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THE HUMAN CELLULAR RESPONSE TO PEANUT *(ARACHIS HYPOGAEA)* AND CROSS-REACTING TREE-NUTS

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TABLE OF CONTENTS

SUMMARY

Peanut and tree-nut allergy are incurable, frequently life-threatening conditions, affecting 1 to 2 percent of the population with evidence suggesting that their prevalence has doubled in the last 5 years. Within the paediatric population, reactions are characterised by both an exacerbation of dermatitis or anaphylaxis, while the latter is the typical reaction among adults. Exposure to minute quantities of peanut and tree-nuts is sufficient to produce anaphylaxis in sensitised individuals and, when compared to other food allergens, anaphylaxis due to these allergens is more likely to be of a life-threatening nature or associated with death. Potential exposures are widespread and frequently hidden, while the coexistence of allergy to multiple tree-nuts or tree-nut and peanut increases further the variety of exposures that might produce anaphylaxis in sensitised subjects. Current strategies for the prevention of reactions are limited to avoidance of exposure and the provision of adrenaline based first aid kits to sufferers. Such strategies have proven at best partially effective, but no cure for nut allergy currently exists. Two studies have provided proof of concept that immunological desensitisation might be possible for peanut allergy but neither study was able to maintain desensitisation, and each was associated with a high prevalence of anaphylactic reactions.

The major allergens of peanut are the seed storage proteins Ara h 1 and Ara h 2. An increasing volume of data exists for tree-nut allergens, within which it has been demonstrated that the major allergens also in general are seed storage proteins. Despite the large quantity of data that exists for peanut and tree-nut allergens with regards to the elicited B cell humoral response, only limited T cell data are available. The presence of the dominant T cell epitopes within allergen immunotherapy extracts is critical for the efficacy of clinical and immunological tolerance induction and hence the availability of such data is crucial to facilitate the development of that therapy.

Within this thesis, data is provided with regards the T cell response to peanut and tree-nuts. A cohort of 32 peanut and tree-nut allergic subjects has been gathered, providing novel information about this disease in its adult form. Of particular note is the high prevalence of multiple nut sensitivity in this cohort. Examination of the humoral immune response to crude peanut extract has confirmed the status of Ara h 1 and Ara h 2 as major allergens, with many other proteins within peanut also demonstrating a high frequency of recognition by peanut allergic sera. Following purification of Ara h 1 and Ara h 2 from unfractionated natural peanut extract, both have been demonstrated to be major T cell allergens. Using overlapping synthetic 20-mer peptides representing the entire Ara h 2 sequence, the major T cell epitope containing regions of Ara h 2 are described, with 62.5 % of Ara h 2 peptide responsive subjects demonstrating reactivity towards Ara h 2 (19-47) and 50% towards Ara h 2 (73-

101). Strong IL-5 cytokine responses were demonstrated towards Ara h 2 (19-38), providing corroborative evidence for this region containing a major T cell epitope. Exploring potential T cell cross-reactive proteins within peanut and hazelnut, it is demonstrated that Ara h 2 is most strongly linked with cross-reactive T cell responses by hazelnut specific T cell lines towards the major allergens of peanut. Such cross-reactivity may occur between Ara h 2 and the major hazelnut allergen Cor a 9, as Ara h 2 (19-38) and Cor a 9 share a region of 50% sequence identity and 70% similarity. This thesis therefore confirms the status of Ara h 1 and Ara h 2 as major allergens, identifies the major T cell epitope containing regions of Ara h 2, and demonstrates that multiple nut sensitivity is associated with cross-reactive T cell responses, within which Ara h 2 is clearly implicated. These data therefore suggest that Ara h 2 is an ideal candidate protein as the basis to future immunotherapeutic approaches to peanut allergy.

DECLARATION

This thesis contains no material that has been accepted for the award of a ıy other degree or diploma in any university or other institution and contains to the best of my knowledge, no material previously published or written by another person, except where due reference is made in the text of the thesis.

Ian Glaspole [redacted] Date 2/4/2004

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CHAPTER 1 LITERATURE REVIEW

1.1 INTRODUCTION

Allergies to peanut and tree nut are incurable and life threatening conditions that affect approximately 1-2% of the general population, whereby exposure to even minute amounts of either may lead to anaphylaxis. They belong to a class of illness known as hypersensitivity reactions, which are those reactions that cause objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal individuals (Johansson *et al.* 2001). Within this group of illnesses, allergic reactions are said to be those "initiated by immunological mechanisms" (Johansson *et al.* 2001). However, because disorders of this nature are frequently characterised by the production of specific IgE to the disease inducing antigen, "allergy" is commonly used only to describe IgE mediated disease.

This review examines the pathology of the allergic response and the key role played in that response by the T lymphocyte. Following a discussion of factors that might induce food allergy, the clinical and immunological features of nut allergy and cross-reactivity within the allergic response are discussed. Finally, allergen immunotherapy, with specific reference to T cell targeted methods that might prove efficacious in food allergic subjects, is discussed.

1.2 THE ALLERGIC RESPONSE

1.2.1 IgE

As noted in the introduction, the presence of specific IgE is the hallmark of allergic disease. A unique serum factor associated with the allergic response was first noted by Prausnitz and Kustner in 1921, who found that serum from a sensitised subject could be transferred to a non-sensitised subject and produce allergic features on challenge (Prausnitz *et al.* 1921). This serum factor was characterized in the 1960s and found to be a novel immunoglobulin, designated immunoglobulin E (Ishizaka *et al.* 1966; Johansson *et al.* 1967). Features unique to IgE include its being in the lowest serum concentration (< 100 ng/ml) of all the secreted immunoglobulin classes and possessing the shortest half life (< 2.5 days) (Galli *et al.* 1999).

IgE is produced by B cells following class switch to production of ε germline transcripts. Two separate signals are required to induce this change, including signals from the cytokines interleukin 4 (IL-4) and IL-13, as well as CD40-CD40 ligand interaction. That IL-4 is crucial in this process has been demonstrated in IL-4 transgenic mice, as well as in mice treated with anti-IL-4 or anti-IL-4 receptor antibodies (Finkelman *et al.* 1986; Tepper *et al.* 1990; Finkelman *et al.* 1991). IL-13 shares many features with IL-4, including its ability to activate

B cells and cause IgE class switch (Punnonen *et al.* 1993). Both cytokines activate the Iε promoter region of B cell DNA, which induces the activation of Stat6, along with NFκB and B cell-specific activator protein (BSAP), which are required to act coordinately with Stat6 to induce the production of ε germline transcripts (Delphin *et al.* 1995; Thienes *et al.* 1997; Oettgen 2000). Studies using anti-CD40 antibodies, CD40 ligand protein, and knockout mice for both components have confirmed the importance of the interaction of CD40 with CD40 ligand in the activation of IgE production (Jabara *et al.* 1990). CD40 exists on the B cell surface membrane while CD40 ligand is expressed predominantly on T lymphocytes but may also be found on the surface of allergic effector cells including mast cells, basophils, and eosinophils; the role of these effector cells in the induction of IgE production is unclear, as IgE production occurs in their absence (Ha *et al.* 1986; Takeishi *et al.* 1991).

1.2.2 IgE Receptors

1.2.2.1 FcεRI

IgE possesses two receptors which, apart from sharing the same ligand, differ significantly in structure and function. Fcε receptor I (FcεRI), as it exists in human antigen presenting cells (APC), mast cells and basophils, is made up of an α chain and two γ chains, while a separate form possessing an additional β chain is also present on mast cells and basophils (Galli *et al.* 1999). Functionally, the α chain interacts with IgE via its Cε2 and Cε3 regions; the γ chain, which sits predominantly within the cytoplasm, transmits activation signals; and the β chain, which traverses the cytoplasmic membrane four times, acts as a signal amplifier (Malveaux *et al.* 1978; Sterk *et al.* 1982; Blank *et al.* 1989; Jouvin *et al.* 1995; Lin *et al.* 1996; Daeron 1997). FcεRI is expressed constitutively in high levels on mast cells and basophils and its role in IgE mediated responses is fundamental; in studies where the molecule is destroyed, systemic anaphylaxis has proven impossible to induce (Dombrowicz *et al.* 1993). In mice however, FcεRI-independent anaphylaxis may also be possible via stimulation of FcγRIII through IgG1 binding (Miyajima *et al.* 1997). Only several hundred of the approximately 1×10^5 IgE molecules on the mast cell or basophil cell surface need be cross-linked to produce cell activation (Dembo *et al.* 1979). FcεRI expression is upregulated by IgE itself, along with IL-4 and IL-13, and is downregulated by co-aggregation with the immunoreceptor tyrosine-based inhibitory motif bearing receptors FcγRIIB, GP49 molecules – members of the inhibitory receptor superfamily – and mast cell function-associated antigen (Ono *et al.* 1996; Takai *et al.* 1996; Sihra *et al.* 1997; Scharenberg 1999; Yamaguchi *et al.* 1999).

1.2.2.2 FcεRII

FcεRII, also called CD23, occurs in two forms. FcεRIIa is found on B cells, while FcεRIIb is only found on B cells stimulated with IL-4, along with other cells including monocytes, eosinophils, platelets, T cells, dendritic cells (DC) and some thymic epithelial cells (Yokota *et al.* 1988). A number of roles have been proposed for FcεRII but none has been proven. They include regulation of IgE synthesis, B cell activation and homing, IgE dependent phagocytosis, cytokine like actions, and antigen focusing within which IgE mediated enhancement of antibody responses occurs (Fujiwara *et al.* 1994; Payet *et al.* 1999; Tsicopoulos *et al.* 2000). FcεRII is also able to bind a number of other molecules, including CD21, the receptor for complement component C3b and the Epstein-Barr virus; interactions with FcεRII on T lymphocytes may act to enhance T cell responses to specific antigen.

1.2.3 Effector Cells of the Allergic Response

1.2.3.1 Mast cells

Mast cells are derived from CD34+ haematopoeitic progenitor cells, and mature following relocation from bone marrow to their typical distribution beneath epithelial surfaces and near or within peripheral nerves (Kirshenbaum *et al.* 1992). Mast cells can be classified according to the presence or absence of mast cell chymase, although phenotypic commitment within this classification does not appear fixed (Galli 1990). The chymase negative variety are associated with the seasonal migration of mast cells to the nasal mucosa seen in sufferers of seasonal rhinitis and may therefore represent inducible cells activated with allergic disease (Bentley *et al.* 1992). Their development and survival is dependent on interaction with their major growth factor, stem cell factor (SCF), and its receptor c-kit on the mast cell surface (Galli *et al.* 1994). The latter is biologically active in both soluble and cell surface bound forms; cells upon which it is found include stromal cells, endothelial cells and fibroblasts, suggesting that mast cells may be locally activated within peripheral tissues (Costa *et al.* 1996). Other cytokines contributing to mast cell development include the T_H2 cytokines such as IL-4 as well as IL-3 (Madden *et al.* 1991).

1.2.3.2 Basophils

Basophils are also derived from $CD34^+$ progenitor cells, but mature in bone marrow and are located predominantly within the circulation, where they represent the smallest cell fraction (Galli *et al.* 1999). Their major developmental factor is IL-3 (Lantz *et al.* 1998). Apart from their effector function, it is speculated that basophils are important early sources of IL-4,

which may play a part in skewing the T lymphocyte response towards a T_H2 phenotype; this is discussed in detail below.

1.2.3.3 Eosinophil and mast cell derived mediators

Mast cell and basophil activation occurs following cross-linking of IgE bound to FcεRI, as described above. Other routes to activation include biological or physical means, including IL-1, IL-3, IL-8, and GM-CSF, substance P, complement C3a and C5a, platelet-activating factor (PAF), hyperosmolarity, and physical stimuli such as vibration, heat and cold (Bachert 2002). Systemic symptoms occurring via non-IgE mediated effector cell activation are termed anaphylactoid, while IgE mediated activation produces anaphylaxis. Mediators released from basophils and mast cells are classified according to whether they are preformed when cell activation occurs, or are newly synthesized upon activation. Histamine is a key preformed mediator, and basophils and mast cells represent its major source within the circulation and peripheral tissues, respectively (Galli *et al.* 1999). Its actions include direct effects at the time of the allergic response as well as immunomodulatory and proinflammatory actions. The direct actions of histamine are mediated predominantly by the H_1-receptor in allergic disease, and include increased vascular permeability, smooth muscle contraction, vasodilatation and flushing, mucus secretion and pruritus. Other receptors also exist for histamine, but their role in the allergic response is minor or unclear (Bachert 2002). Histamine receptors are expressed on basophils, mast cells and eosinophils; the action of histamine on the former two cell types appears inhibitory while, in low concentrations, it appears to upregulate eosinophil chemotaxis and receptor expression (Bachert 2002). Histamine also acts on vascular endothelial cells to upregulate expression of adhesion molecules and hence cellular diapedesis (Saito *et al.* 1996). Histamine receptors are expressed on DC, and recently it has been demonstrated that immature DC cultured in the presence of histamine differentiate into DC2 type cells, enhancing the production of T_H2 cells and hence leading to an allergic phenotype (Caron *et al.* 2001). Finally, histamine can act directly on lymphocytes, and appears to inhibit T_H1 responses (Lagier *et al.* 1997).

Tryptase is a preformed mediator that is a neutral protease and exists in both α and β forms. It is specific to mast cells, and is the major enzyme stored in cytoplasmic granules. Tryptase plays several important roles within the allergic response; *in vivo* studies in experimental animals and in humans show that tryptase inhibitors block allergen-induced early- and late-phase bronchoconstriction and airway hyperresponsiveness (Krishna *et al.* 2001). Tryptase acts on prekalikrein and generates kinins which in turn produce enhanced vascular permeability, a feature of anaphylaxis, although the extent to which tryptase contributes to anaphylaxis has not been measured (Imamura *et al.* 1996). Other significant preformed mediators include proteoglycans, which assist in storage of preformed mediators within the

cell and modulate the kinetics of mediator release upon degranulation, and lysophospholipase, which is released from basophils as well as eosinophils and forms Charcot-Leyden crystals (Galli *et al.* 1999). The major newly synthesized mediators include the cyclooxygenase and lipoxygenase metabolites of arachidonic acid, being prostaglandin D2, I2, A1 and E1 derived predominantly from mast cells, and leukotriene C4, D4 and E4, derived from both cell types (Galli *et al.* 1995). The effects of these mediators during anaphylaxis are mainly on vascular tone and permeability. Their relative contribution is not well established but, based on a study using a canine model of ragweed induced anaphylaxis within which each pathway was blocked pharmacologically, it appeared that only the cyclooxygenase derivatives mediated hypotension and reduced cardiac output (Mink *et al.* 1999). The other major classes of mediator released from mast cells and basophils upon degranulation are cytokines, predominantly TNF-α, along with many others, such as IL-4, IL-5, IL-6, IL-8, IL-13, βFGF, and VPF/VEGF (Galli *et al.* 1999).

1.2.3.4 Eosinophils

Eosinophils represent the other major effector cell of the allergic response, but because they constitutively express only low amounts of FcεRI, as opposed to basophils and mast cells, their role in food induced anaphylaxis is likely to be limited (Bochner *et al.* 1991). They are derived from $CD34^+$ progenitor cells and mature within the bone marrow before receipt of chemotactic signals leads to their relocation to the circulation and peripheral tissues, predominantly along mucosal surfaces. Two key signals include IL-5, which leads to their relocation to the peripheral circulation, and eotaxin, which appears to be the major chemokine for eosinophils (van Rensen *et al.* 2001; Menzies-Gow *et al.* 2002). Eosinophils express numerous receptors and their activation is therefore likely to occur in response to a large number of stimuli. Upon activation, they release a number of mediators, including major basic protein, eosinophil cationic protein, eosinophil derived neurotoxin, eosinophil peroxidase, lysosomal hydrolases and lysophospholipase (Galli *et al.* 1999). Additionally, they secrete lipid mediators, predominantly the leukotrienes, as well as numerous chemokines and cytokines involved in cell recruitment and augmentation of the immune response (Galli *et al.* 1999).

1.2.4 Phases of the Allergic Response

1.2.4.1 Early phase reaction

The early phase reaction of the allergic response refers to those features that develop typically within the first few minutes of effector cell activation, and reflects the release of preformed mediators (Figure 1.1). It appears that this phase is dependent on mast cell

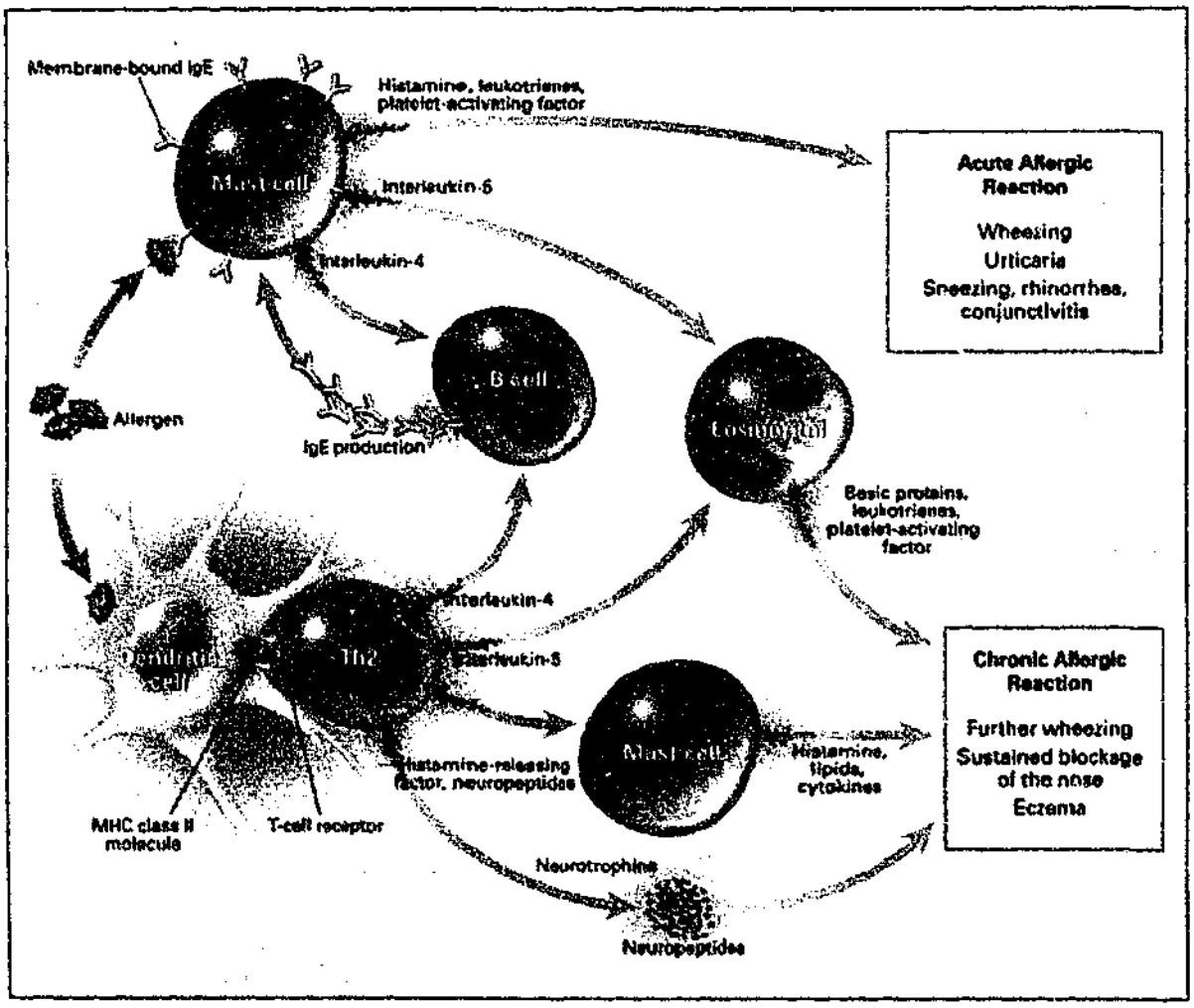


Figure 1.1 Pathways Leading to Acute and Chronic Allergic Reactions.

From: Kay 2001.

activation, as passive cutaneous anaphylaxis cannot be induced in mast cell deficient mice, but can be in those same mice with reconstituted mast cell numbers (Wershil *et al.* 1987; Lantz *et al.* 1998). Debate exists as to the role of mast cells in the early phase response associated with food induced anaphylaxis as tryptase is generally not detectable following food induced anaphylaxis (Sampson *et al.* 1992). However, histamine is also difficult to detect, suggesting that these findings may in fact reflect problems with assay sensitivity (Lin *et al.* 2000). Upon cross-linking of IgE on the FcεRI receptor on the mast cell surface, immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic surface of the β and γ chains of the FcεRI receptor causes activation of Lyn and subsequent phosphorylation of Syk (Beaven *et al.* 1996). At least two cascades are activated through Syk: the protein kinase C cascade for regulation of secretion, and the mitogen-activated protein kinase cascade for the regulation of phospholipase A2 (Beaven *et al.* 1996). In addition to these pathways, FcεRI receptor cross-linking also leads to activation of phosphatidylinositol 3-kinase (Beaven *et al.* 1996). These cascades lead to the release of preformed, granule-associated histamine and tryptase and the membrane-derived lipid mediators leukotrienes, prostaglandins, and platelet-activating factor (Kay 2001). These mast-cell mediators have a critical role in anaphylaxis, rhinoconjunctivitis, and urticaria, but are of less relevance to chronic allergic conditions such as dermatitis and asthma (Kay 2001).

1.2.4.2 Late phase reaction

The late phase reaction refers to a recrudescence of allergy symptoms that occurs 6 to 12 hours following allergen exposure and reflects the actions of newly synthesized mediators, cytokines and chemokines producing activation and tissue infiltration by effector cells, particularly eosinophils (Kay 2001). In the context of food induced anaphylaxis, this is thought to correlate with rebound anaphylaxis, whereby anaphylactic symptoms may recur, typically within 12 hours of the initial insult. Studies using peptide immunotherapy have shown that late phase reactions need not follow an early reaction, strongly suggesting that such responses are not IgE dependent (Larche 2000). However, late phase reactions occur in mice sensitized entirely through passive cutaneous transfer, suggesting IgE dependent mechanisms also exist (Martin *et al.* 1993).

1.3 T CELL CONTROL OF THE ALLERGIC RESPONSE

1.3.1 The T_H1:T_H2 Paradigm

In 1986, Mosmann *et al.* described the existence in mice of two distinct T helper cell subsets, which they termed T_H1 and T_H2 cells, based on their profile of cytokine production (Mosmann *et al.* 1986). In response to antigenic or non-specific stimulation, cells of a T_H1 phenotype produced IL-2, IFN-γ, IL-3 and GM-CSF, while T_H2 cells produced IL-4 and IL-5 but not IFN-γ (Mosmann *et al.* 1986). Subsequently, the spectrum of T_H2 cytokines has grown to now include IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 (Abbas *et al.* 1996).

T_H1 and T_H2 cells appear to represent the two ends of a continuum of cytokine responses and each is able to suppress the other's upregulation (Maggi *et al.* 1992). The existence of these subsets is based predominantly on murine data but, as is discussed below, has since been shown to exist in humans as well (Robinson 2000). Prominent within the CD4+ T cell subset of humans, but also present within mice, is a third T helper cell type termed T_H0, which produces both IL-4 and IFN-γ (Paliard *et al.* 1988). The functional relevance of the T_H0 cell to allergic disease is uncertain, particularly as the function of IL-4 is abrogated by the presence of IFN-γ; it may represent a precursor for the other T cell phenotypes (Paliard *et al.* 1988; Kamogawa *et al.* 1993).

Additional sources of T_H2 cytokines have been delineated since the original description by Mosmann *et al.*, including various effector cells, $CD8^+$ T cells and NKT cells. Mast cells, basophils and eosinophils are all able to produce IL-4 and probably function to enhance the inflammatory cascade (Plaut *et al.* 1989; Brunner *et al.* 1993; Moqbel *et al.* 1995). Arguments that these cells play a role in the development of an IgE mediated response appear somewhat circular, with the production of IL-4, from mast cells and eosinophils at least, being dependent on the cross-linking of already established IgE bound to FcεRI on their cell surface. A role for basophils in the development of an allergic phenotype is discussed below.

Type 1 and type 2 cytokine producing subsets have also been found amongst $CD8^+$ cells isolated from human lymphocytes (Maggi *et al.* 1994). In general, such T cells produce a type 1 cytokine pattern, but when cultured in the presence of IL-4, may be induced to produce a type 2 cytokine pattern (Coyle *et al.* 1996). Whether these cells are relevant to the development of allergic disease is unclear. The clearest linkage to allergic disease can be made with virus induced asthma exacerbations, where the $CD8^+$ T cell response is important in the clearance of the viral pathogen. Certain viruses, such as respiratory syncytial virus, induce lung eosinophilia and bronchial hyper-reactivity and $CD8^+$ T cells have been shown to be critical within that response (Schwarze *et al.* 1999). Additionally, IL-5 producing $CD8^+$

T cells can induce airway eosinophilia with stimulation by viral peptide (Coyle *et al.* 1995). Other $CD8^+$ T cell responses appear inhibitory of T_H2 responses, and their role in the development of allergic disease remains ill-defined. NKT cells are discussed below.

Apart from their pattern of cytokine secretion, efforts to distinguish T cell phenotypes have also been made on the basis of cell surface markers, including co-stimulatory molecules, cytokine receptors and chemokine receptors. CD30 is a member of the tumor necrosis superfamily and has been suggested as a marker for T_H2 cells, having been found to be associated specifically with T_H2 cytokine secreting clones from human subjects allergic to grass pollen (Del Prete *et al.* 1995). Cow's milk specific T cell clones from subjects allergic to cow's milk also express higher levels of this cell surface molecule; CD30 expression returned to normal levels with the spontaneous development of tolerance (Schade *et al.* 2002). However, CD30 has also been found to be upregulated on T cells in response to *Mycobacterium tuberculosis* infection, and hence is not exclusively expressed by T_H2 cells (Munk *et al.* 1997). ST2L, a transmembrane protein similar in structure to the IL-1 receptor, has been reported as another cell surface marker specific for T_H2 cells. In research also encompassing studies of the IL-18 receptor as a T_H1 cell surface marker, murine and more recently human peripheral blood mononuclear cells (PBMC) and T cell lines (TCL) have shown a significant positive correlation between the presence of these markers and the presence on intra-cellular cytokine staining of IL-4 and IL-5 or IFN-γ respectively (Chan *et al.* 2001) Additionally, mice deficient in this molecule have impaired T_H1 responses while antibodies to IL-18R can induce T_H2 responses (Xu *et al.* 1998; Xu *et al.* 1998; Yoshimoto *et al.* 1998). However, IL-18 may have some role in the enhancement of T_H2 responses as well: IL-18 has recently been found to act as a cofactor to induce IL-4, IL-10 and IL-13 as well as IFN-γ production in T cells in the presence of anti-CD3 monoclonal antibodies (mAb), while administration of IL-18 alone or with IL-2 *in vivo* significantly increased serum IgE levels in mice (Hoshino *et al.* 2000). Finally, the IL-12 receptor has also been put forward as a marker for T_H1 cells. T cells cultured in the presence of unopposed IL-4, lose IL-12Rβ2 expression, while those cultured in the presence of IFN-γ retain IL-12Rβ2; each is associated with the development of T_H2 and T_H1 responses, respectively (Rogge *et al.* 1997; Szabo *et al.* 1997).

Certain chemokine receptors are linked to each T cell subtype. For T_H1 cells, these are CCR5 and CXCR3, while for T_H2 cells, CCR3, CCR4 and CRTH2 are the most strongly associated receptors (Sallusto *et al.* 1997; Bonecchi *et al.* 1998; Cosmi *et al.* 2000). CCR5 and CXCR3 have been found in high levels on the T cells located at sites of inflammation in the T_H1 mediated diseases rheumatoid arthritis and multiple sclerosis (Qin *et al.* 1998; Balashov *et al.* 1999). Both CCR3 and CRTH2 are expressed on other leucocytes associated with allergic inflammation, while CCR3 and CCR4, may be weakly expressed on the alternative

phenotype, or not expressed at all, and as such these molecules are not ideal discriminators of cell polarity (Cosmi *et al.* 2000; Chtanova *et al.* 2001).

In addition to cell surface markers, various intra-cellular transcription factors are upregulated in each phenotype. One such marker is Stat-6, a transcription factor that upregulates the IL-4 gene and hence development of a T_H2 phenotype (Takeda *et al.* 1997). Recently, several studies have shown that such a phenotype can develop in Stat-6 or IL-4 receptor deficient mice, raising the possibility of other pathways to T_H2 differentiation (Finkelman *et al.* 2000; Jankovic *et al.* 2000). Other transcription factors associated with T_H2 development include GATA-3, c-Maf and JunB, the latter selectively acting on IL-4 transcription, while GATA-3 acts on several T_H2 cytokines (Asnagli *et al.* 2001). GATA-3 is able to induce T_H2 cytokine expression in the absence of Stat-6 and IL-4R, suggesting it may act as a downstream mediator of Stat-6 (Asnagli *et al.* 2001). Additionally, GATA-3 can be activated independently of Stat-6, via CD28 activation, perhaps providing the pathway to T_H2 cytokine expression seen in Stat-6 and IL-4 deficient mice (Rodriguez-Palmero *et al.* 1999). T_H1 cytokine expression is strongly linked to Stat-4 expression although, as for Stat-6 and T_H2 cytokine expression, some T_H1 cytokines, such as IFN-γ, remain detectable in Stat-4 deficient mice (Murphy *et al.* 2000). T-bet is a T_H1 specific transcription factor that may be the T_H1 equivalent of GATA-3: it strongly induces the production of T_H1 cytokines and its expression is mutually exclusive of GATA-3 (Szabo *et al.* 2000).

1.3.2 Physiological Role of T helper Cell Subsets

The differing cytokine profiles of the two T cell subsets confer differing actions on the immune system. T_H1 cells, via IFN-γ production, activate macrophages and stimulate IgG production, CD8+ T cell activation, and recruit other inflammatory cells. Intra-cellular organisms and pathogens destroyed by phagocytosis are most susceptible to this type of response (Chtanova *et al.* 2001). Whether a T_H2 cytokine response is an absolute requirement in the immune response to any infection is debated, although this response is visible during helminthic infestation, and may be the optimal response to extracellular metazoan infections (Romagnani 1997). A T_H2 cytokine profile is also seen in the protective immune response following fetal implantation (Romagnani 1997). Each of the T_H2 cytokines has numerous actions with certain actions being of fundamental importance. As has been discussed, IL-4 is critical for the B cell class-switch to IgE production (Vercelli *et al.* 1989). The predominant action of IL-5 is on eosinophils, causing their production and mobilization (Tavernier *et al.* 2000). IL-13 has actions very similar to those of IL-4 but has also been shown to act directly on end-organs such as the bronchial wall to induce mucus production and smooth muscle contraction (Corry 1999). Both types of T cell response act to augment

the production of cells of the same phenotype and inhibit the opposite phenotype (Maggi *et al.* 1992).

1.3.3 Pathological Associations of T helper Cell Subsets

Aberrant immune responses of each type are associated with disease. A T_H1 profile is the characteristic feature of auto-immune diseases such as rheumatoid arthritis and multiple sclerosis (Qin *et al.* 1998; Balashov *et al.* 1999). That allergic disease is associated with a T_H2 response is based on several sources of data. Numerous allergic diseases have demonstrated a T_H2 predominant response at the site of end-organ involvement or within peripheral blood, including asthma, allergic rhinitis, and eczema (Robinson *et al.* 1992; Christodoulopoulos *et al.* 2000; Tamaki *et al.* 2001). For example, T helper cells within broncho-alveolar lavage fluid from asthmatic subjects demonstrates a T_H2 pattern and IL-4 and IL-5 mRNA levels correlate with measures of disease severity such as FEV_1 (Robinson *et al.* 1993). Additionally, successful immunotherapy of these diseases has been associated with the replacement of this response with a T_H1 predominant response (Wachholz *et al.* 2002). Further evidence is provided by allergen challenge, which in allergic individuals produces the infiltration of the site of challenge with T_H2 cells (Varney *et al.* 1993). The T_H2 response appears to be the dominant response amongst food allergic subjects as well, co-culture of T cell clones from nut allergic subjects with allergen demonstrating T cell proliferation and T_H2 cytokine profile while non-allergics demonstrate a T_H1 profile (Higgins *et al.* 1995).

In summary, since the recognition of the two poles of the CD4+ T cell subset by Mossmann *et al.* in 1986, much has been established, including their cytokine profile, cell surface and intra-cellular markers, and their roles in the normal and aberrant immune response. The T_H2 predominant response has been established as that associated with allergic disease. However, much remains to be established, with the fundamental question as to why such an aberrant response should occur still unresolved. This question, with specific reference to food allergy is the basis to the next section.

1.4 PRIMING OF THE IMMUNE RESPONSE IN FOOD ALLERGY

1.4.1 Normality: Oral Tolerance to Food Allergens

The typical response of the gut to both commensal organisms and dietary antigens is that of oral tolerance, whereby there is the induction of systemic non-responsiveness to the putative antigen, accompanied by local, mucosal IgA mediated immunity (Nagler-Anderson 2001).

Several investigators have demonstrated within mice that following oral administration of soluble protein antigens, there is a marked reduction in T cell proliferative responses and antibody responses after systemic challenge (Mowat 1985). Within human subjects, Husby *et al.* have demonstrated that oral delivery of a neoantigen produces suppression of delayed type hypersensitivity responses, without the reduction in antibody secretion characteristic of mice (Husby *et al.* 1995). However, as for other mucosal surfaces, the mechanism by which oral tolerance occurs remains fundamentally unexplained.

1.4.2 Gastro-Intestinal Immune System Anatomy

1.4.2.1 Physical barriers

Gastro-intestinal tract (GIT) immune defences consist of physical barriers to antigen penetration, along with innate and acquired immune responses (Figure 1.2). Although only one cell layer thick, enterocytes efficiently exclude antigens from penetrating the gut wall via a number of mechanisms, including intercellular tight junctions, anti-microbial peptides called defensins, secretory IgA, mucins and trefoil peptides (Nagler-Anderson 2001). Furthermore, the filamentous brush border glycocalyx on enterocyte microvilli which functions to allow digested nutrients to gain access to the body is relatively impermeable to macromolecules or bacteria (Nagler-Anderson 2001).

1.4.2.2 Antigen presentation

Soluble proteins such as food allergens are able to penetrate the epithelial barrier, both via enterocytes, as well as via M cells, which transport antigen from the follicle associated epithelium to APC in the underlying gut associated lymphoid tissue (GALT), where the innate and acquired immune systems are encountered. M cells appear to have an antigen-sampling role, and neither standard enterocytes nor M cells act as APC, despite the latter expressing MHC class I and in some instances MHC class II molecules (Nagler-Anderson *et al.* 2001). As to which APC preferentially interact with M cells, interestingly in the region beneath M cells where initial antigen encounter is said to occur, known as the M-cell pocket, macrophages and DC are present in only scanty numbers, while the area is packed with B and T cells. B cells in this region express high levels of CD80/CD86, while juxtaposed T cells express CD40L, suggesting cognate interactions in the antigen-presenting process (Farstad *et al.* 1994).

