nut extracts and the percentage of activated basophils (upper quadrant) was calculated. **(c)** A no antigen negative control was included in the assay to ascertain the percentage of spontaneously activated basophils (upper quadrant). Whole blood was also incubated with fMLP and anti-IgE antibody as positive controls. $CD63^{+}$ cells were gated based on the discrimination of negative and positive control staining.

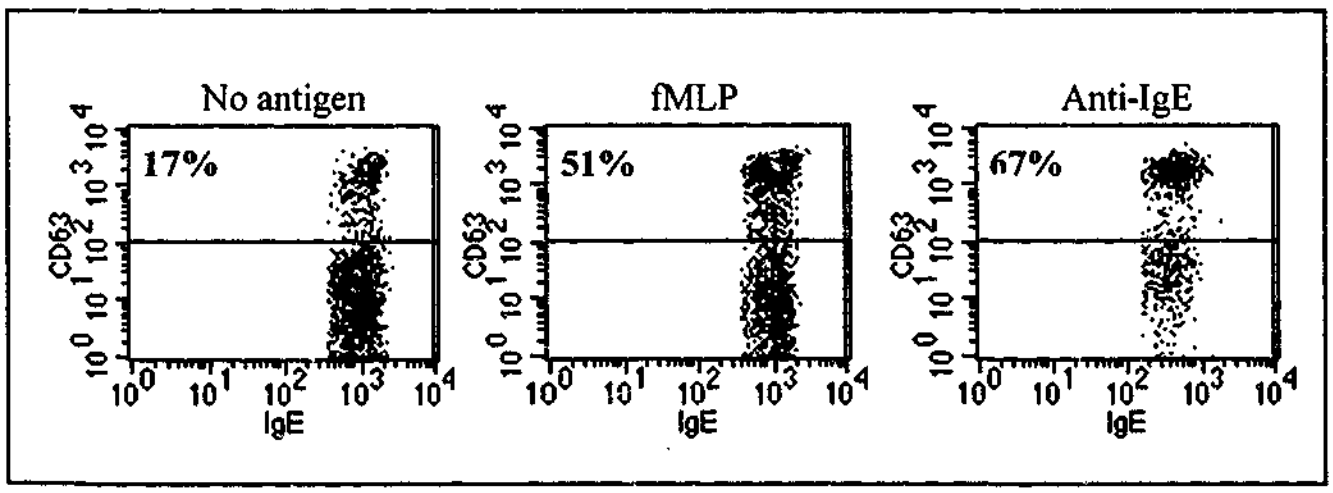
(a)


Side scatter
250
0
0 50 100 150 200 250
Forward scatter
Side scatter
250
0
10^0 10^1 10^2 10^3 10^4
7AAD
Side scatter
250
0
10^0 10^1 10^2 10^3 10^4
IgE-FITC

(b)


Peanut
88%
Almond
31%
Brazil nut
25%
Cashew
53%
Hazelnut
79%
CD63
IgE
10^0 10^1 10^2 10^3 10^4

(c)


No antigen
17%
fMLP
51%
Anti-IgE
67%
CD63
IgE
10^0 10^1 10^2 10^3 10^4

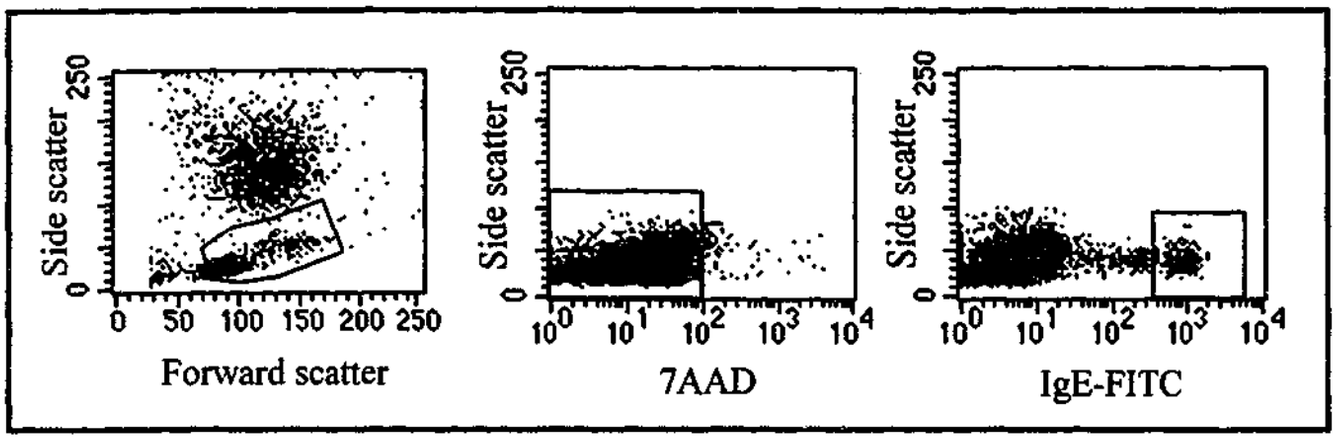
(Figure 3.4b), with the no antigen negative control demonstrating 17% activation (Figure 3.4c).

As a negative control, whole blood from a house dust mite (HDM) allergic, non-peanut/tree nut allergic control subject (NA3) was incubated with the same concentration of peanut and tree nut extract which resulted in minimal basophil activation (1%; Figure 3.5b) which was similar to the no antigen negative control (2%; Figure 3.5c). Incubation of whole blood with the HDM positive control extract (1 μg/ml) resulted in the activation of 73% of basophils (Figure 3.5b). Additional positive controls that were used in this assay included incubation of whole blood with *N*-formyl-Met-Leu-Phe (fMLP) peptides and rabbit anti-IgE antibodies (Figures 3.4c and 3.5c). Anti-IgE was used to demonstrate the expression of CD63 following cross-linking of surface IgE on basophils whereas stimulation with fMLP demonstrated IgE independent CD63 expression, confirming the viability and functionality of the basophils present in patient whole blood.

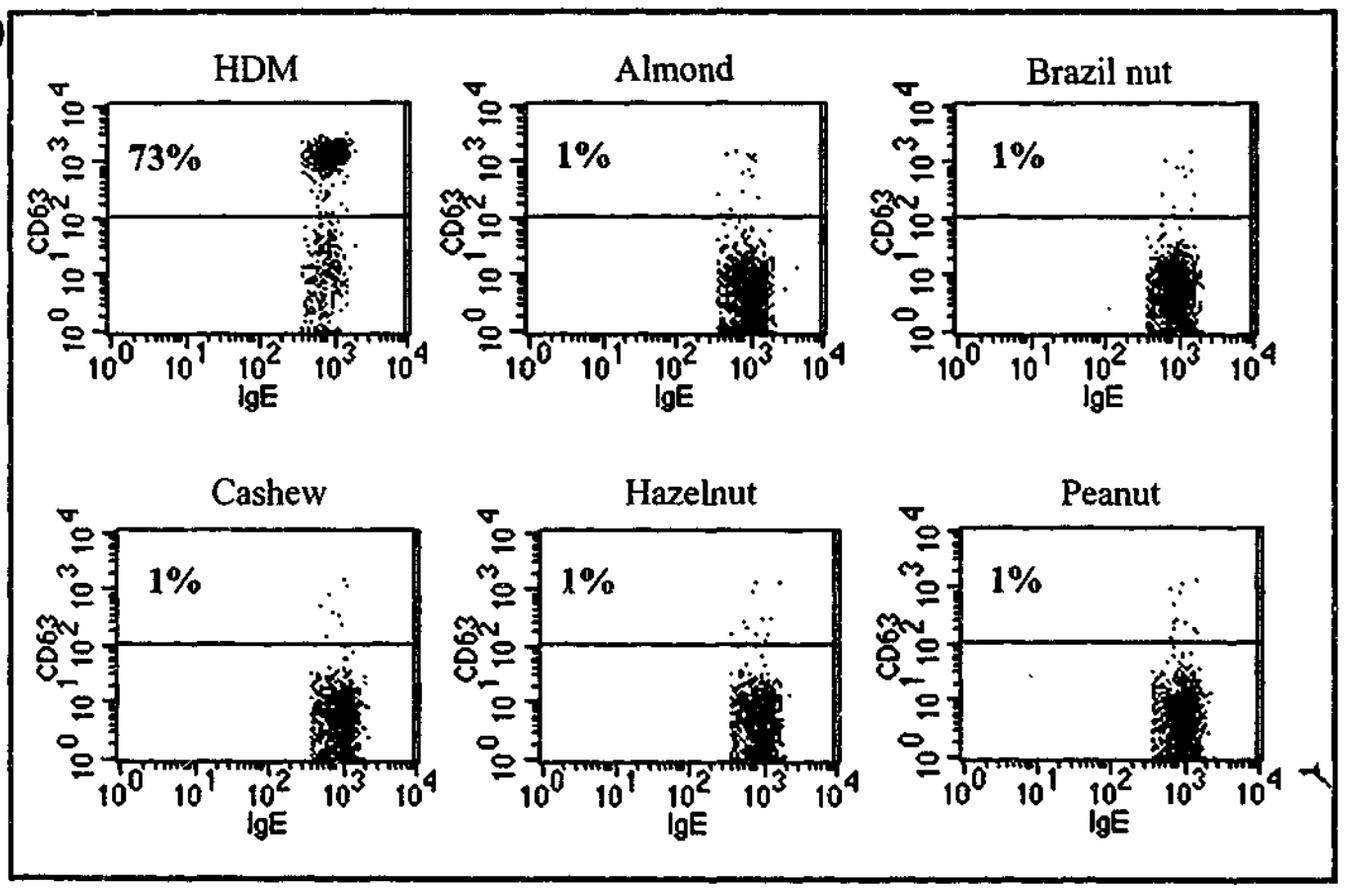
Figure 3.5 Analysis of basophil activation in a house dust mite, non-nut allergic subject following incubation of whole blood with peanut and tree nut extracts

Whole blood from a house dust mite (HDM), non-peanut/tree nut allergic subject (NA3) was incubated with 1 µg/ml of roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut, roasted peanut and HDM extract (positive control). Activation of basophils as indicated by CD63 was analysed as follows: **(a)** Cells were gated based on forward scatter and side scatter. Live cells within this gate were selected via 7AAD exclusion and cells were analysed for high expression of IgE. **(b)** IgE^{hi} cells were analysed for CD63 expression following incubation with peanut and tree nut extracts and the percentage of activated basophils (upper quadrant) was calculated. **(c)** A no antigen negative control was included in the assay to ascertain the percentage of spontaneously activated basophils (upper quadrant). Whole blood was also incubated with fMLP and anti-IgE as positive controls. $CD63^{+}$ cells were gated based on the discrimination of negative and positive control staining.

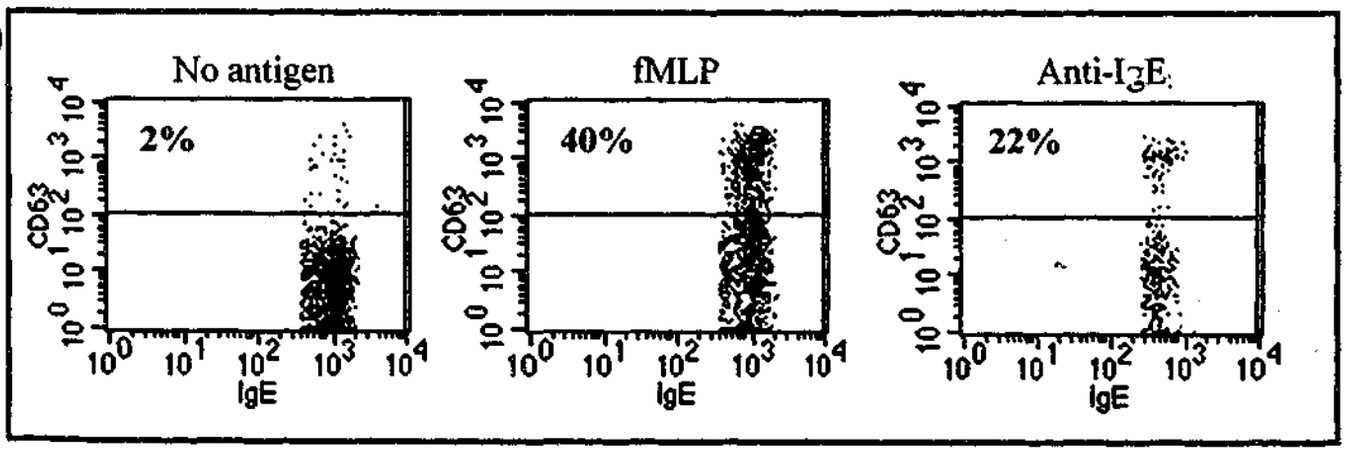
(a)


Side scatter
250
0
0 50 100 150 200 250
Forward scatter
Side scatter
250
0
10^0 10^1 10^2 10^3 10^4
7AAD
Side scatter
250
0
10^0 10^1 10^2 10^3 10^4
IgE-FITC

(b)


HDM
73%
Almond
1%
Brazil nut
1%
Cashew
1%
Hazelnut
1%
Peanut
1%
CD63
IgE
10^0 10^1 10^2 10^3 10^4

(c)


No antigen
2%
fMLP
40%
Anti-IgE
22%
CD63
IgE
10^0 10^1 10^2 10^3 10^4

3.4 DISCUSSION

This chapter has described the preparation and characterisation of raw and roasted almond, Brazil nut, cashew, hazelnut and peanut extracts. The effect of heat treatment on the allergenicity of the peanut and tree nut extracts was first investigated. As assessed by Western immunoblotting and ELISA, minimal differences in IgE binding to raw and roasted peanut and tree nut proteins were observed. This was also reported by Kleber-Janke and colleagues (1999) for peanut extract whereby roasting of peanuts (160°C, 40 minutes) did not significantly alter IgE binding to peanut proteins. Almond proteins from raw and roasted extracts have also been demonstrated to be equally effective at binding serum IgE from almond allergic subjects, although an increase in IgE binding was observed by prolonged roasting at high temperatures (Venkatachalam *et al.*, 2002).

Other studies, however, have reported an increase in the allergenicity of peanut proteins upon heat treatment. These studies have focused on the Maillard reaction, a non-enzymatic reaction between a protein and a reducing sugar that occurs during thermal processing and cooking (Namiki, 1988). The amino groups of proteins become glycosylated, forming Amadori products, which degrade into dicarbonyl intermediates. These intermediary compounds react with amino groups of proteins to form stable end-products known as advanced glycation end products (AGE) which are thought to be allergenic. A number of studies have identified the Maillard reaction as a contributing factor to the allergenicity of peanuts (Chung and Champagne, 1999, Maleki *et al.*, 2000a, Chung *et al.*, 2002). Chung and Champagne (1999) demonstrated that AGEs, formed by heating a non-allergenic peanut protein such as lectin in the presence of

sugars, can effectively compete with untreated peanut allergens for serum IgE antibodies. This suggests that the Maillard reaction can convert a non-allergenic protein into a potentially allergenic protein. Similarly, Maleki and colleagues (2000a) observed that roasted peanut proteins inhibited IgE binding to raw peanut proteins more effectively (90-fold higher) than raw peanut proteins. Both studies attributed this finding to the presence of AGEs in heat-treated peanuts that contribute to its overall allergenicity.

Discrepancies between the results from the above studies and those obtained in this study may be due to the different methods used for the thermal processing of peanuts. Chung and Champagne (1999) and Maleki and colleagues (2000a) carried out Maillard reactions by heating peanut proteins at 50-55°C in the presence of sugars such as glucose, fructose, mannose, xylose and arabinose, some of which are known to be present in peanuts. In contrast, the peanuts used in this study were heated at 180°C for 10 minutes. Also, this study compared the IgE reactivity of raw and roasted peanut extracts using direct IgE binding assays which examined whether there was any increase or decrease in IgE binding. The previously mentioned studies utilised competitive IgE-binding assays which assessed the affinity of IgE antibodies for proteins in heat-treated and untreated peanut proteins. It is also not known whether there are any differences in the biological activity of raw and roasted peanut and tree nut extracts as determined by effector cell function. This should be considered in future studies to provide further insight into the effects of heat treatment on the allergenicity of peanut and tree nut proteins.

The results from this study indicate that the IgE-binding epitopes of the majority of peanut and tree nut allergens are heat-stable. This was demonstrated clearly by

Koppelman and colleagues (1999) for Ara h 1 whereby purified Ara h 1 from peanuts heat-treated at temperatures ranging from 50°C-200°C for 15 minutes, was shown to have similar IgE-binding properties to Ara h 1 from untreated peanuts. However, the latter study also demonstrated using spectroscopic measurements that Ara h 1 undergoes significant heat-induced denaturation at the molecular level, suggesting that conformational epitopes may be less relevant for IgE binding to this allergen or that the epitopes are located in areas of the molecule that are heat-stable (Koppelman *et al.*, 1999). Whether other peanut and tree nut allergens exhibit similar conformational changes upon heating is not known and requires further investigation.

The unfractionated peanut extract prepared for use in this study and in subsequent chapters was also shown by SDS-PAGE to contain protein bands with molecular masses corresponding to the previously reported peanut allergens Ara h 1, Ara h 2 and Ara h 3. These allergens also bound serum IgE antibodies from peanut allergic subjects. Both Ara h 1 and Ara h 2 have been classified as major peanut allergens with studies suggesting that these two allergens are recognised in peanut extract by approximately 70-90% of peanut allergic individuals (Burks *et al.*, 1991, Burks *et al.*, 1992, Clarke *et al.*, 1998) although IgE reactivity to Ara h 1 can be as low as 35% (de Jong *et al.*, 1998). IgE reactivity to the *N*-terminal breakdown product of Ara h 3 (~14 kDa) has been shown to occur in only 36% of peanut allergic individuals thus classifying this protein as a minor allergen (de Jong *et al.*, 1998). The frequency of reactivity to these peanut allergens within a population of peanut allergic patients was assessed in this study with similar findings (Chapter 5). For these reasons, the unfractionated peanut extract preparation described in this chapter was deemed satisfactory for use in subsequent cross-reactivity studies.

In addition to direct IgE binding ELISA, an effector cell-based *in vitro* test was used to further characterise the peanut and tree nut extracts. The basophil activation test uses CD63 expression as a marker of activated basophils which can be detected by flow cytometry following allergen challenge. This test has previously been utilised as a tool for diagnosing immediate type allergy to foods such as carrot, celery and hazelnut (Erdmann *et al.*, 2003). In this chapter, the basophil activation test was used as a biologically relevant IgE binding assay for the characterisation of allergen preparations. Using this test, the unfractionated peanut and tree nut preparations were shown to activate basophils from a subject demonstrating sensitisation to both peanuts and tree nuts but not from a HDM, non-peanut/tree nut allergic subject. The ability of these peanut and tree nut extracts to activate basophils from a larger population of peanut and tree nut allergic subjects and atopic, non-peanut/tree nut allergic subjects was also assessed in this study and will be discussed in Chapter 5. That the peanut and tree nut extracts did not activate basophils from a non-peanut/tree nut allergic subject indicates that the extracts did not directly induce activation in a non-IgE dependent manner. It is particularly important to establish this to ascertain the clinical relevance of these allergen preparations. Ideally, skin prick tests using these extracts would be conducted, however, this carries the risk of anaphylaxis in sensitive individuals. Consequently, effector cell-based assays such as the basophil activation test provide a much safer option.

In conclusion, almond, Brazil nut, cashew, hazelnut and peanut extracts were produced and subsequently shown to be IgE reactive, making them useful allergen preparations for the investigation of IgE cross-reactivity between peanut and tree nuts. Basophil activation tests also demonstrated that these antigen preparations were capable of activating basophils from a peanut and tree nut allergic subject but not a non-peanut/tree

nut allergic subject, thus confirming the immunological relevance of the allergens present in these extracts.

CHAPTER 4

CLONING AND CHARACTERISATION OF RECOMBINANT PEANUT ALLERGENS

4.1 INTRODUCTION

The increasing prevalence of peanut allergy within the general population and the potential severity of the allergic reactions have highlighted the need to understand the mechanisms behind this type of food allergy. Initial studies focused on the identification of the allergens responsible for inducing hypersensitivity reactions in peanut-sensitive subjects. Several allergens have been identified, however, only three of these have been well characterised. Ara h 1 and Ara h 2 were the first peanut allergens to be identified and were subsequently classified as major allergens with >90% reactivity in peanut allergic subjects (Burks *et al.*, 1991, Burks *et al.*, 1992, Burks *et al.*, 1995, Kleber-Janke *et al.*, 1999). In contrast, Ara h 3 has been classified as a minor peanut allergen, with approximately 44% reactivity in peanut allergic subjects (Eigenmann *et al.*, 1996, Rabjohn *et al.*, 1999). The extent to which these peanut allergens contribute to IgE cross-reactivity between peanut and tree nuts is evaluated in this project.

This chapter details the production of recombinant peanut allergens for use in cross-reactivity studies. The major peanut allergens, Ara h 1 and Ara h 2, were cloned from peanut cDNA using PCR technology. In contrast, Ara h 3 was previously isolated as an

IgE-binding clone following immunoscreening of a λgt11 peanut cDNA library using serum from a peanut allergic subject (de Leon, 1999). All three recombinant allergens were expressed using a prokaryotic expression system. These recombinant allergens were characterised for IgE reactivity using IgE-binding assays such as Western immunoblotting and ELISA. The basophil activation test was utilised to assess the biological activity of these recombinant allergen preparations by measuring *in vitro* activation of basophils from peanut allergic subjects.

4.2 METHODS

4.2.1 PCR amplification of cDNA encoding Ara h 1 and Ara h 2 from peanut cDNA

Primers incorporating restriction enzyme sites for directional subcloning were designed based on the published sequences of Ara h 1 (Burks *et al.*, 1995) and Ara h 2 (Stanley *et al.*, 1997) (Table 4.1). These primers were used to amplify the corresponding cDNA from various peanut cDNA preparations that were previously made (de Leon, 1999). PCR reactions were set up as outlined in Table 4.2 and loaded onto a PCR thermal cycler (Perkin Elmer, USA). A negative control reaction containing no cDNA template was also included for each PCR reaction.

4.2.2 DNA agarose gel electrophoresis

PCR and restriction enzyme digest products were visualised by electrophoresis on 1% agarose gels containing ethidium bromide (1μg/ml). The appropriate amount of DNA

Table 4.1 Primer sequences for Ara h 1, Ara h 2 and pPROEX™ HT plasmid

		Restriction enzyme	Primer sequence (5' to 3')
Ara h 1	Forward	*Sal* I	GCG GCG TCG ACG ATG AGA GGG AGG GTT TCT
	Reverse	*Xba* I	CGC TCT AGA TCA GTT AAA AGC CTT CAA
Ara h 2	Forward	*Eco*RI	GCG GAA TTC CTC ACC ATA CTA GTA GCC
	Reverse	*Pst* I	CGC CTG CAG TTA GTA TCT GTC TCT GCC
Plasmid	Forward (M13 reverse)	-	AGC GGA TAA CAA TTT CAC ACA GG
	Reverse (pPROEX HT reverse)	-	TGA TTT AAT CTG TAT CAG G

Table 4.2 PCR reactions for the amplification of Ara h 1 and Ara h 2 cDNA from peanut cDNA

REACTION	TEST (µl)	NEGATIVE CONTROL (µl)
PCR Supermix	45	45
Forward primer (50 pmol/µl)	2.5	2.5
Reverse primer (50 pmol/µl)	2.5	2.5
Peanut cDNA	5	*
Mineral oil	50	50
TOTAL VOLUME	105	105

* - 5 µl sterile H_2O as a negative control

sample together with DNA sample loading buffer was then loaded into the appropriate wells alongside 8 µl of GeneRuler™ DNA Ladder Mix. DNA samples were resolved at 90 V for approximately 20-30 minutes and visualised using a UV lamp. DNA bands of interest were excised from the gel and stored at -20°C.

4.2.3 Isolation of DNA from agarose gel slices

DNA was purified from agarose gel slices using the QIAquick™ Gel Extraction Kit, following the manufacturer's instructions. DNA eluate was stored at -20°C.

4.2.4 Restriction enzyme digests

DNA restriction enzyme digests were carried out according to the manufacturer's instructions. The recommended amount of DNA was digested with the appropriate enzyme in 10 µl reactions which were incubated at 37°C for 2 hours and reactions were stopped by incubation at 70°C for 10 minutes. Digestion products were visualised by 1% agarose gel electrophoresis and DNA bands of interest were excised from the gel and purified as outlined in Section 4.2.3.

4.2.5 Subcloning of Ara h 1, Ara h 2 and Ara h 3 cDNA into pPROEX™ HT plasmid

The restriction enzyme digested cDNA encoding Ara h 1, Ara h 2 and Ara h 3 were subcloned into the pPROEX™ HT plasmid following the manufacturer's instructions. The appropriate amount of insert and vector DNA (ratio 1:3) along with T_4 DNA ligase and T_4 DNA Ligase buffer (final concentration of 1 X) were incubated overnight at 12°C. The reaction was stopped by incubation at 70°C for 10 minutes.

4.2.6 Preparation of calcium competent *E. coli* cells

A single colony of *E. coli* was placed in 10 ml Luria-Bertani (LB) broth containing 200 µl 20% maltose and incubated overnight at 37°C. The following day, 150 µl of the overnight culture was diluted in 15 ml LB broth and incubated at 37°C until $OD_{600\ nm}$ was 0.3-0.4. Cells were centrifuged at 693 x g at 4°C for 10 minutes. Pellet was resuspended in 3.75 ml 50 mM calcium chloride (stored at 4°C) and incubated on ice for 10 minutes. Cells were again centrifuged as described above and pellet was resuspended in 375 µl 50 mM calcium chloride. Bacterial cells were then incubated on ice for a minimum of 2 hours before transformation.

4.2.7 Transformation of plasmid DNA constructs into calcium competent *E. coli* cells

Calcium chloride competent *E. coli* cells (200 µl; prepared as outlined in Section 4.2.6) were added to 10 µl of ligation reaction and incubated on ice for 60 minutes. Bacterial cells were heat-shocked by incubation at 42°C for 2 minutes. 500 µl of LB broth were added to the cells and this was incubated at 37°C for 90 minutes. Cells (200 µl) were then spread onto LB-Ampicillin agar plates which were incubated overnight at 37°C.

4.2.8 Preparation of electrocompetent *E. coli* cells

A single colony of *E. coli* was placed in 10 ml LB broth and incubated overnight at 37°C, with shaking. Approximately 3 ml of the overnight culture were placed in 250 ml LB broth and incubated at 37°C until OD_{600} of 0.5-1.0. Cells were chilled on ice and then pelleted by centrifugation at 693 x g at 4°C for 10 minutes. The supernatant was discarded and cells were resuspended in an equal volume (250 ml) of ice cold sterile

H_2O followed by centrifugation as described above. The supernatant was again discarded and cells were resuspended in 125 ml ice cold sterile H_2O followed again by centrifugation as previously described. The supernatant was again discarded and cells were resuspended in 40 ml ice cold sterile 10% glycerol and pelleted by centrifugation as described above. Finally, cells were resuspended in 1 ml ice cold 10% glycerol and stored in aliquots at -70°C until use.

4.2.9 Transformation of plasmid constructs into electrocompetent *E. coli* cells by electroporation

Following ligation, 1 µl of Dextran Blue was added to 10 µl of ligation reaction to allow DNA to become more visible when pelleted. The DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 3 x volume 100% ethanol (stored at -20°C) to the ligation reaction and incubating this overnight at -20°C. DNA was centrifuged at 16060 x g for 15 minutes at 4°C to pellet DNA. The pellet was washed with 70% ethanol (stored at -20°C) and centrifuged as described above for 5 minutes. The ethanol was removed and the pellet dried for 1 hour after which it was dissolved in 10 µl of sterile H_2O at 37°C for 10 minutes. 2 µl of the ligation reaction was transformed into 50 µl of electrocompetent cells (prepared as outlined in Section 4.2.8) by electroporation using a Bio-Rad Micropulser™ (Bio-Rad, USA). 1 ml of LB broth was added to the cells which were subsequently incubated at 37°C for 1 hour, with shaking. 100 µl of cells were spread onto LB-Ampicillin agar plates and incubated overnight at 37°C.

4.2.10 Purification of plasmid DNA from *E. coli* cells

Bacterial colonies were selected from LB-Ampicillin agar plates and each colony was grown overnight at 37°C in 10 ml LB broth containing 10 µl ampicillin stock. Plasmid DNA was purified using the QIAGEN® Plasmid Midi Kit following the manufacturer's instructions. Plasmid DNA from transformants were screened for the presence of the correct size cDNA insert by restriction enzyme digestion (Section 4.2.2) or by PCR using the same protocol outlined in Section 4.2.4. The plasmid DNA from positive transformants were submitted for sequencing (The Baker Institute, Australia) using M13 reverse and pPROEX HT reverse primers (Table 4.1).

4.2.11 Timecourse expression of recombinant peanut allergens

Separate 50 ml tubes containing 10 ml LB broth containing 10 µl ampicillin stock were inoculated with single colonies containing the recombinant plasmid construct and non-recombinant plasmid and incubated overnight at 37°C, with shaking. The overnight culture was diluted 1/10 with LB broth containing ampicillin (prepared as described above) and incubated at 37°C with shaking until OD_{600} was 0.5-1.0. 1 ml of culture was collected and cells were pelleted by centrifugation at 16060 x g for 5 minutes and subsequently resuspended in PBS ($OD_{600\,nm}$ 1.0 = 200 µl PBS). Protein expression was induced by inoculating cultures with isopropyl-β-D-thiogalactopyranoside (IPTG) for a final concentration of 1 mM (10 µl 1 M IPTG per 10 ml culture). Cells were incubated at 37°C (with shaking) and samples were collected 2, 4 and 6 hours following induction of recombinant protein expression. Cells from each sample were pelleted and resuspended in PBS as described above. Timecourse samples were then analysed by SDS-PAGE (see Section 2.6.1) for recombinant protein expression.

4.2.12 Determination of the solubility of expressed recombinant proteins

Tubes containing 10 ml LB broth and 10 µl ampicillin stock were inoculated with single colonies containing the cDNA-pPROEX HT plasmid construct and pPROEX HT plasmid alone and incubated overnight at 37°C, with shaking. The overnight culture was diluted 1/10 with LB broth containing ampicillin (prepared as described above) and incubated at 37°C with shaking until $OD_{600\ nm}$ was 0.5-1.0. Protein expression was induced by inoculating cultures with IPTG for a final concentration of 1 mM (10 µl 1 M IPTG per 10 ml culture). Cells were incubated at 37°C for 4 hours with shaking. Cells were pelleted by centrifugation at 4300 x g. Soluble proteins were obtained by sonicating cell pellets in PBS for 20 seconds using a Branson Sonifier® (Branson Ultrasonics Corporation, USA) and cooling pellets on ice for another 20 seconds. This was repeated a further 5 times. Cell debris was pelleted by centrifugation at 16060 x g and supernatant was collected. Insoluble proteins were obtained by resuspending sonicated cell pellets in PBS. The soluble and insoluble fractions were analysed by SDS-PAGE (outlined in Section 2.6.1) and anti-His_6 tag immunoblotting for the expression of the recombinant protein.

4.2.13 Large scale expression of recombinant proteins

A flask containing 250 ml LB broth containing ampicillin (0.1 mg/ml) was inoculated with a single colony containing the recombinant plasmid construct and grown overnight at 37°C. The overnight culture was diluted 1/10 for a final volume of 2 L and cells were incubated at 37°C (with shaking) until $OD_{600\ nm}$ was 0.5-1.0. Protein expression was induced by adding IPTG to the cultures for a final concentration of 1 mM (2 ml of 1 M IPTG) and expression was carried out for 4 hours. Cultures were centrifuged at 4300 x g for 15 minutes and pellets were resuspended in denaturing lysis buffer (2-5 ml per

gram wet weight). Pellets were sonicated as outlined in Section 4.2.12. Solution was centrifuged at 20000 x g for 10 minutes to pellet cell debris. The total lysate was subsequently used for the purification of recombinant proteins by nickel chelate chromatography as described in the following sections.

4.2.14 Purification of recombinant proteins under denaturing conditions using pH elution (pH method)

5 ml of nickel-nitrilotriacetic acid (Ni-NTA) resin was packed in a 25 ml Econo-Pac® disposable chromatography column (Bio-Rad, USA) and equilibrated with 5 column volumes of denaturing lysis buffer. The total lysate containing the recombinant protein (50 ml) was applied to the column and the flow through was collected. The column was washed twice with 5-10 column volumes of denaturing wash buffer and wash fractions were collected. The recombinant protein was then eluted from the column in 4 x 5 ml fractions using denaturing elution buffer. Fractions were analysed by SDS-PAGE and Coomassie brilliant blue staining (see Section 2.6.1). Elution fractions containing the recombinant protein were then dialysed (dialysis cassettes with 3 kDa cut-off, Pierce, USA) overnight against two changes of PBS to remove the 8 M urea.

4.2.15 Purification of recombinant proteins under denaturing conditions using imidazole (imidazole method)

The purification of recombinant protein under denaturing conditions using imidazole was performed as described in Section 4.2.14. Following the application of the cleared lysate to the column, the column was washed with 5-10 column volumes of denaturing wash buffer containing imidazole and fractions were collected. The recombinant

protein was then eluted from the column in 4 x 5 ml fractions using denaturing elution buffer containing imidazole. Fractions were analysed by SDS-PAGE and Coomassie brilliant blue staining as outlined in Section 2.6.1. Elution fractions containing the recombinant protein were then dialysed (dialysis cassettes with 3 kDa cut-off, Pierce, USA) overnight against two changes of PBS at room temperature to remove the 8 M urea.

4.2.16 Sulfonation and refolding of rAra h 2

Expression of rAra h 2 was carried out as described in Section 4.2.13. Cell pellets were sonicated in sulfonation buffer as described in Section 4.2.12. The recombinant protein was then purified using both the pH and imidazole methods (Sections 4.2.14 and 4.2.15) and fractions were analysed by SDS-PAGE and Coomassie brilliant blue staining as described in Section 2.6.1.

The refolding of rAra h 2 was conducted firstly by diluting sulfonated rAra h 2 to 0.25 mg/ml in sulfonation buffer followed by incubation with shaking at room temperature for 2 hours. The sulfonated protein was dialysed (dialysis cassettes with 3 kDa cut-off, Pierce, USA) against refolding buffer to remove the sulfonating reagents. The sulfonated rAra h 2 in refolding buffer was then dialysed overnight at 4°C against refolding buffer containing 0.2 mM oxidised glutathione and 1 mM reduced glutathione (Clark, 1998). Finally, the solution was dialysed against PBS (as described above) at 4°C. The refolded protein was analysed by SDS-PAGE and Coomassie brilliant blue staining as described in Section 2.6.1.

4.2.17 Anti-His_6 tag immunoblotting

Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes as outlined in Sections 2.6.1 and 2.6.2. Membranes were blocked with 10% blocking solution for at least 1 hour and membranes were washed once in 0.05% PBS-Tween and twice in PBS, 5 minutes for each wash. His_6-tagged proteins were detected by incubating membranes overnight in anti-His_6 tag monoclonal antibodies (diluted 1/200 with 0.5% BSA in PBS). Membranes were washed as described above and incubated in sheep anti-mouse Ig HRP-conjugated antibodies (diluted 1/2000 with 0.5% BSA in PBS). Antibody binding was detected using the substrate 4-chloro-1-naphthol as described in Section 2.6.2.

4.2.18 Inhibition immunoblotting

Crude roasted peanut extract and Bermuda grass pollen extract (BGP) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes as described in Sections 2.6.1 and 2.6.2. Membranes were blocked with 10% blocking solution for at least 1 hour and membranes were washed once in 0.05% PBS-Tween and twice in PBS, 5 minutes for each wash. The subject sera used for the inhibition studies were initially titrated against both roasted peanut and BGP extracts by serially diluting sera in 0.5% BSA in PBS followed by incubation with nitrocellulose strips electroblotted with these extracts and measuring IgE binding (outlined in Section 2.6.2). Inhibition immunoblotting was performed by pre-incubating subject sera (in the appropriate dilution) with 0.2, 1, 5, 25 and 125 µg/ml of purified rAra h 3 protein for 1 hour at room temperature, with shaking. Nitrocellulose strips of roasted peanut extract and BGP

extract were then incubated overnight with the subject sera. IgE binding was subsequently detected as outlined in Section 2.6.2.

4.3 RESULTS

4.3.1 Cloning and characterisation of recombinant peanut allergens

4.3.1.1 Amplification of cDNA encoding Ara h 1 from peanut cDNA

Primers based on the 5' and 3' ends of the Ara h 1 sequence published by Burks *et al.* (1995) were used to PCR amplify the corresponding cDNA from peanut cDNA. A fragment of approximately 2000 bp was isolated and subcloned into the pPROEX™ HT plasmid vector for expression of Ara h 1 as a His_6-tagged recombinant protein. DNA sequencing of the resulting plasmid construct and subsequent comparisons showed 100% identity with the published sequence (Genbank accession no. L34402).

4.3.1.2 Isolation of cDNA encoding Ara h 2 from peanut cDNA

Primers based on the 5' and 3' ends of the Ara h 2 sequence published by Stanley and colleagues (1997) were used to PCR amplify the corresponding cDNA from peanut cDNA. A 500 bp fragment was isolated and subcloned into the pPROEX™ HT plasmid vector for expression as a His_6-tagged protein. DNA sequencing of the plasmid construct and a subsequent comparison showed 100% identity with the published sequence (Genbank accession no. L77197).

4.3.1.3 Sequencing of cDNA from an IgE-reactive clone isolated from a peanut cDNA library

An IgE-reactive clone was isolated from a λgt11 peanut cDNA library following immunoscreening using serum from a peanut allergic subject (de Leon, 1999). The cDNA from this clone (approximately 1315 bp) was subcloned into pPROEX™ HT plasmid and sequenced. DNA and amino acid sequence comparisons showed identity with previously cloned peanut proteins (Figures 4.1 and 4.2). As summarised in Table 4.3, this clone has a high DNA sequence identity (99%) with a glycinin-type peanut seed storage protein. Comparisons also showed 96% and 94% DNA sequence identity with the previously cloned peanut allergens, Ara h 3 and Ara h 4, respectively. Both these allergens also belong to the glycinin/legumin family of seed storage proteins. A high percentage of identity was also obtained at the amino acid sequence level (Table 4.3). It was concluded that this IgE-reactive clone is an Ara h 3-like peanut glycinin allergen although sequence comparisons indicate that it encodes only the partial cDNA, with up to 450 bp missing from the 5' end (Figure 4.1). Several attempts were made to obtain the missing 5' sequence using RT-PCR but these were unsuccessful.

4.3.2 Expression of recombinant peanut allergens

4.3.2.1 Expression of rAra h 1 in *E. coli* cells

The Ara h 1-pPROEX™ HT plasmid construct was transformed into electrocompetent Epicurian Coli® BL21-CodonPlus® (DE3)-RIL *E. coli* cells and calcium competent ER1793 *E. coli* cells. The expression of the encoded His_6-tagged protein was induced by adding IPTG to the bacterial cultures and samples collected 2, 4 and 6 hours following induction were analysed by SDS-PAGE (Figure 4.3). Expression of rAra h 1

```
Clone          9 tgagggtgatctcattgcagttcccaccggtgttgctctctggatgtacaacgaccatga 68
Glycinin     463 ..........................................t.................. 518
Ara h 3      384 ..................................t.....c.c...........c.. 443
Ara h 4      453 ..................................t.....c.............c.. 512
Ara h 3/4   1452 ..................................t.....................  1511

Clone         69 cactgatgttgttgctgtttctcttactgacaccaacaacaacgacaaccagcttgatca 128
Glycinin     519 ..........................................................  578
Ara h 3      444 ..........................................................  503
Ara h 4      513 ..........................................................  572
Ara h 3/4   1512 ..........................................................  1571

Clone        129 gttccccaggagattcaatttggctggaaaccacgagcaagagttcttaagatcccagca 188
Glycinin     579 ..................................................a.......  638
Ara h 3      504 ........................g...acg.................g.a.......  563
Ara h 4      573 ........................g.......................g.a.......  632
Ara h 3/4   1668 ..................g.........................a.......  1718
Ara h 3/4   1572 ..........                                              1581

Clone        189 acaaagca---------gac---------gaagaagcttaccatatagcccatacagccc 230
Glycinin     639 ........---------...---------.............................  680
Ara h 3      564 ........gacaaagca...---------.............................  614
Ara h 4      633 ........---------...aaagcagac.............................  683
Ara h 3/4   1719 ........---------...---------.............................  1760

Clone        231 gcaaagtcagcctagacaagaagagcgtgaatttagccctcgaggacagcacagccgcag 290
Glycinin     681 ..........................................................  740
Ara h 3      615 ..........................................................  674
Ara h 4      684 ...t....g........g................c.......................  743
Ara h 3/4   1761 .....c........a.........c.............................g.....  1820

Clone        291 agaacgagcaggacaagaagaagaaaacgaaggtggaaacatcttcagcggcttcacgcc 350
Glycinin     741 ..........................................................  800
Ara h 3      675 ..........................................................  734
Ara h 4      744 ........................g.................................  803
Ara h 3/4   1821 ..................c.....................................  1880

Clone        351 ggagttcctggcacaagccttccaggttgacgacagacagatagtgcaaaatctaagagg 410
Glycinin     801 ..........................................................  860
Ara h 3      735 ..........a.............................................c.....  794
Ara h 4      804 ..........a.............................t...........gt.g..  863
Ara h 3/4   1881 ..........................................t.......c.....  1940

Clone        411 cgagaacgagagtgaggaagagggagccattgtgacagtgaagggaggcctcagaatctt 470
Glycinin     861 ..........................................................  920
Ara h 3      795 .....c.........a.........................g.................  854
Ara h 4      864 ..............a....................g....g.................  923
Ara h 3/4   1941 ..............c...c....................g.................  2000

Clone        471 gagcccagatagaaagagaggtgccgacgaagaagaggaatccgatgaagatgaatatga 530
Glycinin     921 ..........................................a.................  980
Ara h 3      855 ..................c.....................a.................  914
Ara h 4      924 ..........g...c.........................a.........c.......  983
Ara h 3/4   2001 ...................                                        2019
Ara h 3/4   2039                          ..........a.................  2075

Clone        531 atacgatgaagaggatagaaggcgtggcaggggaagcagaggcaggggggaatggtattga 590
Glycinin     981 ..........................................................  1040
Ara h 3      915 ..........................................................  974
Ara h 4      984 ....c.....c.....g.........................g.................  1043
Ara h 3/4   2101                ..................................c..c.....c.....  2147
Ara h 3/4   2076 ...t...........                                            2090
```

Figure 4.1 DNA sequence comparisons between IgE-reactive λgt11 clone and other peanut proteins in Genbank database

```
Clone       591 agagacgatctgcacagcaagtgttaaaaagaacattggtagaacagatcccctgacat 650
Glycinin   1041 ............................................................ 1100
Ara h 3     975 ..............c.......c..................................... 1034
Ara h 4    1044 ..............c...t.....................g..............c.... 1103
Ara h 3/4  2148 g.....c........c......t..................................... 2207

Clone       651 ctacaatcctcaa-gctggttcactcaaaactgccaacgatctc------aaccttctaa 703
Glycinin   1101 ............-.....................................------..... 1153
Ara h 3    1035 ......c.....-.....................................------..... 1087
Ara h 4    1104 ....g.......gc.....................-.......------..... 1156
Ara h 3/4  2208 ......c......-.....................g...cagctt..... 2266

Clone       704 tccttaggtggcttggacttagtgctgaatatggaaatctctacaggaatgcattgtttg 763
Glycinin   1154 ............................................................ 1213
Ara h 3    1088 .a..............c..................................... 1147
Ara h 4    1157 ............................................................ 1216
Ara h 3/4  2481                                                 ..... 2494
Ara h 3/4  2267 ..................................... 2313

Clone       764 tccctcactacaacaccaacgcacacagcatcatatatgcattgaggggacgggctcacg 823
Glycinin   1214 ............................................................ 1273
Ara h 3    1148 ..g.....................................cg..... 1207
Ara h 4    1217 ............................................................ 1276
Ara h 3/4  2495 ..............g..... 2554

Clone       824 tgcaagtcgtggacagcaacggcaacagagtgtacgacgaggagcttcaagagggtcacg 883
Glycinin   1274 ............................................................ 1333
Ara h 3    1208 ............................................................ 1267
Ara h 4    1277 .......g..... 1336
Ara h 3/4  2555 .......g.............g.........t.................a..... 2614

Clone       884 tgcttgtggtgccacagaacttcgccgtcgctggaaagtcccagagcgacaactttgaat 943
Glycinin   1334 ............................................................ 1393
Ara h 3    1268 ..................................................g.....c.... 1327
Ara h 4    1337 .t.........................g.....g.............g.....c.... 1396
Ara h 3/4  2615 ..........................g.....t.....g..... 2674

Clone       944 acgtggcattcaagacagactcaaggcccag:atagcc..ctcgccggtgaaaactcca 1003
Glycinin   1394 ..............................a.........t..... 1453
Ara h 3    1328 ..............................................................g 1387
Ara h 4    1397 ..................t.....................t.t..............t 1456
Ara h 3/4  2675 ..........................................a.....a.........t 2734

Clone      1004 tcatagataacttgccggaggaggtggttgcaaattcatatggcctcccaagggagcagg 1063
Glycinin   1454 ..................a..a..... 1513
Ara h 3    1388 ..........c.....a..... 1447
Ara h 4    1457 ..........c..... 1516
Ara h 3/4  2735 ..... 2794

Clone      1064 caaggcagcttaagaacaacaacccccttcaagttcttcgttccaccgtctcaacagtctc 1123
Glycinin   1514 ..... 1573
Ara h 3    1448 ..................g..... 1507
Ara h 4    1517 ..................t.t...g..... 1576
Ara h 3/4  2795 ..................g..... 2854

Clone      1124 tcagggctgtggcttgaaaacaagcgtgacatgtatgtgtgttatccactacatacatac 1183
Glycinin   1574 .....  1590
Ara h 3    1508 cg.....  1522
Ara h 4    1577 cg.....a.....  1597
Ara h 3/4  2855 .g.....a..... 2914

Clone      1184 tttttgccacaactactgaataatacatattaataacgaccgagaataatgtagttttaa 1243
Ara h 4    1671                                                 ..... 1688
Ara h 3/4  2915 .....---.....a.....----.....t 2967

Clone      1244 ttttgtagtgtcaataagaatacaaaagggcattcatgccttttttgtttaagct 1297
Ara h 4    1689 ....-....a.g.....g.....g.....  1739
Ara h 3/4  2968 .....g.....g.....g..... 3021
```

```
Clone        4 EGDLIAVPTGVALWMYNDHDTDVVAVSLTDTNNNDNQLDQFPRRFNLAGNHEQEFLR--- 60
Glycinin   154 ..............F.......................................--- 210
Ara h 3/4  152 ..............F.......................................--- 208
Ara h 3    129 ............F.L.....................................T......YQQ 188
Ara h 4    152 ............F.L.....................................T......YQQ 211

Clone       61 SQQQSRRRSLPYSPYSPQSQPRQEEREFSPRGQHSRRERAGQEEENEGGNIFSGFTPEFL 120
Glycinin   211 Y.............................................................. 270
Ara h 3/4  209 Y................T..K..D.........G........Q.................. 268
Ara h 3    189 QSR............................................................ 248
Ara h 4    212 QSR............H.R..R.....R..............D.................. 271

Clone      121 AQAFQVDDRQIVQNLRGENESEEEGAIVTVKGGLRILSPDRKRGADEEE---------ES 171
Glycinin   271 ..................................................---------.Y 321
Ara h 3/4  269 ...........L.........D.Q......R............RQQY.RPDEEEEYDEDE 328
Ara h 3    249 E................T...........R............R.....---------.Y 299
Ara h 4    272 E..............W.............R.........GT.......---------.Y 322

Clone      172 DEDEYEYDEEDRRRGRGSRGRGNGIEETICTASVKKNIGRNRSPDIYNPQAGSLKTAND- 230
Glycinin   322 ..................................................... 380
Ara h 3/4  329 Y.YDE.ERQQ.........S............F.....................EL 388
Ara h 3    300 ..................................A.....................- 358
Ara h 4    323 ...Q...H.Q.G........G...........C......G....H..D..RWFTQNCH.- 381

Clone      231 -LNLLILRWLGLSAEYGNLYRNALFVPHYNTNAHSIIYALRGRAHVQVVDSNGNRVYDEE 289
Glycinin   381 -..................................................... 439
Ara h 3/4  389 Q.....................................................D..F... 448
Ara h 3    359 -..........P.............A..........:.R..................... 417
Ara h 4    382 -..................................................... 440

Clone      290 LQEGHVLVVPQNFAVAGKSQSDNFEYVAFKTDSRPSIANLAGENSIIDNLPEEVVANSYG 349
Glycinin   440 ....................................N...F..................... 499
Ara h 3/4  449 ......................E.....................F.............. 508
Ara h 3    418 ......................E.....................V.............. 477
Ara h 4    441 ......................E................F.....F.............. 500

Clone      350 LPREQARQLKNNNPFKFFVPPSQQSLRAVA 379
Glycinin   500 ............................... 529
Ara h 3/4  509 .......................E....... 538
Ara h 3    478 .Q........................P.... 507
Ara h 4    501 ......................F...P.... 530
```

Figure 4.2 Amino acid sequence comparison between IgE-reactive λgt11 clone and other peanut proteins in Genbank database

Table 4.3 Sequence identity of IgE-reactive clone with other peanut proteins

		DNA			Protein		
Peanut protein	**Accession no.**	**Length (bp)**	**Overlap**	**Identity (%)**	**Length (AA)**	**Overlap**	**Identity (%)**
Glycinin	AF125192	1590	1121/1128	99	529	304/376	80
Ara h 3 (glycinin)	AF093541	1524	1097/1139	96	507	293/379	77
Ara h 4 (glycinin)	AF086821	1853	1085/1146	94	530	281/379	74
Ara h 3/Ara h 4	AF510854	3825	523/548	95	538	292/387	78
			340/352	96			
			129/130	99			
			50/52	96			

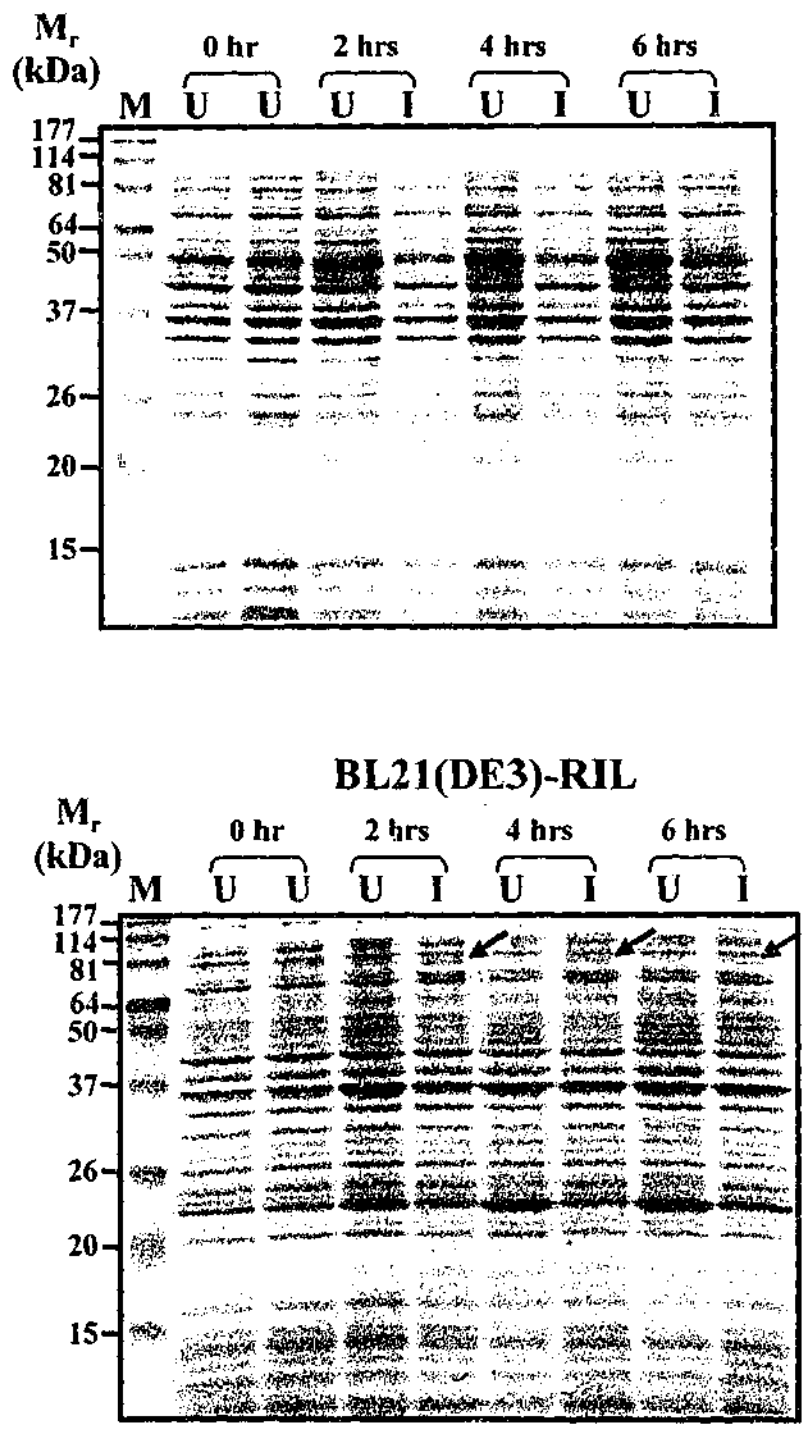


Figure 4.3 Expression of rAra h 1 in two different *E. coli* strains

The Ara h 1-pPROEX HT construct was transformed into *E. coli* cells (ER1793 and BL21(DE3)-RIL) and expression was induced using IPTG. Samples were collected from uninduced (U) and induced (I) cultures before IPTG induction and 2, 4 and 6 hours following induction. Samples were then analysed by 14% SDS-PAGE (reducing conditions) followed by Coomassie blue staining and arrow indicates the position of the expressed rAra h 1. M indicates position of molecular mass markers (M_r).

was obtained with BL21 (DE3)-RIL *E. coli* cells with a molecular mass of approximately 70 kDa (indicated by arrows) which is in accordance with the predicted molecular mass based on the DNA sequence, although expression levels were very low. Expression appeared to be optimal at 4 hours after induction. No expression of rAra h 1 could be detected in ER1793 *E. coli* cells.

4.3.2.2 Expression of rAra h 2 in *E. coli* cells

The Ara h 2-pPROEX™ HT plasmid construct was transformed into different *E. coli* strains and the expression of the encoded His_6-tagged protein was induced by the addition of IPTG to the bacterial cultures. Samples from induced and uninduced cultures were collected before induction and 2, 4 and 6 hours after induction and protein expression was analysed by SDS-PAGE (Figure 4.4). rAra h 2 expression was obtained in ER1793 *E. coli* cells with the presence of a protein with a molecular mass of approximately 18 kDa in the induced cultures, which corresponds to the predicted molecular weight. Expression of rAra h 2 could be detected 2 hours after induction but optimal expression occurred after 4 hours with protein expression still detectable after 6 hours. Also, most of the rAra h 2 protein was present in the insoluble fraction (data not shown) and consequently protein purification had to be conducted under denaturing conditions. No expression of rAra h 2 was detected with the other *E. coli* strains.

4.3.2.3 Expression of rAra h 3 in *E. coli* cells

The Ara h 3-pPROEX™ HT plasmid construct was transformed into electrocompetent Epicurian Coli® BL21-CodonPlus® (DE3)-RIL *E. coli* cells and a timecourse expression of the encoded protein was conducted. Protein expression was induced by the addition

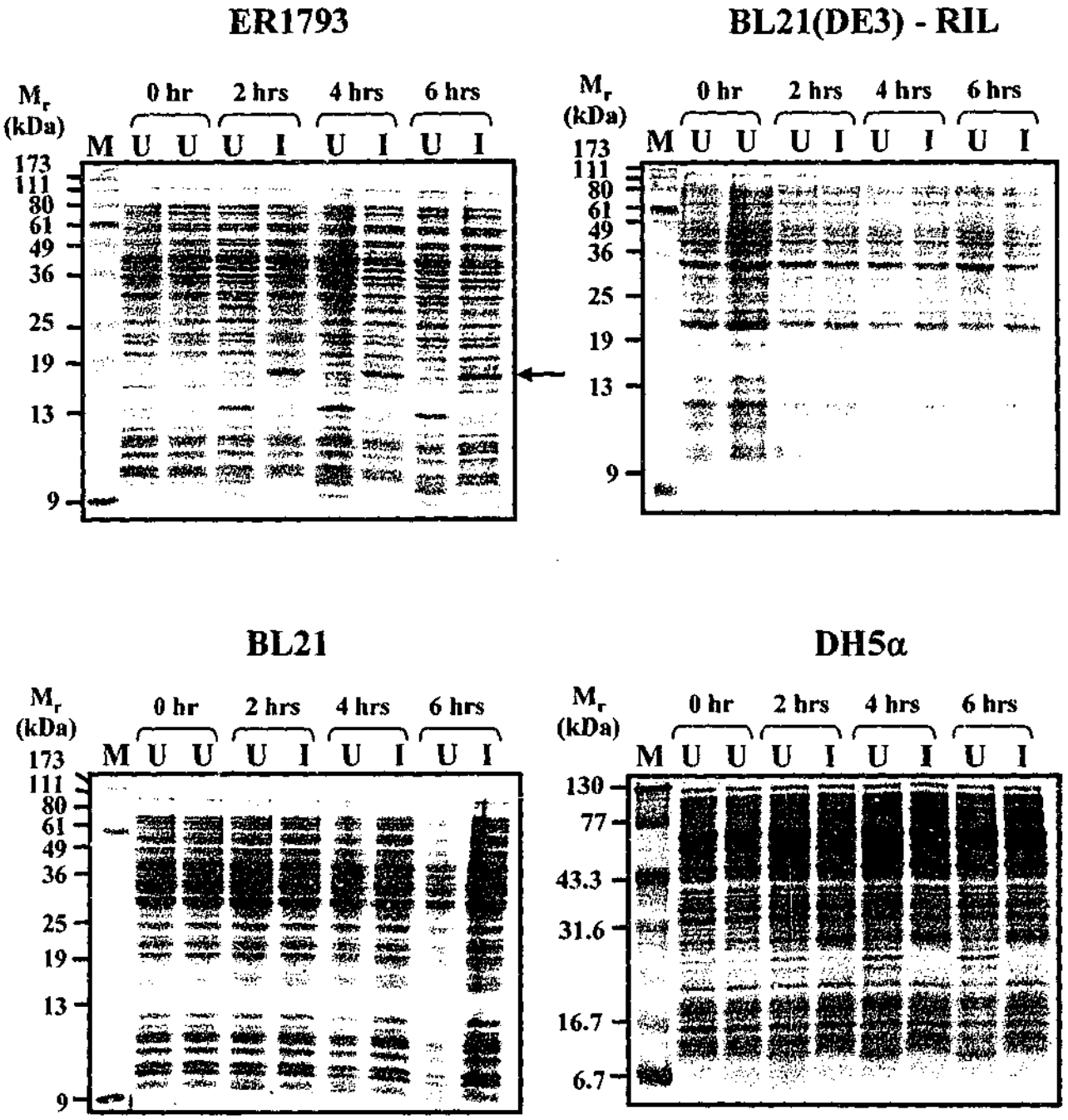


Figure 4.4 Expression of rAra h 2 in different *E. coli* strains

The Ara h 2-pPROEX HT construct was transformed into *E. coli* cells (ER1793, BL21(DE3)-RIL, BL21 and DH5α) and expression was induced using IPTG. Samples were collected from uninduced (U) and induced (I) cultures before IPTG induction and 2, 4 and 6 hours following induction. Protein expression was analysed by 14% SDS-PAGE (reducing conditions) followed by Coomassie blue staining and arrow indicates the position of the expressed rAra h 2. M indicates position of molecular mass markers (M_r).

of IPTG to the cultures and samples were taken before induction and 2, 4 and 6 hours after induction. Protein samples were analysed by SDS-PAGE, as shown in Figure 4.5. It can be seen that expression of rAra h 3 was obtained in these *E. coli* cells as indicated by the overexpression of a ~50 kDa protein in the induced cultures, 2 hours after induction. The molecular mass of this protein is higher compared to the predicted molecular weight mass based on the open reading frame of the DNA sequence (~40 kDa). Maximal expression occurred at approximately 4 hours however expression could still be detected after 6 hours. Similar to rAra h 2, the majority of the expressed rAra h 3 protein was present in the insoluble fraction (data not shown) and thus purification had to be performed under denaturing conditions.

4.3.3 Purification of recombinant peanut allergens

4.3.3.1 Purification of rAra h 1

Following expression in *E. coli* cultures, rAra h 1 was purified using Ni^{2+} resin which has an affinity for the His_6-tag located at the *N*-terminus of the expressed protein. Due to low expression levels, rAra h 1 was purified under denaturing conditions to obtain the maximum yield. This protein was purified using the pH method which involved the use of denaturing buffers with different pH values to wash and elute proteins from the nickel resin. Figure 4.6 shows a Coomassie-stained gel of fractions collected during the purification of rAra h 1 from a 2 L culture. It can be seen that the elution fractions (Figure 4.6, lanes 4-7) contain a protein with a molecular mass of approximately 70 kDa which represents the Ara h 1 monomer and the majority of this protein is present in the first two elution fractions. However, it can also be seen that there are lower molecular mass proteins present in the elution fractions, most notably a protein at approximately

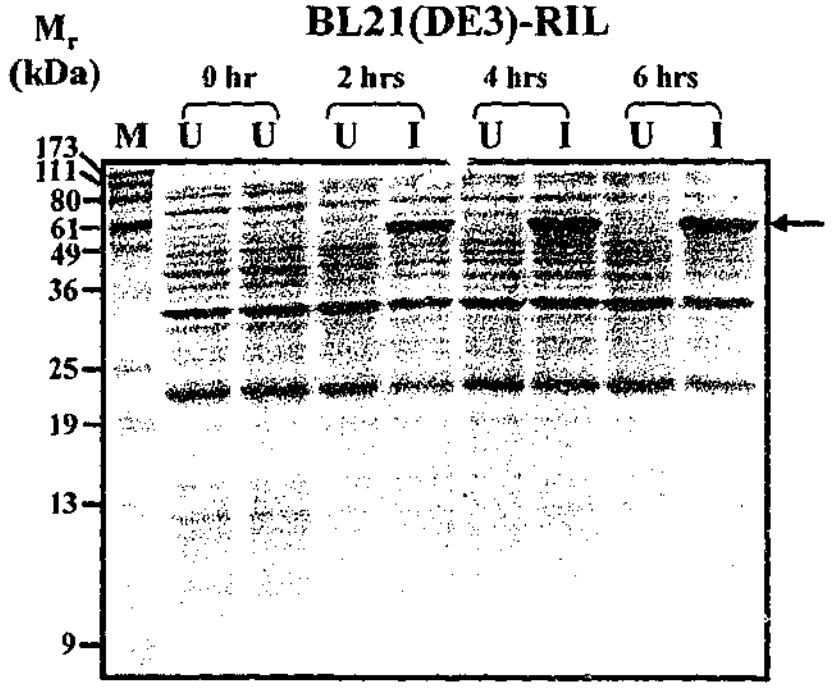


Figure 4.5 Expression of rAra h 3 in *E. coli* cells

The Ara h 3-pPROEX HT construct was transformed into *E. coli* cells (BL21(DE3)-RIL) and expression was induced using IPTG. Samples were collected from uninduced (U) and induced (I) cultures before IPTG induction and 2, 4 and 6 hours following induction. Protein expression was analysed by 14% SDS-PAGE (reducing conditions) followed by Coomassie brilliant blue staining. M indicates the position of molecular mass markers (M_r) and arrow indicates the position of the expressed rAra h 3.