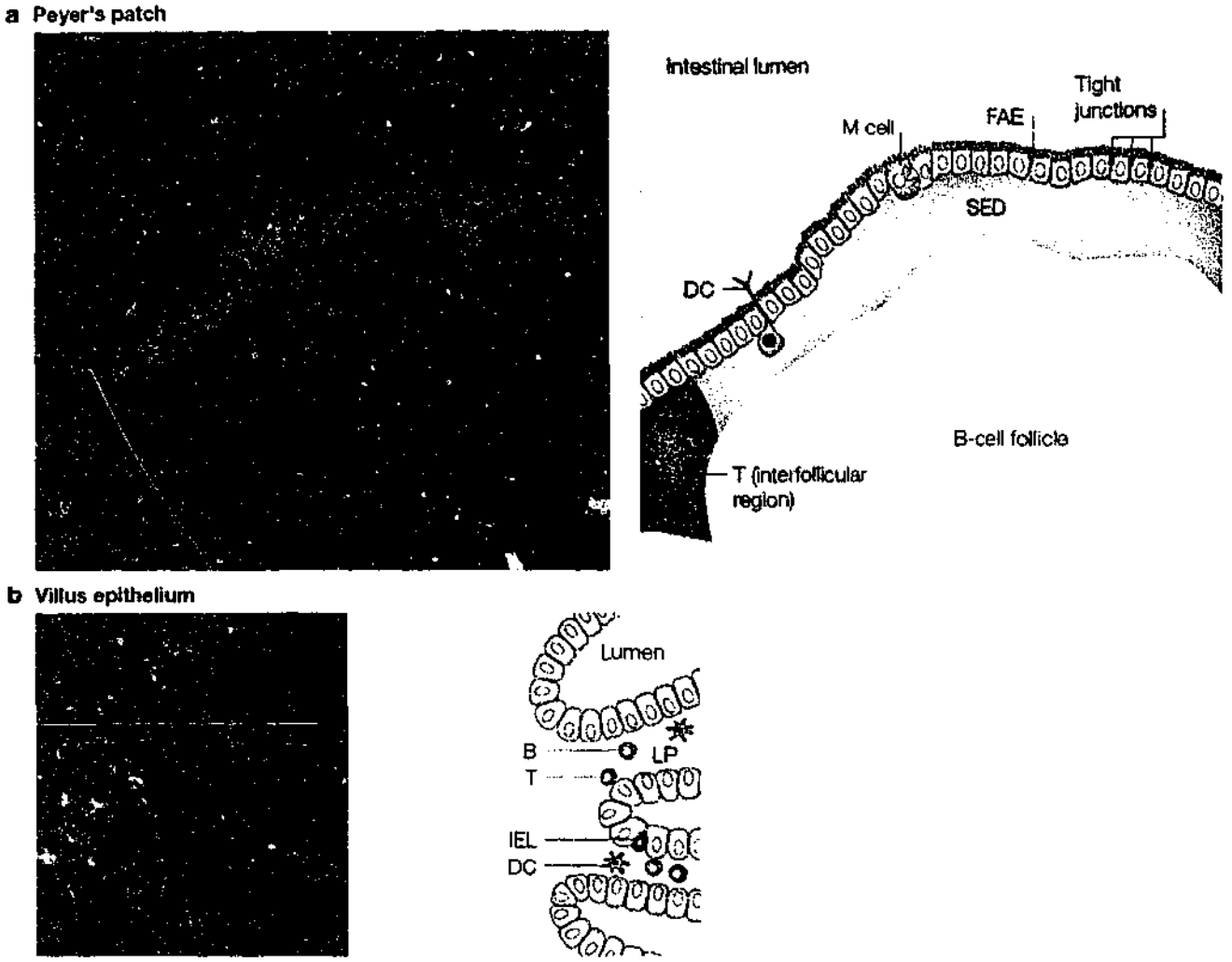


Figure 1.2 Gut-associated lymphoid tissue. (Nagler-Anderson 2001)
The gut-associated lymphoid tissue (GALT) is divided into inductive (Peyer's patch) and effector (lamina propria) sites. In the immunofluorescence images shown, T cells are green and dendritic cells (DCs) are red.
a. Similar to lymph nodes, the Peyer's patch contains B-cell follicles. The follicle-associated epithelium (FAE) covers the dome of the Peyer's patch. Transport across the epithelium occurs through both specialized M cells and by DCs that extend their processes through epithelial tight junctions. DCs are present in both the subepithelial dome (SED) and the interfollicular T-cell areas and are visible as stellate (red) cells in these sites.
b. The intestinal villus epithelium contains an unusual population of intraepithelial lymphocytes (IELs) which reside above the epithelial basement membrane. Scattered lamina propria (LP) effector cells — T cells (T), IgA-secreting B cells (B) and DCs — are located within the villi.

DC and macrophages are abundant within the GALT below the follicle associated epithelium and are also present within the GIT lamina propria. Rather than relying on delivery of antigen via M cells, DC may extend dendrite-like processes through epithelial tight junctions and sample antigen directly (Rescigno *et al.* 2001).

1.4.2.3 Immunoanatomy

Gastro-intestinal lymphoid tissue has been divided into inductive and effector sites (lamina propria). Inductive sites are made up predominantly of Peyer's patches, which comprise at least five B cell follicles with germinal centres surrounded by areas that contain predominantly T cells (Nagler-Anderson *et al.* 2001). They are primarily located in humans within the distal ileum where they number approximately 250 in adulthood (Brandtzaeg *et al.* 2001). Effector sites within the lamina propria are more loosely organised and comprised principally of IgA secreting plasma cells and memory T-effector cells (Farstad *et al.* 2000). Additional to these two sites, a further site for GALT is within the crypt lamina propria where T cell progenitors are found. These cells are thought to be important for the thymus independent development of intra-epithelial lymphocytes, although a clear role for these cells with regards to oral tolerance is yet to be described (Suzuki *et al.* 2000; Hayday *et al.* 2001).

The relative importance of GALT in the development of mature T cell responses has been seriously questioned recently, following a recent study demonstrating that μMT mice, lacking B cells, M cells or Peyer's patches, were still able to generate normal systemic T cell responses to oral antigen, that were identical to those demonstrated by wild type mice (Alpan *et al.* 2001; Spahn *et al.* 2001). It seems likely therefore that other lymph node sites possess this function; one suggested site is the mesenteric lymph node (Nagler-Anderson *et al.* 2001). In a mouse model within which Peyer's patches or Peyer's patches plus mesenteric lymph node development were selectively disrupted, oral tolerance, on the basis of IFN-γ and delayed type hypersensitivity responses, could still be induced in mice lacking Peyer's patches, but not in those mice lacking both (Spahn *et al.* 2002). However, the prevailing milieu within the Peyer's patch in fact seems to be suppressive of immune responses, T cells entering the Peyer's patch becoming chemokine hyporesponsive, and demonstrating suppressed responses to mitogens such as ConA and PMA (Kellermann *et al.* 2001). Furthermore, cytokine expression within Peyer's patches is predominantly of a T_H2 profile whereas within peripheral lymph nodes, a T_H1 profile exists (Kellermann *et al.* 2001). Whether these findings can be extrapolated to human allergic immune responses remains to be determined.

1.4.2.4 Mucosal IgA

The secretion of mucosal IgA has long been recognised as an important gastrointestinal defence mechanism, and appears to act both via preventing antigen entry across the gastrointestinal epithelium, as well as actively transporting antigen that has penetrated the epithelium back to the epithelial surface membrane (Robinson *et al.* 2001). IgA is secreted as a dimer which binds to a secretory piece allowing its transport across the gastrointestinal mucosa. Up to 90% of B cells within the gastrointestinal lamina propria are involved in IgA secretion (Brandtzaeg *et al.* 2001). Despite their abundance in the GALT, evidence exists that in mice, up to 50% of IgA secreting B cells within the lamina propria are derived from the peritoneal cavity (Brandtzaeg *et al.* 2001). Class-switch of B cells to an IgA secreting phenotype is dependent on signals received by transforming growth factor receptor-β receptor type II on the B cell surface, with T_H2 cytokines then controlling the B cell development into an IgA secreting plasma cell (van Ginkel *et al.* 1999; Cazac *et al.* 2000). Interestingly, class-switch to an IgA secreting phenotype appears to occur, at least in mice, through both T cell dependent and independent means, with the latter appearing to occur via signalling from lamina propria stromal cells acting on precommitted IgM secreting cells (Fagarasan *et al.* 2001).

1.4.3 Sites and Mechanisms of Aberrant Immune Function in Food Allergy

1.4.3.1 Antigen and adjuvant

Factors influencing the determination of the T cell phenotype are summarised in Figure 1.3. Of these, antigenic structure is likely to be a highly important. This is highlighted by the fact that, despite the abundance of different food types eaten by man, the great majority of all food allergies in Western society are produced by only one of seven foods (Sicherer 2002). Several references highlight the fact that allergens tend to possess enzymatic activity, and it has recently been demonstrated that Der p 1, through its cysteine protease activity, is able to cleave CD25 and produce diminished IFN-γ production, thereby favouring IgE synthesis (Ghaemmaghami *et al.* 2001). However, if bee venom phospholipase A_2 (PLA_2) is rendered enzymatically inactive, it remains capable of producing an allergic response and hence enzymatic activity is by no means mandatory for a protein to be allergenic (Wymann *et al.* 1998). Antigen tertiary structure contributes to the nature of the T cell response, Akdis *et al.* demonstrating that correctly folded PLA_2 induces high IL-4, IL-5 and IL-13 levels in T cell culture, whereas non-refolded recombinant PLA_2 ($rPLA_2$) induced more IFN-γ and IL-2, along with higher proliferative responses. This difference appeared to relate to the involvement of different APC, as $rPLA_2$ lost its IgE binding epitopes and hence was not presented by B cells, instead exclusively by monocytes (Akdis *et al.* 1998). Both allergic and

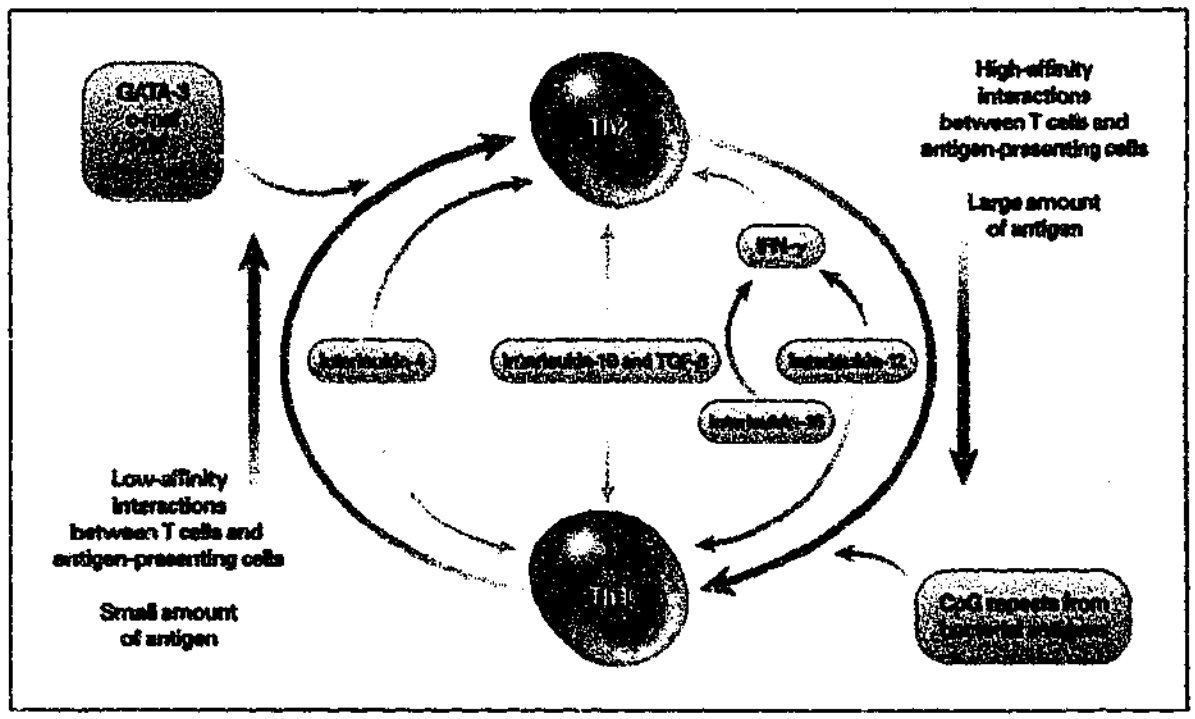


Figure 1.3 Immunologic and Cellular Factors Regulating the Expression of Th1 and Th2 Cells.

From: Kay 2001.

non-allergic subjects recognise the same T cell epitopes of PLA_2, and hence features specific to the primary structure of allergens do not appear to play a role in determining the phenotype of the T cell response (Blaser *et al.* 1998).

The major allergens of peanuts are glycoproteins, raising the possibility that their carbohydrate side chain is important in determining the immune response that these proteins induce. That carbohydrate side chains are important for the generation of T cell responses has been speculated upon by several authors as one of the causes for the frequently seen finding of T cell responses to natural but not recombinant antigen (Dudler *et al.* 1995; Wurtzen *et al.* 1999). Carbohydrate interactions with the T cell to provoke an immune response may potentially occur through both MHC dependent and non-MHC dependent means. Dudler *et al.* were able to generate HLA-DR restricted T cell clones responsive to natural but not deglycosylated PLA_2, and could abrogate proliferative responses to the natural protein in a dose-dependent fashion using anti-carbohydrate antibodies (Dudler *et al.* 1995). On the other hand, Corinti *et al.* were able to generate CD8+ T cell clones responsive to *Parietaria* pollen extract which showed diminished proliferative and cytokine responses when carbohydrate was destroyed, yet for which MHC restriction could not be demonstrated (Corinti *et al.* 1997). The importance of carbohydrate side chains in the generation of the T_H2 response has been highlighted by several authors, but in greatest depth by Okano *et al.* who have demonstrated carbohydrate mediated effects in responses to schistosomiasis, bee venom and Japanese cedar pollen (Velupillai *et al.* 1997; Okano *et al.* 1999; Okano *et al.* 1999; Okano *et al.* 2001; Okano *et al.* 2001). With regards allergic disease, the latter group have demonstrated that T_H2 proliferative and cytokine responses are greatly diminished when carbohydrate is removed from the sensitising allergen (Okano *et al.* 2001). They have ascribed adjuvant activity to carbohydrate side chains in producing the T_H2 response by demonstrating that in a mouse model of sensitisation to schistosoma egg antigen, T_H2 profile cytokine responses were absent and IgE production greatly diminished when carbohydrate side chains were destroyed with periodate (Okano *et al.* 1999). Additionally, T_H2 responses could be induced to neo-glycoproteins produced when the carbohydrate side chain of *Shistosoma mansonii* egg antigen was linked to human serum albumin (Okano *et al.* 2001).

Mechanisms by which carbohydrate might influence the T cell response have been speculated to include direct activity as a T cell epitope, alteration of peptide structures to induce antigenicity, or binding to carbohydrate cell surface receptors to increase efficiency of antigen presentation. The latter has been somewhat explored and dismissed in the case of the major Japanese cedar pollen allergen Cry j 1 by Okano *et al.*, who demonstrated that mAb to glycosylated immunogenic Cry j 1 peptides bound with equal avidity to deglycosylated forms and that blockade of the mannose receptor did not alter responses to glycoprotein (Okano *et al.* 2001).

Differences in APC type may also explain the observed differences in T cell phenotype produced by changes in antigen concentration. Low dose PLA_2 used to stimulate T cells produced high levels of IL-4 production, whereas high concentrations produced a trend towards increased IFN-γ production (Secrist *et al.* 1995; Carballido *et al.* 1997). As B cells present low concentration antigen 1000-10000 times more efficiently than macrophages, it is likely this effect on IL-4 production is influenced by their involvement at low concentrations, and indeed this effect could be diminished or enhanced by lowering or raising B cell numbers in the total APC pool (Secrist *et al.* 1995). These dose dependent differences in T cell response appear to translate *in vivo* to the resultant B cell response, as mice immunised with minute doses of PLA_2 or keyhole limpet haemocyanin produced high quantities of IgE, while this was diminished amongst mice immunised with higher doses (Kolbe *et al.* 1995).

Similar dose effects have been described for food antigens. Two methods of inducing oral tolerance have been described and include either persistent feeding with low doses of antigen or feeding with high dose antigen. Sensitised mice continuously fed micro-doses of ovalbumin for 14 days show a reduction in T_H2 but not T_H1 responses (Wu *et al.* 1998; Marth *et al.* 2000). Anergy of both types of responses has been shown for high dose antigen feeding models (Friedman 1996; Mowat *et al.* 1996; Kang *et al.* 1999). In a further study, mice fed the highest dose of ovalbumin or fed continuously at low doses showed suppressed immune responses, while those fed intermittent low dose or intermediate doses of ovalbumin showed enhanced immune responses (Marth *et al.* 2000). Such tolerance appears to be associated with enhanced production of both transforming growth factor-β (TGF-β) and IL-10 (Marth *et al.* 2000; Tsuji *et al.* 2001). The extent to which tolerance occurs is reduced in primed as opposed to naïve mice (Leishman *et al.* 2000).

Adjuvant environmental structures delivered to the immune system at the time of antigen exposure may also lead to an altered immune response to that antigen. Changes in the quantities of such environmental exposures that have occurred across the latter part of the twentieth century form the basis to the "hygiene hypothesis" for the increasing prevalence of allergic disease (Strachan 1989). These signals are described below with regards their interaction with antigen presenting cells.

1.4.3.2 Physical defences

For an antigen to invoke an allergic response, it must be able to reach those cells that determine the nature of that response. Compromise of the physical defences of the gut, for example via GIT infection or inflammation, such that penetration of antigens is increased may therefore allow greater antigen sampling by immune cells and potentially the

development of altered immune responses via, for example, changes in antigen concentration. In a biopsy study of sensitised rats, increased penetration of the gut wall by ingested antigen was demonstrated, relative to non-sensitised subjects (Majamaa *et al.* 1996). This feature may be mediated via IgE bound to FcεRII on gastrointestinal mucosal cells, as the administration of intra-intestinal anti-CD23 antibody abolishes this finding, while it can be transferred amongst sensitised animals using whole sera, but not IgE depleted sera (Yang *et al.* 2000). Peanut allergens are also remarkably resistant to the acid pH of the stomach and are therefore likely to remain intact as they enter the small intestine where absorption would occur (Astwood *et al.* 1996).

1.4.3.3 Antigen presenting cells

Dendritic cells have claims to being pivotal in the polarisation of immune responses to environmental antigens, on both anatomical and functional grounds. Their location at epithelial surfaces leads them to be among the first immune cells to interact with antigens, whereupon they contribute to both the innate and acquired immune response (Mellman *et al.* 2001). However, it is their functional capability as professional APC to stimulate naïve T cells that allows them to drive these cells into distinct classes of effectors, while their additional ability to create T regulatory cells whose primary functions are to suppress the immune response adds further to their orchestration of the immune response (Weiner 2001). In that setting, aberrant function of DC is likely to be an important contributor to the generation of the allergic response.

DC are derived from bone marrow $CD34^+$ stem cells, and several phenotypes have been distinguished experimentally to date using a variety of stimulation methods (Caux *et al.* 1992; Sallusto *et al.* 1994). The two major precursor phenotypes that have been described include myeloid DC, so called because of their monocytic origins, and lymphoid DC derived from plasmacytoid cells (Liu 2001; von Bubnoff 2001). The latter cells are found in the thymus and, in mice, in secondary lymphoid organs.

According to their location DC vary in their function and possess corresponding changes in their expression of cell surface molecules (Novak *et al.* 1999). Immature DC are located primarily at epithelial surfaces where they are attracted to sites of inflammation via their chemokine receptors CCR1, CCR2, CCR5, and CCR6. Their function at these sites is antigen uptake, a function for allergens likely to be facilitated by FcεRI expression, particularly so in atopic individuals, within whom this molecule is expressed on DC in high quantities (Lanzavecchia *et al.* 2001). Following antigen uptake, DC migrate to draining lymph nodes via the expression of different chemokine receptors including CCR4, CCR7, CXCR4, SLC, and ELC, where they interact with T cells in the paracortical areas (Novak *et*

al. 1999). As this process occurs, antigen uptake ability is lost and instead MHC class I and II molecules, to this point expressed but located predominantly intracellularly within late endocytic compartments, are loaded with peptide and relocated to the cell surface. Additionally co-stimulatory molecules such as CD80 and CD86 are upregulated (von Bubnoff 2001).

Mature DC have been classified in one model as DC1 and DC2 subtypes respectively (von Bubnoff 2001). DC1 cells, myeloid DC, carry the CD11c myeloid cell surface antigen and are able to induce T_H1 polarisation via IL-12 production, while DC2 cells, lymphoid DC, are $CD11c^-$ and induce T_H2 polarisation through unknown mechanisms (Rissoan *et al.* 1999). An alternative view is that DC1 cells are able to induce both forms of p · isation, depending on the amount of IL-12 that they produce (Kalinski *et al.* 1999). This view has been strengthened by recent evidence that murine DC can be induced to produce signals leading to production of T cells of both T_H1 and T_H2 phenotypes, depending on whether they are presenting helminthic or bacterial antigens, with bacterial induced IL-12 production inhibited by the presence of helminthic antigens (Cervi *et al.* 2004). Production of IL-12 is dependent on maturation signals received by the DC as well as the local milieu of cytokines and other chemical mediators (Lanzavecchia *et al.* 2001). Maturation signals include substances interacting with Toll-like receptors such as microbial lipopolysaccharide, CpG DNA or dsDNA, TNF-α, IL-1β or CD40 ligation: these patterns of signals have been collectively described within the danger model of immune responses, described above (Matzinger 1994). The presence of other substances within the local milieu can also influence DC function, with IFN-γ or IL-4 enhancing IL-12 production, while IL-10 and TGF-β, histamine, PGE2, vitamin D3, extracellular ATP and measles virus inhibit its production (Caron *et al.* 2001; Lanzavecchia *et al.* 2001; Mazzoni *et al.* 2001).

Two models for tolerance induction have been described for DC. A discrete subpopulation of gastrointestinal DC have been identified that take up apoptotic epithelial cells and constitutively migrate to local draining lymph nodes and present environmental or self-antigen to T cells; in the absence of costimulatory signals expressed on their cell surface, T cells may be anergised and this may be one route through which tolerance ensues (Mellman *et al.* 2001). Alternatively, IL-10 production may also be a critical factor in tolerance induction; in a mouse model of ovalbumin hypersensitivity, pulmonary DC secreting IL-10 could induce tolerance in adoptive transfer experiments, while their absence in IL-10 deficient mice lead to an inability to induce tolerance (Akbari *et al.* 2001; Weiner 2001). However, in the gut the main inhibitory signal appears to be TGF-β. Akbari *et al.* have shown that DC from mesenteric lymph nodes of mice tolerant of ovalbumin produce TGF-β and drive the proliferation of regulatory T helper type 3 cells (T_H3) cells producing further TGF-β, while those derived from the pulmonary tree produce IL-10 predominantly and

stimulate the production of T regulatory type 1 cells (T_R1) cells, producing further IL-10 (Akbari *et al.* 2001).

It remains to be elucidated whether DC function, and in particular their secretion of the inhibitory cytokines IL-10 and TGF-β, is disturbed in allergic disease. However, DC derived from Peyer's patches preferentially stimulate the differentiation of T_H0 clones towards a T_H2 phenotype, suggesting additional events occur to produce allergic disease (Everson *et al.* 1997).

1.4.3.4 Regulatory and suppressor T cells

Following antigen presentation there are a number of cell types that have been described which might regulate the subsequent immune response, including CD4+CD25+ T cells, T_H3 cells, Tr1 cells, γδ T cells, anergic T cells, and CD4+ NKT cells. Within the context of food antigens, it is the T_H3 cell that is best described as being relevant, with its TGF-β secretory function having being shown to be important in both immune suppression within oral tolerance models as well as the class switch of B cells to an IgA secreting phenotype (Coffman *et al.* 1989; Chen *et al.* 1994). However, there is a dearth of information with regards regulatory T cell function in the development of allergic responses, and the aberrant function of each listed cell type could be implicated in allergic disease.

T_H3 cells are generated with oral administration of antigen, and TGF-β is critical in their function (Chen *et al.* 1994). Both CD4+ and CD8+TGF-β secreting cells have been described within the gut in mice and rat models (Chen *et al.* 1994). CD4+ TGF-β secreting cells also secrete variable amounts of IL-4 and IL-10, but their function seems independent of these cytokines, as TGF-β secreting cells retain suppressive properties in their absence (Powrie *et al.* 1996). Such cells have been shown to be suppressive of inflammatory responses, including T_H1 mediated disease such as experimental auto-immune encephalomyelitis (EAE) (Chen *et al.* 1994). Their relationship to the suppression of T_H2 responses and allergic disease has not yet been demonstrated. Indeed, in a mouse model of oral tolerance induced by feeding with high dose ovalbumin, tolerance could still be achieved in TGF-β null mice, suggesting other mechanisms for oral tolerance also exist (Barone *et al.* 1998).

The CD4+CD25+ T cell has been described as being of particular importance in the control of the T cell response, with roles established for the control of auto-immunity, anti-tumour activity, and allograft tolerance, together with *in vitro* evidence for their ability to prevent the expansion of other T cell subsets, and *in vivo* functionality in preventing T cell activation (Read *et al.* 2001). These cells have been demonstrated to be of thymic lineage, and may also be peripherally induced (Akbar *et al.* 2003; Bach *et al.* 2003). They appear to require

direct cell contact for both their activation by APC and subsequent immune suppression of T cells. How this is mediated is unclear, with evidence to suggest a role for TGF-β as well as via interaction with CTLA-4 on the CD4+CD25+ T cell surface membrane (Bach *et al.* 2003). Their role in oral tolerance has recently been established in a murine model for ovalbumin sensitivity, whereby transgenic mice for the ovalbumin TCR were fed ovalbumin and showed a proportional and absolute increase in functionally immune suppressing CD4+CD25+ T cell numbers in GIT draining lymph nodes (Zhang *et al.* 2001). Evidence for CD4+CD25+ T cell function within allergic disease is limited to their role in asthma, whereby it has been demonstrated that they may in fact upregulate a T_H2 response; antigen induced eosinophil recruitment could be abrogated by CD4+CD25+ T cell depletion in a murine model, while neutrophil and T cell recruitment was enhanced, and T_H2 type cytokine production in the airway was diminished (Suto *et al.* 2001). This confusing paradox in function highlights the need for further research into CD4+CD25+ T cell function in allergic disease.

As IL-4 has been shown to be of critical importance in directing T cell precursors towards a T_H2 phenotype, the $NK1.1^+$ T cell subset has been focused upon, given its function as a major source of early IL-4 production (Le Gros *et al.* 1990; Swain *et al.* 1990; Seder *et al.* 1992; Yoshimoto *et al.* 1994). However, the data with regards the importance of these cells in the generation of *in vivo* T_H2 responses have been contradictory. While mice deficient in NK1.1 cells do not produce early IL-4 mRNA upon anti-CD3 treatment and no IgE in response to anti-IgD antibodies, they are still able to produce T_H2 responses to ovalbumin and keyhole limpet hemocyanin, have no impairment in resistance to parasite infection, nor inhibition of immediate cutaneous hypersensitivity (Yoshimoto *et al.* 1995; Brown *et al.* 1996; von der Weid *et al.* 1996). More recently, it has been shown that NKT cells expressing the semi-invariant TCR Vα14 can be induced to produce IL-4 by stimulation with α-galactoseramide or analogues of that molecule, with consequent ablation of T_H1 associated diseases, such as type 1 diabetes and EAE (Miyamoto *et al.* 2001; Sharif *et al.* 2001). However, the effect of stimulation of NKT cells through this route appears minor with regards to the development of T_H2 responses to standard stimuli, mice deficient in these cells being shown to produce IgE to both parasite infection and ovalbumin immunisation. Indeed, IFN-γ production is also induced by this form of stimulation and, perhaps secondary to this phenomenon, the predominant effect on the T_H2 response, as for the T_H1 response, appears to be inhibitory (Cui *et al.* 1999; Nakayama *et al.* 2001).

γδ T cells appear important in the production of IgA as mucosal synthesis is lowered in mice deficient in these cells (Fujihashi *et al.* 1996). They are usually CD4-CD8-, and are skewed towards the production of a T_H1 cytokine profile with antigen stimulation (Hayday *et al.* 2000). Their role within the gut appears to be in regulation of the response by αβ T cells to

infection; mice deficient in the δ chain are susceptible to gut pathology due to an excess response to pathogens (Roberts *et al.* 1996). These cells are disproportionately abundant in young animals and it has been suggested they play a role in defining subsequent T_H1 responses of the αβ T cell pool, perhaps thereby reducing the susceptibility to allergy (Hayday *et al.* 2000). However, no clear association between aberrant γδ T cell function and food allergy has yet been made.

Anergic $CD4^+$ T cells appear also to have a suppressor effect on T cell responses, through mechanisms mediated via the APC (Vendetti *et al.* 2000). In studies using anergic human T cells cocultured with immature DC and responder T cells, the upregulation of costimulatory molecules and HLA-DR was nearly completely abolished on immature DC, and apoptosis was induced in mature DC. This resulted in the suppression of proliferative responses amongst the responder T cell population (Frasca *et al.* 2002).Whether such cells exist within the gut, or play a role in food allergy, remains undefined.

T_R1 cells appear less relevant to the control of the GIT immune response, having not been demonstrated within that location to date. Functionally however, they behave in a manner that bares close resemeblance to current models of tolerance and successful immunotherapy. They are produced through repetitive stimulation of naïve CD4+ cells *in vitro* in the presence of IL-10; following antigen stimulation, these cells can inhibit both T_H1 and T_H2 responses, a function for which IL-10 secretion appears critical (Weiner 2001); (Read *et al.* 2001).

1.4.3.5 Cytokine milieu

Within the milieu surrounding naïve T cells at the time of antigen presentation, many factors have been shown to influence the nature of subsequent polarisation. Two cytokines that appear particularly important, based upon murine data, are IL-4 and IL-12. Initial evidence with regards IL-4 and IL-12 demonstrated that they were able to act as autocrine growth factors, while suppressing the proliferation of the opposite cell type (Fernandez-Botran *et al.* 1988; Gajewski *et al.* 1988). Subsequently, two groups have reported that IL-4 is crucial in the development of murine T_H2 cells (Le Gros *et al.* 1990; Swain *et al.* 1990). Swain *et al.* found that murine lymphocytes stimulated to proliferate by IL-2 in the absence of IL-4, produced IL-2 and IFN-γ without IL-4 or IL-5, while those cells cultured in the presence of both IL-2 and IL-4 progressively lost the ability to secrete IFN-γ, and developed the ability to secrete IL-4 and IL-5 (Swain *et al.* 1990). Le Gros *et al.* were able to induce IL-4 production following stimulation with anti-IgD, if IL-4 and IL-2 were present in the culture system (Le Gros *et al.* 1990). Evidence that these findings represent polarisation of the T cell response rather than the selective development of different T cell subsets stems from experiments with pigeon cytochrome C, whereby murine naïve pigeon cytochrome C

specific TCR transgenic T cells that were stimulated with a peptide derived from pigeon cytochrome C in the absence of IL-4 developed a T_H1 phenotype while priming in the presence of IL-4 on the other hand led to cells of a T_H2 phenotype (Seder *et al.* 1992).

The importance of IL-12 in the determination of T cell polarisation is suggested by experiments demonstrating that neutralising antibodies to IL-12 promote the production of T_H2 cells (Manetti *et al.* 1993). Furthermore, Hsieh *et al.* were able to show that naïve ovalbumin specific TCR transgenic T cells differentiated into T_H1 cells when stimulated with ovalbumin presented by matched B cells in the presence of IL-12 (Hsieh *et al.* 1993). On the other hand, the presence of IL-4 at the time of T cell induction inhibits the expression of IL-12Rβ2 on activated CD4+ cells, leading to the loss of IL-12 signalling, preventing T_H1 differentiation (Szabo *et al.* 1997).

The simultaneous presence of IFNγ overrides the above phenomenon and restores functionality of IL-12 expression. IFNγ is able to inhibit the proliferation of T_H2 but not T_H1 cells, by acting through the IFNγRβ which is expressed only by T_H2 cells (Pernis *et al.* 1995). This suggests that the presence of IL-4 is important for the differentiation of T cells towards a T_H2 phenotype, but this must be in the absence of IFNγ as otherwise a T_H1 phenotype will exist.

1.4.3.6 Genetic factors

Multiple genetic polymorphisms have been proposed as predisposing to allergic disease. These have involved genes encoding the FcεRI β subunit, IL-4, IL-13, HLA molecules, CC chemokines and their receptors and molecules involved in lineage commitment and signalling pathways (Toda *et al.* 2002). Such studies, typically based on genome wide screening followed by candidate gene characterisation methods, have been difficult to replicate across different populations and have therefore frequently failed to carry significant weight. The most recently reported of these associations was that of the ADAM33 gene for asthma (Van Eerdewegh *et al.* 2002). ADAM33 is one of the ADAM genes, which represent a subfamily of the zinc-dependent metalloproteinase superfamily (Shapiro *et al.* 2002). These molecules are important in the maintenance and repair of the extracellular matrix and it seems unlikely therefore that they play a significant role in the causation of food allergy (Shapiro *et al.* 2002). A genetic influence on the incidence of peanut allergy has been strongly suggested in studies of monozygotic and dizygotic twins (Sicherer *et al.* 2000). HLA class II genetic polymorphisms have been reported for peanut allergy in one study, with four class II genotypes, DRB1*08, DRB1*08/12 tyr 16, DQB1*04, DPB1*0301, found to be increased among peanut allergic subjects or their families; the first three were found to be increased specifically in peanut allergic subjects (Howell *et al.* 1998). However, peanuts

contain multiple allergens, each with multiple T cell epitopes, such that it is likely that the presence of many rather than single HLA polymorphisms determines the presence of peanut allergy.

1.4.3.7 T cell receptor

Just as diminished TCR signalling through lowered antigen concentration is associated with a T_H2 response, so too are changes to the TCR that diminish affinity with its MHC-peptide ligand (Pfeiffer *et al.* 1995; Faith *et al.* 1999; Blander *et al.* 2000; Boyton *et al.* 2002). Specific T cell receptors are not associated with particular functional responses, as T cell clones can be driven in different directions (Hsieh *et al.* 1992). However, changes to peptides at their contact residues with the T cell receptor, so called altered peptide ligands, can lead to alterations in cytokine profile, with those leading to diminished affinity generally being associated with a T_H2 phenotype, suggesting that TCRs that bind MHC-peptide with low affinity are also likely to lead to this phenomenon (Pfeiffer *et al.* 1995; Faith *et al.* 1999). Indeed, TCR belonging to T_H2 cells appear to have changes in their α chain facilitating low affinity interactions; recently, TCRα chain complementarity determining region 3 has been shown to be elongated in T_H2 cells, this change producing less optimal interaction between TCR and MHC-peptide (Boyton *et al.* 2002).

1.4.3.8 Costimulation

Costimulation is critical in the activation of both T_H1 and T_H2 responses, and in its absence anergy may occur (Gause *et al.* 1997). The major costimulatory system defined to date is that involving CD28 on the T cell, and its ligands CD80 and CD86 on APC. Ligation of CD28 on T cells by CD80 (B7.1) or CD86 (B7.2) enhances the production and stability of several cytokine transcripts, including IL-2, so that costimulation of the T cell response occurs (Lindstein *et al.* 1989). Alternatively, ligation of CTLA4 on the T cell by these molecules provides a negative signal, limiting immune responses (Chambers *et al.* 1997). The kinetics and avidity of binding are such that it is likely that the interaction of CD86 with CD28 dominates in costimulation, whereas the interaction of CTLA4 and CD80 governs negative signalling (Bugeon *et al.* 2000). It has been suggested that CD80- and CD86-mediated costimulation differentially produce a T_H1 and T_H2 response respectively, but this has not been a universal finding (Kuchroo *et al.* 1995; Bugeon *et al.* 2000). In the strongest data relating to this finding, Kuchroo *et al.* administered anti-B7 antibodies to mice with EAE. Anti-B7.1 reduced the incidence of disease while anti-B7.2 increased disease severity in association with alterations in cytokine profile. Administration of anti-B7.1 at immunisation resulted in predominant generation of T_H2 clones whose transfer both prevented induction of EAE and abrogated established disease.

The inducible costimulator molecule (ICOS) is a recently described novel member of the CD28 family expressed on activated cells. ICOS binds to a novel ligand of the B7-like molecule, termed B7 related protein 1 (B7RP-1) (Mages *et al.* 2000). ICOS costimulation has been shown to induce $CD4^+$ T cells to produce a predominantly T_H2 response, characterised by IL-4, IL-5, and IL-10 production, although with some IFN-γ production as well; the latter is enhanced with loss of ICOS costimulation. Unlike CD28, ICOS stimulation does not lead to IL-2 production (Hutloff *et al.* 1999; Riley *et al.* 2001). Further evidence for the importance of ICOS in the determination of the T_H2 immune response stems from the fact that, along with naïve T cells, T_H2 cells maintain its expression, while T_H1 cells have diminished expression (McAdam *et al.* 2000). Within a mouse model of allergic lung inflammation, ICOS blockade lead to a loss of T_H2 effector function, such that greatly diminished levels of T_H2 cytokines were produced (Tesciuba *et al.* 2001). More recently however, ICOS has been demonstrated to be an important regulator of both the T_H1 and T_H2 response, loss of ICOS function abrogating both IL-4 and IFN-γ levels, and impairing class-switch to IgG1, IgG2a and IgE (Greenwald *et al.* 2002).

The suppressor of cytokine signaling (SOCS) family is also likely to be important in the development of a T_H2 response, being expressed in high levels in such cells. SOCS-3 transgenic mice show increased T_H2 responses and features characteristic of asthma, while dominant-negative mutant SOCS-3 transgenic mice, as well as mice with a heterozygous deletion of SOCS-3, have decreased T_H2 development (Seki *et al.* 2003). Disturbances of the function of this molecule may therefore lead to enhanced T_H2 responses.

In summary, there are many levels at which an altered immune response might be induced, leading to a food allergy. These include features unique to the allergen, host structural abnormalities in gastrointestinal defences, cellular abnormalities in APC, regulatory cells or the T lymphocyte itself, and genetic abnormalities producing aberrant molecular function. However, to date no consistent abnormality has been noted in any allergic disease, and it seems likely that a combination of factors must exist for such diseases to occur.

1.5 NUT ALLERGY

1.5.1 Clinical Features

The typical age of onset of all food allergies, including nut allergy, is in early life and the mean for peanut allergy has been reported as 22 months (Sicherer *et al.* 1999). However, unlike milk and egg allergy, which is generally lost between the ages of 3 and 5, nut allergy is likely to be lifelong (Dannaeus *et al.* 1981; Host *et al.* 1995). Recently, it has been suggested that up to 20% of peanut allergic subjects may become tolerant of nuts, but this

has never been assessed in a prospective study (Hourihane *et al.* 1998). As to why nut allergy should persist through life remains unresolved.

Nut allergic subjects are almost universally atopic, with 53 out of 55 subjects being so in Ewan's landmark study in which a large cohort of nut allergic subjects were characterised (Ewan 1996). Not surprisingly therefore, peanut allergic subjects are at greater risk of having current asthma and eczema (Woods *et al.* 2002). A family history of atopy, particularly among maternal relatives is also more common, although other immediate family members having nut allergy increases one's risk of nut allergy only moderately, with the prevalence of peanut allergy amongst siblings of a peanut allergic subject being only 7% (Hourihane *et al.* 1996). Maternal diet, both ante-natal and post-natal during breast feeding, has been described as an additional risk factor as over 80% of initial presentations are said to occur with the first known exposure to nuts (Hourihane *et al.* 1996). However, in trials of maternal exclusion diets, whereby women were prevented from eating nuts during pregnancy and while breast-feeding, the onset of nut allergy appeared to be delayed but not prevented, with the prevalence of nut allergy being similar beyond the first year of life (Hattevig *et al.* 1989; Zeiger *et al.* 1989). Risk factors for life-threatening reactions include the presence of asthma and having had severe reactions in the past (Ewan 1996).

The incidence of food allergy is directly related to exposure within that region, and it is unlikely that peanut allergy posed a significant problem before the turn of the twentieth century within Western society. At that time peanut plants were introduced to cotton farms to allow crop rotation and subsequently peanut has become a ubiquitous food additive as well as being used in a variety of other products including pharmaceuticals and skin care products. Australians consume approximately 1.5 kg of peanut/capita/year (Douglas *et al.* 1997). The utilisation of peanut in a wide range of food products no doubt contributes to the frequency of accidental exposures to peanut, which occur in over 50% of nut allergic subjects over a 5 year period (Sicherer *et al.* 1998). Additionally, only small quantities of peanut need be consumed to produce reactions, with one well constructed study using randomised placebo controlled double blind challenges demonstrating that subjective reactions occur at doses as low as 100 μg peanut, with clear cut objective reactions occuring at a dose as low as 2 mg of peanut (Hourihane *et al.* 1997). Finally, peanut allergens are remarkably hardy, remaining allergenic despite both harsh acidic treatment reminiscent of the stomach and thermal denaturation mimicking cooking (Burks *et al.* 1992; Astwood *et al.* 1996; Koppelman *et al.* 1999). Indeed, as a result of the Maillard reaction whereby, in a process characteristic of the browning of foods, proteins heated in the presence of carbohydrates become glycosylated, IgE binding avidity may in fact be increased (Maleki *et al.* 2000).

Food allergy varies in its presentation according to age. In both adults and children it may produce anaphylaxis, while children may also suffer from subacute or chronic modes of presentation such as atopic dermatitis or gastrointestinal manifestations (Sicherer 2002). Nut induced anaphylaxis is characterised by symptoms developing within minutes to a few hours after ingestion of the food, generally beginning with oral or pharyngeal "tingling" or pruritus (Sampson 2002). Subjects then may develop a sensation of airways tightening, colicky abdominal pain, nausea and vomiting, and cutaneous features such as urticaria, angioedema, and flushing (Sampson 2002). Fatal cases typically develop hypotension, arrhythmia, and progressive respiratory symptoms, while death due to laryngeal oedema is apparently rare (Sampson 2002). Approximately 35% of children with moderate to severe atopic dermatitis have food allergy (Sicherer 2002).

1.5.2 Epidemiology

Estimates of the prevalence of food allergy within the community vary, dependent upon definition. Sensitisation, the presence of specific IgE towards foods unrelated to whether or not the subject has food related symptoms, has an incidence of approximately 6% within the general community (Bjornsson *et al.* 1996). True food allergy, whereby anaphylactic symptoms occur following exposure to a specific food with associated IgE demonstrable within the subject's sera, has been estimated to occur in up to 8% of children and 2% of adults in the Western population (Bjornsson *et al.* 1996). The prevalence of allergy of nuts, including tree nuts and peanuts, in 1999 was reported to be around 1.1% of the US population, based upon a telephone survey using a validated questionnaire, with the prevalence of peanut allergy being approximately 0.6% and tree nut allergy being approximately 0.5% (Sicherer *et al.* 1999). A similar prevalence has been demonstrated for an Australian adult population (Woods *et al.* 2002). Since that time, Sicherer *et al.* have performed a further telephone survey and found, within the paediatric subgroup, that the incidence of nut allergy appears to have doubled since the last survey was done, with corroborative data for that impression provided by Canadian researchers (Kagan *et al.* 2003; Sicherer *et al.* 2003).

The frequency of allergy to individual tree nuts has varied between authors. In Ewan's nut allergic group, peanuts were the commonest cause of allergy (76% of subjects), followed by Brazil nut (29%), almond (23%), hazelnut (21%), walnut (13%) and cashew (5%) (Ewan 1996). In Sicherer's group, peanuts again were the commonest allergen (84% of subjects), but amongst the tree nuts, walnuts produced reactions in over 50% subjects, while Brazil nut was the least common nut to produce reactions (7%) (Sicherer *et al.* 1998). The median age of onset of reactions appears higher for tree nuts than peanuts, the majority of children being sensitised to peanut within the first two years of life, as opposed to five years old for tree

nuts (Sicherer *et al.* 1998). However, the pattern of IgE reactivity to peanuts and tree nuts on RAST appears similar in all age groups, suggesting that rather than reflecting immunological differences, these findings are due to age and region related differences in exposure to different nut varieties (Pumphrey *et al.* 1999).

The incidence of multiple nut sensitivity has varied widely between studies. In Sicherer's randomly sampled telephone survey group, of subjects that could ascribe a particular causal nut to their symptoms, approximately 35% felt they were allergic to tree nuts alone, 43% felt they were allergic to peanuts alone, and only a small proportion of subjects, 4 out of 164 (2.4%) subjects, reported multiple nut sensitivity (Sicherer *et al.* 1999). This figure is significantly lower than that reported within other studies and may reflect the difference in sampling techniques. In the study by Ewan *et al.*, 40% of the 62 consecutive nut allergic subjects seen in an allergy clinic had sensitivity to more than one nut, while 31% of peanut allergic subjects had allergy to tree nuts as well (Ewan 1996). In a similar study of children with either tree nut or peanut allergy, 34% of peanut allergic subjects were allergic to other nuts, while 37% of tree nut allergic subjects reacted to more than one form of tree nut (Sicherer *et al.* 1998).

A single Australian paper exists with regards to out of hospital anaphylaxis presentations to a large adult tertiary referral centre's emergency department (Brown *et al.* 2001). Within that study, food induced reactions were the third most common cause for presentation with anaphylaxis, representing 17% of all causes, behind drug induced reactions then insect induced reactions. Of the food induced reactions, the commonest cause was seafood, with nuts producing only 4 of 22 such presentations. These data are somewhat surprising based on the world data, although they may reflect the exclusion of paediatric cases from the sample population, and lack of consideration of out of hospital deaths due to anaphylaxis. In a Dutch community, the out of hospital incidence of anaphylaxis has been estimated at 3.2 cases per 100000 inhabitants per year, with 5% of those cases being fatal (Sorensen *et al.* 1989). Based on data collected at the Mayo Clinic, approximately one third of such presentations are due to food (Yocum *et al.* 1994). These data can be extrapolated to the Australian population, and suggest that around 600 cases of out of hospital anaphylaxis occur each year, 200 of which are due to food, and ten of which will be fatal (Burks *et al.* 1999).

It has been estimated that nut allergy produces at least 25% of food induced anaphylactic reactions, but the majority of severe reactions (Burks *et al.* 1999). Of seven fatal food induced reactions observed by Yunginger *et al.* in a 16 month period, four were due to peanut with an additional case being due to pecan (Yunginger *et al.* 1988). In 1992, Sampson *et al.* reported that, of 13 cases of fatal or near fatal food induced anaphylaxis collected over a 14 month period, 10 were induced by either tree nuts or peanuts, including 5 out of the 6

fatal cases reported. Of these, peanut induced 3 of the 6 fatal cases, and 1 of the near fatal cases (Sampson *et al.* 1992).

1.5.3 Diagnosis and Current Management of Nut Allergy

Nut allergy is diagnosed on the basis of clinical history in the presence of either specific IgE towards nuts or a positive food challenge (Sampson 2002). History alone, when properly recorded has been shown to carry excellent positive predictive value but in general confirmation of specific IgE is sought to allow proper advice on allergen avoidance, and to exclude other potential allergens (Sicherer *et al.* 1998). Commonly used *in vitro* assays for specific IgE are generally one of the variants of radio allergosorbent testing (RAST), whereby antigen is fixed to a solid phase, incubated with sera, then assayed for the presence of specific IgE via enzyme linked or radio labelled anti-human IgE antibodies. The accuracy of such tests is clearly dependent on the extract used for IgE detection, but although SDS-PAGE profiles of commercially available extracts differ, each carries roughly similar abilities to bind IgE and elicit wheal and flare reactions at skin prick testing (Hefle *et al.* 1995). All such methods carry excellent sensitivity and negative predictive value, but suffer through their lack of specificity and positive predictive value in an unselected population: where the prevalence of food allergy is assumed to be 10%, the positive predictive value of RAST for nuts was only 15% in a study by Sampson et al., making clinical history paramount so as to ascertain the level of pre-test probability (Sampson *et al.* 1997; Clark *et al.* 2003).

Skin prick testing for the diagnosis of nut allergy, in an unselected population, is similarly poorly specific, with Pucar *et al.* showing that 46 of 64 patients with a positive skin prick test demonstrated a negative food challenge (Pucar *et al.* 2001). The specificity of skin prick testing can be improved by increasing the threshold size at which a test is considered positive With the caveat that such a threshold is dependent on both the patient population and extract used, Hill *et al.* have been able to determine a wheal size above which skin prick tests show a perfect correlation with food challenge (Hill *et al.* 2001). Similar adjustments can be made to RAST to provide improved specificity (Sampson *et al.* 1997) Unfortunately, as for RAST, skin prick testing is not 100% sensitive and up to 15% of patients may be expected to have a negative test (Sampson *et al.* 1997; Baker *et al.* 1999). Not surprisingly, RAST and skin prick testing show excellent correlation (Sampson *et al.* 1997). Discrepancies between the two tests, whereby RAST is positive and skin prick testing and clinical history is negative seem likely to reflect binding of IgE to cross-reactive carbohydrate determinants, as preincubation of sera with such molecules before peanut RAST produces near complete loss of positivity (van der Veen *et al.* 1997). Skin prick testing is probably negative in this situation because IgE binding to these carbohydrate

determinants appears to carry poor biologic activity, producing 1000 fold less basophil histamine release than IgE from truly allergic sera (van der Veen *et al.* 1997).

The gold standard for the diagnosis of food allergy is the oral food challenge (Sicherer 2002). Blinded oral food challenges carry a false negative rate of approximately 3% and therefore are always followed by an open challenge. Of course, nut allergy can induce severe anaphylaxis and in general, as avoidance does not carry equivalent nutritional or social deficits, challenge testing is only carried out where less invasive testing has yielded a likely false positive result (Sicherer 2002).

Present day management of peanut allergy is limited to meticulous avoidance of nut containing foods and counselling in the first aid management of anaphylaxis. Avoidance, as discussed above, is extremely difficult over a prolonged period and associated with considerable stress related morbidity (Primeau *et al.* 2000). The mainstay of anaphylaxis management is adrenaline, and self-injectable forms are given to patients routinely as part of emergency treatment kits. However, proper use of such kits amongst food allergic subjects is low, with a recent study showing that of patients stating they had adrenaline with them at all times, only 71% of patients in fact did and only 55% carried a kit within its expiry date (Sicherer *et al.* 2000). Strategies aimed at prevention via alteration of maternal and infant diet have not been shown to alter the prevalence of nut allergy at 24 months of age, and lead to lowered foetal and maternal birth weights (Zeiger *et al.* 1989). Efforts at immunotherapy for nut allergy are discussed below.

1.5.4 Allergenic Proteins within Peanuts and Tree Nuts

1.5.4.1 Peanut allergens *(Arachis hypogaea)*

1.5.4.1.1 Human B cell response to peanut allergens

Peanuts contain many proteins, with the number of distinct bands visible on SDS-PAGE depending on the methodology used; up to 53 bands are visible following high pressure liquid chromatography (de Jong *et al.* 1998). Only two of these proteins warrant classification as major allergens using standard criteria, whereby IgE reactivity occurs within greater than 50% of the peanut allergic population; these proteins are termed Ara h 1 and Ara h 2 (Burks *et al.* 1998). Another five minor allergens have been described. Data regarding the peanut allergens are summarised in Table 1.1.

Ara h 1 belongs to the vicilin seed storage family, one of the most abundant proteins found in legumes. Seed storage proteins are thought to be important energy and nitrogen sources for the seed as it undergoes germination, as well as possessing some protective properties

Table 1.1 Allergenic proteins within peanut (Burks *et al.* 1991; Burks *et al.* 1992; Kleber-Janke *et al.* 1999; Rabjohn *et al.* 1999)

	Ara h 1	Ara h 2	Ara h 3	Ara h 4	Ara h 5	Ara h 6	Ara h 7
Seed storage protein family	vicilin	conglutin	glycinin	glycinin	profilin	conglutin	conglutin
Protein type	7S globulin	2S albumin	11S globulin	11S globulin		2S albumin	2S albumin
Molecular weight	63.5	17	14	36	14	14.5	15.8
Isoelectric point	4.55	5.2		5.5	4.6	5.2	5.6
Allergenicity	major allergen	major allergen	minor allergen	minor allergen	minor allergen	minor allergen	minor allergen
Linear epitopes	23	10	4				
Immunodominant linear epitopes	4	3	1				

against pathogens. Ara h 1 has been reported in studies of differing quality as being an important peanut allergen. Burks *et al.* noted that 6 out of 6 patients carried specific IgE on western blotting towards purified natural Ara h 1, while neither of two controls demonstrated specific IgE (Burks *et al.* 1991). Other groups have not reported the same frequency of IgE binding to this allergen. Clarke *et al.* performed immunoblots of crude peanut extract using sera from 91 subjects, and found Ara h 1 was the most immunoreactive protein within crude extract, with 73% of subjects demonstrating IgE towards it (Clarke *et al.* 1998). Using a recombinant form of Ara h 1 for western blotting, Kleber-Janke demonstrated that 65% of subjects possessed Ara h 1 specific IgE (Kleber-Janke *et al.* 1999). de Jong *et al.* reported that in their population of 14 peanut allergic subjects, Ara h 1 was not a major allergen, with only 35% of peanut allergic subjects recognising this protein on western blotting of crude extract (de Jong *et al.* 1998).

Within linear epitope mapping studies of Ara h 1, 23 different linear IgE binding epitopes were identified, with 4 of the peptides appearing to be immunodominant, being recognised by > 80% patients, and binding greater quantities of IgE than the other epitopes (Burks *et al.* 1997). Structural features that might explain the allergenicity of Ara h 1 have been explored by a number of groups. Using a homology based model, Ara h 1 has been shown to be a trimer with its linear epitopes clustered in two main regions, representing sites of monomer-monomer linking, so that IgE cross-linking might be more likely due to their close proximity (Shin *et al.* 1998; Maleki *et al.* 2000). Linear epitope data may be irrelevant to the *in* vivo immune response, where proteins in their native form retain tertiary structure, such that some linear epitopes may not be accessible to antibody. A space filling model has confirmed this phenomenon for Ara h 1, whereby 10 of the 23 linear epitopes revealed by Burks *et al.* are in fact buried by the tertiary structure (Burks *et al.* 1997). The primary structure of the linear epitopes of Ara h 1 plays a major role in their allergenicity, with single amino acid substitutions with alanine or methionine reducing IgE binding to approximately 1% of that of the native primary structure (Shin *et al.* 1998).

Ara h 2 is a glycoprotein, with 20% of its molecular mass representing carbohydrate side chains. It is a member of the conglutin seed storage family, and migrates as a doublet on SDS-PAGE with an average molecular mass of 17.5 kDa (Burks *et al.* 1992). It has been characterised as a major allergen by Burks' group upon western blotting, on the basis of its reactivity with 6 out of 6 sera tested (Burks *et al.* 1992) Others have confirmed its importance, Clarke demonstrating within that group's large cohort, 71% of subjects possessed specific IgE to Ara h 2 upon western blotting of crude peanut extract. Kleber-Janke *et al.* have demonstrated that 85% of subjects possessed IgE towards their recombinant form upon western blotting, and de Jong's group have shown that approximately 78% of their group demonstrate specific IgE to purified natural Ara h 2 (Clarke *et al.* 1998; de Jong

et al. 1998; Kleber-Janke *et al.* 1999). Linear epitope mapping has demonstrated 10 IgE binding epitopes throughout Ara h 2, with 3 likely to be immunodominant. As for Ara h 1, IgE binding to Ara h 2's 3 immunodominant linear epitopes could be significantly diminished with single amino acid substitutions (Stanley *et al.* 1997). Ara h 2 has 8 cysteine residues and could form up to 4 disulphide bonds. Digestion of disulphide bonds as may occur in the gut, leads to the production of a 10 kDa fragment that contains several of the intact immunodominant IgE binding epitopes, providing a physical basis to their immunodominance (Sen *et al.* 2002).

Ara h 3 is one of a number of minor allergens within peanut, and was first identified using soy absorbed sera, so as to lower the probability of spuriously identifying immunologically irrelevant IgE reactive protein fractions as important (Eigenmann *et al.* 1996). Subsequently, Ara h 3 has been cloned and found to react with sera from 8 out of 18 peanut allergic subjects upon western blotting (Rabjohn *et al.* 1999). Ara h 3 has a molecular mass of 14 kDa and is a member of the glycinin seed storage family. Its cDNA codes for a protein of approximately 57 kDa, suggesting that Ara h 3 is either cleaved from a precursor protein, a typical feature of glycinin family proteins, or is a breakdown product (Rabjohn *et al.* 1999). Using peptides based upon the 57 kDa protein coded for by Ara h 3 cDNA, four IgE binding regions have been found, with one region appearing immunodominant (Rabjohn *et al.* 1999).

Only limited information is available with regards the other peanut allergens and this is summarised in Table 1.2.

1.5.4.1.2 Human T cell response to peanut allergens

Published work evaluating the T cell response to peanut allergens is limited to evaluation of the response to crude peanut extract, and no study to date has demonstrated conclusive evidence for T cell epitopes of any of the major peanut allergens. Several papers have explored PBMC responses to crude peanut and demonstrated that peanut allergic subjects have greater proliferative responses than non-allergic controls, and that such a response is due to expansion of the CD4+ T cell subset and is MHC class II restricted (de Jong *et al.* 1996(Higgins, Lamb et al. 1995); Laan *et al.* 1998). However, the distinction between allergic and non-allergic groups based on proliferative responses is not clear cut and certainly not of any use diagnostically (Higgins *et al.* 1995). Furthermore, proliferative responses are not proportional to the degree of clinical severity, or measures of specific IgE such as skin prick testing or RAST titre (Hourihane *et al.* 1998). Studies of PBMC responses to other allergens have also shown that non-allergic subjects may have proliferative responses to both pure allergens and crude extracts, with the more useful distinction between the two groups being the pattern of associated cytokine secretion (O'Hehir *et al.* 1993; Ng *et*

Table 1.2 Allergenic proteins within tree nuts (references in text).

	Mr	Protein name	Storage family	Protein classification	Allergenicity	Homologous proteins
Hazelnut	18	Cor a 1.04		Bet v 1 homologous pathogenesis related protein	major allergen	Bet v 1
	9	Cor a 8		LTP	associated with anaphylaxis	peach LTP
	47	Cor a 11	vicilin	7S globulin	major allergen	soybean sucrose binding protein
	32			2S-albumin	major allergen	soybean napin 2S albumin
	35		legumin		major allergen	legumin fragment from conifer
	40	Cor a 9	legumin	11 S globulin	major allergen	Ara h 3, almond allergens
	14	Cor a 2		profilin		
Brazil nut	9	Ber e 1		2S-albumin	major allergen	Jug r 1
	29	Ber e 2				
Walnut	14	Jug r 1		2S-albumin	major allergen	
	47	Jug r 2	vicilin		major allergen	Ara h 1; minimal IgE cross-reactivity,despite 70% sequence homology.
	9	Jug r 3		Lipid transfer protein		
		Jug r 4	legumin	11 S globulin		
Cashew	50	Ana o 1	vicilin		major allergen	Ara h 1; linear epitope studies revealed no significant homology between epitopes
	53, 33	Ana o 2	legumin	11 S globulin		
Almond	66				major allergen	
	50				major allergen	
	39-44				minor allergen	
	37				associated with anaphylaxis	
	45		conglutin γ			
	12		2S-albumin			Jug r 1

al. 2002). Information with regards the pattern of cytokine secretion from such studies amongst the peanut literature has suggested that allergic subjects show a T_H2 biased phenotype, Laan *et al.* showing that mRNA expression for IL-4 but not IFN-γ showed a significant positive correlation with proliferative responses (Laan *et al.* 1998).

Studies that have generated T cell clones to crude peanut extract have been limited. de Jong *et al.* generated clones from allergic donors that produced both IL-4 and IL-5 but little or no IFN-γ, whereas clones generated by Higgins *et al.* from allergic subjects produced both IL-4 and IFN-γ, although IL-4 was present in approximately 7 times greater concentrations (Higgins *et al.* 1995; de Jong *et al.* 1996). Although this difference may be ascribed to differences in the time at which supernatants were sampled, the presence of preserved IFN-γ secretion has been described for other food allergens as well, and suggests that both types of responses are present simultaneously in food allergic subjects (Ng *et al.* 2002).

1.5.4.2 Tree nut allergens

1.5.4.2.1 Hazelnut *(Corylus avellana)*

In a study of 65 hazelnut allergic subjects suffering predominantly from oral allergy syndrome, major allergens were located at 18 kDa, 32 kDa, 35 kDa and 47 kDa on IgE immunoblotting under reducing conditions (Pastorello *et al.* 2002). Sequencing of these proteins revealed that the 18 kDa protein, recognised by greater than 95% of subjects, was homologous to the birch *(Betula verrucosa)* pollen major allergen Bet v 1 and the hazel pollen allergen Cor a 1, an unsurprising finding in this group suffering oral allergy syndrome. Four isoforms of Cor a 1 have been described, with Cor a 1.01, 1.02 and 1.03 occurring in hazel pollen and Cor a 1.04 in hazelnut (Luttkopf *et al.* 2002). Of interest with regards cross-reactive responses to peanut is that the 47 kDa protein, also recognised by greater than 95% of sera tested, is a member of the vicilin superfamily to which Ara h 1, and Jug r 2 (a major walnut allergen) also belong. The 35 kDa protein was found to be a legumin and the 32 kDa protein a 2S-albumin, the latter family also including Ara h 2. Sera from seven patients suffering life-threatening reactions to hazelnut were also used for immunoblotting and in all cases showed reactivity to a 9 kDa protein, that was not recognised by subjects with oral allergy syndrome, and found to be a lipid transfer protein on sequencing (Pastorello *et al.* 2002). This protein, subsequently being termed Cor a 8, is not associated with birch pollenosis, and appears particularly associated with anaphylactic reactions to hazelnut (Pastorello *et al.* 2002).

Recently, further hazelnut allergens have been described. Cor a 9 exists within a 40 kDa fraction on a 2D gel, and was recognised by serum IgE from 12 out of 14 hazelnut allergic

subjects. Internal sequencing suggested the protein was an 11S globulin, a family which also contains Ara h 3 as well as allergens from soy bean, almond, and oak (Beyer *et al.* 2002). Cor a 11 is listed within the Allergen Nomenclature Registry but no published literature with regards its characterisation is available.

1.5.4.2.2 Brazil nut *(Bertholletia excelsa)*

Immunoblotting of Brazil nut extract revealed that all 11 subjects with anaphylaxis to Brazil nut tested possessed specific IgE towards a 9 kDa protein, designated Ber e 1, which when sequenced was shown to be a large chain subunit of a 2S albumin, the molecular mass of which the authors predicted to be around 13 kDa (Pastorello *et al.* 1998). While reactivity to this protein was only seen in subjects with anaphylaxis to Brazil nut, reactivity to proteins of higher molecular weight was seen in both symptomatic and asymptomatic subjects, throwing their significance in the causation of Brazil nut allergy into question. Ber e 2 is listed on the Allergen nomenclature registry but no published literature with regards its characterisation is available.

1.5.4.2.3 Walnut *(Juglans regia)*

The major allergen of walnut, Jug r 1, has been produced recombinantly via a cDNA library derived from *Juglans regia* (English walnut) embryo. Following its expression from a cDNA clone, it was found to react with sera from 12 out of 16 walnut allergic subjects (Teuber *et al.* 1998). Furthermore, 7 of the 16 subjects had reduction of IgE binding on ImmunoCAP by greater than 50% when sera were preincubated with rJug r 1. Sequencing of rJug r 1 demonstrated that the protein is a 2S albumin with closest homology to the Brazil nut major allergen Ber e 1. Upon SDS-PAGE, the molecular weight of the recombinant protein was 15-16 kDa, but inhibition immunoblots using rJug r 1 as the inhibitor produced reduced binding to proteins at 14 kDa, 10-12 kDa and 5 kDa within walnut extract (Teuber *et al.* 1998). This suggests that Jug r 1 behaves like other 2S albumin seed storage proteins, and consists of large and small subunits, the 14 kDa protein representing the intact hetero-dimer. Linear epitope studies have demonstrated IgE reactivity to 3 peptides on the large subunit, each containing a common subunit, but none of the small subunit: the contribution of these epitopes to conformational epitopes is unclear, as significant quantities of rJug r 1 reactive IgE remained on dot blots, after preincubation of walnut allergic sera with a peptide containing the IgE reactive common subunit (Robotham *et al.* 2002).

A second walnut major allergen, Jug r 2 has been described by the same group (Teuber *et al.* 1999). This protein, also recognised via screening of a cDNA expression library, was recognised by sera from 9 out of 15 walnut allergic subjects. Sequencing suggested that Jug r

2 is a vicilin seed storage protein, with 70% sequence similarity to Ara h 1. The molecular mass of the mature protein is likely to be 48 kDa, following cleavage of a 170aa region from a 66 kDa proprotein, as occurs with other vicilins. Inhibition studies using recombinant Jug r 2 as the inhibitor demonstrated loss of IgE reactivity to bands at 44 or 47 kDa, depending on the %SDS gel used, and sequencing of this protein revealed it to be Jug r 2 (Teuber *et al.* 1999). Despite both Ara h 1 and Jug r 2 being vicilin seed storage proteins, preincubation of sera with natural peanut extract did not affect binding to Jug r 2 on western blotting of walnut kernel extract, suggesting that there is little IgE cross-reactivity between these two proteins (Teuber *et al.* 1999).

1.5.4.2.4 Almond *(Prunus dulcis)*

The major almond protein is amandin, and accounts for 65% of total aqueous extractable protein. It is a relatively stable protein after food processing procedures such as being subjected to blanching, roasting, autoclaving at high temperatures and microwaving (Venkatachalam *et al.* 2002). Amandin has a molecular weight on native gel of 460 kDa and breaks down to multiple bands under reducing conditions on SDS-PAGE. Major IgE reactive bands are present at 66 kDa (9/14 subjects), 50 kDa (9/14 subjects) and 39-44 kDa (4/14 subjects); less frequently IgE-reactive bands occur at bands found only in total almond extract: at 28 and 37 kDa (1/14 subjects) and 10 kDa (3/14 subjects) (Roux *et al.* 1999; Roux *et al.* 2001)

A recent study using sera from 5 subjects with almond allergy confirmed by open food challenge has shown major IgE reactive proteins at 12 kDa and 45 kDa (Poltronieri *et al.* 2002). N-terminal sequencing of these proteins revealed sequence similarity with English walnut and Brazil nut 2S-albumins, and lupine seed conglutin γ, respectively (Poltronieri *et al.* 2002). In a further small study, IgE specific to proteins lying in the 37 kDa molecular mass range was found to be specific for the presence of allergic reactions; while IgE specific to proteins lying in the 50-62 kDa range was found in subjects without clinical reactions (Pasini *et al.* 2000).

1.5.4.2.5 Cashew *(Anacardium occidentale)*

The major cashew allergen, Ana o 1, was characterised using a recombinant form derived from screening a cDNA expression library (Wang *et al.* 2002). Immunoblotting using sera from 20 cashew allergic subjects revealed Ana o 1 to be a major allergen, with 10 subjects demonstrating specific IgE towards the cloned protein. Inhibition experiments of western blotted cashew extract showed that the recombinant protein inhibited IgE reactivity to a band at 50 kDa. Analysis of the deduced protein sequence suggested that Ana o 1 was a member

of the vicilin seed storage family. However, comparison of its linear epitopes with those of Ara h 1 revealed that, amongst its 11 IgE binding epitopes, none showed significant sequence homology with those reported for Ara h 1. Knowledge of the structure of the conformational epitopes of both allergens would allow a far more robust comparison.

Ana o 2, a member of the legumin seed storage protein family, has also been characterised in a similar manner by the same authors (Wang *et al.* 2003). It is recognised by 62% of cashew allergic subjects' specific IgE and is therefore a major allergen. On SDS-PAGE, it has a major band at approximately 33 kDa and a minor band at approximately 53 kDa. It appears to have seven regions of significant IgE binding in linear IgE epitope mapping studies, several of which were similar in position, but not structure, to IgE linear epitopes of Ara h 3 (Wang *et al.* 2003).

1.5.4.2.6 Others

Apart from those nuts described in detail above, little data exist with regards to the characterisation of allergenic proteins present within other nut species. In a case report describing reactivity to macadamia nut *(Macadamia integrifolia)*, strong IgE binding to a protein of 17.4 kDa was demonstrated (Sutherland *et al.* 1999). Pistachio nut *(Pistacia vera)* proteins recognised by IgE from three subjects experiencing pistachio induced anaphylaxis were demonstrated at 41 kDa, 34 kDa, 52 kDa and 60 kDa, with the 41 kDa protein being recognised by all 3 subjects (Parra *et al.* 1993). Descriptions of reactions to cooked but not raw pecan nut *(Carya illinoinensis)* have characterised the allergenic protein as being 15 kDa in mass, and present only in the cooked food (Malanin *et al.* 1995). Reactions such as this may represent modification in protein structure due to the Maillard reaction as described above (Maleki *et al.* 2000).

1.6 CROSS-REACTIVITY OF NUT ALLERGENS

1.6.1 General Principles of Cross-Reactive Allergic Responses

Cross-reactivity between allergens is defined differently according to one's bias towards clinical or laboratory based medicine (Aalberse 2000). Clinicians use the term to associate allergens to which clinical sensitivity, such as anaphylaxis or rhinitis, is commonly shared, while laboratory based immunologists use the term to associate allergens to which there is a common measurable immunological response, whether or not there is an associated clinical response. Cross-reactivity is classified according to the putative development of the underlying immune response: type 1 reactions are those to which the immune response has been mounted directly (e.g. peanut allergy), while type 2 reactions are cross-reactive

responses with initial sensitisation to an alternative allergen (e.g. oral allergy syndrome) (Sicherer 2001).