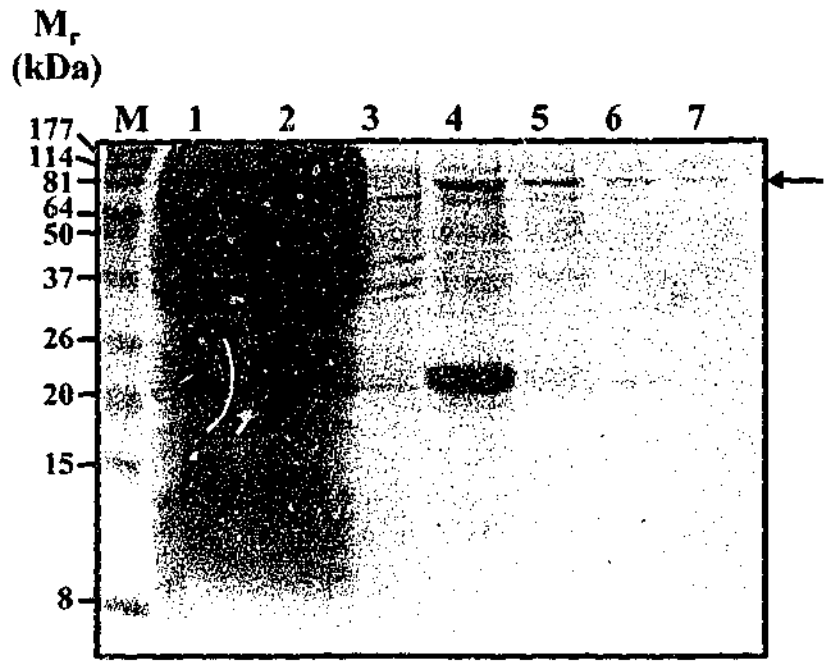


Figure 4.6 Purification of rAra h 1

rAra h 1 was expressed in *E. coli* cells and purified under denaturing conditions using nickel chelate chromatography. Purification was carried out using the pH method and fractions were analysed by 14% SDS-PAGE (reducing conditions) followed by Coomassie blue staining. Arrow indicates the position of the rAra h 1 monomer. **Lanes:** M – molecular mass markers (M_r), 1 – total lysate, 2 – flow through, 3 – wash, 4-7 – elution fractions 1-4.

20 kDa. The first two Ara h 1 elution fractions were pooled together for use in subsequent studies.

4.3.3.2 Purification of rAra h 2

4.3.3.2.1 Optimisation of the purification of rAra h 2 by nickel chelate chromatography

rAra h 2 was purified as for rAra h 1 using Ni^{2+} resin. Due to the insoluble nature of the expressed rAra h 2, purification was conducted under denaturing conditions. Two methods were initially assessed to determine the optimum purification of this protein. Initially, the pH method was employed and fractions collected during this purification procedure were analysed by SDS-PAGE. As shown in Figure 4.7a, purification of rAra h 2 using this method yielded minimal amounts of recombinant protein in the elution fractions (lanes 5-7), with much of the protein still bound to the resin (lane 8). Consequently, a second method was utilised which involved the addition of imidazole to the wash and elution buffers (imidazole method). Imidazole is a ring-like structure that forms part of the structure of histidine. At low concentrations, imidazole can prevent non-specific, low-affinity binding of background proteins and His_6-tagged proteins by binding to the nickel ions in the resin and disrupting the binding of dispersed histidine residues in non-tagged background proteins. SDS-PAGE analysis of the fractions from this method of purification showed successful elution of rAra h 2 from the Ni^{2+} resin (Figure 4.7b). The elution fractions (Figure 4.7b, lanes 5-7) contained increased amounts of rAra h 2 although some residual protein could still be detected in the resin. There also appeared to be some high molecular mass contaminants (48-111 kDa) present in the elution fractions which may have been *E. coli* proteins.

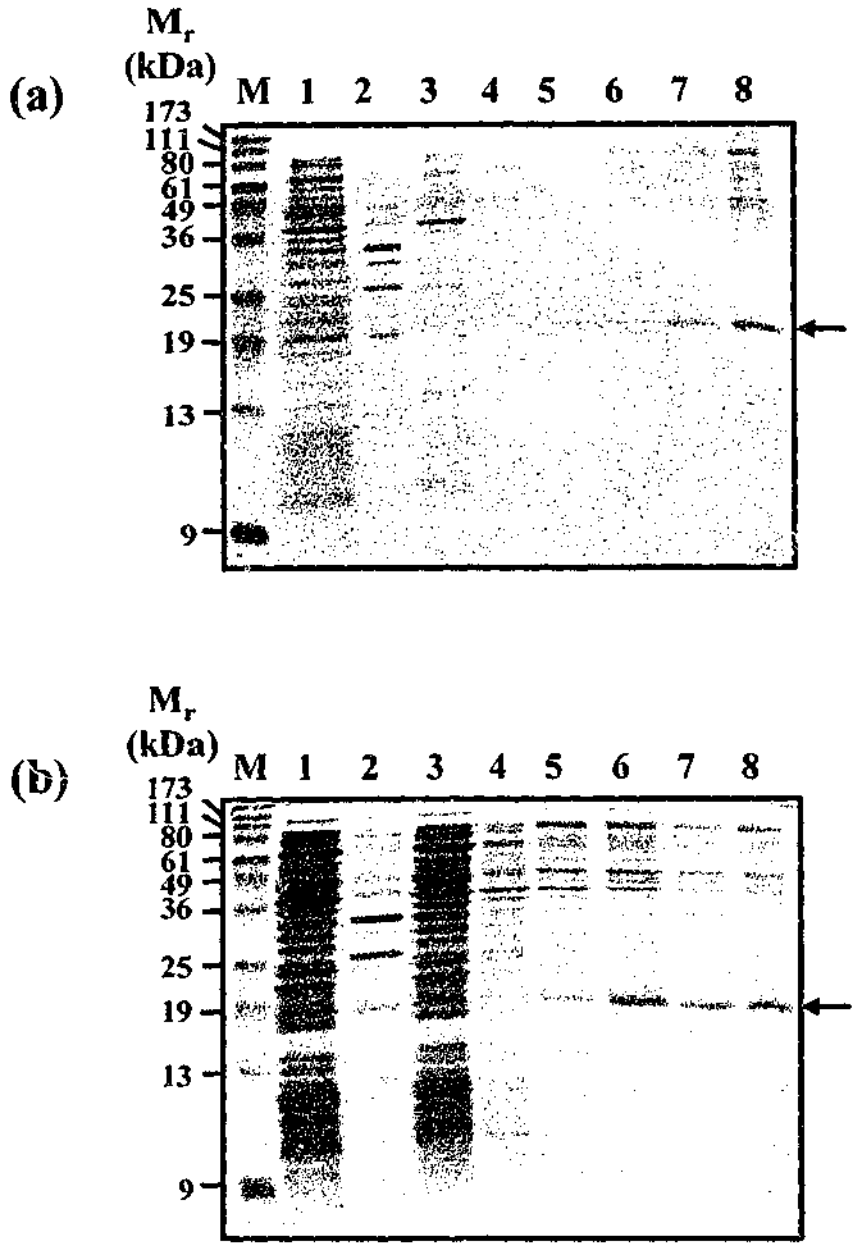


Figure 4.7 Purification of rAra h 2 by nickel chelate chromatography

rAra h 2 was purified using Ni^{2+} resin under denaturing conditions. Two methods were assessed for optimum purification: **(a)** purification using the pH method (pH 6.3 for the wash buffer and pH 4.5 for the elution buffer and **(b)** purification using the imidazole method (20 mM imidazole for the wash buffer and 250 mM imidazole for the elution buffer). Fractions were analysed by 14% SDS-PAGE (reducing conditions) and proteins were stained with Coomassie brilliant blue. Arrows indicate the position of rAra h 2. **Lanes:** M – molecular mass markers (M_r), 1 – total lysate, 2 – cell pellet, 3 – flow through, 4 – wash, 5-7 – elution fractions 1-3, 8 – resin.

To minimise the presence of contaminants in the elution fractions, further optimisation was performed using an imidazole concentration gradient to determine the optimal concentration of imidazole in the wash and elution buffers. The concentration of imidazole added to the buffers ranged from 30-50 mM for the wash buffer and 150-500 mM for the elution buffer. Fractions were analysed by SDS-PAGE and Western immunoblotting using an anti-His_6 tag monoclonal antibody. Figure 4.8a shows a Coomassie-stained SDS-PAGE gel of the wash and elution fractions and it can be seen that the elution of rAra h 2 from the nickel resin was achieved using the minimal imidazole concentration of 150 mM, with maximum yield at 500 mM (lanes 6-8). Concentrations of 30-50 mM imidazole in the wash buffer did not appear to elute any recombinant protein from the resin (Figure 4.8a, lanes 3-5). These results were confirmed by Western immunoblotting (Figure 4.8b) and consequently, concentrations of 50 mM and 500 mM imidazole were chosen for the wash and elution buffers, respectively, to obtain the maximum yield with minimal contaminants.

The Western immunoblotting results also showed that some of the high molecular mass proteins in the purification fractions also bound the anti-His_6 tag monoclonal antibody, indicating that these proteins may be multimers of rAra h 2 (Figure 4.8b, red arrows). Similarly, there appears to be some rAra h 2 breakdown products from the purification process as indicated by low molecular mass proteins which bound the anti-His_6 tag monoclonal antibody (Figure 4.8b, blue arrow). However, these rAra h 2 multimers and breakdown products appear to be in low abundance as indicated by the intensity of antibody binding when compared to the monomer. Further attempts were made to minimise the presence of high molecular mass protein aggregates in the elution fractions with the addition of glycerol and/or Tween-20 in the wash and elution buffers. Glycerol acts by reducing the hydrophobic interactions between proteins while detergents such as

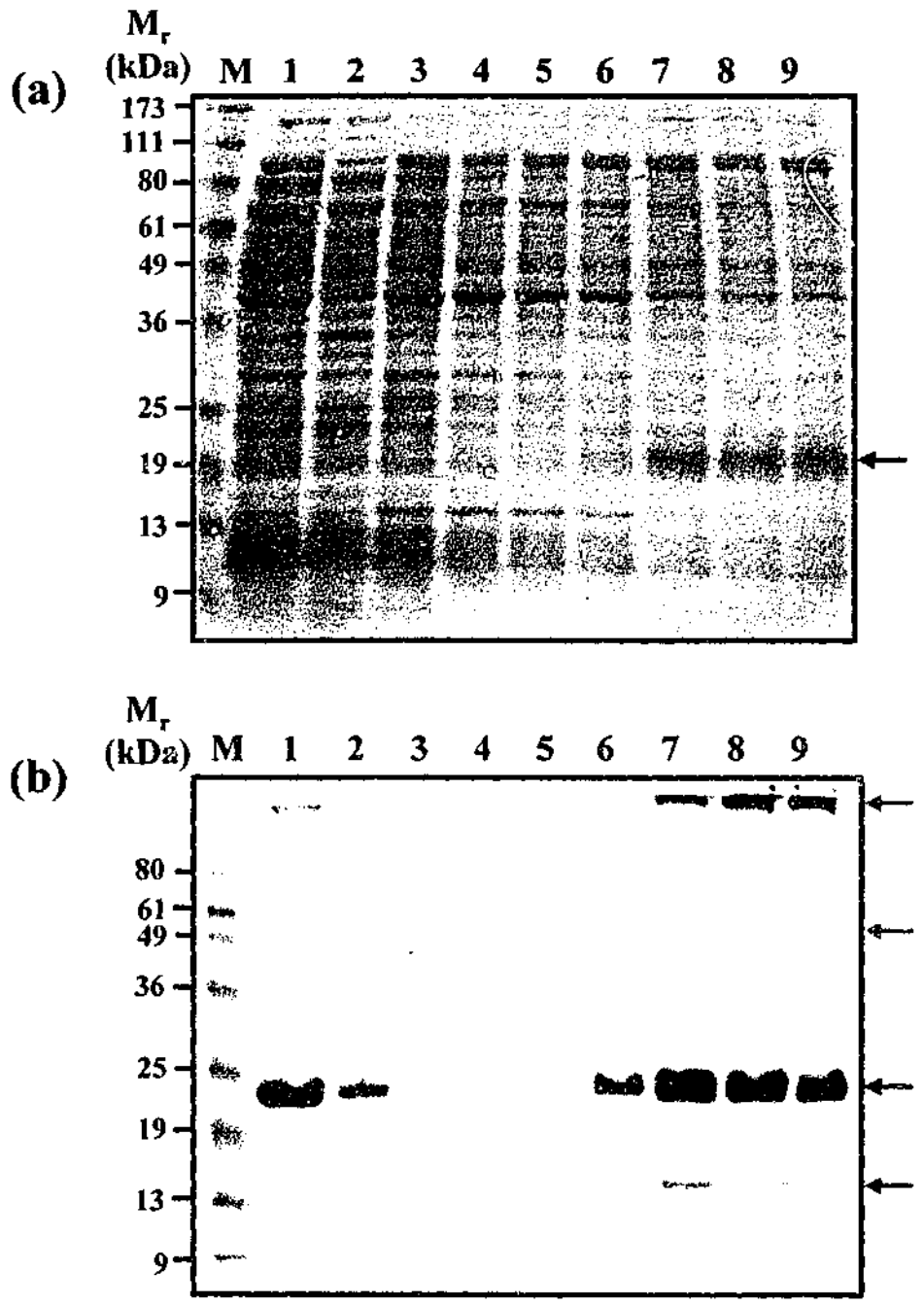


Figure 4.8 Purification of rAra h 2 using an imidazole concentration gradient

An imidazole concentration gradient was used to determine the optimum concentration of imidazole for the wash (30-50 mM) and elution (150-500 mM) buffers. **(a)** Fractions were analysed by 14% SDS-PAGE under reducing conditions and proteins were stained with Coomassie brilliant blue. **(b)** Separated proteins were also electroblotted onto nitrocellulose membranes and anti-His_6 tag monoclonal antibody was used to detect the elution of rAra h 2 from resin. Black arrows indicate position of rAra h 2 monomer, red arrows indicate position of possible rAra h 2 multimers and blue arrow indicates position of possible rAra h 2 breakdown products. **Lanes:** M – molecular mass markers (M_r), 1 – total lysate, 2 – flow through, 3 – 30 mM imidazole, 4 – 40 mM imidazole, 5 – 50 mM imidazole, 6 – 150 mM imidazole, 7 – 250 mM imidazole, 8 – 500 mM imidazole, 9 – resin.

Tween-20 can reduce non-specific interactions between background proteins and the His_6-tagged protein. However, the use of glycerol and Tween-20 did not reduce the number of contaminants (data not shown).

Using denaturing buffers containing imidazole, large scale purification of rAra h 2 was performed using 2 L *E. coli* cultures and fractions from this procedure were analysed by SDS-PAGE, as shown in Figure 4.9b. It can be seen that the majority of rAra h 2 protein is present in the first 2 elution fractions (Figure 4.9b, lanes 6-7) with a yield of approximately 1 mg/L of culture. High molecular mass contaminants were also present but the rAra h 2 monomer is the most abundant protein in these fractions. The wash fractions (Figure 4.9b, lanes 3-5) also contained minimal amounts of rAra h 2. Consequently, the first 2 elution fractions were pooled and dialysed against PBS, with rAra h 2 remaining soluble after dialysis, for use in future experiments.

4.3.3.2.2 Sulfonation of rAra h 2

The effect of sulfonation on the yield and purity of rAra h 2 from *E. coli* cultures was investigated. Sulfonation results in the 'capping' of cysteine residues within the protein, preventing the formation of disulfide bonds (Clark, 1998). In this procedure, rAra h 2 was expressed and cell pellets were sonicated in sulfonation buffer. Purification was carried out using both the optimised imidazole method and the pH method. Figure 4.10 shows an SDS-PAGE analysis of the purification fractions. rAra h 2 was successfully eluted from the nickel resin using both methods and the yield was noticeably higher following sulfonation (2.5 mg/L culture). However, using the optimised imidazole method, rAra h 2 is eluted in fractions 1 and 2 (Figure 4.10a, lanes 6-7) whereas in the pH method, it can be found in fractions 2 and 3 (Figure 4.10b, lanes 7-8). To maintain

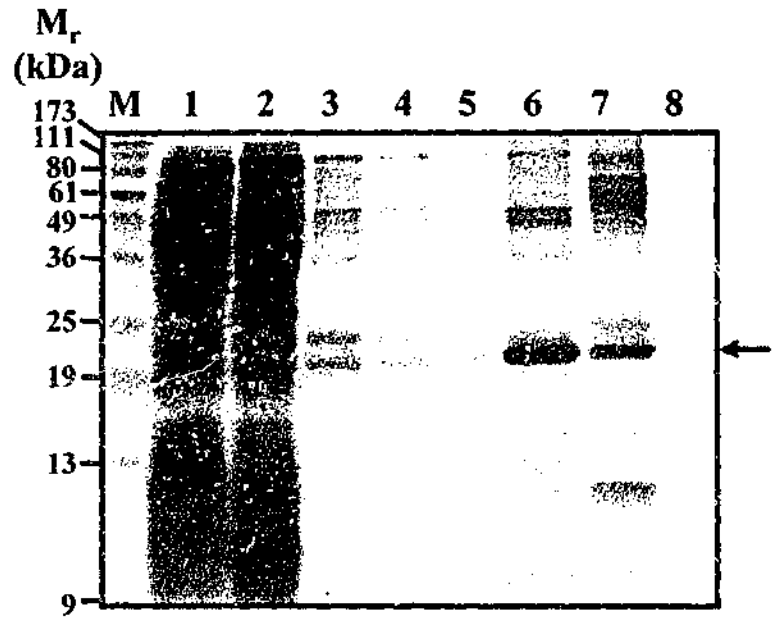


Figure 4.9 Large-scale purification of rAra h 2 under optimised conditions

rAra h 2 was purified using Ni^{2+} resin under optimised conditions and fractions were analysed by 14% SDS-PAGE under reducing conditions followed by Coomassie brilliant blue staining. Arrow indicates position of rAra h 2 monomer. **Lanes:** M – molecular mass markers (M_r), 1 – total lysate, 2 – flow through, 3-5 – wash fractions 1-4, 6-8 – elution fractions 1-3.

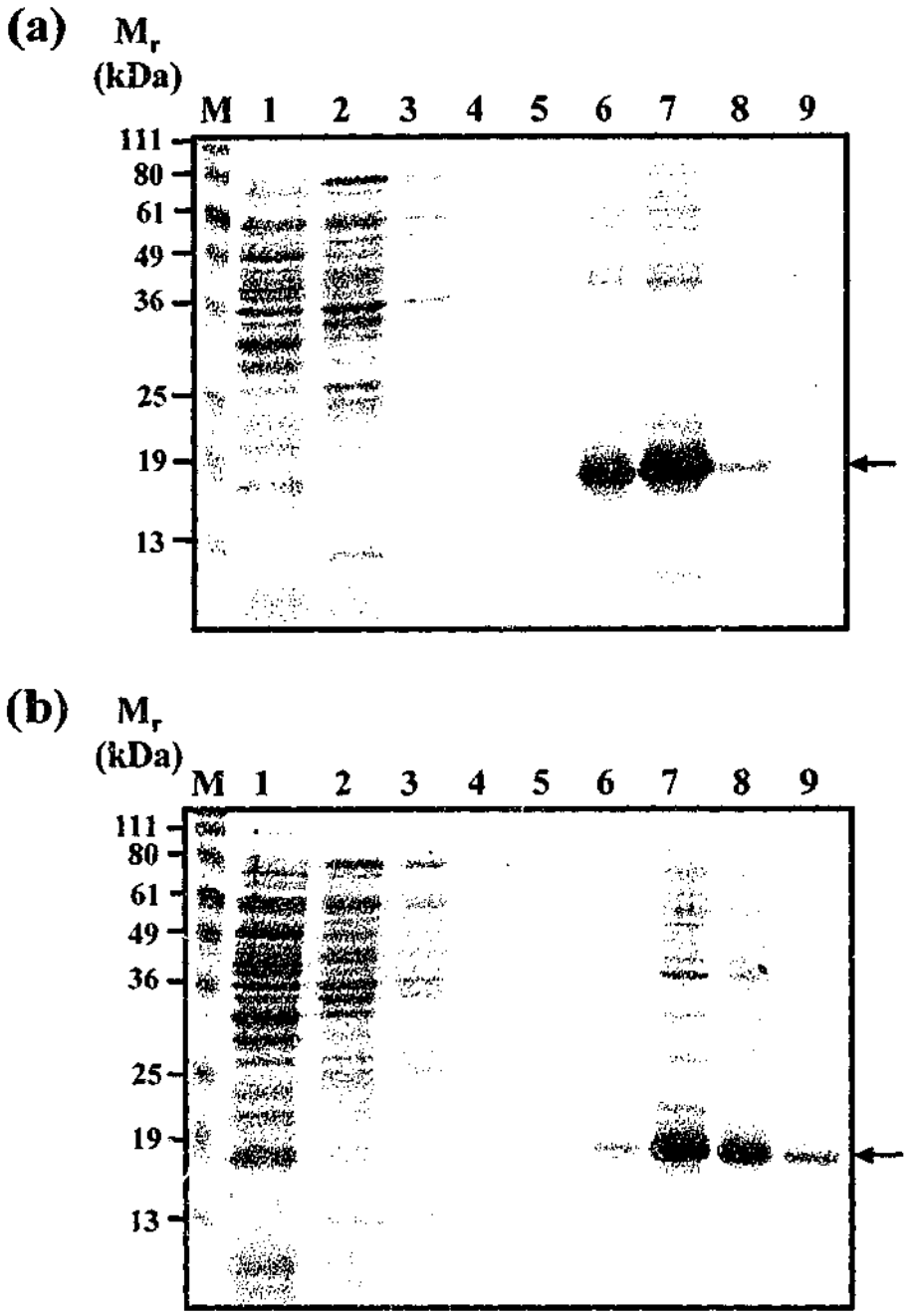


Figure 4.10 Purification of sulfonated rAra h 2

rAra h 2 was expressed using 2 L *E. coli* cultures and cell pellet was sonicated in sulfonation buffer. Two methods of purification were used: **(a)** purification using previously optimised buffers containing imidazole and **(b)** purification using the pH method. Fractions were resolved by 14% SDS-PAGE under reducing conditions and proteins were stained with Coomassie brilliant blue. Arrows indicate position of rAra h 2 monomer. **Lanes:** M – molecular weight markers (M_r), 1 – flow through, 2-5 – wash fractions 1-4, 6-9 – elution fractions 1-4.

uniformity, sulfonated rAra h 2 was purified using the optimised imidazole method. It should be noted that high molecular mass contaminants were still present in the elution fractions.

4.3.3.3 Purification of rAra h 3

Purification of rAra h 3 was carried out under denaturing conditions due to its insoluble nature. Similar to rAra h 2, pilot purification of rAra h 3 was performed using the pH method and the imidazole method. Fractions from both methods of purification were analysed by SDS-PAGE (Figure 4.11). Purification using the pH method (Figure 4.11a) yielded higher amounts of rAra h 3 (lanes 5-7) compared to the imidazole method (Figure 4.11b), as indicated by the intensity of staining. Consequently, the pH method was used in the large scale purification (Figure 4.12) and it can be seen that the majority of purified rAra h 3 was present in the first two elution fractions (Figure 4.12, lanes 4-5). However, the wash fraction also contained a high amount of unbound rAra h 3 (Figure 4.12, lane 3). This indicates that rAra h 3 is expressed in high abundance such that the amount of protein from a 2 L culture exceeds the binding capacity of the column. Protein estimations indicated that a 2 L culture can express up to 100 mg/L of rAra h 3 protein.

4.3.4 IgE reactivity of recombinant peanut allergens

4.3.4.1 IgE reactivity of rAra h 1

Western immunoblotting under reducing conditions was used to investigate the IgE reactivity of purified rAra h 1, as well as characterise the protein contaminants present in the purified fractions (Figure 4.13). It can be seen that the rAra h 1 monomer bound

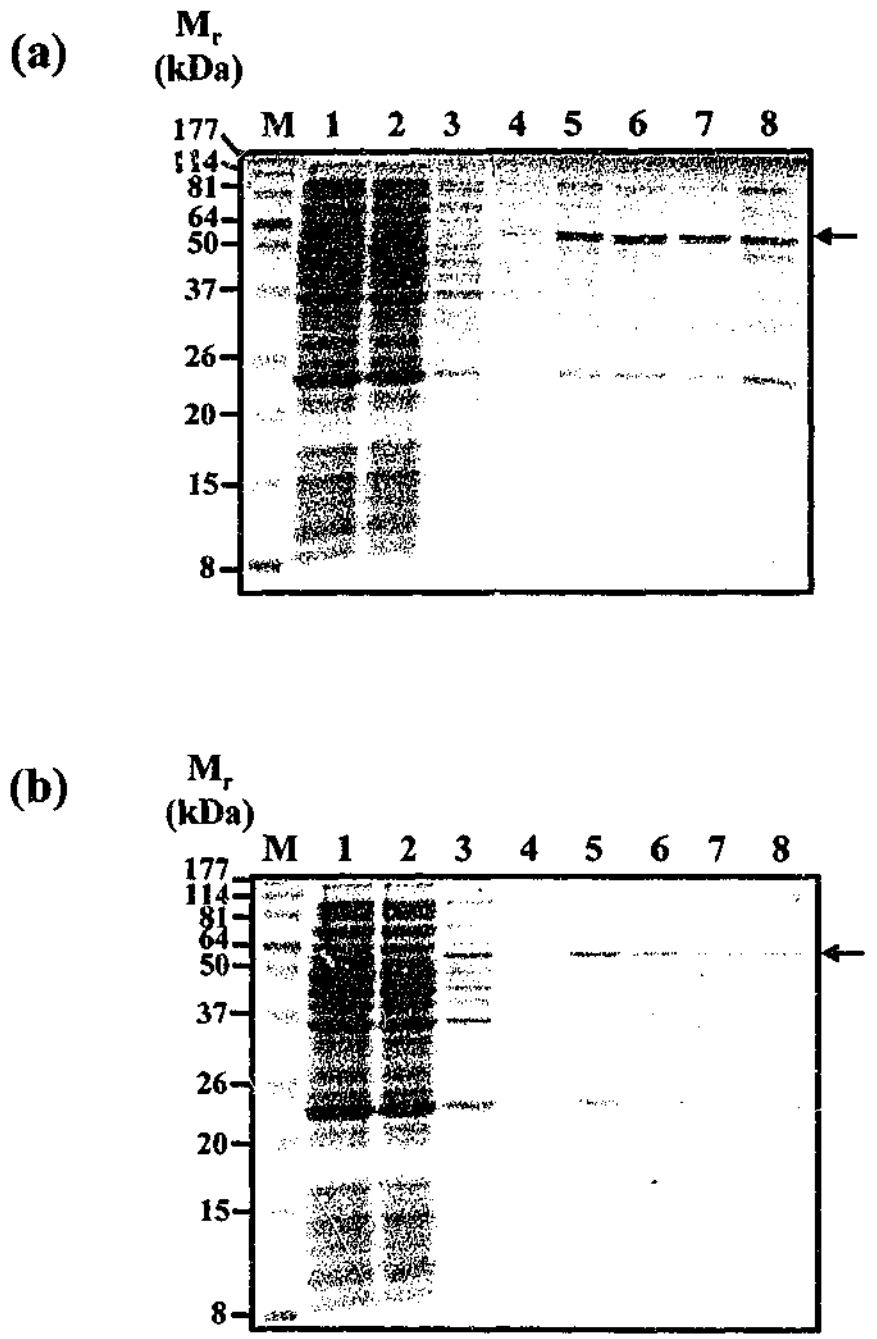


Figure 4.11 Purification of rAra h 3

rAra h 3 was expressed in *E. coli* cells and purified under denaturing conditions using nickel chelate chromatography. Two methods were used: **(a)** pH method and **(b)** imidazole method. Fractions were resolved by 14% SDS-PAGE under reducing conditions and proteins were stained with Coomassie brilliant blue. Arrows indicate the position of rAra h 3 monomer. **Lanes:** M – molecular mass markers (M_r), 1 – total lysate, 2 – flow through, 3-4 – wash fractions 1-2, 5-7 – elution fractions 1-3, 8 – resin.

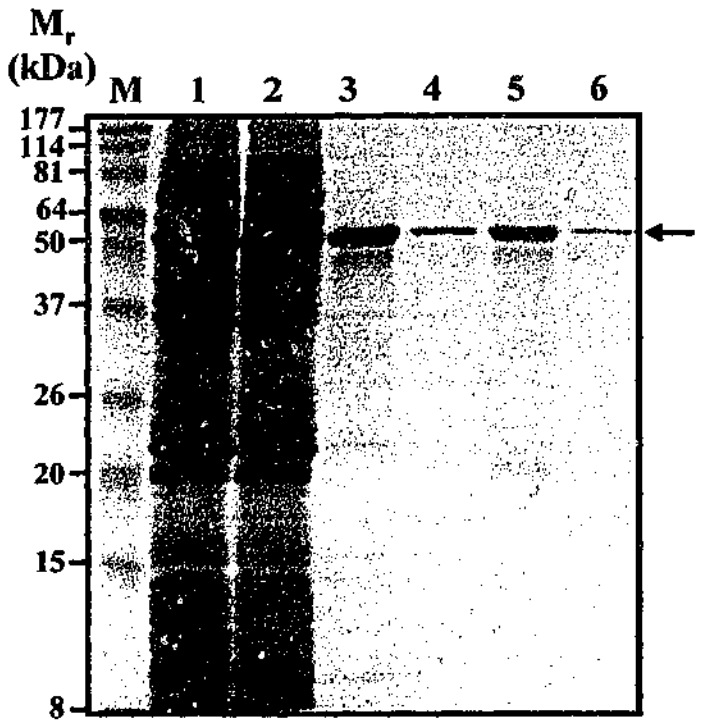


Figure 4.12 Large-scale purification of rAra h 3

rAra h 3 was purified using Ni^{2+} resin from a 2 L culture under denaturing conditions using the pH method. Fractions were resolved by 14% SDS-PAGE under reducing conditions and proteins were stained with Coomassie brilliant blue. Arrow indicates position of rAra h 3 monomer. **Lanes:** M – molecular mass markers (M_r), 1 – total lysate, 2 – flow through, 3 – wash fraction, 4-6 – elution fractions 1-3.

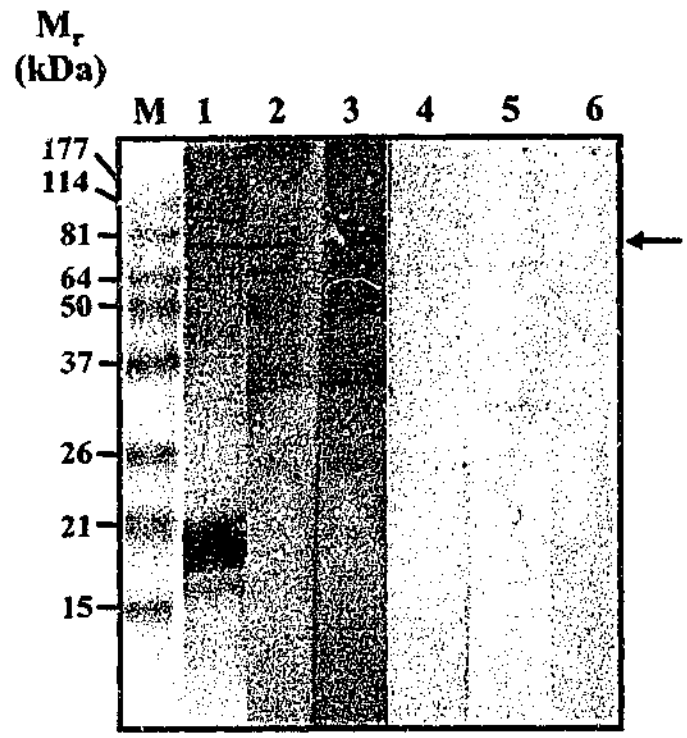


Figure 4.13 Analysis of serum IgE reactivity to purified rAra h 1 by immunoblotting

Purified rAra h 1 (2.5 μg) was resolved by 14% SDS-PAGE under reducing conditions followed by Coomassie blue staining (lane 1). IgE reactivity was assessed by Western immunoblotting using sera from 2 peanut allergic subjects (lanes 2 and 3). Sera from 2 atopic, non-peanut allergic subjects were used as negative controls (lanes 4 and 5). A secondary and tertiary antibody control was also included (lane 6). M indicates the position of the molecular mass standards (M_r) and arrow indicates the position of rAra h 1 monomer.

IgE antibodies from 2 peanut allergic subjects tested (Figure 4.13, lanes 2-3), confirming its allergenicity. IgE binding to the rAra h 1 monomer was accompanied by IgE reactivity to the lower molecular mass proteins (25-65 kDa), indicating that these proteins were likely to be breakdown products of the intact rAra h 1. However, the 20 kDa protein contaminant did not bind serum IgE from the peanut allergic subjects. This may be an *E. coli* protein that shows some affinity to the nickel resin. No IgE binding to the intact rAra h 1 or its breakdown products was observed when sera from 2 atopic, non-peanut allergic subjects were used (Figure 4.13, lanes 4-5).

The IgE reactivity of rAra h 1 under reducing and non-reducing conditions was also compared to examine the role of disulfide bonds in the conformation of this rAra h 1 preparation (Figure 4.14). As shown in Figure 4.14b, rAra h 1 forms high molecular mass multimers (lane 1) under non-reducing conditions. This is likely to be due to intermolecular disulphide bond formation between Ara h 1 monomers or with other proteins present in the preparation. These high molecular mass multimers, however, were still capable of binding serum IgE from a peanut allergic subject and appeared to be more IgE reactive under non-reducing conditions (Figure 4.14b, lane 2).

The IgE reactivity of the rAra h 1 preparation was also assessed quantitatively by ELISA. As shown in Figure 4.15, IgE binding to rAra h 1 was observed in 5 out of 8 peanut allergic subjects (63%), thus confirming the IgE reactivity of this recombinant allergen. Minimal IgE binding was observed among the atopic, non-peanut allergic and non-atopic control subjects.

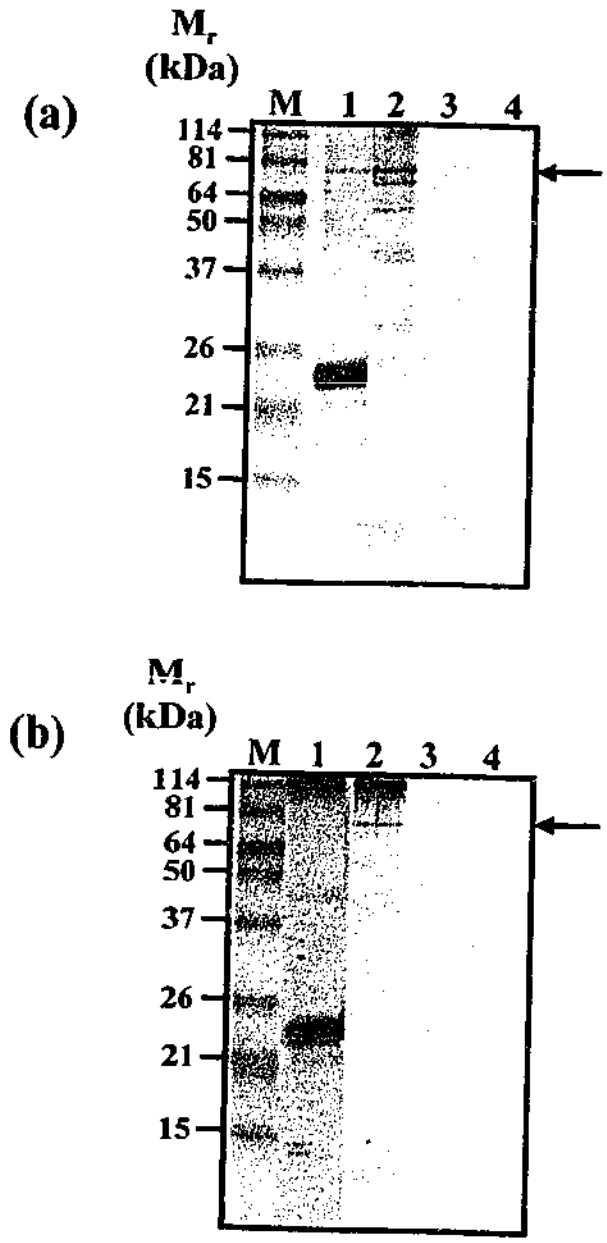


Figure 4.14 IgE reactivity of rAra h 1 under reducing and non-reducing conditions

Purified rAra h 1 (2.5 μg) was resolved under **(a)** reducing and **(b)** non-reducing conditions by 14% SDS-PAGE and stained with Coomassie blue (lane 1) or electroblotted onto nitrocellulose membranes. The IgE reactivity of rAra h 1 under reducing and non-reducing conditions was assessed. Lane 2 was probed with serum from a peanut allergic subject and lane 3 was probed with serum from an atopic, non-peanut allergic control subject. Lane 4 was incubated with the secondary and tertiary antibodies. M indicates the position of molecular mass standards (M_r) and arrow indicates position of rAra h 1 monomer.

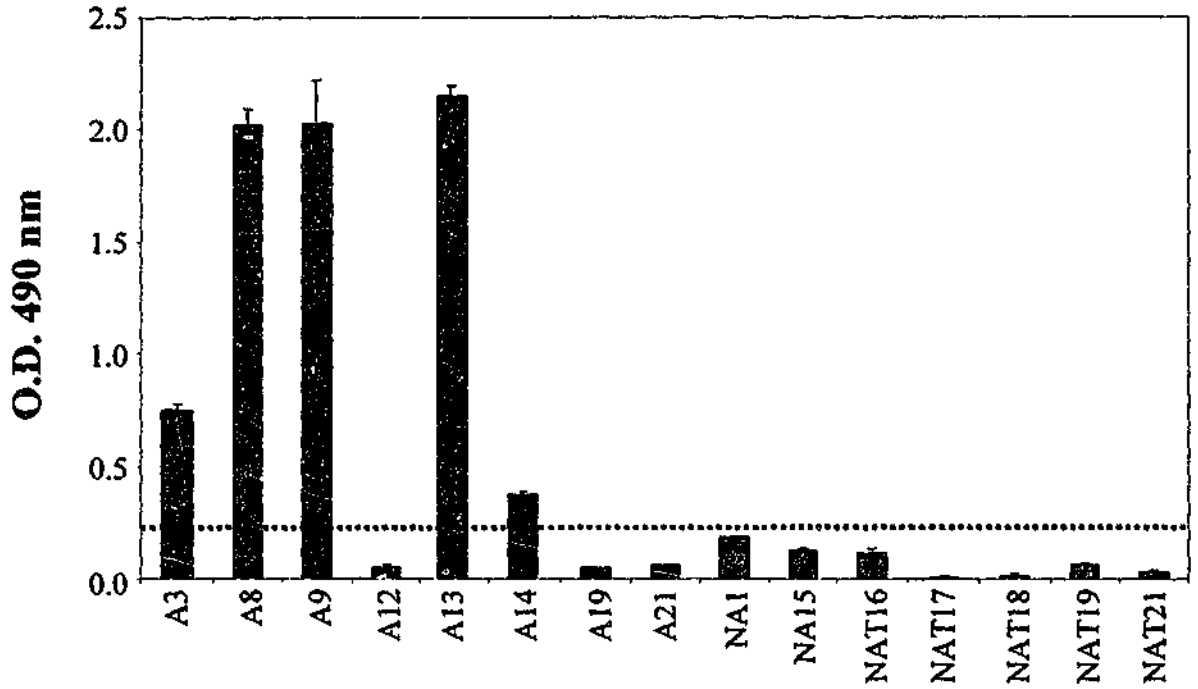


Figure 4.15 IgE reactivity of rAra h 1 as assessed by ELISA

ELISA plates were coated with 1 µg/ml of rAra h 1 and IgE binding was assessed using sera from 8 peanut allergic subjects (A). IgE binding was also assessed using sera from 2 atopic, non-peanut allergic subjects (NA) and 5 non-atopic subjects (NAT) as negative controls. The absorbance from control wells containing no antigen was subtracted from antigen-coated wells. Mean of triplicate values are shown and the standard deviation is indicated by the error bars. The positive cut-off was calculated as the mean + 2SD of the negative control subject data and is indicated by ..... .

4.3.4.2 IgE reactivity of non-sulfonated, sulfonated and refolded rAra h 2

The IgE reactivity of the non-sulfonated, sulfonated and refolded forms of rAra h 2 was initially assessed by Western immunoblotting under reducing and non-reducing conditions to examine disulfide bond formation in these protein preparations. The refolded form was obtained by dialysing sulfonated rAra h 2 against refolding buffer in the presence of oxidising and reducing agents. Figure 4.16a shows a Coomassie stained gel of all three forms of rAra h 2 resolved under reducing conditions. It can be seen that the non-sulfonated, sulfonated and refolded rAra h 2 proteins are present as single protein bands at ~20 kDa. These protein bands also bound IgE antibodies from a peanut allergic subject, demonstrating that all three forms of rAra h 2 are IgE reactive. However, under non-reducing conditions the non-sulfonated, sulfonated and refolded rAra h 2 appear to form high molecular mass multimers with other proteins present in the preparations although a single protein band at 20 kDa could still be detected with the sulfonated form (Figure 4.16b). Immunoblots revealed that these multimers were able to bind serum IgE antibodies from a peanut allergic subject. These high molecular mass multimers of rAra h 2 may be the result of intermolecular disulphide bond formation between rAra h 2 monomers or *E. coli* protein contaminants. IgE binding to rAra h 2 also appears to be greater under non-reducing conditions as indicated by the intensity of staining. No IgE binding was observed when serum from an atopic, non-peanut allergic subject was used as a probe, demonstrating the specificity of the observed IgE binding.

The IgE reactivity of all three forms of rAra h 2 was further assessed quantitatively by ELISA using sera from 8 peanut allergic subjects as well as 2 atopic, non-peanut allergic subjects and 5 non-atopic subjects as negative controls. It can be seen from Figure 4.17 that the non-sulfonated, sulfonated and refolded rAra h 2 bound IgE

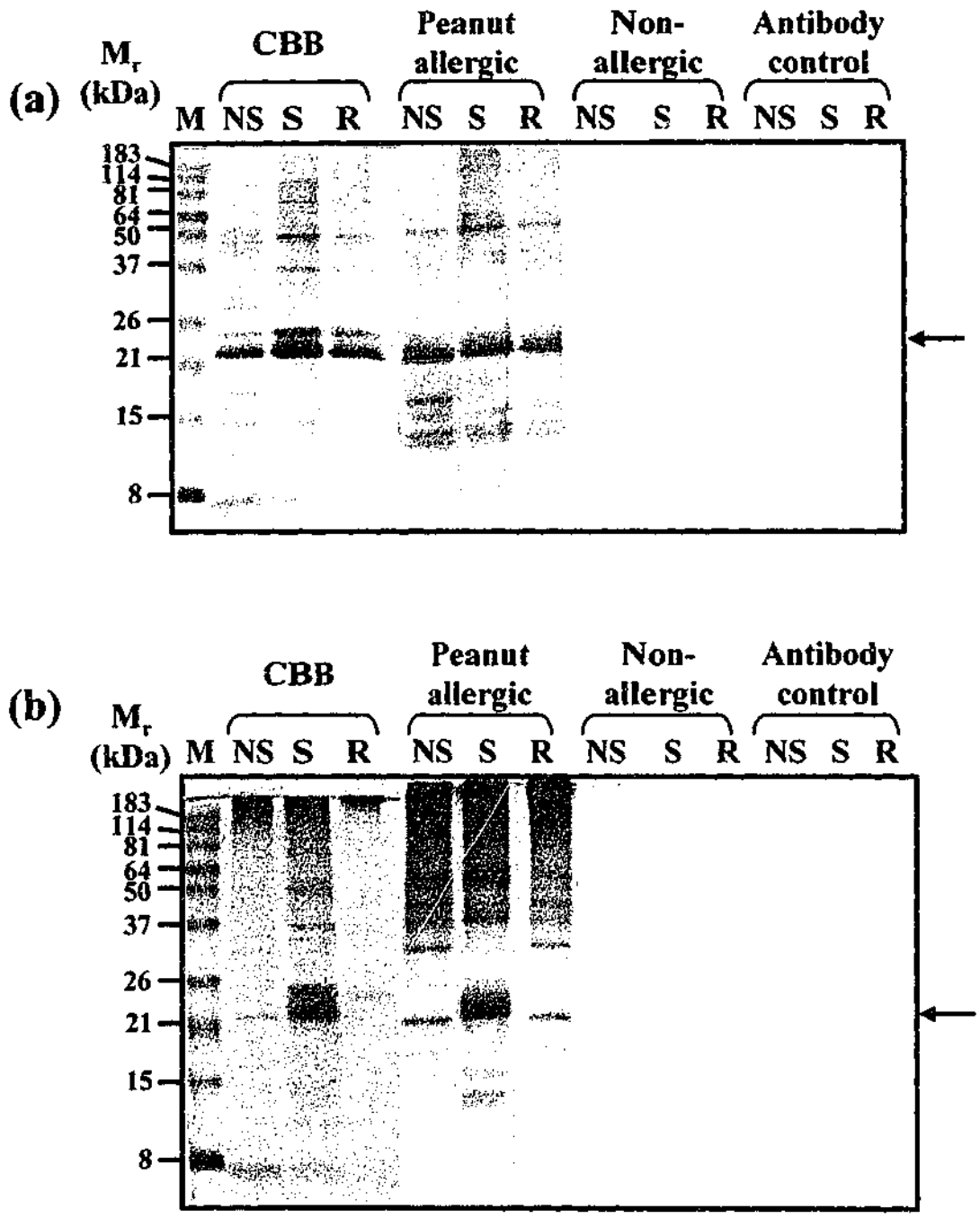


Figure 4.16 IgE reactivity of non-sulfonated, sulfonated and refolded rAra h 2 under reducing and non-reducing conditions

2 μg of purified non-sulfonated (NS), sulfonated (S) and refolded (R) rAra h 2 were resolved by 14% SDS-PAGE under **(a)** reducing and **(b)** non-reducing conditions and proteins were stained with Coomassie brilliant blue (CBB). IgE reactivity was assessed by Western immunoblotting using sera from a peanut-allergic subject and an atopic, non-peanut allergic subject. Membranes were also incubated with the secondary and tertiary antibodies as a control. M indicates position of molecular mass markers (M_r) and arrows indicate position of the rAra h 2 monomer.

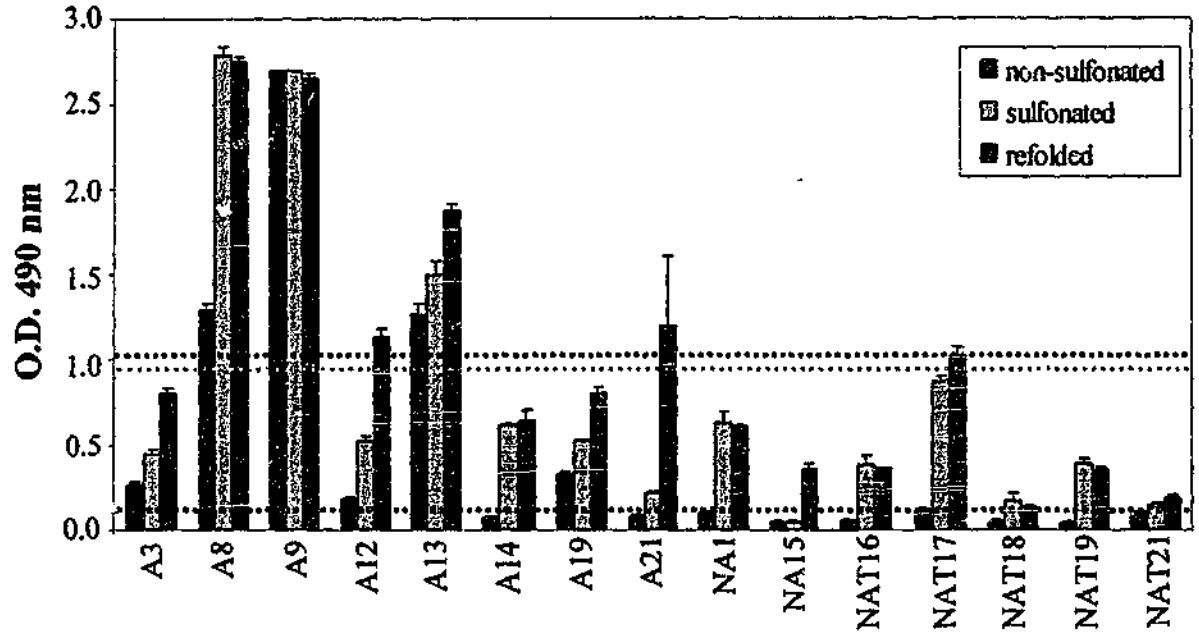


Figure 4.17 IgE reactivity of non-sulfonated, sulfonated and refolded rAra h 2 by ELISA

ELISA plates were coated with 1 μg/ml of non-sulfonated, sulfonated and refolded rAra h 2 and IgE binding was assessed in 8 peanut allergic subjects (A). IgE binding was also assessed using sera from 2 atopic, non-nut allergic subjects (NA) and 5 non-atopic subjects (NAT) as negative controls. The absorbance from control wells containing no antigen was subtracted from antigen-coated wells. Mean of triplicate values are shown and bars represent standard deviation. Dotted lines represent positive cut-off of mean + 2SD of non-peanut allergic negative control data.

antibodies from peanut allergic subjects. However, the sulfonated and refolded forms showed high levels of background IgE reactivity (i.e. bound serum IgE from non-allergic and non-atopic subjects) in contrast to the minimal IgE binding for the non-sulfonated rAra h 2. It was also demonstrated that the highest percentage of IgE reactivity was obtained with non-sulfonated rAra h 2, with 6 out of 8 (75%) subjects tested showing IgE binding to this protein. Consequently, the non-sulfonated form of rAra h 2 was selected for use in further studies.

4.3.4.3 IgE reactivity of purified rAra h 3

The IgE reactivity of purified rAra h 3 was initially assessed by Western immunoblotting under reducing conditions. As shown in Figure 4.18, sera from the 2 peanut allergic subjects tested demonstrated IgE binding to rAra h 3 at ~52 kDa (lanes 2-3). IgE binding was also observed with some lower molecular mass proteins which may be breakdown products of the rAra h 3 monomer. In contrast, minimal IgE binding was observed when sera from 2 atopic, non-peanut allergic subjects were used as probes.

The IgE reactivity of rAra h 3 under reducing and non-reducing conditions was also compared (Figure 4.19). Under reducing conditions, the rAra h 3 protein is present as a 52 kDa monomer. However, under non-reducing conditions the rAra h 3 protein forms higher molecular mass multimers (Figure 4.19b, lane 1). In contrast to rAra h 1 and rAra h 2, the majority of the rAra h 3 is still present as a monomer. Western immunoblotting under non-reducing conditions revealed that these rAra h 3 multimers are IgE reactive (Figure 4.19b).

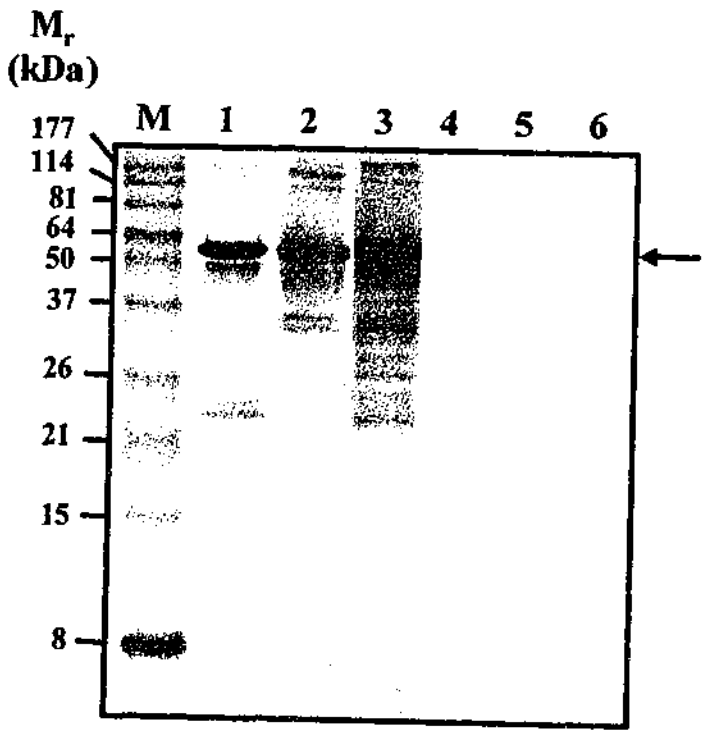


Figure 4.18 IgE reactivity of rAra h 3 using sera from peanut allergic subjects

Purified rAra h 3 (3 μg) was resolved by 14% SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue (lane 1). IgE reactivity was assessed by Western immunoblotting using sera from 2 peanut allergic subjects (lanes 2 and 3). Sera from 2 atopic, non-peanut allergic subjects were used as the negative control (lanes 4 and 5). A secondary and tertiary antibody control was also included (lane 6). M indicates position of molecular mass markers (M_r) and arrow indicates position of rAra h 3 monomer.

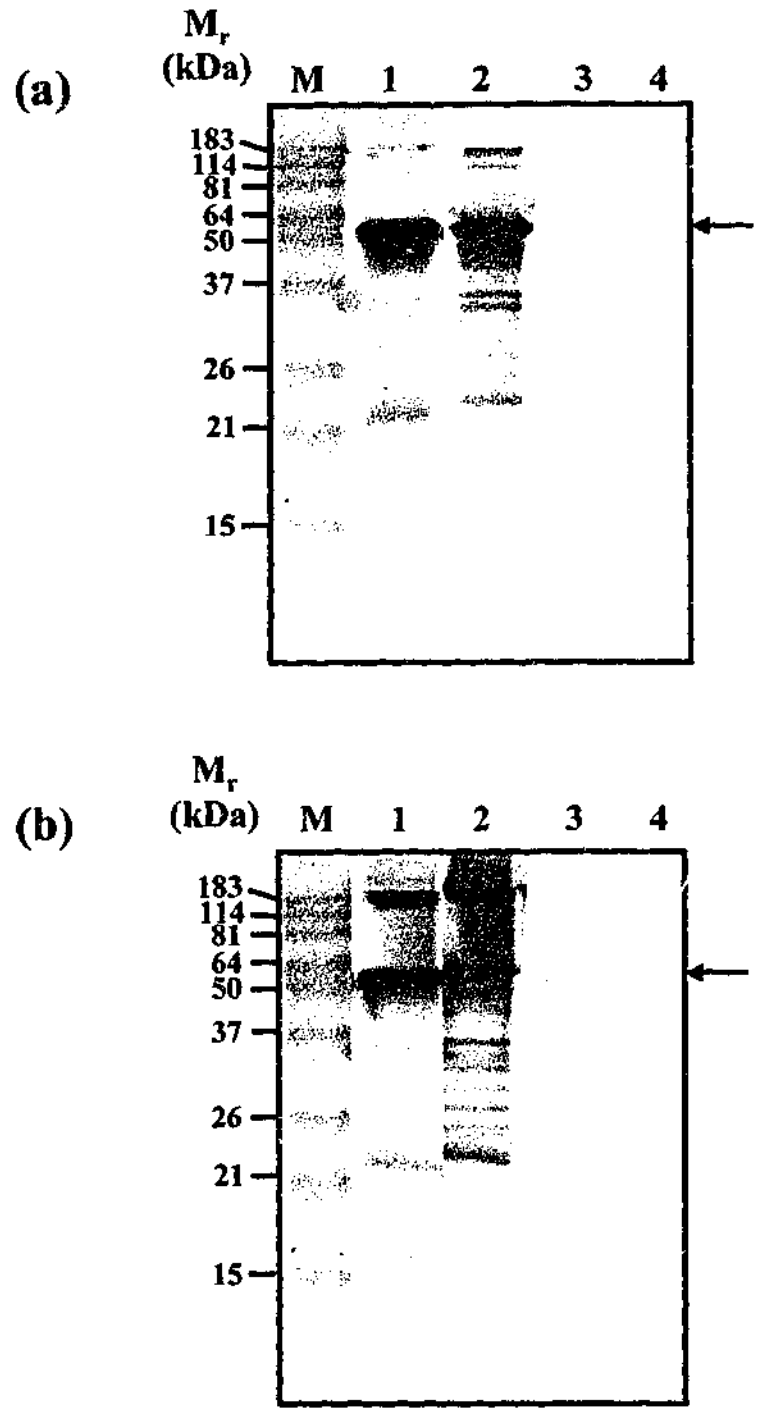


Figure 4.19 IgE reactivity of rAra h 3 under reducing and non-reducing conditions

Purified rAra h 3 (3 μg) was resolved under reducing **(a)** and non-reducing conditions **(b)** by 14% SDS-PAGE and stained with Coomassie brilliant blue (lane 1) or electroblotted onto nitrocellulose membranes. The IgE reactivity of rAra h 3 under reducing and non-reducing conditions was assessed. Lane 2 was probed with serum from a peanut allergic subject and lane 3 was probed with serum from an atopic, non-peanut allergic control subject. Lane 4 was incubated with secondary and tertiary antibody. M indicates the position of molecular mass standards (M_r). Arrow indicates position of rAra h 3 monomer.

ELISA was also used to quantitatively analyse the IgE reactivity of rAra h 3. Following dialysis against PBS to remove the urea, the purified rAra h 3 became insoluble although some remained soluble. The insoluble proteins were resuspended in denaturing lysis buffer and the IgE reactivity of both the soluble and insoluble fractions was subsequently assessed by ELISA. As shown in Figure 4.20, the level of IgE binding among 8 peanut allergic subjects was similar for both the soluble and insoluble fractions, with 3/8 (38%) subjects showing positive IgE reactivity. Minimal or no IgE binding was observed when sera from atopic, non-allergic and non-atopic subjects were used.

4.3.5 Identification of Ara h 3 in crude peanut extract

Ara h 3 was initially cloned by Rabjohn and colleagues (1999) following *N*-terminal sequencing of a 14 kDa IgE-reactive protein in crude peanut extract. Cloning experiments using primers based on this *N*-terminal sequence revealed that the size of the intact Ara h 3 protein was approximately 57 kDa (Rabjohn *et al.*, 1999). The rAra h 3 cDNA isolated in this study encodes only part of the sequence published by Rabjohn and colleagues (1999). To determine the size of this allergen in crude peanut extract, inhibition immunoblotting studies were carried out. Serum from a subject previously demonstrated to have rAra h 3-specific IgE was titrated against roasted peanut extract by Western immunoblotting (Figure 4.21a) and a serum dilution of 1/20 was selected.

The diluted subject serum was subsequently pre-incubated with different concentrations of rAra h 3 and incubated with nitrocellulose strips electroblotted with crude roasted peanut extract proteins. IgE binding was subsequently detected. Figure 4.21b shows that in the absence of rAra h 3 inhibitor, IgE antibodies bound to protein in peanut

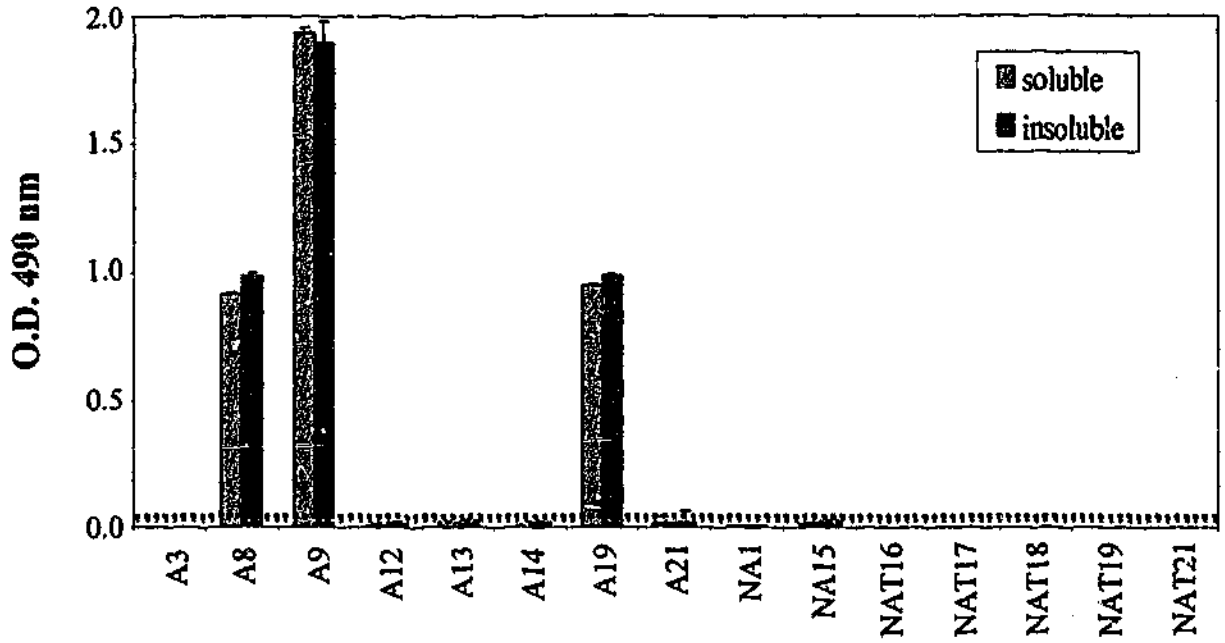


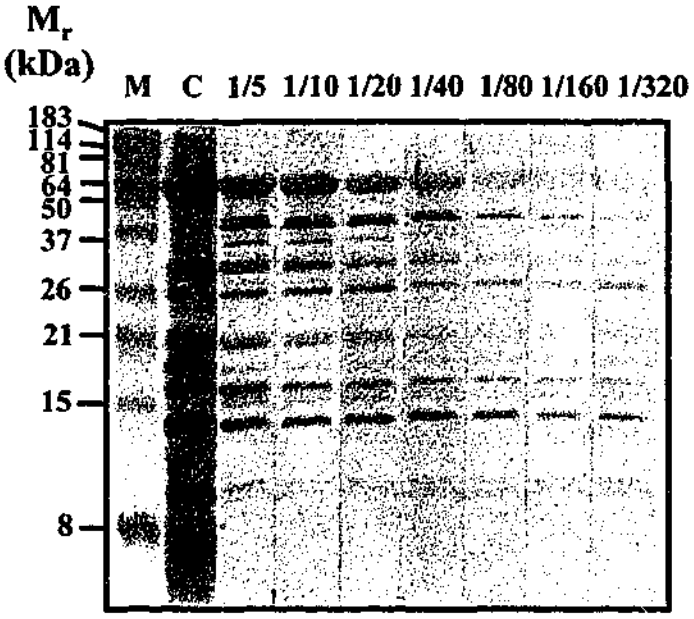
Figure 4.20 IgE reactivity of soluble and insoluble rAra h 3 as assessed by ELISA

ELISA plates were coated with 1 μg/ml of soluble and insoluble rAra h 3 and IgE binding was assessed in 8 peanut allergic subjects (A). IgE binding was also assessed using sera from 2 atopic, non-nut allergic subjects (NA) and 5 non-atopic subjects (NAT) as negative controls. The absorbance from control wells containing no antigen was subtracted from antigen-coated wells. Mean of triplicate values are shown and bars represent standard deviation. Dotted lines represent positive cut-off of mean + 2SD of non-peanut allergic negative control data.

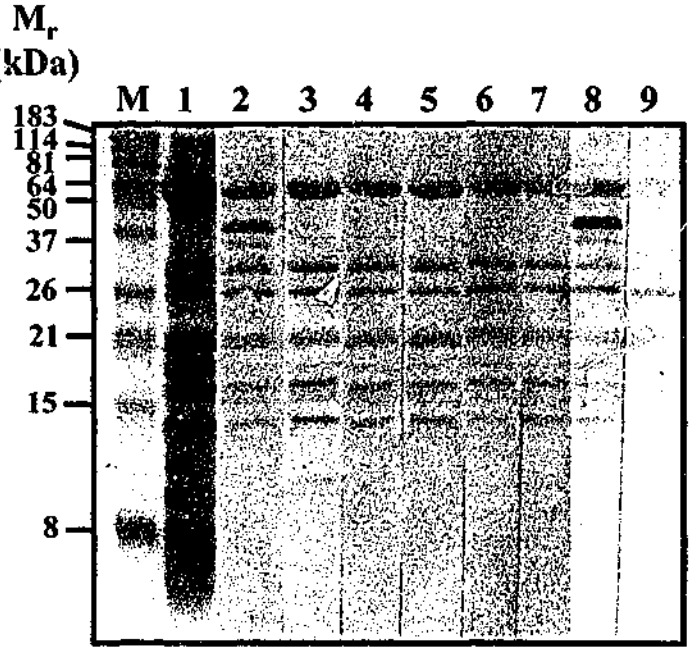
Figure 4.21 Inhibition of IgE binding to peanut extract using rAra h 3 as the inhibitor

Roasted peanut extract (30 μg) was resolved by 16% SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue or electroblotted onto nitrocellulose membranes. **(a)** Serum from subject A1 was initially titrated against roasted peanut extract to determine the optimum serum dilution for inhibition immunoblotting. A dilution of 1/20 was selected. **(b)** Inhibition immunoblotting was performed whereby serum from subject A1 was pre-incubated with 0.2-125 μg/ml of rAra h 3 and IgE binding to roasted peanut extract was assessed in comparison to the no inhibitor control. Subject serum was also pre-incubated with keyhole limpet haemocyanin (KLH) as a negative control. A secondary and tertiary antibody control immunoblot was also included.