As stated above, although IgE and to a lesser extent T cell cross-reactivity has been described for many allergens, this does not always translate to clinical sensitivity. Hence the demonstration of cross-reacting IgE or T cell responsiveness cannot be used with great accuracy to recommend food avoidance and clinicians continue to rely on epidemiological data to make these recommendations. As to why immunological cross-reactivity should be clinically silent, no clear answer exists: possibilities include the fact that methods of determining IgE cross-reactivity such as immunoblotting or ELISA produce relatively linearised proteins so that epitopes that are buried within the native protein become exposed, allowing IgE binding (Aalberse 2000; Lehmann *et al.* 2003). Alternatively, assays used to detect specific IgE can be extremely sensitive, so that low affinity reactions that may not be associated with a physiological response may be detected.

1.6.2 Cross-reactive B cell Responses

The majority of data regarding the immunological basis to allergic cross-reactivity have involved the study of IgE using methods such as inhibition ELISA and inhibition immunoblotting (Caballero *et al.* 1997; Fernandez-Rivas *et al.* 1997; Sutherland *et al.* 1999; Figueredo *et al.* 2000; Hemmer *et al.* 2001). From these studies, a number of conclusions have been possible with regards the structure of cross-reacting proteins. Cross-reacting proteins appear to share similar primary and tertiary structures, giving them a similar fold: cross-reactivity is apparently rare where there is less than 50% identity and in most cases this is greater than 70% (Aalberse 2000). Analysis of protein folds suggests that allergens can be classified into four cross-reacting structural families, although by no means will all proteins of this structure cross-react, so that other factors are also likely to be important. These four structures and examples of each are:

1. antiparallel β-structures, including Ara h 1 and soybean-trypsin inhibitor;
2. antiparallel β-sheet intimately associated with one or more α-helices, examples including β-lactoglobulin and profilin;
3. α+β structures within which the α and β structures are not intimately associated, an example being lysozyme;
4. α-helical structures, examples including seed 2S proteins and parvalbumin (Aalberse 2000).

Protein cross-reactivity may exist at a variety of levels, including between isoforms of proteins within the same source, between similar proteins present through a number of

sources, and between proteins both exogenous and endogenous to the host. Interestingly, despite their close similarity in structure, isoform cross-reactivity is not necessarily the rule, isoforms with a proline substitution showing only limited cross-reactivity to other hazel pollen allergens in one study (Luttkopf *et al.* 2002). The largest body of work is that characterising pan-allergens, IgE reactive proteins which occur across a number of different species. A number of such proteins have been described within food allergy, including the pathogenesis related (PR) proteins such as lipid transfer proteins and chitinases, as well as proteins with other biological functions such as the profilins and seed storage proteins. Additionally certain molecular features of allergens have been explored, such as the carbohydrate side chains of glycoproteins.

The pathogenesis related proteins accumulate in plants in response to environmental stresses such as infection or wounding and include 14 different families of protein. Those implicated in food allergy are listed in Table 1.3 (Breiteneder *et al.* 2000; Midoro-Horiuti *et al.* 2001). The most frequent clinical syndrome associated with cross-reacting IgE antibodies is the oral allergy syndrome, which is typically associated with sensitisation to Bet v 1, the major allergen of birch. This protein is a member of the PR-10 family and has homologues within clinically cross-reactive fruits and vegetables, such as apple, pear and stone fruit, celery, potato and carrot, along with hazel pollen and the N-terminus of the major hazelnut allergen (Breiteneder *et al.* 2000).

Another important group of cross-reactive proteins are the lipid transfer proteins, which form the PR-14 family of pathogenesis related proteins, and are found amongst the *Rosaceae* and *Prunoideae* fruits, and cereals such as maize and rice (Pastorello *et al.* 1999; Pastorello *et al.* 2000; Pastorello *et al.* 2000; Pastorello *et al.* 2001). These proteins are a family of 9 kDa polypeptides, many of which contain 8 conserved cysteines forming 4 disulphide bridges, making them highly resistant to temperature and pH changes (Diaz-Perales *et al.* 2000). Many of these foods are also implicated in the oral allergy syndrome, but where pollinosis does not occur, it appears that it is sensitisation to this class of proteins that is responsible for this form of food allergic disease (Pastorello *et al.* 1999).

Apart from the pathogenesis related proteins many other proteins are implicated in cross-reactive allergic responses towards food (see Table 1.4). Of these, profilins, and the seed storage proteins are the most relevant to nut allergy. Profilins are 12-15 kDa monomeric actin-binding proteins which act to maintain the cytoskeleton of plants and are widespread through the plant kingdom (Breiteneder *et al.* 2000). Within peanut, Ara h 5 is a profilin. These proteins have also been associated with clinical cross-reactivity between hazel pollen and nut, the celery-mugwort-spice syndrome, between Bet v 2 and foods implicated in the oral-allergy syndrome, and between zucchini and pollen (Hirschwehr *et al.* 1992; Kleber-

Table 1.3 Plant food allergens homologous to PR-type proteins

Allergen homologous to	Protein classification	Allergen source/allergen
PR-2 type proteins	β-1,3-Gluconases	Fruits, vegetables
PR-3 type proteins	Basic class I chitinases	Avocado (Pers a 1), chestnut, banana
PR-4 type proteins	Chitinases similar to protein Win proteins	Turnip, elderberry
PR-5 type proteins	Thaumatin-like proteins	Cherry (Pru av 2), apple (Mal d 2), bell pepper (P23)
PR-10 type proteins	Bet v 1-homologous proteins	Apple (Mal d 1), cherry (Pru av 1), apricot (Pru ar 1), pear (Pyr c 1), celery (Api g 1), carrot (Dau c 1), parsley (pcPR), potato (pSTH)
PR-14 type proteins	Lipid transfer proteins	Peach (Pru p 3), apple (Mal d 3), soybean (Gly m 1), barley

Table 1.4 Other plant food allergens with known biologic functions

Protein classification	Allergen source/allergen
Inhibitors of proteases and α-amylases	Soybean: Kunitz trypsin inhibitor family; cereals: trypsin/α-amylase inhibitors; barley: Hor v 1/BMAI-1, CMb, BDP; wheat: CM16; rye: Sec c 1,RDAI-1, RDAI-3; rice: RAP
Peroxidases	Wheat, barley
Profilins	Peanut: Ara h 5; soybean: Gly m 3; celery: Api g 4; pear: Pyr c 4; hazelnut, apple, carrot, lychee, tomato, pumpkin seeds
Seed storage proteins	
2S albumins	Yellow mustard: Sin a 1; oriental mustard: Bra j 1; oilseed rape: BnlI1; Brazil nut: Ber e 1; English walnut: Jug r 1
Vicilins	Peanut: Ara h 1; English walnut: Jug r 2
Conglutins	Peanut: Ara h 2, Ara h 6, Ara h 7
Glycinins	Peanut: Ara h 3, Ara h 4; soybean
Beta-conglycinins	Soybean
Thiol-proteases	Papaya: papain; fig: ficin; pineapple: bromelain; kiwi: actinidin/Act c 1;
	soybean: Gly m 1
Lectins	Peanut: agglutinin

Janke *et al.* 1999; Breiteneder *et al.* 2000; Reindl *et al.* 2000). The seed storage proteins include the albumins and globulins which make up the seed storage proteins of all angiosperms apart from cereals. These proteins, which differ according to their solubility at low and high salt concentrations, are also characterised according to their sedimentation coefficient, allowing their classification as 2S albumins, and 7S and 11S globulins (Pastorello *et al.* 2001). As has been discussed, many of the major allergens of peanut and tree nut are seed storage proteins.

Several groups have demonstrated that IgE cross-reactivity amongst glycosylated allergens is dependent in some instances on the carbohydrate side chain of these molecules, but their relevance to clinically significant IgE responses is debated (Reindl *et al.* 2000; Anliker *et al.* 2001; Hemmer *et al.* 2001). Antibodies to carbohydrate side-chains has been shown to significantly confound the interpretation of *in vitro* assays for specific IgE assays to grass pollens, by producing cross-reactive responses not confirmed clinically or via skin prick testing (Mari *et al.* 1999). That this IgE response is due to cross-reacting carbohydrate determinants has been convincingly explored by van der Veen *et al.*, who showed that in a group of grass pollen allergic subjects with IgE reactivity to peanut yet no clinical history of peanut sensitivity, this reactivity could be abolished nearly completely with use of the grass pollen carbohydrate side chain as an inhibitor (van der Veen *et al.* 1997). Importantly, this group had 1000 fold higher threshold to histamine release in basophil histamine release assays. Cross-reactive binding of IgE specific to birch pollen, mugwort pollen, and celery was also lost by the deglycosylated form (Bublin *et al.* 2003). As discussed previously, not all carbohydrate mediated IgE binding is likely to be immunologically irrelevant. Loss of the carbohydrate component of the major olive allergen Ole e 1 has been shown to diminish both IgE binding as well as histamine release, and similar findings have been demonstrated for the celery allergen Api g 5 (Batanero *et al.* 1996; Batanero *et al.* 1999); (Bublin *et al.* 2003).

1.6.3 Cross-reactive T cell Responses

While much data have been generated with regards cross-reacting proteins within food recognised by IgE, far less data exist for the T cell response to food allergens, with the major focus being upon the determination of the extent of amino acid sequence similarity required to produce responses to peptides derived from different sources. Research using altered peptide ligands has demonstrated that single amino acid substitutions may produce marked alteration in T cell responses (Faith *et al.* 1999). The need for high sequence homology is reiterated in data demonstrating that not all T cell clones are reactive to differing isoforms of allergens, despite only minor amino acid variation (Hales *et al.* 1997; Sparholt *et al.* 1997; Muller *et al.* 1998; Wurtzen *et al.* 1999). Minimal epitope mapping has demonstrated that cross-reactivity occurs where identical amino acid sequences exist (Ohno *et al.* 2000). On

the other hand, cross-reactive T cell responses have been demonstrated for peptides from *D. farinae* and *D. pteronyssinus* where sequence homology was only approximately 81%, for apple and birch where sequence homology was only 50% and for celeriac and birch where sequence similarity was 72% (Fritsch *et al.* 1998; Bohle *et al.* 2000; Hales *et al.* 2000; Bohle *et al.* 2003). Interestingly, for the major latex allergens *(Hevea brasiliensis)* Hev b 1 and Hev b 3, where sequence similarity is only 47%, cross-reactivity is lost (Bohle *et al.* 2000). The data with regards the degree of sequence identity required for cross-reactive T cell responses are thus conflicting, but suggest that provided core epitopes remain unaltered, significant sequence variation elsewhere within the molecule may occur for cross-reactivity to be preserved.

Some data suggest that MHC type may also be important in determining whether cross-reactivity occurs. Kingetsu *et al.* demonstrated the importance of genetic factors in a mouse model of Japanese cedar and cypress sensitivity whereby B10.S (H-2S) mice immunized with Cry j 1 or Cha o 1 generated T cells and antibodies reactive to both antigens, but other mouse species could generate responses specific to only one or neither antigen (Kingetsu *et al.* 2000). However, while *D. farinae* reactive human T cell clones have been shown to be MHC class II restricted, no specific MHC haplotype has so far been demonstrated as being involved in leading to T cell cross-reactive responses (O'Hehir *et al.* 1988; Muller *et al.* 1998).

Only limited work has been performed characterising the TCR of cross-reactive T cell clones. Amongst T cell clones reacting to *Lolium perenne* and *Poa pratensis*, examination of the TCR suggested that while these clones used distinct Jα genes, 9 out of 10 clones examined possessed the Vα13 gene suggesting that cross-reactivity is due in part to restricted usage of TCR Vα genes (Mohapatra *et al.* 1994). On the other hand, Vβ gene usage amongst grass cross-reactive clones did not appear restricted, although appeared so in a group of house dust mite (HDM) reactive T cell clones (O'Hehir *et al.* 1991; Schenk *et al.* 1995).

1.6.4 Cross-reactive immune responses to peanut and tree-nuts

The clinical data presented above have shown that in up to 40% of nut allergic subjects, multiple sensitivities occur. Early studies examining causes for this finding highlighted the presence of IgE cross-reactive proteins in phylogenetically similar plants (Bernhisel-Broadbent *et al.* 1989). However, such *in vitro* associations have not been supported epidemiologically, and more recently attempts to delineate sites of cross-reactivity have focussed on the presence of similar proteins within those species to which sensitivity is commonly shared (Wurtzen *et al.* 1998; Rodriguez *et al.* 2000).

Several studies have demonstrated cross-reactivity between nuts but not sought to characterise cross-reacting proteins beyond rudimentary levels. Peanut has been shown in immunoblotting experiments to completely inhibit in 1 subject and nearly completely in another subject, IgE binding to walnut crude extract (Teuber *et al.* 1999). Inhibition immunoblots using albumin fractions from walnut and hazelnut as inhibitors suggested significant cross-reactivity exists between this fraction and almond conglutin γ, with lesser cross-reactivity also demonstrated for almond 2S albumin (Poltronieri *et al.* 2002). Partial cross-reactivity between hazelnut and macadamia nut has been demonstrated by Sutherland *et al.*, with sub-total diminution of IgE binding to a 17.4 kDa protein with preincubation of sera with hazelnut extract (Sutherland *et al.* 1999). 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 (Hirschwehr *et al.* 1992; Vocks *et al.* 1993; Luttkopf *et al.* 2002) Pistachio nut appears to cross-react with cashew nut, RAST inhibition studies suggesting that IgE binding to pistachio was diminished by preincubation with cashew, although inhibitory concentrations were high (Fernandez *et al.* 1995). de Leon *et al.* have used ELISA with unfractionated nut extracts to demonstrate that IgE cross-reactivity exists between peanut and the tree nuts hazelnut, almond, and Brazil nut, but not cashew (de Leon *et al.* 2003).

B cell data highlighting proteins belonging to the same protein family are discussed in relation to the major tree nut allergens above and demonstrate that membership of similar families need not lead to immunological cross-reactivity. Cross-reactive T cell responses to nut allergens have been explored in only one paper to date. Higgins *et al.* produced five T cell clones from a subject with specific IgE to peanut but not hazelnut. One of the five clones they generated proliferated in response to stimulation with both hazelnut and peanut and demonstrated a T_H0 profile on cytokine analysis (Higgins *et al.* 1995). This suggested that T cells specific to nut do demonstrate cross-reactivity, although whether such cross-reactivity occurs for epitopes associated with a T_H2 response remains to be elucidated. Demonstration of the latter is likely to be important in the development of T cell vaccines for nut allergy.

1.7 ALLERGEN IMMUNOTHERAPY

1.7.1 Introduction

First performed in 1911 by Noon and Freeman for the treatment of pollinosis, allergen immunotherapy (SIT) refers to the administration of gradually increasing doses of allergen extract to an allergic subject in an attempt to ameliorate allergic symptoms (Noon 1911; Bousquet *et al.* 1998). It is clearly efficacious in the treatment of hymenoptera venom

allergy, as well as for inhalant allergen induced rhinitis or asthma (Douglass *et al.* 1997). However broader indications for diseases such as eczema or for allergens such as the nuts, other food allergens, and latex have been limited by the induction of serious adverse reactions during therapy, along with a lack of efficacy (Oppenheimer *et al.* 1992). In this setting, an enhanced understanding of the mechanisms of SIT has become imperative, so that modified approaches to therapy that permit treatment of these indications become possible (Bousquet *et al.* 1998; Rolland *et al.* 2000).

1.7.2 Efficacy and Current Indications for Allergen Immunotherapy

The most detailed analysis of the efficacy of SIT remains the meta-analysis by Abramson *et al.*, who analysed the benefit of SIT within the treatment of asthma (Abramson *et al.* 2000). On the basis of 54 trials of desensitisation included within the analysis - 25 for HDM, 13 for pollen, 8 for animal dander, 2 for Cladosporium and 6 using multiple allergens - a significant reduction in asthma symptoms and medication following immunotherapy was demonstrated. No similar meta-analysis exists for the use of SIT for other indications, but randomised controlled trials have confirmed its efficacy in treating allergic rhinitis and hymenoptera and vespid allergy (van der Zwan *et al.* 1983; Durham *et al.* 1999).

The recent WHO position statement on the use of SIT stresses the need to consider treatment indications based upon the sensitising allergen rather than the pattern of underlying organ involvement, as allergic disease is frequently a multi-organ process (Bousquet *et al.* 1998). Stratifying according to sensitising allergen, clear efficacy has been shown for numerous tree, grass and weed pollens producing allergic rhinitis, for grass pollen induced asthma, and for grass pollen and ragweed induced conjunctivitis. HDM desensitisation has been shown to be efficacious for similar indications for those listed for pollen induced disease, while only limited evidence exists for desensitisation towards moulds or animal dander. Insect venom desensitisation is effective therapy in the vast majority of venom allergic subjects (van der Zwan *et al.* 1983). The optimal dose of allergen, producing the most favourable therapeutic ratio, appears to be 5-20 μg of major allergen based on immunotherapy studies with well standardised extracts of ragweed, grass, cat, mite, and venom (Bousquet *et al.* 1998). The optimal duration of therapy for persistent responses to immunotherapy appears to be at least 3 years, based upon studies performed using standardised HDM vaccine or grass pollen extract (Durham *et al.* 1999).

1.7.3 Problems with Current Allergen Immunotherapy

Safety concerns have limited the uptake of allergen immunotherapy in some parts of the world (Larche 2000). Additionally, problems exist via the lack of proper standardization of

allergen content in preparations, the relative complexity of its administration, and unpredictable and limited efficacy amongst its recipients. Concerns with regards safety arose via two retrospective studies from the United Kingdom and the USA of SIT induced morbidity and mortality. In 1986, the British Committee on Safety of Medicines reported that there had been 26 immunotherapy induced deaths from 1957, with 5 in the preceding 18 months: acute asthma was particularly highlighted as a cause of death (Medicines 1986). In 1987, the AAAAI reported on fatalities in the USA where 46 deaths occurred between 1945 and 1984, 30 with sufficient data for analysis: 6 were associated with SPT, 24 with SIT. Highlighted risk factors included asthma, β-blocker usage, and higher degrees of allergen sensitivity based on skin prick testing or RAST (Reid *et al.* 1993).

While these safety concerns still allow cautious application of immunotherapy for certain indications, applications of standard immunotherapy approaches to food allergy have not been possible. Two clinical trials of desensitisation towards peanuts have occurred to date, with neither producing acceptable levels of efficacy or safety. Nelson *et al.* administered a rush protocol to six patients with peanut allergy, followed by one year of maintenance therapy (Nelson *et al.* 1997). All patients experienced systemic reactions through both the build up and maintenance phases of therapy with a median of 4 epinephrine injections required for their control during the build up phase. There was increased tolerance of peanut on oral challenge and skin prick test to peanut, but this was lost in three patients unable to remain on the top dose of maintenance therapy due to side effects. The only other trial of desensitisation to peanuts was tragically marred by a death due to anaphylaxis in a placebo randomised patient wrongly administered peanut extract, and again demonstrated a high frequency of systemic reactions amongst those receiving active therapy (Oppenheimer *et al.* 1992).

1.7.4 Mechanisms of Allergen Immunotherapy

1.7.4.1 Changes in the humoral immune response to the allergen

Allergen specific IgE levels in the early phase of SIT may actually increase, although in the long term fall to baseline levels (Lichtenstein *et al.* 1973). As discussed below, this does not appear to impact on effector cell function, or target organ sensitivity (Malling *et al.* 1982). Serum IgG levels, particularly IgG4, increase constantly during SIT and until recently have been regarded as unimportant in the efficacy of therapy (Birkner *et al.* 1990; Ebner 1999). In particular, IgG4 does not mediate antibody-dependent effector mechanisms and does not act as blocking antibody as it is present in both responders and non-responders. Furthermore, rush SIT for hymenoptera allergy is effective after only hours, well before igG levels begin

to rise; and following which successfully treated patients may have normal levels of IgG (Durham *et al.* 1998). However, Wacholz *et al.* have rekindled interest in so-called "blocking antibodies" by demonstrating that following successful SIT, IgG antibodies are produced that inhibit, in an antigen specific manner, IgE binding to CD23 on B cells, and subsequent antigen presentation to T cells (Wachholz *et al.* 2003). Furthermore certain patterns of IgG subclass response may signal the efficacy of SIT: high IgG4 levels have been associated with failure of immunotherapy with inhalant allergens, while high resting IgG1 levels are associated with the presence of a late phase response to allergen provocation (Djurup *et al.* 1987).

1.7.4.2 Changes in effector cell function post allergen immunotherapy

The effector cell response to allergen is profoundly altered post SIT. Creticos *et al.* have shown using nasal challenge in ragweed allergic asthmatic subjects that there is a large reduction in the concentration of inflammatory mediators such as histamine, (TAME)-esterase, and prostaglandin D_2 following SIT within nasal fluid (Creticos *et al.* 1985). Similarly, effector cell numbers are decreased in affected end organs post immunotherapy, an example being the reduction in mast cell and eosinophil numbers seen in the nasal mucosa of rhinitic patients post HDM and pollen desensitisation respectively (Furin *et al.* 1991; Otsuka *et al.* 1991). Additionally effector cells appear to undergo phenotypic change post SIT. In a study of peripheral blood basophil surface molecule expression post venom immunotherapy, there was a significant decline in numerous markers of activation. These included CD23, CD63, CD40L, HLA-DR, IL-2Rβ, IL-4Rα, IL-5R, among others (Siegmund *et al.* 2000). Strikingly, these changes were detectable within a week of the end of a 7 day course of rush immunotherapy. These effects may be modulated by IFN-γ and IL-10 as, in an *in vitro* model of birch pollen desensitisation, the addition of neutralizing anti-IFN-γ antibodies or anti-IL-10 antibodies increased CD23 expression (Roever *et al.* 2003).

1.7.4.3 Changes in the cellular immune response to allergen

1.7.4.3.1 Alteration in cytokine profile: T_H1 to T_H2 profile

Numerous studies have addressed the changes in T-lymphocyte phenotype that occurs with immunotherapy, with the resounding conclusion being that there is a shift from a T_H2 to a T_H1 phenotype once the maintenance dose phase is achieved (Durham *et al.* 1998; Ebner 1999). The fundamental change repeatedly demonstrated is a reduction in the IL-4 (or IL-5):IFN-γ ratio, although how this is brought about has varied between studies, with some demonstrating a reduction in IL-4 or IL-5, while others show either an increase in IFN-γ

alone or a combination of both changes (Secrist *et al.* 1993; Jutel *et al.* 1995; McHugh *et al.* 1995; Bellinghausen *et al.* 1997; Ebner *et al.* 1997; Meissner *et al.* 1999). The induction of phenotypic change appears to be dependent on neither the individual allergen used, nor the protocol used for desensitization; trials demonstrating a T_H1 phenotypic shift have used bee or wasp venom, grass pollen, cat, and HDM SIT, and standard, rush and ultra-rush protocols (see Table 1.5). The kinetics of the change in T cell phenotype has been addressed in several studies where subjects have been followed longitudinally, with the universal finding being that phenotypic shift is demonstrated after the maintenance dose of SIT is achieved, with one study suggesting a biphasic response, such that the IL-4:IFN-γ ratio is increased during the build up phase of therapy (McHugh *et al.* 1995; Bellinghausen *et al.* 1997; Ebner *et al.* 1997; Benjaponpitak *et al.* 1999). Jannsen *et al.* have shown that effective immunotherapy in a mouse model was dependent on an early vigorous T cell proliferative response occurring in both local draining lymph nodes as well as the systemic circulation (Janssen *et al.* 2000). While most studies have concentrated on finding changes within lymphocytes derived from peripheral blood, one group has confirmed that lymphocytes derived from end organs also showed a T_H1 phenotypic shift, demonstrating that lymphocytes derived from both the skin and nasal mucosa produce increased amounts of IFN-γ and possibly diminished amounts of IL-4 and IL-5 following successful grass pollen immunotherapy (Varney *et al.* 1993; Durham *et al.* 1996).

Of interest are two studies that rather than demonstrating a reduction in the IL-4:IFN-γ ratio, demonstrate a blanket reduction in both cytokines as well as proliferative responses, suggesting that the key action of immunotherapy is the induction of anergy of all allergen specific lymphocyte responses (Akdis *et al.* 1996; O'Brien *et al.* 1997). Most studies demonstrating a shift in T cell phenotype also demonstrate a profound reduction in T cell proliferative responses, giving at least some support to this postulate (see Table 1.5). How both a loss of responsiveness and the presence of cells of an altered phenotype may co-exist has been suggested by one group who, having demonstrated an initial anergic response to allergen challenge, could then induce proliferative and cytokine responses of either phenotype depending on the cytokine used whilst culturing short term TCLs (Akdis *et al.* 1996). This finding suggests that, in addition to the loss of the aberrant T_H2 response, the reconstitution of a normal T cell response must also occur, and this is dependent on the milieu of cytokines present within the local microenvironment (Figure 1.4) (Akdis *et al.* 1996; Akdis *et al.* 2000).

Table 1. 5 Immunological changes associated with immunotherapy.

Allergen	Immunotherapy regimen	Tissue examined	Cytokine change						Proliferative change	Reference
			IL-4	IL-5	IFN-γ	IL-4/IFNγ ratio	IFNγ	IL-10		
HDM	Standard	PBMC	↓	NR	↓	NR	NR			(O'Brien *et al.* 1997)7)
Bee venom	Ultra-rush	PBMC	↓	↓	↑	↓		no change	↓	(Jutel *et al.* 1995)5)
Bee or wasp venom	Rush	PBMC	↓	NR	↑	↓		↑	↓	(Bellinghausen *et al.* 1997)7)
Grass pollen	Standard	Skin biopsy	↓$^{\Psi}$	↓$^{\Psi}$	↑	↓*		NR		(Varney *et al.* 1993)3)
HDM or grass	Standard	T cell line	↓$^{\Psi}$	NR	no change	↓*		NR	↓	(Secrist *et al.* 1993)3)
Bee venom	Rush	T cell line	↓	↓	↓	NR		NR	↓	(Akdis *et al.* 1996)6)
Bee venom	Standard or Rush	PBMC	↓	NR	↑	↓*		NR	↓	(McHugh *et al.* 1995)5)
Cat	Standard	PBMC	no change	↓	no change	NR		NR	NR	(Benjaponpitak *et al.* 1999)9)
Grass pollen	Standard	T cell lines and clones	NR	NR	NR	↓		NR	↓	(Ebner *et al.* 1997)7)
HDM or grass	Standard	T cell line	NR	NR	NR	↓		NR	NR	(Meissner *et al.* 1999)9)

NR: not recorded; ↓: decreased; ↑: increased; *extrapolation of data presented in paper; $^{\Psi}$not statistically significant; cytokine change: change in cytokine levels either post induction or completion of immunotherapy.

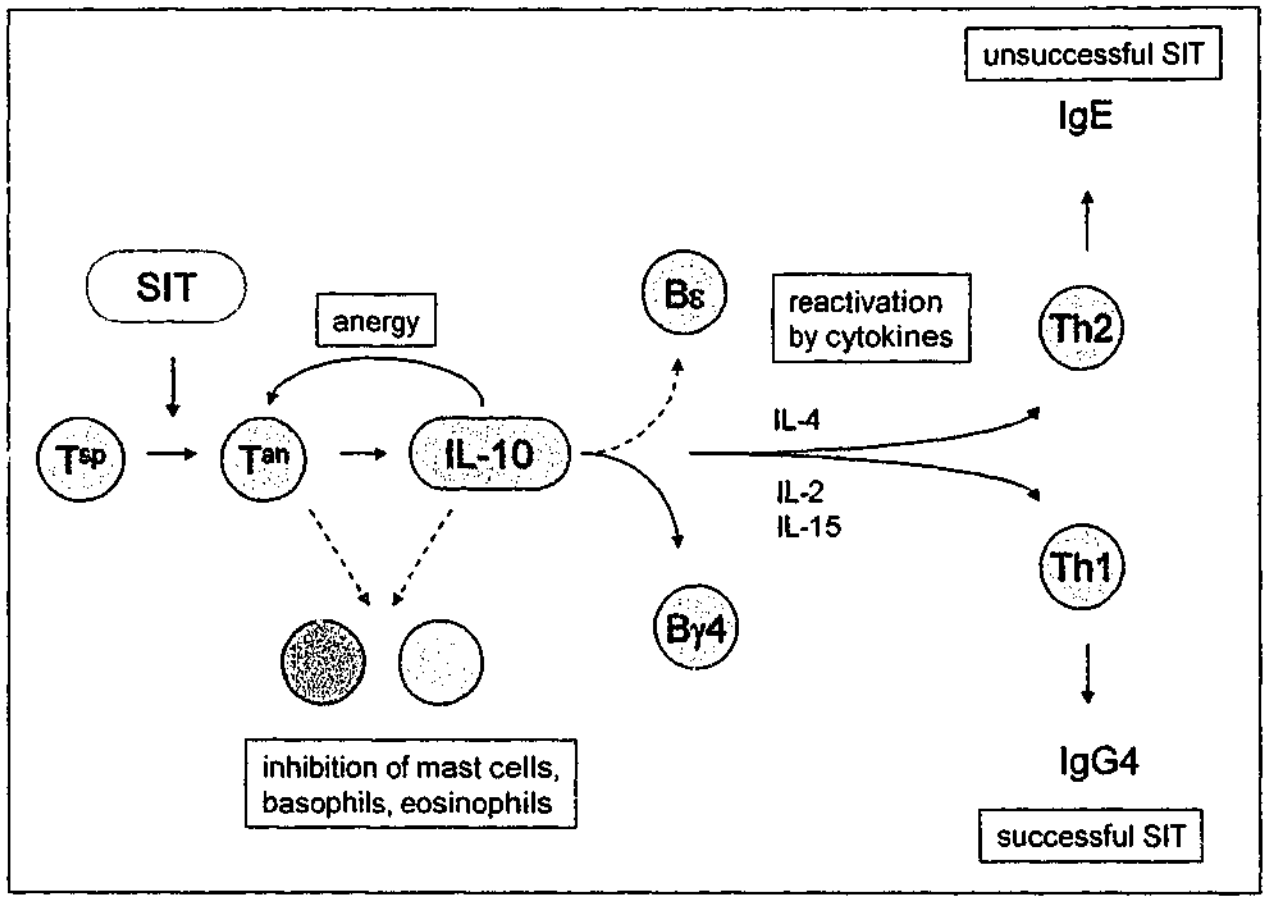


Figure 1.4 Proposed mechanism of allergen immunotherapy(Larche 2000).
Continuous treatment with high doses of allergen establishes a state of aliergen-specific anergy in peripheral T-Cells (T^{an}), which is characterised by suppressed proliferative and cytokine responses, together with an increase in IL-10 production. IL-10 suppresses specific T-cells (T^{sp}) in an autocrine fashion. It also suppresses IgE production and enhances IgG_4. Subsequent activation, priming and survival of allergic inflammatory effector cells are down-regulated. The anergic T cell can be re-activated by cytokines from the tissue micro-environment. In successful SIT, anergic T-cells recover by the influence of micro-environmental IL-2 and/or IL-15 to produce Th0/Th1 cytokines. In an atopic individual, IL-4 may reconstitute a Th2 cytokine pattern and re-activate an allergic response.

1.7.4.3.2 Action on T lymphocytes: Deletion versus anergy versus immune deviation

Although the latter model suggests that anergy is the initial fate of allergen specific T-lymphocytes during immunotherapy this is the subject of debate, with clonal deletion and immune deviation being suggested as alternative fates (Rolland *et al.* 1998). Both deletion and anergy would be expected to produce the decreased proliferative responses seen in the studies listed above; evidence for deletion as being a significant contributor to this phenomenon would require features of apoptosis to be demonstrated. To date, although mucosally delivered high dose antigen has been demonstrated to produce apoptosis of specific clones, this has not been demonstrated within models of allergic disease nor following specific immunotherapy (Chen *et al.* 1995).

Anergy of T_H2 responsive cells, the reversible loss of proliferative and cytokine responses to specific allergen stimulation, has been strongly argued by Akdis and colleagues (Akdis *et al.* 2000). The above study within bee venom allergic individuals is weakened by the use of polyclonal TCLs (Akdis *et al.* 1996). However, additional evidence for anergy is provided by Cao *et al.*, who showed that vaccination of mice with a multi-epitope recombinant allergen lead to marked reduction in proliferation and IL-2 production, along with diminished production of IL-4 and to a lesser extent IFN-γ (Cao *et al.* 1997). An intracellular mechanism for diminished responsiveness amongst anergic allergen specific T cells has been suggested by Faith *et al.*, who showed that anergic PLA_2-specific T cell clones respond to anti-CD3 stimulation with abrogated $p56^{lck}$ and ZAP-70 tyrosine kinase activities (Faith *et al.* 1997).

Arguments for immune deviation center around the observed differences in T cell phenotype that can be induced by alteration of the dose of allergen, site of allergen exposure, nature of the APC, co-stimulatory signal and cytokine milieu that have previously been listed while discussing determinants of the T cell response. An additional factor that may be of importance to subcutaneous SIT include the presence of local type I collagen; DC were able to induce T_H1 responses among short term TCLs when cocultured with type I collagen and allergen and which in its absence were of a T_H2 phenotype (Brand *et al.* 1999).

Cytokines particularly highlighted in the induction of these responses are IL-10 for anergy and IL-12 for immune deviation. IL-10 has been shown to be elevated 7 days after specific immunotherapy, reaching maximal levels 28 days after specific immunotherapy in studies involving rush immunotherapy for wasp and bee venom allergy (Bellinghausen *et al.* 1997; Akdis *et al.* 1998). As discussed in more detail previously, IL-10 is thought of as a general inhibitor of both T_H1 and T_H2 immune responses, producing anergy of both proliferative and cytokine responses (Moore *et al.* 2001). This role appeared important in the induction of

such responses for these forms of immunotherapy, as neutralization of endogenous IL-10 lead to restoration of PLA_2-specific proliferative and cytokine responses (Akdis *et al.* 1998). This function may be mediated via inhibition of co-stimulation by APC, or via inhibition of the actions of CD28 (Akdis *et al.* 2000). IL-10 may also be important in the early efficacy of rush immunotherapy as it has been shown to have inhibitory actions on allergic effector cells, including reduced mast cell production of inflammatory cytokines in response to IgE cross-linking, and enhanced eosinophil cell death (Takanaski *et al.* 1994; Arock *et al.* 1996).

IL-12 has been shown to alter the cytokine profile of polyclonal T_H2 lymphocytes towards a T_H1 phenotype, in association with other cell surface or intracellular changes also suggestive of immune deviation (Annunziato *et al.* 2001; Smits *et al.* 2001). However, only limited evidence exists for the elevation of IL-12 during immunotherapy; IL-12 mRNA containing cells have been shown to be increased in late phase cutaneous responses post allergen challenge following grass pollen immunotherapy, with colocalisation studies demostrating the major source of this cytokine to be macrophages (Hamid *et al.* 1997). Increases in IL-12 mRNA showed a positive correlation with increases in IFN-γ and a negative correlation with IL-4, suggesting an association between these changes. More recently, monocytes cultured from subjects undergoing bee venom immunotherapy have also demonstrated increased IL-12 production in comparison to an untreated control population (Magnan *et al.* 2001).

1.7.5 Approaches to Overcoming Problems with Allergen Immunotherapy

Numerous approaches to improving the efficacy of immunotherapy and diminishing its adverse effects are currently being explored. Detailed discussion of these is beyond the context of this review, but examples include the use of CpG motifs within allergen vaccines as a T_H1 adjuvant, and DNA vaccines, which have been successfully employed within a mouse model of peanut allergy to induce tolerance (Chu *et al.* 1997; Roy *et al.* 1999; Tighe *et al.* 2000). This discussion is limited to those therapies that may be available via the elucidation of dominant T cell epitopes, the most important of which to date has been peptide immunotherapy.

Peptide immunotherapy has been attempted using cat, bee, and ragweed allergen models. Within the cat allergic model, a large amount of data have been derived using two large peptides based on the structure of Fel d 1, termed IPC-1 and IPC-2. Norman *et al.* stratified subjects to 3 dose groups and demonstrated diminished lung and nasal symptoms amongst those subjects receiving 4 injections of 750 μg peptide, the highest dose of therapy (Norman *et al.* 1996). Marcotte *et al.* demonstrated that this was associated with a reduction in IL-4 production by IPC-1/IPC-2 specific TCLs (Marcotte *et al.* 1998). Two other authors have shown efficacy for this therapy amongst cat allergic asthmatics: Pene *et al.* demonstrated a

reduction in PD_{20} for those subjects immunised with the high or medium dose regimen, while Maguire demonstrated an improvement in FEV1 in subjects receiving 8 doses of 750 μg (Pene *et al.* 1998; Maguire *et al.* 1999). More recently, a detailed clinical study using 16-mer overlapping peptides including the entire sequence of both chains of Fel d 1 have been performed (Oldfield *et al.* 2002). Within that study, immunotherapy was associated with the induction of non-responsiveness of PBMC following immunotherapy, along with elevated IL-10 levels. Clinically, subjects receiving active therapy demonstrated diminished early and late phase reactions on intra-dermal testing and stated they tolerated exposure to cat significantly better in comparison to pre-therapy levels (Oldfield *et al.* 2002). There was however no difference between placebo and active groups with regards bronchial responsiveness, or between the two groups with regards symptom scores (Oldfield *et al.* 2002).

Bee venom peptide immunotherapy has demonstrated efficacy in a study by Muller *et al.* (Muller *et al.* 1998). Five bee venom allergic subjects were given an equimolar mixture of three major epitopes of PLA_2, the major allergen within bee venom, subcutaneously to a dose of 100 μg. No systemic side effects occurred during the immunotherapy schedule and challenge to 10 μg subcutaneous PLA_2 produced no systemic response in any of the 5 subjects, while 3 out of 5 showed no systemic response to live bee sting. This clinical response was associated with diminished proliferative responses of short term TCLs stimulated with PLA_2 or the peptide mixture, and a reduction of all cytokine responses including IFN-γ, IL-4, IL-5 and IL-13.

Thus, efficacy has been demonstrated within both cat allergic and bee venom allergic subjects for peptide immunotherapy. Whether similar models of immunotherapy will be efficacious within the food allergic population remains unclear as no food allergic model has yet been used for peptide based treatments.

The basis to the efficacy for peptide immunotherapy appears to stem from the retained presence of T cell epitopes. Litwin *et al.* have demonstrated that immunotherapy does not require the presence of IgE reactive epitopes to retain efficacy, a pepsin treated form of PLA_2 leading to a substantial decrease in specific anti-PLA_2 IgE and IgG antibodies as well as a decline in skin test sensitivity to PLA_2 (Litwin *et al.* 1988). While therapy with T cell epitopes down-regulates the immune response, immunodominant T cell epitopes are maximally suppressive (Gammon *et al.* 1991; Hoyne *et al.* 1997). Further evidence for this action being T cell dependent has been provided by Haselden *et al.* Noting that numerous peptide immunotherapy studies demonstrated late allergic responses, they speculated that this was a T cell dependent response and indicated T cell activation. Immunising using smaller Fel d 1 derived peptides than those used in previous studies that did not cross-link

IgE or produce basophil histamine release, they demonstrated no immediate reactions, but late asthmatic reactions in 9 out of 40 subjects (Haselden *et al.* 1999). Subsequently these subjects demonstrated hyporesponsiveness to Fel d 1 peptides. These phenomena were felt by the authors to reflect T cell activation as previously they had shown these peptides could produce T cell activation *in vitro* and those subjects demonstrating a late asthmatic response expressed a DR1, DR4, or DR13 allele suggesting such responses were MHC class II restricted (Larche 2000).

A criticism of peptide based immunotherapy is that the human population is outbred, expressing a range of MHC molecules and T cell receptors, so that multiple peptides are likely to be required within vaccines to cover all T cell epitopes producing allergic disease. To overcome this, it has been successfully demonstrated that longer overlapping peptides encompassing the entire molecule of PLA_2 and therefore including all potential T cell epitopes retain their T cell immunogenicity and ability to induce T cell non-responsiveness, a T_H1 phenotype and IL-10 production (Fellrath *et al.* 2003). A similar approach has been used for Fel d 1, as described above (Oldfield *et al.* 2002). However, data exists that despite the human population's diverse range of such molecules, there are only a few dominant epitopes within allergenic proteins suggesting these epitopes demonstrate promiscuity both in their MHC presentation as well as TCR recognition. As noted above, Haselden *et al.* demonstrated that subjects reacting to a 17-mer Fel d 1 peptide expressed either DR1, DR4 or DR13; subjects also showed plasticity at the level of the T cell receptor, with a single TCR recognising Fel d 1 in the context of two different HLA-DRB1 subtypes (Haselden *et al.* 1999). Furthermore, murine studies suggested that therapy with the dominant epitopes of allergenic proteins produces suppression of immune responses to the entire protein including its minor epitopes, so-called intra-molecular suppression. Hoyne *et al.* demonstrated in mice immunised intra-nasally with Der p 1 peptides that tolerance to other epitopes within Der p 1 also occurred, provided that previous exposure to the intact Der p 1 had also occurred (Hoyne *et al.* 1997).

Although peptide immunotherapy is the clearest application stemming from the mapping of T cell epitopes, other approaches that bypass IgE binding also rest on this information. One such approach is the manufacture of hypoallergenic B cell epitope mutants, whereby alteration of decisive amino acids in B cell epitopes is performed producing lowered IgE binding while retaining T cell reactivity (Ferreira *et al.* 1998; Swoboda *et al.* 2002). Such an approach has been performed for Ara h 3 (Rabjohn *et al.* 2002). In that study, point mutations were introduced into Ara h 3 cDNA at codons encoding critical amino acids in IgE epitopes. IgE binding to the modified protein decreased approximately 35-85% in comparison to IgE binding to wild-type Ara h 3 but the modified Ara h 3 protein retained the capacity to stimulate T-cell proliferation to the same extent as the wild-type protein in 3 out

of 5 subjects tested. Studying Bet v 1, Ferreira and colleagues used site directed mutagenesis to replace six amino acid residues characteristic of a high IgE binding isoform with those present at the same positions in a low IgE binding isoform. While this produced greatly reduced IgE binding in 13 birch pollen allergic subjects, T cell proliferation was unchanged (Ferreira *et al.* 1998). In a similar study by Swoboda *et al.*, dominant linear B cell epitopes of Lol p 5 were mutated in recombinant Lol p 5 resulting in variably diminished IgE binding, basophil histamine release and immediate skin prick test reactivity, while T cell proliferative responses were maintained in Phl p 5 specific clones (Swoboda *et al.* 2002). More recently, mutation of the major latex allergen Hev b 6 has demonstrated similar *in vivo* correlates of efficacy and safety (Karisola *et al.* 2004). No examination of cytokine responses is made within any of these papers, and clinical data with regards attempts to induce hyporesponsiveness using this method are not currently available.

T cell epitope mapping also yields material for the creation of altered peptide ligands, whereby single amino acid alterations are created in T cell epitope peptides. As discussed previously, peptide modifications that produce alterations in the affinity of binding to the TCR may lead to diminished T cell activation and cytokine production. Faith *et al.* have shown that alanine substitutions within 2 dominant T cell epitopes of PLA_2 resulted in a diminished T cell response, inhibition of IL4 production with no alteration in the production of IFNγ (Faith *et al.* 1999). Such an approach may prove useful in the generation of genetically modified foods, lacking T cell epitopes.

In conclusion, SIT has been available for nearly 100 years, but its most probable method of action, via the induction of T cell anergy in association with IL-10 production and subsequent activation of a T_H1 response, has only recently been described. Knowledge of this paradigm allows the pursuit of therapies which specifically act on these mediators while avoiding IgE binding. Proper characterisation of the T cell response and its epitopes is a critical part of this pursuit.

1.8 SUMMARY AND AIMS

Nut allergy is an incurable and life threatening disorder affecting between 1 and 2% of the population with evidence suggesting that it is increasing in prevalence in tandem with the other allergic diseases. Efforts at curing these disorders have been universally unsuccessful due to anaphylaxis during the build up and maintenance phases of immunotherapy.

Hope for new approaches to immunotherapy for nut allergy is increasing as the allergenic proteins within the nuts are recognised. While much work has been done in this area with regards IgE reactivity towards the peanut major allergens, very little has been published with

regards T cell responses to these proteins. Clearly, the T cell response is crucial both in the regulation of the allergic response and as a therapeutic target for immunotherapy. As such, in the first set of experiments in this thesis, Ara h 1 and Ara h 2 are purified and their T cell proliferative and cytokine responses characterised.

Numerous novel approaches exist that may circumvent IgE cross-linking and consequent anaphylaxis and thus allow safe immunotherapy for nut allergy. One such method is the use of non-IgE reactive peptides containing the major T cell epitopes. Using this technique with non-IgE reactive peptide fragments of PLA_2 and Fel d 1, SIT has been successfully carried out for hymenoptera and cat allergy, respectively, providing hope that this may be a route towards immunotherapy for peanut allergy. Within this thesis, regions containing the immunodominant T cell epitopes of the major peanut allergen Ara h 2 are demonstrated, so as to identify important epitopes that may be included in future immunotherapy.

An understanding of the mechanisms of cross-reactivity between the nuts is important to ensure future therapies are curative of allergy towards potentially cross-reactive epitopes in other nut species. IgE cross-reactivity is likely to be caused by reactivity to homologous proteins and pan-allergens. This is highlighted in a case report included in this thesis demonstrating IgE cross-reactivity between orange seeds, other citrus seeds and peanut. At present, no published work demonstrates T cell cross-reactivity between peanut and tree nuts. The demonstration of such data is crucial for the development of immunotherapy for nut allergy. Within this thesis it is demonstrated that cross-reactivity exists between peanut and hazelnut on the basis of proliferative and cytokine responses of short term hazelnut specific TCLs, and that such cross-reactivity may be mediated particularly via Ara h 2 and Cor a 9.

CHAPTER 2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Tissue Culture Reagents

Ara h 2 20-mer peptides	Chiron Mimotopes, Australia
Ficoll-Paque (research grade)	Amersham Biosciences, Sweden
Foetal Bovine Serum (FBS)	Gibco BRL, USA
Human AB+ serum	Sigma Chemical Company, USA
Penicillin-Streptomycin-Glutamine	Gibco BRL, USA
Phytohaemagglutinin (PHA)	Wellcome Diagnostics, England
Recombinant human IL-2 (rIL-2)	Cetus Corporation, USA
RPMI 1640 (glutamine free)	Gibco BRL, USA
Sodium heparin (preservative free)	David Bull Laboratories, Australia
Tetanus toxoid	Laboratory stocks

2.1.2 Cell Lines and Bacterial Transfectants

rAra h 2 transfected BL-21 *E. coli*	Kind gift of Ms M de Leon

2.1.3 Immunoblotting Reagents

Nitrocellulose membrane (BA 0.45 μm)	Schleicher and Schuell, Germany
Rabbit anti-mouse/human immunoglobulin-horseradish peroxidase (HRPO) conjugate	Dako Corporation, Denmark
Skim milk powder	Diploma, Australia
Ponceau S	Sigma Chemical Company, USA
Enhanced chemiluminescence reagent	Pierce, USA

2.1.4 ELISA Reagents

Biotinylated rat-anti-human IFN-γ	Endogen, USA
Biotinylated rat-anti-human IL-5	PharMingen, USA

Human recombinant IFN-γ	Endogen, USA
Human recombinant IL-5	PharMingen, USA
Mouse anti-human IFN-γ	Endogen, USA
Mouse anti-human IL-5	PharMingen, USA
Streptavidin-biotinylated HRPO complex	Amersham, USA
Enhanced chemiluminescence reagent	Perkin Elmer, USA

2.1.5 Flow Cytometry Reagents

Mouse anti-human CD4-FITC	PharMingen, USA
Mouse anti-human CD4-FITC/CD8-PE	Becton Dickinson, USA
Mouse anti-human CD3-FITC/CD19-PE	Becton Dickinson, USA
Mouse anti-human CD45-FITC/CD14-PE	Becton Dickinson, USA
Mouse IgG_1-FITC/IgG_1-PE isotype control	Becton Dickinson, USA
Mouse IgG_1-APC isotype control	PharMingen, USA
Mouse IgG_1-FITC isotype control	PharMingen, USA
Mouse IgG_1-PE isotype control	PharMingen, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma Chemical Company, USA

2.1.6 General Reagents

Dialysis tubing (MW cut off 3.5 kDa)	Pierce, USA

2.1.7 General Chemicals

Ammonium persulphate (AP)	Bio-Rad Laboratories, USA
Ampicillin	Sigma Chemical Company, USA
Aqueous counting scintillant (ASCII)	Ajax Chemicals, Australia
Bis-acrylamide, electrophoresis purity	Bio-Rad Laboratories, USA
Bovine Serum Albumin (BSA)	Sigma Chemical Company, USA
Bovine γ-globulin (BGG)	Sigma Chemical Company, USA
Bromophenol blue	BDH Laboratory Supplies, UK
Coomassie Brilliant Blue R-250	Bio-Rad Laboratories, USA

Dimethylpimelimidate (DMP)	Sigma Chemical Company, USA
Dimethylsulphoxide (DMSO)	Sigma Chemical Company, USA
Dithiothreitol (DTT)	Sigma Chemical Company, USA
Ethanol, 95% (absolute)	Ajax Chemicals, Australia
Glacial acetic acid	Ajax Chemicals, Australia
Glycerol	Ajax Chemicals, Australia
Glycine	Ajax Chemicals, Australia
Hydrogen chloride	Ajax Chemicals, Australia
Hydrogen peroxide (H_2O_2) (30%)	Ajax Chemicals, Australia
Hydrogen sulphate (H_2SO_4), analytical grade	Ajax Chemicals, Australia
Isopropyl-β-D-thiogalactoside (IPTG)	Sigma Chemical Company, USA
Methanol	Ajax Chemicals, Australia
Polyethylene glycol (average MW 20,000)	BDH Laboratory Supplies, UK
Ponceau S	Sigma Chemical Company, USA
Protein assay dye reagent	Pierce Laboratories, USA
prestained markers	
Sodium azide (NaN_3)	BDH Laboratory Supplies, UK
Sodium carbonate (Na_2CO_3)	Ajax Chemicals, Australia
Sodium chloride (NaCl)	Ajax Chemicals, Australia
Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	Sigma Chemical Company, USA
Sodium dodecyl sulphate (SDS)	Bio-Rad Laboratories, USA
Sodium hydrogen carbonate ($NaHCO_3$)	BDH Laboratory Supplies, UK
Sodium phosphate (Na_2HPO_4)	Sigma Chemical Company, USA
N,N,N',N'-Tetramethylethylene-diamine (TEMED)	Bio-Rad Laboratories, USA
^{3}H-thymidine (methyl)	DuPont, USA
Trichloroacetic acid (TCA)	BDH Laboratory Supplies, UK
Triethanolamine	BDH Laboratory Supplies, UK
Tris (hydroxymethyl) aminomethane (Tris)	BDH Laboratory Supplies, UK

Trypan blue	Calbiochem, USA
Tryptone	Becton Dickinson, USA
Tween 20 (Polyxyethylenesorbitan monolaurate)	Sigma Chemical Company, USA
Yeast extract	Sigma Chemical Company, USA

2.2 METHODS

2.2.1 Buffers and Solutions

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

2.2.1.1 Acrylamide - Bis (50% w/v)

A commercially available premixed preweighed acrylamide/bis powder was diluted in 162 ml water (Bio-Rad Laboratories, USA) to yield a 50% solution. This was stored at room temperature, protected from light.

2.2.1.2 Ammonium persulphate (10% w/v)

A 10% ammonium persulphate solution was prepared by adding 1 g ammonium persulphate to 10 ml H_2O. The solution was stored at 4°C for up to 3-4 days.

2.2.1.3 Binding buffer

A 0.1 M Na_2HPO_4 solution, adjusted to pH 9.0, was prepared and stored at room temperature.

2.2.1.4 10% Milk powder

10 g of skim milk powder dissolved in 100 ml PBS.

2.2.1.5 BGG standard for the Pierce protein assay

1.45 mg BGG was dissolved in 1 ml H_2O, stored at -20°C in 100 µl aliquots and used for the standard curve in the Pierce protein assay (Pierce, USA) according to the manufacturer's instructions.

2.2.1.6 Coomassie Brilliant Blue R-250

A 0.2% (w/v) Coomassie Brilliant Blue R-250 (Coomassie Blue) solution was prepared by dissolving 0.2 g of Coomassie Brilliant Blue R-250 in 100 ml of 50% (v/v) methanol, 10% (v/v) glacial acetic acid in H_2O. This solution was filtered through Whatman #1 filter paper and stored at room temperature in the dark.

2.2.1.7 Destaining solution

Coomassie Blue stained gels were destained using 7% glacial acetic acid. The destaining solution was prepared by diluting 70 ml glacial acetic acid to 1 L with H_2O.

2.2.1.8 ELISA coating buffer, pH 9.6

This buffer consisted of 0.86 g Na_2CO_3 and 1.72 g $NaHCO_3$ dissolved in 100 ml H_2O and was stored at 4°C. The pH of this solution was as indicated and did not need to be adjusted.

2.2.1.9 Elution buffer

A 500 ml solution containing 500 mM imidazole, 50 mM NaH_2PO4, 300 mM NaCl, and 8 M urea was prepared and stored at room temperature.

2.2.1.10 FACS wash buffer (1% FBS/0.02% NaN3/PBS)

5 ml of FBS and 1.54 ml of 1 M NaN_3 solution was added to 443.46 ml of PBS and the solution stored at 4°C.

2.2.1.11 FBS-15% DMSO

To 50 ml of FBS, 8.8 ml of DMSO was added to yield a 15% DMSO solution. This was aliquoted into 10 ml polypropylene tubes and stored at –20 °C until required.

2.2.1.12 Luria-agar (L-agar)

To 1 L of L-broth 15 g of agar was added and the solution sterilised by autoclaving.

2.2.1.13 Luria-broth (L-broth)

L-broth was prepared by the addition of 5 g of tryptone, 2.5 g of yeast extract and 2.5 g of NaCl to 450 ml of water. The pH was then adjusted to 7.0 and the solution made up to 500 ml. The medium was then sterilised by autoclaving.

2.2.1.14 Lysis buffer

A 500 ml solution containing 100 mM NaH_2PO4, 10 mM Tris-HCl, 8 M urea, adjusted to a pH of 8.0 using HCl was prepared and stored at room temperature.

2.2.1.15 Phosphate buffered saline (PBS), pH 7.2

A 10x stock solution was prepared by dissolving 85 g NaCl, 3.9 g $NaH_2PO_4.2H_2O$ and 10.7 g Na_2HPO_4 in 1 L H_2O. This was stored at room temperature and diluted ten-fold when required.

2.2.1.16 PBS/0.1% BSA

0.1 g of BSA was dissolved in 100 ml of PBS, and used on the day of preparation.

2.2.1.17 PBS/Tween 20 (0.05% v/v)

PBS/Tween 20 (0.05% v/v) was prepared by adding 2.5 ml Tween 20 to 5 L PBS. This solution was stored at room temperature.

2.2.1.18 Phytohaemagglutinin (PHA) (400 µg/ml)

2 mg of lyophilised PHA was reconstituted with 5 ml H_2O under sterile conditions to give a final concentration of 400 µg/ml. This was then aliquoted into 50 µl lots and stored at -20°C.

2.2.1.19 Resolving gel buffer, pH 8.8

This buffer was prepared by adding 182 g Tris, 4.0 g SDS and H_2O to 950 ml. The pH was adjusted to 8.8 with HCl and made up to 100 ml with H_2O. This buffer was stored at 4°C.

2.2.1.20 RPMI 1640/Penicillin-Streptamycin-Glutamine (wash medium)

5 ml of Penicllin-Streptamycin-Glutamine (Gibco, USA) was added to 500 ml of RPMI 1640 medium to yield final concentrations of 2mM L-glutamine and 100 U/ml of penicillin/streptamycin. The medium was stored at 4°C protected from light.

2.2.1.21 RPMI 1640/10 U/ml sodium heparin (heparinised medium)

5000 units of sterile preservative free sodium heparin and 5 ml of Penicillin-Streptamycin-Glutamine (Gibco, USA) was added to 500 ml of RPMI 1640 medium to yield final concentrations of 10 U/ml sodium heparin, 2 mM L-glutamine and 100 U/ml of penicillin/streptamycin. The medium was stored at 4°C protected from light.

2.2.1.22 RPMI 1640/5% human AB+ serum (complete medium)

To 500 ml of RPMI 1640 medium, heat inactivated human AB+ serum and Penicillin-Streptamycin-Glutamine (Gibco, USA) were added to yield final concentrations of 5% human serum, 2 mM L-glutamine and 100 U/ml of penicillin and streptamycin. The medium was stored at 4°C protected from light.

2.2.1.23 SDS running buffer

This buffer was prepared by dissolving 6.06 g Tris, 28.8 g glycine and 2 g SDS in 2 L H_2O. This solution was stored at room temperature.

2.2.1.24 SDS reducing sample buffer

A stock solution was prepared by the addition of 3.8 g Tris, 11.5 g SDS, 50 ml glycerol and 100 mM (1.56 g) DDT to 500 ml H_2O. 0.1% bromophenol blue was added to allow tracking of the protein dye front. The buffer was stored at –20 °C and thawed just prior to use .

2.2.1.25 1 M Sodium azide

6.5 g of NaN_3 was carefully added to 100 ml of H_2O in a fume hood and the resulting solution stored at room temperature.

2.2.1.26 Stacking gel buffer, pH 6.8

This buffer was prepared by the addition of 30 g Tris and 2.0 g SDS to 450 ml H_2O. Following adjustment of the pH with HCl the volume was made up to 500 ml with H_2O and the buffer stored at 4°C.

2.2.1.27 Transfer buffer

This buffer was prepared by adding 5.81 g Tris, 2.93 g glycine, 0.375 g SDS to 800 ml H_2O. This solution was stored at room temperature in the dark.

2.2.1.28 Trypan blue (0.25% w/v)

This stain was prepared by dissolving 0.25 g trypan blue in 100 ml H_2O. This was filtered through Whatman #1 filter paper and stored at room temperature.

2.2.1.29 Wash buffer

A 500 ml solution containing 50 mM imidazole, 50 mM NaH_2PO4, 300 mM NaCl, and 8 M urea was prepared and stored at room temperature.

2.2.2 Study Population

Peanut and tree nut allergic individuals were recruited from the Alfred Hospital Allergy Clinic and the Department of Pathology and Immunology, Monash University. Donors were chosen on the basis of a history of clinical symptoms of nut allergy, positive peanut or tree nut specific IgE (CAP-Pharmacia, score > 1) or skin prick test (wheal > 5 mm). The study was approved by the Alfred Hospital Ethics Committee and informed consent was obtained from all donors before blood was obtained.

2.2.3 Protein Concentration Determination

To determine the protein concentration of nut and seed extracts a Pierce micro protein assay, based upon bicinchoninic acid induced detection of protein induced reduction of cuprous ion, was used (Smith *et al.* 1985). A 1450 µg/ml stock of BGG was used to establish a standard curve in the range of 725 – 6 µg/ml. Samples for testing and BGG standard were diluted in MilliQ water and plated in triplicate (25 µl/well) in a 96 well flat bottom plate (Linbro, USA). 200 µl of Pierce Micro-Protein Assay dye concentrate was added to each well and mixed well. Plates were then read in a Bio-Rad 3550 microplate reader (Bio-Rad Laboratories, USA) at 595 nm and sample concentrations extrapolated from the standard curve using Microplate manager software.

2.2.4 Endotoxin Estimation

Endotoxin estimation was performed using a Biowhittaker Pyrogent Multi-test kit (Cambrex, USA) as per the manufacturer's instructions.

2.2.5 Antigens

2.2.5.1 Preparation of crude peanut extract, Ara h 1 and Ara h 2

Preparation of crude peanut extract, purification of natural Ara h 1 and Ara h 2 fractions, and expression of recombinant Ara h 2 is described in chapter 3.

2.2.5.2 Ara h 2 peptides

All Ara h 2 peptides were synthesized based on the published amino acid sequence deduced from the cDNA sequence of Ara h 2 (Stanley *et al.* 1997). The Ara h 2 20-mer peptides (11-18 amino acid overlap) were purchased from Mimotopes, Clayton, Australia. Lyophilised peptides were reconstituted in sterile PBS to a concentration of 1 mg/ml and filter sterilised by passage through a 0.2 µm sterile filter. Hydrophobic peptides which would not

reconstitute properly were first dissolved in a small volume (10 µl/mg peptide) of DMSO and then brought to the appropriate volume with sterile PBS and sterilised as above.

2.2.6 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Gel Staining

2.2.6.1 Preparation of 14% SDS-polyacrylamide mini gels

A 14% resolving gel solution was prepared by mixing 7 ml 50% acrylamide/BIS, 9.4 ml resolving bel buffer, 250 µl 10% SDS, 7.7 ml water, 6.25 µl TEMED and 625 µl ammonium persulphate. The gel solution was transferred into a minigel casting unit (Novex, USA) up to a point approximately 2 cm from the top of the gel plates. H_2O was then overlaid onto the gel solution to produce a flat gel surface and exclude atmospheric oxygen which interferes with polymerisation. Upon gel polymerisation (approximately 60 minutes), the gel surface was rinsed with H_2O and a 4% stacking gel added. A solution sufficient for 4 minigels comprised 4.2 ml stacking gel buffer, 1.0 ml 50% acrylamide, 4.2 ml stacking gel buffer, 125 µl of 10% SDS, 6.3 ml H_2O, 5.0 µl TEMED and 1 ml ammonium persulphate. A plastic template was then inserted into the stacking gel and the gel was allowed to stand for 30 minutes to polymerise. If not used immediately, gels were stored at 4°C in a moistened sealed plastic bag for up to 14 days.

2.2.6.2 SDS-PAGE

Gels were loaded on an electrophoresis unit (Novex, USA) and running buffer was added to the inner and outer chambers of the tank. Samples were prepared by diluting to a maximum concentration of 1.5 mg/ml in SDS-sample buffer and water and boiled for 5 minutes to ensure protein reduction, before loading onto gels. Generally 20 µl (for 10 well gels) and 10 µl (for 15 well gels) of sample was loaded per well. Where SDS-PAGE was being carried out for nitrocellulose transfer of Ara h 1, the Ara h 1 solution was diluted 1 in 10, to avoid non-specific binding of serum IgE. Low molecular weight standards (Invitrogen, USA) for gels to be stained with Coomassie Brilliant Blue were inserted at a volume of 5 µl/well and 8 µl/ml for nitrocellulose transfer. Gels were electrophoresed at a constant current of 125 volts per gel until the dye front reached the bottom of the gel.

2.2.6.3 Coomassie Brilliant Blue staining of SDS-PAGE gels

Gels were stained in 0.2% Coomassie Brilliant Blue for 1 hour at room temperature and destained in destaining solution at room temperature.

2.2.7 Immunoblotting

SDS-PAGE gels were assembled into stacks in transfer buffer as follows: blotting paper, nitrocellulose membrane, gel and blotting paper. Sample proteins and pre-stained molecular weight markers were electrophoretically transferred from SDS-PAGE gels to nitrocellulose at 30 volts for 3 hours in an Xcell II blot module (Novex, USA). Following transfer, the nitrocellulose membrane was stained with Ponceau S to ensure adequate protein transfer, then cut into strips and blocked by incubation with 10% milk powder-PBS for 1 hour at room temperature. The blocked nitrocellulose membrane was then washed in PBS and incubated with sample sera diluted 1 in 5 with 1% milk powder-PBS-Tween for all proteins except for Ara h 1, whereupon a 1 in 10 serum dilution was used. Incubation took place overnight, following which the blot was washed 3 times in PBS-Tween, 5 minutes/wash. A rabbit anti-human IgE HRPO conjugate (Dako, Denmark) diluted 1 in 500 dilution in 1% milk powder-PBS-Tween was then applied and incubated 1-2 hours at room temperature. Following washing 3 times in PBS-Tween, then 3 times in PBS for 5 minutes/wash, the membrane was incubated for 1 minute with freshly prepared chemiluminescence substrate (Du Pont, USA). Excess reagent was drained and the blot placed between two sheets of plastic transparency film. The nitrocellulose was then photographed and analysed using Labworks image acquisition software (UVP Laboratory Products, UK).

2.2.8 Inhibition Immunoblotting

To explore IgE cross-reactivity, inhibition immunoblotting was carried out. Sera for immunoblotting were diluted 1 in 5 with 1% milk powder PBS-0.05% Tween and incubated with a range of concentrations of antigen (25-100μg/ml sera) for two hours. To exclude false reduction in IgE binding in subsequent immunoblotting experiments via protease induced destruction, protease inhibitor tablets were added to the diluent (Roche, Germany). Immunoblotting was then carried out as described above.

2.2.9 Cell Culture

2.2.9.1 Cryopreservation and thawing of PBMCs and TCLs

PBMC and TCL were cryopreserved in FBS/15% DMSO in polypropylene cryovials (Greiner, Germany). Following pelleting by centrifugation, 329 x g, 10 minutes, cells were resuspended in ice cold FBS/15% DMSO at a concentration of 0.5-1 x 10^7 cells/ml. Vials were placed into a "Mr Frosty" freezing container (Nalgene, USA), and placed in a -80°C freezer overnight before transfer to liquid nitrogen (-180 °C) for long term storage.

Thawing took place via placement of the frozen cell suspension within a 37°C water bath. When thawed, the suspension was transferred to a 25 ml tube and wash medium was added drop-wise with continual mixing until 15 ml had been added. Cells were recovered by centrifugation at 329 x g for 10 minutes followed by resuspension in complete medium.

2.2.9.2 Mononuclear cell separation from peripheral blood

Peripheral blood (80-100 ml) was collected by venipuncture in 50 ml syringes flushed with preservative free sodium heparin to prevent clotting. Blood was then diluted 1:1 with warm (37°C) heparinised medium. 25 ml of diluted blood was gently layered onto 15 ml of Ficoll-Paque in a 50 ml polypropylene tube. Samples were centrifuged at 725 x g for 25 minutes at room temperature, brake off. The PBMC layer (buffy coat) was harvested using a sterile disposable plastic pipette, placed into a fresh 50 ml tube and washed with RPMI/heparin medium (30 ml RPMI:20 ml PBMC). Cells were pelleted at 500 x g for 15 minutes and washed in 20 ml of plain RPMI-1640, 329 x g for 10 minutes. PBMC were then resuspended in RPMI/5% human AB^+ serum (complete medium) and the viable cell number determined by trypan blue exclusion using a haemocytometer. These cells were then used for culture immediately of cryopreserved as described above.

2.2.9.3 PBMC proliferation assays

1 x 10^6 PBMC/well were cultured in 96-well U bottom plates (Linbro ICN Biomedicals, USA) in 200 µl complete medium along with antigen, medium alone, tetanus toxoid and PHA (2 µg/ml) as negative and two positive controls respectively for 7 days at 37°C in a humidified incubator, 5% CO_2. Cultures were pulsed for the last 16 hours with ^{3}H-thymidine (1 µCi/well) and harvested onto printed glass fibre filters (Wallac, U.K.) using a 96 well automatic cell harvester (Skatron, UK). ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy with a Wallac 1205 β-counter.

2.2.9.4 Generation of short term T cell lines

Freshly harvested or frozen stored PBMC were cultured in 24-well plates (Greiner Biotechnik, Germany) at 2.5 x 10^6 cells per well (2 ml volume) in complete medium with antigen at an optimised concentration for 7 days at 37°C in a 5% CO_2 humidified incubator. In the case of frozen PBMC the cells were first washed once in wash medium, 1 in 10 dilution of PBMC to medium, and pelleted at 329 x g for 10 minutes to remove DMSO. At day 7 cells were washed once and resuspended at 1 to 1.5 x 10^6/ml and added together with 1 x 10^6/ml washed irradiated (3000 rads; Gammacell 1000 Elite, Nordion International, Inc.) autologous PBMC (from liquid nitrogen stocks) and antigen into fresh 24-well plates. At day

2 following restimulation, 25 U/ml of recombinant human interleukin-2 (rIL-2) was added and at day 4, 1 ml of culture medium was removed and replaced with fresh medium and 25 U/ml rIL-2. For some experiments due to low cell numbers at 2 weeks, 3 week TCLs were generated by restimulation with antigen and rIL-2 as above for a further week. In all experiments T cells were rested for 6 to 7 days after the last addition of antigen and APC.

2.2.9.5 Short term T cell line proliferation assays

5.0 x 10^4/well TCL cells were cultured in triplicate in 96-well U bottom plates (Linbro ICN Biomedicals, USA) with antigen or medium alone and rIL-2 as negative and positive controls respectively, in the presence of autologous irradiated (3000 rads) PBMC (5.0 x 10^4/well). Cultures were incubated for 3 days. In the last 16 hours of culture, wells were pulsed with ^{3}H-thymidine (1 μCi/well), then harvested onto printed glass fibre filters with a 96-well automatic cell harvester. ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy.

2.2.9.6 Assessment of tissue culture reagent mitogenicity and toxicity

Mitogenic and toxic potential of tissue culture antigens as well as human sera used for preparation of complete medium was assessed using short term house dust mite specific TCL generated in a manner analogous to nut specific TCL. Mitogenicity was assessed via 3 days stimulation of this TCL with increasing concentrations of the relevant reagent, in the presence of autologous irradiated PBMC as APC, along with control wells containing either cells alone or house dust mite and IL-2 as negative and two positive controls respectively. For the last 16 hours of culture, cells were pulsed with ^{3}H-thymidine (1 μCi/well) and harvested onto printed glass fibre filters with a 96-well automatic cell harvester. In the last 16 hours of culture, wells were pulsed with ^{3}H-thymidine (1 μCi/well), then harvested onto printed glass fibre filters with a 96-well automatic cell harvester. ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy. Toxicity was assessed in a similar manner, except that, in addition to antigen, cells were co-cultured with 25 U/ml of rIL-2, in the absence of APC.