(a)



(b)



M – Molecular mass markers (M_r)

1 – Coomassie brilliant blue staining

2 – no inhibitor

3 – 0.2 µg/ml rAra h 3

4 – 1 µg/ml rAra h 3

5 – 5 µg/ml rAra h 3

6 – 25 µg/ml rAra h 3

7 – 125 µg/ml rAra h 3

8 – 125 µg/ml KLH

9 – 2° + 3° antibody control

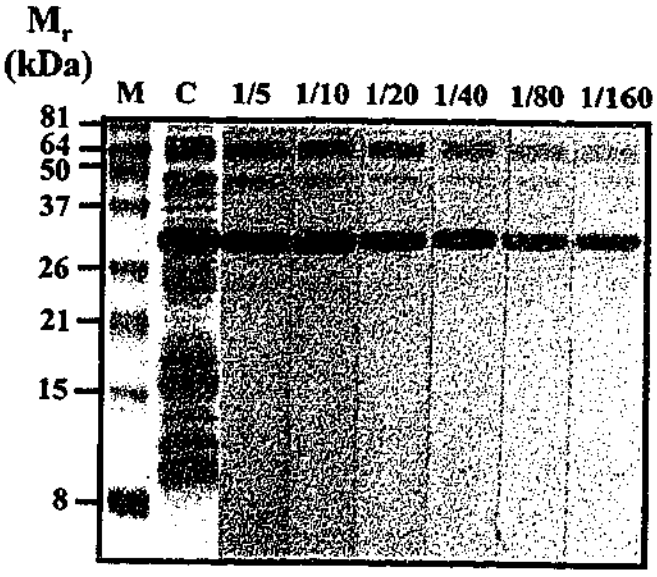
extract with a molecular mass of ~40 kDa (lane 2). However, pre-incubation of serum with as little as 0.2 µg/ml of rAra h 3 abolished IgE binding to this 40 kDa protein (Figure 4.21b, lane 3). As expected, no inhibition of IgE binding to the 14 kDa breakdown product of Ara h 3, which encodes the missing 5' DNA sequence, was observed when serum was pre-incubated with rAra h 3. As a negative control, serum was pre-incubated with an irrelevant protein, in this case KLH, which did not abolish IgE binding to the 40 kDa protein (Figure 4.21b, lane 8). No antibody binding was observed with the secondary and tertiary detection antibodies alone (Figure 4.21b, lane 9).

The specificity of the observed inhibition was investigated further by inhibition immunoblotting using crude Bermuda grass pollen (BGP) extract. Again, serum from a BGP allergic, non-peanut allergic subject was titrated against BGP extract (Figure 4.22a) and a dilution of 1/40 was selected. The subject serum at this dilution was then pre-incubated with rAra h 3 and IgE binding to nitrocellulose strips electroblotted with BGP proteins was assessed. As shown in Figure 4.22b, pre-incubation of serum with different concentrations of rAra h 3 did not inhibit IgE binding to crude BGP extract. In contrast, pre-incubation of serum with 125 µg/ml BGP extract inhibited IgE binding to high molecular mass proteins (Figure 4.22b, lane 8). This result further confirms the specificity of the observed inhibition of IgE binding to the 40 kDa protein in peanut extract by rAra h 3.

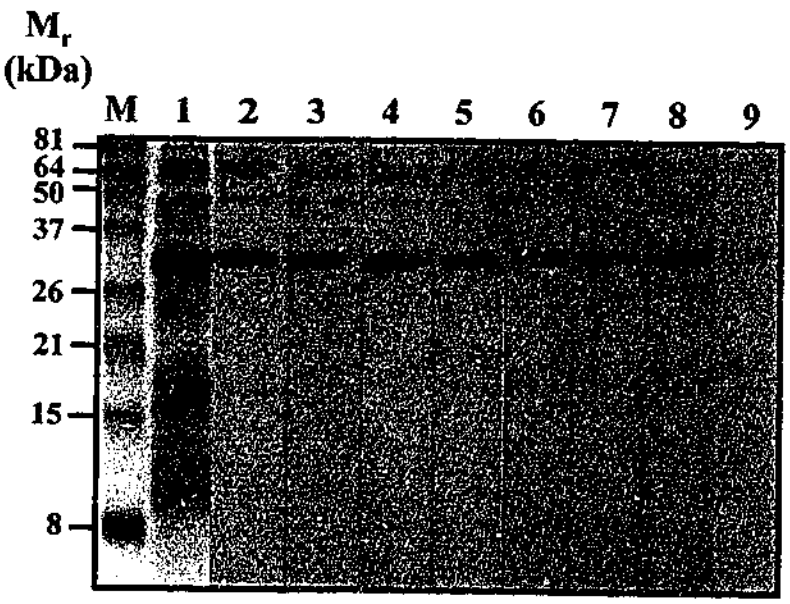
Figure 4.22 Non-specific inhibition of IgE binding to BGP extract using rAra h 3 as an inhibitor

BGP extract (30 μg) was resolved by 14% SDS-PAGE and stained with Coomassie brilliant blue or electroblotted onto nitrocellulose membranes. **(a)** Serum from a BGP allergic subject, with no history of peanut allergy (NA6), was intially titrated against roasted peanut extract to determine the optimum serum dilution for inhibition immunoblotting. A dilution of 1/40 was selected. **(b)** Inhibition immunoblotting was performed whereby serum from subject NA6 was pre-incubated with 0.2-125 μg/ml of rAra h 3 and IgE binding to BGP extract was assessed in comparison to the no inhibitor control. Subject serum was also pre-incubated with BGP extract at the maximum inhibitor concentration as a positive control. A secondary and tertiary antibody control immunoblot was also included.

(a)



(b)



M – Molecular mass markers

1 – Coomassie brilliant blue staining

2 – no inhibitor

3 – 0.2 µg/ml rAra h 3

4 – 1 µg/ml rAra h 3

5 – 5 µg/ml rAra h 3

6 – 25 µg/ml rAra h 3

7 – 125 µg/ml rAra h 3

8 – 125 µg/ml BGP extract

9 – 2° + 3° antibody control

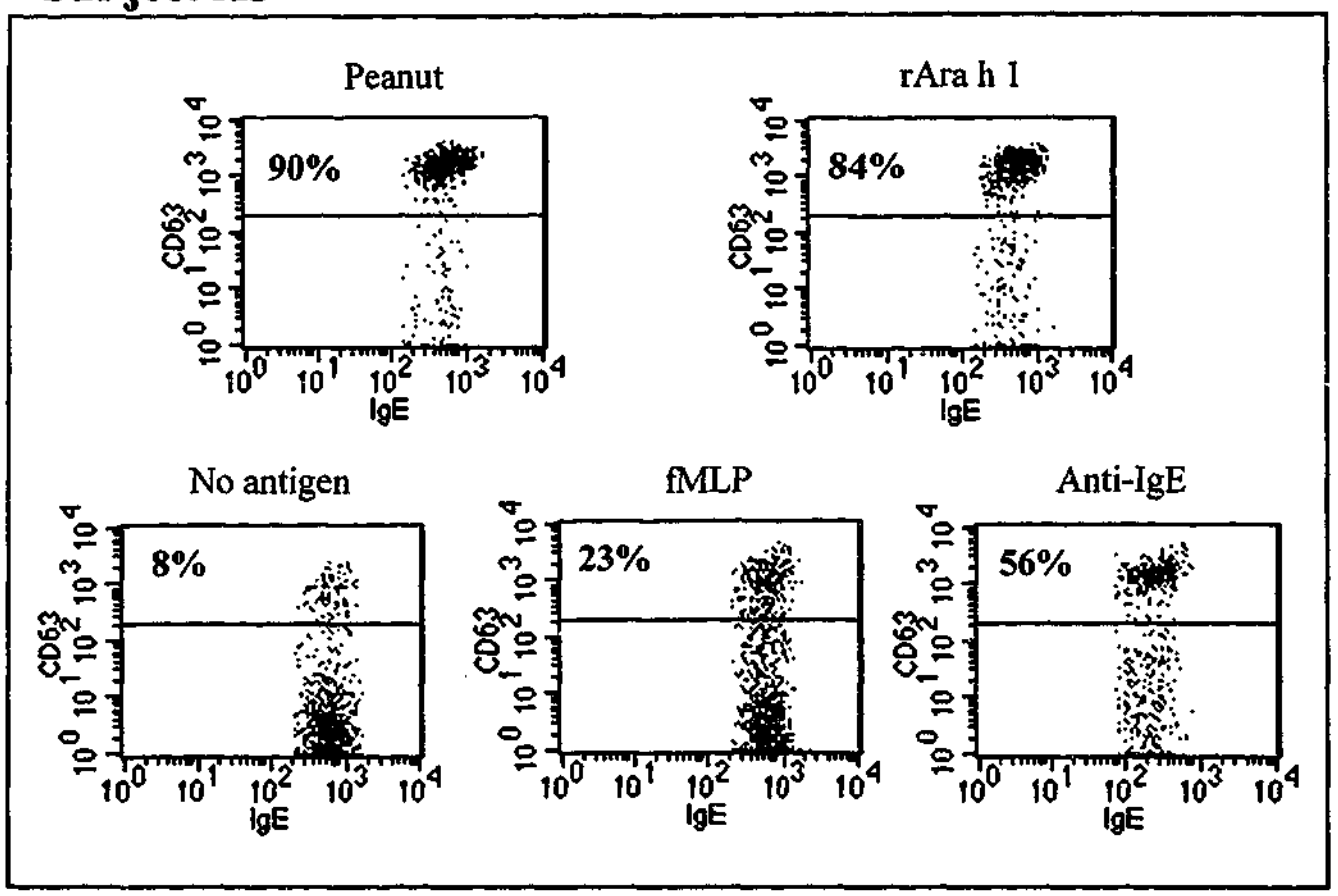
4.3.6 Biological activity of recombinant peanut allergens

4.3.6.1 Activation of basophils using rAra h 1, rAra h 2 and rAra h 3

Given that the recombinant peanut allergens prepared in this study were demonstrated to be IgE reactive, it was also deemed useful to show that these allergens were able to activate basophils from peanut allergic subjects. This was important in validating the biological activity of the recombinant allergen preparations. In this *in vitro* test, whole blood from 3 peanut allergic subjects (A3, A8 and A17) with serum IgE reactivity to rAra h 1, rAra h 2 or rAra h 3 was incubated with the recombinant allergens and unfractionated roasted peanut extract and the percentage of activated basophils, as indicated by CD63 expression, was determined by flow cytometry and analysed as outlined in Section 3.3.4. There was a high percentage of activated basophils (82-90%) following incubation of whole blood from subjects A3, A8 and A9 with roasted peanut extract (Figure 4.23). This figure also shows that incubation of whole blood with each of the recombinant peanut allergens resulted in a high percentage of activated basophils (84-87%), indicating that the rAra h 1, rAra h 2 and rAra h 3 preparations were biologically active. A similar percentage of activated basophils was obtained following stimulation of whole blood with both the soluble and insoluble fractions of rAra h 3 (Figure 4.23c) which correlates with the observed similarity in IgE reactivity of these two fractions. With all three allergen preparations, the percentage of activated basophils was higher compared to the positive controls, fMLP and anti-IgE stimulation. In contrast, the no antigen negative control stimulation produced lower percentages of activated basophils (3-34%), demonstrating the specificity of this assay. These results indicate that the recombinant allergen preparations were biologically active.

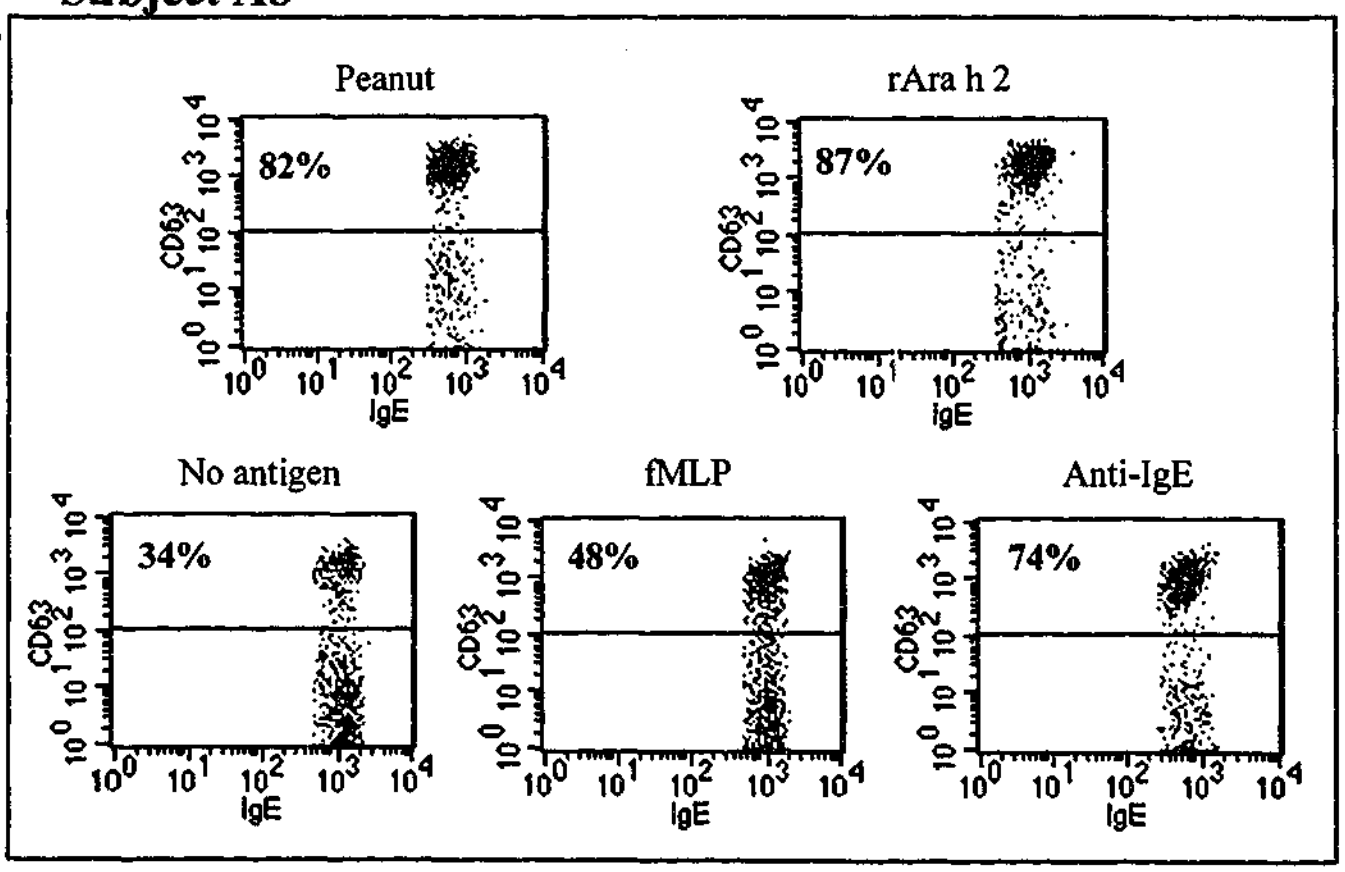
Subject A3

(a)



Subject A8

(b)



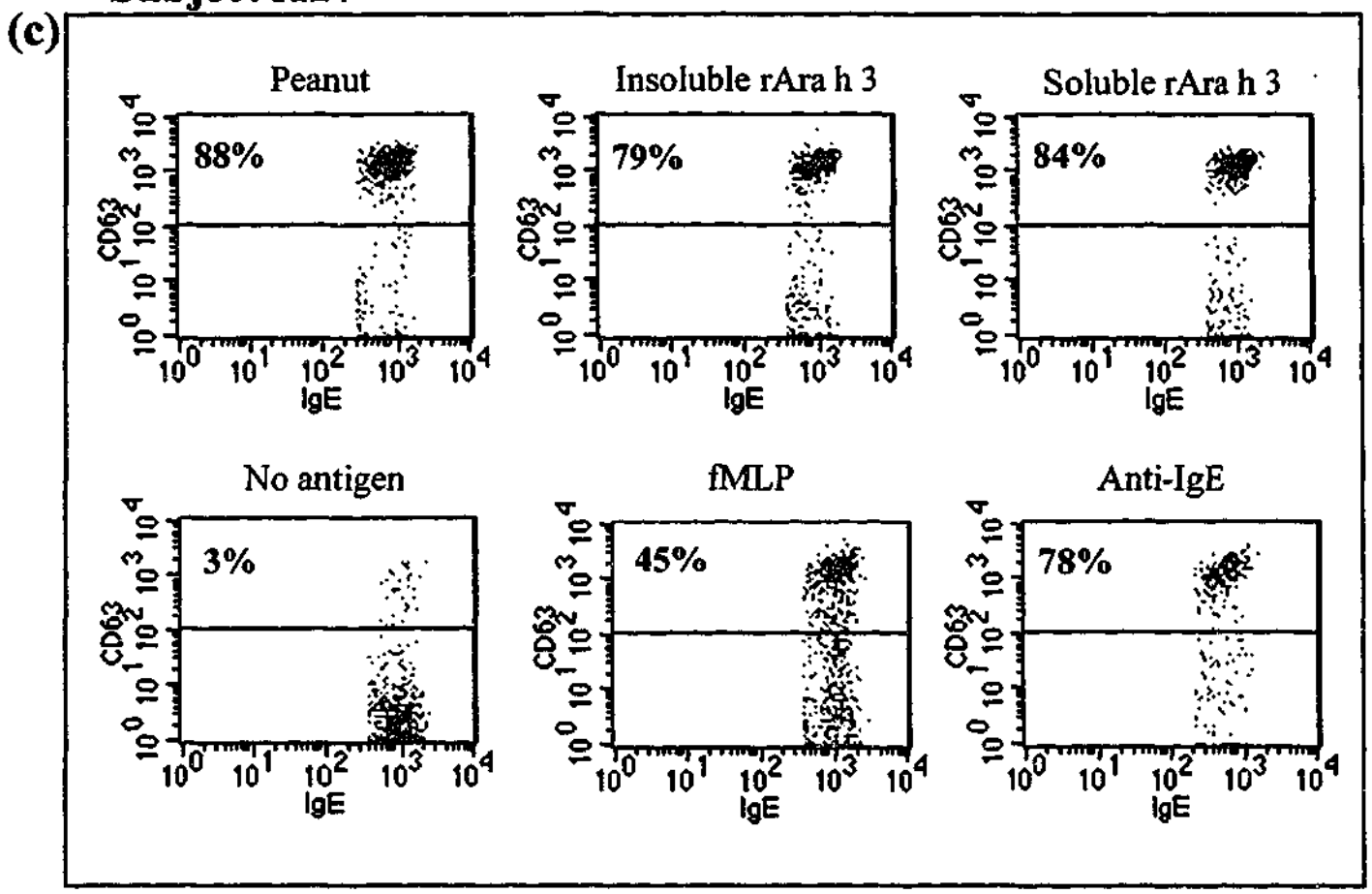


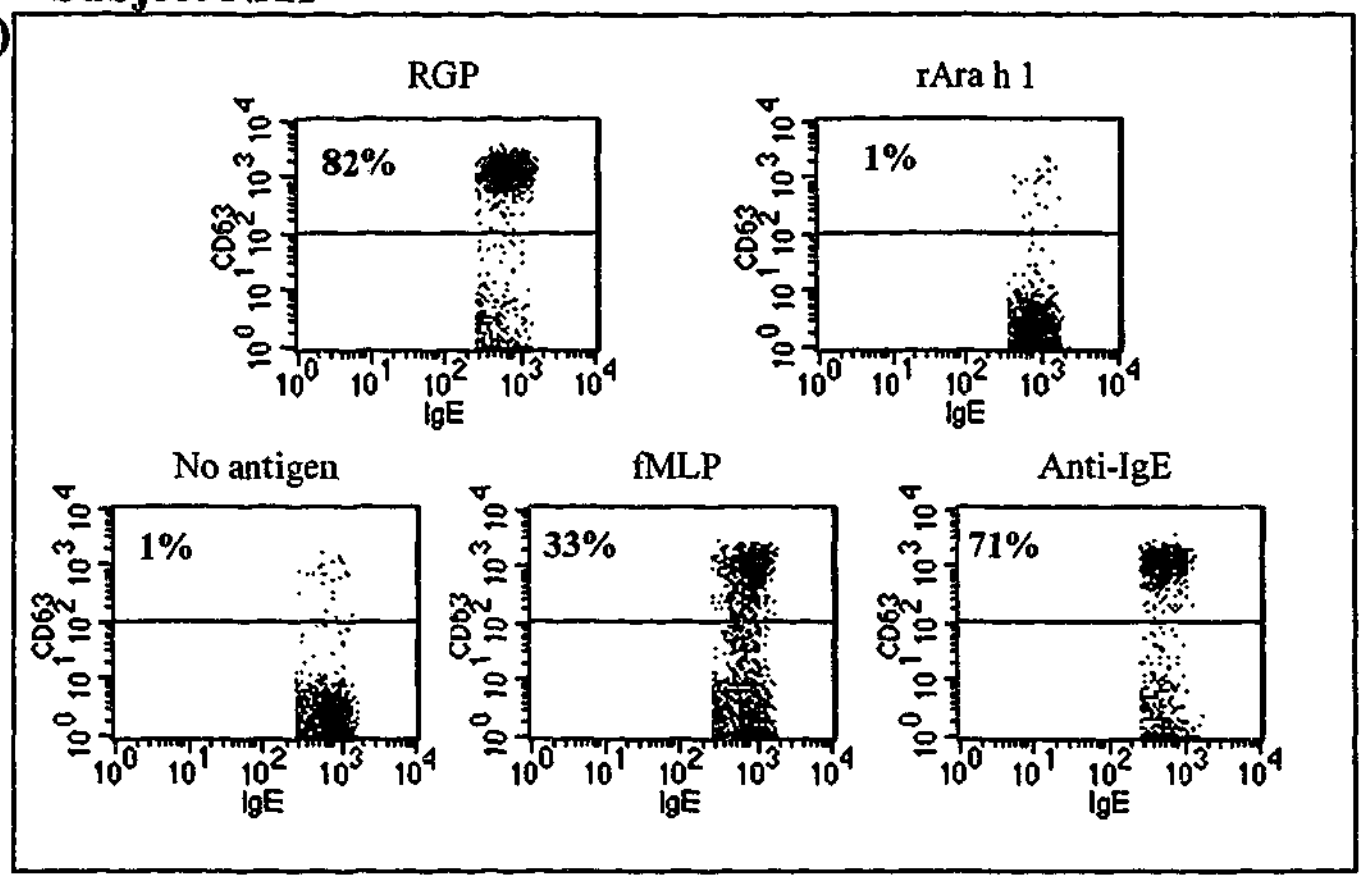
Figure 4.23 Activation of basophils from peanut allergic subjects following stimulation with recombinant peanut allergens

Whole blood from 3 peanut allergic subjects (A3, A8 and A17) were incubated with 1 μg/ml of roasted peanut extract (positive control) and purified **(a)** rAra h 1, **(b)** rAra h 2 and **(c)** rAra h 3 and the percentage of activated basophils (upper quadrant) was analysed. A no antigen negative control was included in the assay to ascertain the percentage of spontaneously activated basophils (upper quadrant). Whole blood was also incubated with fMLP and anti-IgE antibody as positive controls. $CD63^+$ cells were gated based on the discrimination of negative and positive control staining.

As an additional control for specificity, rAra h 1, rAra h 2 and rAra h 3 were also used to stimulate basophils from HDM, non-peanut allergic and rye grass pollen (RGP), non-peanut allergic subjects. It can be seen from Figure 4.24 that incubation of whole blood with these recombinant peanut allergens resulted in minimal activation of basophils in comparison to the peanut allergic subjects (Figure 4.23). In contrast, stimulation with the positive control allergens (HDM or RGP) resulted in high percentages of activated basophils (82-83%) which were again higher compared to the positive controls, fMLP and anti-IgE. Minimal basophil activation (1-4%) was obtained in the absence of antigen stimulation (Figure 4.24).

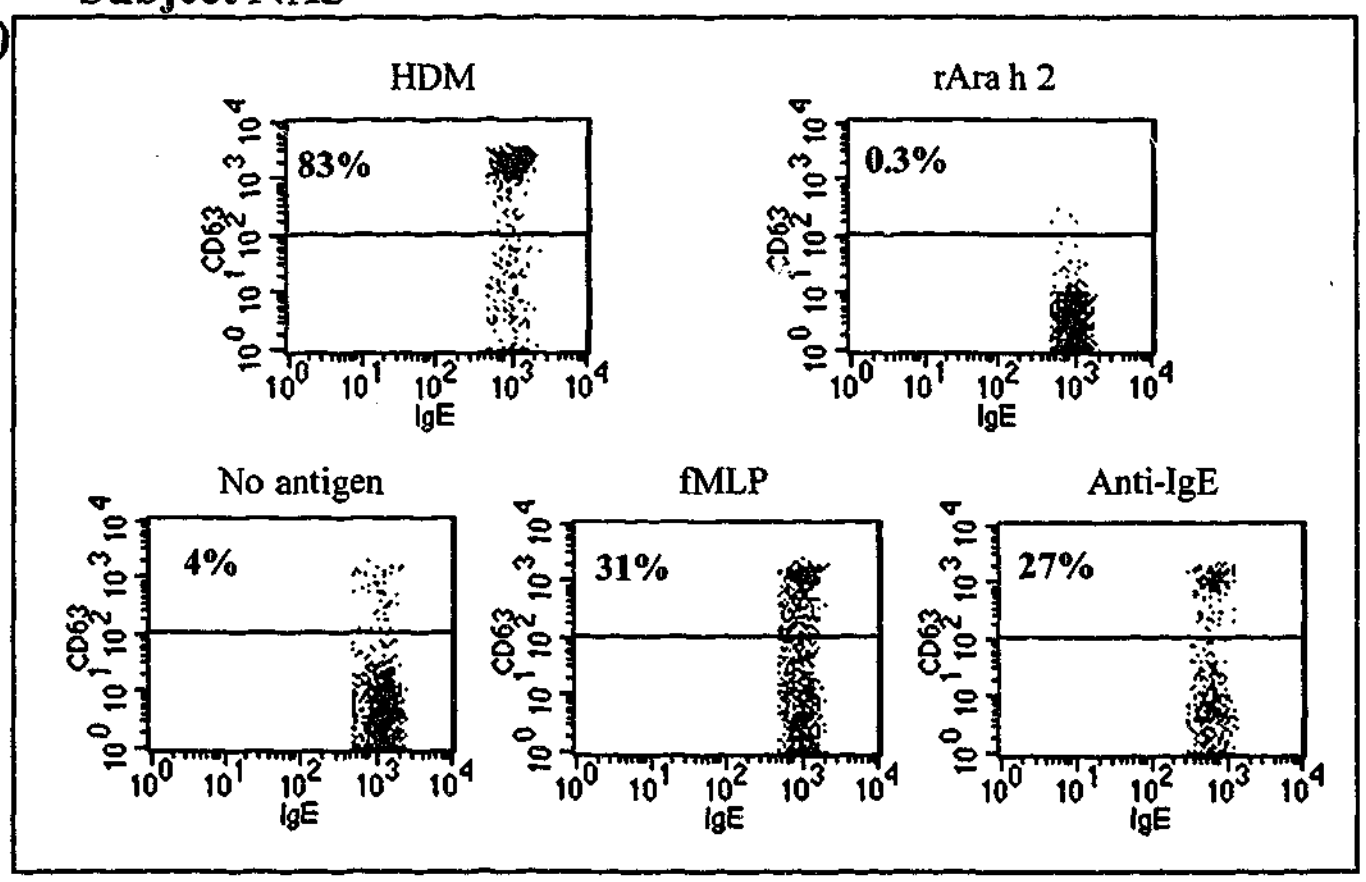
Subject NA1

(a)



Subject NA3

(b)



Subject NA1

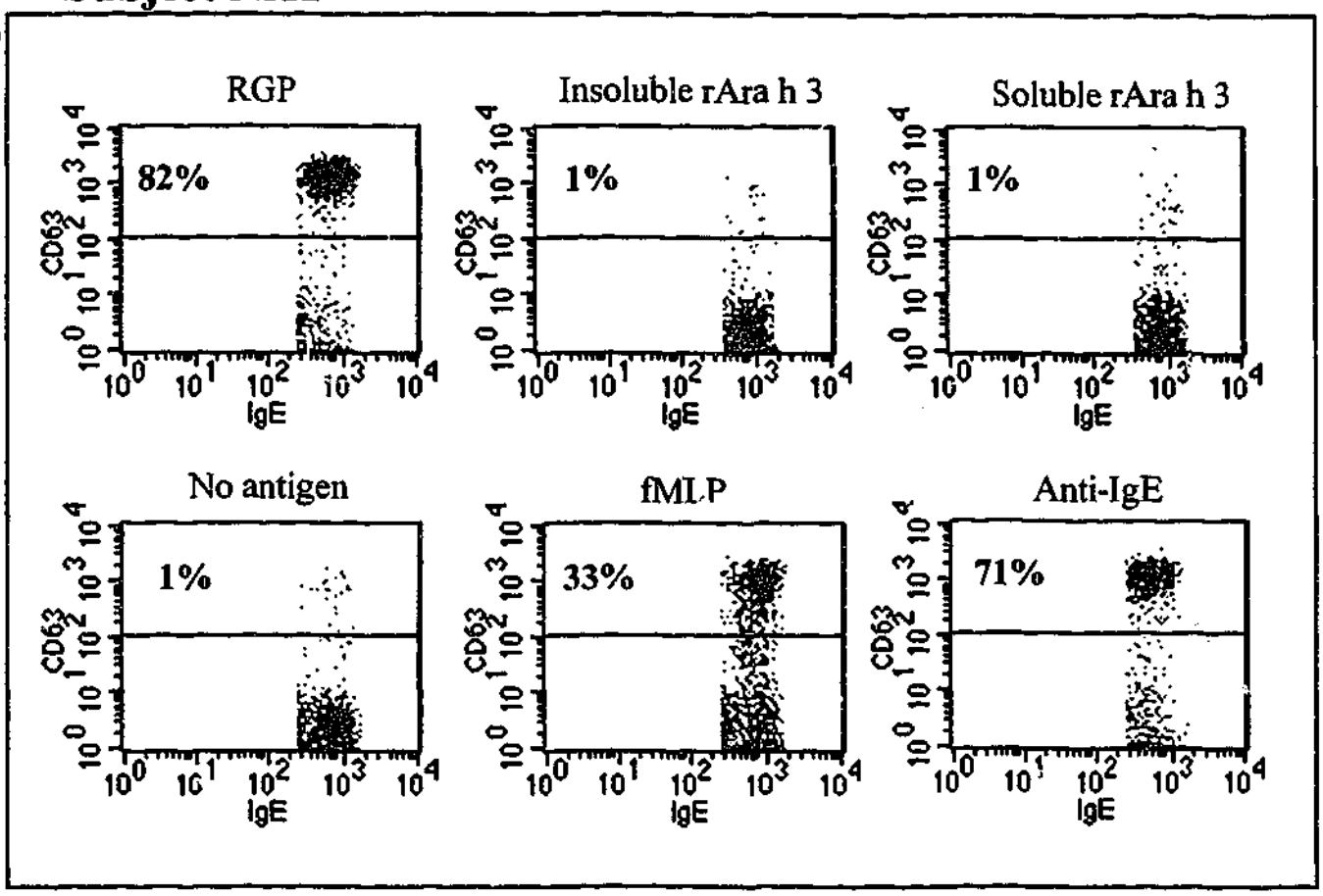


Figure 4.24 Activation of basophils from non-peanut allergic subjects following stimulation with recombinant peanut allergens

Whole blood from a HDM allergic, non-peanut allergic subject (NA3) and a RGP allergic, non-peanut allergic subject (NA1) were incubated with 1 μg/ml of HDM or RGP extract (positive control) and purified **(a)** rAra h 1, **(b)** rAra h 2 and **(c)** rAra h 3 and the percentage of activated basophils (upper quadrant) was analysed. A no antigen negative control was included in the assay to ascertain the percentage of spontaneously activated basophils (upper quadrant). Whole blood was also incubated with fMLP and anti-IgE antibody as positive controls. $CD63^+$ cells were gated based on the discrimination of negative and positive control staining.

4.4 DISCUSSION

This chapter describes the cloning, expression and purification of the recombinant forms of the peanut allergens Ara h 1, Ara h 2 and Ara h 3. All three recombinant allergens were expressed in *E. coli* cells with the aim of using these proteins in the investigation of allergenic B cell cross-reactivity between peanut and tree nuts which will be reported in subsequent chapters.

rAra h 1 was successfully expressed in *E. coli* cells although numerous truncated products of Ara h 1 were present in the purified fractions. This finding was also described by Burks *et al.* (1995) who attributed it to the inefficient translation of the amino terminal portion of this protein which may be due to rare codons (Kane, 1995), numerous cysteine residues or the secondary structure of the mRNA. Such causes of poor translation may also have contributed to the low yields of rAra h 1 generated in this study.

The amount and quality of the expressed recombinant protein can, however, be improved by using other *E. coli* strains that contain extra copies of rare codons (e.g. Rosetta strain) that are frequently used in the Ara h 1 gene, for example AGG/AGA (arginine). These were the first rare codons reported to be detrimental to protein expression (Spanjaard and Van Duin, 1988, Spanjaard *et al.*, 1990). *E. coli* strains with mutations in both the thioredoxin reductase and glutathione reductase genes (e.g. Origami, BL21trxB-) are also available which greatly enhance disulfide bond formation in the cytoplasm, resulting in greater conformational stability and increased intact expression of proteins. Such methods have been employed in other studies to obtain

efficient expression of recombinant proteins (Lauber *et al.*, 2001, Loyevsky *et al.*, 2003) and may allow more efficient translation of the Ara h 1 gene, resulting in the expression of intact proteins and improved protein yields. The use of other expression vectors may also improve protein yields as demonstrated in a recent study by Lehmann and colleagues (2003) which reported varying expression levels of rAra h 2 depending on the *E. coli* strain and vector used. Nevertheless, the IgE reactive rAra h 1 preparation generated in the current study will still be a useful tool for studying the allergic response to peanut and tree nut allergens.

Ara h 2, a major peanut allergen, was first described by Burks *et al.* (1992). The high-level expression and purification of the recombinant form of this peanut allergen was only recently described in a study by Lehmann *et al.*, (2003). In this chapter, Ara h 2 was successfully expressed in *E. coli* cells, with moderate yields, and the expressed protein was also shown to be IgE reactive. Attempts were made to improve the yield and IgE reactivity of rAra h 2 by sulfonating and subsequently refolding this protein. Such experiments have been previously used to generate recombinant allergens that assume the same conformation as the native derivative, thus enhancing IgE binding (Su *et al.*, 1999). The sulfonation of rAra h 2 did result in greater yields and subsequent refolding improved its IgE reactivity however IgE binding was also detected using sera from subjects that were not sensitive to peanut. This may be due to incorrect refolding of rAra h 2, leading to the formation of irrelevant IgE binding epitopes. The presence of *E. coli* protein contaminants may also have interfered in the refolding process as demonstrated by the formation of rAra h 2 multimers. Successful refolding may be achieved by obtaining a 'cleaner' protein preparation using gel filtration or ion-exchange chromatography to remove unwanted proteins. Protein expression using a modified *E. coli* strain that has an oxidising cytoplasm that promotes the formation of

disulfide bonds (e.g. Origami (DE3)) can also produce a properly folded rAra h 2 molecule (Lehr. *et al.*, 2003). In contrast, non-sulfonated rAra h 2 was shown to be IgE reactive in subjects with confirmed peanut allergy but not in non-peanut allergic subjects and as a consequence, this form of rAra h 2 was used in subsequent cross-reactivity studies.

The Ara h 3 used in the current was initially isolated as an IgE-reactive clone following immunoscreening of a λgt11 peanut cDNA library using serum from a peanut allergic subject (de Leon, 1999). This clone was sequenced and shown to encode a partial cDNA of Ara h 3. Attempts were made using RT-PCR to obtain the 5' DNA sequence corresponding to the published sequence from Rabjohn *et al.* (1999) but these were unsuccessful. However, expression of this partial Ara h 3 cDNA produced an IgE-reactive protein. Inhibition immunoblotting studies also demonstrated that this protein may exist in unfractionated peanut extract as an IgE-reactive 40 kDa protein. *N*-terminal sequencing of this protein to confirm its identity was not possible due to its low abundance in crude peanut extract. Consequently, further studies are required to determine whether this 40 kDa protein is indeed natural Ara h 3.

It could be argued that the expression of Ara h 1, Ara h 2 and Ara h 3 using a prokaryotic system is not an accurate reflection of their natural derivatives in peanut, which are produced in a eukaryotic system. Post-translational modifications (e.g. glycosylation) which may be required for IgE recognition do not occur in a prokaryotic expression system. Whether this is relevant for peanut allergens is not known and thus cannot be excluded, however the IgE reactivity of the recombinant allergens shown in this chapter indicate that at least some of the relevant IgE-binding epitopes are present in these recombinant preparations.

It is also useful to demonstrate that these recombinant peanut allergen preparations are biologically active. *In vitro* assays such as histamine release or basophil activation have been widely used to ascertain the biological relevance of recombinant allergens (Boutin *et al.*, 1997, Iacovacci *et al.*, 2002, Diaz-Perales *et al.*, 2003, Westphal *et al.*, 2003). In this chapter, each recombinant allergen was assessed for its ability to activate basophils from peanut allergic subjects by flow cytometry as indicated by cell surface CD63 expression. It was demonstrated that recombinantly expressed Ara h 1, Ara h 2 and Ara h 3 were able to activate basophils from peanut allergic subjects. Given that these recombinant allergens have the tendency to form multimers in solution, it is unclear whether basophil activation was due to the presence of all of the IgE binding epitopes on the surface of the protein or the aggregation of IgE binding epitopes following multimer formation. A comparison of the biological activity of the recombinant and natural forms of Ara h 1, Ara h 2 and Ara h 3 should be considered in future studies to assess the true biological relevance of the recombinant allergen preparations. This may also provide further insight on whether carbohydrate groups contribute to the biological activity of peanut allergens. Nevertheless, together with the IgE binding assays, these data confirm that these recombinant peanut allergen preparations contain some if not all of the relevant IgE binding epitopes.

In conclusion, the peanut allergens Ara h 1, Ara h 2 and Ara h 3 were successfully cloned from peanut cDNA and expressed in *E. coli* cells as recombinant proteins. These recombinant allergens bound serum IgE antibodies from peanut allergic subjects and were able to activate basophils, confirming their biological activity. The availability of purified peanut allergens will be useful in determining whether or not these allergens are involved in IgE cross-reactivity between peanuts and tree nuts, thus contributing to the manifestation of multiple-nut sensitivities in the peanut allergic population

CHAPTER 5

CHARACTERISATION OF THE B CELL RESPONSE TO PEANUT AND TREE NUT ALLERGENS IN PEANUT ALLERGIC SUBJECTS

5.1 INTRODUCTION

In the previous two chapters, the generation of unfractionated peanut and tree nut extracts as well as purified recombinant peanut allergens for use in cross-reactivity studies is described. However, before use in cross-reactivity studies, it important to characterise the IgE antibody response of peanut allergic subjects to these allergen sources. In particular, it is necessary to establish that many peanut allergic subjects are sensitised to at least one tree nut type as this forms the basis for investigating allergenic cross-reactivity between peanut and tree nuts. It is also of interest to characterise subject serum IgE levels to the recombinant peanut allergens to confirm that these allergens are involved in the manifestation of peanut allergy and thus, will be useful tools in subsequent cross-reactivity studies.

The aim of this chapter was to investigate the B cell response to unfractionated peanut, almond, Brazil nut, cashew and hazelnut extracts and to the recombinant peanut allergens Ara h 1, Ara h 2 and Ara h 3 in a panel of peanut allergic subjects, atopic, non-peanut/tree nut allergic subjects and non-atopic subjects. Assays for IgE reactivity were used to compare the responses of peanut allergic and non-peanut/tree nut allergic

subjects. The presence of specific IgE to the unfractionated peanut and tree nut extracts as well as to the recombinant peanut allergens was also compared with *in vitro* activation of basophils following stimulation with the same extracts.

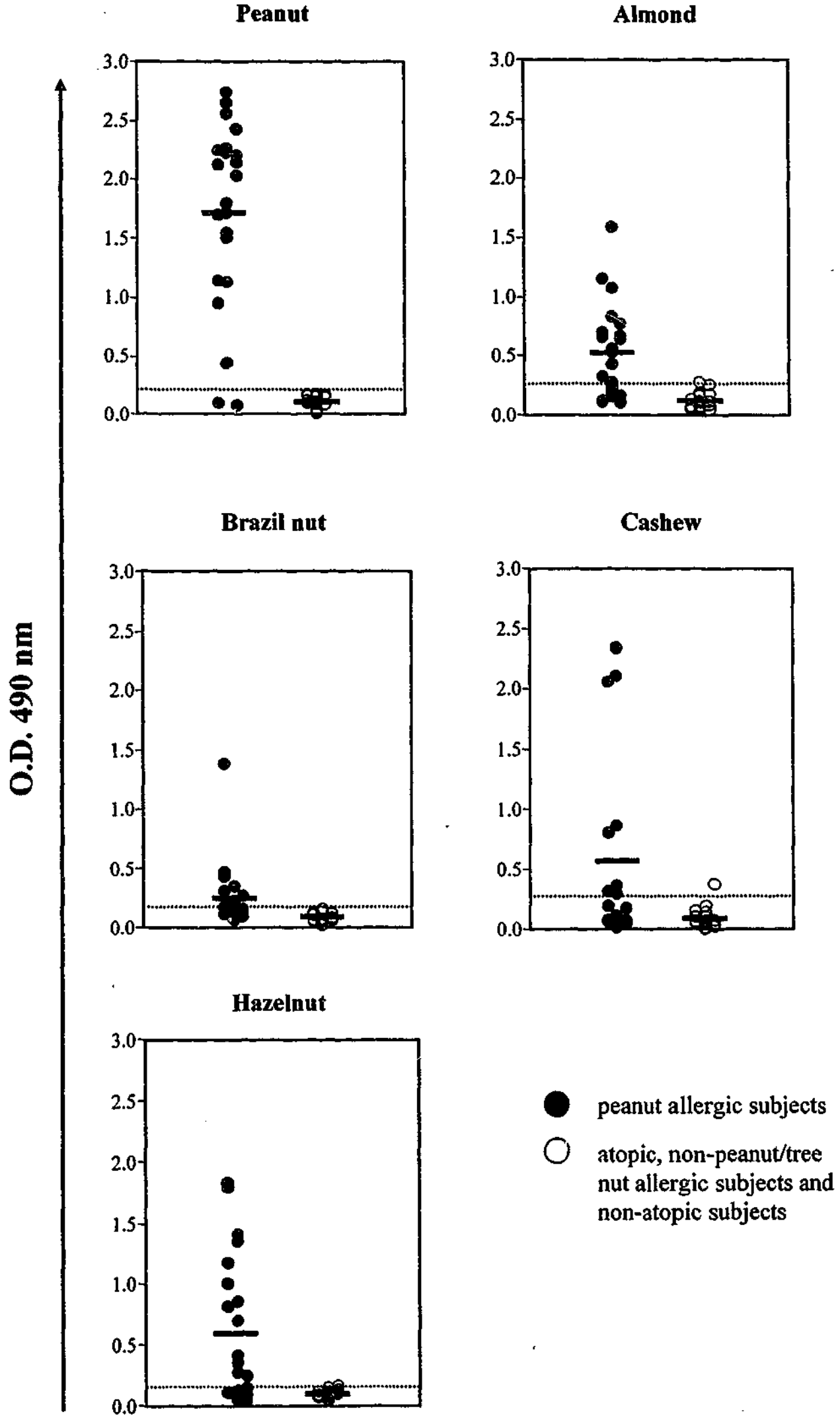
5.2 RESULTS

5.2.1 ELISA for IgE reactivity to almond, Brazil nut, cashew, hazelnut and peanut extracts in peanut allergic and control populations

The serum IgE reactivity of 22 peanut allergic, 15 atopic, non-peanut/tree nut allergic and 6 non-atopic subjects to the peanut and tree nut extracts was analysed quantitatively by ELISA to confirm the observation that the majority of peanut allergic subjects also have specific IgE to at least one tree nut. Of these 22 peanut allergic subjects, 16 are known to be clinically sensitive to at least one tree nut (see Table 2.1). It was earlier established that there was little difference in the IgE binding capacity of raw and roasted peanut and tree nut extracts (Section 3.3.2) and consequently the forms (raw or roasted) chosen for each extract was according to that most commonly consumed. A comparison of the magnitude of serum IgE binding to roasted peanut, roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extracts for peanut allergic subjects, atopic, non-peanut allergic subjects and non-atopic subjects is shown in Figure 5.1. The highest level of IgE binding was observed for peanut followed by hazelnut, cashew, almond and Brazil nut, as indicated by the mean of the $OD_{490\ nm}$ readings for the peanut allergic subjects.

Figure 5.1 Combined results of IgE reactivity to unfractionated peanut, almond, Brazil nut, cashew and hazelnut for peanut allergic, non-peanut/tree nut allergic and non-atopic subjects

ELISA plates were coated with 1 μg/ml of roasted peanut, roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extracts and serum IgE binding was assessed for 22 peanut allergic subjects, 15 atopic, non-peanut/tree nut allergic subjects and 6 non-atopic subjects was assessed. ——— indicates mean of data and ····· is the positive cut-off as indicated by the mean + 2SD of the atopic, non-peanut/tree nut allergic and non-atopic data.


Peanut
Almond
Brazil nut
Cashew
Hazelnut
O.D. 490 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
peanut allergic subjects
atopic, non-peanut/tree nut allergic subjects and non-atopic subjects

The frequency of IgE reactivity to peanut and tree nut extracts among the peanut allergic population was also analysed. Table 5.1 summarises the reactivity of all the peanut allergic subject sera tested. Of the sera tested, 20/22 (91%) peanut allergic subjects were positive for IgE binding to peanut extract. Among the tree nut extracts, almond showed the highest frequency of IgE binding with 15/22 (68%) peanut allergic subjects showing reactivity followed by hazelnut where IgE binding was observed in 13/22 (59%) subjects. Brazil nut and cashew shared the same frequency of reactivity with IgE binding to these extracts observed in 9/22 (41%) subject sera. Overall, 18/22 (82%) peanut allergic subjects had detectable IgE levels to at least one tree nut.

The IgE reactivity of each subject to unfractionated peanut and tree nut extracts by ELISA was also compared with their CAP-FEIA scores and clinical history (Table 5.2). The mean $OD_{490\ nm}$ values for each subject were given an arbitrary score of 1 to 6 to depict the magnitude of IgE binding to the peanut and tree nut extracts. In general, a history of clinical sensitivity to almond, cashew, hazelnut or peanut correlated with a positive CAP-FEIA and/or ELISA score to these nut extracts. However, lack of known clinical sensitivity to any of the tree nuts tested did not preclude a positive CAP-FEIA or ELISA score. It is difficult to ascertain whether these positive CAP-FEIA and ELISA scores also equate with clinical sensitivity since food challenges were not performed with these subjects due to the high risk of anaphylaxis. It is interesting to note that subjects A7 and A22 appeared to be the only true peanut monoreactors in this peanut allergic population, as indicated by clinical history and specific IgE results. These data demonstrate that the majority of peanut allergic subjects also have IgE antibodies that bind to proteins in tree nut extracts.

Table 5.1 Frequency of IgE binding to peanut and tree nut extracts by ELISA in a population of peanut allergic subjects

Subject no.	Peanut	Almond	Hazelnut	Brazil	Cashew
A1	+	+	+	+	-
A2	+	+	-	+	+
A3	+	+	+	-	-
A4	+	+	+	-	+
A5	+	-	-	-	+
A6	+	+	+	+	+
A7	+	-	-	-	-
A8	+	+	-	+	-
A9	+	+	+	+	-
A10	+	+	+	+	-
A11	+	+	+	+	-
A12	+	-	-	-	-
A13	+	+	+	-	+
A14	+	+	+	+	+
A15	+	+	+	+	+
A16	+	+	-	-	-
A17	+	+	-	-	-
A18	-	-	+	-	+
A19	+	+	+	-	+
A20	-	-	-	-	-
A21	+	-	+	-	-
A22	+	-	-	-	-
% IgE reactivity	91	68	59	41	41

Table 5.2 Comparison of clinical history, CAP-FEIA and ELISA scores to peanut and tree nuts in peanut allergic subjects

Subject no.	Known clinical sensitivities	Peanut		Almond		Brazil nut		Cashew		Hazelnut	
		CAP-FEIA	ELISA	CAP-FEIA	ELISA	CAP-FEIA	ELISA	CAP-FEIA	ELISA	CAP-FEIA	ELISA
A1	P	3	5	ND	1	ND	1	1	0	ND	4
A2	P, B, C, H	2	3	ND	1	0	1	3	5	ND	0
A3	P, A	3	5	0	2	0	0	0	0	0	1
A4	P	1	1	1	1	1	0	ND	1	1	1
A5	P, B, C, H	3	4	0	0	0	0	0	1	0	0
A6	P, A, H	3	5	ND	2	ND	1	ND	1	3	2
A7	P	2	4	ND	0	ND	0	ND	0	ND	0
A8	P, C, H	5	5	ND	2	2	1	2	0	1	0
A9	P	6	5	2	3	2	1	1	0	3	3
A10	P, H	2	3	3	3	ND	3	ND	0	4	4
A11	P, C, H	6	6	2	2	ND	1	2	0	2	1
A12	P, A, H	3	5	ND	0	ND	0	ND	0	3	0
A13	P, A, C	5	6	1	1	1	0	3	5	2	1
A14	P, H	2	4	2	2	3	1	2	2	3	3
A15	P, A, C, H	2	3	2	4	2	1	3	2	2	3
A16	P, A	2	5	0	2	0	0	0	0	ND	0
A17	P	4	6	0	2	0	0	ND	0	2	0
A18	P, H	1	0	2	0	2	0	ND	5	3	3
A19	P, A, B, C, H	3	5	0	2	0	0	3	5	2	2
A20	P, H	0	0	0	0	0	0	0	0	0	0
A21	P	2	4	1	0	0	0	1	0	2	2
A22	P	2	2	0	0	0	0	0	0	0	0

ELISA scores ($OD_{490\,nm}$ values): **0** ≤ cut-off; **1** cut-off-0.49; **2** 0.50-0.99; **3** 1.00-1.49; **4** 1.50-1.99; **5** 2.00-2.49; **6** 2.50-3.00
P – peanut; **A** – almond; **B** – Brazil nut; **C** – cashew; **H** – hazelnut; **ND** – not done

CAP-FEIA and/or ELISA score correlates with positive clinical sensitivity

CAP-FEIA and ELISA scores do not correlate with positive clinical sensitivity

5.2.2 Comparison of clinical sensitivity and specific IgE with the activation of basophils to peanut and tree nuts

Basophil activation following stimulation with peanut and tree nut extracts was compared with clinical sensitivity and specific IgE by ELISA and/or CAP-FEIA in a sub-population of peanut allergic subjects. These peanut and tree nut extracts were previously demonstrated to be biologically active following activation of basophils from a peanut and tree nut allergic subject (Section 3.3.4). Whole blood from 9 peanut allergic subjects was incubated with roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut and roasted peanut extracts and the percentage of activated basophils was subsequently analysed as outlined in Section 3.3.4. Atopic, non-peanut/tree nut allergic subjects previously shown to have positive basophil activation with either HDM or RGP (data not shown), were included as negative controls. Figure 5.2 shows a comparison of the percentage of activated basophils following stimulation with peanut and tree nut extracts for peanut allergic and atopic, non-pea ·ıt/tree nut allergic subjects. The cut-off for positive basophil activation for each extract was defined as the highest percentage of basophil activation obtained with the non-peanut/tree nut allergic subjects. It can be seen from Figure 5.2 that the highest level of basophil activation was observed with peanut followed by almond, hazelnut, Brazil nut and cashew, as indicated by the mean percentage of activated basophils for the peanut allergic subjects.

The frequency of positive basophil activation was also analysed and is summarised in Table 5.3. All 9 peanut allergic subjects (100%) demonstrated positive basophil activation to peanut, almond and Brazil nut. In contrast, positive basophil activation to hazelnut and cashew was observed in 8/9 (89%) and 6/9 (67%) of peanut allergic subjects, respectively. Clinical sensitivity to peanuts and tree nuts correlated with

Figure 5.2 Combined results of basophil activation to unfractionated peanut, almond, Brazil nut, cashew and hazelnut extract for peanut allergic subjects and atopic, non-peanut/tree nut allergic subjects.

Whole blood from 9 peanut allergic subjects and 5 atopic, non-peanut/tree nut allergic subjects were incubated with 1 μg/ml of roasted peanut, roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extract and the percentage of activated basophils was calculated. The percentage of activated basophils in the absence of antigen stimulation was subtracted from the percentage of activated basophils obtained with the test antigens. The highest percentage of basophil activation obtained with the non-allergic control subjects was used as the positive cut-off and is represented by ····· . Mean of data is represented by ——— .

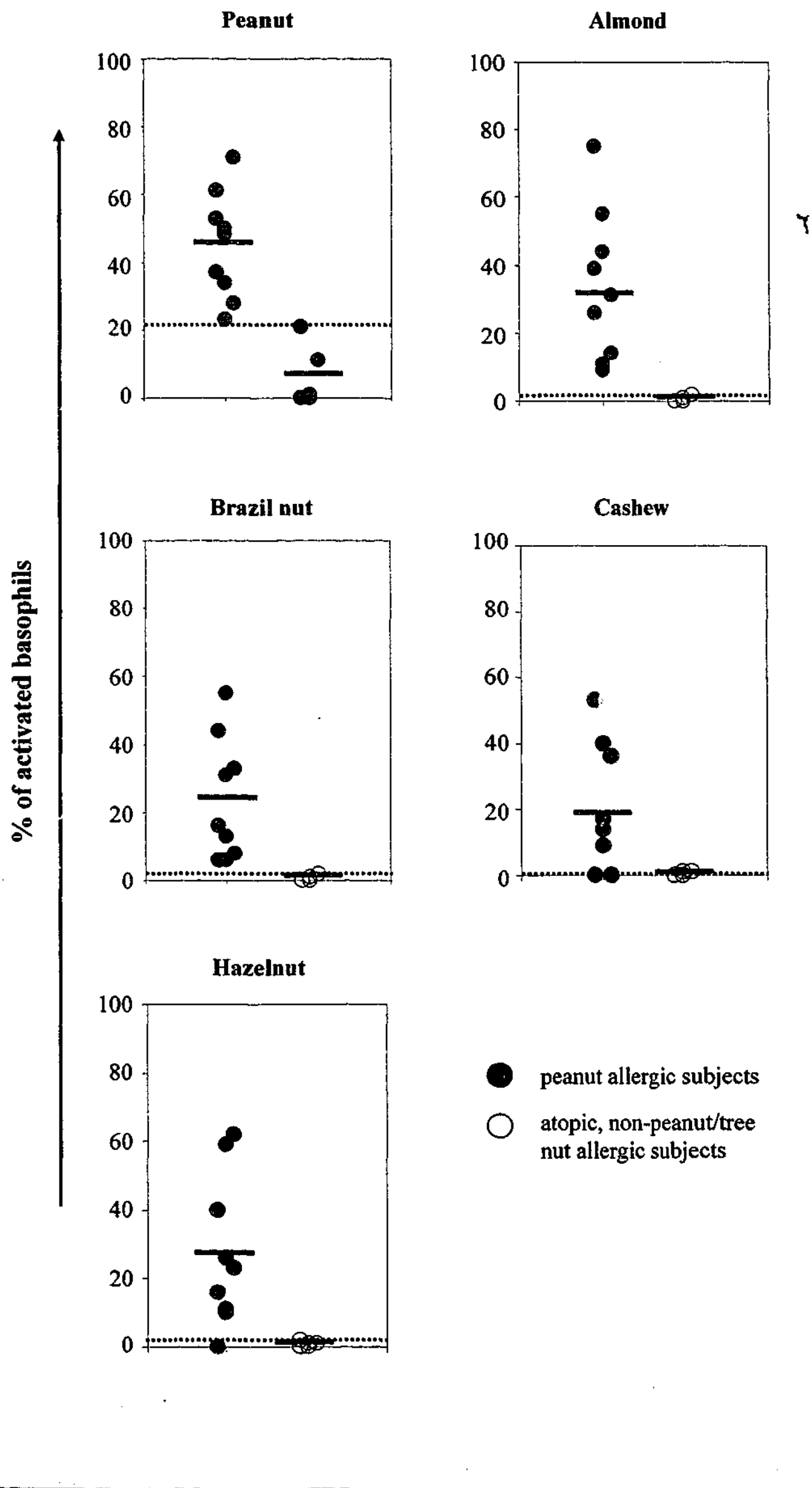

Peanut
Almond
Brazil nut
Cashew
Hazelnut
100
80
60
40
20
0
% of activated basophils
peanut allergic subjects
atopic, non-peanut/tree nut allergic subjects

Table 5.3 Frequency of positive basophil activation in a sub-population of peanut allergic subjects following incubation of whole blood with 1 µg/ml of peanut and tree nut extracts

Subject no.	Known clinical sensitivities	Basophil activation				
		Peanut	Almond	Brazil	Cashew	Hazelnut
A3	P, A	+	+	+	-	+
A8	P, C ,H	+	+	+	+	+
A9	P	+	+	+	-	+
A11	P, C, H	+	+	+	+	+
A12	P, A, H	+	+	+	-	-
A13	P, A, C	+	+	+	+	+
A14	P, H	+	+	+	+	+
A15	P, A, C, H	+	+	+	+	+
A19	P, A, B, C, H	+	+	+	+	+
% Positive		100	100	100	67	89

P – peanut; **A** – almond; **B** – Brazil nut; **C** – cashew; **H** – hazelnut

Positive clinical history correlates with basophil activation data

Positive clinical history does not correlate with basophil activation data

positive basophil activation in the majority of patients. All of the peanut allergic subjects tested demonstrated positive basophil activation to at least two tree nut types.

The presence of serum IgE specific for the peanut and tree nut extracts tested correlated with positive basophil activation in the majority of patients (Table 5.4). In most subjects, the presence of serum IgE to peanut, almond, Brazil nut, cashew and/or hazelnut (as measured by RAST and/or ELISA) translated to positive basophil activation. In some subjects (e.g. A12 and A19), a history of clinical sensitivity to a particular nut type did not equate with positive IgE binding to that nut extract by CAP-FEIA or ELISA. However, basophils from these subjects became activated when whole blood was incubated with that particular nut extract. Thus, the basophil activation test is more sensitive than the ELISA or CAP-FEIA. This is further supported by the positive basophil activation obtained for Brazil nut with subjects A3 and A12, in the absence of detectable levels of specific IgE. Interestingly, subject A12 had specific IgE to hazelnut by CAP-FEIA and a positive clinical history but was by negative ELISA and basophil activation to that extract. This may be due to differences between the hazelnut extract used for the CAP-FEIA and the extract prepared in this study. Again, it is difficult to determine whether positive basophil activation data to some of the tree nuts correlate with clinical sensitivity since food challenges were not performed due to the risk of anaphylaxis. Positive basophil activation in the absence of a history of sensitivity to the tree nuts tested may indicate sensitisation to these nuts and consequently a potential for an allergic reaction to occur upon subsequent exposure.

5.2.3 Serum IgE reactivity to different peanut allergens by immunoblotting

Sera from 22 peanut allergic subjects, 2 atopic, non-peanut/tree nut allergic subjects and 3 non-atopic subjects were examined for allergen-specific IgE binding to roasted peanut

Table 5.4 Comparison of IgE reactivity by CAP-FEIA and ELISA with activation of basophils for peanut and tree nut extracts in peanut allergic subjects

Subject no.	Known clinical sensitivities	Peanut			Almond			Brazil nut			Cashew			Hazelnut		
		CAP-FEIA	ELISA	BAT	CAP-FEIA	ELISA	BAT	CAP-FEIA	ELISA	BAT	CAP-FEIA	ELISA	BAT	CAP-FEIA	ELISA	BAT
A3	P, A	+	+	+	-	+	+	-	-	+	-	-	-	-	+	+
A8	P, C, H	+	+	+	ND	+	+	+	+	+	+	-	+	+	-	+
A9	P	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+
A11	P, C, H	+	+	+	+	+	+	ND	+	+	+	-	+	+	+	+
A12	P, A, H	+	+	+	ND	-	+	ND	-	+	ND	-	-	+	-	-
A13	P, A, C	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
A14	P, H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A15	P, A, C, H	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A19	P, A, B, C, H	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+
% Positive		100	100	100	71	89	100	67	56	100	86	44	67	89	78	89

P – peanut; **A** – almond; **B** – Brazil nut; **C** – cashew; **H** – hazelnut; **ND** – not done

IgE reactivity by CAP-FEIA and/or ELISA does not correlate with basophil activation data

Positive clinical history does not correlate with basophil activation data

Positive clinical history does not correlate with CAP-FEIA and ELISA data

proteins immobilised onto nitrocellulose membranes. Negligible IgE binding to roasted peanut proteins was observed for atopic, non-peanut/tree nut allergic sera and non-atopic control sera, however, numerous proteins in roasted peanut extract bound IgE antibodies from peanut allergic sera. Examples of the IgE-binding profiles of 3 peanut allergic and 2 control subjects (one atopic, non-peanut/tree nut allergic and one non-atopic) are depicted in Figure 5.3. Ara h 1 and Ara h 2 were the most frequently recognised proteins with 12/22 (55%) and 13/22 (59%) subjects showing reactivity to these proteins, respectively. In contrast, the 14 kDa *N*-terminal breakdown product of Ara h 3 and the Ara h 3-like 40 kDa protein were recognised by 10/22 (45%) and 8/22 (36%) subjects, respectively. The IgE-binding profiles of the majority of peanut allergic subjects in this study were not restricted to Ara h 1, Ara h 2 and Ara h 3. Specifically, 21/22 (95%) subjects had serum IgE that bound to other peanut proteins although the identity of these proteins is not known. A summary of the profiles for all peanut allergic sera tested is given in Table 5.5.