2.2.9.7 Harvesting of T cell culture supernatants for cytokine testing

Supernatants (70-75 μl/well of triplicate cultures) were harvested and pooled from T cell proliferation assay cultures at 48 hours (IL-5, IFN-γ) and frozen at -80°C. Harvested supernatants were replaced with warm (37°C) complete medium and cells subsequently pulsed and harvested as described previously.

2.2.10 Flow Cytometry

T cells from in vitro culture were washed once in cold (4°C) FACS wash buffer prior to staining. Cells ($0.5x10^6$/tube) were stained with appropriate fluorochrome-labelled monoclonal antibodies or relevant isotype controls (10 µl/tube) for 15 minutes on ice and protected from light. Cells were washed once by addition of cold wash buffer, pelleted by centrifugation (329 x g 5 minutes, 4°C) and resuspended in wash buffer. The percentage and mean fluorescence intensity of stained cells was determined from 100,000 events using a Becton Dickinson FACScalibur flow cytometer and "Cell Quest" software.

2.2.11 Cytokine ELISA

IL-5 and IFN-γ levels in culture supernatants were measured by sandwich ELISA. White Costar (Corning, USA) ELISA plates were coated with capture mAb (IL-5 and IFN-γ, 2 µg/ml; 30µl/well) diluted in binding buffer overnight at 4°C. Plates were then washed three times in PBS/0.05% Tween (wash buffer) and wells blocked with 100 µl/well of 1% BSA/PBS (blocking buffer) for 1 hour at room temperature. Following three washes in wash buffer, 30 µl/well of serial dilutions of recombinant human IL-5 or IFN-γ (5000-0.15 pg/ml) in blocking buffer-0.05% Tween or culture supernatants were added and incubated overnight at 4°C. Following four washes in wash buffer, plates were incubated with biotinylated detection mAb (IL-5, 1 µg/ml; IFN-γ, 0.5 µg/ml; 50 µl/well) diluted in blocking buffer for 1 hour at room temperature. Plates were then washed 6 times in wash buffer and incubated with a 1 in 2000 dilution of streptavidin-peroxidase (50 µl/well) in blocking buffer for 45 minutes at room temperature. Following 8 washes in wash buffer, 100 µl/well of freshly prepared chemiluminescent substrate (Perkin-Elmer, USA) was used and plates read in a Lumicount microplate glow luminometer (Packard Instrument Company, USA), 0.5 seconds/well, automatic sensitivity setting. Standard curve construction and determination of unknown cytokine levels was performed using Packard Instruments software. IL-5 and IFN-γ ELISA sensitivities were 2 pg/ml and 4 pg/ml, respectively.

2.2.12 Statistical Analysis

All statistical analysis was performed using SPSS statistical software (SPSS, USA). For non-normally distributed data, a Mann-Whitney test was employed to assess the level of significance of differences between values for a particular parameter for any two groups. For continuous variables, linear regression analysis (Pearson's) was performed to assess the degree of correlation between two parameters while logistic regression analysis was performed for categorical data.

CHAPTER 3 PEANUT ALLERGEN PREPARATION AND PURIFICATION

3.1 INTRODUCTION

Ara h 1 and Ara h 2 have previously been established as major allergens of peanut on the basis of serum IgE binding by peanut allergic cohorts, although the nature of the T cell response to these allergens remains essentially uncharacterised (Burks *et al.* 1991; Burks *et al.* 1992; Clarke *et al.* 1998; de Jong *et al.* 1998; Kleber-Janke *et al.* 1999). These allergens represent only 2 of up to 53 proteins within crude peanut extract (CPE) on the basis of SDS-PAGE, many of which might act as allergens, mitogens or prove toxic within cell culture (de Jong *et al.* 1998). Hence, for reliable experimental work to take place, a method of purification of Ara h 1 and Ara h 2 is required that adequately excludes contaminating proteins, while producing sufficient quantities of each allergen to allow completion of experiments within a suitable time frame.

A variety of methods of purification of Ara h 1 and Ara h 2 have previously been described and have included techniques based upon high performance liquid chromatography of CPE (Burks *et al.* 1991; Burks *et al.* 1992; de Jong *et al.* 1998), along with production from a cDNA or genomic DNA library (Kleber-Janke *et al.* 2000; Viquez *et al.* 2001). Within our laboratory, recombinant Ara h 2 (rAra h 2) production has been successfully achieved by Ms Maria de Leon after cloning of Ara h 2 DNA in a pPROEX-HTa vector (Invitrogen, USA) expressed in BL21-Codon Plus® *E. coli* (Stratagene, USA) and used for experiments exploring the nature of the IgE response to this allergen. This protein was initially used in attempts to drive Ara h 2 specific T cell lines but, as detailed in data presented below, with limited success, likely due to the high concentrations of endotoxin within that extract which proved impossible to remove adequately without loss of Ara h 2. The presence of endotoxin would be likely to skew the T cell response to antigen present within the same extract (Tough *et al.* 1997; Matsui *et al.* 2001). Additionally, as the recombinant Ara h 2 was produced within a prokaryotic vector, it remained unglycosylated, posing additional problems for T cell assays, such structures potentially being important in the generation of T_H2 responses as well as for the generation of cross-reactive T cell responses (Batanero *et al.* 1996; Okano *et al.* 1999; Okano *et al.* 2001). In this setting, a method of purification for Ara h 1 and Ara h 2 (nAra h 1 and nAra h 2, respectively) was established using CPE.

This chapter describes different strategies for the purification of Ara h 1 and Ara h 2 and details reasons for the choice of the final preferred method. Characterisation of those extracts

used for T cell experiments, including CPE, nAra h 1 and nAra h 2, and rAra h 2, are detailed.

3.2 METHODS

3.2.1 Preparation of Crude Peanut Extract

Peanut protein extraction was carried out using a protocol established by Ms Maria de Leon in our laboratory, and a similar protocol applied to the extraction of protein from tree nuts and citrus seeds. Raw peanuts were obtained from the Australian Peanut Board and stored at –80°C until use. For preparation of CPE, 10 gm of nut meal was placed in a 50 ml polypropylene tube and defatted by mixing with 25 ml acetone. After centrifugation at 2100 x g for 10 minutes, the pellet was washed with diethyl ether. Following centrifugation at 2100 x g for 10 minutes, this step was repeated five times. Subsequently, the pellet was vacuum filtered for approximately 10 minutes to yield dried defatted nut meal, which was then ground to a powder under liquid nitrogen using a mortar and pestle. This was placed in a 50 ml polypropylene tube and mixed with 30 ml of PBS overnight at 4 ^{0}C using an orbital rocker. After centrifugation at 12000 x g for 10 minutes the supernatant was collected and placed in 25 ml ultracentrifuge tubes and centrifuged at 19000 x g for 45 minutes to obtain a clear supernatant. To remove residual ether, the supernatant was dialysed using a 3.5 kDa cutoff dialysis membrane (Pierce, USA) in 5 L of PBS at 4 ^{0}C with 3 changes of dialysate overnight. For cell culture, the supernatant was then filter sterilised through a 0.2 µm filter before being aliquoted into 1 ml cryovials for preservation at –30 ^{0}C. All extracts used for cell culture were characterised with regards lymphocyte mitogenic and toxic potential, while SDS-PAGE, protein estimation and western blotting for IgE reactivity was carried out on all extracts (see relevant sections in this chapter).

3.2.2 High Performance Liquid Chromatography for Fractionation of Peanut Extract

3.2.2.1 Size exclusion chromatography

2 ml of CPE 5 mg/ml was concentrated to approximately 300 µl volume in an Amicon YM-3 3 kDa pore size centrifugal filtration device (Millipore, USA). Using a Pharmacia Superdex 75 HR10/30 size exclusion column (Amersham Pharmacia, Sweden) in a Biorad Biologic HPLC circuit (Biorad, USA), 250 µl of the concentrated CPE was loaded onto the column and 0.5 ml fractions collected in a 50 ml elution volume, using filtered PBS as the elution

buffer. Protein containing fractions were resolved on a 14% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue.

3.2.2.2 Cation exchange chromatography

CPE at a concentration of 5 mg/ml was thawed and re-equilibrated in 10 mM $NaPO_4$ buffer, pH 6.7. Three 1 ml aliquots of CPE were loaded onto a Pharmacia Mono S 2 ml cation exchange column (Amersham Pharmacia, Sweden) equilibrated in 10 mM $NaPO_4$ buffer, pH 6.7 (buffer A), and the run-off collected for subsequent nAra h 2 purification. After washing the column with 20 ml of 10 mM $NaPO_4$ buffer, pH 6.7, HPLC was performed using a 26 ml three step gradient of increasing concentration of 1 M NaCl in 10 mM Tris HCl pH 6.7 as the elution buffer (buffer B). 23% buffer B was run for 5 ml to elute contaminating proteins, then 40% buffer B was run to elute relatively pure Ara h 1 with eluted protein collected in 1 ml fractions. Protein containing fractions were then resolved on a 14% SDS polyacrylamide gel. After centrifugal filtration at 3000 x g in a Sartorius Vivaspin 50 kDa pore size 6 ml filtration unit (Sartorius, Germany) to remove contaminating low molecular weight proteins, pooled nAra h 1 containing fractions were re-equilibrated in PBS and filter sterilised using a 0.2 µm filter before characterisation and storage at –20 ^{0}C.

3.2.2.3 Anion exchange chromatography

Flow-through collected during loading of CPE onto the cation exchange column was found to be relatively abundant in nAra h 2 and was therefore used for its further purification. Pooled flow-through from CPE used for cation exchange was re-equilibrated in 10 mM Tris HCl pH 7.6. Six ml of the run off was loaded onto a Bio-Scale Q 2 ml column (Bio-Rad Laboratories, USA) equilibrated in 10 mM Tris HCl pH 7.6 (buffer A). Using 1 M NaCl in 10 mM Tris HCl pH 7.6 (buffer B), a 20 ml linear gradient from 0% to 50% buffer B was established to elute bound protein in 1 ml fractions. Protein containing fractions were then resolved on a 14% SDS polyacrylamide gel. Fractions high in nAra h 2 were pooled, re-equilibrated in PBS and filter sterilised using a 0.2 µm filter before characterisation and storage at –20 ^{0}C.

3.2.3 Recombinant Ara h 2 Preparation

3.2.3.1 Expression of rAra h 2 in BL21-Codon Plus® *E. coli* (Stratagene, USA)

BL21-Codon Plus® *E. coli* (Strategene, USA) were transformed with Ara h 2 cDNA by Ms Maria de Leon as part of her doctoral research, and stocks of these transformed

bacteria stored in glycerol were a kind gift from Ms de Leon. Streaking for single colonies was performed on L-agar plates containing 1 ml/L of 10% ampicillin. Plates were incubated overnight in a 37°C warm room. Cultures were then established from single colonies in 100 ml L-broth containing 100 µg/ml of ampicillin, overnight at 37°C with shaking. Cultures were diluted 1 in 10 in L-broth/100 µg/ml ampicillin and incubated with shaking at 37°C for 3-4 hours or until the $OD_{(600\ nm)}$ was between 0.5-1. Expression of rAra h 2 was induced by addition of 600 mM (final concentration) IPTG and cultures continued for 3-4 hours. Induced cultures were then pelleted, 1500 x g for 10 minutes at room temperature, and resuspended in lysis buffer at a concentration of 5 ml/g wet weight. Resuspended pellets were then sonicated 6 times in 20 second intervals to break apart bacterial cells and release the soluble rAra h 2 into the supernatant. The insoluble fraction of the sonicate was pelleted, 1500 x g for 10 minutes, and the supernatant containing the soluble rAra h 2 was harvested. Small amounts of both the pellet and supernatant were kept for analysis by SDS-PAGE as described.

3.2.3.2 Nickel column affinity purification of rAra h 2

A commercially available nickel column kit (Novagen, Inc.) was used to purify rAra h 2 from induced bacterial cell sonicates. A 50% Ni-NTa resin with a 5 ml bed volume was equilibrated using 5 column volumes of lysis buffer. The sonicate containing soluble rAra h 2 was then run through the column, followed by 10 volumes of wash buffer to remove weakly bound bacterial proteins. The bound protein was then eluted from the column with 4 column volumes of elution buffer and collected in separate 5 ml fractions. The unbound sonicate, washes and eluate were analysed by SDS-PAGE, immunoblotting, and protein estimation.

3.2.4 Acetone Precipitation

Five ml of 6.5 mg/ml CPE was thawed and placed in a salt water ice bath to maintain a temperature less than –2° C. 1.25 ml of acetone was added to the CPE to create a 20% acetone solution, and allowed to stand in the ice bath for 15 minutes. Precipitated protein was then pelleted via centrifugation at 750 x g for 10 minutes and the supernatant decanted, before resuspension of the protein pellet in 5 ml PBS. Further acetone was added to the supernatant to create a 25% acetone solution and the process repeated in 5% acetone increments until a 50% acetone solution was achieved. Protein precipitated at each concentration and the associated supernatant was analysed for protein content via resolution on a 14% SDS-polyacrylamide gel.

3.2.5 Ammonium Sulphate Precipitation

A saturated solution containing ammonium sulphate was prepared and stored at 4° C. CPE, 4 mg/ml, was thawed and placed in aliquots of between 100 µl and 700 µl in Eppendorf tubes, and the saturated ammonium sulphate solution added slowly with stirring to make up the volume of each to 1 ml, creating a range of concentrations of ammonium sulphate between 30% and 90%. These solutions were allowed to stand for 20 minutes at 4° C before pelleting of precipitated protein via centrifugation at 1900 x g for 10 minutes. Supernatants were then collected and pelleted protein resuspended in fresh 1 ml PBS. Both supernatant and resuspended protein were then analysed via resolution on a 14% SDS polyacrylamide gel.

3.2.6 Gel Excision

In 500 µg aliquots, CPE was resolved on three 1 mm 10% SDS polyacrylamide mini-gels (Novex, USA) and stained using Coomassie Brilliant Blue dye. After visualisation of the resolved protein extract on a light box, the region of the gel corresponding to nAra h 1 was excised using a clean razor blade. Each excised band was then stacked onto a 1.5 mm 10% SDS polyacrylamide mini-gel and electrophoresed until the dye front had run into the running buffer within the gel tank. The gel was then macerated and placed in 2.5 ml 0.1 M Tris HCl, pH 9.5. After 2 hours, the Tris HCl solution was decanted and a second elution performed overnight using a further 2.5 ml of 0.1 M Tris HCl, pH 9.5.

A second method of nAra h 1 extraction was also evaluated, whereby after the dye front had run into the running buffer from the secondary gel, the running buffer was replaced with freshly prepared running buffer in the lowest volume possible to achieve electrophoresis within the gel tank, representing approximately 120 ml. Electrophoresis was then continued for a further 150 minutes and the running buffer collected, and acetone precipitation performed of protein within the running buffer. After centrifugation, the pelleted protein was resuspended in PBS. Protein concentration was determined and resolution on a 14% SDS-polyacrylamide gel carried out.

3.2.7 Ultrafiltration

Ultrafiltration was attempted with CPE using Amicon Centriprep regenerated cellulose filtration devices in 10 kDa and 50 kDa pore sizes (Millipore, USA). For the 10 kDa nominal molecular mass cut-off filter, 2 ml of 5 mg/ml CPE was placed in a 2 ml filtration unit and subjected to 6000 x g for 70 minutes. The 400 µl retentate was then made up to 2 ml, resolved on a 14% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue dye.

For the 50 kDa pore size filter, 1 ml of CPE was diluted to 15 ml in PBS, then subjected to 3500 x g producing a retentate of 2.5 ml after 30 minutes. This process was repeated in 3 further cycles and the retentate and CPE protein profile compared after resolution on a 14% SDS polyacrylamide gel and staining with Coomassie Brilliant Blue.

3.3 RESULTS

3.3.1 Analysis of CPE

3.3.1.1 SDS-PAGE

CPE was resolved on a 14% SDS-PAGE gel, illustrated within Figure 3.1, and demonstrated 16 protein bands with molecular masses ranging from 8 kDa to 180 kDa. The most prominent protein within the extract had a molecular mass of 61 kDa, consistent with Ara h 1 (Burks *et al.* 1991). A second protein with an average molecular mass of 15 kDa and visualised as two closely migrating bands on SDS-PAGE was consistent with Ara h 2 (Burks *et al.* 1992). Although slightly lower than its reported molecular mass of 17 kDa, sequencing of the purified protein, as described below, confirmed its identity as Ara h 2 (Burks *et al.* 1992; Stanley *et al.* 1997). Ara h 3, Ara h 5, Ara h 6 and Ara h 7 are reported to have similar molecular masses of approximately 14 to 16 kDa and may each have been present in a band immediately below Ara h 2, although as for Ara h 2, are running lower than reported on 14% SDS-PAGE (Kleber-Janke *et al.* 1999; Rabjohn *et al.* 1999). A large band of 36 kDa molecular mass may have represented Ara h 4 (Kleber-Janke *et al.* 1999). The peanut extract also contained proteins of lower molecular mass, consistent with the known panallergen lipid transfer protein (Pastorello *et al.* 2001).

3.3.1.2 Western blotting for IgE reactivity

Both Ara h 1 and Ara h 2 in the CPE bound IgE extensively among the 30 peanut allergic subjects used for these experiments, being recognised by 97% and 93% of sera from the peanut allergic subjects, respectively (Figure 3.2). However, Ara h 1 was extensively recognised by IgE in non-peanut allergic sera, and when western blotting was reoptimised to minimise this phenomenon the percentage of peanut allergic sera demonstrating IgE reactivity with Ara h 1 fell to 70%. These findings are discussed in detail in chapter 4. Interestingly, a number of other bands were frequently associated with IgE binding, as indicated in Figure 3.2. Those which were recognised by IgE from greater than 50% of the peanut allergic cohort had molecular masses of 89, 36, 26, 25, 24, 20, 11 and 9 kDa.

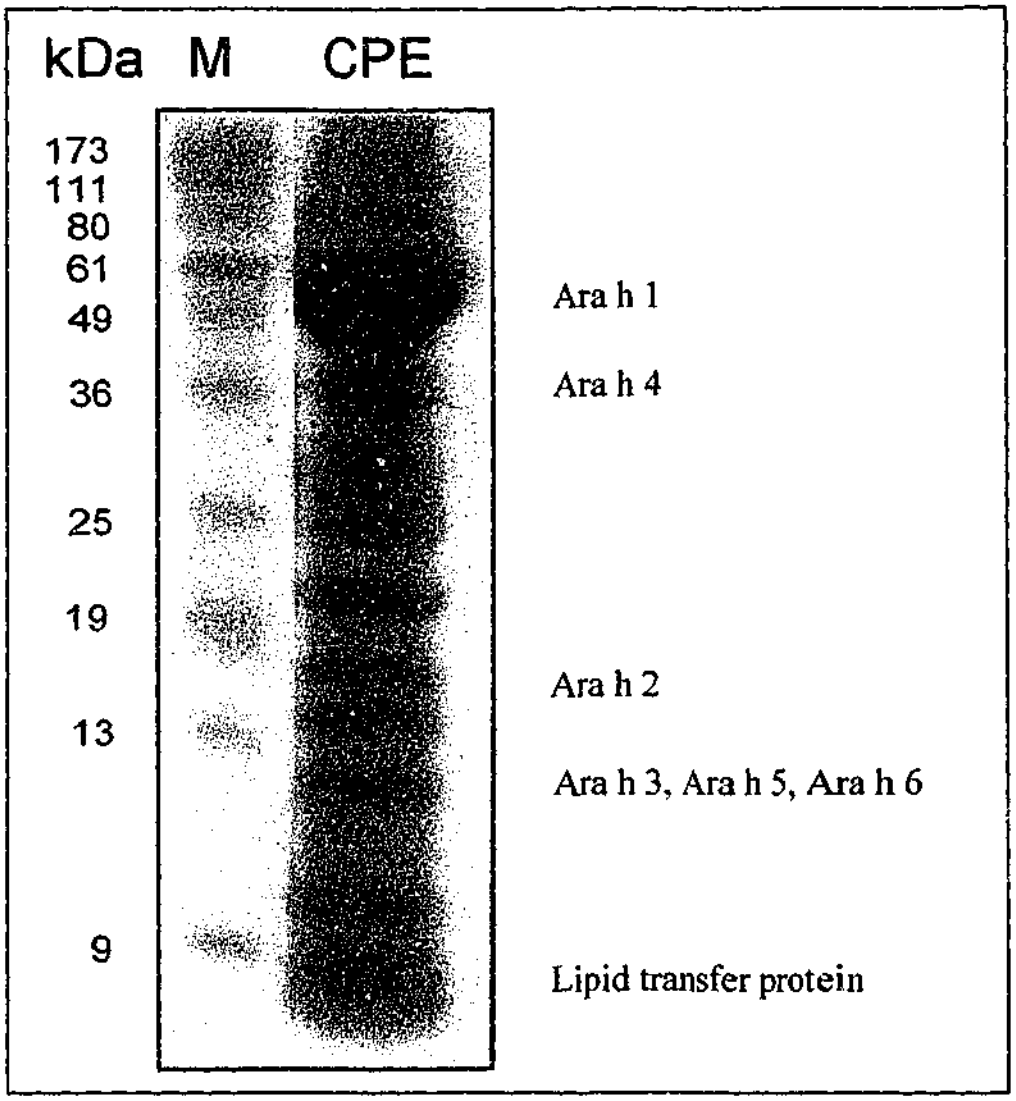


Figure 3.1 SDS-PAGE analysis of crude peanut extract

kDa: relative molecular mass; M: molecular mass markers; CPE: crude peanut extract. CPE was resolved on a 14% SDS-PAGE gel and proteins visualised with CBB staining. Known peanut allergens and pan-allergens are displayed adjacent to their predicted positions within the resolved extract.

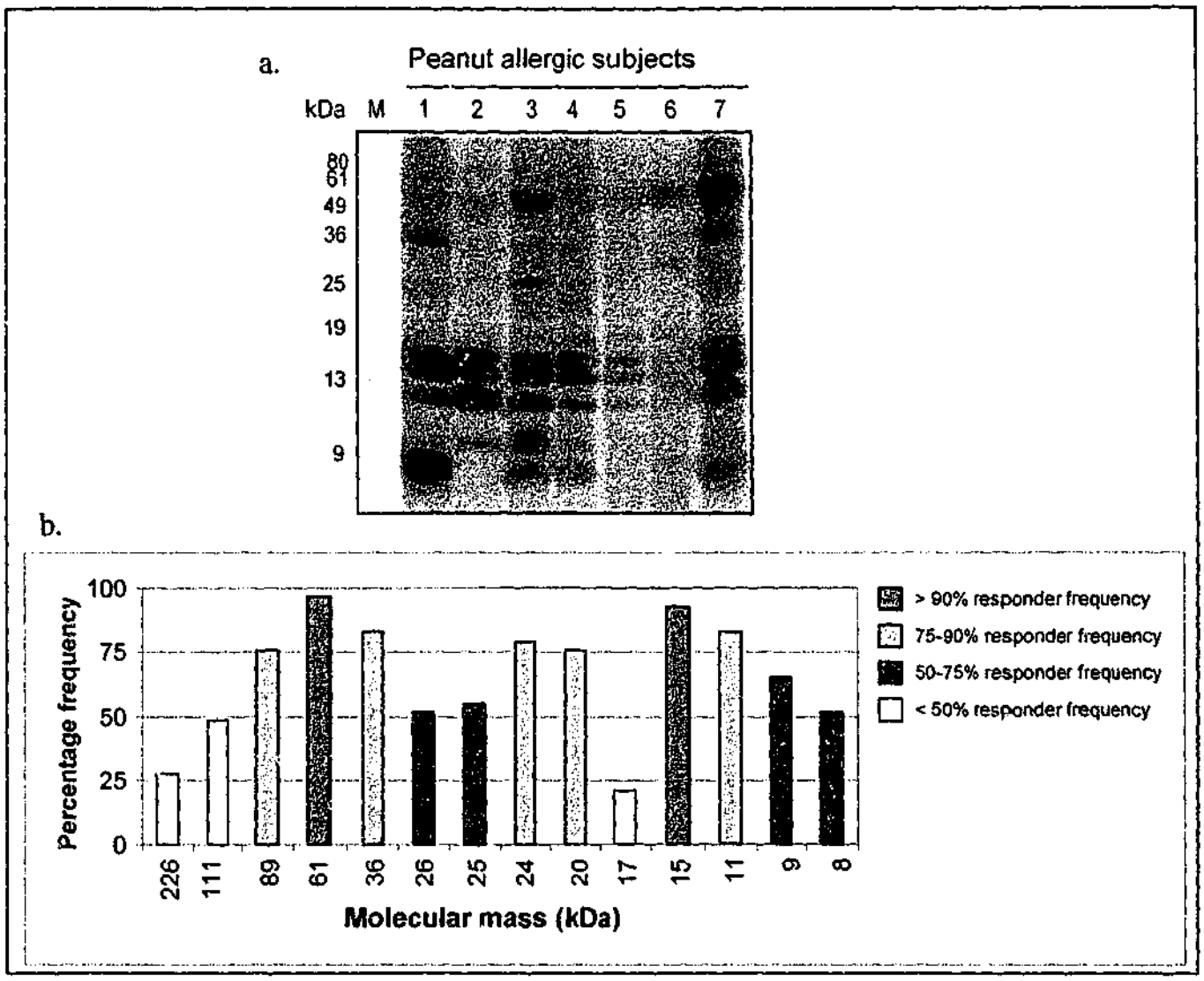


Figure 3.2 (a) Representative western blot and (b) percentage frequency of IgE reactivity among peanut proteins

(a) Western blots for peanut protein specific IgE from 7 representative peanut allergic subjects. (b) Percentage of individuals with IgE reactivity to individual peanut proteins visible on western blotting, amongst a cohort of 30 peanut allergic subjects. Proteins likely to represent Ara h 1 (61 kDa) and Ara h 2 (15 kDa) were recognised by IgE from greater than 90% of donors.

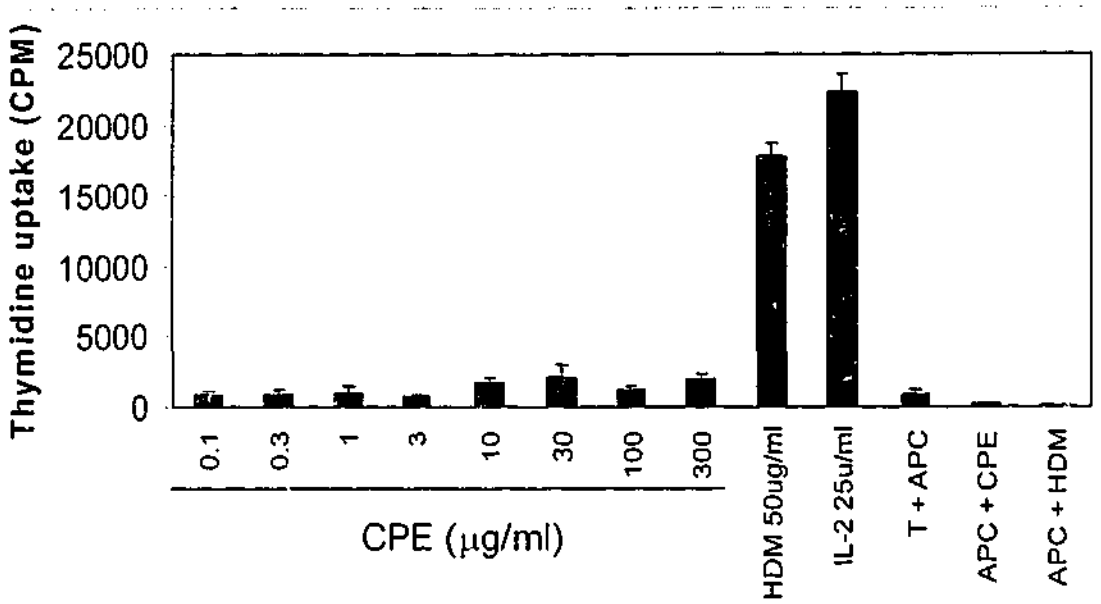
3.3.1.3 T cell mitogenicity and toxicity

To ensure that CPE was suitable for use in T cell assays, mitogenicity, toxicity and endotoxin estimation assays were carried out. These data are summarised in Figure 3.3. Using a three week oligoclonal T cell line specific for house dust mite (kind gift of Ms Leanne Gardner), it can be seen that no significant proliferation occurs when these cells were stimulated with CPE, while there is limited suppression of proliferation demonstrated amongst the same cell line when culture with CPE occurred in the presence of recombinant IL-2. Endotoxin estimation using a limulus amebocyte lysate assay (Bio-Whittaker, USA) revealed only low concentrations, 23.6 EU/ml or 0.0079 EU/µg protein being demonstrated. Hence, significant mitogenic or toxic potential, along with endotoxin concentrations, were absent within the CPE. Proliferation assays using PBMC from both allergic and non-allergic donors demonstrated dose-dependent responses and are outlined within chapter 4.

3.3.2 Analysis of Natural Ara h 1

For a total quantity of 12 mg CPE loaded onto the cation exchange column, 1.44 mg nAra h 1 was collected, providing a yield of 12%. The chromatogram during cation exchange and corresponding appearance of collected fractions after resolution on a 14% SDS-polyacrylamide gel are illustrated in Figure 3.4. Fractions used for further purification of nAra h 1 (indicated by # within the figure) revealed the expected protein band of 61 kDa molecular mass, as well as a further band of 30 kDa molecular mass, and low abundance proteins of approximately 8 kDa molecular mass. The latter proteins were removed via filtration through a 50 kDa centrifugal filtration device (Vivaspin, Sartorius, Germany) These proteins were only faintly visible in prefiltration samples when resolved on a 14% SDS-PAGE gel (data not shown). The final extract contained the 61 kDa band in greater than 95% relative abundance of the total protein within the extract, but continued to demonstrate the 30 kDa band in minimal quantities (Figure 3.5). This band could not be removed from nAra h 1 containing extracts by any of the other methods of purification attempted. Western blotting of the extract demonstrated that the 61 kDa protein was recognised by IgE within donor peanut allergic sera but not non-peanut allergic controls. Unfortunately, commercial N-terminal protein sequencing could not be performed. This was likely to be due to the technique used for sequencing and elution of protein from the PVDF substrate, as despite large quantities of protein being presented for sequencing, extremely low levels of protein were reported as being detected. However, based on size, abundance, and its IgE binding properties, this protein was likely to represent nAra h 1, and alternative methods of identification were not performed.

(a) mitogenicity assay



(b) toxicity assay

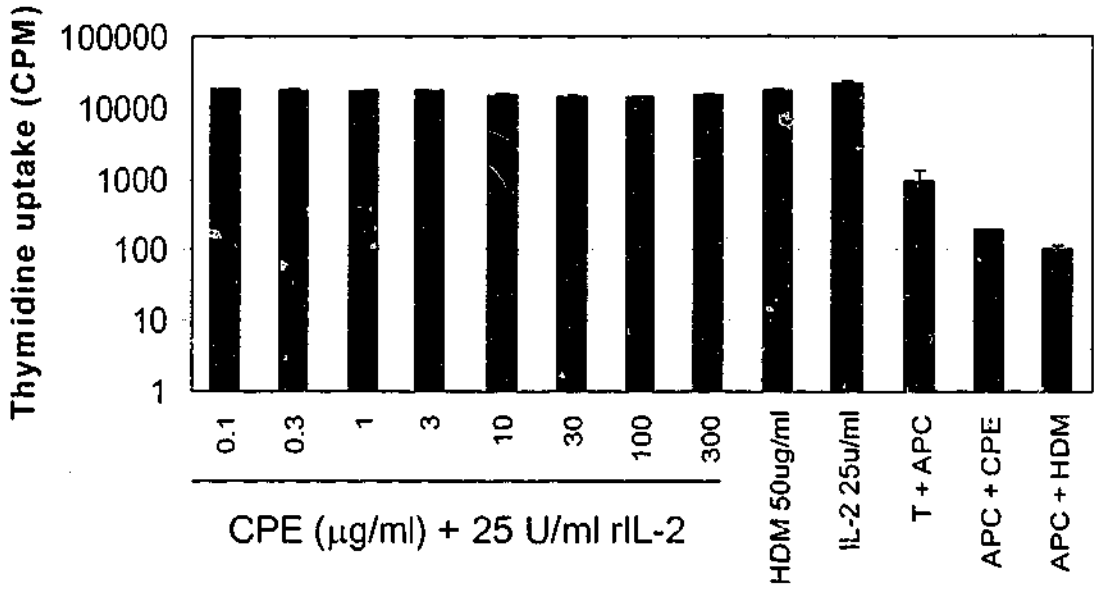
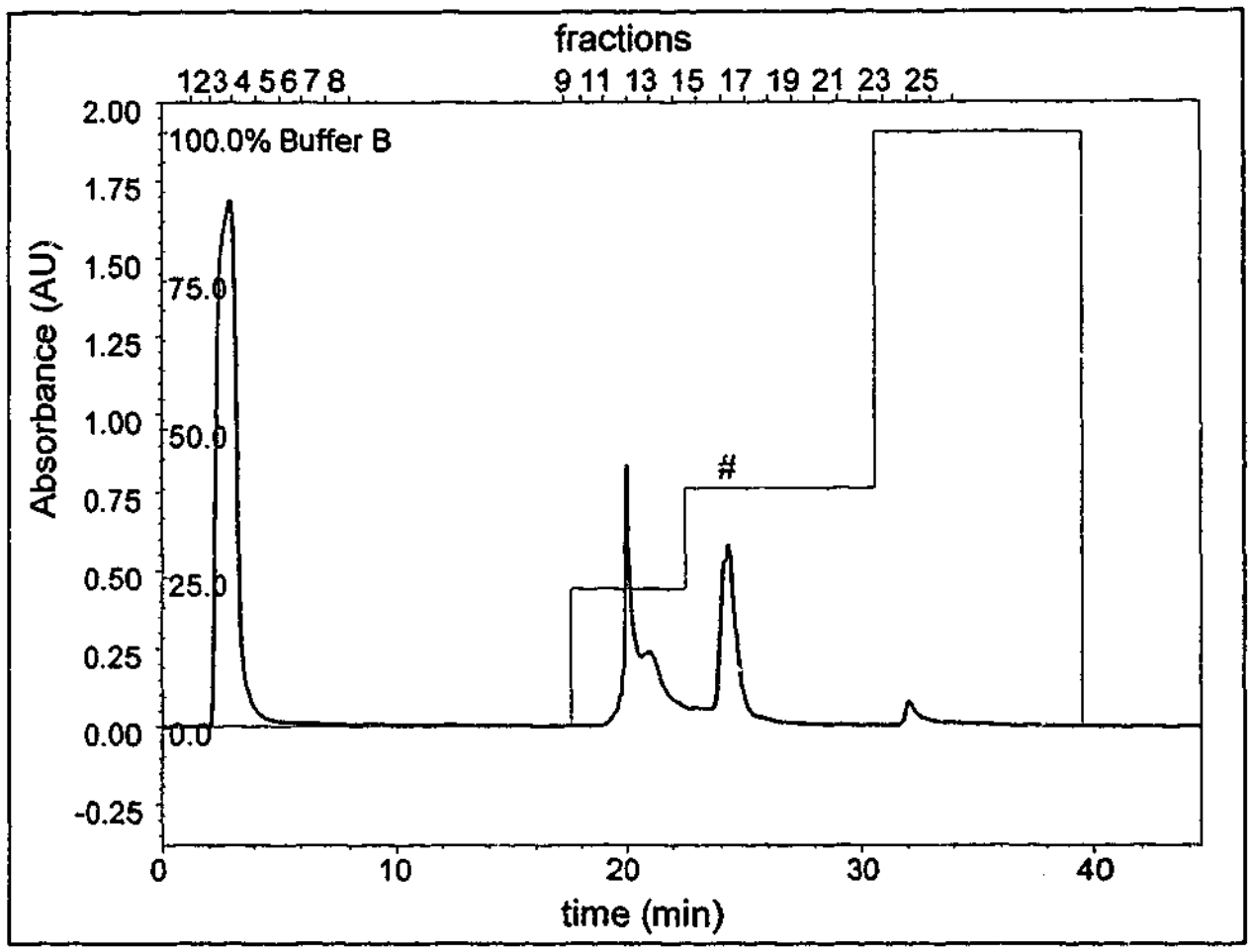


Figure 3.3 CPE T cell mitogenicity and T cell toxicity assays

(a) T cell mitogenicity assay: Triplicate cultures of 5 x 10^4 cells/well of a 3 week HDM specific TCL plus APC, stimulated with a range of concentrations of CPE for 72 hours.