The serum IgE reactivity of 22 peanut allergic subjects, 15 atopic, non-peanut/tree nut allergic subjects and 6 non-atopic subjects to recombinant peanut allergens was also assessed by ELISA. Comparisons of the magnitude of IgE binding to these allergens are shown in Figure 5.4. The highest level of IgE binding was observed for rAra h 1, followed by rAra h 2 and rAra h 3 as indicated by the mean $OD_{490\ nm}$ readings for the peanut allergic subjects. The frequency of IgE reactivity to these recombinant peanut allergens was also analysed. Table 5.6 summarises the reactivity of all the peanut allergic sera tested. Of the three recombinant allergens, Ara h 2 showed the highest frequency of reactivity with 14/20 (70%) subjects showing reactivity followed by Ara h 1 where IgE binding was demonstrated in 13/22 (59%) subjects. These data confirm the observation that Ara h 1 and Ara h 2 are major peanut allergens (Burks *et al.*, 1991,

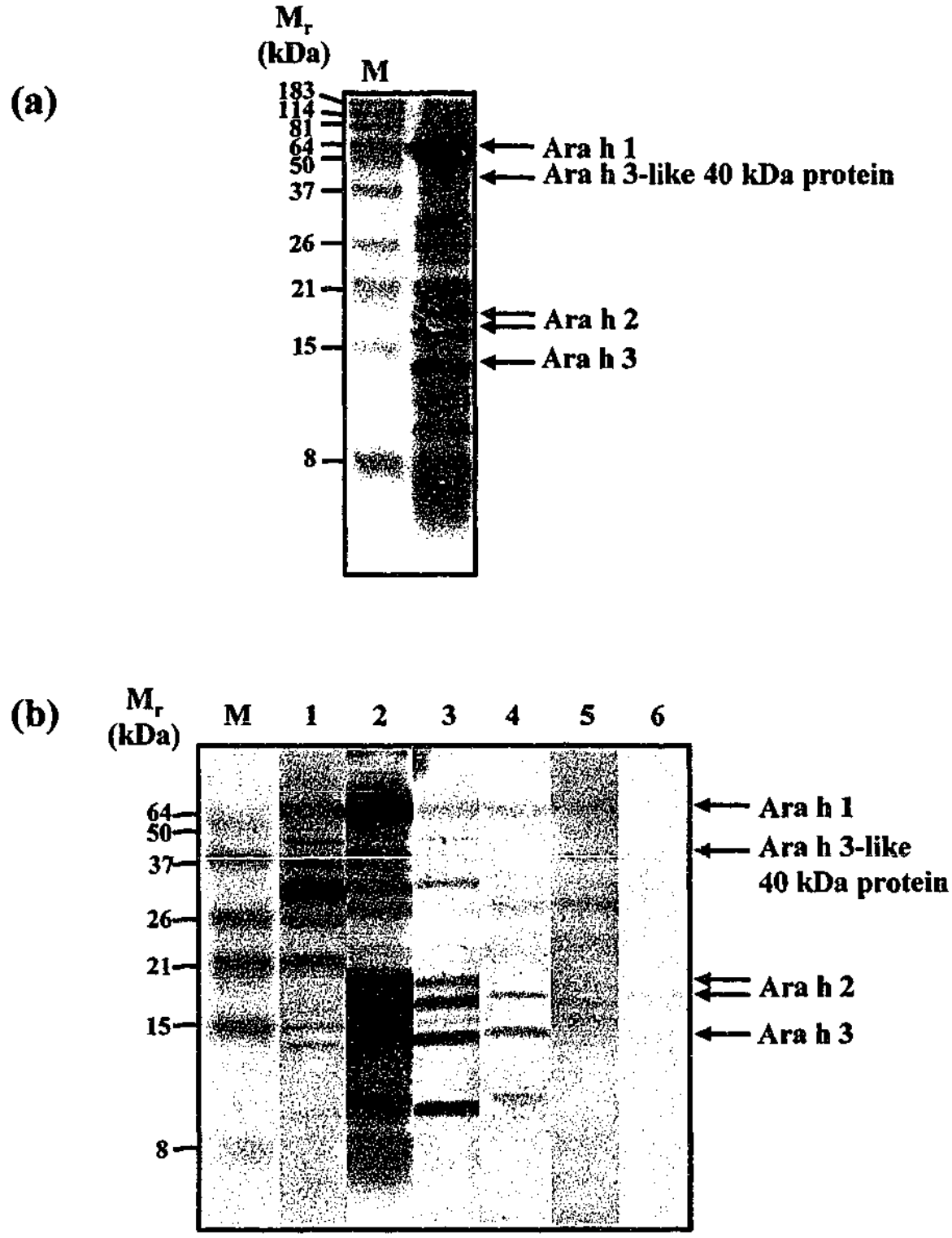


Figure 5.3 Identification of IgE-binding peanut proteins by immunoblotting

(a) Roasted peanut extract (30 μg) was resolved by 16% SDS-PAGE under reducing conditions and stained with Coomassie brilliant blue. **(b)** Proteins were then electroblotted onto nitrocellulose membranes and probed with sera from peanut allergic subjects A4, A9 and A16 (lanes 1-3), atopic, non-peanut/tree nut allergic subject NA1 (lane 4) and non-atopic subject NAT18 (lane 5). Lane 6 shows a membrane incubated in 0.5% BSA in PBS which served as the secondary and tertiary antibody control. M indicates position of molecular mass markers (M_r).

Table 5.5 Frequency of IgE binding to peanut allergens in a population of peanut allergic subjects as assessed by Western immunoblotting

Subject no.	Ara h 1	Ara h 2	Ara h 3 (14 kDa)	Ara h 3 (40 kDa)	Other peanut allergens
A1	+	-	-	+	+
A2	-	-	-	+	+
A3	+	+	-	-	+
A4	+	-	-	-	+
A5	+	-	-	+	+
A6	+	-	-	-	+
A7	-	+	+	-	+
A8	+	+	+	+	+
A9	+	+	+	+	+
A10	-	-	+	-	+
A11	+	+	+	+	+
A12	+	+	+	-	+
A13	+	+	+	-	+
A14	+	+	+	-	+
A15	-	+	-	-	-
A16	-	+	+	-	+
A17	+	+	+	+	+
A18	-	-	-	-	+
A19	-	+	-	-	+
A20	-	-	-	-	+
A21	-	-	-	-	+
A22	-	+	-	+	+
% IgE reactivity	55	59	45	36	95

Figure 5.4 Combined results of IgE reactivity to rAra h 1, rAra h 2 and rAra h 3 for peanut allergic subjects, non-peanut/tree nut allergic subjects and non-atopic subjects

ELISA plates were coated with 1 μg/ml of rAra h 1, rAra h 2 and rAra h 3 and IgE binding was assessed in 22 peanut allergic subjects (closed circles). 15 atopic, non-peanut/tree nut allergic subjects and 6 non-atopic subjects (open circles) were also included as negative controls. ——— indicates mean of data and ····· is the positive cut-off as indicated by the mean + 2SD of the atopic, non-peanut/tree nut allergic and non-atopic data.

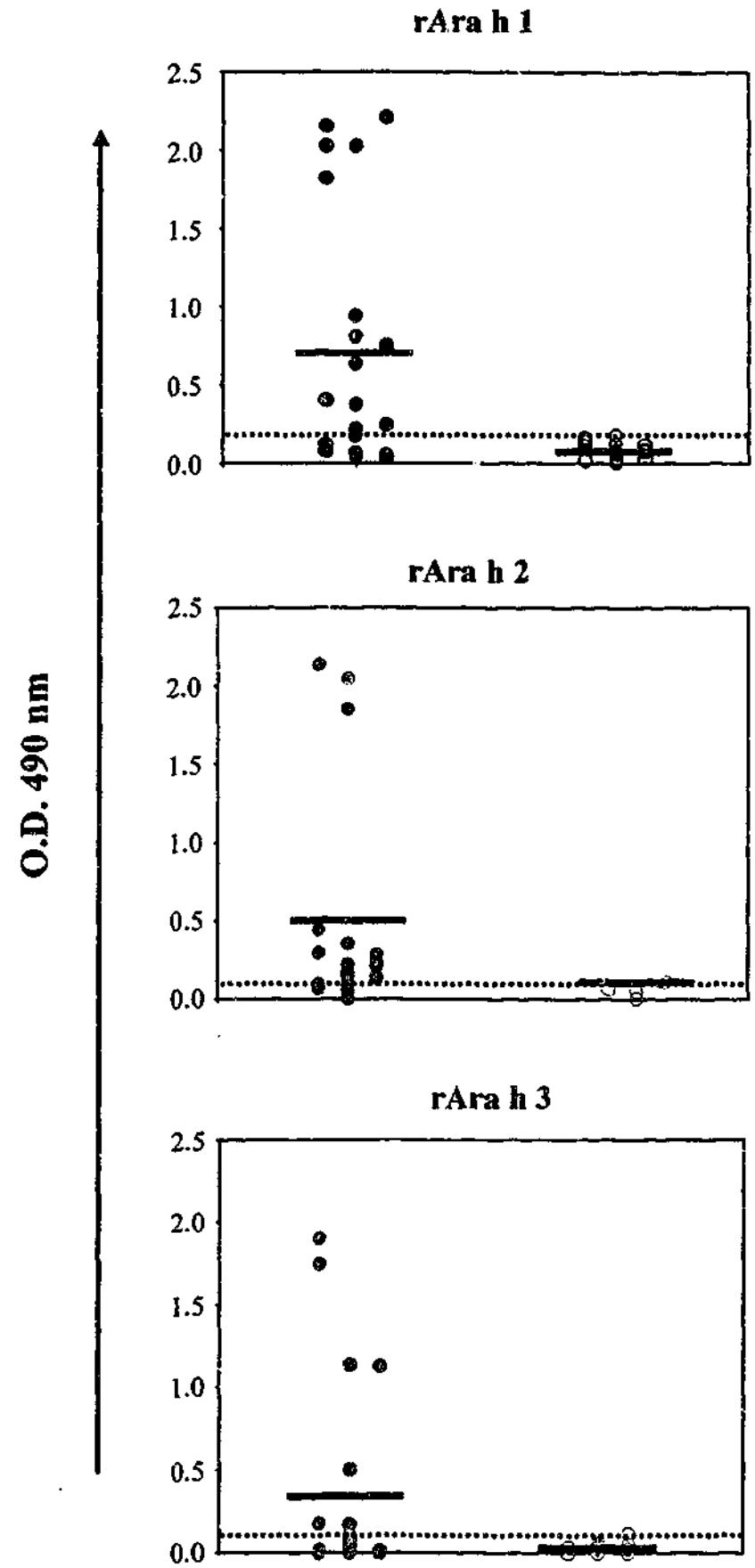

rAra h 1
2.5
2.0
1.5
1.0
0.5
0.0
rAra h 2
2.5
2.0
1.5
1.0
0.5
0.0
rAra h 3
2.5
2.0
1.5
1.0
0.5
0.0
O.D. 490 nm

Table 5.6 Frequency of IgE binding to recombinant peanut allergens by ELISA

Subject no.	ELISA		
	rAra h 1	rAra h 2	rAra h 3
A1	-	+	+
A2	+	+	+
A3	+	+	-
A4	-	-	-
A5	+	-	+
A6	+	+	-
A7	-	+	-
A8	+	+	+
A9	+	+	+
A10	-	+	-
A11	+	ND	+
A12	-	+	-
A13	+	+	-
A14	+	-	-
A15	+	+	-
A16	+	+	-
A17	+	ND	+
A18	+	-	-
A19	-	+	-
A20	-	-	-
A21	-	-	-
A22	-	+	-
% IgE reactivity	59	70	32

ND – not done

Burks *et al.*, 1992, Burks *et al.*, 1995, Kleber-Janke *et al.*, 1999). In contrast, only 7/22 (32%) subjects had IgE antibodies specific for Ara h 3, classifying this protein as a minor allergen.

A comparison of the IgE reactivity of the natural and recombinant forms of Ara h 1, Ara h 2 and Ara h 3 was also conducted. IgE reactivity to the natural allergen was assessed by Western immunoblotting using roasted peanut extract while IgE reactivity to the recombinant form was assessed by ELISA. As summarised in Table 5.7, 12/22 (55%) and 13/22 (59%) subjects demonstrated IgE reactivity to natural and recombinant Ara h 1, respectively. IgE reactivity to Ara h 2 was slightly higher, with 13/22 (59%) subjects recognising natural Ara h 2 and 14/20 (70%) recognising the recombinant form. In contrast, natural and recombinant Ara h 3 bound IgE antibodies from 8/22 (36%) and 7/22 (32%) of peanut allergic subjects, respectively. These data demonstrate that in the majority of peanut allergic subjects, IgE binding to the natural form of the allergen in unfractionated peanut extract correlated with IgE reactivity to the recombinant form. Specific IgE as measured by ELISA also appears to be more sensitive than Western immunoblotting as demonstrated by the higher frequency of IgE reactivity obtained with Ara h 1 and Ara h 2.

5.2.4 Comparison of specific IgE with the activation of basophils using recombinant peanut allergens

The activation of basophils from peanut allergic subjects using recombinant peanut allergens was compared with the presence of specific IgE as measured by ELISA. These recombinant allergens were previously demonstrated to be biologically active following activation of basophils from peanut allergic subjects (Section 4.3.6). Whole blood from a sub-population of peanut allergic subjects were stimulated with rAra h 1,

Table 5.7 Frequency of IgE binding to peanut allergens by Western immunoblotting and ELISA in peanut allergic subjects

Subject no.	Ara h 1		Ara h 2		Ara h 3	
	Western (natural)	ELISA (recombinant)	Western (natural)	ELISA (recombinant)	Western (natural)	ELISA (recombinant)
A1	++	-	-	+	+++	++++
A2	-	++	-	+	++	++
A3	+++	++	+	+	-	-
A4	++	-	-	-	-	-
A5	+	+	-	-	+/-	+
A6	+	++	-	+	-	-
A7	-	-	+	+	-	-
A8	+++	+++++	+++	++++	+	+++
A9	+++	+++++	+++	+++++	+	++++
A10	-	-	-	+	-	-
A11	+++	+++++	+++	ND	++	+++
A12	+	-	+	+	-	-
A13	+/-	+++++	++	+++++	-	-
A14	+/-	+	+	-	-	-
A15	-	+	+	+	-	-
A16	-	++	++	+	-	-
A17	+++	++++	+++	ND	++	++
A18	-	+	-	-	-	-
A19	-	-	+	+	-	-
A20	-	-	-	-	-	-
A21	-	-	-	-	-	-
A22	-	-	+	+	+/-	-
% IgE reactivity	55	59	59	70	36	32

ND – not done

Western blot scores:

-	no reactivity
+/-	weak reactivity
+	moderate reactivity
++	strong reactivity
+++	very strong reactivity

ELISA scores ($OD_{490\ nm}$):

-	⩽ cut-off value
+	cut-off value – 0.49
++	0.50 – 0.99
+++	1.00 – 1.49
++++	1.50 – 1.99
+++++	2.00 – 2.49
++++++	2.50 – 3.00

Western blot and ELISA data do not correlate

rAra h 2 and rAra h 3 and the percentage of activated basophils was analysed as outlined in Section 3.3.4. Atopic, non-peanut/tree nut allergic subjects previously demonstrated to have positive basophil activation with HDM and/or RGP (data not shown) were included as negative controls. Figure 5.5 is a comparison of the percentage of activated basophils following stimulation with 1 µg/ml of rAra h 1, rAra h 2 and rAra h 3. The cut-off for positive basophil activation was defined as the highest percentage of basophil activation obtained for the non-peanut/tree nut allergic subjects. It can be seen that the highest level of positive basophil activation was obtained for rAra h 1 followed by rAra h 3 and rAra h 2, as indicated by the mean percentage of activated basophils.

The frequency of positive basophil activation to these recombinant peanut allergens was also analysed and is summarised in Table 5.8. The highest frequency of basophil activation was obtained with rAra h 1, with 5/5 (100%) subjects tested demonstrating positive basophil activation. This was followed by rAra h 2 where 6/7 (86%) subjects tested were positive for basophil activation. In contrast, 3/6 (50%) subjects tested showed positive basophil activation to rAra h 3.

Specific IgE to the recombinant allergens as measured by ELISA was compared with basophil activation. These data are summarised in Table 5.9. For rAra h 1 and rAra h 3, all subjects with specific IgE for these recombinant allergens demonstrated positive basophil activation to these allergens. The absence of specific IgE to rAra h 3 also equated with the absence of positive basophil activation. Similar results were obtained for rAra h 2 where positive basophil activation was obtained with the majority of subjects showing IgE reactivity to this recombinant allergen. However, basophils from subject A12, previously demonstrated to have specific IgE to rAra h 2, were not

Figure 5.5 Combined results of basophil activation to rAra h 1, rAra h 2 and rAra h 3 for peanut allergic subjects and atopic, non-peanut allergic subjects

Whole blood from 10 peanut allergic subjects and 6 atopic, non-peanut allergic subjects were incubated with 1 µg/ml of rAra h 1, rAra h 2 and rAra h 3 and the percentage of activated basophils was calculated. The percentage of activated basophils in the absence of antigen stimulation was subtracted from the percentage of activated basophils obtained with the test antigens. The highest percentage of activation obtained with the non-allergic control subjects was used as the positive cut-off and is represented by ····· . The mean percentage of basophil activation for peanut allergic and non-peanut allergic subjects is indicated by —— .

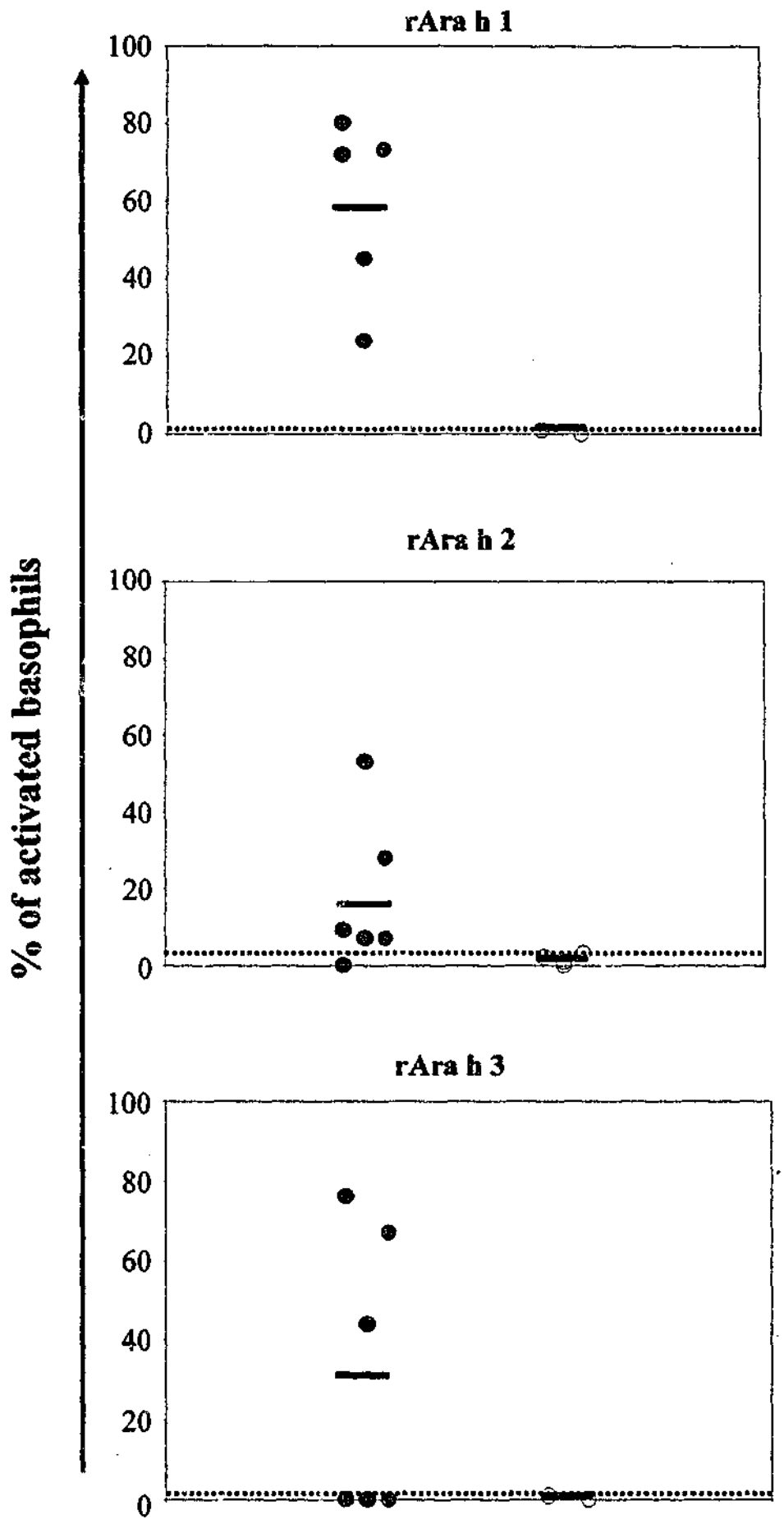

rAra h 1
rAra h 2
rAra h 3
100
80
60
40
20
0
% of activated basophils

Table 5.8 Frequency of positive basophil activation to recombinant peanut allergens in peanut allergic subjects

Subject no.	Basophil activation		
	rAra h 1	rAra h 2	rAra h 3
A3	+	+	-
A8	ND	+	ND
A9	+	+	+
A11	+	ND	+
A12	ND	-	ND
A13	+	ND	-
A14	ND	+	ND
A15	ND	+	-
A17	+	ND	+
A19	ND	+	ND
% Positive	100	86	50

ND – not done

Table 5.9 Comparison of IgE reactivity by ELISA with the activation of basophils to recombinant peanut allergens in peanut allergic subjects

Subject no.	rAra h 1		rAra h 2		rAra h 3	
	ELISA	BAT	ELISA	BAT	ELISA	BAT
A3	+	+	+	+	-	-
A8	+	ND	+	+	+	ND
A9	+	+	+	+	+	+
A11	+	+	ND	+	+	+
A12	-	ND	+	-	-	ND
A13	+	+	+	+	-	-
A14	+	ND	-	+	-	ND
A15	+	ND	+	+	-	-
A17	+	+	ND	ND	+	+
A19	-	ND	+	+	-	ND

ND – not done

ELISA data does not correlate with basophil activation data

activated following stimulation with this allergen. In contrast, basophils from subject A14, previously demonstrated to have no specific IgE for Ara h 2, were activated following stimulation with the same allergen. Both subjects have weak IgE reactivity to natural and/or recombinant Ara h 2 (see Table 5.7) which may have contributed to the contrasting results. Nevertheless, serum IgE reactivity and basophil activation to the recombinant peanut allergens correlated in the majority of peanut allergic subjects.

5.3 DISCUSSION

Using serum IgE assays, reactivity to the tree nuts almond, Brazil nut, cashew and hazelnut was investigated in a population of peanut allergic subjects. It was observed that the majority of peanut allergic subjects in this study had IgE antibodies specific to at least one other tree nut. Of the tree nuts examined, almond showed the highest frequency of IgE binding among the peanut allergic subjects, followed by hazelnut, Brazil nut and cashew. Sicherer and colleagues (1998) examined the serology of peanut allergic subjects using the CAP system quantitative antibody fluoroscein-enzyme immunoassay (FEIA) and found significant correlations between the level of peanut-specific IgE and tree nut-specific IgE antibodies in 111 subjects. In particular, peanut-specific IgE levels correlated with IgE levels for hazelnut, Brazil nut and almond (Sicherer *et al.*, 1998). Basophil activation following stimulation with tree nut extracts was also observed in the present study among a sub-population of peanut allergic subjects. These data confirm the clinical observation that peanut allergic subjects are commonly sensitised to at least one tree nut type (Sampson *et al.*, 1992, Ewan, 1996, Sicherer *et al.*, 2001).

Serum IgE reactivity to unfractionated peanut extract was assessed in a population of peanut allergic subjects using Western immunoblotting. Ara h 1 and Ara h 2, classified as major peanut allergens, were recognised by approximately 55% and 59% of peanut allergic subjects, respectively. However, IgE reactivity to the recombinant forms of these two allergens as assessed by ELISA produced a higher frequency, with 59% and 70% of subjects having specific IgE to rAra h 1 and rAra h 2, respectively, which may be due to the sensitivity of the assay. Alternatively, the reduction of the peanut

allergens following SDS-PAGE may lead to the loss of epitopes, resulting in a lower frequency of IgE reactivity. Previous studies have demonstrated that these two peanut allergens are recognised in crude peanut extract by 70-90% of peanut allergic subjects (Burks *et al.*, 1991, Burks *et al.*, 1992, Clarke *et al.*, 1998) although reactivity to natural Ara h 1 can be as low as 35% as reported by de Jong and colleagues (1998). A reactivity of >50% among the subjects involved in this study confirms that Ara h 1 and Ara h 2 are major peanut allergens. IgE reactivity to the *N*-terminal breakdown product of natural Ara h 3 was slightly lower, with approximately 43% of subjects having IgE antibodies specific for this allergen. This is consistent with previously reported data by de Jong and colleagues (1998) where approximately 36% of peanut allergic subjects showed IgE binding to a 14 kDa protein in crude peanut extract by Western immunoblotting. The current study also showed that 36% of peanut allergic subjects demonstrated IgE binding to a 40 kDa Ara h 3-like protein in crude peanut extract, with 32% of subjects recognising the recombinant form. Previous studies have reported an IgE-reactive 40 kDa protein in crude peanut extract that is recognised by approximately 27-55% of peanut allergic subjects (Clarke *et al.*, 1998, de Jong *et al.*, 1998). However, it is difficult to ascertain without *N*-terminal sequence data if this protein also corresponds to the Ara h 3-like allergen reported in this study.

Western immunoblotting studies demonstrated that IgE reactivity to peanut extract was not restricted to Ara h 1, Ara h 2 and Ara h 3. Nearly all of the subjects involved in this study had IgE antibodies specific for other peanut proteins, revealing the diversity of the IgE response to peanut extract among these subjects. It also indicates the presence of numerous allergenic proteins in peanut extract, an observation that has been previously reported (Barnett *et al.*, 1983, Clarke *et al.*, 1998, de Jong *et al.*, 1998). Thus, other

peanut proteins which are involved in the sensitisation of peanut allergic subjects are yet to be identified.

The presence of allergen-specific IgE correlated with basophil activation following stimulation with the same allergen. This is not surprising given that circulating allergen-specific IgE antibodies are primarily involved in the sensitisation of basophils which become activated upon subsequent exposure to the allergen, resulting in the release of mediators that are responsible for the clinical symptoms associated with allergy. The sensitivity of the basophil activation test also appears to be greater compared to the IgE-binding assays despite optimisation of the latter assay. This effector cell-based *in vitro* assay was useful in confirming subject sensitivity to an allergen source in cases where there was positive clinical history but no detectable specific IgE. Ideally, confirmation of food allergy is conducted through DBPFCs but this carries a substantial risk of anaphylaxis. Consequently, the basophil activation test may provide an alternative to food challenges for use as a confirmatory test (Erdmann *et al.*, 2003).

The high frequency of IgE reactivity to the recombinant peanut allergens Ara h 1, Ara h 2 and Ara h 3 confirmed that these allergens are involved in the sensitisation of peanut allergic subjects. However, it was also necessary to demonstrate that the recombinant form exhibits similar IgE reactivity to the natural form of these allergens. This is of importance as many allergens in their natural state are classified as glycoproteins, characterised by the presence of carbohydrate moities. In contrast, recombinant allergens expressed using a prokaryotic system do not undergo post-translational modifications such as glycosylation. This can affect the IgE reactivity of the recombinant allergen especially if carbohydrate groups are required for IgE binding

(Smith *et al.*, 1996, Westphal *et al.*, 2003). In this study, the frequency of IgE reactivity to Ara h 1, Ara h 2 and Ara h 3 in a population of peanut allergic subjects was similar for both the natural and recombinant forms of these allergens. Basophils from a sub-population of peanut allergic donors were also activated following stimulation with rAra h 1, rAra h 2 and rAra h 3. Thus, it appears that carbohydrate moieties may play a minor role in IgE binding to these peanut allergens and are not necessary for basophil activation as measured in this study using the basophil activation test.

In summary, the assessment of IgE reactivity to tree nut extracts confirmed that the majority of peanut allergic subjects have IgE antibodies to at least one tree nut type. Electroblotting of peanut proteins confirmed the existence of previously reported allergens and their prevalence of recognition in a population of peanut allergic subjects. The prevalence of IgE reactivity to the recombinant forms of Ara h 1, Ara h 2 and Ara h 3 was shown to be similar to their natural derivatives in unfractionated peanut extract. The presence of specific IgE to the recombinant peanut allergens was shown to correlate with the activation of basophils from peanut allergic subjects to the same allergen. It is evident that the peanut and tree nut allergen extracts used in this chapter are clinically relevant in the peanut allergic population, in particular the recombinant peanut allergens, and thus will be useful in the investigation of IgE cross-reactivity between peanut and tree nut allergens, as reported in subsequent chapters.

CHAPTER 6

IMMUNOLOGICAL ANALYSIS OF ALLERGENIC CROSS-REACTIVITY BETWEEN PEANUTS AND TREE NUTS

6.1 INTRODUCTION

Peanut and tree nuts are a common cause of fatal and near-fatal food-induced anaphylaxis in sensitive individuals. Peanut allergy is a more frequent presentation than tree nut allergy but co-sensitisation to both is a common clinical observation. Whether this is due to cross-reactive peanut and tree nut allergens is not known.

IgE cross-reactivity has previously been demonstrated between some tree nuts (Parra *et al.*, 1993, Fernandez *et al.*, 1995, de las Marinas *et al.*, 1998, Sutherland *et al.*, 1999, Poltronieri *et al.*, 2002). In contrast, very little information on IgE cross-reactivity between peanut and tree nuts is available, with the exception of a study by Teuber *et al.* (1999) which demonstrated the absence of IgE cross-reactivity between walnut and peanut. Considering the prevalence of multiple peanut and tree nut allergy, it is of great interest to determine whether this can be attributed to cross-reactive allergens present in peanut and tree nuts. Such information is important for patient management and may lead to simplified diagnosis and improved therapy.

The aim of the studies in this chapter was to investigate IgE cross-reactivity between peanut and tree nuts. Unfractionated peanut, almond, Brazil nut, cashew and hazelnut

extracts as well as the recombinant forms of the peanut allergens, Ara h 1, Ara h 2 and Ara h 3 were used in inhibition assays to establish cross-reactivity between peanut and tree nuts. Affinity-purified antibodies specific for the recombinant peanut allergens were subsequently used to identify potential cross-reactive homologues in almond, Brazil nut, cashew and hazelnut.

6.2 METHODS

6.2.1 Inhibition ELISA using peanut, tree nut extracts and recombinant peanut allergens

Inhibition experiments were conducted by coating 96-well polystyrene plates with roasted peanut extract, rAra h 1, rAra h 2 or rAra h 3 (diluted to 1 µg/ml in ELISA coating buffer) and blocking as described in Section 2.7. Subject sera (diluted in 1% blocking solution for a previously determined $OD_{490\ nm}$ reading of ~1.0 for the coating antigen) were pre-incubated with peanut and tree nut extracts, rAra h 1, rAra h 2, rAra h 3 or, as a control, keyhole limpet hemocyanin (KLH) in the presence of protease inhibitors at a final antigen concentration of 0.2, 1, 5, 25 and 125 µg/ml, at room temperature for 1 hour. The inhibition mixtures (including sera with no inhibitor as positive controls) were then dispensed into wells (50 µl/well) and incubated at 37°C for 2 hours. IgE binding was then measured as described in Section 2.7. Percentage inhibition was calculated using the following formula:

$$\% \text{ inhibition} = 100 - \left(\frac{OD_{490} \text{ of serum with inhibitor}}{OD_{490} \text{ of serum without inhibitor}} \times 100 \right)$$

Lower inhibitor concentrations (0.04, 0.008, 0.0016 and 0.00032 µg/ml) were also used for some extracts to enable the calculation of the concentration required for 50% inhibition of IgE binding (IC_{50}).

6.2.2 Measurement of non-specific inhibition of IgE binding using latex-glove extract and rHev b 6.01 inhibition ELISA

This inhibition assay was performed to measure the non-specific inhibition of IgE binding by the peanut and tree nut extracts as well as the recombinant peanut allergens. 96-well polystyrene plates were coated with latex-glove extract or rHev b 6.01 (diluted to 1 µg/ml in ELISA coating buffer) as described in Section 2.7. The assay was then continued as described in the previous section using serum from a latex allergic, non-peanut/tree nut allergic control subject with almond, Brazil nut, cashew, hazelnut, peanut extracts and recombinant peanut allergens as the inhibitors. Positive control inhibitors included latex-glove extract and rHev b 6.01. KLH was used as the negative control. Inhibition of IgE binding to latex-glove extract or rHev b 6.01 was then measured and calculated as outlined in the previous section.

6.2.3 Affinity purification of allergen-specific antibodies

96-well polystyrene plates were coated with allergen extract (diluted to 1 µg/ml in ELISA coating buffer; 50 µl/well) and incubated overnight at 4°C. Plates were washed 5 times with 0.05% PBS-Tween and blocked with 5% blocking solution (200 µl/well) for 1 hour at 37°C. Plates were again washed 5 times with PBS-Tween and incubated with subject serum (diluted 1/10 with 1% blocking solution; 50 µl/well) at 37°C for 2 hours. Plates were washed 5 times with 0.05% PBS-Tween. Antibodies were eluted by adding 50 µl of glycine buffer containing 1% BSA (pH 2.6) into each well followed by a 10 minute incubation at room temperature, with shaking. The antibody solution was

collected and neutralised to pH ~7.4 with 2 M NaOH. A second antibody elution was conducted and the specificity of the eluted antibody fractions was assessed by ELISA as described in Section 2.7.

6.2.4 Western immunoblotting using affinity purified antibodies

Peanut, tree nut and rye grass pollen extracts were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes and blocked as outlined in Sections 2.6.1 and 2.6.2. Membranes were incubated with affinity purified antibodies and whole serum as a positive control and IgE binding was detected as described in Section 2.6.2.

6.3 RESULTS

6.3.1 IgE cross-reactivity between peanut and tree nuts

Inhibition ELISA was performed to determine whether cross-reactive allergens were present in the peanut and tree nut extracts. In this assay, the capacity of roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extracts to inhibit IgE binding to roasted peanut extract immobilised on an ELISA plate was assessed. As only minimal differences in allergenicity were observed between raw and roasted extracts (Figures 3.1, 3.2 and 3.3), the form (raw or roasted) in which the extracts were used as inhibitors was determined according to that most commonly consumed.

Prior to investigating cross-reactivity between peanut and tree nuts, the ability of the roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut and roasted peanut extracts to non-specifically inhibit IgE binding was tested using a latex glove extract-

specific IgE ELISA (Figure 6.1). Negligible inhibition of IgE binding to latex glove extract (up to 20%) was observed when serum from a latex, non-peanut/tree nut allergic subject (NA6) was pre-incubated with 0.2-125 µg/ml of peanut and tree nut extracts. In contrast, pre-incubation of subject serum with 0.2 µg/ml of the positive control latex-glove extract resulted in almost 100% inhibition of IgE binding to latex-glove extract. Consequently, the cut-off for positive inhibition was set at 20%.

The investigation of IgE cross-reactivity between unfractionated peanut and tree nut extracts was performed using sera from 7 subjects with a history of multiple-nut (peanut and tree nuts) sensitivity and/or specific IgE to peanut and at least one tree nut. Sera from these subjects (A1, A3, A8, A9, A14 and A21) were used in ELISA inhibition assays and the results are shown in Figure 6.2. The specificity of this assay was demonstrated by the strong inhibition obtained with roasted peanut extract (positive control) in all of the sera tested while the negative control extract (KLH) induced minimal or no inhibition of IgE binding to roasted peanut extract.

From the results shown in Figure 6.2, it can be seen that for 5 out of 7 subjects tested, roasted almond showed the highest level of inhibition of IgE binding to roasted peanut extract followed by raw Brazil nut and roasted hazelnut (Figures 6.2b(i), c(i), e(i), f(i), and g(i)). All 5 subjects also had a positive history of allergy, specific IgE or positive basophil activation to these tree nuts (Figures 6.2b(ii), c(ii), e(ii), f(ii) and g(ii)). Subject A9 showed slightly higher inhibition with raw Brazil nut at the highest inhibitor concentration followed by roasted almond and roasted hazelnut (Figure 6.2d(i)). This subject also had specific IgE and positive basophil activation to these tree nut extracts (Figure 6.2d(ii)). In contrast, subject A1 demonstrated the highest level of inhibition of IgE binding to roasted peanut extract with raw Brazil nut and roasted hazelnut (Figure

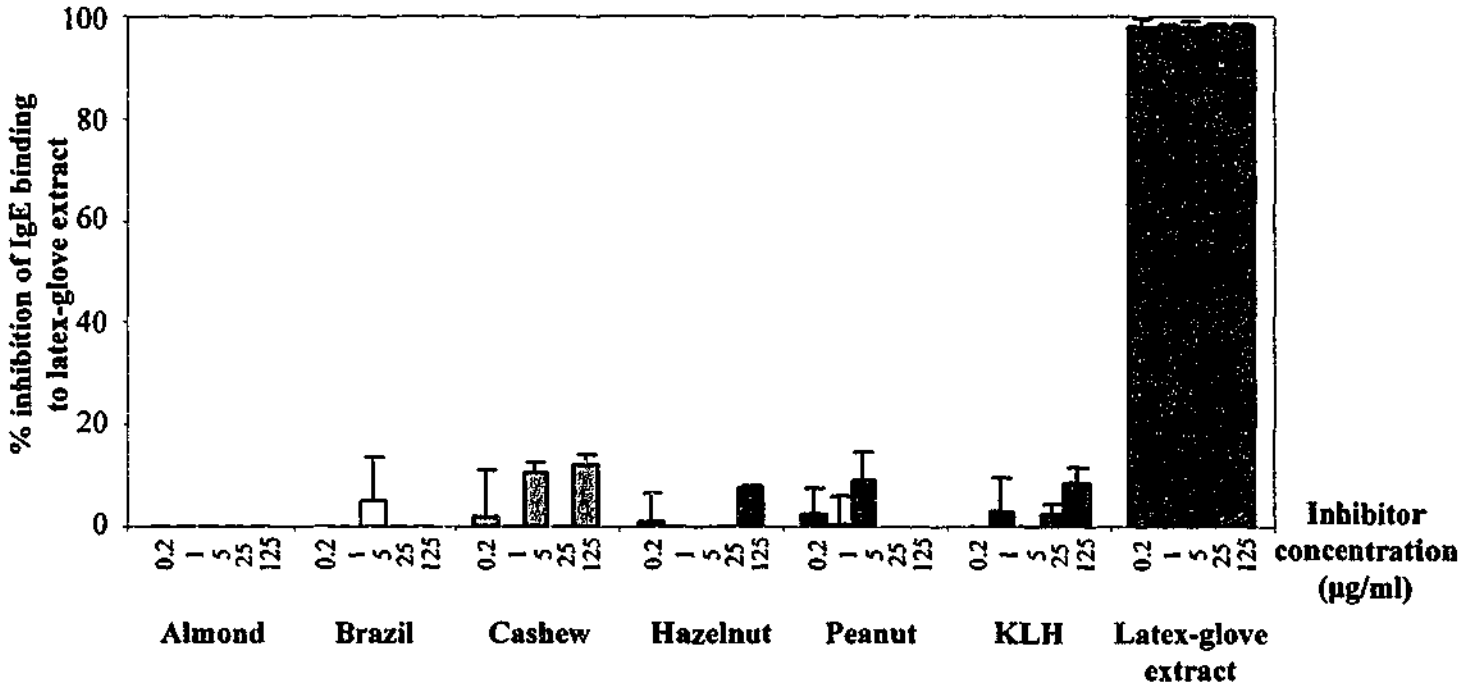


Figure 6.1 Measurement of non-specific inhibition of IgE binding to latex-glove extract using peanut and tree nut extracts

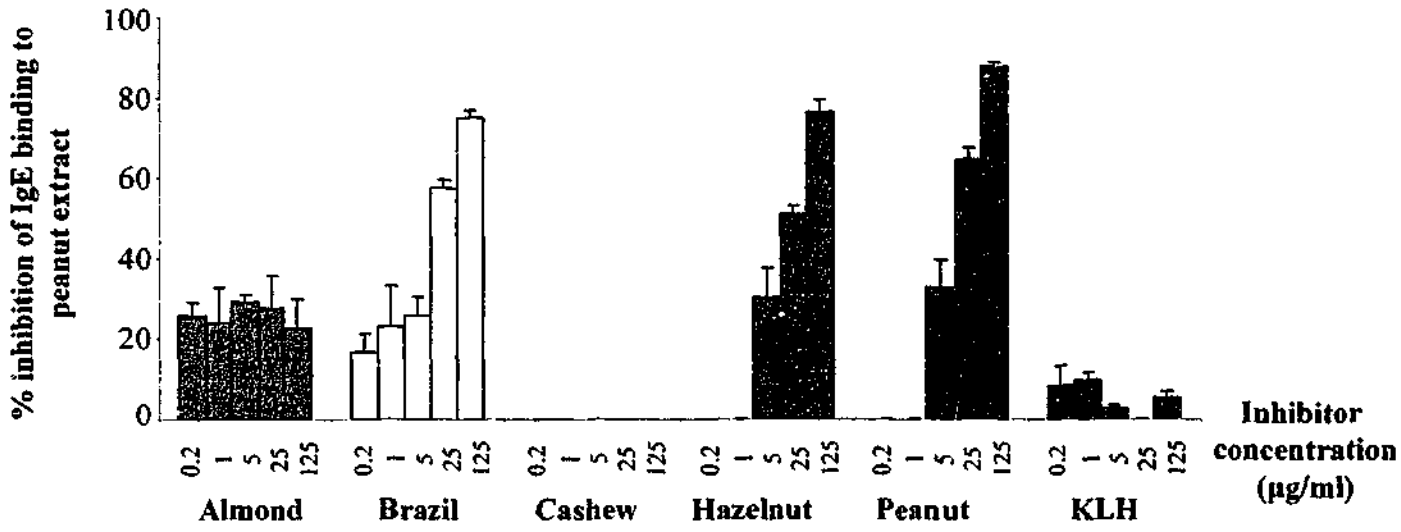
Serum from a latex, non-peanut/tree nut allergic control subject (NA6) was pre-incubated with different concentrations of unfractionated roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut and roasted peanut extract and IgE binding to latex-glove extract immobilised on ELISA plates was measured. Latex-glove extract and KLH were used as the positive control and negative control inhibitors, respectively. Results are expressed as percentage inhibition of IgE binding to latex-glove extract. Mean values for triplicates are shown and the standard deviation is indicated by the error bars.

Figure 6.2 ELISA for inhibition of serum IgE binding to peanut by tree nut extracts

Sera from 7 peanut and tree nut allergic subjects (A1, A3, A8, A9, A14, A19, A21) were pre-incubated with different concentrations of roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut and IgE binding to roasted peanut extract immobilised on ELISA plates was measured. Roasted peanut extract was used as the positive control and KLH was included as a negative control extract. The results for each subject are presented as separate panels **(A-G)**. **(i)** The percentage inhibition of IgE binding to roasted peanut extract for each inhibitor. Mean values for triplicates are shown and the standard deviation is indicated by error bars. **(ii)** Summary of clinical sensitivity, specific IgE (CAP-FEIA and ELISA) and basophil activation data for almond, Brazil nut, cashew, hazelnut and peanut for each subject. **Legend:** NK – not known; ND – not done.

A

(i)

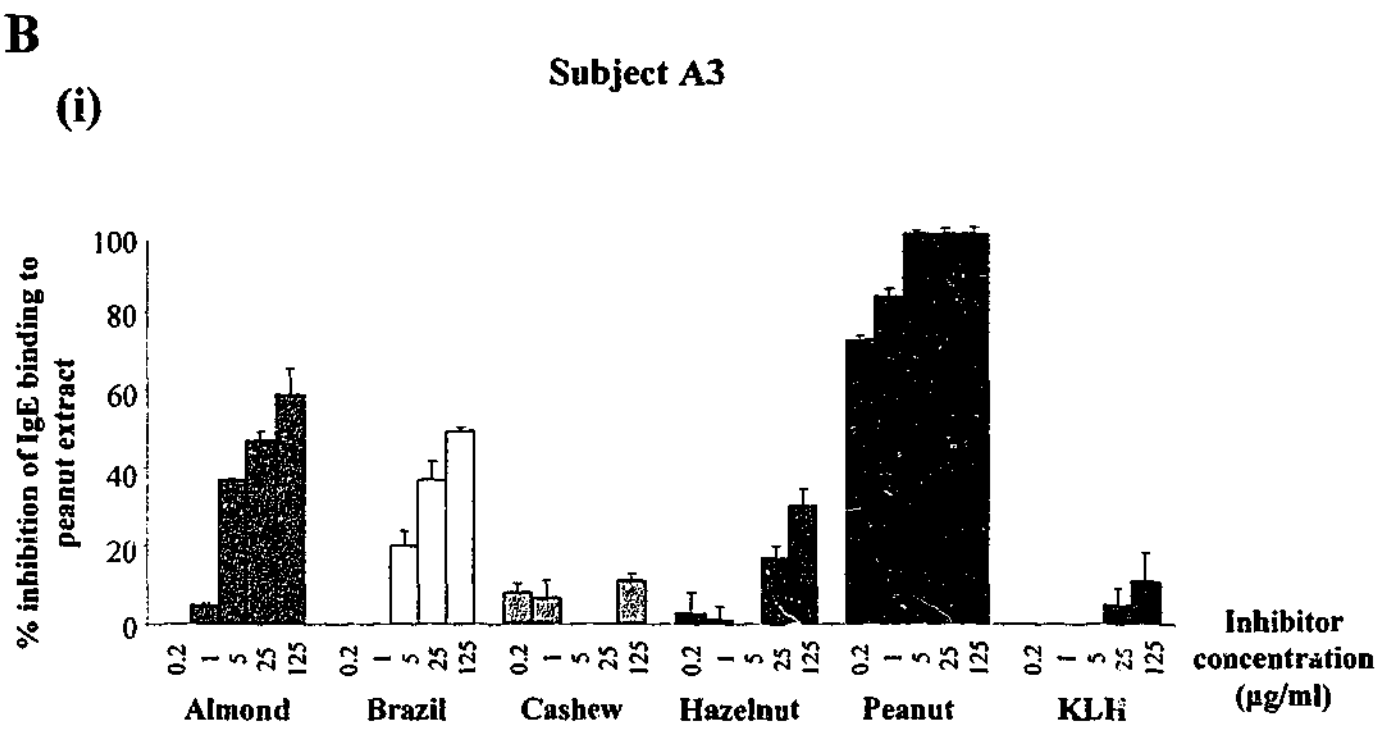


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut
Clinical history	NK	NK	NK	NK	+
CAP-FEIA	ND	ND	+	ND	+
ELISA	+	+	-	+	+
BAT	ND	ND	ND	ND	ND

B

(i)

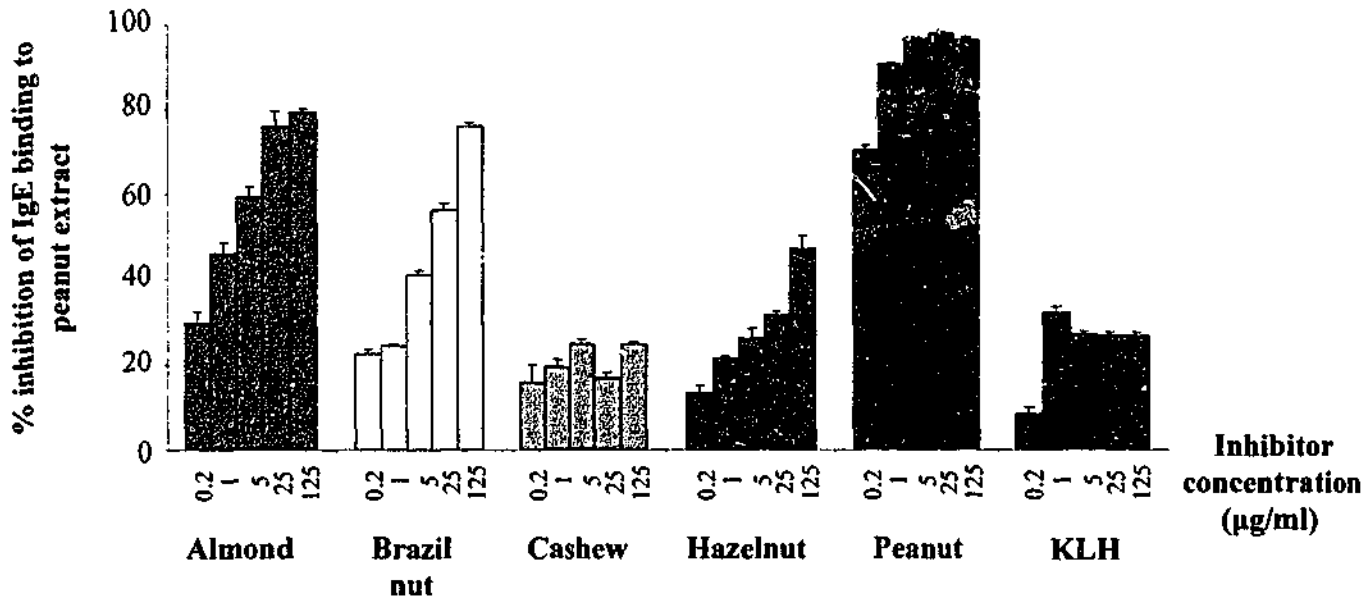


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut
Clinical history	+	NK	NK	NK	+
CAP-FEIA	-	-	-	-	+
ELISA	+	-	-	+	+
BAT	+	+	-	+	+

C

(i)

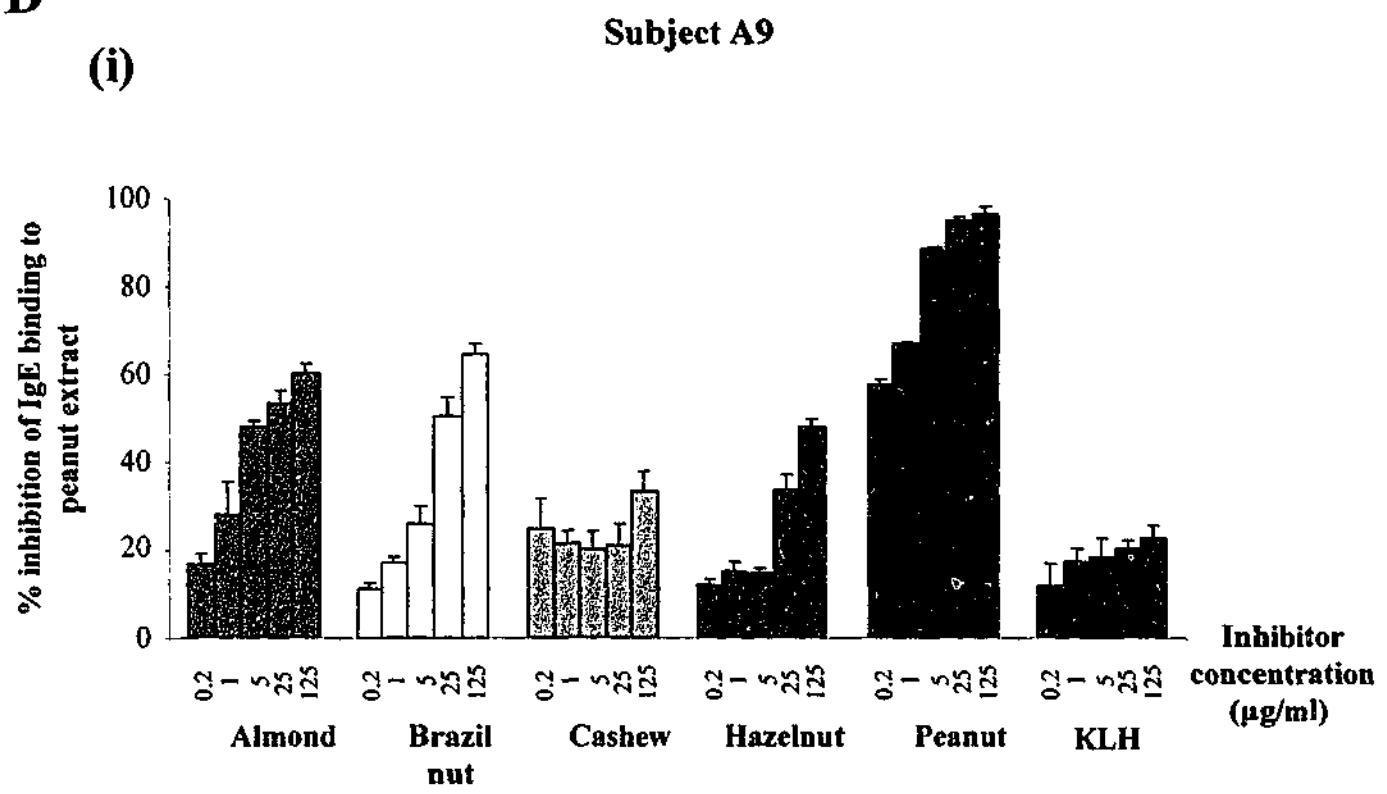


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut
Clinical history	NK	NK	+	+	+
CAP-FEIA	ND	+	+	+	+
ELISA	+	+	-	-	+
BAT	+	+	+	+	+

D

(i)

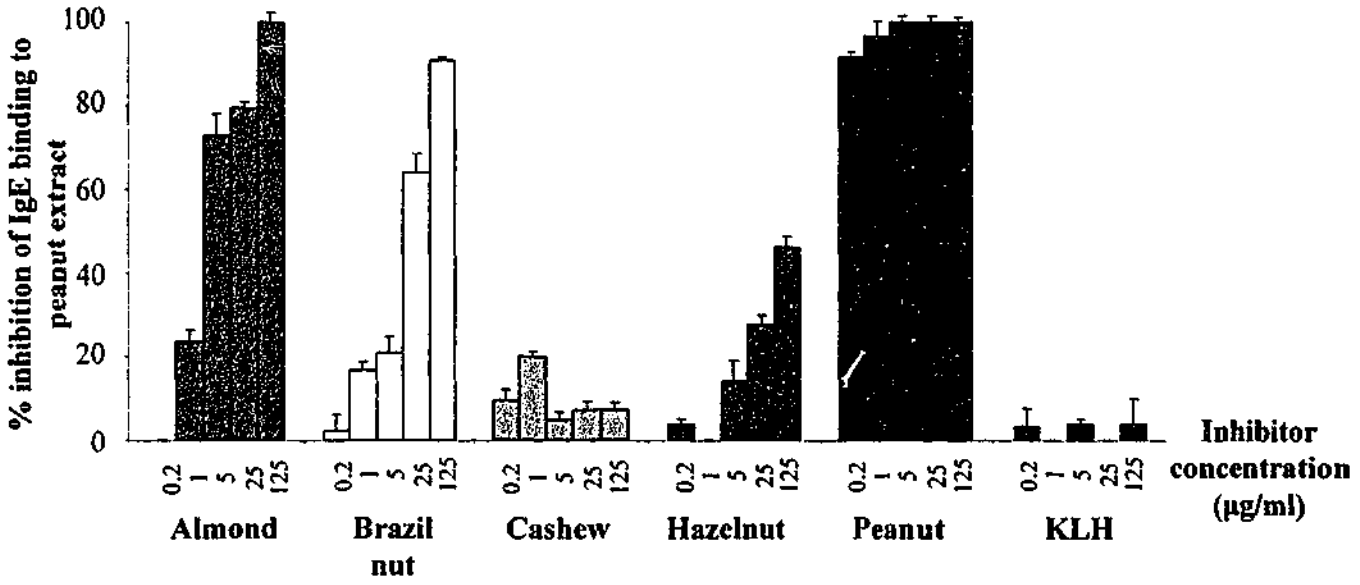


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut
Clinical history	NK	NK	NK	NK	+
CAP-FEIA	+	+	+	+	+
ELISA	+	+	-	+	+
BAT	+	+	-	+	+

E

(i)

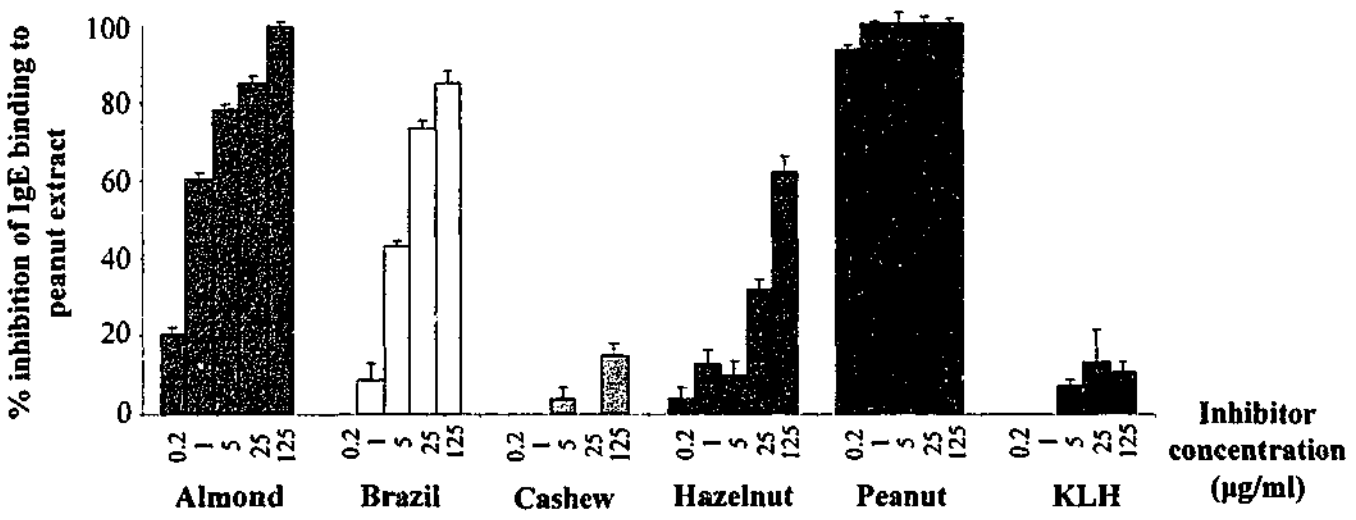


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut
Clinical history	NK	NK	NK	+	+
CAP-FEIA	+	+	+	+	+
ELISA	+	+	+	+	+
BAT	+	+	+	+	+

F

(i)



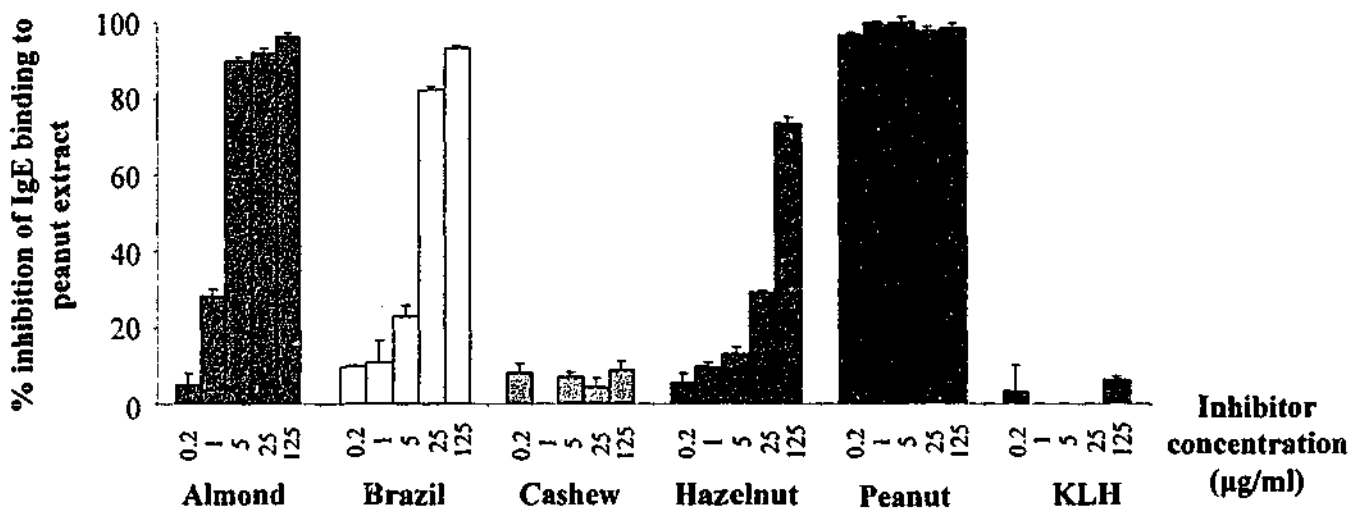
(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut
Clinical history	+	+	+	+	+
CAP-FEIA	-	-	+	+	+
ELISA	+	-	+	+	+
BAT	+	+	+	+	+

G

Subject A21

(i)



(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut
Clinical history	NK	NK	NK	NK	+
CAP-FEIA	+	-	+	+	+
ELISA	-	-	-	+	+
BAT	ND	ND	ND	ND	ND

6.2a(i)). This subject also had measurable specific IgE to these tree nuts (Figure 6.2a(ii)). These data are consistent with the presence of allergens in almond, Brazil nut, hazelnut that cross-react with peanut. Subject A1 showed negligible IgE cross-reactivity between roasted almond and roasted peanut extract even in the context of a positive specific IgE to almond. This indicates that, in contrast to the other 6 subjects, peanut-specific IgE antibodies from this subject do not cross-react with almond proteins.

Similar results were also obtained for roasted cashew extract. All subjects, with the exception of subject A3, had specific IgE to cashew as measured by CAP-FEIA and/or ELISA (Figure 6.2a(ii), c(ii), d(ii), e(ii), f(ii) and g(ii)). However, roasted cashew extract did not demonstrate a dose-dependent inhibition of IgE binding to roasted peanut extract in any of these subjects (Figure 6.2a(i), c(i), d(i), e(i), f(i) and g(i)), with minimal inhibition even at the highest inhibitor concentration of 125 μg/ml, indicating a lack of cross-reactive allergens in this extract. Therefore IgE reactivity to cashew in these subjects is likely to be due to unique cashew allergens.

To quantitate the degree of inhibition observed with the peanut and tree nut extracts, the inhibitor concentration required for 50% inhibition of IgE reactivity (IC_{50}) to roasted peanut extract was determined (outlined in Section 6.2.1) and is summarised in Table 6.1. As expected, the roasted peanut extract (positive control) gave the lowest IC_{50} in all subjects, with concentrations ranging from 0.006-15 μg/ml. Six out of seven subjects achieved 50% inhibition of IgE binding to roasted peanut with roasted almond extract as the inhibitor, although IC_{50} values were much higher than the positive control, ranging from 0.6-45 μg/ml. Within the range of inhibitor concentrations used in this

Table 6.1 Inhibitor concentration required for 50% inhibition of IgE binding to roasted peanut extract

Subject no.	Inhibitor concentration (μg/ml)					
	Roasted almond	Raw Brazil	Roasted cashew	Roasted hazelnut	Roasted peanut[+]	KLH[#]
A1	•	20.5	†	23.0	15.0	†
A3	45.0	*	†	*	0.058	†
A8	1.8	15.9	•	*	0.022	•
A9	7.0	24.5	•	*	0.088	•
A14	3.1	18.6	•	*	0.006	†
A19	0.6	8.2	†	82.0	0.015	†
A21	2.4	14.0	†	71.0	0.015	†

* - did not reach 50% inhibition at the maximum inhibitor concentration of 125 μg/ml
† - did not show inhibition above the levels obtained in non-specific inhibition assay
• - did not demonstrate a dose-dependent inhibition of IgE binding to peanut
+ - positive control inhibitor
# - negative control inhibitor

study, raw Brazil nut extract showed 50% inhibition of IgE binding to roasted peanut extract in 6 out of 7 subjects, while roasted hazelnut extract reached 50% inhibition in only 3 out of 7 subjects. In contrast, both roasted cashew extract and KLH demonstrated negligible inhibition levels, similar to that obtained in the non-specific inhibition ELISA (Figure 6.1).

6.3.2 IgE cross-reactivity between recombinant peanut allergens and tree nuts

6.3.2.1 Measurement of non-specific inhibition by recombinant peanut allergens

Prior to investigating IgE cross-reactivity between peanut and tree nuts using purified recombinant peanut allergens, a non-specific inhibition assay was established to determine whether these recombinant allergen preparations can non-specifically inhibit IgE binding to an unrelated but similarly expressed protein. In this assay, inhibition of IgE binding to rHev b 6.01, was measured using sera from a latex, non-peanut/tree nut allergic subject (NA6). As illustrated in Figure 6.3, pre-incubation of serum with increasing concentrations of rAra h 1, rAra h 2 and rAra h 3 produced minimal inhibition of IgE binding to rHev b 6.01 in comparison to the rHev b 6.01 positive control. Negligible non-specific inhibition was also obtained with the roasted peanut, roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extracts.