(b) T cell toxicity assay: Triplicate cultures of 5 x 10^4 cells/well of a 3 week HDM specific TCL, stimulated with a range of concentrations of CPE extract in the presence of 25 U/ml of rIL-2 for 72 hours.



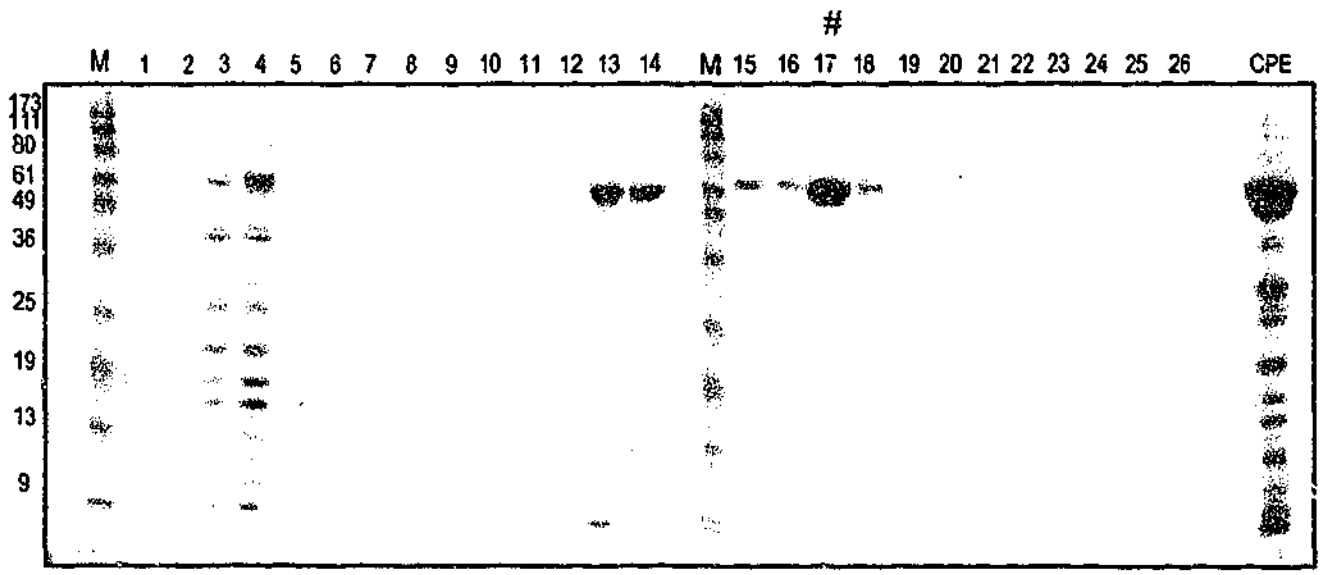


Figure 3.4 Chromatogram and 14% SDS-PAGE of eluted fractions from cation exchange purification of nAra h 1.

kDa: kilodaltons; M: molecular mass markers; 1-25: 0.5 ml fractions collected during anion exchange; CPE: crude peanut extract

5 mg/ml CPE was equilibrated in 10 mM Tris HCl buffer, pH 7.6. A 3 step isocratic protocol was established, using 1 M NaCl in 10 mM Tris HCl as the elution buffer (buffer B). Three 5 mg/ml 1 ml aliquots of CPE were loaded onto a Pharmacia Mono S 2 ml cation exchange column and the run-off collected for subsequent Ara h 2 purification. After washing the column with 20 ml of 10 mM Tris HCl, 23% buffer B was run for 5 ml to elute contaminating proteins, then 40% buffer B was run to elute relatively pure nAra h 1 (indicated by #). 0.5 ml fractions collected were collected and resolved on a 14% SDS-polyacrylamide gel then stained with CBB. Those fractions containing relatively pure nAra h 1 were collected for ultrafiltration and use in T cell experiments.

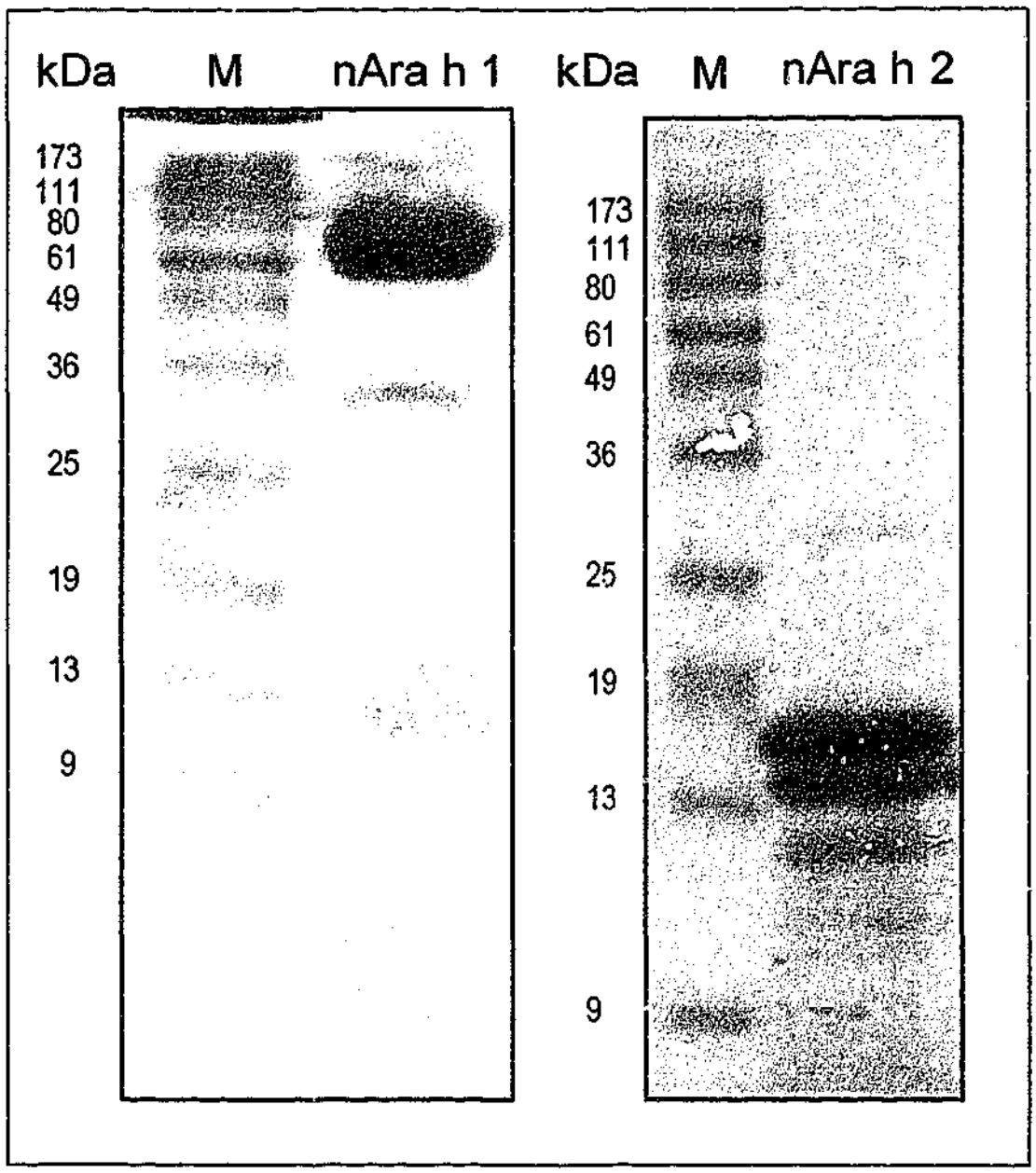


Figure 3.5 14% SDS PAGE profile of purified nAra h 1 and nAra h 2 extracts

M: molecular mass markers; kDa: molecular mass

Cation exchange and ultrafiltration purified nAra h 1, and cation and anion exchange purified nAra h 2 were resolved on a 14% SDS polyacrylamide gel and stained with CBB. These extracts were used for subsequent T cell experiments.

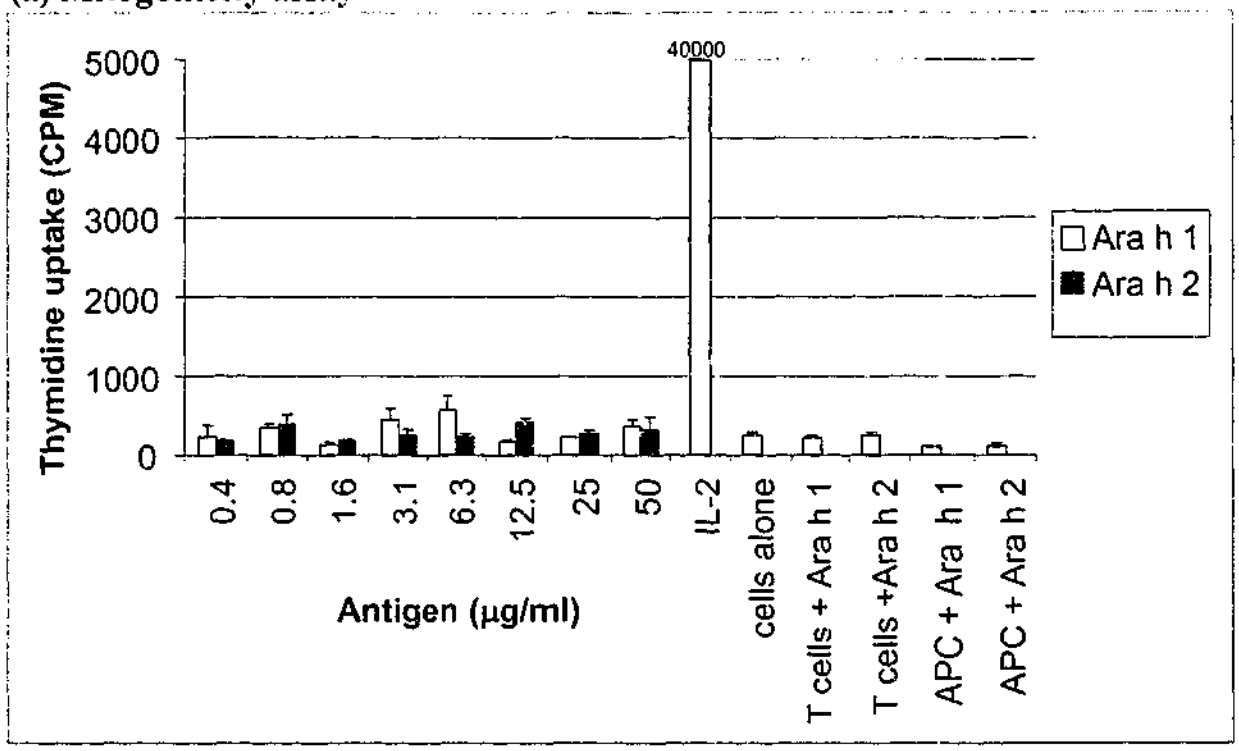
T cell mitogenicity and toxicity assays demonstrated that the extract did not possess either of these confounding properties, as illustrated in Figure 3.6. A dose response was demonstrated amongst PBMC from both peanut and non-peanut allergic donors, and these experiments are described in chapter 4. Estimation of endotoxin concentration demonstrated that only low quantities were contained within the extract, being approximately 5 EU/ml and 0.0024 EU/μg. Western blotting demonstrated that a subject with highly reactive IgE to CPE (subject A1) bound lower molecular weight contaminant proteins within the extract (Figure 3.7). These proteins, of low relative abundance, and weaker IgE reactivity than nAra h 1, were not sequenced, and it is therefore not clear whether this binding represents recognition of breakdown products, co-migration of nAra h 1 with these lower molecular weight proteins, or the presence of unique IgE-reactive proteins. Subjects with weaker IgE reactivity, typical of the profile demonstrated by the peanut allergic cohort, did not react to these lower molecular weight proteins.

3.3.3 Analysis of Natural Ara h 2

Production of nAra h 2 from CPE via a 2 step purification protocol including cation then anion exchange provided a yield of approximately 4%. Following anion exchange of the run-off collected upon loading CPE on to a cation exchange column, collected fractions were resolved on a 14% SDS-polyacrylamide gel (Figure 3.8). Those fractions rich in nAra h 2 (indicated by * within the figure) were pooled and resolved on a 14% SDS-polyacrylamide gel, upon which nAra h 2 demonstrated a relative abundance of greater than 95% (Figure 3.5). nAra h 2 formed a doublet within the gel, unlike its recombinant form, with a molecular mass of approximately 15 kDa. These bands underwent N-terminal sequencing and the first five amino acids demonstrated an identical sequence to the published sequence for nAra h 2 (Stanley *et al.* 1997). Western blotting confirmed that these proteins retained their capacity to bind IgE amongst peanut allergic subjects, in the absence of such binding by non-allergic controls (Figure 3.7). Several low abundance contaminant proteins of lower molecular weight could also be seen on staining the gel with Coomassie Brilliant Blue dye. As for nAra h 1, serum from a subject with exquisite IgE reactivity to all proteins within CPE, but not sera from other subjects, bound these lower molecular weight proteins (Figure 3.7).

nAra h 2 was subjected to T cell mitogenicity and toxicity assays which demonstrated that the extract possessed neither confounding feature (Figure 3.6). Proliferation assays, described in detail in chapter 4, demonstrated a dose-response amongst both allergic and non-allergic controls. This was not due to the presence of endotoxin, assays for this demonstrating 2.5 EU/ml of extract and 0.0011 EU/μg.

(a) Mitogenicity assay



(b) toxicity assay

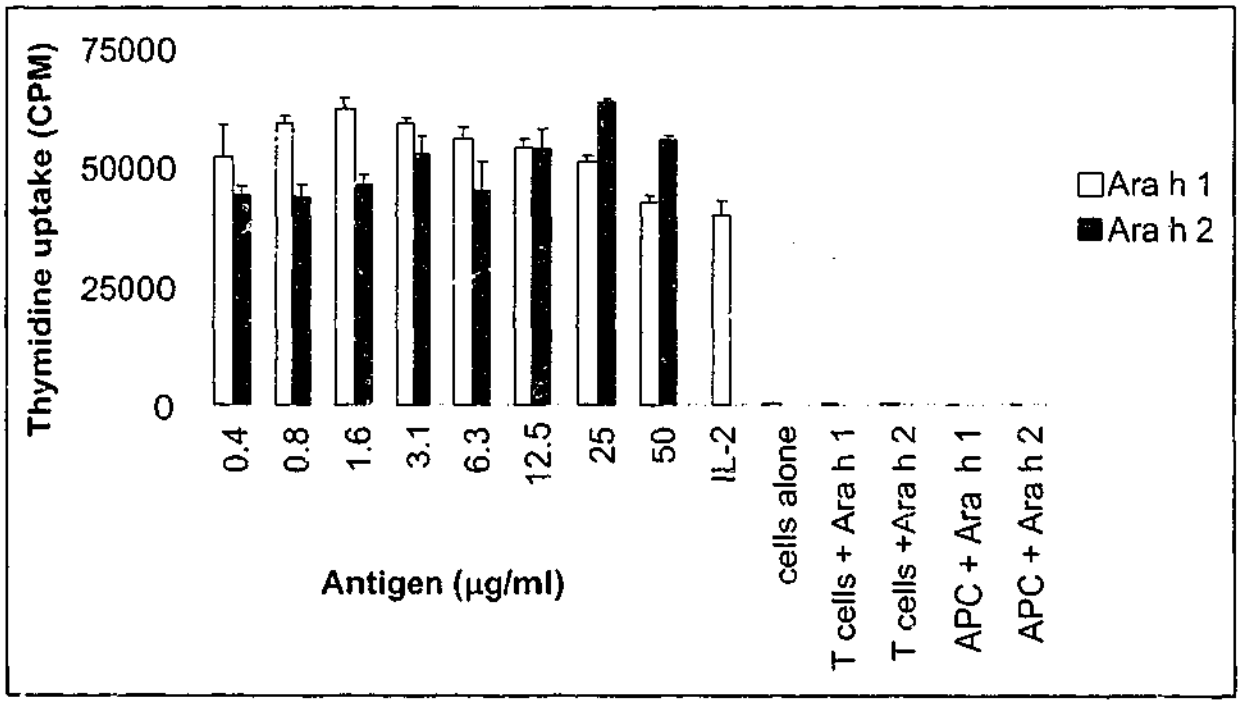


Figure 3.6 HPLC purified Ara h 1 and Ara h 2 T cell mitogenicity and T cell toxicity assays

a. **T cell mitogenicity assay:** Triplicate cultures of 5 x 10^4 cells/well of a 3 week HDM specific TCL plus APC, stimulated with a range of concentrations of either Ara h 1 or Ara h 2 for 72 hours.
b. **T cell toxicity assay:** Triplicate cultures of 5 x 10^4 cells/well of a 3 week HDM specific TCL plus APC, stimulated with a range of concentrations of either Ara h 1 or Ara h 2 extract in the presence of 25u/ml of rIL-2 for 72 hours.

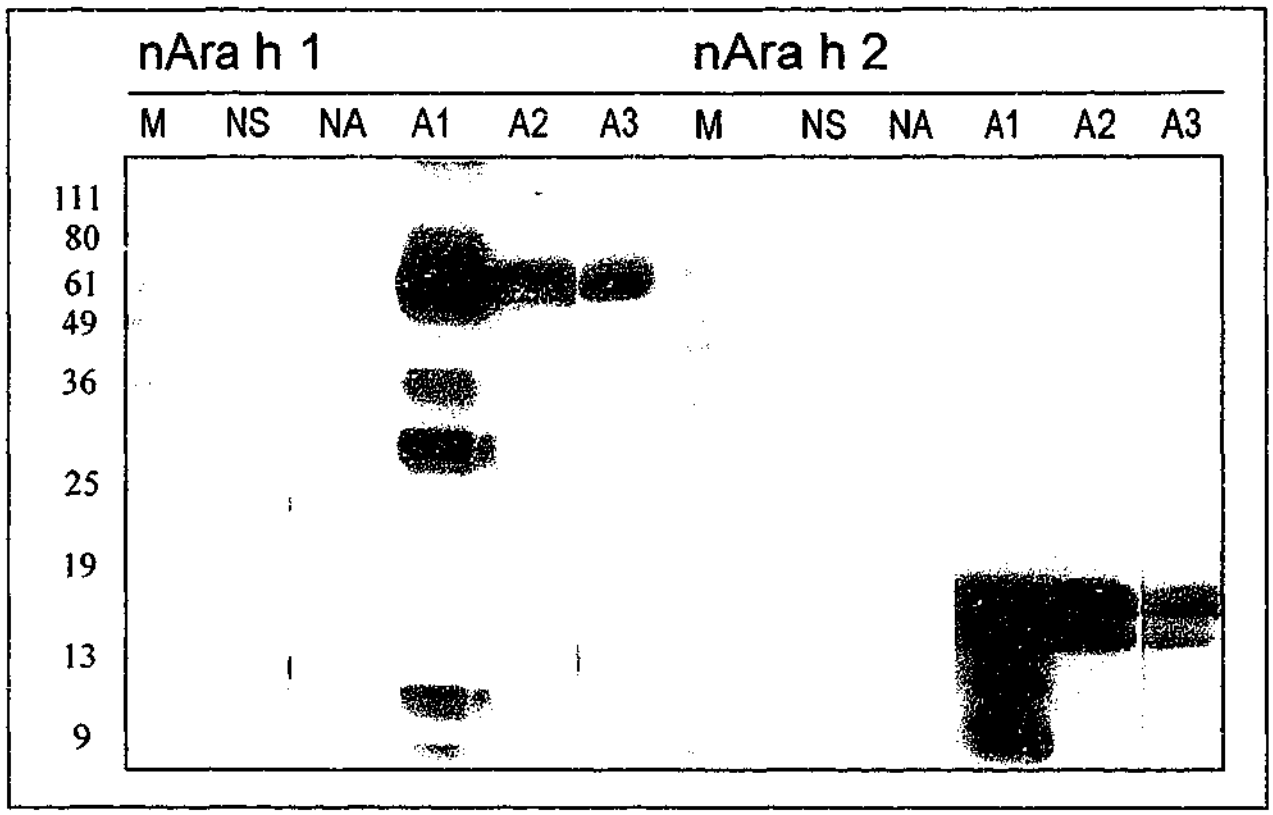
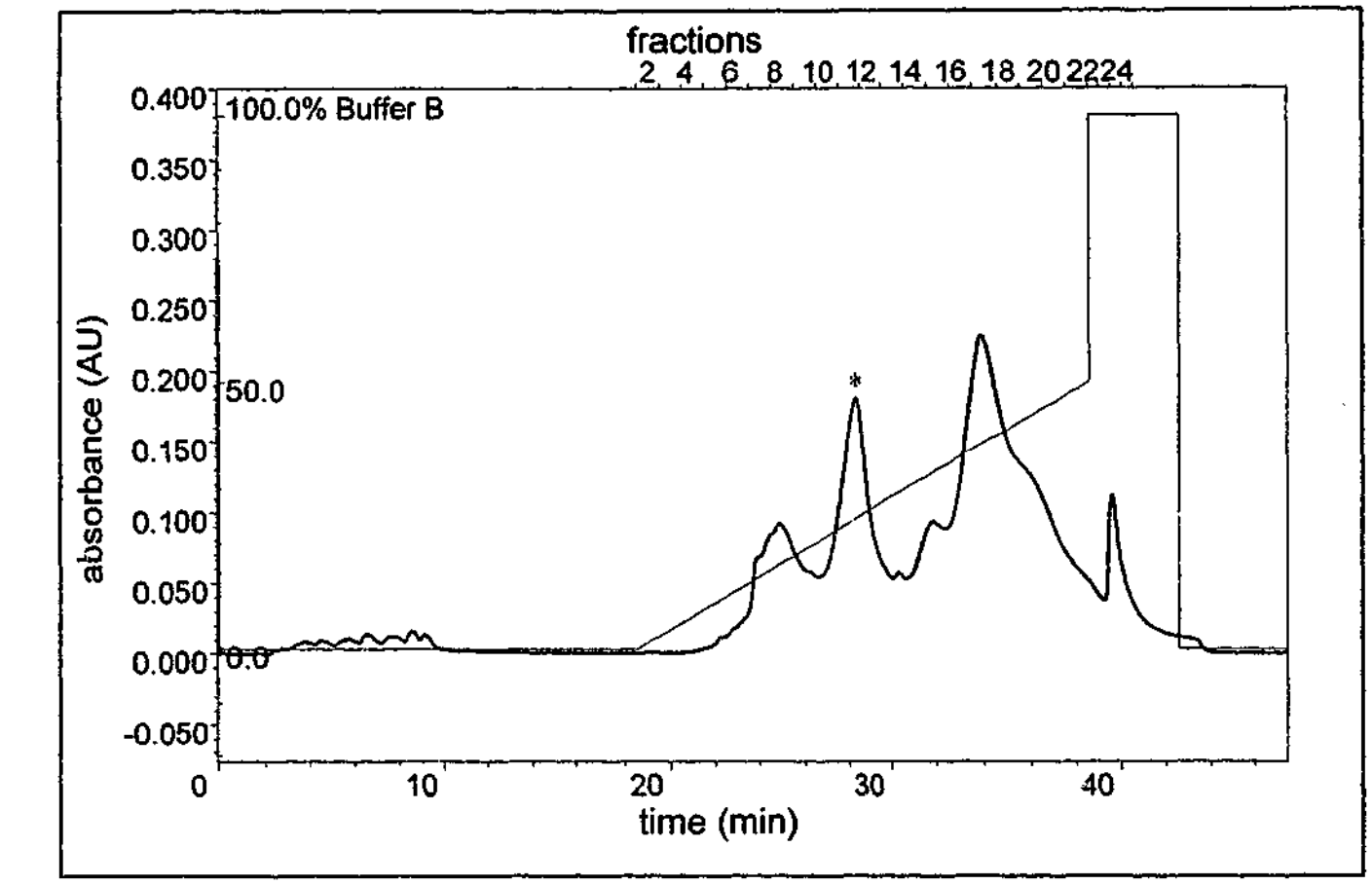


Figure 3.7 Western blot of purified Ara h 1 and Ara h 2

M: molecular mass markers; NS: no sera; NA: non peanut allergic subject; A1: highly sensitised peanut allergic subject; A2, A3:, peanut allergic subjects.

Purified nAra h 1 and nAra h 2 were resolved on a 14% SDS polyacrylamide gel then transferred to nitrocellulose and immunoblotted with peanut allergic and non-allergic sera, along with a no serum control. Subject A1, selected for detection of immunoreactive proteins within the extracts, had previously demonstrated exquisite sensitivity to all proteins within CPE, and was similarly reactive to both purified proteins and contaminants. The other peanut allergic subjects demonstrated reactivity only to nAra h 1 and nAra h 2.



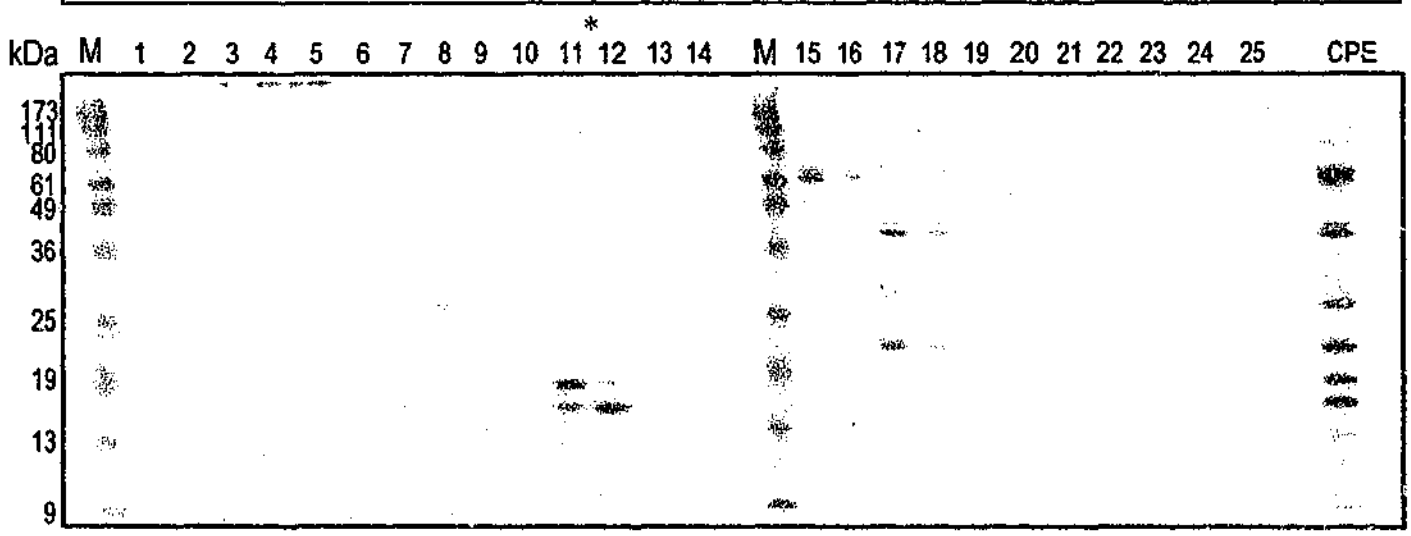


Figure 3.8 Chromatogram and 14% SDS-PAGE of anion exchange purification of nAra h 2

kDa: kilodaltons; M: molecular mass markers; 1-25: 0.5 ml fractions collected during anion exchange; CPE: crude peanut extract

Run-off from cation exchange of CPE was collected and used for purification of nAra h 2. For each ml of CPE loaded onto the Pharmacia Mono S cation exchange column, 2 ml of run-off relatively high in nAra h 2 and low in nAra h 1 was collected. These fractions were pooled and re-equilibrated in 10 mM $NaPO_4$ buffer, pH 6.7. 6ml of the run off solution was loaded onto a Biorad Q 2 ml anion exchange column. After washing the column with 20 ml of 10 mM $NaPO_4$ (buffer A), a 20 ml linear gradient was established between 0% and 50% 1 M NaCl in 10 mM $NaPO_4$ (buffer B) for elution of the bound proteins. A relatively pure nAra h 2 containing band occurred at approximately 22.5% buffer B (indicated by *). 0.5 ml fractions were collected and resolved on a 14% SDS-polyacrylamide gel then stained with CBB. Those fractions containing relatively pure nAra h 2 were collected for use in T cell experiments.

3.3.4 Analysis of Recombinant Ara h 2

Resolution of rAra h 2 on a 14% SDS-polyacrylamide gel demonstrated a dominant band with a molecular mass of approximately 17 kDa, illustrated in Figure 3.9. The plasmid construct for this protein was sequenced within our laboratory and found to match the corresponding published sequence for rAra h 2, while upon ELISA, rAra h 2 was recognised by serum IgE from 14 out of 20 peanut allergic subjects (experiment performed by Ms Maria de Leon).

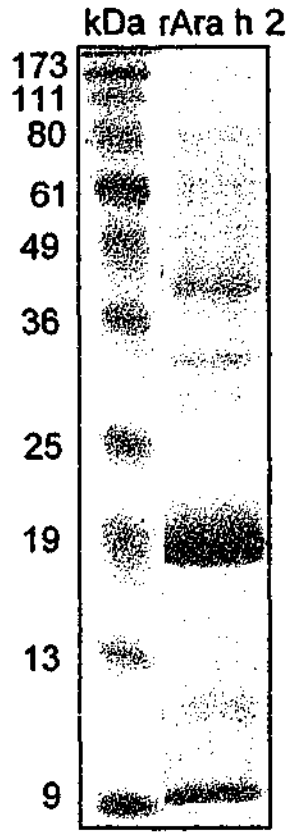
T cell mitogenicity and toxicity assays were carried out using rAra h 2 and are illustrated in Figure 3.9, within which it is demonstrated that rAra h 2 possessed neither confounding feature. Hence rAra h 2 was used to stimulate TCL for use within Ara h 2 epitope mapping experiments. Within these experiments, detailed in chapter 5, TCL initially stimulated with CPE at days 1 and 8, followed by stimulation with rAra h 2 at day 15 were less likely to produce peptide responses than those stimulated with CPE throughout their production, and those that were associated with peptide responses demonstrated responses in an indiscriminate manner. To explore causes for this phenomenon, TCL were produced using rAra h 2 for stimulation at days 1, 8, and 15. Cell lines produced in this manner demonstrated a response to rAra h 2 but not to CPE in subsequent assays, suggesting that the rAra h 2 within our extract may not be the principal antigen producing T cell stimulation within these assays (Figure 3.10).

To explore sources of contamination within the extract, an endotoxin assay was performed and demonstrated that high quantities of endotoxin were present within the extract, at levels greater than 2,000 EU/ml or 0.28 EU/μg extract. Using a polymixin B endotoxin extracting column (Pierce, USA), endotoxin removal was attempted. However, after passage of 1 ml 200 μg/ml rAra h 2 extract across the column, no protein could be demonstrated in the flow-through. This suggests that endotoxin and the rAra h 2 were avidly bound to one another leading to their combined sequestration within the column, as has been demonstrated for other recombinant proteins produced in *E. coli* cells (Wilson *et al.* 2001).

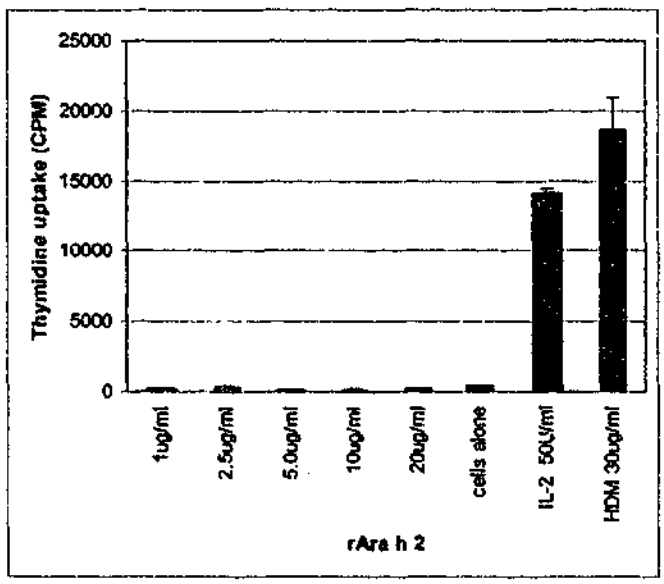
3.3.5 Other Methods of Purification of nAra h 1 and nAra h 2

Experiments exploring acetone precipitation of CPE demonstrated that the majority of protein within the extract precipitated at a concentration of 20% acetone, including Ara h 1 and Ara h 2 (Figure 3.11). However, bands immediately surrounding Ara h 1, with molecular masses of approximately 80 and 49 kDa, along with an 8 kDa protein band, remained in solution until acetone reached a concentration of approximately 30%. Using ammonium sulphate precipitation, a concentration of 50% ammonium sulphate lead to precipitation of

a. SDS PAGE profile of rAra h 2



b. mitogenicity assay



c. toxicity assay

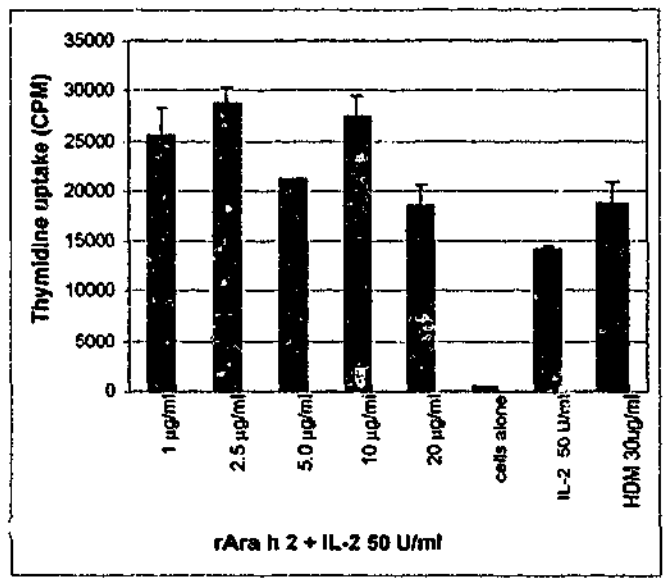


Figure 3.9 Recombinant Ara h 2 SDS-PAGE, T cell mitogenicity and toxicity assays

a. **SDS PAGE profile of recombinant Ara h 2:** rAra h 2 was resolved on a 14% SDS polyacrylamide gel and protein visualised via staining with CBB.

b. **mitogenicity assay:** triplicate cultures of 5×10^4 cells/well of a 3 week HDM specific TCL plus APC, stimulated with a range of concentrations of either Ara h 1 or Ara h 2 for 72 hours.

c. **toxicity assay:** triplicate cultures of 5×10^4 cells/well of a 3 week HDM specific TCL, stimulated with a range of concentrations of rAra h 2 extract in plus 50 U/ml of rIL-2 for 72 hours.