6.3.2.2 IgE cross-reactivity between the major peanut allergen, Ara h 1, and tree nuts

The role of the major peanut allergen, Ara h 1, in the observed cross-reactivity between peanut and tree nuts was investigated. Inhibition ELISA was performed to assess the ability of tree nut extracts to inhibit IgE binding to rAra h 1 using sera from 3 peanut

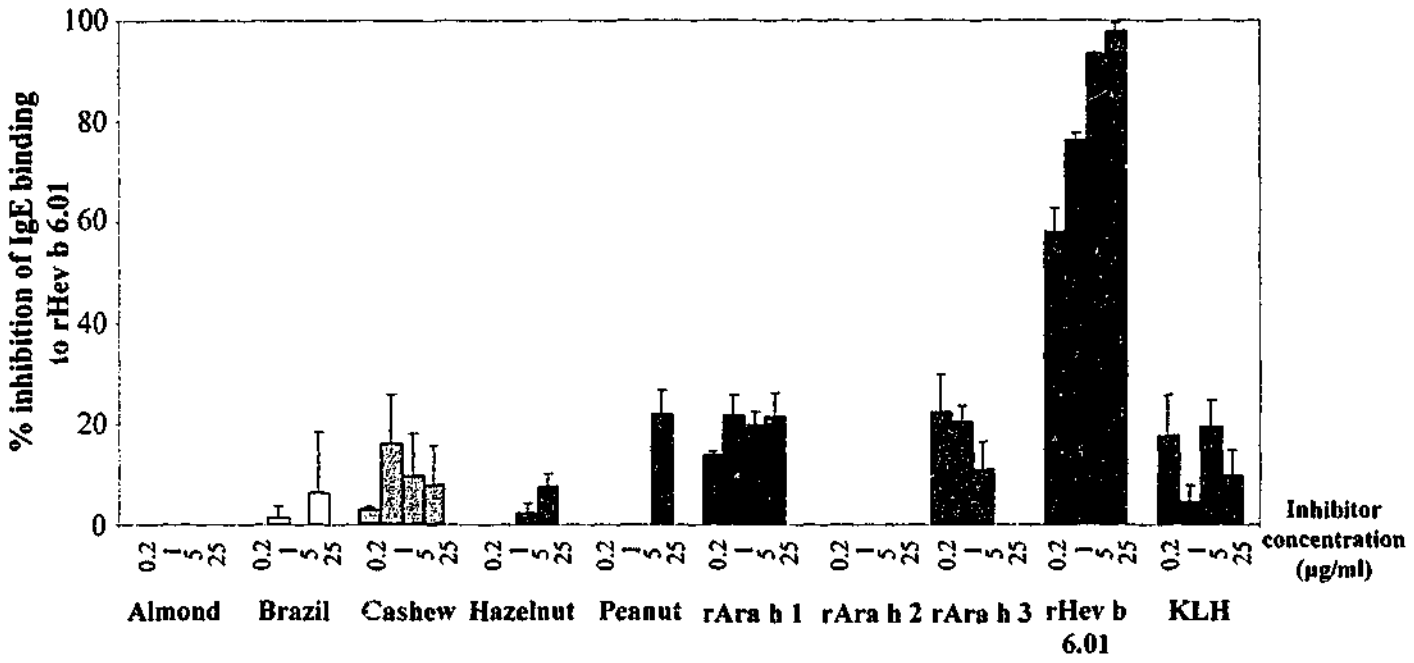


Figure 6.3 Measurement of non-specific inhibition of IgE binding to rHev b 6.01 by rAra h 1, rAra h 2, rAra h 3, peanut and tree nut extracts

Serum from a latex, non-peanut/tree nut allergic control subject (NA6) was pre-incubated with different concentrations of unfractionated roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut, roasted peanut extracts as well as recombinant peanut allergens and IgE binding to rHev b 6.01 immobilised on ELISA plates was measured. rHev b 6.01 and KLH were used as the positive control and negative control inhibitors, respectively. Results are expressed as percentage inhibition of IgE binding to rHev b 6.01. Mean values for triplicates are shown and the standard deviation is indicated by the error bars.

allergic subjects (A3, A8 and A9) previously demonstrated to nave specific IgE to rAra h 1 ($OD_{490\ nm} \geq 1.0$) and some tree nuts. As shown in Figure 6.4, the specificity of this assay can be seen with the high level of inhibition obtained with rAra h 1 (positive control) for all of the subject sera while the negative control inhibitor (KLH) induced minimal or no inhibition of IgE binding to rAra h 1. A high level of inhibition with roasted peanut extract as the inhibitor was also observed in all 3 subjects, confirming the presence of Ara h 1 in this extract.

Of the tree nut extracts tested in this assay, only roasted almond extract inhibited IgE binding to rAra h 1 although a clear dose-dependent inhibition of IgE binding was observed in only one subject (A9; Figure 6.4c(i)) previously shown to have specific IgE to almond (Figure 6.4c(ii)). These inhibition levels were also considerably lower compared to the positive control extracts. All 3 subjects tested previously demonstrated inhibition of serum IgE reactivity to peanut by almond at the crude extract level (Figure 6.2b(i), c(i) and d(i)) but this inhibition was much greater compared to that observed between rAra h 1 and almond, suggesting that cross-reactivity between peanut and almond is not solely due to Ara h 1. However, the low-level inhibition observed between rAra h 1 and almond indicates that there may be an Ara h 1 homologue present in almond extract that contributes to low-level IgE cross-reactivity between peanut and almond.

6.3.2.3 Identification of a potential Ara h 1 homologues in tree nut extracts

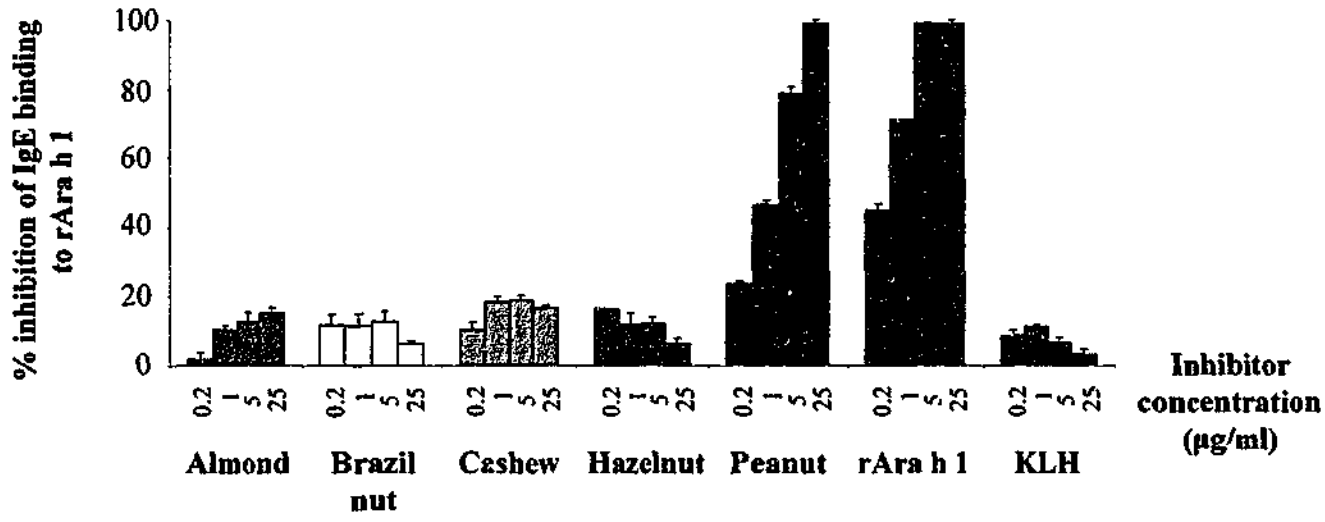
In this section, the presence of cross-reactive proteins in almond extract fractionated by SDS-PAGE is demonstrated using affinity purified rAra h 1-specific antibodies. 96-well polystyrene plates coated with rAra h 1 were used to purify rAra h 1-specific

Figure 6.4 Inhibition of IgE binding to rAra h 1 by peanut and tree nut extracts as measured by ELISA

Sera from 3 peanut and tree nut allergic subjects (A3, A8 and A9) were pre-incubated with different concentrations of roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut and IgE binding to rAra h 1 immobilised on ELISA plates was measured. rAra h 1 and roasted peanut extract were used as the positive controls and KLH was included as the negative control extract. The results for each subject are presented as separate panels **(A-C)**. **(i)** The percentage inhibition of IgE binding to rAra h 1 for each inhibitor. Mean values for triplicates are shown and the standard deviation is indicated by error bars. **(ii)** Summary of clinical sensitivity, specific IgE (CAP-FEIA and ELISA) and basophil activation data for almond, Brazil nut, cashew, hazelnut, peanut and rAra h 1. **Legend:** NK – not known; NA – not applicable.

A

(i)

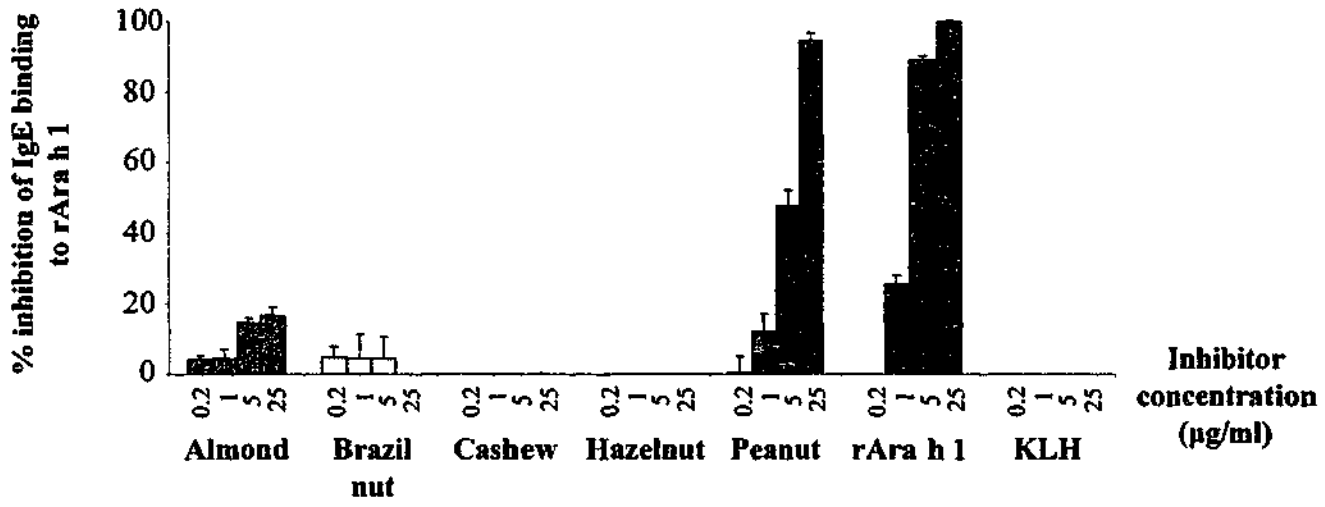


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut	rAra h 1
Clinical history	+	NK	NK	NK	+	NA
CAP-FEIA	-	-	-	-	+	NA
ELISA	+	-	-	+	+	+
BAT	+	+	-	+	+	+

B

(i)

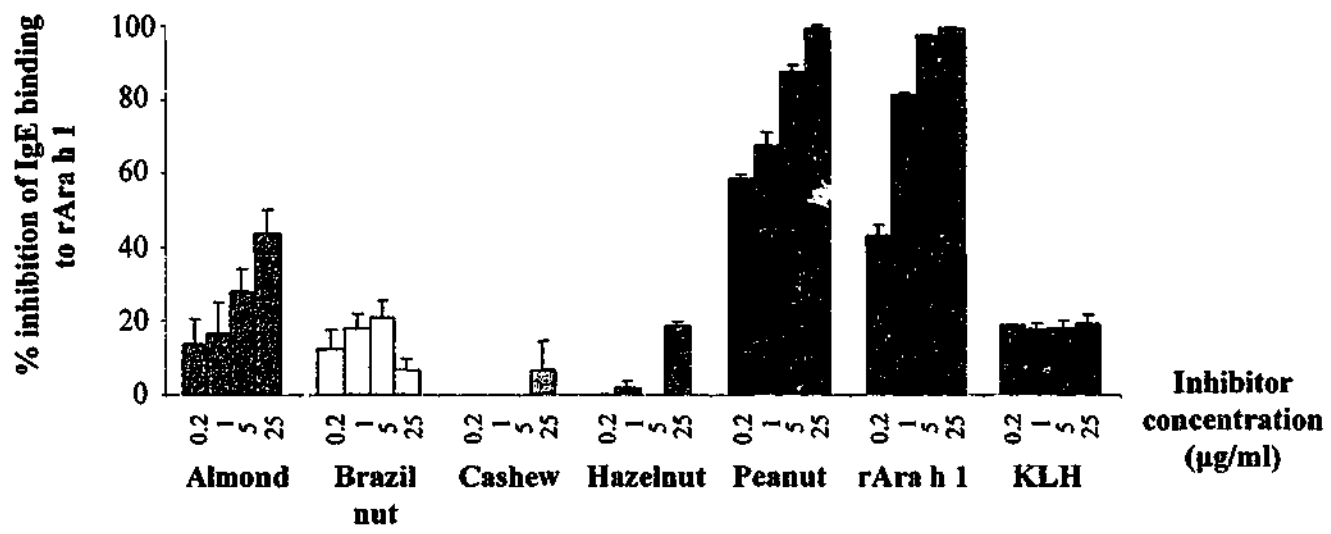


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut	rAra h 1
Clinical history	NK	NK	+	+	+	NA
CAP-FEIA	ND	+	+	+	+	NA
ELISA	+	+	-	-	+	+
BAT	+	+	+	+	+	ND

C

(i)



(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut	rAra h 1
Clinical history	NK	NK	NK	NK	+	NA
CAP-FEIA	+	+	+	+	+	NA
ELISA	+	+	-	+	+	+
BAT	+	+	-	+	+	+

antibodies from a peanut allergic subject (A9). The specificity of the purified antibodies, in particular the IgE antibodies, was assessed by ELISA. As shown in Figure 6.5, whole serum from subject A9 contained high levels of IgE antibodies to rAra h 1, as well as IgE antibodies to house dust mite (HDM) and rye grass pollen (RGP) extract. Following affinity purification using rAra h 1, strong IgE reactivity to rAra h 1 was maintained, but there were negligible levels of IgE binding to HDM and RGP extract. The first purified antibody fraction was subsequently tested for cross-reactivity by Western immunoblotting.

The specificity of the purified rAra h 1-specific antibodies was initially tested by incubating antibodies with roasted peanut nitrocellulose strips (Figure 6.6a). This resulted in prominent IgE binding to a band corresponding to the molecular mass of Ara h 1 (~65 kDa; Figure 6.6a, lane 3). IgE binding to a high molecular mass band at approximately 180 kDa was also observed which may be the trimeric form of Ara h 1, previously reported to occur with this allergen (Maleki *et al.*, 2000b). Ara h 1-specific IgE antibodies also bound to a smear of lower molecular mass peanut proteins (<8 kDa) that have not been previously identified which may be a series of Ara h 1-related proteins or breakdown products of Ara h 1. Negligible IgE reactivity was observed when purified antibodies were incubated with RGP nitrocellulose strips (Figure 6.6b, lane 3), further validating the specificity of the purified antibodies.

To identify tree nut allergens cross-reactive with Ara h 1 and confirm the observed cross-reactivity from the inhibition ELISA, purified anti-rAra h 1 antibodies were incubated with nitrocellulose membranes which had been immobilised with roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut proteins. rAra h 1-specific IgE antibodies bound to a 49 kDa almond protein which may be a homologue of Ara h 1

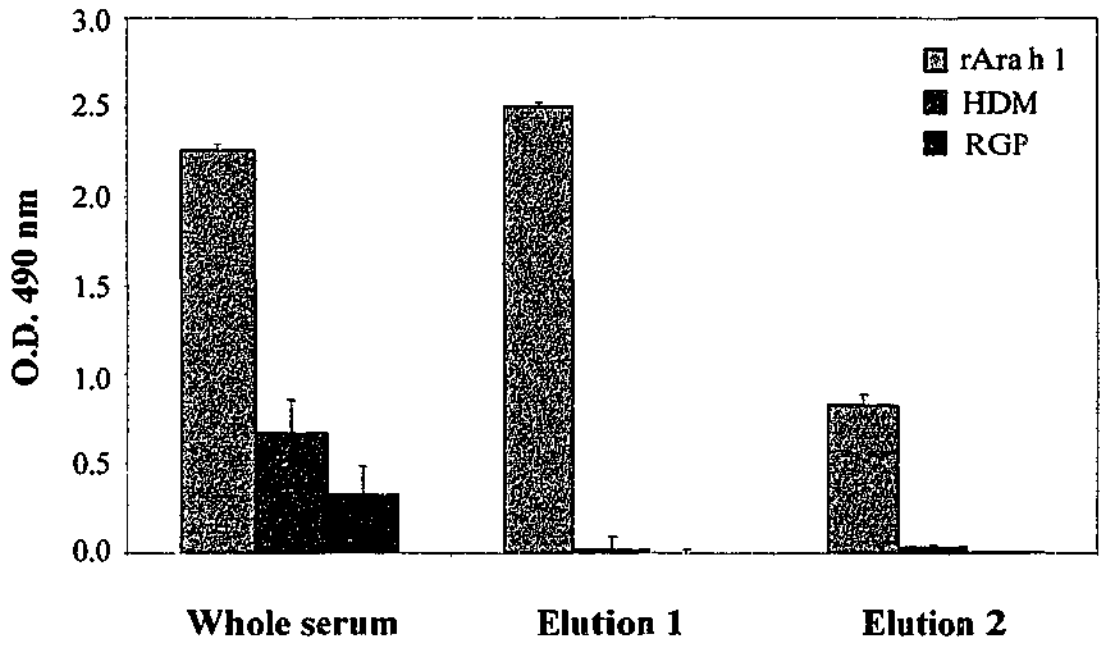
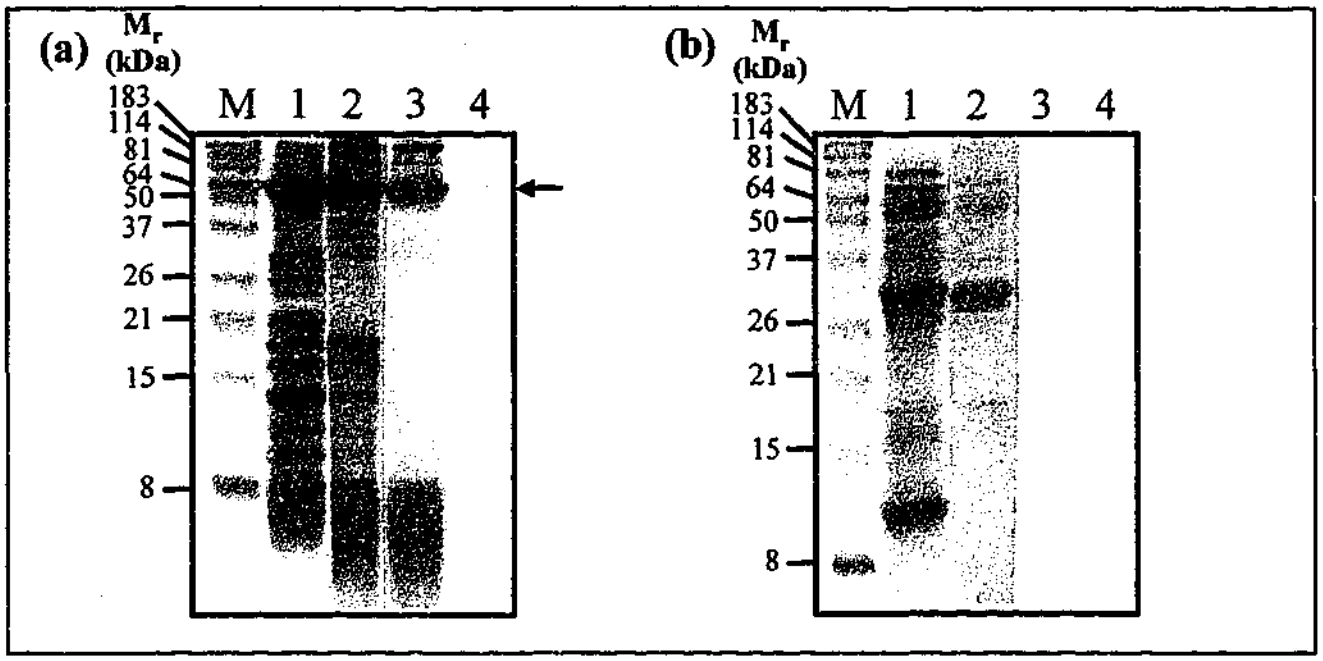


Figure 6.5 Specificity of affinity purified anti-rAra h 1 antibodies

ELISA plates were coated with 1 μg/ml of rAra h 1, house dust mite (HDM) and rye grass pollen (RGP) extracts. IgE binding using whole serum (diluted 1/10) from subject A9 was compared with affinity purified anti-rAra h 1 antibodies (neat; equivalent to 1/10 dilution of whole serum) from subject A9 serum. The absorbance in control wells containing no antigen was subtracted from antigen coated wells. Mean values for triplicates are shown and the standard deviation is indicated by the error bars.

Figure 6.6 Identification of cross-reactive allergens in tree nuts using affinity-purified anti-rAra h 1 antibodies

Anti-rAra h 1 antibodies were purified from subject A9 serum and the specificity of the eluted IgE antibodies was tested by incubation with **(a)** roasted peanut extract and **(b)** RGP nitrocellulose strips. Arrow indicates position of Ara h 1 monomer. **Lanes:** M – molecular mass markers (M_r); 1 – Coomassie-stained gel; 2 – whole serum (diluted 1/10); 3 – anti-rAra h 1 antibodies (neat; equivalent to 1/10 dilution of whole serum); 4 – no serum control blot. Cross-reactive tree nut allergens were identified by incubation of anti-rAra h 1 antibodies with roasted almond (A), raw Brazil nut (B), roasted cashew (C) and roasted hazelnut (H) extracts immobilised onto nitrocellulose membranes followed by detection of IgE binding. **(c)** Coomassie-stained gel of tree nut extracts. **(d)** Incubation of tree nut extracts with subject A9 whole serum (diluted 1/10). **(e)** Incubation of tree nut extracts with anti-rAra h 1 antibodies (neat; equivalent to 1/10 dilution of whole serum). **(f)** No serum negative control blot. Arrows indicate position of the cross-reactive allergen. M indicates position of molecular mass markers (M_r).


(a)
M_r (kDa)
183
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M 1 2 3 4
(b)
M_r (kDa)
183
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M 1 2 3 4
(c)
M_r (kDa)
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81
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M A B C H
(d)
M_r (kDa)
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M A B C H
(e)
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26
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M A B C H
(f)
M_r (kDa)
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26
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M A B C H

(Figure 6.6e). This band was also recognised by IgE antibodies in the whole serum of subject A9, in addition to other bands (Figure 6.6d). A comparison of the intensity of IgE binding to this protein using whole serum (Figure 6.6d) and rAra h 1-specific IgE antibodies (Figure 6.6e) suggests that there are other IgE antibodies present in subject A9 serum that bind to this almond allergen. These IgE antibodies may be specific for unique epitopes within this potential Ara h 1 homologue. The binding of rAra h 1-specific IgE antibodies to Ara h 1 in roasted peanut extract (Figure 6.6a, lane 3) was also more intense compared to the 49 kDa almond protein (Figure 6.6e). This suggests that rAra h 1-specific IgE antibodies may have low affinity for this almond allergen or that only a small proportion of these antibodies are cross-reactive. Minimal IgE binding was observed for Brazil nut, cashew and hazelnut following incubation with the purified Ara h 1-specific antibodies (Figure 6.6e), although IgE binding to some proteins was obtained using whole serum (Figure 6.6d). These immunoblotting results correlated with the inhibition ELISA data with almond extract showing weak inhibition of serum IgE binding to rAra h 1, pointing to the presence of an Ara h 1 homologue in almond that exhibits low-level cross-reactivity with this peanut allergen.

6.3.2.4 IgE cross-reactivity between the major peanut allergen, Ara h 2 and tree nuts

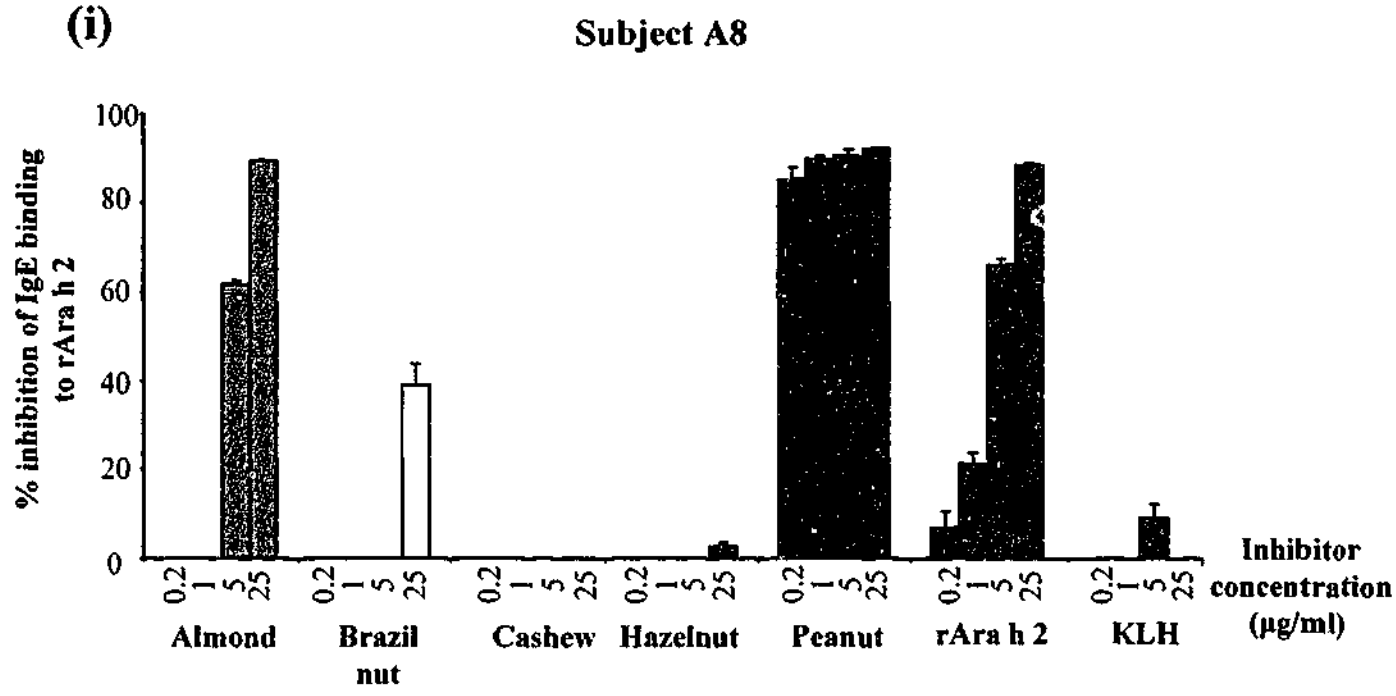
The second major peanut allergen, Ara h 2, was also used in inhibition assays to determine if this allergen is involved in the observed IgE cross-reactivity between peanuts and tree nuts. The ability of tree nut extracts to inhibit IgE binding to rAra h 2 was assessed using sera from 2 peanut allergic subjects (A8 and A9) previously demonstrated to have high levels of specific IgE to rAra h 2 ($OD_{490\ nm} \geq 1$) as well as to some tree nuts. As shown in Figures 6.7a(i) and b(i), high levels of inhibition were

Figure 6.7 ELISA for inhibition of serum IgE binding to rAra h 2 by peanut and tree nut extracts

Sera from 2 peanut and tree nut allergic subjects (A8 and A9) were pre-incubated with different concentrations of roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut and IgE binding to rAra h 2 immobilised on ELISA plates was measured. rAra h 2 and roasted peanut extract were used as the positive controls and KLH was included as the negative control extract. The results for each subject are presented as separate panels **(A and B)**. **(i)** The percentage inhibition of IgE binding to rAra h 2 for each inhibitor. Mean values for triplicates are shown and the standard deviation is indicated by error bars. **(ii)** Summary of clinical sensitivity, specific IgE (CAP-FEIA and ELISA) and basophil activation data for almond, Brazil nut, cashew, hazelnut, peanut and rAra h 2. **Legend:** NK – not known; ND – not done; NA – not applicable.

A

(i)

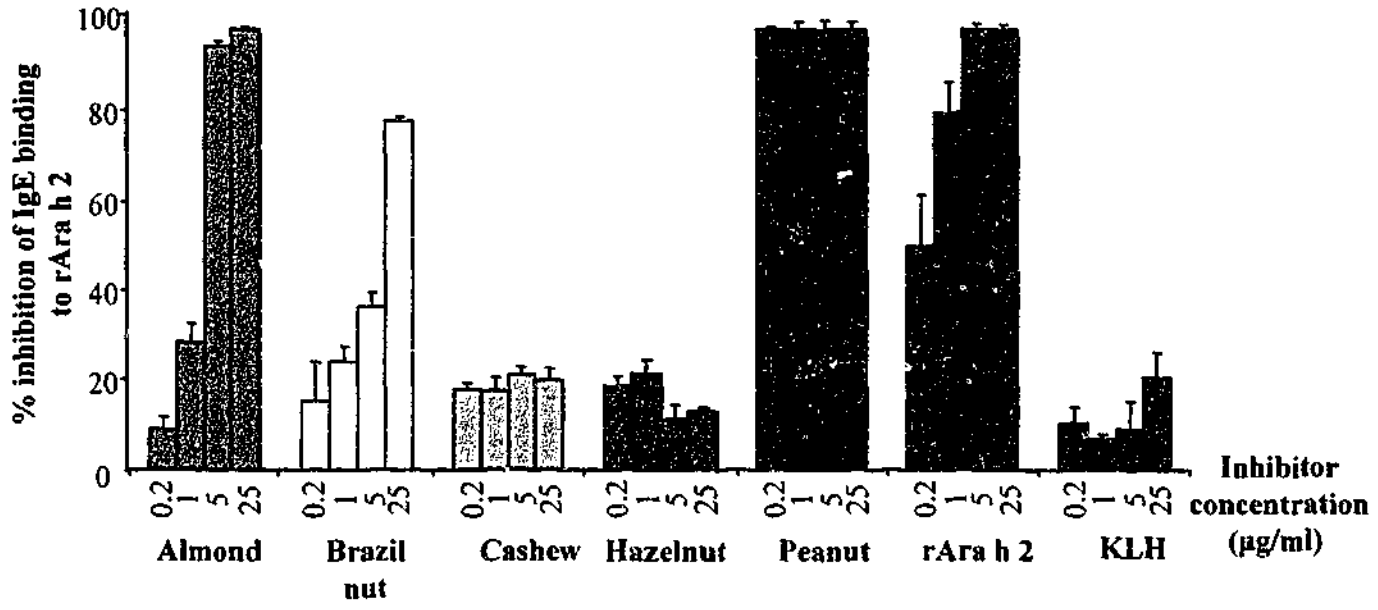


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut	rAra h 2
Clinical history	NK	NK	+	+	+	NA
CAP-FEIA	ND	+	+	+	+	NA
ELISA	+	+	-	-	+	+
BAT	+	+	+	+	+	+

B

(i)



(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut	rAra h 2
Clinical history	NK	NK	NK	NK	+	NA
CAP-FEIA	+	+	+	+	+	NA
ELISA	+	+	-	+	+	+
BAT	+	+	-	+	+	+

obtained with the rAra h 2 positive control in both subjects, demonstrating the specificity of this assay, with minimal inhibition observed with the negative control extract, KLH. Interestingly, higher levels of inhibition were obtained with roasted peanut extract compared to rAra h 2. This indicates that serum IgE antibodies from both subjects may have a higher affinity for the natural form of Ara h 2 present in crude peanut extract. Alternatively, the tendency of rAra h 2 to form multimers (see Sections 4.3.3.2.1 and 4.3.4.2) may mask some of the IgE binding epitopes on the protein, resulting in less efficient inhibition of IgE binding to plate-immobilised rAra h 2. The conformation of rAra h 2 in this preparation may also be different to the natural form of Ara h 2 in peanut extract which may contribute to the decreased efficiency in IgE binding.

Of the tree nut extracts tested for IgE cross-reactivity, roasted almond showed the highest inhibition of IgE binding to rAra h 2, followed by raw Brazil n · extract (Figures 6.7a(i) and b(i)). Both subjects also had specific IgE and positive basophil activation to these tree nuts (Figures 6.7a(ii) and b(ii)). This correlates with the crude extract inhibition ELISA data for both subjects which demonstrated IgE cross-reactivity between peanut, almond and Brazil nut (Figures 6.2c(i) and d(i)), further confirming the role Ara h 2 plays in the observed cross-reactivity between peanut and these tree nuts. IC_{50} values also indicate that a lower concentration of roasted almond extract (2.2-4.2 µg/ml) was required to inhibit 50% of IgE binding to rAra h 2 compared to raw Brazil nut extract (Table 6.2), demonstrating a higher level of cross-reactivity between Ara h 2 and almond allergens. Negligible inhibition of IgE binding was obtained when subject sera were pre-incubated with roasted cashew and roasted hazelnut extracts (Figures 6.7a(i) and b(i)) even in the presence of specific IgE to these tree nuts (Figures 6.7a(ii) and b(ii), indicating the absence of cross-reactivity between Ara h 2 and these tree nuts.

Table 6.2 Inhibitor concentration required for 50% inhibition of IgE binding to rAra h 2

Subject no.	Inhibitor concentration (μg/ml)						
	Roasted almond	Raw Brazil	Roasted cashew	Roasted hazelnut	Roasted peanut	rAra h 2[+]	KLH[#]
A8	4.2	*	†	†	0.11	3.5	†
A9	2.2	11.0	†	†	0.10	0.2	†

* - did not reach 50% inhibition at the maximum inhibitor concentration of 25 μg/ml
† - did not show inhibition above the levels obtained in non-specific inhibition assay
+ - positive control inhibitor
# - negative control inhibitor

6.3.2.5 Identification of potential Ara h 2 homologues in tree nut extracts

Affinity-purified antibodies specific for rAra h 2 were used to identify the proteins responsible for the observed cross-reactivity between Ara h 2 and the tree nuts, almond and Brazil nut. These antibodies were purified following incubation of rAra h 2 immobilised on plates with serum from a peanut allergic subject (A9). Initially, the specificity of the purified antibody fractions was tested by ELISA using rAra h 2, HDM and RGP extracts (Figure 6.8). Whole serum from subject A9 demonstrated IgE binding to rAra h 2, HDM and RGP extracts. However, the first eluted fraction of rAra h 2-specific antibodies from the same serum demonstrated a high level of IgE binding to rAra h 2, with little or no IgE binding to the HDM and RGP extracts. The second eluted antibody fraction was also tested for specificity but minimal IgE reactivity to rAra h 2 was attained. Consequently, the first antibody fraction was used for Western immunoblotting.

Initially, the rAra h 2-specific antibodies were used to probe a blot containing roasted peanut extract as a positive control and, as shown in Figure 6.9a (lane 3), IgE antibodies bound to a protein doublet with a molecular mass of 17-19 kDa which corresponds to the molecular mass of Ara h 2 (see Section 3.3.3). IgE binding was also detected to other peanut proteins of differing molecular masses but not to Ara h 1, indicating that there may be other Ara h 2-like proteins present in peanut extract. Incubation of the purified antibodies with RGP nitrocellulose strips (as a negative control) did not exhibit any IgE binding (Figure 6.9b, lane 3), further confirming the specificity of the purified antibodies.

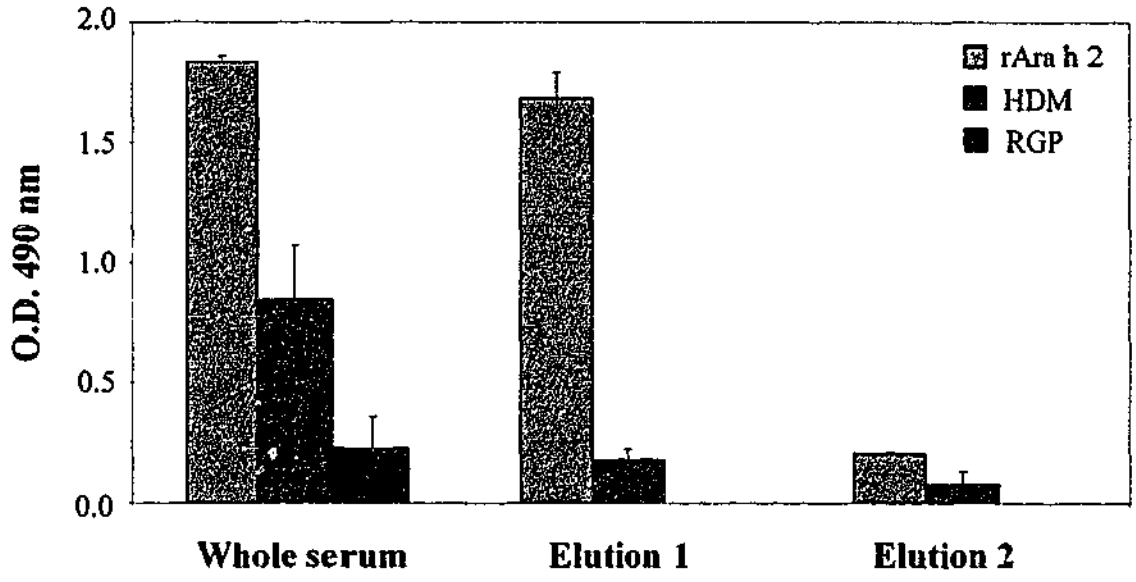
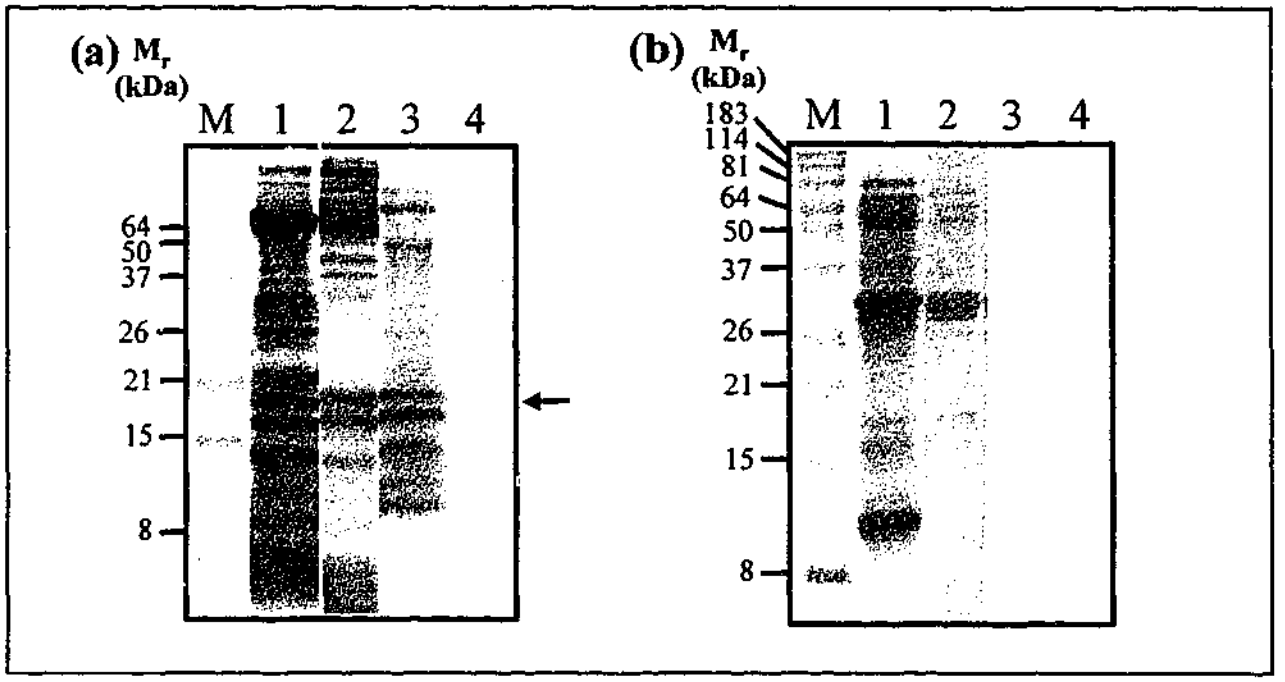


Figure 6.8 Specificity of affinity purified anti-rAra h 2 antibodies

ELISA plates were coated with 1 μg/ml of rAra h 2, house dust mite (HDM) and rye grass pollen (RGP) extracts. IgE binding using whole serum (diluted 1/10) from subject A9 was compared with affinity purified anti-rAra h 2 antibodies (neat; equivalent to 1/10 dilution of whole serum) from subject A9 serum. The absorbance in control wells containing no antigen was subtracted from antigen coated wells. Mean values for triplicates are shown and the standard deviation is indicated by the error bars.

Figure 6.9 Identification of cross-reactive allergens in tree nuts using affinity-purified anti-rAra h 2 antibodies

Anti-rAra h 2 antibodies were purified from subject A9 serum and the specificity of the eluted IgE antibodies was tested by incubation with **(a)** roasted peanut extract and **(b)** RGP nitrocellulose strips. Arrow indicates position of Ara h 2 monomer. **Lanes:** M – molecular mass markers (M_r); 1 – Coomassie-stained gel; 2 – whole serum (diluted 1/10); 3 – anti-rAra h 2 antibodies (neat; equivalent to 1/10 dilution of whole serum); 4 – no serum control blot. Cross-reactive tree nut allergens were identified by incubation of anti-rAra h 2 antibodies with roasted almond (A), raw Brazil nut (B), roasted cashew (C) and roasted hazelnut (H) extracts immobilised onto nitrocellulose membranes followed by detection of IgE binding. **(c)** Coomassie-stained gel of tree nut extracts. **(d)** Incubation of tree nut extracts with subject A9 whole serum (diluted 1/10). **(e)** Incubation of tree nut extracts with anti-rAra h 2 antibodies (neat; equivalent to 1/10 dilution of whole serum). **(f)** No serum negative control blot. Arrows indicate position of cross-reactive allergens. M indicates position of molecular mass markers (M_r).


(a) M_r (kDa)
M 1 2 3 4
64
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(b) M_r (kDa)
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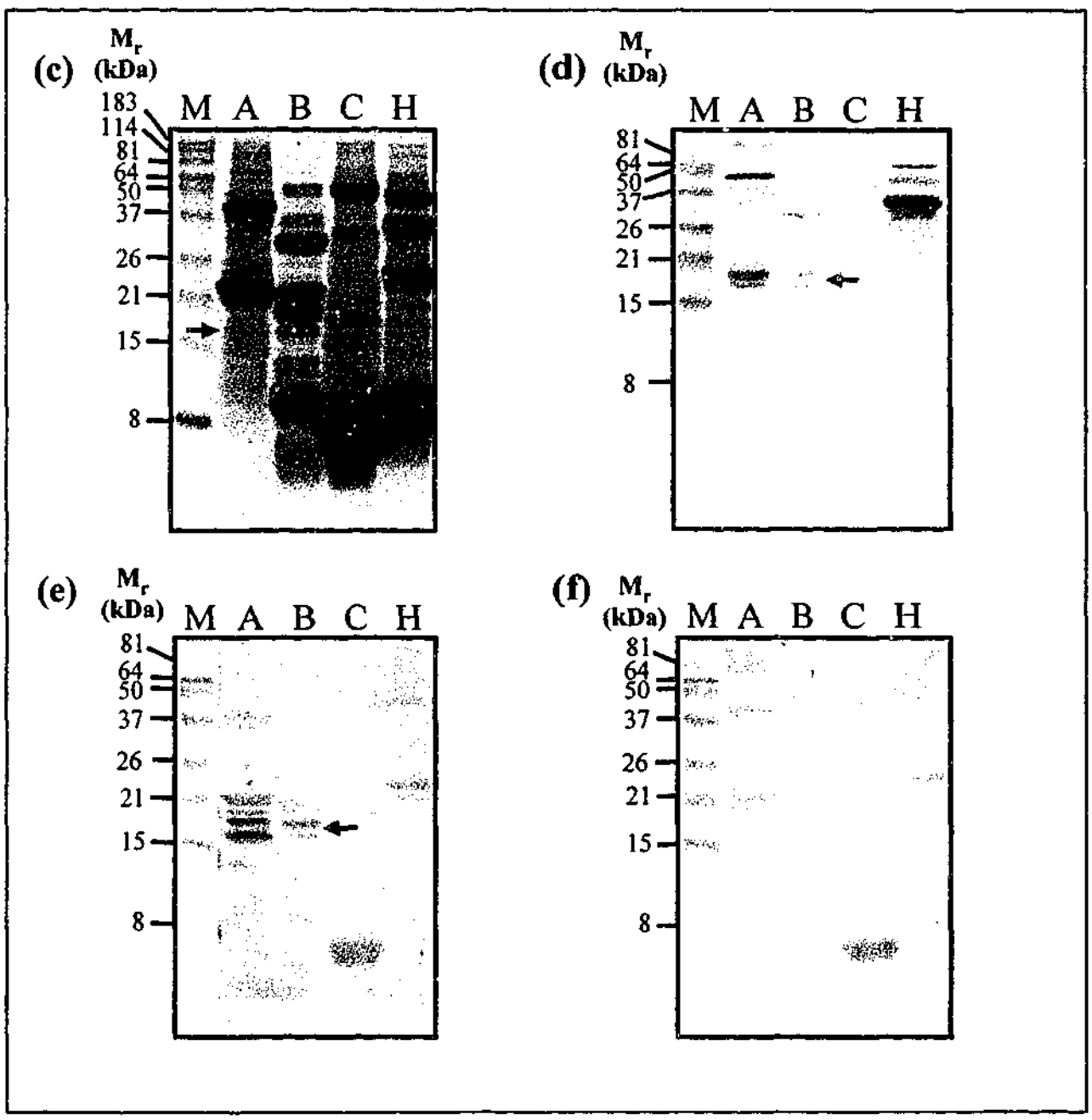
(c) M_r (kDa)
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M A B C H
(d) M_r (kDa)
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50
37
26
21
15
8
M A B C H
(e) M_r (kDa)
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64
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21
15
8
M A B C H
(f) M_r (kDa)
81
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8
M A B C H

The purified rAra h 2-specific antibodies were incubated with nitrocellulose strips of roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut to identify cross-reactive allergens. The secondary and tertiary antibodies showed some reactivity to tree nut proteins as demonstrated by the no serum control blot (Figure 6.9f) and consequently this was used as the control to determine positive serum and purified antibody reactivity to tree nut proteins. As shown in Figure 6.9e, rAra h 2-specific IgE antibodies bound to protein doublets present in roasted almond and raw Brazil nut extract with molecular masses of approximately 16-18 kDa. This is similar to the molecular mass of the Ara h 2 doublet in the peanut extract (Figure 6.9a). These proteins also bound IgE antibodies from subject A9 whole serum (Figure 6.9d) with similar intensity to the purified antibodies (Figure 6.9e), indicating that the majority of serum IgE binding to these almond and Brazil nut allergens may be attributed to cross-reactive Ara h 2-specific IgE antibodies.

The similarity in IgE-binding intensity of the purified rAra h 2-specific antibodies to Ara h 2 in roasted peanut extract (Figure 6.9a, lane 3) and the protein doublet in roasted almond extract (Figure 6.9e) confirms the high level of IgE cross-reactivity detected in the inhibition ELISA studies between rAra h 2 and almond extract (Figure 6.7b(i)). Minimal IgE binding was observed with roasted cashew and roasted hazelnut proteins, confirming the absence of cross-reactivity detected in inhibition assays (Figure 6.7b(i)). These data demonstrate that at least part of the IgE cross-reactivity between peanut and the tree nuts almond and Brazil nut is due to cross-reactive allergens that may be homologues of the major peanut allergen, Ara h 2.

6.3.2.6 IgE cross-reactivity between the peanut allergen Ara h 3 and tree nuts

Although the Ara h 3 clone obtained in this study encoded only the partial cDNA and was classified as a minor peanut allergen based on recognition by the peanut allergic subjects tested, it was still deemed useful to establish whether or not this allergen contributes to IgE cross-reactivity between peanuts and tree nuts. The ability of tree nut proteins to inhibit IgE binding to rAra h 3 was examined using sera from 3 peanut allergic subjects with measurable amounts of specific IgE to rAra h 3 ($OD_{490\ nm} \geq 1$) and tree nuts. As shown in Figures 6.10a(i), b(i) and c(i), pre-incubation of serum with increasing concentrations of roasted almond, raw Brazil nut and roasted cashew extract did not demonstrate a dose-dependent inhibition of IgE binding to rAra h 3 for any of the 3 subjects even in the context of positive specific IgE to these tree nuts (Figures 6.10a(ii), b(ii) and c(ii)). A dose-dependent inhibition of IgE binding to rAra h 3 using roasted hazelnut extract was only observed in one subject (A1; Figure 6.10a(i)) previously shown to have specific IgE to hazelnut (Figure 6.10a(ii)). This, however, was minimal compared to the positive control (rAra h 3). Minimal inhibition was also observed for the positive control roasted peanut extract which may be due to the low abundance of Ara h 3 in peanut extract (see Section 4.3.5). As such, it is difficult to determine whether the absence of cross-reactivity between rAra h 3 and tree nut allergens is valid given that minimal inhibition of IgE binding to rAra h 3 was obtained with the peanut extract positive control in this inhibition assay.

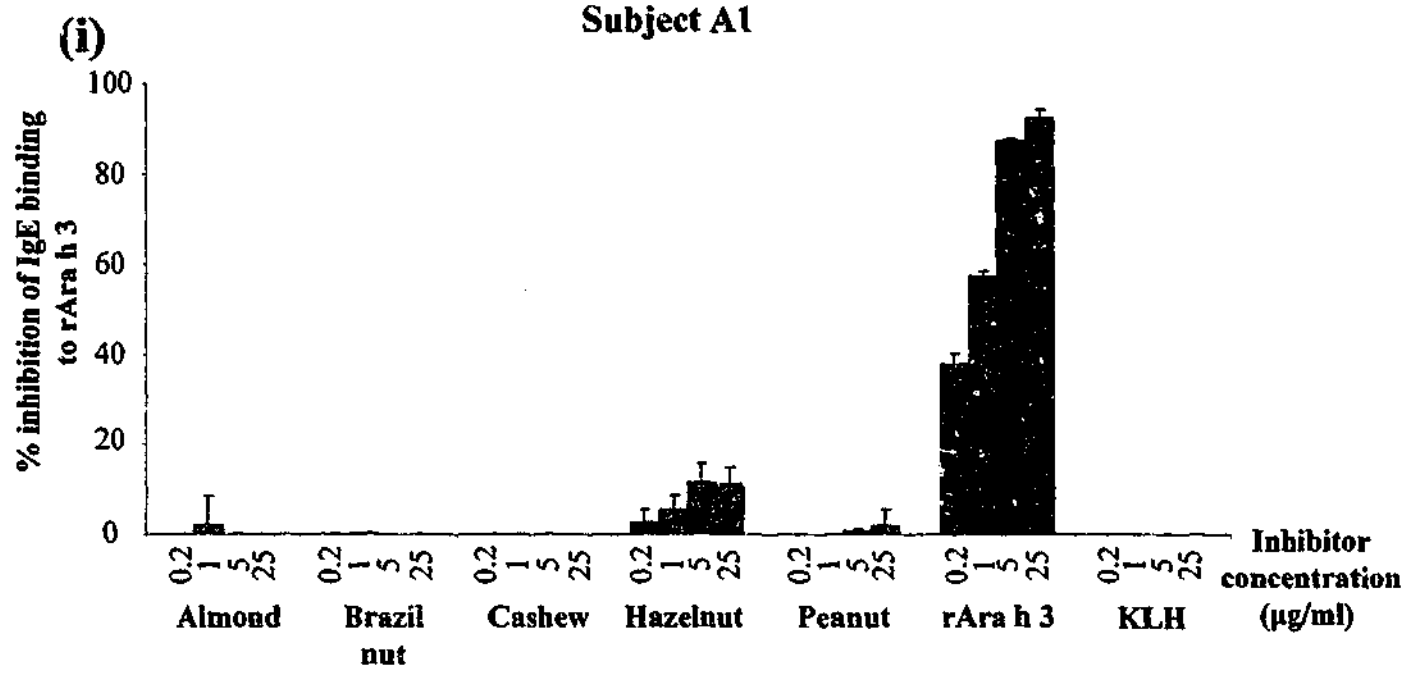
Consequently, an additional control assay was established whereby the inhibition of serum IgE binding to roasted peanut extract using rAra h 3 as the inhibitor was investigated. As depicted in Figure 6.11, significant inhibition of IgE binding to peanut extract was only demonstrated for one subject (subject A1). This may be due to the fact

Figure 6.10 Inhibition of IgE binding to rAra h 3 by peanut and tree nut extracts as measured by ELISA

Sera from 3 peanut and tree nut allergic subjects (A1, A8 and A9) were pre-incubated with different concentrations of roasted almond, raw Brazil nut, roasted cashew, roasted hazelnut and IgE binding to rAra h 3 immobilised on ELISA plates was measured. rAra h 3 and roasted peanut extract were used as the positive controls and KLH was included as the negative control extract. The results for each subject are presented as separate panels (A-C). **(i)** The percentage inhibition of IgE binding to rAra h 3 for each inhibitor. Mean values for triplicates are shown and the standard deviation is indicated by error bars. **(ii)** Summary of clinical sensitivity, specific IgE (CAP-FEIA and ELISA) and basophil activation data for almond, Brazil nut, cashew, hazelnut, peanut and rAra h 3. **Legend:** NK – not known; ND – not done; NA – not applicable.

A

(i)

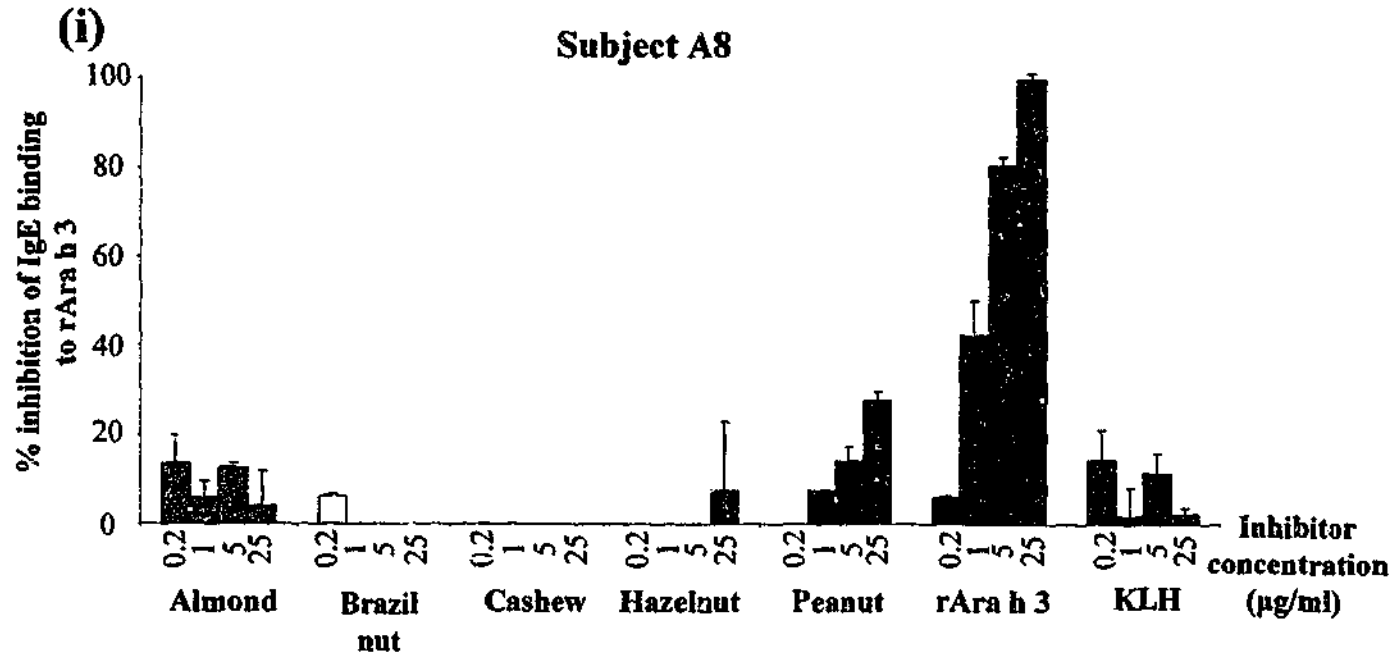


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut	rAra h 3
Clinical history	NK	NK	NK	NK	+	NA
CAP-FEIA	ND	ND	+	ND	+	NA
ELISA	+	+	-	+	+	+
BAT	ND	ND	ND	ND	ND	ND

B

(i)

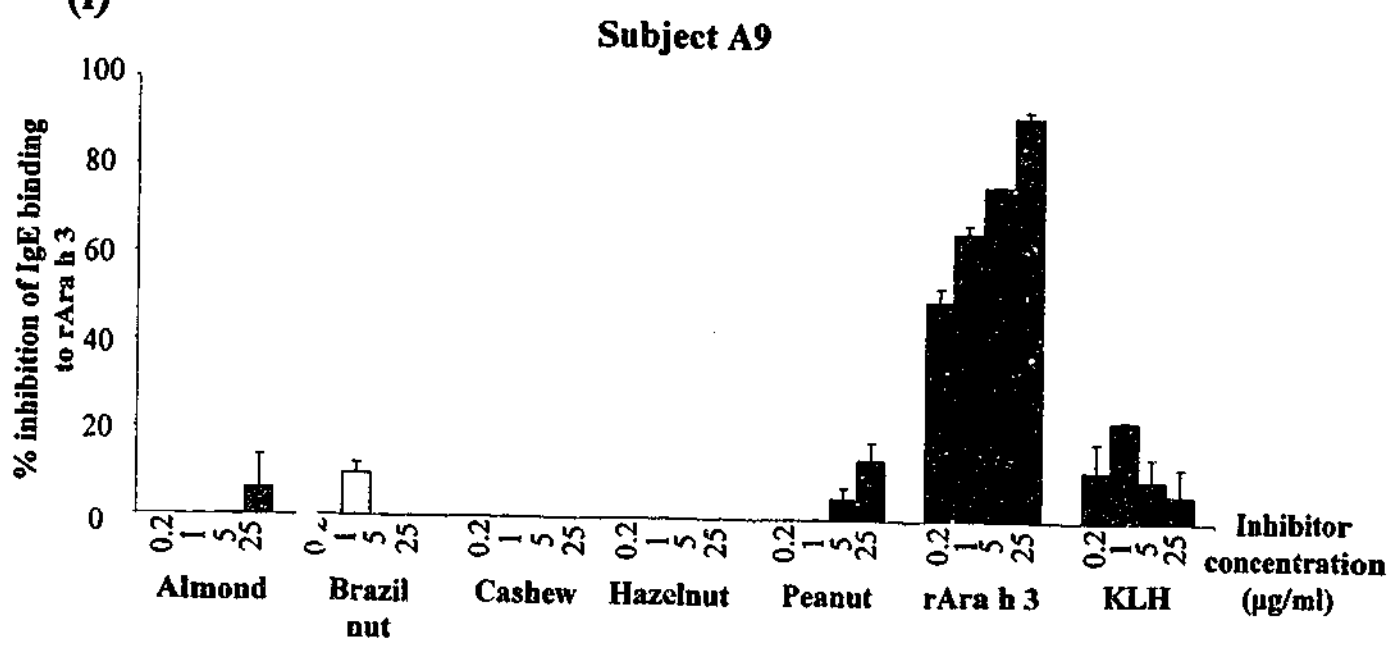


(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut	rAra h 3
Clinical history	NK	NK	+	+	+	NA
CAP-FEIA	ND	+	+	+	+	NA
ELISA	+	+	-	-	+	+
BAT	+	+	+	+	+	ND

C

(i)



(ii)

	Almond	Brazil nut	Cashew	Hazelnut	Peanut	rAra h 3
Clinical history	NK	NK	NK	NK	+	NA
CAP-FEIA	+	+	+	+	+	NA
ELISA	+	+	-	+	+	+
BAT	+	+	-	+	+	+

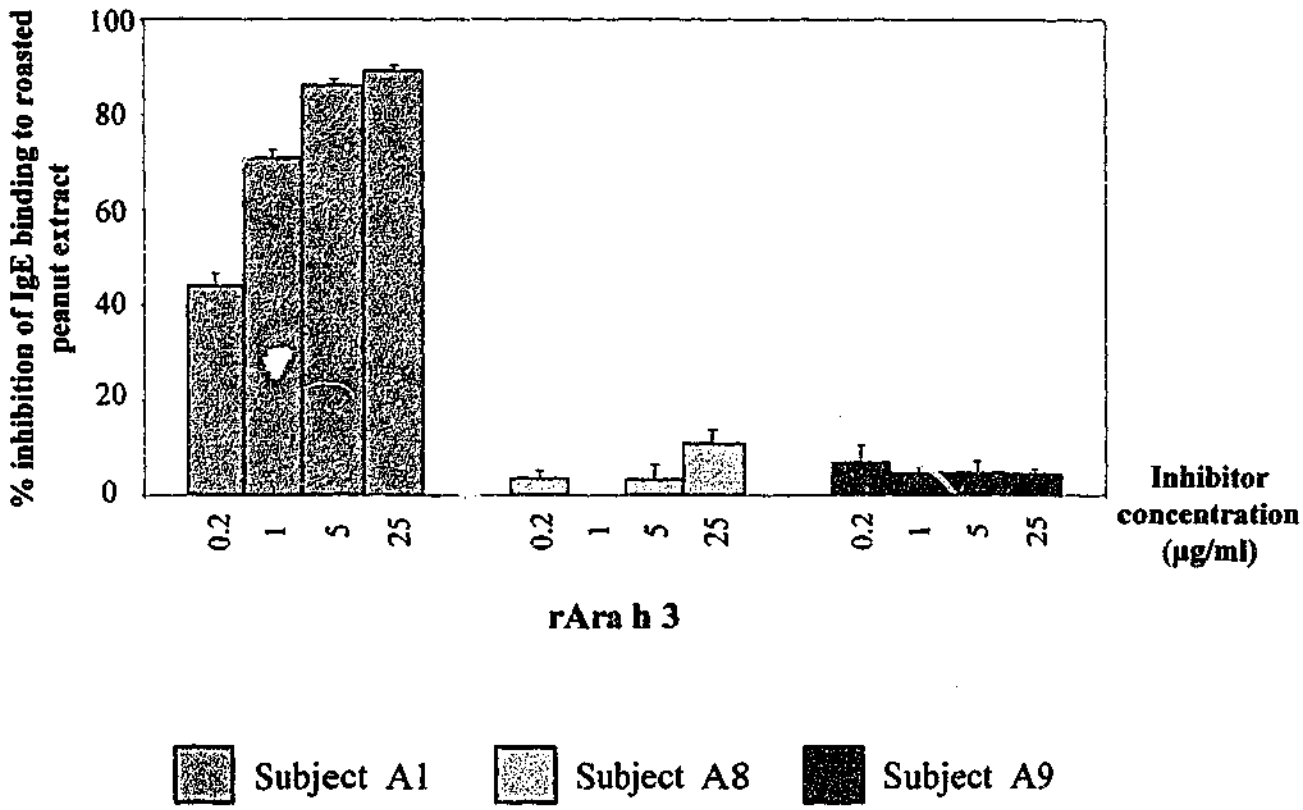


Figure 6.11 Inhibition of IgE binding to peanut by rAra h 3 as measured by ELISA

Sera from 3 known Ara h 3-sensitive subjects were pre-incubated with different concentrations of rAra h 3 and IgE binding to roasted peanut extract immobilised on ELISA plates was measured. Results are expressed as the percentage inhibition of IgE binding to roasted peanut extract. Mean values for triplicates are shown and the standard deviation is indicated by error bars.

that the IgE reactivity of this subject to unfractionated peanut extract is mainly to the 40 kDa Ara h 3-like protein (see Table 5.7). In contrast, subjects A8 and A9 demonstrated weak reactivity to this protein but strong reactivity to Ara h 1 and Ara h 2, thus making it difficult to detect any inhibition of IgE binding to roasted peanut extract using rAra h 3 as the inhibitor since reactivity to peanut extract will still be maintained through IgE binding to Ara h 1 and Ara h 2. Therefore, the weak inhibition of IgE binding to rAra h 3 following pre-incubation of serum with peanut extract is most likely due to the low abundance of this allergen in unfractionated peanut extract.

6.3.2.7 Identification of potential Ara h 3 homologues in tree nuts

Antibodies specific for rAra h 3 were used to identify cross-reactive allergens in almond, Brazil nut, cashew and hazelnut. The specificity of the purified antibodies was initially tested. As shown in Figure 6.12, the first elution fraction of anti-rAra h 3 antibodies purified from subject A9 serum demonstrated IgE binding to rAra h 3 but not to HDM and RGP extract which bound IgE antibodies using whole serum from the same subject. The second eluted antibody fraction demonstrated minimal IgE binding to rAra h 3, HDM and RGP extract. Consequently, the first antibody fraction was used for Western immunoblotting (Figure 6.13). As a positive control, the purified anti-rAra h 3 antibodies were incubated with roasted peanut extract which resulted in IgE binding to a 40 kDa protein (Figure 6.13a, lane 3) which may correspond to natural Ara h 3 (see Section 4.3.5). The purified antibodies were also incubated with RGP extract immobilised on a nitrocellulose membrane which resulted in negligible IgE binding (Figure 6.13b, lane 3), thus confirming antibody specificity.

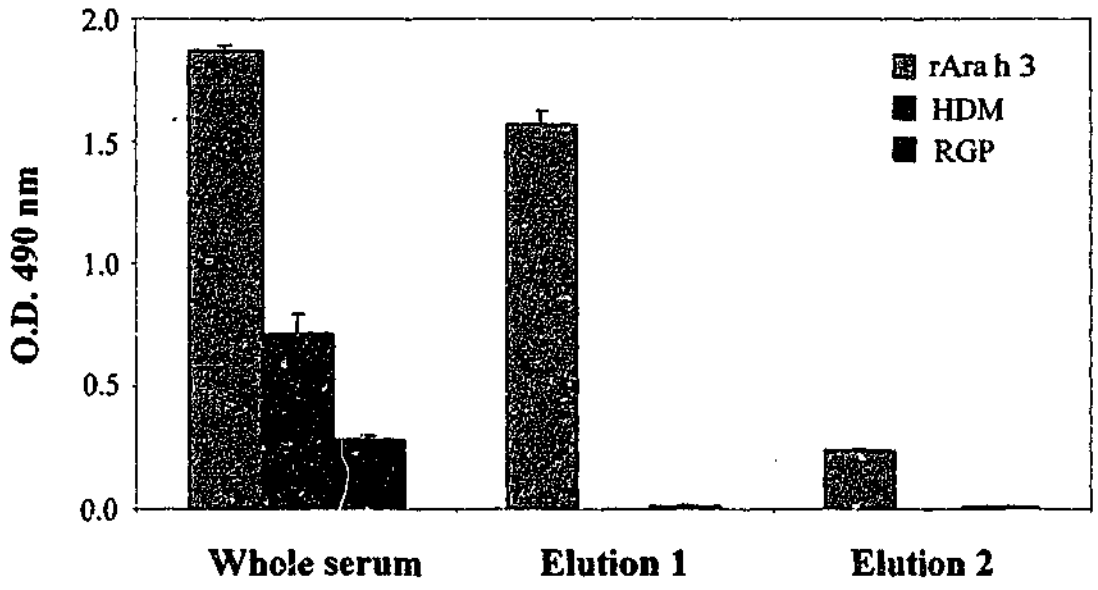
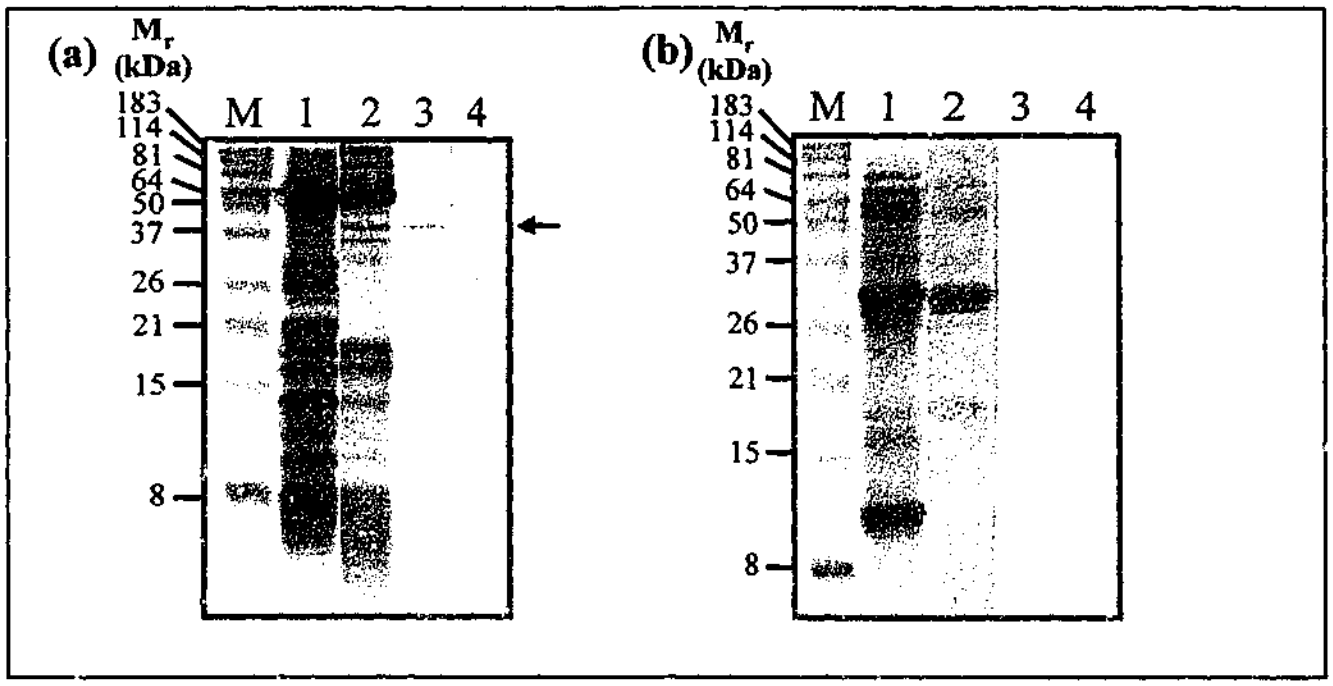


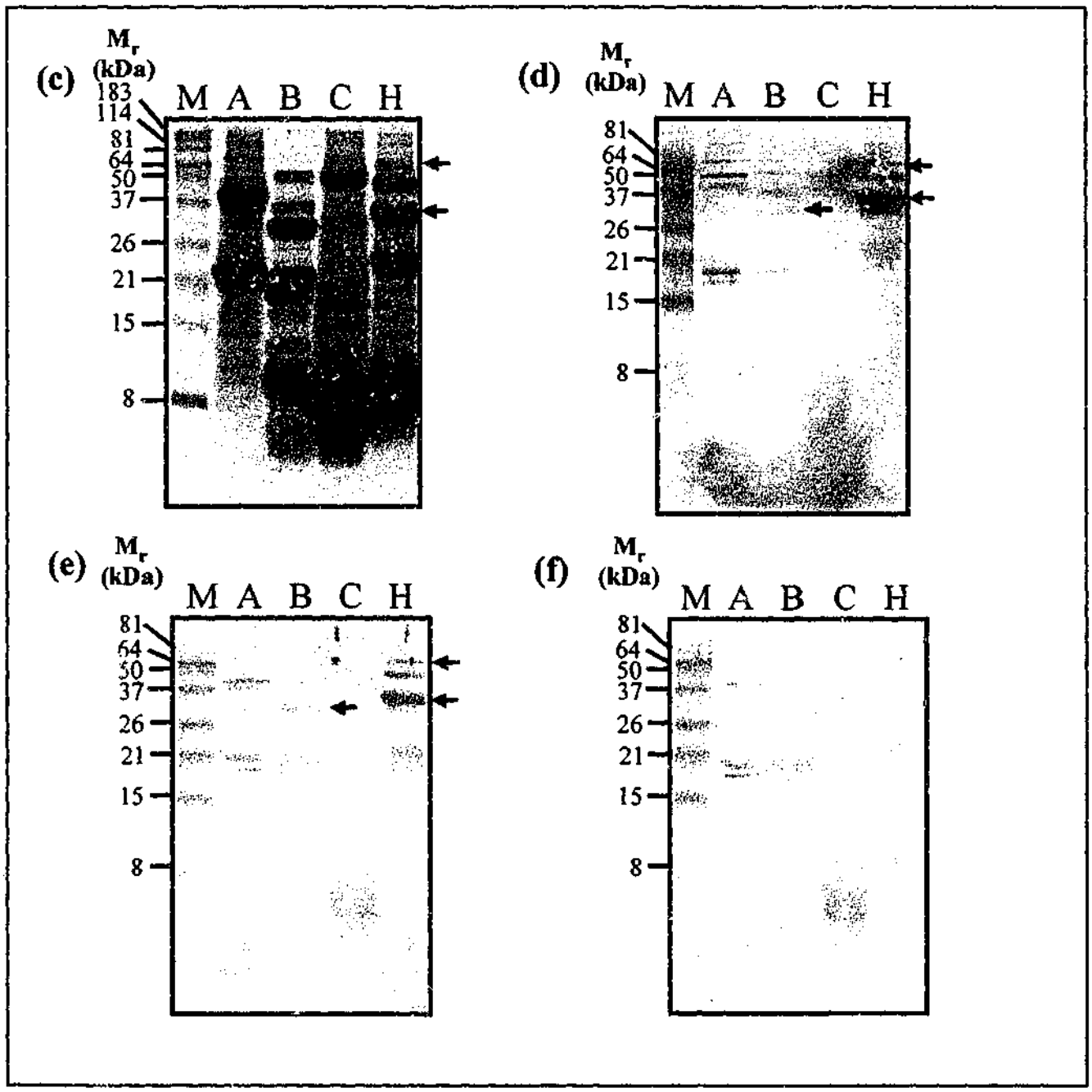
Figure 6.12 Specificity of affinity purified anti-rAra h 3 antibodies

ELISA plates were coated with 1 μg/ml of rAra h 3, house dust mite (HDM) and rye grass pollen (RGP) extracts. IgE binding using whole serum (diluted 1/10) from subject A9 was compared with affinity purified anti-rAra h 3 antibodies (neat; equivalent to 1/10 dilution of whole serum) from subject A9 serum. The absorbance in control wells containing no antigen was subtracted from antigen coated wells. Mean values for triplicates are shown and the standard deviation is indicated by the error bars.

Figure 6.13 Identification of cross-reactive allergens in tree nuts using affinity-purified anti-rAra h 3 antibodies

Anti-rAra h 3 antibodies were purified from subject A9 serum and the specificity of the eluted IgE antibodies was tested by incubation with **(a)** roasted peanut extract and **(b)** RGP nitrocellulose strips. Arrow indicates position of Ara h 3 monomer. **Lanes:** M – molecular mass markers (M_r); 1 – Coomassie-stained gel; 2 – whole serum (diluted 1/10); 3 – anti-rAra h 3 antibodies (neat; equivalent to 1/10 dilution of whole serum); 4 – no serum control blot. Cross-reactive tree nut allergens were identified by incubation of anti-rAra h 3 antibodies with roasted almond (A), raw Brazil nut (B), roasted cashew (C) and roasted hazelnut (H) extracts immobilised onto nitrocellulose membranes followed by detection of IgE binding. **(c)** Coomassie-stained gel of tree nut extracts. **(d)** Incubation of tree nut extracts with subject A9 whole serum (diluted 1/10). **(e)** Incubation of tree nut extracts with anti-rAra h 3 antibodies (neat; equivalent to 1/10 dilution of whole serum). **(f)** No serum negative control blot. Arrows indicate position of cross-reactive allergens. M indicates position of molecular mass markers (M_r).


(a)
M_r (kDa)
183
114
81
64
50
37
26
21
15
8
M 1 2 3 4
(b)
M_r (kDa)
183
114
81
64
50
37
26
21
15
8
M 1 2 3 4


(c)
M_r (kDa)
183
114
81
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50
37
26
21
15
8
M A B C H
(d)
M_r (kDa)
81
64
50
37
26
21
15
8
M A B C H
(e)
M_r (kDa)
81
64
50
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26
21
15
8
M A B C H
(f)
M_r (kDa)
81
64
50
37
26
21
15
8
M A B C H

The purified anti-rAra h 3 antibodies were subsequently incubated with roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extract immobilised on nitrocellulose membranes to identify any cross-reactive allergens. Antibody binding was observed with the no serum control blot (Figure 6.13f) indicating that the secondary and tertiary detection antibodies react to some of the tree nut proteins. This immunoblot was used as the control for the determination of positive serum and purified antibody reactivity to the tree nut extracts. As shown in Figure 6.13e, anti-rAra h 3 IgE antibodies bound to two proteins present in hazelnut extract with molecular masses of approximately 64 and 35 kDa. IgE binding to a 30 kDa protein present in raw Brazil nut extract was also detected. None of these proteins bound the secondary and tertiary control antibodies (Figure 6.13f).

A comparison of the IgE-binding intensity of the purified antibodies with whole serum showed that the reactivity of the purified rAra h 3-specific IgE antibodies to the highly abundant 35 kDa protein in hazelnut extract (Figure 6.13e) was much weaker compared to the whole serum control immunoblot (Figure 6.13d). This suggests that there are other IgE antibodies present in subject A9 whole serum that are specific for this hazelnut allergen. It may also suggest that only a small proportion of rAra h 3-specific IgE antibodies are cross-reactive with this 35 kDa hazelnut allergen. In contrast, whole serum and purified antibody reactivity to the 30 kDa allergen in Brazil nut extract (Figures 6.13d and e) was similar, indicating that the majority of IgE binding to this allergen can be attributed to rAra h 3-specific antibodies. This IgE cross-reactivity between rAra h 3 and proteins present in Brazil nut and hazelnut was not detected using inhibition ELISA (Figure 6.10c(i)). This is probably due to the different amounts of protein used in the two assays, with the use of higher total amounts of tree nut proteins (30 µg) for immunoblotting enabling the detection of low-level cross-reactive

interactions. Nevertheless, these data suggest that the peanut allergen Ara h 3 contributes to IgE cross-reactivity between peanut, Brazil nut and hazelnut. It is interesting to note that for subject A1, whose reactivity to peanut extract can be attributed mainly to the 40 kDa Ara h 3-like protein (Table 5.7), hazelnut produced the highest inhibition of IgE reactivity to peanut at the crude extract level (Figure 6.2a(i)).

6.4 DISCUSSION

Allergy to at least one tree nut is a common clinical observation in the peanut allergic population and past studies have suggested the presence of cross-reactive allergens (Parra *et al.*, 1993, Vocks *et al.*, 1993, Fernandez *et al.*, 1995, de las Marinas *et al.*, 1998, Teuber and Peterson, 1999). This chapter demonstrated serum IgE cross-reactivity between allergens present in peanut, almond, Brazil nut and hazelnut, which are the most common causes of peanut and tree nut allergy (Ewan, 1996). No IgE cross-reactivity was detected between cashew and peanut. It was also shown that some of the observed IgE cross-reactivity between peanuts and tree nuts can be attributed to the peanut allergens Ara h 1, Ara h 2 and Ara h 3.

Differences in the degree of inhibition were observed between peanut and the different tree nuts as shown by the IC_{50} for inhibition of peanut reactivity. For the majority of subjects, almond inhibited IgE binding to peanut at lower concentrations compared to Brazil nut and hazelnut, although these values were considerably higher when compared to the IC_{50} for the peanut control extract. The high inhibition of IgE binding to peanut by the peanut extract positive control compared with the tree nut extracts suggests that the level of cross-reactivity between peanut and tree nuts is low. This may reflect differences in the abundance of cross-reactive allergens or epitopes in peanut and tree nut extracts and/or differences in the affinity of peanut-specific IgE antibodies for proteins in almond, Brazil nut and hazelnut extracts (Aalberse *et al.*, 2001b). However, the abundance of cross-reactive allergens in the extract appears to play a minor role as demonstrated by immunoblotting studies using allergen-specific antibodies. rAra h 2-specific IgE antibodies were highly cross-reactive to potential homologues in almond

and Brazil nut that were of low abundance whereas rAra h 3-specific IgE antibodies demonstrated low reactivity to a highly abundant hazelnut and Brazil nut protein.

It is more likely that epitope similarity and antibody affinity contributed to the differing levels of cross-reactivity observed in this study. The extent to which these two attributes contribute to cross-reactivity between allergens is largely determined by the level of sequence homology. A high level of overall sequence homology is likely to result in IgE-binding epitopes that are of high sequence similarity that would lead to high affinity, cross-reactive IgE antibody interactions. In contrast, low sequence similarity may yield fewer, low affinity cross-reactive IgE-binding epitopes. The higher level of cross-reactivity observed between Ara h 2 and almond and Brazil nut allergens certainly suggests that this peanut allergen may have a higher sequence similarity with homologous proteins in almond and Brazil nut, in comparison to Ara h 1 and Ara h 3 which both exhibited low level cross-reactivity with tree nut allergens. Such issues can be addressed by obtaining the sequences of the potential Ara h 1, Ara h 2 and Ara h 3 homologues in almond, Brazil nut and hazelnut and comparing them with the known sequences of Ara h 1, Ara h 2 and Ara h 3. Epitope mapping would also provide further insight on the contribution of antibody affinity to differing levels of cross-reactivity.

Further studies investigating IgE cross-reactivity between peanut and tree nuts using both natural and recombinant peanut allergens should be conducted to determine if there are any differences in the level of cross-reactivity to tree nuts between the two forms. Although peanut allergic subjects would normally be exposed to the natural form of peanut allergens, the results from the previous chapter demonstrated that IgE antibodies from peanut allergic subjects recognised epitopes present on the recombinant peanut allergens used in this study. Purified recombinant peanut allergen-specific IgE

antibodies from peanut allergic subjects also reacted to the natural form of the allergen in peanut extract and, as discussed in Chapter 7, basophils resensitised with these antibodies became activated following stimulation with peanut extract. This evidence demonstrates that the rAra h 1, rAra h 2 and rAra h 3 preparations used in this study contain IgE-binding epitopes that are present on the natural forms of these allergens. It also suggests that the IgE cross-reactivity observed between these recombinant peanut allergens and allergens in almond, Brazil nut and hazelnut is likely to be due to relevant IgE-binding epitopes. Whether the natural forms of Ara h 1, Ara h 2 and Ara h 3 contain additional cross-reactive IgE-binding epitopes is not known and should be investigated to further validate the results of this study.

This study also sought to identify of some of the tree nut proteins that cross-react with the major peanut allergens, Ara h 1, Ara h 2 and Ara h 3. IgE cross-reactivity was demonstrated between the major peanut allergen, Ara h 1 and a 49 kDa protein in almond extract. Ara h 1 is a member of the vicilin family of seed storage proteins (Burks *et al.*, 1991, Burks *et al.*, 1995, Burks *et al.*, 1997, Kleber-Janke *et al.*, 1999) and this cross-reactive almond protein may also be a member of the vicilin family although none have been identified thus far. Proteins in almond and Brazil nut were also found to share similar IgE binding epitopes with the major peanut allergen, Ara h 2. These proteins have a similar molecular mass to the Ara h 2 doublet, indicating that these may be Ara h 2 homologues. Ara h 2 is a member of the conglutin family of seed storage proteins which have also been reported to contribute to the allergenicity of almonds (Poltronieri *et al.*, 2002). Poltronieri and colleagues (2002) identified an IgE reactive 45 kDa almond protein and *N*-terminal sequencing showed 40% identity with conglutin from white and narrow-leafed blue lupine. Typically, seed conglutins are processed into two subunits consisting of a 28-30 kDa *N*-terminal subunit and a 17 kDa *C*-terminal

subunit (Kolivas and Gayler, 1993). The potential Ara h 2 homologues identified in almond and Brazil nut extract have molecular masses ranging from 17-19 kDa and thus may correspond to the *C*-terminal subunit.

IgE antibodies specific for Ara h 3, a member of the legumin protein family, were found to cross-react with a 35 kDa protein in hazelnut extract and a 30 kDa protein present in Brazil nuts. Hazelnut allergens belonging to the legumin family have been previously identified and characterised (Beyer *et al.*, 2002, Pastorello *et al.*, 2002). Pastorello *et al.* (2002) identified an IgE-reactive legumin-like protein from hazelnut extract that had a similar molecular mass to the cross-reactive allergen identified in this study. This evidence suggests that legumin proteins may contribute to IgE cross-reactivity between peanut and hazelnut. It is clear from this study that the peanut allergens, Ara h 1, Ara h 2 and Ara h 3 share similar IgE binding epitopes with proteins present in almond, Brazil nut and hazelnut, which contribute to IgE cross-reactivity between peanut and tree nuts. However, further studies such as *N*-terminal sequencing and molecular cloning are required to establish the identity of the cross-reactive proteins to confirm that these are indeed homologues of previously identified peanut allergens.

An absence of IgE cross-reactivity between peanut and cashew was demonstrated in this study. This is in contrast to past studies reporting IgE cross-reactivity between cashew and other tree nuts (Parra *et al.*, 1993, Fernandez *et al.*, 1995). Cashew allergy is rare among peanut and tree nut allergic individuals. The major cashew allergen, Ana o 1, is a member of the vicilin seed storage family (Teuber *et al.*, 1999, Wang *et al.*, 2002) but no IgE cross-reactivity was detected between Ara h 1 and cashew nut proteins in this study. Although Ana o 1 shares 45% amino acid sequence similarity with Ara h 1, no common IgE binding epitopes were identified by Wang and colleagues (2002). This

suggests that membership of the same protein family does not necessarily translate to immunological cross-reactivity, further emphasising the need to establish the identity of the cross-reactive almond, Brazil nut and hazelnut allergens identified in this study to determine whether cross-reactivity can be attributed to proteins with homologous structures.

Serum IgE reactivity to Ara h 1, Ara h 2 and/or Ara h 3 was also shown to correlate with the pattern of cross-reactivity at the crude peanut and tree nut extract level. For example, peanut allergic subjects with high titres of Ara h 1-specific IgE demonstrated cross-reactivity between peanut and almond, the latter shown to contain a potential Ara h 1 homologue. Similarly, subjects with high IgE reactivity to Ara h 2 demonstrated cross-reactivity between peanut and the tree nuts almond and Brazil nut, both of which were shown to contain potential Ara h 2 homologues. One subject in this study who had high IgE antibody levels to Ara h 3 but minimal specific IgE to Ara h 1 demonstrated serum IgE cross-reactivity between peanut, Brazil nut and hazelnut but not almond. This correlated with immunoblotting studies confirming the presence of potential Ara h 3 homologues in Brazil nut and hazelnut. There were, however, peanut allergic subjects included in this study that did not have specific IgE to any of the above peanut allergens but demonstrated serum IgE cross-reactivity between peanut, almond, Brazil nut and hazelnut proteins. This suggests that there are allergens present in peanut extract other than Ara h 1, Ara h 2 and Ara h 3 that contribute to peanut and tree nut cross-reactivity and are yet to be identified.

Carbohydrate moities of allergens may also contribute to IgE cross-reactivity between peanuts and tree nuts, however the results from this study demonstrate that they are not necessary for cross-reaction. The recombinant peanut allergens used in this study were

expressed using a prokaryotic system and thus do not contain carbohydrate groups. However, these recombinant allergens were shown to share similar IgE binding epitopes with proteins present in almond, Brazil nut and hazelnut. This indicates that there are some cross-reactive IgE binding epitopes that are not carbohydrate groups. This study, however, was limited since a comparison of cross-reactivity using purified natural and recombinant allergens was not conducted. Therefore, the role of carbohydrate groups in peanut and tree nut cross-reactivity cannot be completely excluded without further testing.

The clinical significance of carbohydrate-specific antibodies is also doubtful. In a report by van der Veen and colleagues, 29 of 32 grass pollen sensitised subjects had IgE antibodies directed at *N*-linked carbohydrate groups found on peanut proteins, but only one of four subjects with a positive history and diagnosis of peanut allergy showed a similar reactivity (van der Veen *et al.*, 1997). Importantly, grass pollen allergic subjects with cross-reactive IgE antibodies to carbohydrate determinants did not exhibit clinical symptoms of peanut allergy and the concentrations of peanut allergens which induced basophil histamine release for these subjects were 1000-fold higher than control pollen allergens. Thus it appears that carbohydrate moities may play a minimal role in triggering the allergic response to peanut and tree nut allergens.

From a taxonomic perspective, peanut and tree nuts are distantly related. In this study, the observed level of cross-reactivity between peanut, almond, Brazil nut, cashew and hazelnut did not correlate with the plant taxonomic relationship. Peanut and cashew, both belonging to the Rosidae subclass (Table 1.1), did not show IgE cross-reactivity. Similarly, a previous study found no evidence of cross-reactivity between peanut and macadamia (Sutherland *et al.*, 1999) which also belongs to the same subclass. Unlike

grass pollen allergy where cross-reactivity between different grasses correlates highly with taxonomic classification (Suphioglu *et al.*, 1993), peanut and tree nut cross-reactivity cannot be predicted by taxonomic relationship.

In summary, inhibition assays demonstrated the presence of cross-reactive allergens in peanuts and tree nuts including almond, Brazil nut and hazelnut. The plant taxonomic classification of peanuts and the tree nuts used in this study did not predict allergenic cross-reactivity which may be due to the fact that peanut is distantly related to tree nuts. In addition, this study has provided evidence that the major peanut allergens, Ara h 1 and Ara h 2, as well as Ara h 3 are cross-reactive with tree nut allergens as IgE antibodies specific for these proteins cross-react with almond, Brazil nut and hazelnut proteins. Although these findings may explain, in part, the high frequency of tree nut sensitivity among peanut allergic individuals, it provides the basis for further studies allowing the molecular identification and characterisation of the cross-reactive tree nut allergens as well as the corresponding IgE-binding epitopes. Such information should contribute to improved diagnosis and treatment of peanut and tree nut allergy.

CHAPTER 7

CHARACTERISATION OF CROSS-REACTIVE IgE ANTIBODIES

7.1 INTRODUCTION

IgE cross-reactivity has previously been reported to contribute to multiple sensitivities to different allergen sources in some allergic individuals. For example, many grass pollen allergic patients are sensitive to more than one type of grass and this is thought to be due to the presence of cross-reactive allergens in different grass families (Weber, 2003). The latex-fruit syndrome is another example whereby more than half of latex-sensitised individuals reportedly have IgE antibodies specific to proteins from some fruits and vegetables. About one-third of these patients experience Type I hypersensitivity reactions upon ingestion of foods such as avocado, banana, chestnut, kiwi and potato (Brehler *et al.*, 1997, Lavaud *et al.*, 1997, Raulf-Heimsoth *et al.*, 1997, Salcedo *et al.*, 1999).

In the previous chapter, it was reported that peanut-specific IgE antibodies cross-react with allergens present in tree nuts such as almond, Brazil nut and hazelnut. It is, however, unclear whether all cross-reactive IgE antibodies have biologically relevant activity. Cross-reactive carbohydrate determinants (CCDs) have been demonstrated to have little or no biological activity and can contribute to false-positive results on *in vitro* tests that investigate cross-reactivity (van der Veen *et al.*, 1997, Mari *et al.*, 1999, Mari, 2002). Others have reported that CCD-specific IgE from some patients with tomato

allergy can trigger histamine release from basophils and therefore contribute to the manifestation of this type of food allergy (Foetisch *et al.*, 2003, Westphal *et al.*, 2003). Given the controversy surrounding the biological significance of cross-reactive IgE antibodies, this study sought to determine whether peanut-specific IgE antibodies can activate basophils following exposure to cross-reactive tree nut allergens.

In this chapter the establishment of an *in vitro* assay to assess the biological significance of cross-reactive peanut-specific IgE antibodies is described. The assay involved the removal of surface IgE from donor basophils followed by resensitisation with test sera or affinity-purified antibodies. Resensitised cells were stimulated with different allergen extracts and basophil activation, as indicated by CD63 expression, was analysed. Optimisation experiments were performed firstly to obtain partially purified donor basophils. The conditions required for the removal of surface IgE were determined and finally the ability to resensitise basophils with peanut allergic sera or affinity-purified antibodies specific for peanut extract, rAra h 1 and rAra h 2 was investigated. When the test was established, it was used to determine whether basophils resensitised with peanut-specific IgE antibodies can be activated upon exposure to tree nut extracts, thus validating the biological significance of these cross-reactive antibodies.

7.2 METHODS

7.2.1 Affinity purification of allergen-specific antibodies

Antibodies specific to roasted peanut extract and the recombinant peanut allergens, Ara h 1 and Ara h 2 were purified as outlined in Section 6.2.3. A single antibody elution was conducted and the specificity of this fraction was assessed by ELISA as described in Section 2.7.

7.2.2 Purification of peripheral blood mononuclear cells (PBMCs) from whole blood by Ficoll-Paque centrifugation

Peripheral blood (25 ml) collected in heparinised tubes was diluted 1:1 with pre-warmed (37°C) heparinised RPMI containing PSG. 25 ml of diluted blood was layered onto 15 ml of Ficoll-Paque and centrifuged at 642 x g for 25 minutes (brake off). PBMCs were collected from the buffy coat and washed in heparinised medium for 15 minutes at 446 x g (brake on). The cell pellet was resuspended and washed with heparinised medium for 10 minutes at 286 x g (brake on). The pellet was resuspended in 3 ml of RPMI containing 10% FCS and the cell concentration was determined by mixing 10 µl of cell suspension with 10 µl Trypan blue which was then placed on a haemocytometer. The volume of cells was adjusted for a final concentration of 5 x 10^6 cells/ml.

7.2.3 Stripping of surface IgE antibodies from basophils

After adjusting the cell concentration, cells were pelleted by centrifugation at 250 x g for 5 minutes, resuspended in an equal volume of lactic acid buffer (pH 3.9) and incubated on ice for 2, 5, or 10 minutes. The suspension medium of cells was neutralised by adding 2 volumes of HEPES buffer containing 5% FCS and the cells

were pelleted by centrifugation as described above. This stripping and neutralisation procedure was repeated once more. Cells were then washed in HEPES buffer containing 5% FCS.

7.2.4 Resensitisation of basophils with serum and purified antibodies

The resensitisation of basophils was carried out by resuspending IgE stripped cells in test serum (~100 µl per 5 x 10^6 cells) or purified antibodies (~200 µl per 2.5 x 10^6 cells) followed by incubation at 37°C for 1 hour. Cells were washed once in HEPES buffer containing 5% FCS and resuspended in HEPES buffer containing $CaCl_2$ and 5% FCS to give a final concentration of 5 x 10^6 cells/ml.

7.2.5 Activation of basophils

100 µl of cell suspension (~5 x 10^5cells) were placed in a FACS tube and 20 µl of stimulation buffer containing IL-3 and heparin were added to each tube and incubated at 37°C for 10 minutes. Allergen challenge was performed with the addition of 100 µl of allergen extract (diluted with stimulation buffer containing heparin and IL-3 to obtain the desired concentration) to each tube followed by incubation at 37°C for 20 minutes. In some experiments, the cells were also stimulated with 100 µl rabbit anti-human IgE antibody (diluted 1/1000 in stimulation buffer containing IL-3 and heparin) and fMLP (diluted 1/200 in stimulation buffer containing IL-3 and heparin) as positive controls. Activation of basophils was stopped by incubating cells on ice for 5 minutes.

7.2.6 Fluorescent labeling of cells

Following allergen challenge, cells were incubated with normal goat serum on ice for 10 minutes to reduce non-specific binding of fluorescently labelled antibodies to Fc receptors on the surface of cells. Cells were then stained with labelled antibodies as

outlined in Section 2.8.2. Cells were subsequently washed once with FACS wash buffer (3 ml/tube) followed by centrifugation at 250 x g for 5 minutes (4°C). Cell pellets were resuspended in 100 µl FACS wash buffer per tube and 7AAD was added to cells to exclude non-viable cells. Flow cytometric analysis was performed using a FACScalibur flow cytometer (Becton Dickinson, USA) and Cell Quest software (Becton Dickinson, USA). Approximately 150000 events were collected within the 'lymphocyte-monocyte' gate to ensure that a consistent number of basophils was analysed for each test sample. The gating of $CD63^{+}$ cells was based on the discrimination of the negative control staining (no antigen control) and positive control staining (fMLP and anti-IgE stimulation).

7.3 RESULTS

7.3.1 Partial purification of basophils from whole blood

The stripped basophil activation test cannot be performed using whole blood since the presence of serum IgE may interfere with the removal of surface IgE and the resensitisation of basophils with heterologous IgE. Consequently, PBMCs were purified from whole blood using a Ficoll-Paque gradient and the presence of basophils, characterised by high IgE staining, was assessed by staining cells with FITC-conjugated anti-human IgE antibodies. Figure 7.1 is a comparison of IgE staining of whole blood versus PBMCs for a HDM allergic, non-peanut/tree nut allergic subject. The IgE staining intensity of PBMCs was similar to that of whole blood (Figures 7.1a and c). Basophils (IgE^{hi} cells) from whole blood and PBMCs became activated following incubation with HDM extract as indicated by CD63 expression although the percentage

Whole blood

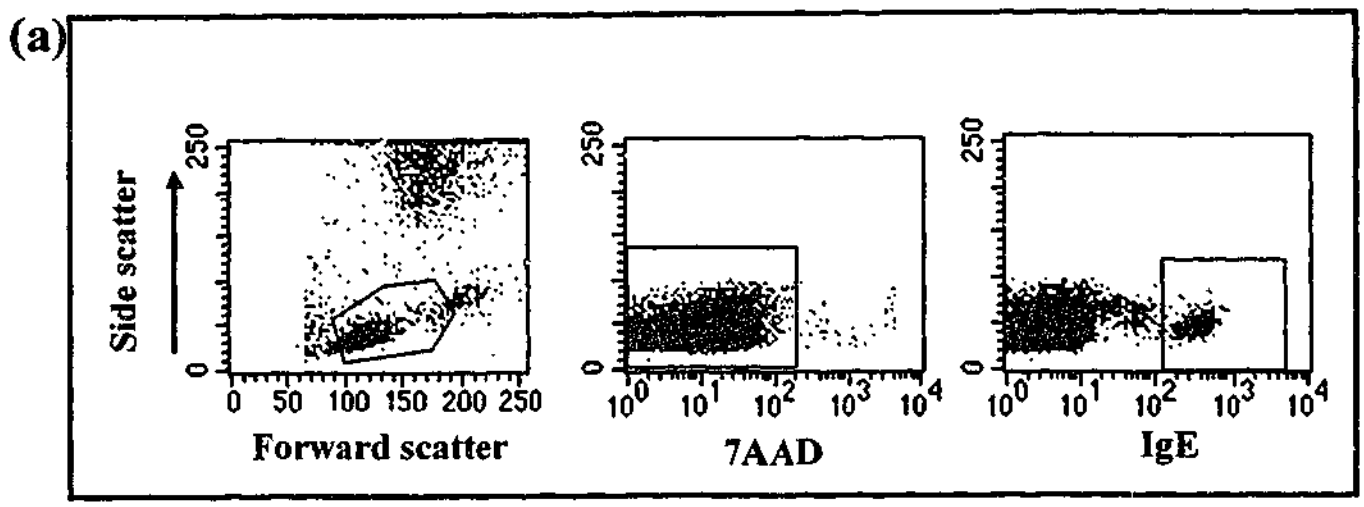
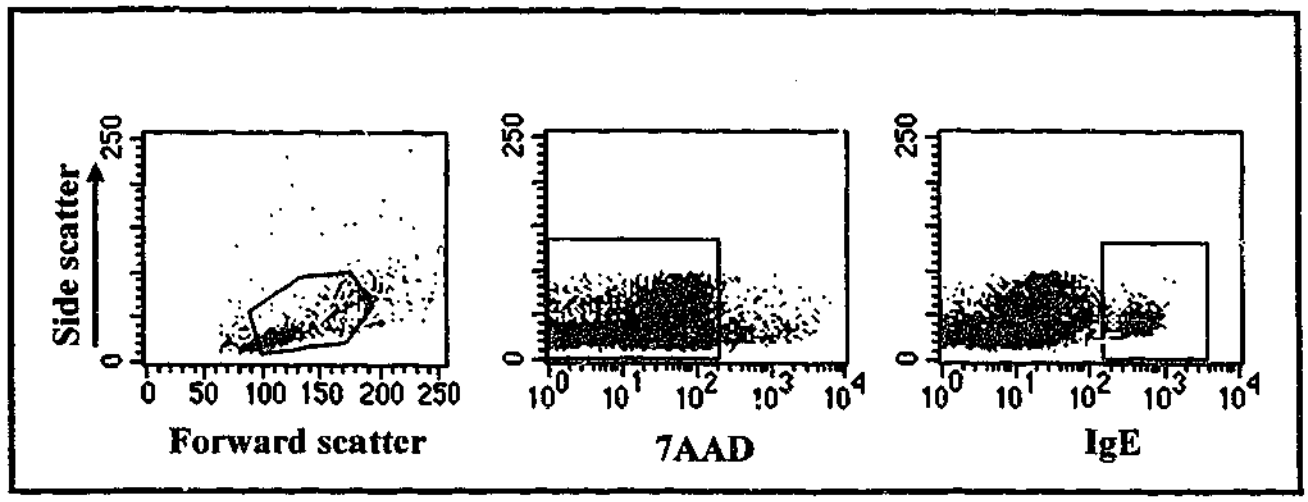


Figure 7.1 Comparison of activated basophils from whole blood and Ficoll-purified leukocytes

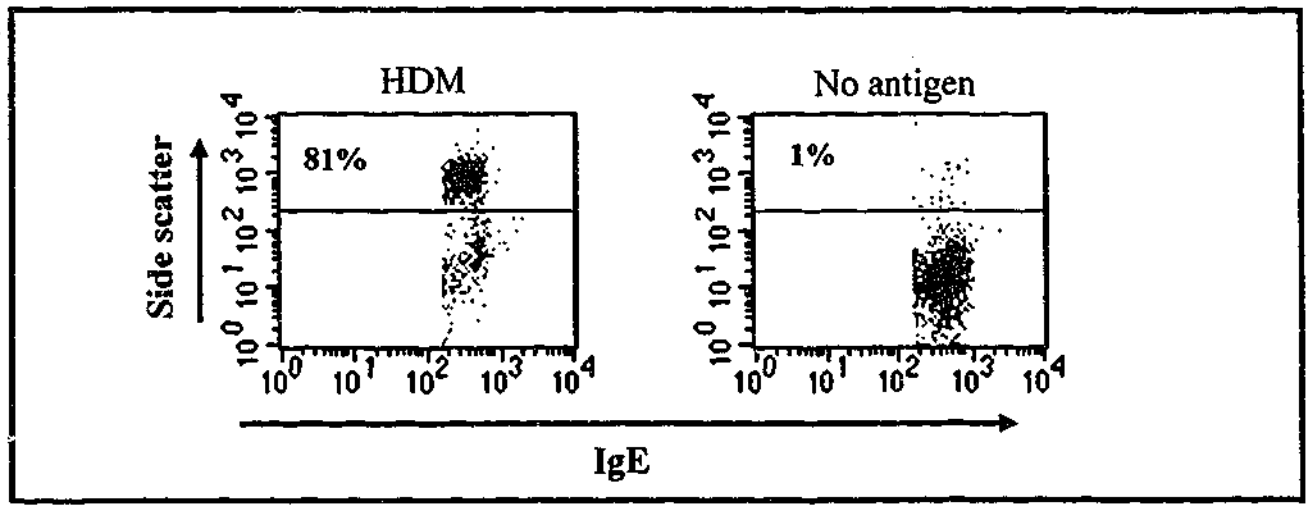
Whole blood **(a and b)** and purified PBMCs **(c and d)** from a HDM allergic, non-peanut/tree nut allergic subject (NA4) were stimulated with 10 μg/ml of HDM extract. Activation of basophils as indicated by CD63 expression was analysed as follows: **(a) and (c)** Cells were gated based on forward scatter and side scatter. Live cells within this gate were selected via 7AAD exclusion and cells were analysed for high expression of IgE. **(b) and (d)** IgE^{hi} cells were analysed for CD63 expression and the percentage of activated basophils was calculated (upper quadrant). A no antigen negative control was included in the assay to ascertain the percentage of spontaneously activated basophils. $CD63^+$ cells were gated based on the discrimination of negative and positive control staining.

Purified PBMCs

(c)



(d)



of activated basophils from PBMCs (81%; Figure 7.1d) was slightly lower compared to whole blood (91%; Figure 7.1b). Monocytes and B cells were shown to be excluded from the IgE^{hi} cell population by CD14 and CD19 staining, respectively and were found to be present in the IgE^{low} population (data not shown). Negligible basophil activation was observed v[illegible]e no antigen control for both whole blood and purified PBMCs (Figures 7.1b an[illegible]). Thus, the purification of PBMCs using a Ficoll-Paque gradient was a satisfactory method for obtaining partially purified basophils which could then be used in the stripped basophil activation test.

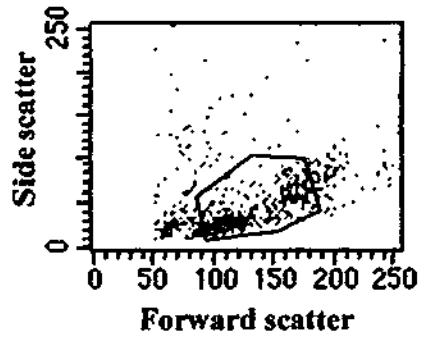
7.3.2 Stripping of surface IgE from basophils

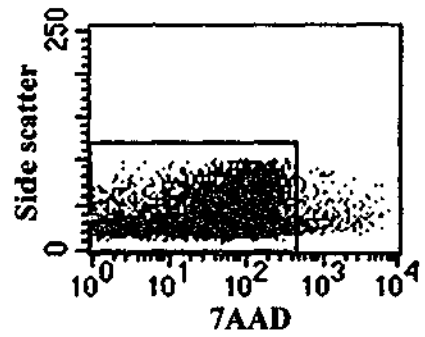
The removal of IgE from the surface of basophils was performed by incubating cells on ice in a low pH buffer (pH 3.9), which has been previously reported (Ishizaka and Ishizaka, 1974, Pruzansky *et al.*, 1983, Nolte *et al.*, 1988, Kleine Budde *et al.*, 2001). The efficient removal of surface IgE is particularly important as this can negatively affect the efficiency of resensitisation. Initially, the effect of incubation time on the dissociation of surface IgE from basophils was examined. Purified PBMCs from a HDM allergic, non-peanut/tree nut allergic subject were resuspended in lactic acid buffer (pH 3.9) and incubated on ice for 2, 5, and 10 minutes followed by allergen challenge. IgE and CD63 expression were subsequently examined. As illustrated in Figure 7.2b, IgE^{hi} cells could still be detected after incubation of cells in lactic acid buffer for 2 minutes. However, dissociation of surface IgE occurred after 5 minutes as indicated by a decrease in the number of IgE^{hi} cells (Figure 7.2b). Interestingly, IgE^{hi} cells could be detected following incubation of cells for 10 minutes in lactic acid buffer (Figure 7.2b). It appears that IgE antibodies are removed from the surface of basophils after incubation in lactic acid buffer for 5 minutes but reassociate after 10 minutes. The basophils also remained functional regardless of the length of time cells were incubated

Figure 7.2 Effect of time on the removal of surface IgE from basophils

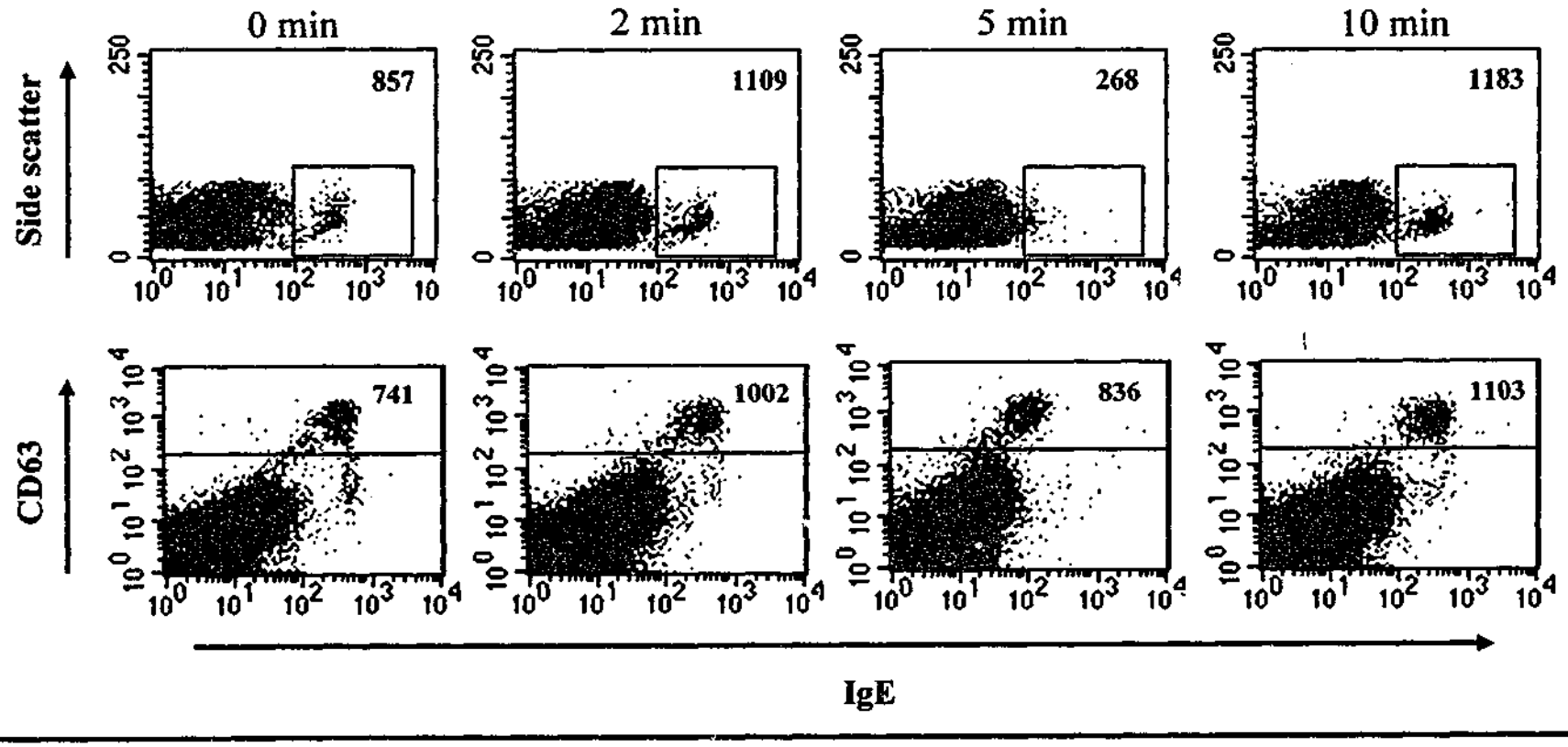
PBMCs from a HDM allergic, non-peanut/tree nut allergic subject (NA4) were incubated in lactic acid buffer (pH 3.9) for 2, 5 and 10 minutes and stimulated with HDM extract (10 μg/ml). IgE staining and CD63 expression was analysed as follows: **(a)** Cells were gated based on forward scatter and side scatter. Live cells within this gate were selected via 7AAD exclusion. **(b)** The number of IgE^{hi} cells and $CD63^{+}$ cells (upper quadrant) was analysed. $CD63^{+}$ cells were gated based on the discrimination of negative and positive control staining.

(a)


Side scatter
250
0
0 50 100 150 200 250
Forward scatter


Side scatter
250
0
10^0 10^1 10^2 10^3 10^4
7AAD

(b)


0 min
2 min
5 min
10 min
Side scatter
CD63
857
1109
268
1183
741
1002
836
1103
IgE

in lactic acid buffer as indicated by CD63 upregulation upon stimulation with HDM extract (Figure 7.2b). Thus, the optimal incubation time for the removal of surface IgE from basophils is 5 minutes, which is in accordance with the results obtained by Pruzansky and colleagues (1983).

It should be also be noted that activation of basophils from a HDM allergic subject still occurred upon HDM stimulation in the absence of an IgE^{hi} cell population, with minimal decrease in the number of $CD63^{+}$ cells. In contrast, basophils from an atopic, non-HDM allergic donor did not become activated upon HDM stimulation (data not shown). This indicates that a high density of surface IgE may not be necessary to obtain basophil activation upon stimulation with allergen extract.

The IgE stripping procedure was repeated to determine whether this results in further removal of IgE from the surface of basophils. In this experiment, cells were washed once after the first stripping procedure and then incubated in lactic acid buffer at 0°C for another 5 minutes followed by allergen challenge. IgE and CD63 expression were then analysed. As shown in Figure 7.3, a second incubation in lactic acid buffer resulted in further removal of surface IgE as indicated by the decrease in the number of IgE^{hi} cells. Again, the basophils remained functional as HDM stimulation resulted in CD63 expression (Figure 7.3). Consequently, the standard procedure that was adopted for the removal of surface IgE from basophils involved two 5 minute incubations in lactic acid buffer, with washes conducted in between incubations.

7.3.3 Sensitisation of basophils with IgE antibodies

Several studies have previously demonstrated that basophils stripped of surface IgE can be resensitised using heterologous sera (Ishizaka *et al.*, 1973, Ishizaka and Ishizaka,

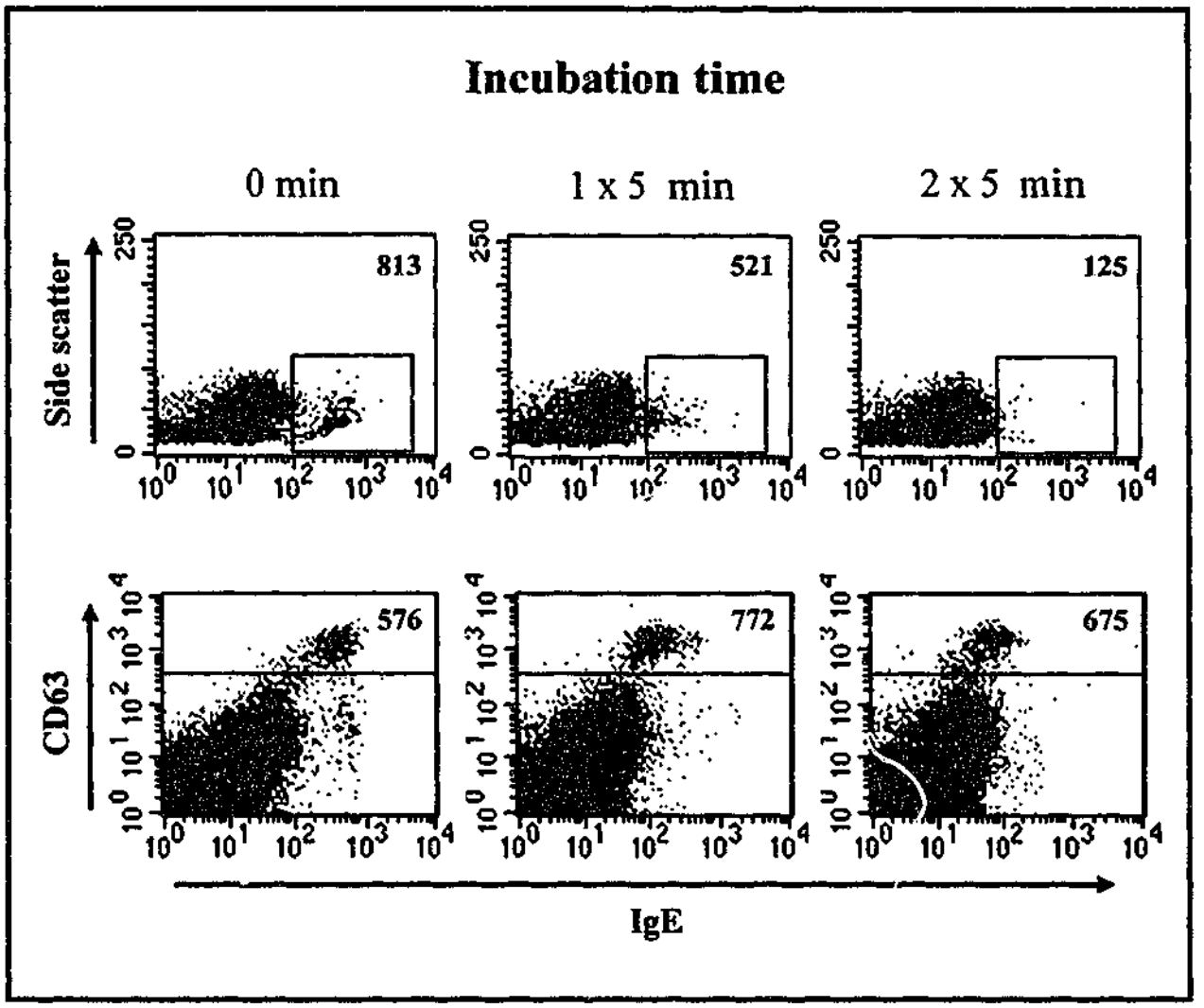


Figure 7.3 Removal of surface IgE after multiple incubations in low pH buffer

PBMCs from a HDM allergic, non-peanut/tree nut allergic subject (NA4) were incubated in lactic acid buffer (pH 3.9) either once or twice and the number of IgE^{hi} cells and $CD63^{+}$ cells (upper quadrant) was analysed following stimulation with 10 μg/ml of HDM extract. $CD63^{+}$ cells were gated based on the discrimination of negative and positive control staining.

1974, Conroy *et al.*, 1979, Pruzansky *et al.*, 1983, Nolte *et al.*, 1988, Kleine Budde *et al.*, 2001, Foetisch *et al.*, 2003). Given that one of the goals of this study is to resensitise basophils with affinity purified peanut-specific IgE antibodies, it was essential to demonstrate that basophils stripped of IgE antibodies could indeed be resensitised. Initial experiments investigated the resensitisation of basophils using serum from a peanut allergic subject. IgE stripped cells were incubated with peanut allergic serum at 37°C (Pruzansky *et al.*, 1983) for 1 hour followed by allergen stimulation. It should be noted that PBMC donors for all experiments were atopic, non-peanut/tree nut allergic subjects to ensure that basophil activation upon exposure to peanut allergens was due to the resensitisation of basophils with IgE antibodies from peanut allergic sera. As shown in Figure 7.4a, incubation of IgE stripped cells in peanut allergic serum at 37°C resulted in increased IgE staining, as shown by the IgE^{hi} cell population. These IgE^{hi} cells also demonstrated increased expression of CD63, in comparison to the IgE stripped cells, following stimulation with 10 µg/ml of roasted peanut extract (Figure 7.4b). Minimal CD63 expression was detected in the absence of allergen stimulation. Thus, it can be seen that IgE stripped basophils can be resensitised with heterologous sera.

The resensitisation of IgE stripped basophils using affinity-purified peanut-specific antibodies was also conducted at 37°C for 1 hour. Basophil activation (expressed as the number of $CD63^{+}$ cells) could be detected following stimulation with roasted peanut extract (Figure 7.5b). This was not obtained with the IgE stripped cells. Minimal basophil activation also occurred in the absence of antigen stimulation. Consequently, these data demonstrate that IgE stripped basophils can be resensitised with affinity-purified antibodies. Due to difficulties in distinguishing the IgE^{hi} cell population from

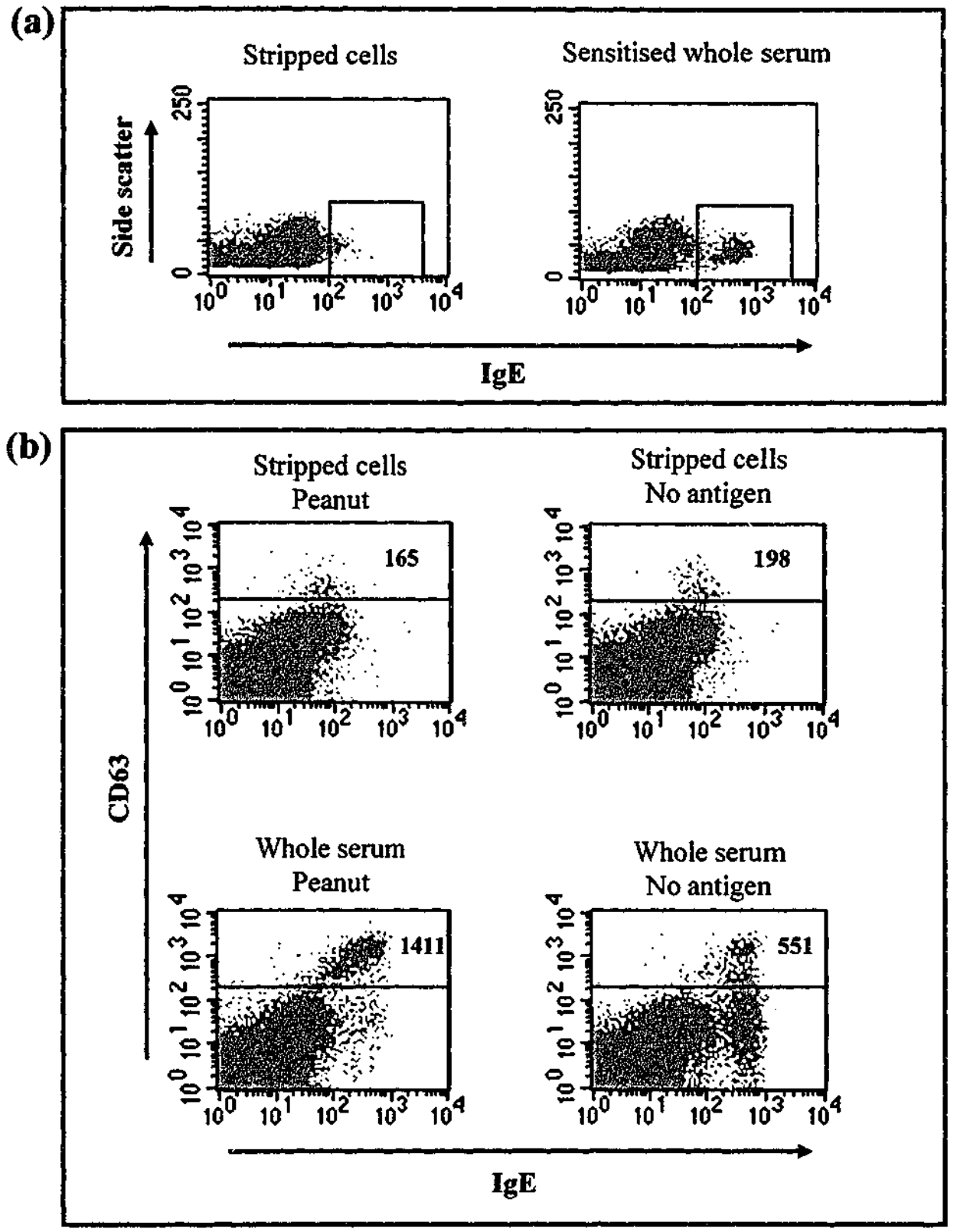


Figure 7.4 Resensitisation of IgE stripped basophils with peanut allergic serum

IgE stripped cells from a HDM allergic, non-peanut/tree nut allergic subject (NA4) were incubated with peanut allergic serum. **(a)** Stripped and resensitised cells were stained with anti-IgE. **(b)** The number of $CD63^+$ cells (upper quadrant) was analysed following stimulation with roasted peanut extract (10 μg/ml). A no antigen control was included to measure background basophil activation. $CD63^+$ cells were gated based on the discrimination of negative and positive control staining.

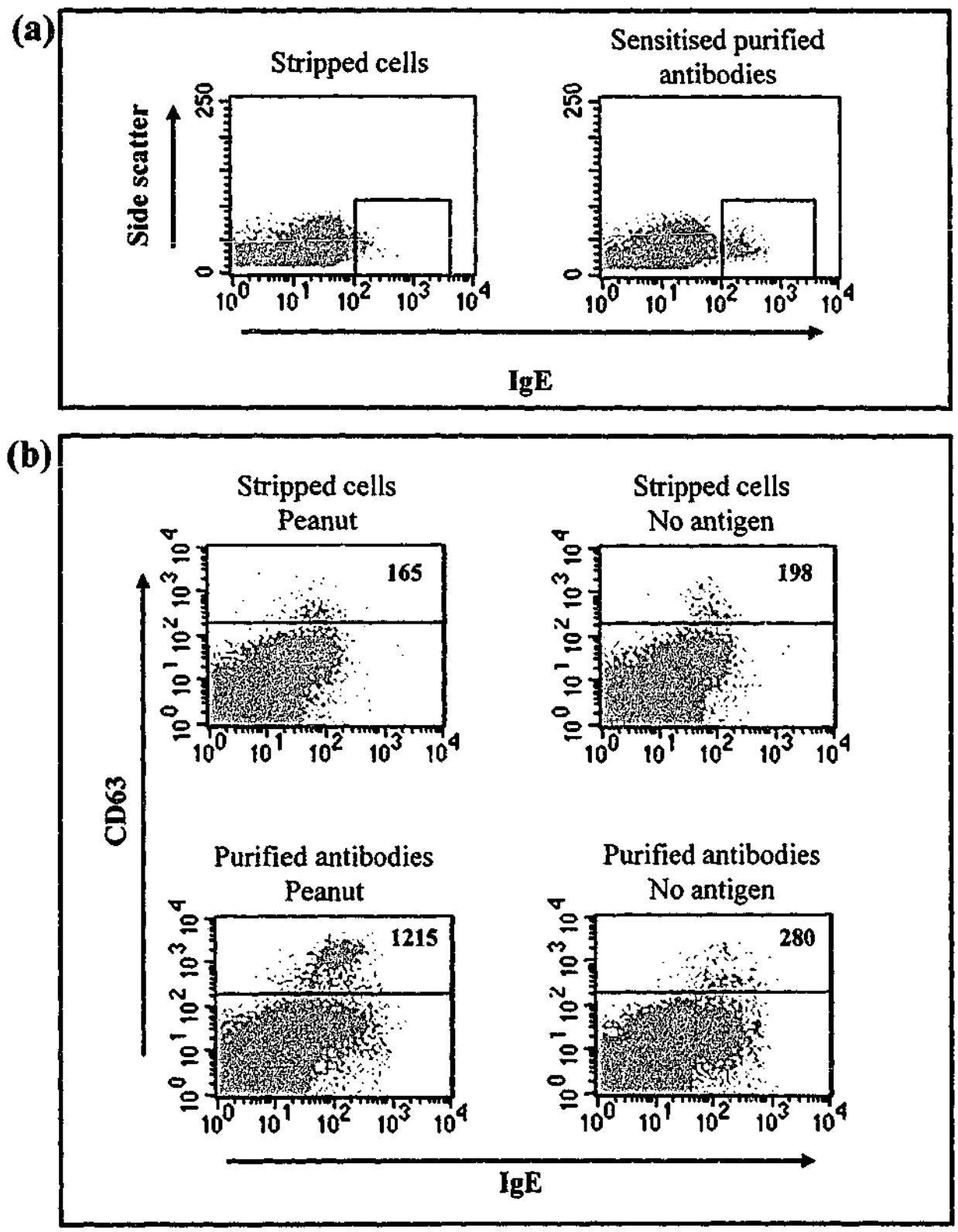


Figure 7.5 Resensitisation of IgE stripped basophils with affinity-purified peanut-specific antibodies

IgE stripped cells from a HDM allergic, non-peanut/tree nut allergic subject (NA4) were incubated with purified peanut-specific antibodies. **(a)** Stripped and resensitised cells were stained with anti-IgE. **(b)** The number of $CD63^+$ cells (upper quadrant) was analysed following stimulation with roasted peanut extract (10 µg/ml). A no antigen control was included to measure background basophil activation. $CD63^+$ cells were gated based on the discrimination of negative and positive control staining.

other IgE-staining cell populations when resensitising cells with purified antibodies, basophil activation was expressed as the number of $CD63^+$ cells.

7.3.4 Biological activity of peanut-specific IgE antibodies

7.3.4.1 Measurement of non-specific basophil activation by peanut and tree nut extracts

The ability of the peanut and tree nut extracts to non-specifically activate resensitised basophils was initially assessed. In this assay, rHev b 6.01-specific antibodies purified from latex allergic, non-peanut allergic subject serum (NA17) were used to resensitise donor basophils followed by stimulation with increasing concentrations of peanut and tree nut extracts. The specificity of the purified antibodies was initially assessed by ELISA. As shown in Figure 7.6, whole serum from subject NA17 had high IgE reactivity to rHev b 6.01 and RGP extract with minimal reactivity to HDM extract. Following affinity purification, IgE reactivity to rHev b 6.01 was maintained while IgE reactivity to HDM and RGP extracts was negligible.

These rHev b 6.01-specific antibodies were subsequently used to resensitise donor basophils. Donor cells for this experiment were obtained from an atopic, non-peanut/tree nut allergic, non-latex allergic subject (NA4) and were stripped of surface IgE. As shown in Figure 7.7a, minimal numbers of activated basophils were obtained when IgE stripped cells were stimulated with 10 µg/ml of rHev b 6.01, latex-glove, peanut and tree nut extracts, levels which were similar to the no antigen control. In contrast, stimulation with the positive controls, anti-IgE and fMLP, resulted in high numbers of activated basophils, demonstrating that these cells were functional and viable. Following resensitisation of IgE stripped cells with rHev b 6.01-specific

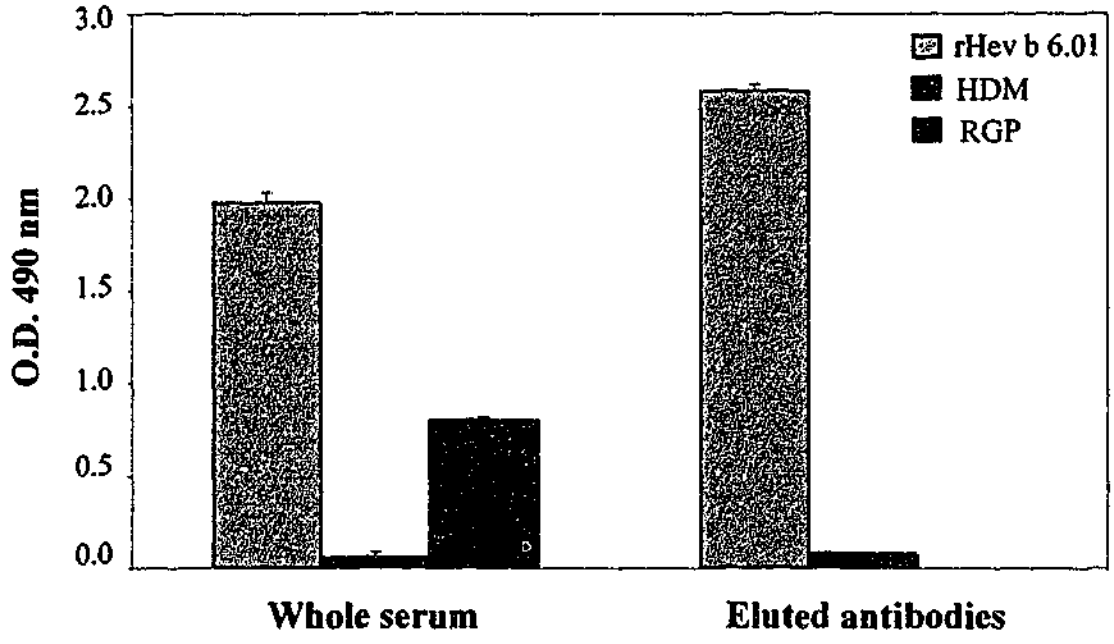


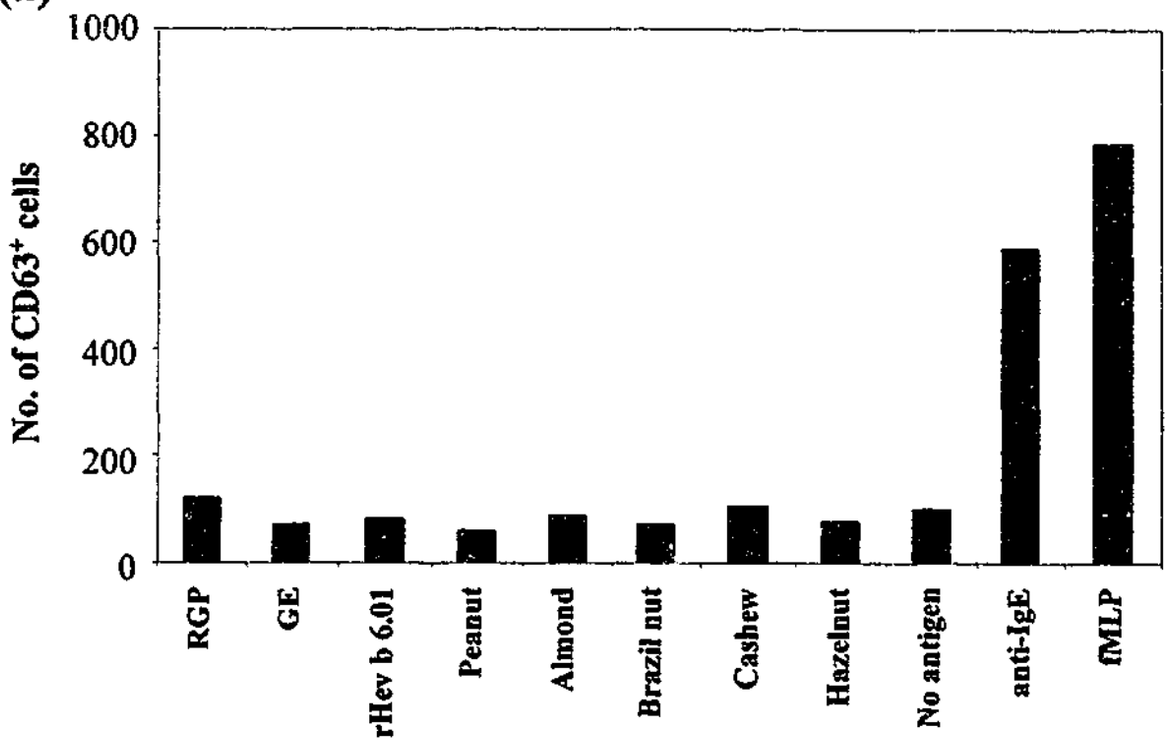
Figure 7.6 Specificity of affinity purified anti-rHev b 6.01 antibodies

rHev b 6.01-specific antibodies were purified from latex and rye grass pollen allergic (RGP) but non-peanut/tree nut allergic serum (subject NA17) and specificity of IgE antibodies was assessed. ELISA plates were coated with 1 μg/ml of rHev b 6.01, house dust mite (HDM) and rye grass pollen (RGP) extracts. IgE binding using whole serum (diluted 1/10) was compared with affinity purified anti-rHev b 6.01 antibodies (neat; equivalent to 1/10 dilution of whole serum). The absorbance in control wells containing no antigen was subtracted from antigen coated wells. Mean values for triplicates are shown and the standard deviation is indicated by the error bars.

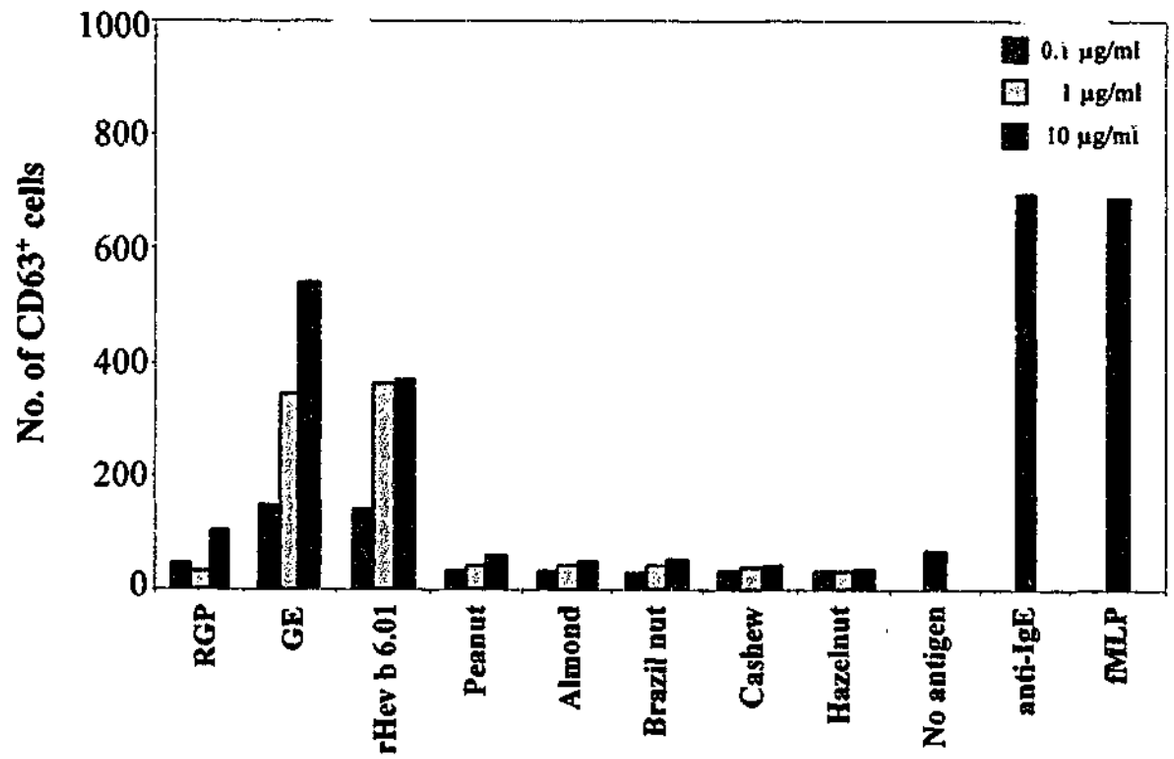
Figure 7.7 Non-specific activation of basophils by peanut and tree nut extracts

Partially purified basophils from an atopic, non-peanut/tree nut allergic, non-latex allergic donor (subject NA4) were stripped of surface IgE and resensitised with rHev b 6.01-specific antibodies purified from latex allergic, non-peanut/tree nut allergic subject serum (NA17). Cells were stimulated with roasted peanut, roasted almond, raw Brazil, roasted cashew and roasted hazelnut extracts and the number of cells expressing CD63 was analysed. RGP extract was included as a negative control and positive controls were rHev b 6.01, latex glove extract (GE), anti-IgE and fMLP stimulation. A no antigen control was used to measure background activation. **(a)** IgE stripped cells from subject NA4 stimulated with allergen extracts (10 µg/ml). **(b)** IgE stripped cells from subject NA4 resensitised with rHev b 6.01-specific antibodies from subject NA17 and stimulated with allergen extracts.

(a)


No. of CD63+ cells
1000
800
600
400
200
0
RGP
GE
rHev b 6.01
Peanut
Almond
Brazil nut
Cashew
Hazelnut
No antigen
anti-IgE
fMLP

(b)


No. of CD63+ cells
1000
800
600
400
200
0
0.1 µg/ml
1 µg/ml
10 µg/ml
RGP
GE
rHev b 6.01
Peanut
Almond
Brazil nut
Cashew
Hazelnut
No antigen
anti-IgE
fMLP

antibodies, stimulation with 0.1, 1 and 10 μg/ml of roasted peanut, roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extracts produced minimal numbers of activated basophils in comparison to the rHev b 6.01 and latex glove extract positive controls (Figure 7.7b). These data demonstrate that the peanut and tree nut extracts used in this study do not non-specifically activate resensitised basophils.

7.3.4.2 Measurement of the biological activity of peanut-specific IgE antibodies

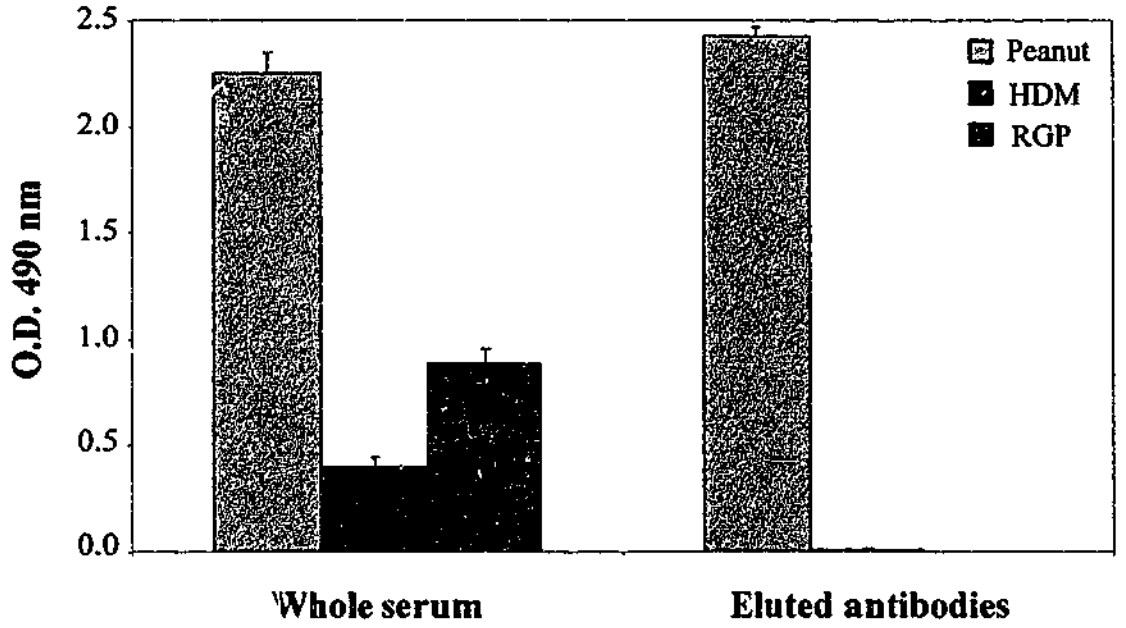
Peanut-specific IgE antibodies have been previously shown to cross-react with the tree nuts almond, Brazil nut and hazelnut (see Section 6.3.1). In this study, the biological activity of cross-reactive anti-peanut antibodies was assessed using the stripped basophil activation test. Peanut-specific antibodies were purified following incubation of roasted peanut extract immobilised on ELISA plates with sera from 2 peanut and tree nut allergic subjects (A8 and A9) that were previously shown to have anti-peanut IgE antibodies that cross-reacted with tree nut proteins (see Figure 6.2). Sera from other peanut allergic subjects were also used to purify peanut-specific antibodies but insufficient amounts for resensitisation were obtained. The specificity of the antibodies purified from these two subjects is shown in Figure 7.8. It can be seen that whole serum from subjects A8 and A9 exhibited IgE reactivity to roasted peanut, HDM and RGP extracts. Following affinity purification, strong IgE reactivity to roasted peanut extract was maintained but there were negligible levels of IgE reactivity to HDM and RGP extract.

To assess the biological activity of cross-reactive anti-peanut IgE antibodies, partially purified donor basophils stripped of surface IgE were sensitised with affinity-purified peanut-specific antibodies. Sensitised cells were challenged with different

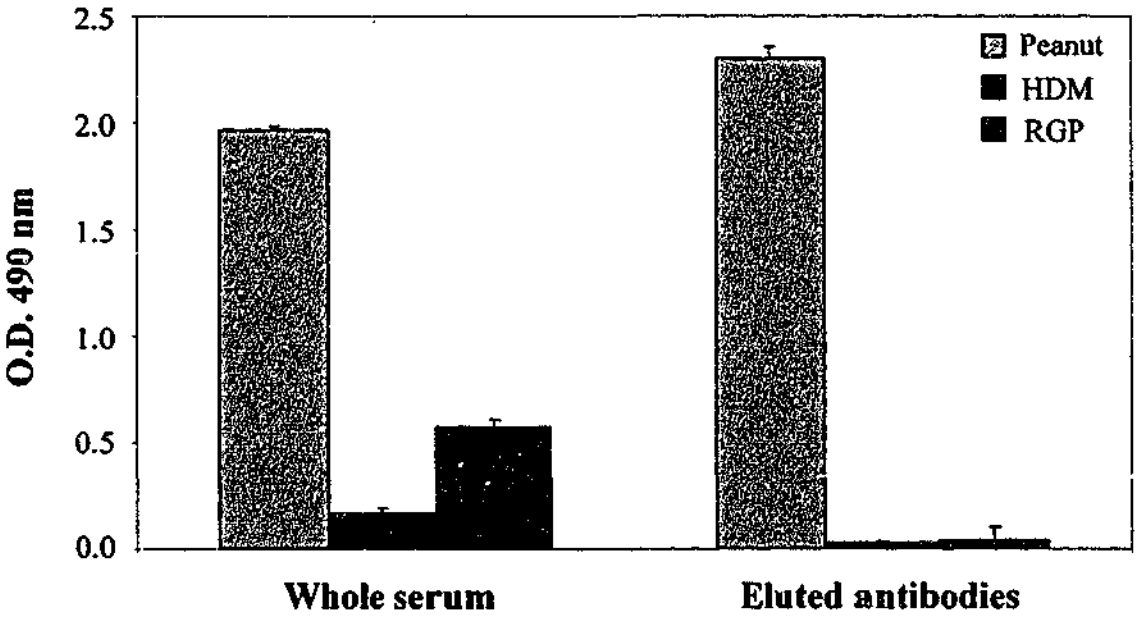
Figure 7.8 Specificity of affinity purified anti-peanut antibodies

Peanut-specific antibodies were purified from 2 peanut allergic subject sera (subjects A8 and A9) and specificity of IgE antibodies was assessed. ELISA plates were coated with 1 μg/ml of roasted peanut, house dust mite (HDM) and rye grass pollen (RGP) extracts. IgE binding using whole serum (diluted 1/10) was compared with affinity purified anti-peanut antibodies (neat; equivalent to 1/10 dilution of whole serum). The absorbance in control wells containing no antigen was subtracted from antigen coated wells. Mean values for triplicates are shown and the standard deviation is indicated by the error bars.

Subject A8



Subject A9



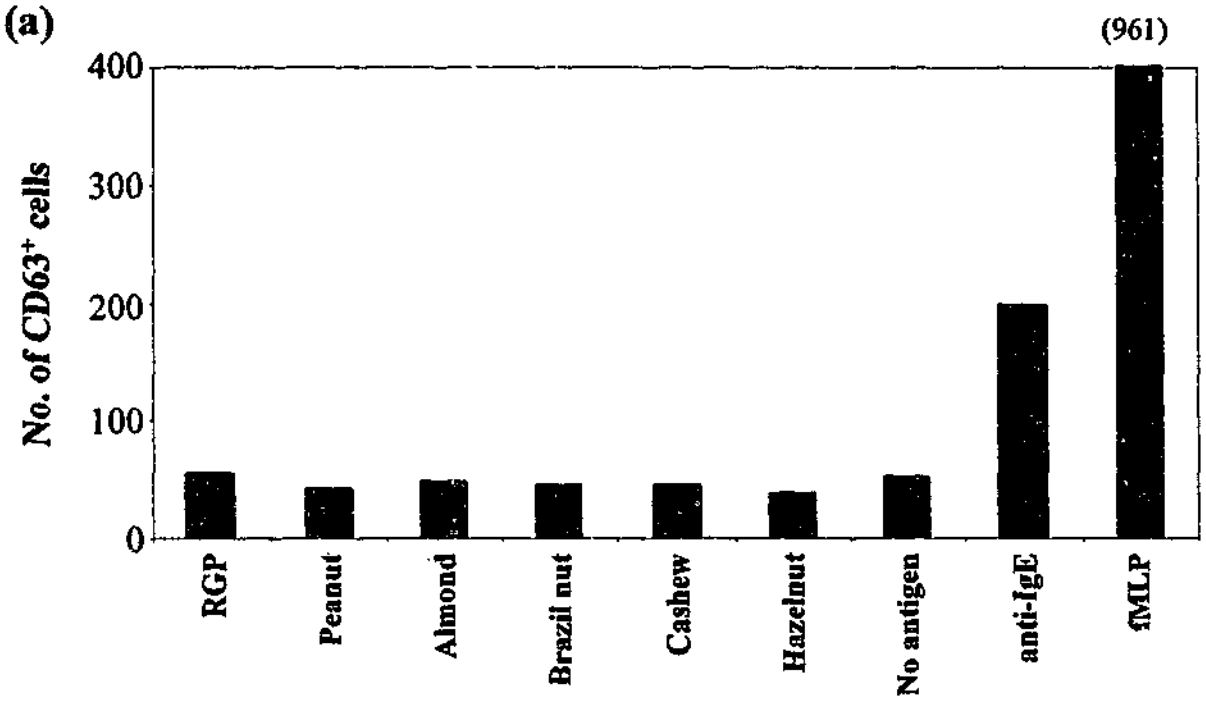
concentrations of roasted peanut, roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extracts and the number of activated basophils ($CD63^+$ cells) was determined. Based on previous optimisation experiments, resensitisation of basophils with IgE antibodies was performed at 37°C using undiluted purified antibodies. In this series of experiments, donor cells were obtained from 2 atopic, non-peanut/tree nut allergic subjects (NA15 and NA16). Initially, donor cells stripped of surface IgE were challenged with the peanut and tree nut extracts (at the highest allergen concentration of 10 µg/ml) to measure background basophil activation. As illustrated in Figures 7.9a and 7.10a, minimal numbers of basophils became activated following stimulation of IgE stripped cells from subjects NA15 and NA16 with peanut and tree nut extracts. In contrast, high numbers of activated basophils were obtained when stripped cells were stimulated with the positive controls, anti-IgE and fMLP, confirming the viability and functionality of these cells.

Following resensitisation with peanut-specific antibodies, cells were stimulated with roasted peanut, roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extracts at concentrations of 0.1, 1 and 10 µg/ml and the number of activated basophils was analysed. Stimulation of cells resensitised with peanut-specific antibodies from subject A8 serum with roasted almond and raw Brazil nut extract resulted in dose-dependent basophil activation that was higher than that obtained for the RGP and no antigen negative controls (Figure 7.9b). A higher concentration was required to obtain a similar level of basophil activation to roasted peanut extract. These data correlate with the previously reported observation that peanut-specific IgE antibodies from this subject cross-react with almond and Brazil nut proteins (see Section 6.3.1; Figure 7.9c) and suggest that these cross-reactive peanut-specific IgE antibodies are biologically active. Subject A8 also had a positive clinical history of sensitivity to Brazil nut as well as

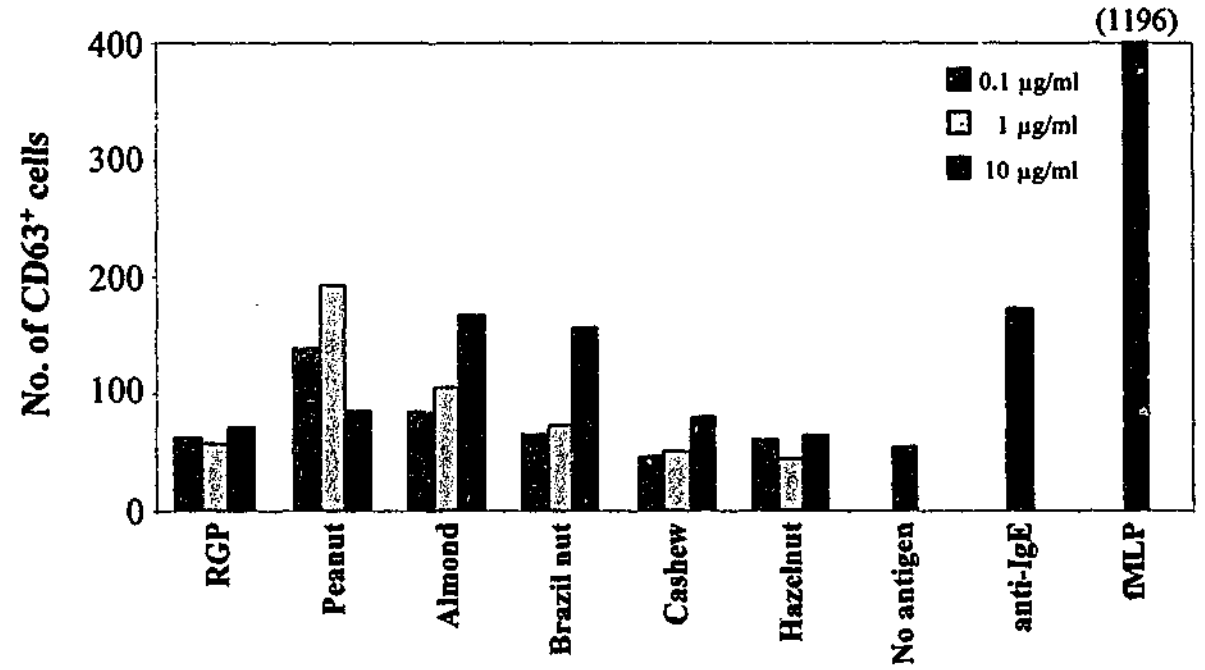
Figure 7.9 Analysis of the biological activity of cross-reactive peanut-specific IgE antibodies from subject A8

Partially purified basophils from an atopic, non-peanut/tree nut allergic donor (subject NA15) were stripped of surface IgE and resensitised with peanut-specific antibodies purified from peanut allergic subject A8 serum. Cells were stimulated with roasted peanut, roasted almond, raw Brazil, roasted cashew and roasted hazelnut extracts and the number of cells expressing CD63 was analysed. RGP extract was included as a negative control and positive controls were anti-IgE and fMLP stimulation. **(a)** IgE stripped cells from subject NA15 stimulated with allergen extracts (10 μg/ml). **(b)** IgE stripped cells from subject NA15 resensitised with peanut-specific antibodies from subject A8 and stimulated with allergen extracts. **(c)** Summary of clinical history, specific IgE, basophil activation and cross-reactivity results for peanut, almond, Brazil nut, cashew, hazelnut and RGP negative control for subject A8. **Legend:** NK – not known; ND – not done; NA – not applicable.

(a)



(b)



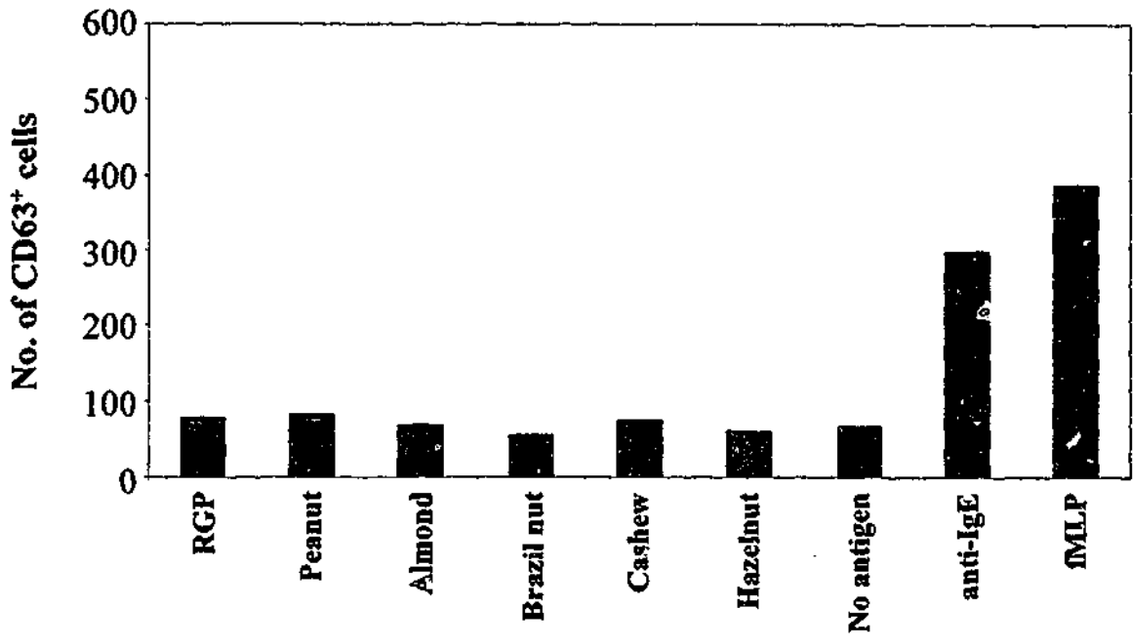
(c)

	RGP	Peanut	Almond	Brazil nut	Cashew	Hazelnut
Clinical history	+	+	NK	+	+	NK
Specific IgE (ELISA)	+	+	+	+	-	-
Basophil activation	ND	+	+	+	+	+
Cross-reactivity with peanut	NA	NA	+	+	-	+

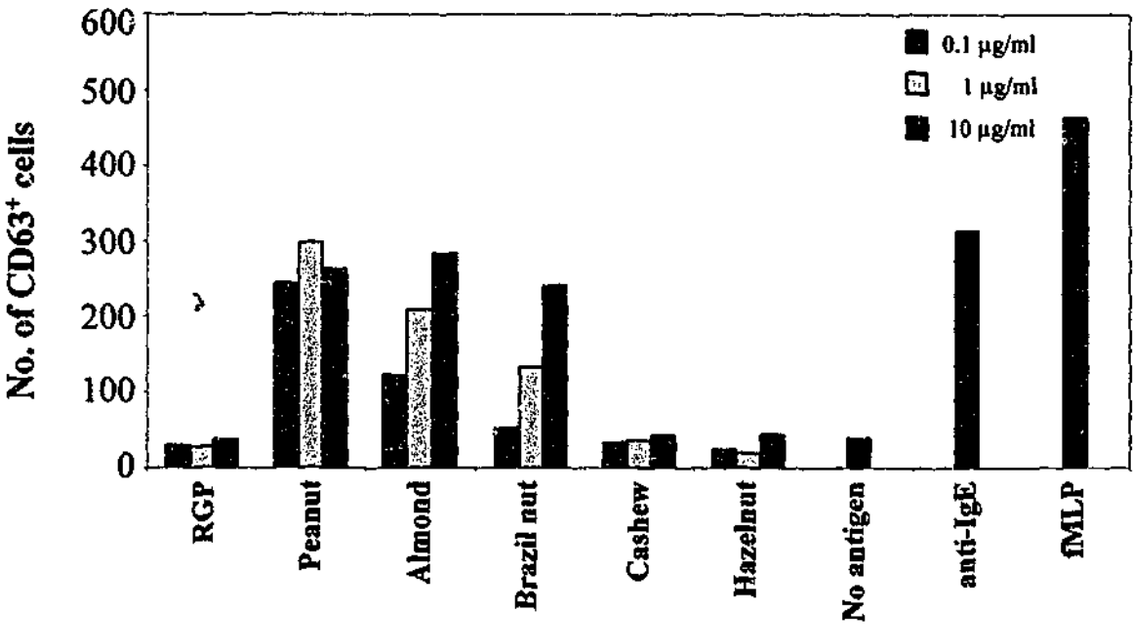
Figure 7.10 Analysis of the biological activity of cross-reactive peanut-specific IgE antibodies from subject A9

Partially purified basophils from an atopic, non-peanut/tree nut allergic subject (NA16) were stripped of surface IgE and resensitised with peanut-specific antibodies purified from peanut allergic subject A9 serum. Cells were stimulated with roasted peanut, roasted almond, raw Brazil, roasted cashew and roasted hazelnut extracts and the number of cells expressing CD63 was analysed. RGP extract was included as a negative control and positive controls were anti-IgE and fMLP stimulation. **(a)** IgE stripped cells from subject NA16 stimulated with allergen extracts (10 µg/ml). **(b)** IgE stripped cells from subject NA16 resensitised with peanut-specific antibodies from subject A9 and stimulated with allergen extracts. **(c)** Summary of clinical history, specific IgE, basophil activation and cross-reactivity results for peanut, almond, Brazil nut, cashew, hazelnut and RGP negative control for subject A9. **Legend:** NK – not known; ND – not done; NA – not applicable.

(a)



(b)



(c)

	RGP	Peanut	Almond	Brazil nut	Cashew	Hazelnut
Clinical history	NK	+	NK	NK	NK	NK
Specific IgE (ELISA)	+	+	+	+	-	+
Basophil activation	ND	+	+	+	-	+
Cross-reactivity with peanut	NA	NA	+	+	-	

positive specific IgE and positive basophil activation to both almond and Brazil nut (Figure 7.9c) which could be due to cross-reactive IgE antibodies for peanut, almond and Brazil nut allergens.

In contrast, negligible basophil activation was obtained when resensitised cells were stimulated with different concentrations of roasted hazelnut extract (Figure 7.9b). The level of activation was similar to the no antigen and RGP negative controls (Figure 7.9b), the latter included as a control for antibody specificity since whole serum from subject A8 was previously shown to contain IgE antibodies specific for this extract (see Figure 7.8). This finding may be due to the lower level of IgE cross-reactivity between peanut and hazelnut allergens in comparison to almond and Brazil nut allergens (see Section 6.3.1). The minimal number of activated basophils obtained with roasted hazelnut extract suggests that low-level cross-reactivity may not translate to positive basophil activation or biological activity. It also indicates that clinical sensitivity and positive basophil activation to hazelnut in subject A8 (Figure 7.9c) may be due to IgE antibodies with unique specificity for hazelnut allergens. Negligible levels of activated basophils were also obtained with cashew extract even though subject A8 had a positive clinical history and positive basophil activation to this tree nut. This confirms the absence of IgE cross-reactivity between peanut and cashew (see Section 6.3.1) and also serves as an additional control for the specificity of this assay.

Similarly, cells resensitised with purified peanut-specific antibodies from subject A9 and subsequently stimulated with different concentrations of roasted almond and raw Brazil nut extract resulted in dose-dependent basophil activation that was higher than the RGP and no antigen negative controls (Figure 7.10b). Again, a higher concentration was required to obtain a similar level of basophil activation to roasted peanut extract.

This confirms the previous observation that peanut-specific IgE antibodies from subject A9 cross-react with allergens in almond and Brazil nut (see Section 6.3.1; Figure 7.10c). Subject A9 also had positive specific IgE and positive basophil activation to almond and Brazil nut, thus confirming sensitisation to these tree nuts which may be partly due to cross-reactive peanut-specific IgE antibodies.

As observed in the previous experiment, negligible basophil activation was obtained when resensitised cells were stimulated with roasted hazelnut extract (Figure 7.10b) even though peanut-specific IgE antibodies from subject A9 previously demonstrated low-level cross-reactivity with hazelnut extract (see Section 6.3.1; Figure 7.10c). The level of activation was similar to the no antigen and RGP negative controls, the latter included as a control for antibody specificity since whole serum from subject A9 was previously shown to contain IgE antibodies specific for this extract (see Figure 7.9). This finding further suggests that low-level cross-reactive IgE antibodies may not be biologically active. Therefore, clinical sensitivity, positive specific IgE and positive basophil activation to hazelnut in subject A9 (Figure 7.10c) is likely to be due to hazelnut-specific IgE antibodies. Negligible levels of activated basophils were also obtained with cashew extract (Figure 7.10b) which confirms the absence of specific IgE and negative basophil activation to cashew in this subject (Figure 7.10c) and also serves as an additional control for the specificity of this assay.

7.3.5 Biological activity of cross-reactive allergen-specific IgE antibodies

7.3.5.1 Measurement of non-specific basophil activation by rAra h 1 and rAra h 2

Prior to investigating the biological activity of cross-reactive rAra h 1 and rAra h 2-specific IgE antibodies, a non-specific stripped basophil activation test was established

to determine whether these recombinant allergens can non-specifically activate resensitised basophils. This experiment was similar to that described in Section 7.3.4.1 except that resensitised cells were stimulated with different concentrations of rAra h 1 and rAra h 2. As shown in Figure 7.11b, stimulation of cells resensitised with rHev b 6.01-specific antibodies with latex-glove extract and rHev b 6.01 produced high numbers of activated basophils compared to the no antigen negative control. These activated basophils were not present prior to resensitisation (Figure 7.11a) demonstrating the specificity of the observed basophil activation. In contrast, minimal numbers of activated basophils were obtained following stimulation with rAra h 1 and rAra h 2, demonstrating negligible non-specific basophil activation by these recombinant allergen preparations.

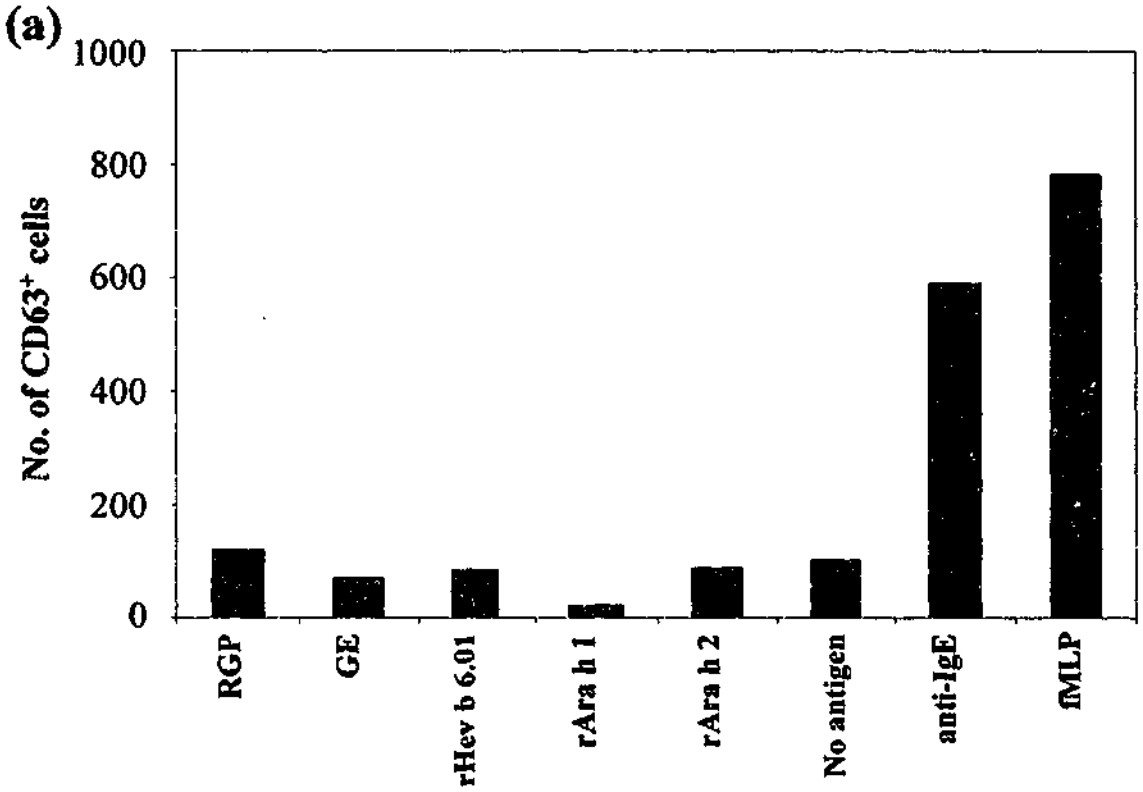
7.3.5.2 Measurement of the biological activity of cross-reactive rAra h 1-specific IgE antibodies

In the previous chapter, cross-reactivity studies using rAra h 1 demonstrated low-level IgE cross-reactivity between this peanut allergen and almond allergens (<50% inhibition of IgE binding to rAra h 1 at the highest inhibitor concentration of almond). In the current study, the biological activity of cross-reactive rAra h 1-specific IgE antibodies was assessed using the stripped basophil activation assay. Different peanut allergic subject sera were used to purify anti-rAra h 1 antibodies but sufficient amounts for resensitisation were obtained from only one subject, namely subject A9, which was previously demonstrated in Section 6.3.2.3. The specificity of the purified anti-rAra h 1 antibodies used in this study was also tested and was similar to that depicted in Figure 6.5.

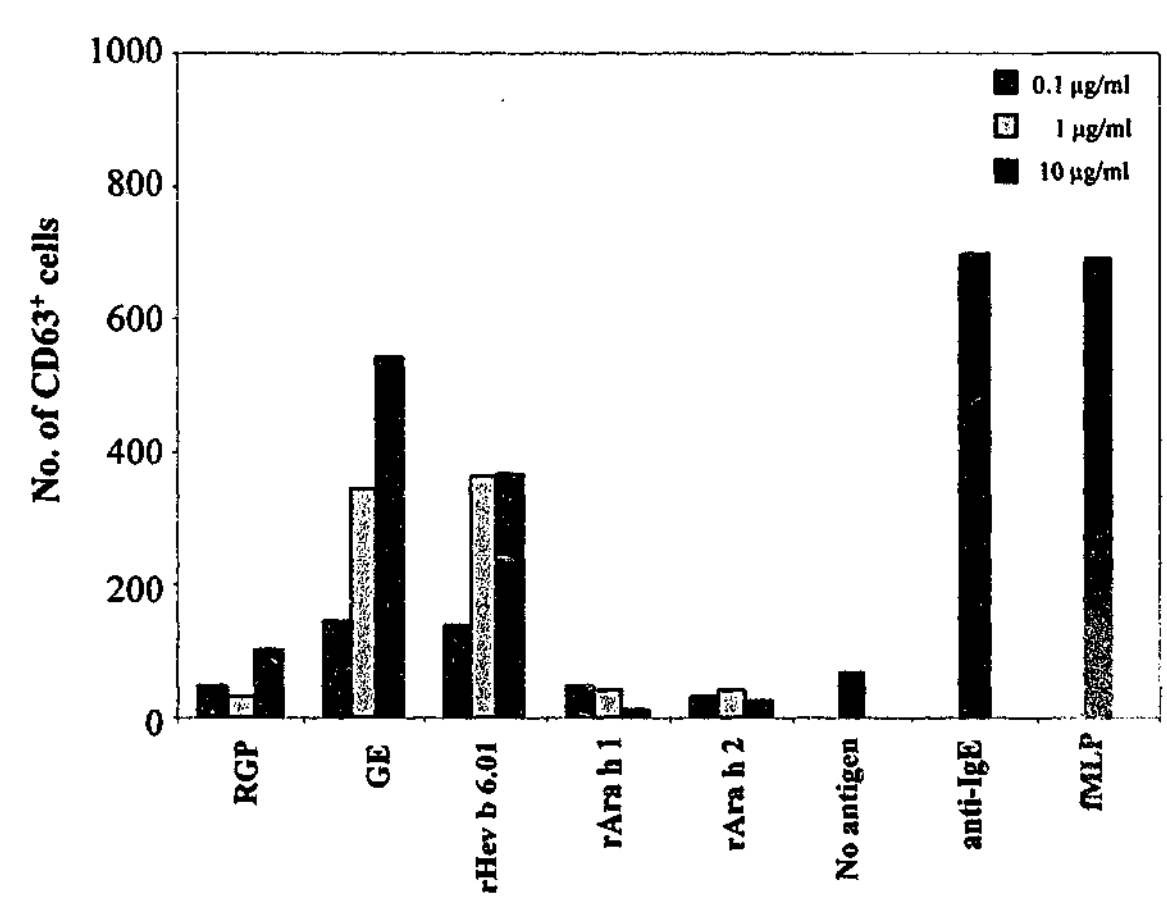
Figure 7.11 Non-specific activation of basophils by rAra h 1 and rAra h 2

Partially purified basophils from an atopic, non-peanut/tree nut allergic, non-latex allergic donor (subject NA4) were stripped of surface IgE and resensitised with rHev b 6.01-specific antibodies purified from a latex allergic, non-peanut/tree nut allergic subject (NA17). Cells were stimulated with rAra h 1 and rAra h 2 and the number of cells expressing CD63 was analysed. RGP extract was included as a negative control and positive controls were rHev b 6.01, latex glove extract anti-IgE and fMLP stimulation. A no antigen control was used to measure background activation. **(a)** IgE stripped cells from subject NA4 stimulated with allergen extracts (10 μg/ml). **(b)** IgE stripped cells from subject NA4 resensitised with rHev b 6.01-specific antibodies from subject NA17 and stimulated with allergen extracts.

(a)


No. of CD63+ cells
1000
800
600
400
200
0
RGP
GE
rHev b 6.01
rAra h 1
rAra h 2
No antigen
anti-IgE
fMLP

(b)


No. of CD63+ cells
1000
800
600
400
200
0
0.1 µg/ml
1 µg/ml
10 µg/ml
RGP
GE
rHev b 6.01
rAra h 1
rAra h 2
No antigen
anti-IgE
fMLP

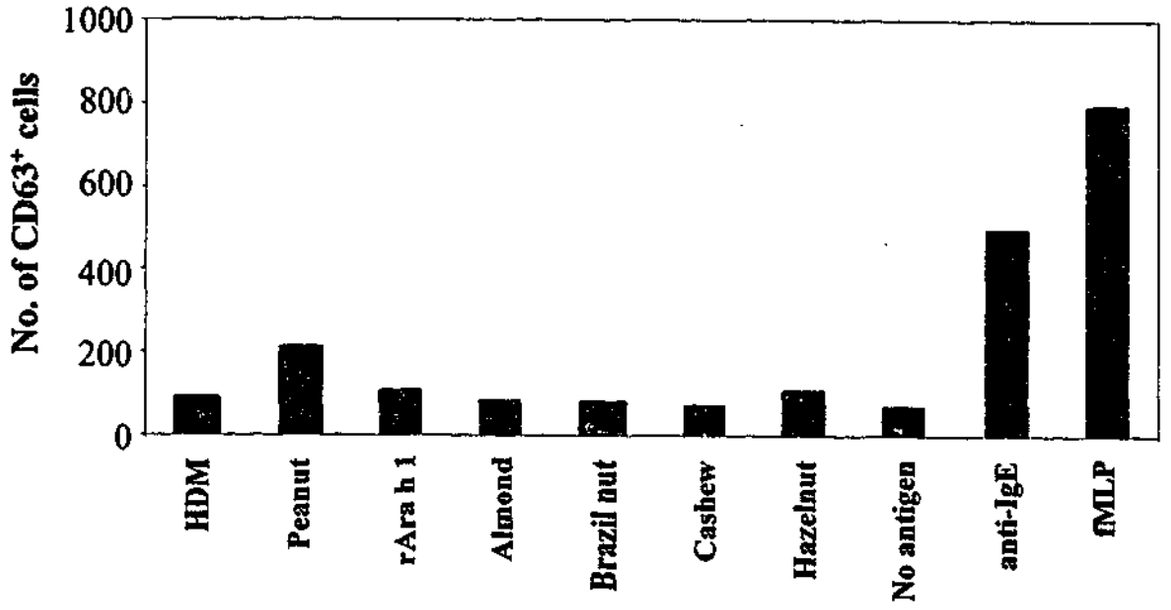
For this experiment, partially purified donor basophils were again obtained from an atopic, non-peanut/tree nut allergic subject (NA1) and surface IgE was removed. Minimal basophil activation (in comparison to the anti-IgE and fMLP positive controls) was obtained when IgE stripped cells were stimulated with rAra h 1 and peanut and tree nut extracts at the highest concentration of 10 µg/ml (Figure 7.12a). However, following resensitisation of cells with affinity-purified rAra h 1-specific antibodies, high numbers of activated basophils were obtained upon stimulation with different concentrations of rAra h 1 in comparison to the HDM and no antigen negative controls (Figure 7.12b). HDM was included as a control for antibody specificity as whole serum from subject A9 was previously shown to contain IgE antibodies specific for this extract (see Figure 6.5). Dose-dependent basophil activation was also obtained with the roasted peanut positive control extract, further confirming the specificity of the purified antibodies.

Stimulation of resensitised cells with different concentrations of roasted almond extract resulted in dose-dependent basophil activation. The level of activation obtained was much less than that with rAra h 1 and roasted peanut extract but greater than the HDM negative control extract (Figure 7.12b). This result suggests that IgE antibodies contributing to the low-level cross-reactivity between rAra h 1 and almond allergens may have low biological activity. The number of activated basophils upon stimulation with raw Brazil nut extract was also similar to that obtained with roasted almond extract. Previous studies did not demonstrate any significant cross-reactivity between rAra h 1 and Brazil nut (see Sections 6.3.2.2 and 6.3.2.3) and therefore the observed basophil activation may be due to the sensitivity of this assay. Minimal basophil activation was observed with roasted hazelnut extract, confirming the previously reported observation that rAra h 1 does not cross-react with hazelnut allergens (see

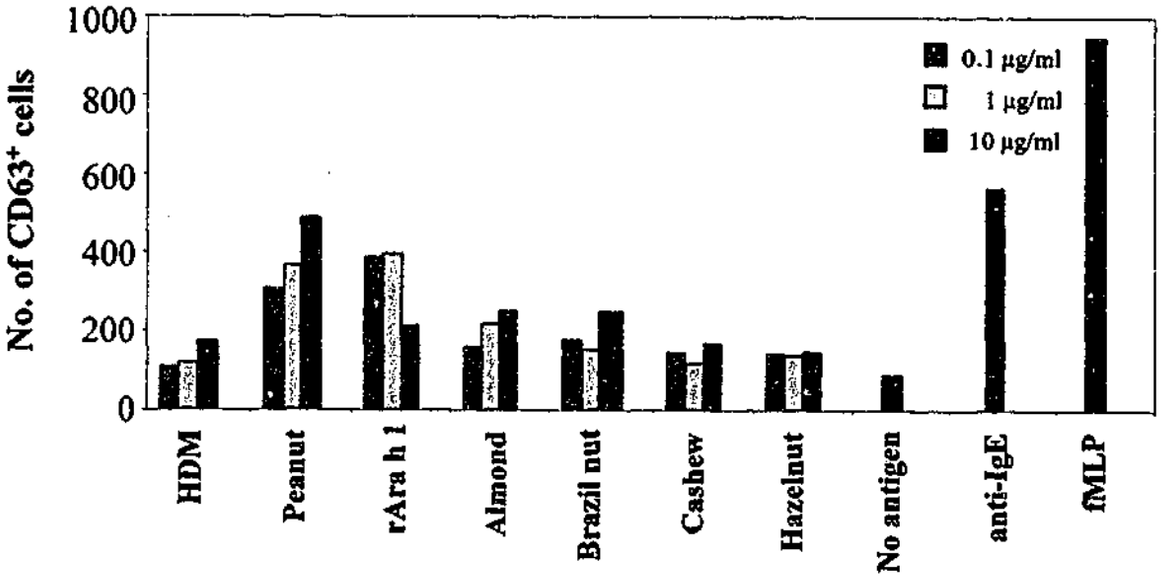
Figure 7.12 Analysis of the biological activity of cross-reactive rAra h 1-specific IgE antibodies

Partially purified basophils from an atopic, non-peanut/tree nut allergic donor (subject NA1) were stripped of surface IgE and resensitised with rAra h 1-specific antibodies purified from peanut allergic subject A9 serum. Cells were stimulated with roasted peanut, roasted almond, raw Brazil, roasted cashew and roasted hazelnut extracts and the number of cells expressing CD63 was analysed. HDM extract was included as a negative control and positive controls were rAra h 1, anti-IgE and fMLP stimulation. A no antigen control was used to measure background basophil activation. **(a)** IgE stripped cells from subject NA1 stimulated with allergen extracts (10 μg/ml). **(b)** IgE stripped cells from subject NA1 resensitised with rAra h 1-specific antibodies from subject A9 and stimulated with allergen extracts. **(c)** Summary of clinical history, specific IgE, basophil activation and cross-reactivity results for rAra h 1, peanut, almond, Brazil nut, cashew, hazelnut and HDM negative control for subject A9. **Legend:** NK – not known; ND – not done; NA – not applicable.

(a)



(b)



(c)

	HDM	Peanut	rAra h 1	Almond	Brazil nut	Cashew	Hazelnut
Clinical history	+	+	NA	NK	NK	NK	NK
Specific IgE (ELISA)	+	+	+	+	+	-	+
Basophil activation	ND	+	+	+	+	-	+
Cross-reactivity with peanut	NA	NA	NA	+	+	-	+

Sections 6.3.2.2 and 6.3.2.3). Similar results were also obtained with roasted cashew extract which correlates with the absence of specific IgE and positive basophil activation to cashew in subject A9 (Figure 7.12c).

7.3.5.3 Measurement of the biological activity of cross-reactive rAra h 2-specific IgE antibodies

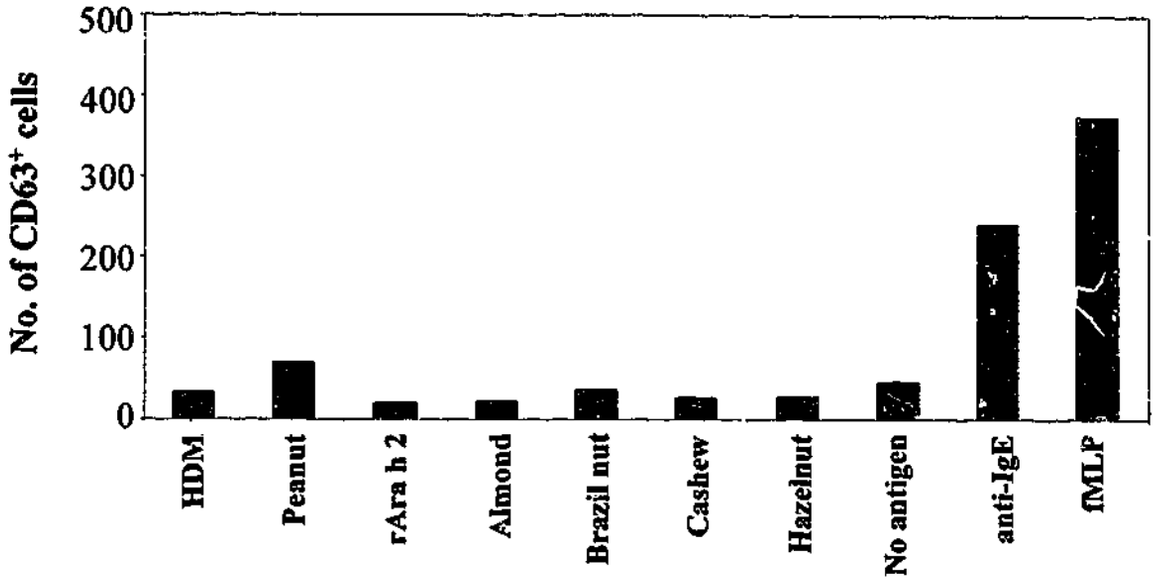
Similar to Ara h 1, the contribution of the major peanut allergen Ara h 2 to the observed IgE cross-reactivity between peanut and tree nuts was investigated in the previous chapter. Using rAra h 2, IgE cross-reactivity was observed between this peanut allergen and allergens present in almond and Brazil nut (Sections 6.3.2.4 and 6.3.2.5). As a consequence, the biological activity of cross-reactive rAra h 2-specific IgE antibodies was assessed. Anti-rAra h 2 antibodies were purified from subject A9 serum as reported previously (Section 6.3.2.5) and the specificity of these antibodies was similar to that shown in Figure 6.8. Again, other peanut allergic subject sera were used to purify rAra h 2-specific antibodies but the amounts obtained were insufficient for the resensitisation of basophils.

Donor cells for this experiment were obtained from an atopic, non-peanut/tree nut allergic subject (NA16) and stripped of surface IgE. As shown in Figure 7.13a, stimulation of IgE stripped cells with 10 μg/ml of rAra h 2, peanut and tree nut extracts resulted in negligible basophil activation, with the number of $CD63^+$ cells similar to the no antigen control. In contrast, anti-IgE and fMLP stimulation produced high numbers of activated basophils, again demonstrating the cells were functional and viable. Following resensitisation of cells with rAra h 2-specific antibodies, stimulation with increasing concentrations of rAra h 2 resulted in basophil activation that was greater

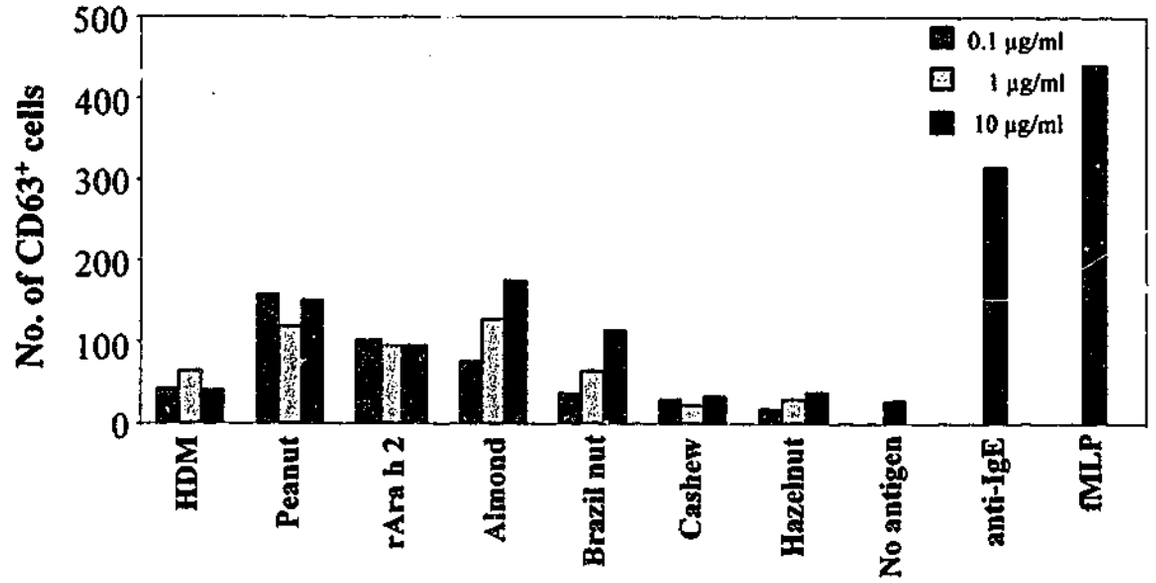
Figure 7.13 Analysis of the biological activity of cross-reactive rAra h 2-specific IgE antibodies

Partially purified basophils from an atopic, non-peanut/tree nut allergic donor (subject NA16) were stripped of surface IgE and resensitised with rAra h 2-specific antibodies purified from peanut allergic subject A9 serum. Cells were stimulated with roasted peanut, roasted almond, raw Brazil, roasted cashew and roasted hazelnut extracts and the number of cells expressing CD63 was analysed. HDM extract was included as a negative control and positive controls were rAra h 2, anti-IgE and fMLP stimulation. A no antigen control was used to measure background basophil activation. **(a)** IgE stripped cells from subject NA16 stimulated with allergen extracts (10 μg/ml). **(b)** IgE stripped cells from subject NA16 resensitised with rAra h 2-specific antibodies from subject A9 and stimulated with allergen extracts. **(c)** Summary of clinical history, specific IgE, basophil activation and cross-reactivity results for rAra h 2, peanut, almond, Brazil nut, cashew, hazelnut and HDM negative control for subject A9. **Legend:** NK – not known; ND – not done; NA – not applicable.

(a)



(b)



(c)

	HDM	Peanut	rAra h 2	Almond	Brazil nut	Cashew	Hazelnut
Clinical history	+	+	NA	NK	NK	NK	NK
Specific IgE (ELISA)	+	+	+	+	+	-	+
Basophil activation	ND	+	+	+	+	-	+
Cross-reactivity with peanut	NA	NA	NA	+	+	-	+

than the no antigen negative control (Figure 7.13b). Basophil activation was also achieved after stimulation of resensitised cells with the peanut extract positive control although the number of $CD63^+$ cells was slightly higher compared to rAra h 2. The lower efficiency of basophil activation obtained with rAra h 2 may be due to incorrect protein refolding or the tendency of this recombinant preparation to form multimers in solution (see Section 4.3.4.2). Nevertheless, the minimal basophil activation following stimulation with the HDM negative control extract (Figure 7.13b), which was included since subject A9 serum showed reactivity to this extract, suggests that the purified antibodies were highly specific to rAra h 2.

Stimulation of resensitised cells with increasing concentrations of roasted almond and raw Brazil nut extract also resulted in dose-dependent basophil activation with levels similar to that obtained with roasted peanut extract. This demonstrates that rAra h 2-specific IgE antibodies are biologically active upon stimulation with cross-reactive allergens from almond and Brazil nut and may contribute to the sensitisation of subject A9 to these tree nuts as indicated by positive specific IgE and positive basophil activation (Figure 7.13c). It also confirms the observed cross-reactivity between rAra h 2 and these tree nuts (see Section 6.3.2.4; Figure 7.13c). Negligible basophil activation was obtained with roasted hazelnut extract which correlates with the absence of cross-reactivity between rAra h 2 and this tree nut even though specific IgE and positive basophil activation to hazelnut was observed with subject A9 (Figure 7.13c). Similar results were also obtained with roasted cashew extract which confirmed the absence of specific IgE and negative basophil activation to cashew in this subject (Figure 7.13c).

7.4 DISCUSSION

The clinical observation that many peanut allergic individuals are sensitive to at least one tree nut type led to this investigation of IgE cross-reactivity between peanut and tree nuts. As outlined in the previous chapter using ELISA, peanut-specific IgE antibodies in peanut allergic subject sera cross-react with allergens present in almond, Brazil nut and hazelnut with the peanut allergens, Ara h 1, Ara h 2 and Ara h 3 contributing to this observed cross-reactivity. However, it was unclear whether these cross-reactive IgE antibodies were biologically active, that is, whether they are involved in effector cell activation upon allergen exposure. It was important to establish this as the IgE-binding assays utilised in the previous chapter measured cross-reactivity using serum IgE antibodies rather than those present on effector cells such as basophils. Therefore it is not clear whether these cross-reactive antibodies are actually involved in a Type I hypersensitivity reaction. It has also been reported that some IgE-reactive allergen molecules are weak inducers of effector cell activation as a consequence of epitope orientation which can prevent cross-linking of surface IgE (Valenta and Kraft, 2001). Therefore, cross-reactive tree nut allergens that bind peanut-specific IgE antibodies may not necessarily be able to mediate cross-linking of effector-cell bound IgE antibodies. Consequently, an *in vitro* assay, the stripped basophil activation test, was established to measure the biological activity of peanut-specific IgE antibodies which was then used to confirm the ability of cross-reactive tree nut allergens to cross-link basophil-bound peanut-specific IgE antibodies.

Using this assay, donor basophils resensitised with anti-peanut IgE antibodies became activated following stimulation with almond and Brazil nut extracts, demonstrating that these cross-reactive antibodies were biologically active. rAra h 2-specific IgE antibodies, which cross-reacted with almond and Brazil nut allergens, were also shown to be biologically active upon stimulation of resensitised basophils with almond and Brazil nut extracts. In contrast, rAra h 1-specific antibodies, which had low-level cross-reactivity with almond allergens, had minimal biological activity, as indicated by the low numbers of activated basophils, following stimulation of resensitised basophils with almond extract. More subjects are, however, required to confirm these observations since this study was limited to two subjects due to difficulties with purifying IgE antibodies from other subject sera. Further experiments using sera from peanut and cashew allergic subjects should also be conducted to confirm the absence of cross-reactivity that was reported in the previous chapter. Nevertheless, the results from this chapter indicate that cross-reactive peanut-specific IgE antibodies may be involved in effector cell activation upon exposure to some tree nut allergens.

The stripped basophil activation assay is a useful assay to ascertain the biological activity of antibodies although past studies have measured histamine release rather than CD63 expression as a function of biological activity (Kleine Budde *et al.*, 2000, Foetisch *et al.*, 2003). This assay has also been previously used to confirm the biological activity of allergen preparations using subject sera (Iacovacci *et al.*, 2002) and can be used to complement serological tests. The main requirement of this type of assay is the availability of donor basophils which must be selected for their ability to become activated upon allergen stimulation. This is particularly important since it has been reported that some subjects have basophils which cannot release histamine by allergen or anti-igE stimulation due to a defect in the signalling pathway (Diamant and

Patkar, 1982, Nguyen *et al.*, 1990, Knol *et al.*, 1992). Such a problem can be overcome by selecting subjects known to be allergic to extracts other than those being tested, which in this study were HDM and RGP.

The ability to purify sufficient amounts of allergen-specific antibodies from subject sera can also be a limitation for this type of assay. From experience, some allergen-specific IgE antibodies have such a high affinity for the allergen that these cannot be purified by low pH elution. In this study, it was possible to purify a moderate amount of peanut-specific IgE antibodies from numerous subject sera but, except in two cases, it was not enough to resensitise basophils and obtain a detectable level of activation following allergen stimulation. In addition, this assay uses purified PBMCs which consist of B cells and monocytes as well as basophils. B cells and monocytes express a low affinity IgE receptor (FcεRII) which can bind IgE antibodies and thus can reduce the amount of purified antibody that can bind to the high affinity IgE receptor (FcεRI) present on basophils. Therefore, the amount of purified antibody plays a crucial role in the successful application of this assay for investigating the biological activity of cross-reactive, allergen-specific IgE antibodies.

The level of cross-reactivity between peanut and tree nut allergens appears to correlate with the biological activity of the cross-reactive IgE antibodies. The number of activated basophils resensitised with peanut-specific antibodies was much higher with almond and Brazil nut in comparison to hazelnut. In previous inhibition assays, almond showed the highest level of cross-reactivity with peanut, followed by Brazil nut and hazelnut. Similarly, basophils resensitised with rAra h 2-specific antibodies became activated upon stimulation with almond and Brazil nut which have been previously shown to contain allergens that were highly cross-reactive with Ara h 2. In contrast, the

low-level cross-reactivity detected between Ara h 1 and almond allergens using inhibition assays was reflected by the minimal basophil activation obtained when basophils resensitised with rAra h 1-specific IgE antibodies were stimulated with almond extract. Thus it appears that a high level of cross-reactivity, as determined by inhibition assays, leads to increased biological activity of cross-reactive IgE antibodies.

This, however, is not surprising given that a high level of cross-reactivity is likely to be due to a high level of sequence homology between cross-reactive allergens which could potentially give rise to multiple cross-reactive IgE-binding epitopes or cross-reactive IgE antibodies. The presence of multiple cross-reactive epitopes would result in high epitope density and subsequent multivalent antigen-antibody interactions which is essential for IgE cross-linking and subsequent triggering of basophils. In contrast, low-level cross-reactivity, as a result of low level sequence homology between two allergens, may give rise to less cross-reactive IgE-binding epitopes, resulting in primarily monovalent antigen-antibody interactions which can be detected using IgE-binding assays but would not be enable efficient cross-linking of effector cell-bound IgE antibodies. Therefore, the biological activity of cross-reactive antibodies as measured by the ability to induce effector cell activation can provide some insight into the degree of sequence homology required between cross-reactive allergens.

Additional factors that may affect the biological activity of cross-reactive IgE antibodies include the abundance of cross-reactive allergens in the crude extract and the affinity of cross-reactive IgE antibodies for the cross-reactive allergen. These may be interrelated in that high antigen concentrations may be required to trigger basophils through low-affinity IgE antibody interactions. This may explain the inability of hazelnut extract to activate basophils sensitised with peanut-specific IgE antibodies even though low-level

cross-reactivity between peanut and hazelnut was detected using inhibition ELISAs. This, however, can be resolved by firstly identifying the cross-reactive allergen in crude extract and subsequently determining its relative abundance. Purified allergens obtained from natural extract or through recombinant protein expression can then be used in antibody affinity studies to determine the role this plays in allergenic cross-reactivity. Epitope mapping and crystallisation studies can also be used to establish whether epitope orientation and density can account for the discrepancy between allergenic cross-reactivity detected using IgE-binding assays and that measured by allergen-induced effector cell activation. This phenomenon has been proposed as a possible reason for the discrepancy between IgE binding and effector cell activation that has been reported for some allergens (Valenta and Kraft, 2001).

Although it was demonstrated in this study that circulating peanut-specific IgE antibodies that cross-reacted with almond and Brazil nut allergens were biologically significant, it is unclear whether these antibodies contribute to the manifestation of almond and Brazil nut allergy in peanut allergic individuals. *In vitro* activation of basophils by cross-reactive tree nut allergens may not necessarily translate to clinical relevance. Although the subjects involved in this study had specific IgE and positive basophil activation to almond and Brazil nut, oral challenges are still required to confirm that these peanut allergic subjects are clinically sensitive to these cross-reactive tree nuts. Even in the context of positive food challenges, it cannot be discounted that IgE antibodies specific for unique almond and Brazil nut epitopes are responsible for the clinical manifestation of this type of tree nut allergy. Therefore it is difficult to assess the exact nature of the contribution of cross-reactive IgE antibodies to peanut and tree nut co-sensitisation in allergic individuals. However, this study demonstrated that

cross-reactive peanut-specific IgE antibodies are biologically active and thus may be involved in the co-sensitisation of allergic individuals to peanut and tree nut allergens.

In summary, this chapter has described the establishment of the stripped basophil activation test as a tool to measure the biological activity of cross-reactive IgE antibodies. Using this assay, it was demonstrated that basophils resensitised with peanut-specific IgE antibodies became activated upon stimulation with almond and Brazil nut extracts which were previously shown to have a high level of IgE cross-reactivity with peanut. Similar results were obtained when basophils resensitised with rAra h 2-specific IgE antibodies were challenged with the same tree nut extracts. In contrast, IgE antibodies involved in low-level cross-reactivity between peanut and hazelnut allergens were not biologically active *in vitro*. A similar observation was also made with rAra h 1-specific IgE antibodies which exhibit low-level cross-reactivity with almond allergens. Thus it appears that the level of cross-reactivity between two allergens is likely to determine the biological activity of the cross-reactive IgE antibodies involved and therefore must be taken into consideration when assessing the immunological relevance of any observed cross-reactivity between different allergen sources.

CHAPTER 8

GENERAL DISCUSSION

8.1 INTRODUCTION

This project investigated allergenic B cell cross-reactivity between peanut and the tree nuts almond, Brazil nut, cashew and hazelnut. The role of the peanut allergens Ara h 1, Ara h 2 and Ara h 3 in this observed cross-reactivity was analysed and potential homologues in the cross-reacting tree nuts were identified. The ability of cross-reactive IgE antibodies to mediate effector cell functions was also assessed. This chapter discusses the mechanisms that may facilitate allergenic cross-reactivity and the clinical significance of the cross-reactive immune response. Finally, avenues for immunotherapy are examined to provide potential strategies for the effective treatment of peanut and tree nut allergy.

8.2 B CELL CROSS-REACTIVITY BETWEEN ALLERGENS FROM DIFFERENT SOURCES

Proteins are termed 'cross-reactive' when an antibody clonotype or T cell clone produced in response to one protein reacts with another related or, in some cases, unrelated protein. In the field of autoimmunity, this is referred to as 'molecular mimicry' which describes the cross-reactivity between antibodies or T cells with host

'self' antigens and microbial determinants from bacteria or viruses (Oldstone, 1998). This mechanism of cross-reactivity between unrelated proteins is postulated to be responsible for autoimmune diseases such as multiple sclerosis, rheumatic fever and insulin-dependent diabetes mellitus (IDDM) (Wucherpfennig and Strominger, 1995, Maclaren and Alkinson, 1997, Guilherme and Kalil, 2002). In allergy, B cell cross-reactivity refers to the ability of an IgE antibody, previously induced by one allergen, to react with another allergen. Allergenic cross-reactivity often reflects the taxonomic or phylogenetic relationship between different allergens. At the molecular level, this equates to high homology at the primary amino acid sequence level resulting in homologous tertiary structures. This suggests that cross-reactivity occurs between closely related allergen sources, as is the case with grass pollen allergens (Weber, 2003), but it has been known for some time that cross-reactivity can also occur between proteins from distantly related species. This thesis has provided evidence that B cell cross-reactive allergens are present in peanut and the tree nuts almond, Brazil nut and hazelnut. The peanut is distantly related to tree nuts as it is classified as a legume but it shares similar functions as seeds in plant development. Given this, it is not surprising that homologous proteins are present in peanuts and tree nuts, some of which are likely to contribute to the high incidence of peanut and tree nut co-sensitisation in allergic individuals.

A number of plant proteins widely distributed throughout the plant kingdom are classified into different protein families. It has been suggested that allergenic cross-reactivity between distantly related organisms is due to proteins that are conserved among many different plants. Examples of these include profilins, pathogenesis-related (PR) proteins, and lipid transfer proteins (LTP). Pollen-associated food allergy has been attributed to a group of proteins known as profilins which are actin-binding proteins

responsible for cytoskeleton formation in plant cells (Breiteneder and Ebner, 2000). The presence of profilin homologues in different plants forms the basis of serum IgE cross-reactivity between birch pollen and fruits and vegetables such as apple, pear, carrot, celery, potato and peach (Ebner *et al.*, 1995, Rodriguez-Perez *et al.*, 2003). Profilins are also thought to be responsible for sensitivity to hazelnuts in hazel pollen-sensitive individuals (Hirschwehr *et al.*, 1992).

Pathogenesis-related proteins, which are accumulated in higher-order plants in response to pathogenic infections, wounding or chemically-induced stress, also contribute to allergenic cross-reactivity (Breiteneder and Ebner, 2000). So far, plant-derived allergens have been identified with sequence similarity to PR-protein families 2, 3, 4, 5, 8, 10 and 14 (Hoffmann-Sommergruber, 2002) some of which are known to be cross-reactive. The basic β-1,3-glucanase isolated from latex, is similar to PR-2 proteins and is cross-reactive with proteins present in banana, potato and tomato (Yagami *et al.*, 1998). Class I chitinases, classified as PR-3 type proteins and characterised by an *N*-terminal hevein domain, are involved in the cross-sensitisation between latex and avocado (Chen *et al.*, 1998, Posch *et al.*, 1999). Oral allergy syndrome (OAS), an association of food allergies to fruits, nuts and vegetables in patients with pollen allergy, is due to allergens homologous to PR-10 type proteins, in particular those homologous to the major birch pollen allergen, Bet v 1. Bet v 1 homologous allergens have been identified in apple (Vanek-Krebitz *et al.*, 1995), celery (Breiteneder *et al.*, 1995, Hoffmann-Sommergruber *et al.*, 1999a) and carrot (Hoffmann-Sommergruber *et al.*, 1999b). More recently, lipid transfer proteins (LTPs), which are homologous to PR-14 type proteins, have also been identified as potential panallergens responsible for cross-reactivity between botanically unrelated plant-derived foods. The major allergen from maize has been shown to cross-react with both peach and rice LTP (Pastorello *et al.*,

2000b) while cross-reactivity has been demonstrated between peach LTP and proteins from walnut and peanut (Asero *et al.*, 2002).

Carbohydrate epitopes on different allergen sources have also been suggested to be involved in the production of cross-reactive IgE antibodies. Early studies demonstrated that the *N*-linked carbohydrate groups of glycoprotein allergens induce the production of IgE antibodies which can cross-react with food and grass pollen allergens (Batanero *et al.*, 1996, Petersen *et al.*, 1996). This was also observed in a study by van der Veen and colleagues (1997) whereby a minority of grass pollen-sensitised individuals had significant serum levels of peanut-specific IgE antibodies directed at cross-reactive carbohydrate determinants (CCDs). However, the clinical significance of these antibodies is doubtful given that these patients did not exhibit clinical sensitivity to peanut. Thus, the exact role of CCDs in allergenic cross-reactivity is yet to be determined.

This thesis has demonstrated allergenic B cell cross-reactivity between peanut and the tree nuts almond, Brazil nut and hazelnut. The identification and characterisation of peanut and tree nut allergens has provided an insight into the potential sources of cross-reactivity between peanut and tree nuts. The seed storage proteins, which are conserved throughout different plant families, are the strong candidates mediating the observed cross-reactivity. The major peanut allergen, Ara h 1, is a member of the vicilin family of seed storage proteins (Burks *et al.*, 1991) along with allergens from walnut, cashew and hazelnut (Teuber *et al.*, 1999, Pastorello *et al.*, 2002, Wang *et al.*, 2002). 2S albumin seed storage proteins have also been implicated as allergens in almond, Brazil nut, hazelnut and walnut (Pastorello *et al.*, 1998, Teuber *et al.*, 1998, Pastorello *et al.*, 2002, Poltronieri *et al.*, 2002) and are also related to conglutin seed storage proteins,

some of which include allergens from almond (Poltronieri *et al.*, 2002) and peanut, namely Ara h 2, Ara h 6 and Ara h 7 (Burks *et al.*, 1992, Kleber-Janke *et al.*, 1999). Legumins or 11S globulins are also seed storage proteins and were shown to be allergenic in peanut, cashew, hazelnut and walnut (Rabjohn *et al.*, 1999, Beyer *et al.*, 2002, Pastorello *et al.*, 2002, Teuber *et al.*, 2003, Wang *et al.*, 2003).

Whether homologous proteins in peanuts and tree nuts are responsible for the observed cross-reactivity in this study is not known since the sequences of the identified cross-reactive allergens have not yet been determined and thus sequence comparisons have not been made. However, the molecular masses of the potential Ara h 2 and Ara h 3 homologues in almond and hazelnut identified in this study corresponded to previously identified allergens in these tree nuts that also belong to the same protein family as these peanut allergens. To date, most studies have investigated cross-reactivity between peanut and tree nuts using whole extracts. In contrast, cross-reactivity studies using purified peanut and/or tree nut allergens are limited with one study demonstrating the absence of cross-reactivity between peanut and the vicilin-like walnut allergen, Jug r 2 (Teuber *et al.*, 1999). It is also evident from this study by Teuber *et al.* (1999) that the presence of proteins from the same family does not necessarily translate to B cell cross-reactivity. This was confirmed in the current study whereby no detectable IgE cross-reactivity was obtained between peanut and cashew even though the major allergens in peanut and cashew, namely Ara h 1 and Ana o 1, are members of the vicilin seed storage family (Burks *et al.*, 1991, Wang *et al.*, 2002).

Although previous studies have indicated the presence of homologous proteins as responsible for cross-reactivity between different organisms, it is likely that the degree of amino acid identity or homology largely determines the potential for cross-reactivity.

Ana o 1 shows 45% amino acid sequence similarity with Ara h 1 yet no common IgE-binding epitopes were identified (Wang *et al.*, 2002). It has been suggested that 50-70% amino acid sequence homology is required for immunological B cell cross-reactivity to occur between proteins (Aalberse, 2000). Yet it has also been suggested that homology between two proteins limited to a small stretch of amino acids can result in cross-reactivity if there are similarities in the tertiary structure (Aalberse *et al.*, 2001a). Such issues can be addressed by performing cross-reactivity studies using purified allergens with known amino acid sequences. This can be achieved given that numerous peanut and tree nut allergens, some of which belong to the same protein family, have been identified, cloned and sequenced. The current study has also identified potential Ara h 1, Ara h 2 and Ara h 3 homologues in almond, Brazil nut and hazelnut which can be further characterised at the molecular level to determine the degree of sequence identity or similarity. Such information would provide further insight into the characteristics required for immunologically relevant cross-reactivity to occur.

8.3 B CELL CROSS-REACTIVITY AND THE IMMUNE RESPONSE

As stated earlier, IgE antibodies can react with highly homologous allergens from different organisms. Exposure to one allergen leads to the production of IgE antibodies that can react with another allergen that is similar in structure to the primary allergen. The humoral arm of the immune system, in particular B cells, has evolved to produce highly specific antibodies to different antigens as part of the rapid response to foreign antigens. Given the specificity of this response, it is interesting that IgE cross-reactivity can still occur between proteins from different organisms, which in some cases can be

distantly related or even unrelated. In this study, IgE antibodies specific for allergens present in peanut, a legume, cross-reacted with allergens present in almond, Brazil nut and hazelnut which are classified as tree nuts. How can this type of B cell cross-reactivity occur in the context of a highly specific immune response?

The initial encounter with an allergen in an atopic individual produces a Th2-type microenvironment through the dominant secretion of IL-4 and IL-13 by Th2 cells which promotes the synthesis of IgE antibodies by B cells. Repeated exposure to the same allergen produces memory responses which become more rapid and are predominantly composed of IgE antibodies that have undergone affinity maturation, thus exhibiting an improved affinity for the allergen. It has been previously suggested that the production of high affinity IgE antibodies is likely to result in increased cross-reactivity (Aalberse *et al.*, 2001a) although one could argue that this is unlikely to occur due to the high specificity of the antibody to a given allergen. However, such a scenario is possible between closely related allergens. Uncontrolled exposure to allergens, which is common for environmental allergens such as grass pollen, is likely to result in the production of highly specific IgE antibodies through multiple cycles of affinity maturation. Yet cross-allergenicity between different grass pollens is a common immunological occurrence and is largely due to the presence of highly homologous allergens (Weber, 2003). The overall sequence identity between group 5 grass pollen allergens is approximately 55-85% while group 2/3 allergens exhibit 85-90% sequence identity between species (Andersson and Lidholm, 2003). With such a high level of sequence identity, it is probable that some of the IgE-binding epitopes of grass pollen allergens are very similar, if not the same, and thus highly specific antibodies for one allergen can still cross-react with a highly homologous allergen. Repeated exposure to the same allergen can also enhance the polyclonality of the IgE response (Aalberse *et*

al., 2001a). The increased diversity of the antibody repertoire is likely to increase the probability that some IgE antibodies recognise the same B cell epitopes from different allergens.

It has been suggested that IgE antibodies of a low affinity may also be involved in allergenic B cell cross-reactivity (Aalberse *et al.*, 2001a). This may be relevant in peanut and tree nut allergen cross-reactivity, especially since these allergen sources are distantly related. Peanut/tree nut allergic patients actively avoid foods containing peanut and tree nut allergens due to the severity of the allergic reactions. As such, IgE antibodies specific for peanut and tree nut allergens in these patients are unlikely to have undergone as many cycles of affinity maturation as grass pollen-specific IgE antibodies. If repeated antigen exposure leads to increased antibody affinity, then it can be expected that less exposure to an antigen could yield antibodies with lower affinity. Thus 'less mature' peanut-specific antibodies may have a lower affinity which could lead to enhanced binding of potential homologues in tree nuts. Previous studies have demonstrated that affinity-matured antibodies elicited against a particular antigen can distinguish between derivatives of that antigen (James and Tawfik, 2003, Yin *et al.*, 2003). In contrast, germline precursor antibodies can bind the primary antigen and its derivatives with similar specificity (James and Tawfik, 2003, Yin *et al.*, 2003). Whether a spectrum of specificity exists between antibodies at varying stages of affinity maturation is not known and requires further investigation but it is likely that antibodies with a lower affinity will exhibit a broader range of specificity compared to those that are of a higher affinity.

An additional mechanism that has been demonstrated to contribute to antibody multispecificity is the existence of different antibody conformations that enable binding

of antibodies to distinct antigens. Using crystallisation studies, James and colleagues (2003) were able to demonstrate that a monoclonal IgE antibody, SPE7, raised against a hapten, 2,4 dinitrophenyl (DNP), assumes two structurally distinct conformations. One form (Ab1) was specific for peptides and proteins whereas the other isomer (Ab2) was specific to haptens (James *et al.*, 2003). Additionally, James and colleagues also demonstrated that the Ab2 isoform possessed a promiscuous, low affinity binding site which bound small aromatic ligands that were similar in structure to DNP. Binding to the aromatic molecules also induced additional rearrangements at the binding site to stabilise the antigen-antibody complex. From this landmark study, it appears that antibody multispecificity can be mediated in two ways: conformational diversity and antibody promiscuity to antigens that mimic the structure of the primary antigen. Whether this occurs *in vivo* is yet to be determined but this model highlights the potential role that conformational diversity may have in triggering autoimmune disease and allergy through cross-reactivity (James *et al.*, 2003).

8.4 ALLERGENIC B CELL CROSS-REACTIVITY AND CLINICAL RELEVANCE

Cross-reactive peanut-specific IgE antibodies were shown in this study to be biologically active. It was demonstrated that basophils sensitised with peanut-specific IgE antibodies became activated following stimulation with almond and Brazil nut extracts. However, the extent to which these cross-reactive antibodies contribute to the clinical manifestation of tree nut allergy is not clear. The majority of peanut allergic subjects in this study had detectable levels of specific IgE to almond, Brazil nut and

hazelnut which were shown in the current study to contain allergens that cross-react with peanut allergens. But one cannot exclude the possibility that allergic reactions upon exposure to these tree nuts are mediated by non-cross-reactive IgE antibodies, that is, IgE antibodies that have a unique specificity for tree nut allergens. Therefore, it is extremely difficult to critically assess the contribution of cross-reactive IgE antibodies to the clinical manifestation of tree nut allergy in peanut allergic individuals. However, it seems likely that peanut-specific IgE antibodies would augment the allergic response to tree nut allergens especially since it has been shown in this study using the stripped basophil activation test that cross-reactive peanut-specific IgE antibodies can induce effector cell activation upon exposure to tree nut allergens.

The use of animal models could provide an avenue for investigating the clinical relevance of cross-reactive antibodies. This is ideal because it allows the investigator to control the sensitisation of the animal to different allergens. Murine models of peanut anaphylaxis have been successfully developed in the past and have been used to investigate immunotherapeutic options for the treatment of peanut allergy (Li *et al.*, 1999, Roy *et al.*, 1999, Li *et al.*, 2000, Lee *et al.*, 2001, Li *et al.*, 2001). However, one group has investigated peanut and tree nut cross-reactivity through the development of a canine model of peanut and tree nut food allergy. Teuber and colleagues (2002) successfully sensitised dogs with peanut, Brazil nut and walnut and found that oral challenges with the sensitising agent elicited allergic symptoms that paralleled those observed in humans. Subsequent cross-reactivity studies demonstrated the absence of any clinical reactions when peanut-sensitised dogs were challenged with Brazil nut or walnut extracts even though specific IgE to Brazil nut and walnut proteins was detected in some of these dogs (Teuber *et al.*, 2002). In contrast, one walnut-sensitised dog with specific IgE to Brazil nut reacted upon challenge with this tree nut (Teuber *et al.*, 2002).

These findings indicate that cross-reactivity between peanut and tree nuts may not be clinically relevant in this dog model, however the results from the current study indicate that additional parameters should be considered before a definite conclusion can be made. In particular, oral challenges using other tree nuts, for example almond, should be conducted especially since the latter was found to have the highest level of cross-reactivity with peanut. Additionally, inhibition studies in this thesis indicated that the level of cross-reactivity between peanut and the tree nuts tested was low and therefore higher challenge doses may be required to obtain clinically relevant cross-reactivity. Nevertheless, this type of animal model, and perhaps murine models, may provide a good basis for the investigation of the clinical relevance of cross-reactive antibodies, which would otherwise be difficult to investigate in humans.

8.5 FUTURE THERAPEUTIC OPTIONS FOR THE TREATMENT OF PEANUT AND TREE NUT ALLERGY

Given the severity and life-threatening nature of allergic reactions to peanut and tree nuts, a number of immunotherapeutic methods are being developed as a form of treatment for peanut and tree nut allergy. Current conventional immunotherapy typically involves a desensitisation protocol of subcutaneously injecting incremental doses of the allergen extract which modifies the immune response, resulting in clinical tolerance to the allergen. This form of immunotherapy has been effective in treating allergies to house dust mite, cat, bee venom and grass pollen, however, it is not recommended for the treatment of peanut and tree nut allergy because of the high level of extreme systemic side effects (Oppenheimer *et al.*, 1992). Consequently, alternative

therapeutic strategies are currently being developed in a bid to reduce the severity of the symptoms associated with this type of food allergy.

One approach directed at limiting the cross-linking of effector cell-bound IgE antibodies is the engineering of recombinant allergens with reduced IgE reactivity and preserved T cell reactivity. Site-directed mutagenesis of B cell epitopes on allergens has been shown to reduce IgE binding to allergens in birch pollen (Ferreira *et al.*, 1998), timothy grass (Schramm *et al.*, 1999), house dust mite (Hakkart *et al.*, 1998) and natural rubber latex (Drew *et al.*, unpublished). This strategy has already been used to modify the peanut allergen, Ara h 3, with approximately a 35-85% reduction of IgE binding to the modified allergen using sera from Ara h 3 allergic subjects (Rabjohn *et al.*, 2002). This hypoallergenic form of Ara h 3 also retained the ability to stimulate T cells from these patients, and therefore has the capacity to induce tolerance. As such, this modified peanut allergen can potentially be used in allergen-specific immunotherapy. The cross-reactive B cell epitopes of peanut allergens can also be potential targets for mutation thus incorporating the treatment of tree nut allergy as well as peanut allergy. However, there is currently no evidence to show that immunotherapy using modified allergens is effective in modifying an established Th2 response. Therefore, future studies will need to address whether immunotherapy using hypoallergenic variants can indeed abrogate Th2 responses, most likely using animal models, before allergen mutant vaccines can be used in the treatment of peanut and tree nut allergy. Also, this type of approach may not be suitable in cases where the patient is sensitised to more than one allergen. Although the major peanut allergens have been identified, the majority of peanut allergic subjects involved in this study had specific IgE to more than one peanut allergen and therefore would require a cocktail of hypoallergenic mutants for this form of treatment to be

effective. Whether it is possible to cover the range of allergens present in crude peanut extract is not known and must be addressed for successful immunotherapy.

Specific immunotherapy using T cell epitope peptides has been shown to have some degree of efficacy in the treatment of certain allergies and is particularly attractive because, similar to hypoallergenic mutants, it minimises the cross-linking of effector cell-bound IgE antibodies. The administration of peptides based on the dominant T cell epitopes of allergens has been shown to induce specific T cell tolerance in mice (Briner *et al.*, 1993, Hoyne *et al.*, 1993). Clinical trials of allergen peptide immunotherapy have been performed for cat and bee venom allergy with variable efficacy. Administration of peptides of the major cat allergen, Fel d 1, induced local reactions in some patients although a decrease in IL-4 production by T cells from these patients was detected (Norman *et al.*, 1996). A more recent study by Oldfield and colleagues (2002) demonstrated that treatment of cat allergic subjects with multiple doses of a Fel d 1 peptide vaccine inhibited early and late phase allergic reactions to the whole allergen. This was shown to be associated with an increase in IL-10 production by peripheral blood mononuclear cells, thus inducing tolerance (Oldfield *et al.*, 2002).

Similarly, immunotherapy using peptides of the major bee venom allergen, phospholipase A_2 (PLA_2), protected patients from challenge with PLA_2 and a decrease in T cell proliferation and cytokine secretion against the whole PLA_2 allergen was detected which was consistent with T cell anergy (Muller *et al.*, 1998). This form of therapy has not been considered for the treatment of peanut allergy largely because the T cell epitopes of peanut allergens have not yet been identified. Therefore, further study is required to identify the dominant T cell epitopes of peanut allergens and to ensure

that peptides representing these epitopes would not induce systemic reactions in sensitive individuals.

Non-specific strategies have also been considered for the treatment of peanut and tree nut allergy. Of these, the most promising approach has been the use of TNX-901, a humanised IgG1 monoclonal antibody specific for an epitope in the CH3 region of the IgE antibody which is responsible for binding to the high affinity FcεRI receptor on mast cells and basophils (Leung *et al.*, 2003). Leung and colleagues conducted a double-blind, randomised trial in 84 peanut sensitive patients whose level of clinical sensitivity was determined by a double-blind, placebo controlled food challenge (DBPCFC) prior to the study. These patients were administered with subcutaneous injections every four weeks of TNX-901 at doses of 150, 300, 450 mg or a placebo for a total of 16 weeks. Subsequent DBPCFCs demonstrated a significant increase in the mean threshold dose of peanut that elicited symptoms in allergic individuals receiving TNX-901. Although this treatment did not cure these patients, it diminished the risk of a severe or fatal reaction after an accidental ingestion. The non-specific nature of this form of treatment is appealing as it may be advantageous in patients with multiple food allergies where a strict diet is difficult to manage, as is the case with peanut and tree nut allergy. It is also ideal for patients that are sensitive to more than one allergen from any given source. Other non-specific mechanisms that have been reported to suppress peanut-induced anaphylaxis include the administration of IL-12 (Lee *et al.*, 2001) and treatment with a Chinese herbal formula, FAHF-1 (Li *et al.*, 2001), although these have only been used in mouse models of peanut allergy and thus the effect in humans is not known.

8.7 CONCLUSIONS

This thesis has confirmed that B cell cross-reactivity exists between peanut and tree nut allergens. The peanut allergens Ara h 1, Ara h 2 and Ara h 3 were shown to contribute to this observed cross-reactivity although other peanut allergens may also be implicated. Peanut-specific IgE antibodies that cross-reacted with tree nut allergens were also demonstrated to be biologically active. Overall, these mechanisms explain, in part, the common clinical observation of co-sensitisation to peanut and tree nuts in the peanut allergic population. Such information can be used to improve the diagnosis as well as the management of peanut allergy through the avoidance of potentially cross-reactive foods. However, further study is still required to fully understand the basis of peanut and tree nut B cell cross-reactivity, including the identification and molecular characterisation of the cross-reactive allergens. Characterisation of the B cell and T cell cross-reactive immune response would also provide further insight into the possible mechanisms involved in the manifestation of multiple allergies although this was not the goal of this thesis. Nevertheless, a number of immunotherapeutic options for the treatment of peanut and tree nut allergy are currently being developed, some of which show some promise in successfully diminishing the severe symptoms associated with this type of food allergy.

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Immunological analysis of allergenic cross-reactivity between peanut and tree nuts

M. P. de Leon*†, I. N. Glaspole*†‡, A. C. Drew*†‡, J. M. Rolland†‡, R. E. O'Hehir*†‡ and C. Suphioglu*†‡

*Departments of *Allergy, Immunology and Respiratory Medicine, The Alfred Hospital, and †Pathology and Immunology, Monash University, Commercial Road, Melbourne, Victoria 3004, Australia and ‡Co-operative Research Centre for Asthma, Sydney, Australia*

Summary

Background Peanut and tree nut allergy is characterized by a high frequency of life-threatening anaphylactic reactions and typically lifelong persistence. Peanut allergy is more common than tree nut allergy, but many subjects develop hypersensitivity to both peanuts and tree nuts. Whether this is due to the presence of cross-reactive allergens remains unknown.

Objective The aim of this study was to investigate the presence of allergenic cross-reactivity between peanut and tree nuts.

Methods Western blotting and ELISA were performed using sera from subjects with or without peanut and tree nut allergy to assess immunoglobulin E (IgE) reactivity to peanut and tree nut extracts. Inhibition ELISA studies were conducted to assess the presence of allergenic cross-reactivity between peanut and tree nuts.

Results Western blot and ELISA results showed IgE reactivity to peanut, almond, Brazil nut, hazelnut and cashew nut for peanut- and tree nut-allergic subject sera. Raw and roasted peanut and tree nut extracts showed similar IgE reactivities. Inhibition ELISA showed that pre-incubation of sera with almond, Brazil nut or hazelnut extracts resulted in a decrease in IgE binding to peanut extract, indicating allergenic cross-reactivity. Pre-incubation of sera with cashew nut extract did not cause any inhibition.

Conclusion These results show that multiple peanut and tree nut sensitivities observed in allergic subjects may be due to cross-reactive B cell epitopes present in different peanut and tree nut allergens. The plant taxonomic classification of peanut and tree nuts does not appear to predict allergenic cross-reactivity.

Keywords allergen avoidance, allergy, cross-reactivity, IgE, inhibition ELISA, multiple nut sensitivity, peanut, tree nuts, taxonomic classification

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Introduction

Peanut and tree nut allergy is characterized by a high frequency of life-threatening anaphylactic reactions and typically lifelong persistence [1]. There appears to be a strong clinical association between peanut and tree nut allergy, although reports regarding the prevalence of multiple nut sensitivity have produced widely disparate findings. Two groups analysing subjects from specialist allergy clinics have reported a prevalence of between 35% and 40% [2, 3]. However, in a randomly sampled population of the general community, only 2.4% of peanut- and tree nut-allergic subjects reported symptoms consistent with allergy to more than one variety [4]. A similar lack of certainty relates to the relative prevalence of allergy among the different varieties of tree nuts. In a study by Ewan [2], Brazil nut was the tree nut to which sensitivity most commonly existed, followed by almond, hazelnut, walnut and cashew, whereas in Sicherer et al.'s [3] group, walnut produced greater than 50% of reactions to tree nuts and Brazil nut was the least commonly sensitizing nut. In both studies, peanut, a groundnut (legume) was the most common sensitizing agent.

Early studies examining the basis of cross-reactivity between foods were able to demonstrate the presence of immunoglobulin E (IgE) cross-reactive proteins in phylogenetically similar plants, but these links were not borne out clinically [5]. More recent studies have concentrated on determining the presence of proteins of homologous structure within peanuts and different varieties of tree nut, although their presence does not necessarily equate with cross-reactivity. The major peanut allergen Ara h 1 and the major walnut allergen Jug r 2 are both members of the vicilin seed storage family, yet they show no significant IgE cross-reactivity on the basis of inhibition immunoblotting experiments [6]. However, in a small study using sera from two walnut-allergic subjects, by pre-incubating with crude peanut extract, IgE binding to a crude walnut extract immunoblot

*Correspondence: Dr Cenk Suphioglu, Department of Allergy, Immunology and Respiratory Medicine, Monash University Medical School, Commercial Road, Melbourne, Victoria 3004, Australia.
E-mail: Cenk.Suphioglu@med.monash.edu.au

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was significantly abrogated, suggesting that cross-reactive proteins do occur within these foods [6]. No other studies have examined cross-reactivity between peanut and tree nuts.

In contrast, IgE cross-reactivity between the various tree nuts has been demonstrated by several authors. Inhibition immunoblots using albumin fractions from walnut and hazelnut as inhibitors suggested that significant cross-reactivity exists between this fraction and almond conglutin 7, with lesser cross-reactivity also demonstrated for almond 2S albumin [7]. Partial cross-reactivity between hazelnut and macadamia nut has been demonstrated by Sutherland et al. [8], with sub-total diminution of IgE binding to a 17.4-kDa protein with pre-incubation of sera with hazelnut extract. Hazelnut has also been shown to possess multiple cross-reacting proteins with sesame seed and poppy seed, apart from a unique allergen of approximately 20 kDa, and also with allergens associated with the oral allergy syndrome, tree pollens and stone fruit [9–11]. Pistachio nut appears to cross-react with cashew nut, with RAST inhibition studies suggesting that IgE binding to pistachio was diminished by pre-incubation with cashew extract, although inhibitory concentrations were high [12]. In another study, Brazil nut-allergic individuals were shown to have IgE antibodies to peanut, hazelnut and walnut [13]. Finally, some have suggested that there may be common allergens between pistachio, peanut, walnut, chestnut, almond and cashew nuts as well as pine nut and almond [12, 14, 15].

Although it has been suggested that cross-reactivity may exist between peanut and different tree nut types, whether this is the case for all nuts to which subjects are commonly sensitized remains unknown. Determination of the immunological relationship between peanut and tree nuts is essential for patient management and the development of safe vaccines for nut allergy. This study was undertaken to determine if peanut-specific IgE antibodies from peanut- and tree nut-allergic subjects cross-react with proteins found in the commonly encountered tree nuts.

Materials and methods

Subjects

Sera from four subjects recruited from the Alfred Hospital Asthma and Allergy Clinic, Melbourne, Australia were used in this study. These subjects had a clinical history of sensitivity to peanut and tree nuts. All had specific IgE to peanut (RAST score of ⩾2, Pharmacia CAP System™, Pharmacia Diagnostics, Uppsala, Sweden). The study was approved by the Alfred Hospital Ethics Committee and informed consent was obtained from all subjects before blood was obtained. The clinical characteristics and nut-specific IgE RAST scores for these subjects are given in Table 1. Sera from two non-peanut- and tree nut-allergic individuals, one atopic (history of grass pollen sensitivity) and one non-atopic, were used as negative controls.

Preparation of crude nut extracts

Peanuts and the tree nuts almond, Brazil nut, cashew and hazelnut were used in this study and the taxonomic

Table 1. Characteristics of peanut and tree nut-sensitive patients

						Clinical reaction to peanut and tree nuts				RAST scores					
Patient no.	Age (years)	Sex	Atopy history	Known nut sensitivities	Other food sensitivities	Clinical features of anaphylaxis	Age at first reaction (years)	Time since last reaction (months)	Total IgE (kU/L)	Peanut	Almond	Brazil nut	Cashew	Hazelnut	Walnut
1	27	F	Eczema	Peanut, almond		Asthma, urticaria, facial oedema	19	11	nd	3	0	0	0	0	nd
2	34	M	Asthma, rhinitis, eczema	Peanut, hazelnut		Asthma, urticaria, facial oedema	8	12	416	2	1	2	2	2	2
3	28	M	Rhinitis, eczema	Peanut, Brazil nut, almond, hazelnut, walnut, cashew, pine nut		GIT upset, laryngeal oedema, facial oedema	1	36	4189	2	0	0	3	2	2
4	54	M	Asthma, eczema	Peanut, walnut	Banana	Asthma, laryngeal oedema, facial oedema	1	120	2492	2	1	0	1	2	1

nd–not done.

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Table 2. Taxonomic classification of peanut and tree nut plants (USDA, NRCS. 2001. The PLANTS Database, Version 3.1. National Plant Data Centre)

Sub-class	Order	Family	Genus, species (Common name)
Rosidae	Fabales	Fabaceae (Pea family)	*Arachis hypogaea* L. (Peanut)
	Proteales	Proteaceae (Protea family)	*Macadamia integrifolia* (Macadamia nut)
	Rosales	Rosaceae (Rose family)	*Ivesia pityocharis* (Pine nut) *Prunus dulcis* (Almond)
	Sapindales	Anacardiaceae (Sumac family)	*Anacardium occidentale* L. (Cashew) *Pistacia vera* L. (Pistachio nut)
Hamamelidae	Fagales	Betulaceae (Birch family)	*Corylus avellana* L. (Hazelnut)
		Fagaceae (Beech family)	*Castanea sativa* (European chestnut) *Quercus ilex* L. (Acorn nut)
	Juglandales	Juglandaceae (Walnut family)	*Carya illinoinensis* (Pecan)
			Juglans regia L. (English walnut)
Dilleniidae	Lecythidales	Lecythidaceae (Brazil nut family)	*Bertholletia excelsa* (Brazil nut)

classification of these plants is shown in Table 2. The extraction of proteins from peanut and tree nuts was conducted using a method similar to the Pharmacia CAP system. Commercially available almonds, Brazil nuts, cashews, hazelnuts and peanuts (either raw or roasted at 180 °C for 15 min; Naytura, Sydney, Australia) were crushed and defatted by adding acetone. Contents were mixed and centrifuged at 1000 *g* and the pellet was resuspended in diethyl ether. This procedure was repeated five times. After the final extraction, the crushed product was separated by vacuum filtration and dried for 10 min under vacuum. The dried, defatted product was then ground to powder using liquid nitrogen and incubated overnight in phosphate-buffered saline (PBS) alone (for Western blotting and ELISA studies) or PBS with Complete™ protease inhibitor cocktail (for inhibition ELISA) (Roche Diagnostics, Mannheim, Germany) at 4 °C, with shaking. The extract was further centrifuged at 1000 *g* to pellet debris and at 20 000 *g* to obtain a clear supernatant. The protein concentration of each nut extract was then determined using the Bio-Rad Micro protein assay (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. A suitable walnut extract for this study could not be obtained due to unsatisfactory protein yields.

Electrophoresis and Western immunoblotting

Gel electrophoresis and immunoblotting were conducted following established protocols [16]. Briefly, proteins from crude roasted peanut extracts were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 16% gels, according to Laemmli [17], under reducing conditions using the Xcell II Mini-Cell apparatus (Invitrogen, Carlsbad, CA, USA) at 125 V for 2 h. Benchmark™ pre-stained protein ladder (Invitrogen) was also resolved and proteins were stained with Coomassie brilliant blue (CBB; Sigma, St Louis, MO, USA).

For Western immunoblotting, peanut proteins separated by 16% SDS-PAGE were transferred onto nitrocellulose membranes (0.45 μm; Schleicher and Schuell Inc., Dassel, Germany) at 25 V for 2 h using Xcell II blotting apparatus (Invitrogen) according to Towbin and Gordon [18]. Membranes were blocked in 1% bovine serum albumin (BSA) in PBS for 1 h and subsequently washed once with PBS containing 0.05% Tween-20 (PBS-T) and twice in PBS alone. IgE reactivity of peanut proteins was determined by incubating the membranes in subject and control sera diluted 1 : 5 with 0.5% BSA in PBS. Membranes were then incubated in rabbit polyclonal anti-human IgE antibody (1 : 200; DAKO, Carpinteria, CA, USA) and horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG antibody (1 : 2000; Promega, Madison, WI, USA) each for 1 h, with washes conducted between incubations as described above. IgE binding was detected using the substrate 4-chloro-1-naphthol (Sigma) in the presence of hydrogen peroxide [16].

Serum IgE ELISA and inhibition ELISA

ELISA studies were performed according to established methods [19]. Briefly, raw and roasted nut extracts were adjusted to 1 μg/mL using 50 mM bicarbonate buffer, pH 9.6, dispensed into 96-well polystyrene plates (50 μL/well; Costar, Acton, MA, USA), and incubated overnight at 4 °C. Plates were washed with PBS-T and blocked with 5% skim milk powder (SMP) in PBS-T (200 μL/well) for 1 h at 37 °C. After washing with PBS-T, 50 μL of subject and control sera, diluted with PBS-T containing 1% SMP, were added to the wells and incubated at 37 °C for 2 h. Plates were washed with PBS-T and incubated with rabbit polyclonal anti-human IgE antibody (1 : 1000; 50 μL/well; DAKO) for 1 h at 37 °C, followed by HRP-labelled goat anti-rabbit IgG antibody (1 : 1000; 50 μL/well; Promega), incubated similarly for 1 h at 37 °C, with PBS-T washes in between incubations. IgE binding was detected using *O*-phenylenediamine tablets (Sigma) dissolved in 0.05 M phosphate-citrate buffer (50 μL/well; Sigma). The reaction was stopped after 10 min with the addition of 4 M hydrochloric acid (50 μL/well), and the absorbance (OD) in each well was measured at 490 nm. The absorbance in control wells containing no antigen was subtracted from the absorbance in antigen-coated wells to account for non-specific binding. Assays were performed in triplicate and mean values with standard deviation are shown.

Inhibition experiments were conducted by coating 96-well polystyrene plates with roasted peanut extract (1 μg/mL), and blocking as described above. Subject and control sera (diluted with 1% SMP in PBS-T for an OD 490 nm reading of ~1.0 for peanut extract) were pre-incubated with nut extracts or, as a control, keyhole limpet haemocyanin (KLH; Sigma) in the presence of protease inhibitors (as described before) at a final concentration of 0.2, 1, 5, 25 and 125 μg/mL, at room temperature for 2 h. The inhibition mixtures (including sera

with no inhibitor as positive controls) were then dispensed into wells (50 µL/well) and incubated at 37 °C for a further 2 h. The assay was then continued as described above. Percentage inhibition was calculated using the following formula:

$$\%\,\text{inhibition} = 100 - \left(\frac{OD_{490}\text{ of serum with inhibitor}}{OD_{490}\text{ of serum without inhibitor}} \times 100\right)$$

Lower inhibitor concentrations (0.04, 0.008, 0.0016 and 0.00032 µg/mL) were also used for roasted peanut extract to enable the calculation of the concentration required for 50% inhibition of IgE binding (IC_{50}).

Results

Western blot analysis of serum IgE reactivity to roasted peanut extract

Roasted peanut extract was separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membrane strips were incubated with sera from four peanut- and tree nut-allergic subjects to examine IgE reactivity to peanut allergens (Fig. 1). Subject 1 showed IgE reactivity to bands corresponding to the molecular mass of the known peanut allergens Ara h 1, Ara h 2 and Ara h 3. All four subjects reacted to a 23-kDa protein that has not been described before. Serum from two control individuals (one atopic and one non-atopic) did not show IgE binding to roasted peanut proteins. Western immunoblotting using almond, Brazil nut, cashew and hazelnut extracts was also performed with these patient sera. Several allergenic proteins were detected for each tree nut; however, the pattern of IgE binding differed for each patient (data not shown).

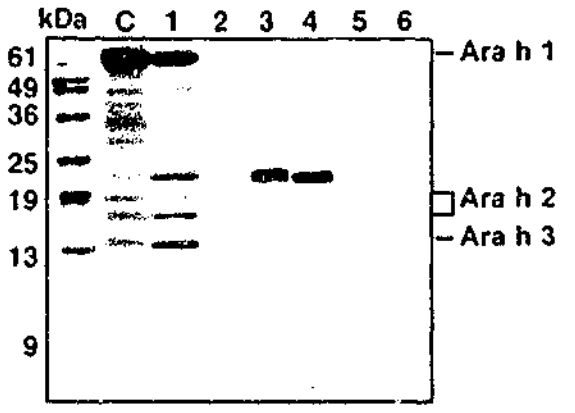

Fig. 1. Immunoblotting for IgE binding to crude roasted peanut extract. Crude roasted peanut extract was resolved by 16% SDS-PAGE as shown by Coomassie staining (C). Proteins were transferred onto nitrocellulose membranes and probed for IgE binding using sera from peanut-allergic (1–4), atopic, non-nut-allergic (5) and non-atopic (6) patients.

ELISA for serum IgE response to crude peanut and tree nut extracts

Direct IgE ELISA was performed to confirm the IgE reactivity of crude peanut, almond, Brazil nut, cashew and hazelnut extracts for use in inhibition assays. The difference in IgE reactivity between raw and roasted peanut and tree nut extracts was also assessed to investigate the effects of roasting on the allergenicity of these extracts. Fig. 2 shows the IgE reactivity of raw and roasted peanut and tree nut extracts, with different levels of IgE binding observed in all four peanut- and tree nut-allergic subjects. Subjects 2 and 3 (Figs 2b,c) showed IgE binding to all raw and roasted peanut and tree nut extracts, although the latter showed minimal IgE reactivity to raw and roasted Brazil nut extracts. Sera from subjects 1 and 4 (Figs 2a,d) had IgE antibodies to raw and roasted almond, hazelnut and peanut extracts, with minimal or no IgE binding to Brazil and cashew nut extracts. Negligible IgE binding was obtained with non-nut allergic and non-atopic control sera (Figs 2e,f). Titration of serum IgE was also conducted for subjects 2 and 3 to determine if there were any changes in IgE binding after roasting of the peanut and tree nut extracts (Fig. 3). In both subjects, the level of IgE binding to the raw and roasted extracts did not differ between serum dilutions. This was further confirmed by Western immunoblotting studies where minimal differences in IgE reactivity were observed for raw and roasted peanut and

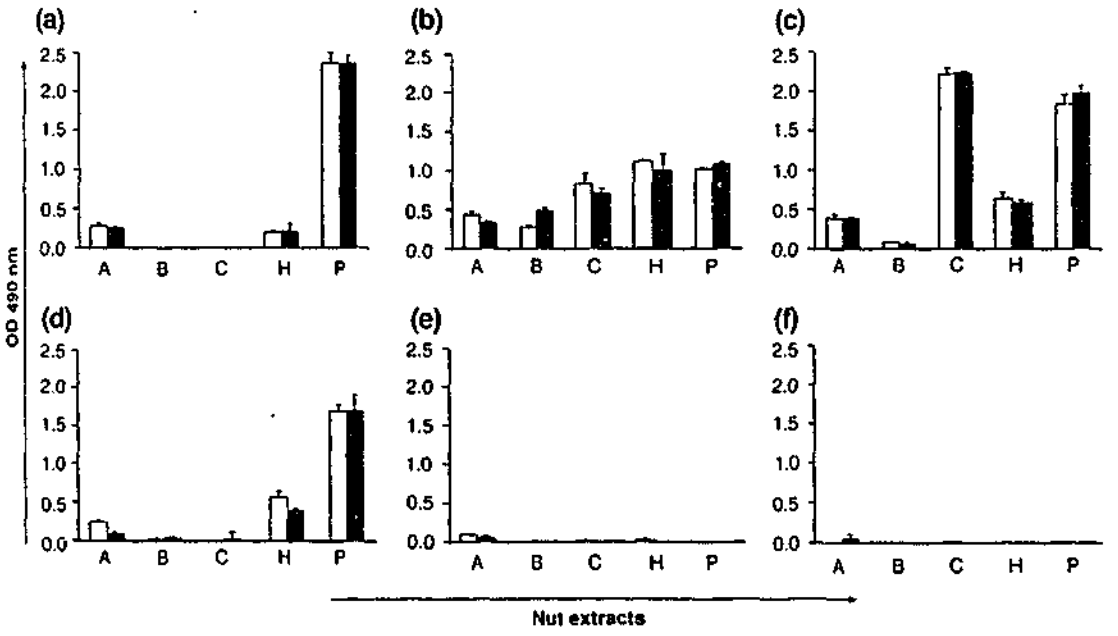

Fig. 2. ELISA for serum IgE binding to different nut extracts. ELISA plate was immobilized with raw (empty bars) and roasted (solid bars) nut extracts (1 µg/mL) including almond (A), Brazil nut (B), cashew (C), hazelnut (H) and peanut (P). IgE binding was assessed among four multiple nut-allergic patients (a–d), one atopic, non-nut-allergic patient (e) and one non-atopic patient (f), at a serum dilution of 1 : 10. The absorbance in control wells containing no antigen was subtracted from antigen-coated wells. The mean values for triplicates are shown and the standard deviation is indicated by error bars.

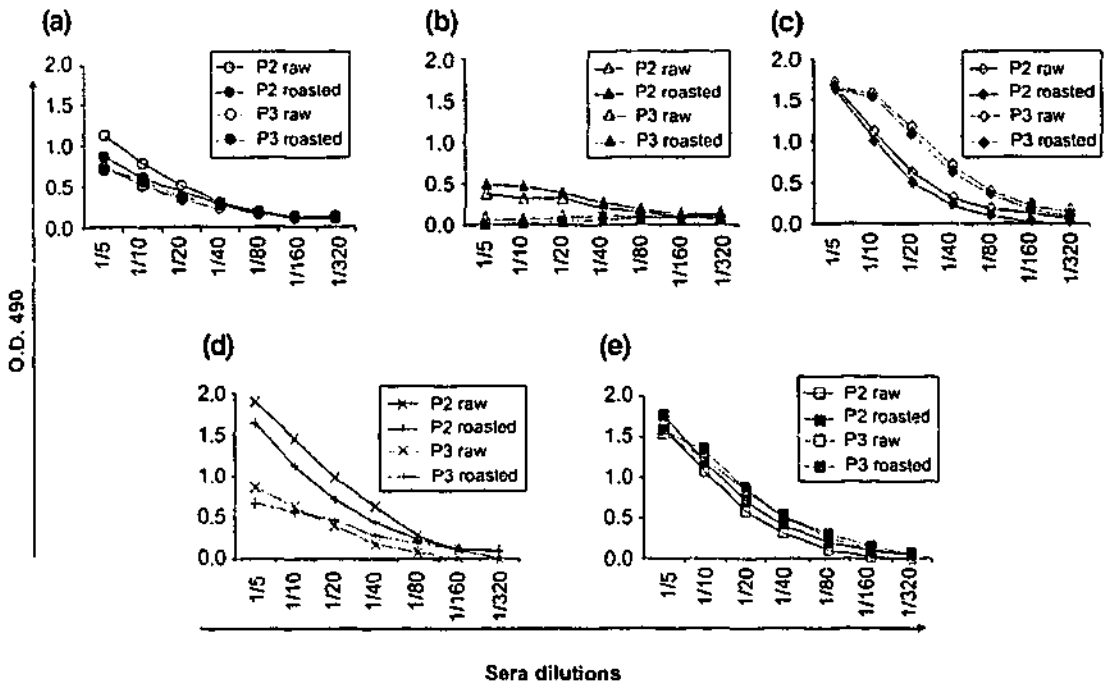

Fig. 3. ELISA for titration of serum IgE against raw and roasted nut extracts. ELISA plate was immobilized with raw and roasted almond (a), Brazil nut (b), cashew (c), hazelnut (d) and peanut (e) extracts. Sera from patient nos. 2 (P2) and 3 (P3) were serially diluted and IgE binding was assessed. The mean values for triplicates are shown. The absorbance in control wells containing no antigen was subtracted from antigen-coated wells.

tree nut proteins (data not shown). Therefore, roasting appears to have little or no effect on the allergenicity of peanut and tree nuts.

Inhibition ELISA

Inhibition ELISA was performed to determine whether cross-reactive allergens were present in the peanut and tree nut extracts. In this assay, the capacity of roasted almond, raw Brazil nut, roasted cashew and roasted hazelnut extracts to inhibit IgE binding to roasted peanut extract immobilized on an ELISA plate was assessed. As only minimal differences in allergenicity were observed between raw and roasted extracts (Figs 2 and 3), the form (raw or roasted) in which the extracts were used as inhibitors was determined according to that most commonly consumed.

The results of the dose-dependent ELISA inhibition assays for all four peanut- and tree nut-allergic subjects are shown in Fig. 4. The specificity of this assay was demonstrated by the strong inhibition obtained with roasted peanut extract (positive control) in all the subject sera, while the negative control extract (KLH) induced minimal or no inhibition of IgE binding to roasted peanut extract. To assess the specificity of the inhibitions observed, a non-specific inhibition ELISA was performed. In this assay, the non-specific inhibitory effect of the peanut and tree nut extracts was evaluated in a latex-glove-extract-specific IgE ELISA. Negligible inhibition of IgE binding to the glove extract was observed when serum from a latex, non-nut allergic patient was pre-incubated with 0.2–125 μg/mL of peanut and tree nut extracts (data not shown), further validating the specificity of this assay. Of the tree nut extracts used in this study, roasted almond showed the highest level of inhibition followed by raw Brazil nut and roasted hazelnut extract. These data are consistent with the presence of cross-reactive allergens in almond, Brazil nut, hazelnut and peanut. In contrast, cashew extract did not demonstrate a dose-dependent inhibition of IgE binding to roasted peanut extract, with minimal inhibition at the maximum inhibitor concentration of 125 μg/mL, indicating the lack of cross-reactive allergens in this extract. The differences in the degree of inhibition observed with the peanut and tree nut extracts were further investigated by determining the IC_{50} to roasted peanut extract, as shown in Table 3. As expected, the roasted peanut extract (positive control) gave the lowest IC_{50} in all subjects, with concentrations ranging from 0.006 to 0.058 μg/mL. All four subjects also achieved 50% inhibition of IgE binding to roasted peanut with roasted almond extract as the inhibitor, although IC_{50} values were much higher than the positive control, ranging from 0.6 to 45 μg/mL. Raw Brazil nut extract showed 50% inhibition of IgE binding to roasted peanut extract in three out of four subjects, while two out of four subjects showed 50% inhibition with roasted hazelnut extract within the range of inhibitor concentrations used in this study. In contrast, both roasted cashew extract and KLH demonstrated negligible inhibition levels similar to that obtained in the non-specific inhibition ELISA (data not shown).

Discussion

Allergy to at least one tree nut is a common clinical observation in the peanut-allergic population, and limited immunological studies suggest the presence of cross-reactive allergens [9, 12, 14, 15, 20]. Here we demonstrate serum IgE cross-reactivity between allergens present in peanut, almond, Brazil nut and hazelnut using inhibition ELISA. No IgE cross-reactivity was detected between cashew and peanut.

In this study, four subjects who were allergic to peanut as well as one or more tree nuts were studied in detail. The RAST scores for almond, Brazil nut, cashew, hazelnut and peanut in all four subjects correlated well with the IgE levels obtained in the serum IgE ELISA. However, in some cases, subject clinical history did not correlate with RAST scores. Subjects 1 and 3, both with a history of sensitivity to almonds, were positive for IgE to almond by ELISA, but negative by RAST. There are no published reports of

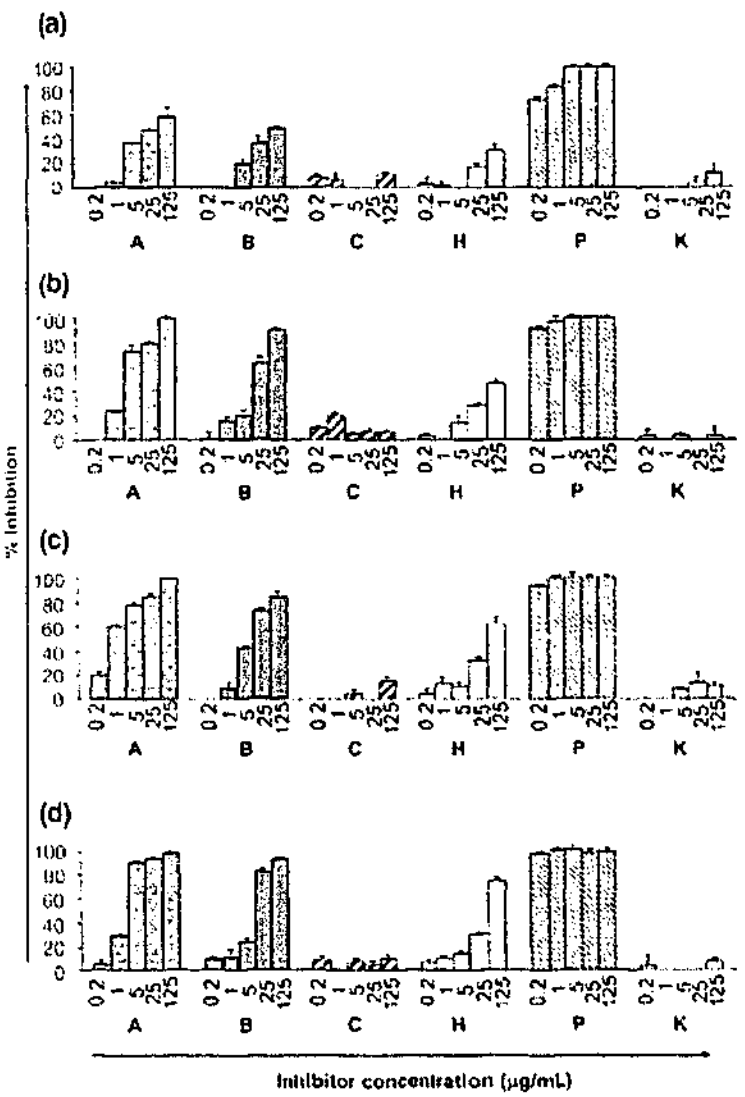

Fig. 4. ELISA for inhibition of serum IgE binding to peanut by other nut extracts. Potential inhibitors of serum IgE binding to plate-immobilized roasted peanut extract include crude roasted almond (A), raw Brazil nut (B), roasted cashew (C), roasted hazelnut (H), roasted peanut positive control (P) and KLH negative control (K) extracts. Inhibition of IgE binding was assessed using four multiple nut-allergic patients (a–d). The mean values for triplicates are shown and the standard deviation is indicated by error bars.

Table 3. Inhibitor concentration required for 50% inhibition of IgE binding to roasted peanut extract

	Inhibitor concentration (μg/mL)					
Patient no.	Roasted almond	Raw Brazil	Roasted cashew	Roasted hazelnut	Roasted peanut*	KLH†
1	45.0	‡	§	‡	0.058	§
2	3.1	18.6	§	‡	0.006	§
3	0.6	8.2	§	82.0	0.015	§
4	2.4	14.0	§	71.0	0.015	§

*Positive control inhibitor.
†Negative control inhibitor.
‡Did not reach 50% inhibition at the maximum inhibitor concentration of 125 μg/mL.
§Did not show inhibition above the levels obtained in non-specific inhibition assay.

the correlation between RAST and ELISA for detection of nut-specific IgE, but our results suggest that ELISA testing is more sensitive than RAST. RAST testing for peanut-specific IgE has previously demonstrated a false-negative rate of approximately 15% [21]. Within our study, several subjects demonstrated a positive RAST score and ELISA in the absence of a clinical history. While this finding may represent false-positive results, the peanut and tree nut sensitivity data are derived from clinical history rather than oral food challenge, and therefore our specific IgE data may reflect clinically unrecognized nut sensitivities.

The effect of roasting on allergenicity of peanut and tree nut proteins was also investigated in this study. At high temperatures, reducing sugars present in peanuts may react with proteins by the Maillard reaction producing protein-sugar end-products with enhanced allergenicity [22–24], and another study showed loss of IgE binding to a low molecular weight protein of almond following roasting [25]. In our study, IgE binding to almond, Brazil nut, cashew, hazelnut and peanut proteins did not differ between the raw and roasted forms, indicating that the IgE-binding epitopes are heat-stable, possibly contributing to their allergenicity. Whether this is due to heat-stable tertiary structures or the presence of linear epitopes in heat-labile allergens is not known and requires further investigation.

Given that tree nut allergy is a common occurrence among peanut-allergic individuals, information regarding the level of allergenic cross-reactivity between peanut and other tree nuts is critical but currently lacking. In this study, inhibition assays demonstrated that IgE cross-reactivity occurs between peanut, almond, Brazil nut and hazelnut, which are the most common causes of peanut and tree nut allergy [2]. This suggests that there may be similarities in the IgE-binding epitopes of proteins present in these foods. There are, however, differences in the degree of inhibition observed between peanut and the different tree nuts as shown by the IC_{50} for inhibition of peanut reactivity. Almond inhibits IgE binding to peanut at lower concentrations than Brazil nut and in turn hazelnut, although these values were considerably higher when compared to the IC_{50} for peanut control extract.

The high inhibition of IgE binding to peanut by the peanut extract positive control compared with the tree nut extracts suggests that the level of cross-reactivity between peanut and tree nuts is low. This may reflect differences in the abundance of cross-reactive allergens or epitopes in peanut and tree nut extracts and/or differences in the affinity of peanut-specific IgE antibodies for proteins in almond, Brazil nut and hazelnut extracts [26]. Although the observed cross-reactivity between peanut and tree nuts is low, it is of high clinical relevance. This study does not address the identity of the cross-reacting proteins between peanut and tree nuts, but it seems likely that sensitivity to Ara h 1 is not the basis for this phenomenon. Teuber et al. [6] demonstrated that IgE cross-reactivity between walnut and peanut is not likely to occur between their respective major allergens Jug r 2 and Ara h 1, despite their 36.1% amino acid sequence identity and belonging to the vicilin seed storage family. Other authors have suggested that 70% amino acid sequence homology is required for immunological cross-reactivity to occur between proteins; structures likely to be shared by peanut and the tree nuts that may show this degree of similarity include the panallergens lipid transfer protein and profilin, along with the other seed storage proteins [27]. Despite being the most probable sources of cross-reactivity between peanut and tree

nuts, the relative concentrations of these proteins, their degree of sequence similarity, their extent of IgE cross-reactivity and the functional significance of these features remain undetermined.

Carbohydrate moieties of allergens may also contribute to IgE cross-reactivity; however, the clinical significance of these antibodies is doubtful and they were not examined in this study. In a report by van der Veen et al. [28], 29 of 32 grass pollen-sensitized individuals had IgE antibodies directed at N-linked carbohydrate groups of peanut proteins, but only one of four patients with a positive history and diagnosis of peanut allergy showed such reactivity. Importantly, the grass pollen-allergic individuals with cross-reactive IgE antibodies to carbohydrate determinants did not exhibit clinical symptoms of peanut allergy, and concentrations of peanut allergens that induced basophil histamine release for these subjects were 1000-fold higher than control pollen allergens.

This study demonstrated a lack of cross-reactivity between peanut and cashew, in contrast to past studies demonstrating IgE cross-reactivity between cashew, and other tree nuts [12, 15]. Cashew allergy is rare among peanut- and tree nut-allergic individuals. The major cashew allergen Ana o 1 is a member of the poorly cross-reactive vicilin seed storage family [6, 29]. Although Ana o 1 shows 45% amino acid sequence similarity with Ara h 1, no common IgE-binding epitopes are identified. The absence of other panallergens may also contribute to the observed lack of IgE cross-reactivity between peanuts and cashew nuts.

Peanut-allergic subjects often have serum IgE antibodies that cross-react with other legumes such as soybean. However, ingestion of such legumes does not necessarily induce a clinical allergic reaction in these subjects, suggesting that this cross-reactivity is clinically irrelevant [5, 30]. In our experience, the overwhelming majority of peanut-allergic subjects also have tree nut clinical allergy, suggesting that the cross-reactivity we have observed between peanut and tree nuts is clinically relevant. Nevertheless, the ability of cross-reactive peanut-specific IgE antibodies to induce a type I hypersensitivity response to tree nut allergens should be tested.

From a taxonomic perspective, peanuts and tree nuts are distantly related. In this study, the observed level of cross-reactivity between peanut, almond, Brazil nut, cashew and hazelnut did not correlate with the plant taxonomic relationship. Peanut and cashew, both belonging to the Rosidae subclass, did not show IgE cross-reactivity. Similarly, a previous study found no evidence of cross-reactivity between peanut and macadamia [8], which also belongs to the same subclass. Unlike grass pollen allergy, where cross-reactivity between different grasses correlates highly with taxonomic classification [31], peanut and tree nut cross-reactivity cannot be predicted safely by a taxonomic relationship.

In summary, this study has provided evidence that cross-reactive allergens are present in peanuts and tree nuts including almond, Brazil nut and hazelnut. This may explain the high frequency of tree nut sensitivity among peanut-allergic individuals. However, unanswered questions remain, including the sites at which cross-reactivity occur, the reasons for differing levels of cross-reactivity and the biological relevance of the cross-reactive IgE antibodies. Inhibition studies and sequence comparisons of different allergens from peanut, walnut and cashew nuts suggest that vicilin seed storage proteins do not play a role in the cross-reactivity of peanut and tree nuts; differences in the relative concentrations of other possible panallergens may be the basis for the different degrees of cross-reactivity observed in this study [27]. Although the findings of the current study contribute to better patient management and diagnosis, further studies allowing the molecular identification and characterization of other potential cross-reactive nut allergens as well as the corresponding IgE-binding epitopes will be a major contribution to improved diagnosis and treatment of peanut and tree nut allergy.

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