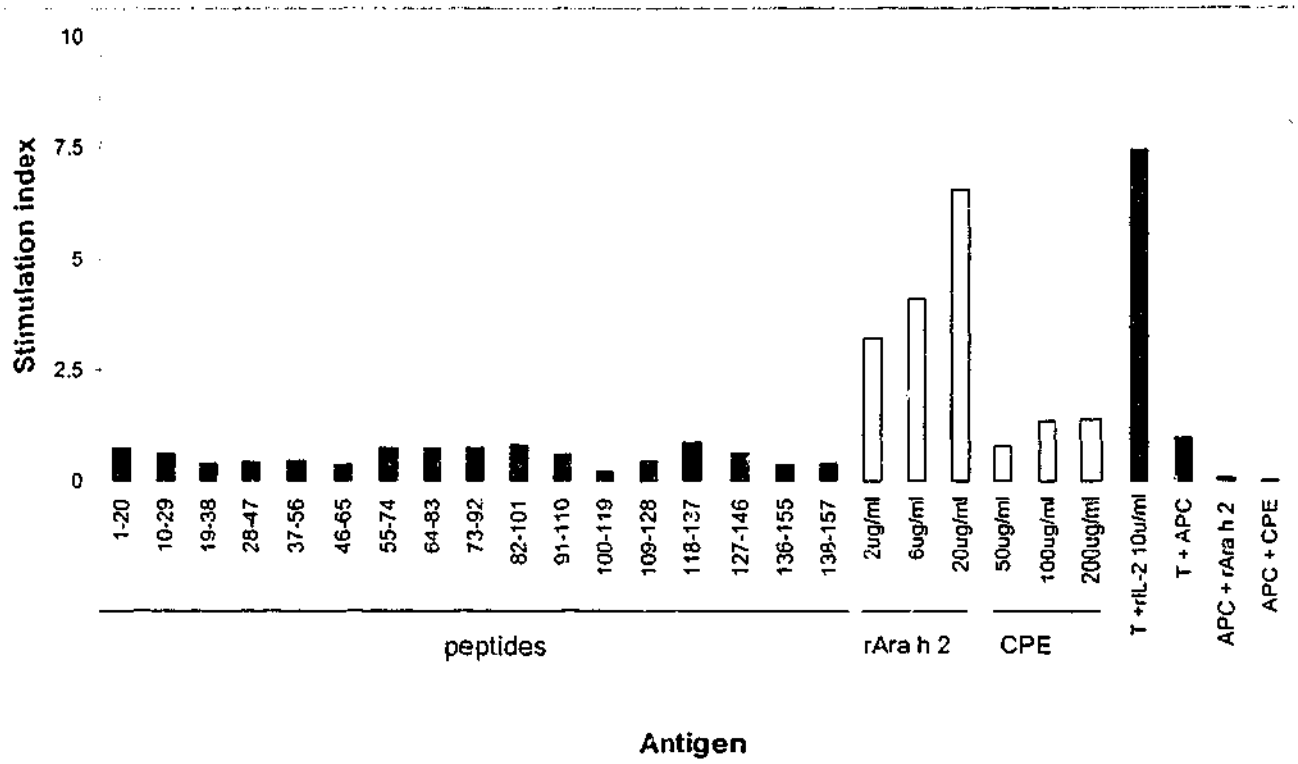


Figure 3.10 Recombinant Ara h 2 specific TCL proliferation assay

A 2 week rAra h 2 specific oligoclonal TCL (5×10^4 cells/well) from an individual with peanut allergy was generated using rAra h 2 stimulation on days 1 and 8, then stimulated with Ara h 2 peptides at a concentration of 10μg/ml in the presence of autologous irradiated PBMC as APC (5×10^4 cells/well) in 3 day cultures. Proliferative responses were assessed by tritiated thymidine incorporation (mean counts per minute [cpm] of triplicate cultures).

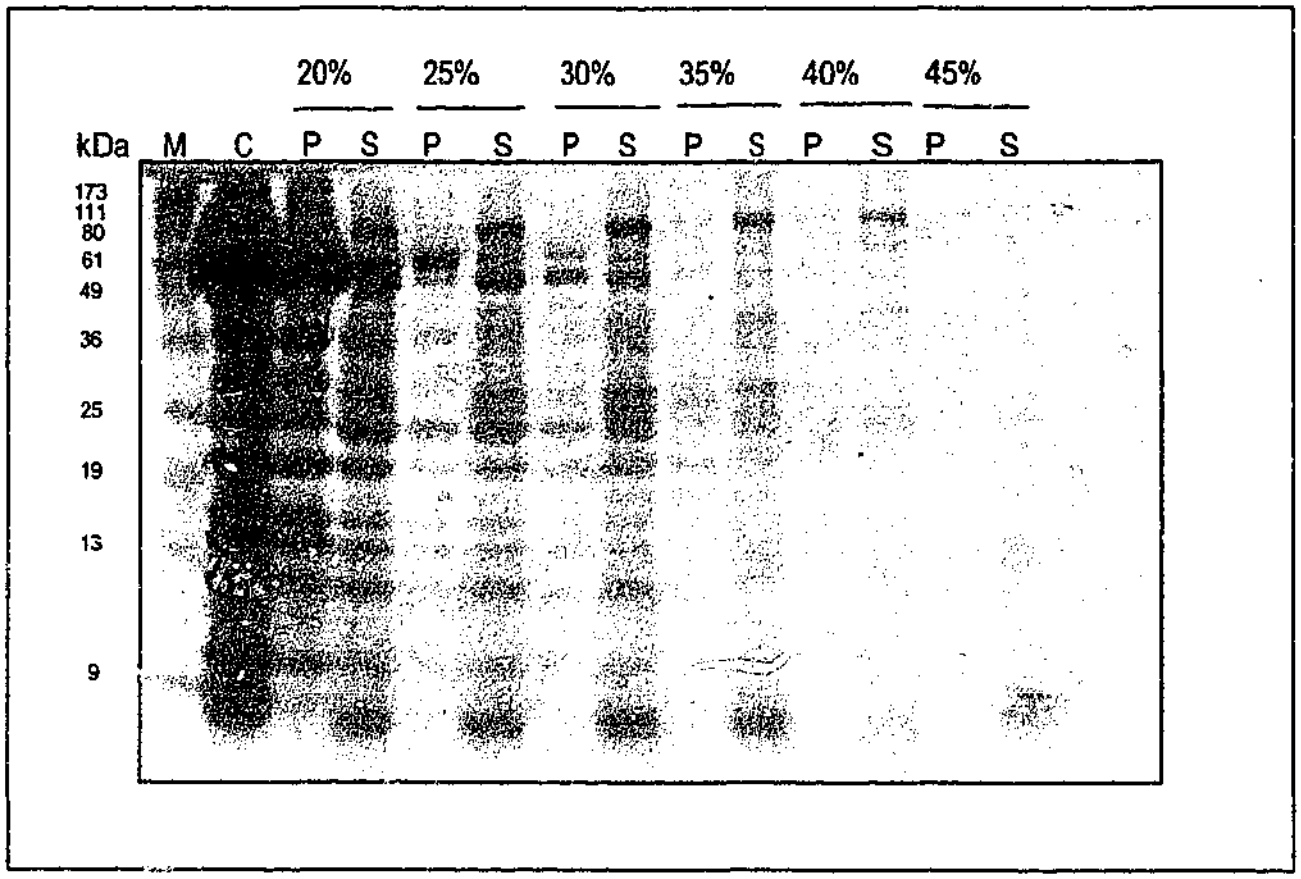


Figure 3.11 SDS-PAGE resolution of samples collected during acetone precipitation of crude peanut extract

C: CPE; P: pellet; SN: supernatant; kDa: kiloDaltons; M: molecular mass markers; 20-45% percent concentration of acetone

CPE, pH 7.5, 6.5 mg/ml was cooled in an ice bath to approximately 0 °C, and increasing concentrations of acetone were established via the progressive addition of acetone to the CPE. Supernatant samples and entire protein precipitates were collected before the addition of further acetone. Samples were resolved on a 14% SDS-polyacrylamide gel and stained with CBB. A 25% acetone solution lead proteins of 80 and 52 kDa molecular mass to remain in solution, while most other proteins were predominantly insoluble at the concentration.

proteins of approximately 36, 28, 25, and 20 kDa, with relative preservation of other proteins within solution (Figure 3.12). Combination of these two approaches demonstrated that a moderately pure nAra h 1 rich extract could be created using the pellet from 20% acetone precipitation followed by collection of the supernatant of 50% ammonium sulphate precipitation (Figure 3.13). Using gel excision methods, an apparently pure nAra h 1 extract was created (Figure 3.14). The yield for this method was approximately 2 %, with the majority of protein not leaving the secondary gel. Ultrafiltration of CPE demonstrated that the majority of protein remained within the retentate, although lower molecular weight proteins were diminished in concentration using the 50 kDa pore size filter (Figure 3.15). Size exclusion chromatography demonstrated the capacity to remove proteins with molecular masses less than 20 kDa from the CPE (Figure 3.16). A relatively pure Ara h 2 containing extract was created, although this was heavily contaminated by lower molecular mass proteins.

3.4 DISCUSSION

In these experiments, methods for the production of relatively pure nAra h 1 and nAra h 2 from CPE have been demonstrated. Neither extract was mitogenic or toxic to T cells in vitro. While potentially contaminating proteins were present in each extract, these proteins were present in extremely low concentrations and vastly reduced relative abundance in comparison to the major peanut allergens. Each extract contained comparable quantities of endotoxin, in low levels. Each extract was recognised by specific IgE and sequencing confirmed that the Ara h 2 extract matched the published sequence. As such, these preparations were suitable for use in T cell experiments, described in subsequent chapters.

CPE was initially prepared using a protocol previously established within our laboratory by Ms Maria de Leon. This extract was fundamental to all subsequent experimental chapters within this thesis and hence its proper characterisation was vital. CPE demonstrated a similar profile on SDS-PAGE to that previously reported (Koppelman *et al.* 2001). As for purified nAra h 1 and nAra h 2, CPE demonstrated IgE reactivity on western blotting using sera from a cohort of peanut allergic subjects. A number of other proteins demonstrated frequent IgE reactivity, including 11, 36, 24, 20, 89, 9, 25, and 8 kDa in over 50% of the cohort. Clarke et al. have demonstrated that IgE binding occurs frequently to bands with molecular masses of 15, 10, 30, 18 and 51 kDa in decreasing order and that reactivity to a 15 kDa protein was found to be higher in patient groups with severe reactions to peanut (Clarke *et al.* 1998). Low molecular mass proteins recognised by our cohort ran below their expected molecular mass on SDS-PAGE gels and the 15 kDa protein reported by Clarke et al. is likely to correspond to the 11 kDa protein seen by our cohort. Distinction of CPE allergens

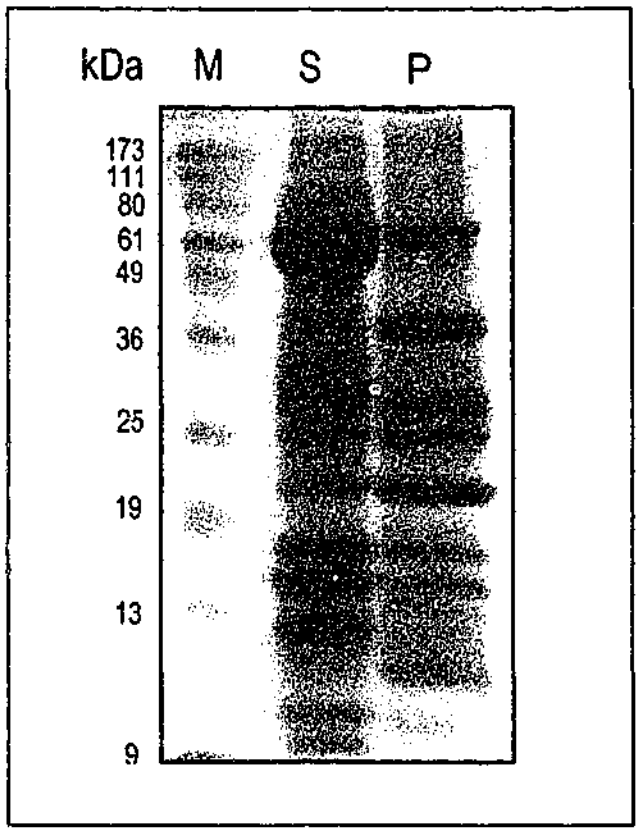


Figure 3.12 50% Ammonium sulphate precipitation of crude peanut extract

S: supernatant; P: pellet; kDa: kiloDaltons; M: molecular mass markers
A saturated ammonium sulphate solution and CPE were cooled to 4 °C. CPE was then slowly diluted with the ammonium sulphate solution to create a range of concentrations between 30% and 90% ammonium sulphate. After standing for 20 minutes at 4 °C, the supernatant was collected and the protein precipitate pelleted then resuspended in PBS. Samples were resolved on a 14% SDS-polyacrylamide gel and stained with CBB. A 50% ammonium sulphate solution lead to precipitation of several proteins of 20-40 kDa molecular mass, but did not produce significant separation of Ara h 1 from Ara h 2.

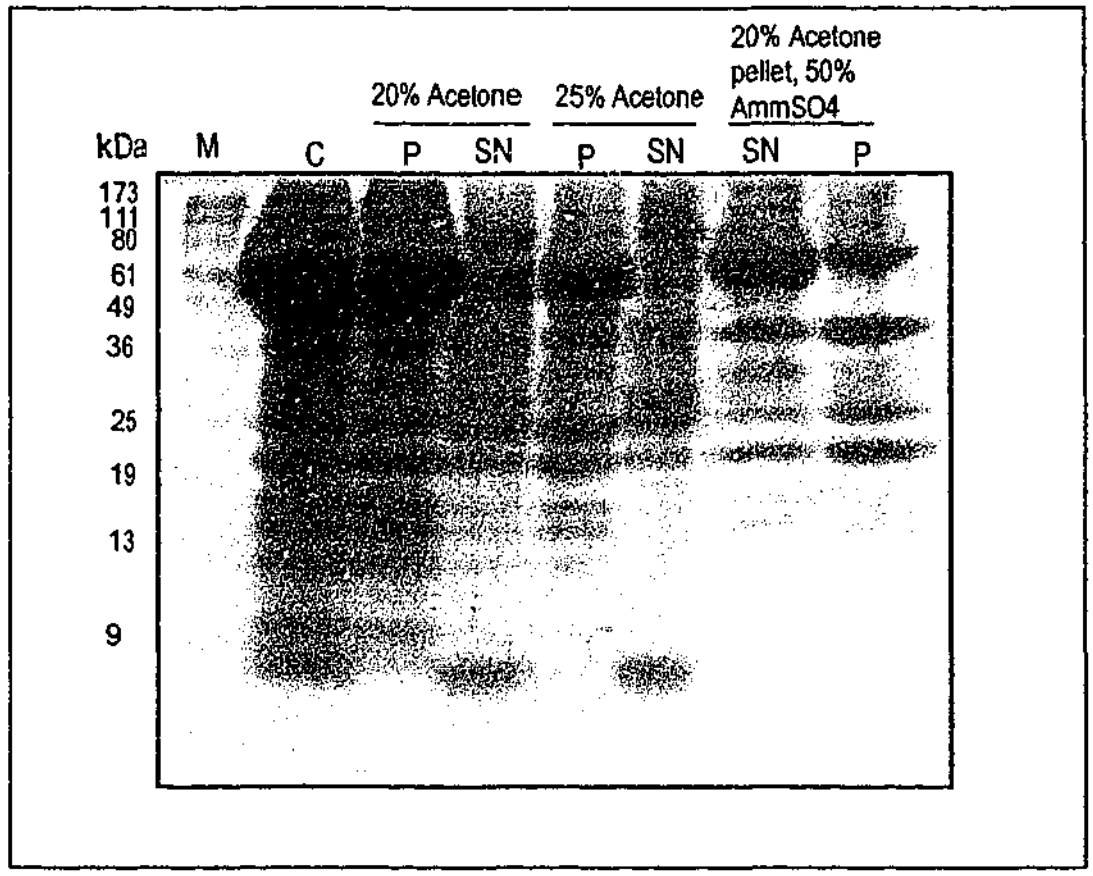


Figure 3.13 Combined acetone and ammonium sulphate precipitation

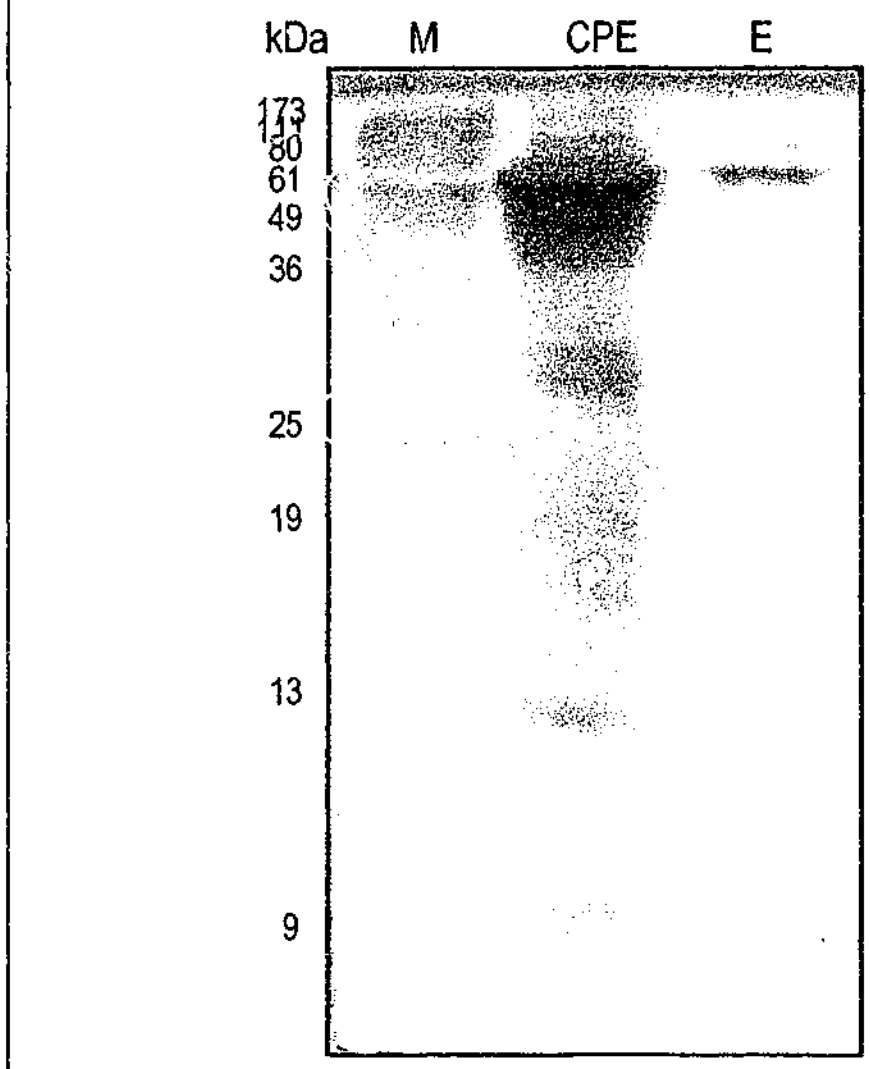
M, molecular weight markers; C, CPE; P, pellet; SN, supernatant.

A 2 step protocol, combining acetone and ammonium precipitation was established. 20% and 25% acetone precipitation of CPE was performed, and the protein precipitate collected and resuspended in PBS. This solution was then subjected to 50% ammonium sulphate precipitation.

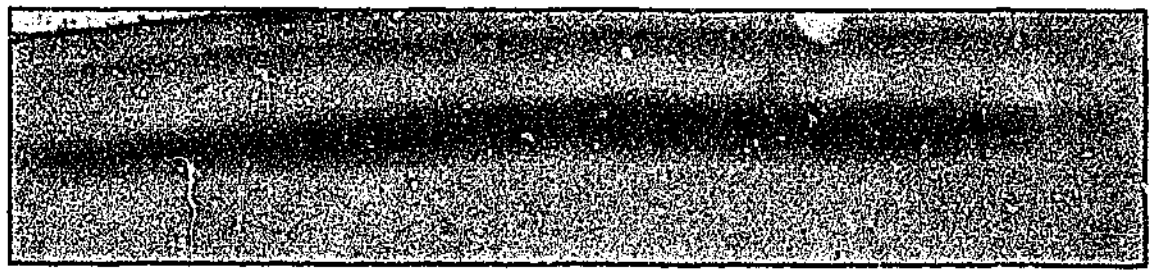
Figure 3.14 SDS-PAGE purification of Ara h 1

CPE (500 μg/gel) was separated on three 10% 1 mm SDS polyacrylamide gels (primary gels) and then stained with Coomassie brilliant blue dye to visualize protein. Bands corresponding with Ara h 1 were excised and loaded onto a 1.5 mm 14% SDS polyacrylamide gel (secondary gel) to allow separation from the dye. The running buffer was then exchanged for fresh buffer and the gel run for a further 150 minutes. The running buffer was then collected and protein within it precipitated using acetone. The collected protein was then dissolved in PBS and separated on **(a)** a 14% SDS polyacrylamide gel. Total yield using this technique was only 2% of the starting quantity. The majority of the excised Ara h 1 could be seen to remain in the **(b)** secondary gel.

(a) Ara h 1 post gel excision



(b) Secondary gel



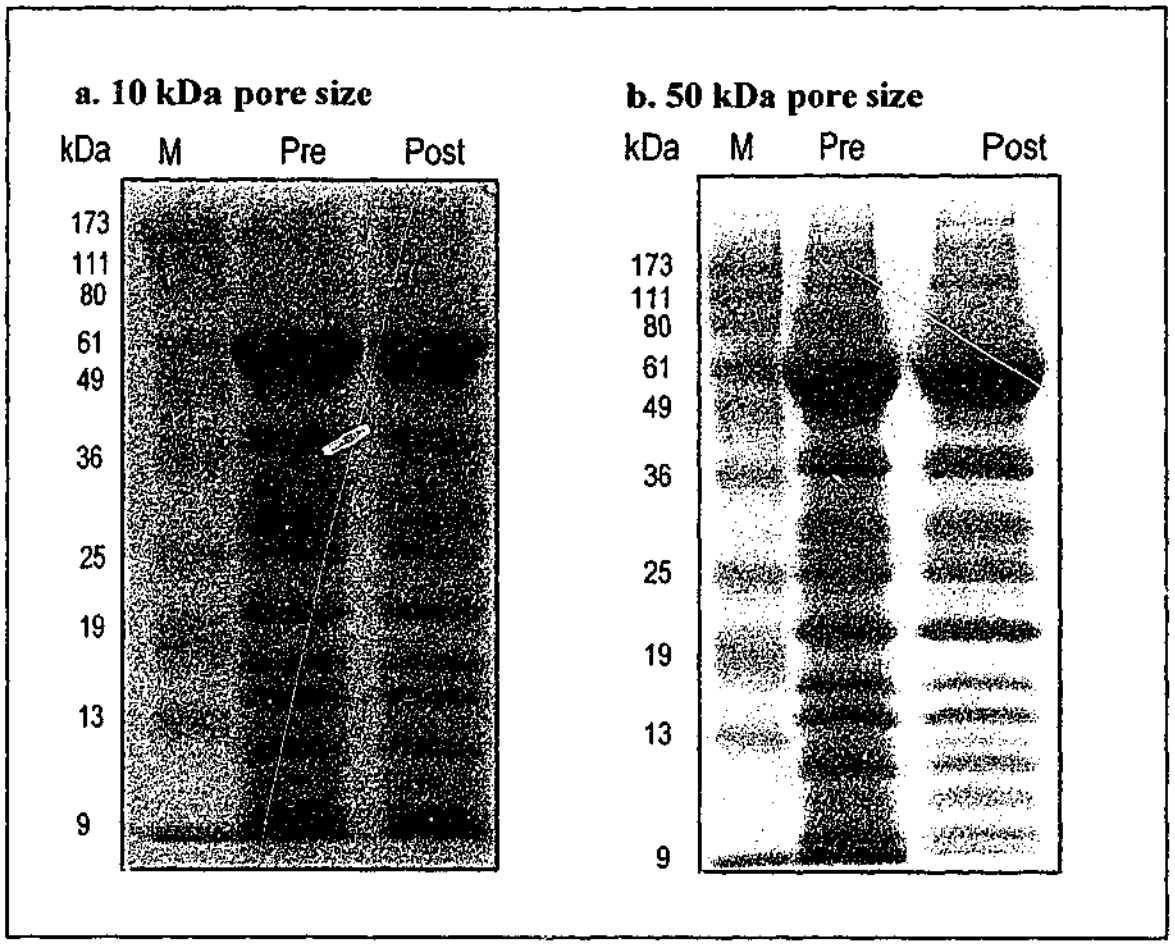


Figure 3.15 YM-10 and YM-50 Amicon Centriprep filtration
a. 10 kDa and b. 50 kDa pore size centrifugal filter; Pre: CPE pre-filtration; Post: CPE post filtration

Ultrafiltration of CPE using Amicon Centriprep centrifugal filtration units of 10kDa and 50kDa pore size was performed. 1 ml of CPE was diluted to 15 ml in PBS, then concentrated to 2.5 ml via centrifugal filtration. This process was repeated in 3 further cycles and the retentate and CPE protein profile compared after resolution on a 14% SDS polyacrylamide gel, stained with CBB. No significant change occurred using the 10 kDa pore size filtration unit, while loss of proteins of less than 19 kDa could be seen with 50 kDa pore size filtration.

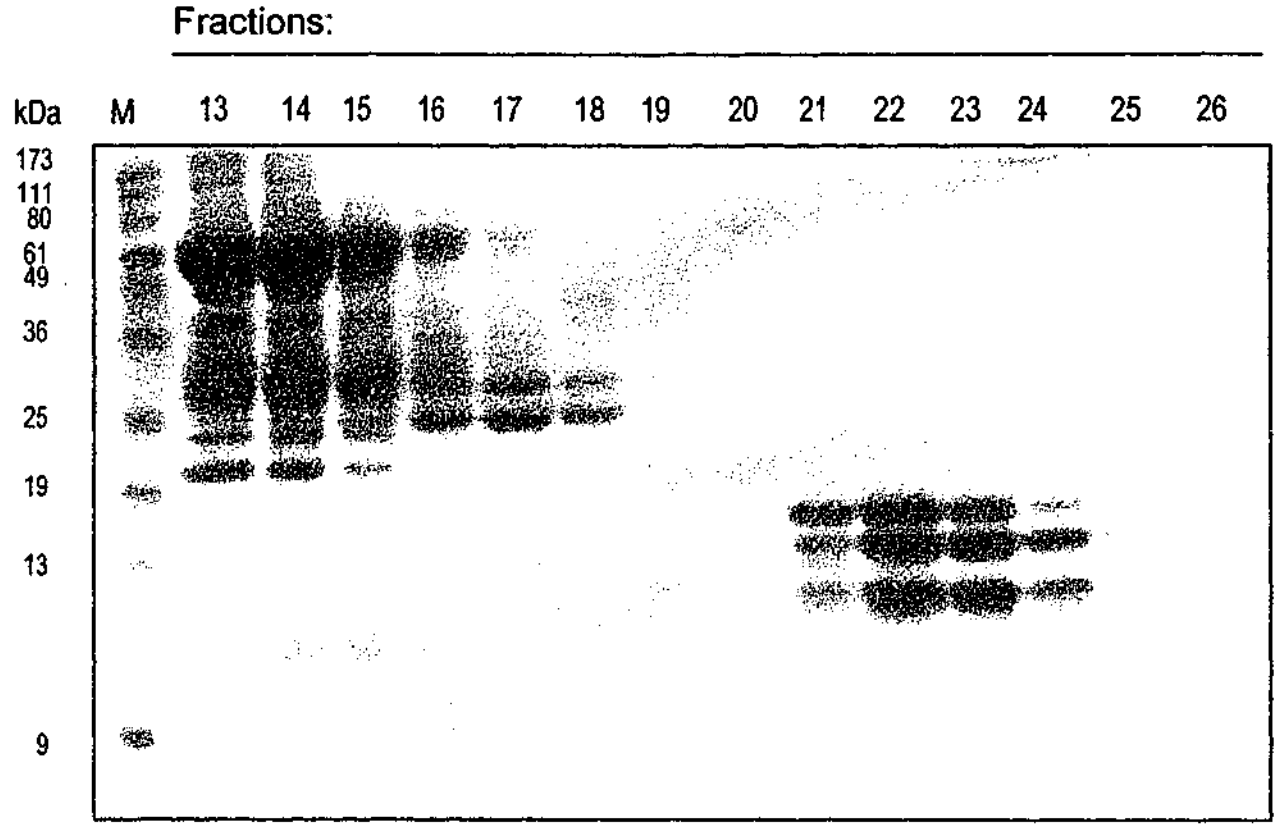


Figure 3.16 Size exclusion chromatography of CPE

2 ml of CPE 5 mg/ml was concentrated to approximately 300 µl volume in an Amicon YM-3 3 kDa pore size centrifugal filtration device. Using a Pharmacia Superdex 75 HR10/30 size exclusion column (Amersham Pharmacia, Sweden), 250 µl of the concentrated CPE was loaded onto the column and 0.5 ml fractions collected in a 50 ml elution volume, using filtered PBS as the elution buffer. Protein containing fractions were resolved on a 14% SDS polyacrylamide gel and stained with CBB.

recognised by our cohort based upon severity is not possible as selection was based upon a history of anaphylaxis. Nevertheless, IgE binding to the 15 kDa protein occurred in 83% of our cohort, more frequently than any other protein apart from Ara h 1 and Ara h 2. CPE was able to induce T cell responses in a dose-dependent manner; these features are discussed in detail in chapter 4. Finally, CPE did not demonstrate mitogenicity or toxicity to T cells and contained negligible quantities of endotoxin. As a consequence, CPE was also suitable for use in T cell experiments.

The method of preparation of natural Ara h 1 and Ara h 2 described in this chapter carries a number of benefits over the use of alternatively prepared extracts. By using preparations derived from CPE, glycosylation of proteins is unaffected. The retention of carbohydrate side chains is likely to be important for the study of T cell responses, for a number of reasons. Processing of proteins by antigen presenting cells may differ between glycosylated and non-glycosylated proteins, such that the potential to produce a T_H1 or T_H2 response may differ (Okano *et al.* 1999; Okano *et al.* 2001). Additionally, carbohydrate side chains may form a component of the T cell epitope and their loss may therefore lead to absence of the T cell response (Dudler *et al.* 1995). Finally, carbohydrate side chains may be the target of cross-reactive responses for IgE, and this may therefore have implications for IgE facilitated antigen presentation and generation of T cell responses (Batanero *et al.* 1996; van Neerven *et al.* 1999).

An additional benefit of naturally derived preparations is the potential to include all isoforms of the allergen of interest in the final preparation. Ara h 1 is reported to have at least 2 isoforms and Ara h 2 is reported to have at least 8 isoforms (Becker 1997; Li *et al.* 2000). While confirmation of the presence of these isoforms in our preparations could be performed via 2-dimensional gel electrophoresis, isoforms frequently carry similar isoelectric points, such that methods of purification dependent on charge such as that used in this chapter are likely to lead to their collection in the same fraction. It has been shown for a number of allergens that T cell responses to individual isoforms vary, both for magnitude of proliferation and the nature of the cytokine response (Hales *et al.* 1997; Sparholt *et al.* 1997; Muller *et al.* 1998; Wurtzen *et al.* 1999). Endotoxin levels within our preparations were negligible and equivalent to one another. In comparison, endotoxin levels in our recombinant extract produced in *E. coli* cells had extremely high levels of endotoxin. The presence of endotoxin may have a number of effects on T cell proliferation, augmenting proliferative responses and altering the nature of cytokine responses (Tough *et al.* 1997; Matsui *et al.* 2001). As such, its absence from the preparations used for subsequent experiments removed a potentially significant confounder to the interpretation of T cell responses both with regards to responses to individual allergens as well as cross-reactive responses.

Another potentially significant problem with regards the use of these preparations is the presence of contaminant proteins. Western blotting of the nAra h 1 and nAra h 2 extracts using serum from a subject demonstrating exquisite IgE reactivity to all proteins within CPE, demonstrated that the contaminant proteins in the nAra h 1 and nAra h 2 extracts bound IgE from that serum. However, subjects with lesser IgE reactivity demonstrated IgE binding to only the proteins of interest. Contaminant proteins may produce their own T cell responses and cross-react with the proteins of interest. However, while these proteins may represent unique structures, they may also represent breakdown products. Within the Ara h 1 extract, the largest contaminant band has a molecular mass of approximately 30 kDa, and therefore could represent a breakdown product approximately half the size of Ara h 1. This could be determined by N-terminal sequencing of that protein. To minimise the presence of contaminant proteins, affinity purification could have been used as an alternative method of purification from crude extract. However, this method requires monoclonal antibodies directed towards the desired protein, which were unavailable during this research.

Other methods of purification attempted in these experiments proved far less powerful in achieving protein separation than anion and cation exchange chromatography. Because ultrafiltration using a 50 kDa nominal molecular mass cut-off filter allowed the removal of lower molecular mass proteins, this step was included in the purification of Ara h 1 via cation exchange. Ara h 1 purification was successfully performed using gel excision, but at the expense of an extremely low yield of only 2% and contamination with agents used within SDS-PAGE, removal of which may have lead to even greater protein loss. Additionally Ara h 2 is of far reduced relative abundance to Ara h 1 within CPE, such that production of sufficient quantities of Ara h 2 would be difficult to practically achieve. In comparison, the yield for Ara h 1 using cation exchange was 12%, and for Ara h 2 was 4%. Recombinant Ara h 2 was successfully expressed and was demonstrated to be IgE reactive on western blotting. However, recombinant Ara h 1 could not easily be expressed, such that proliferation assays comparing Ara h 1 and Ara h 2 would require the use of preparations produced under different conditions and therefore would be liable to lead to confounding responses due to differences in the content of the final protein suspension. As discussed, this was particularly likely to occur on the basis of the large difference in endotoxin concentration between the recombinant Ara h 2 and natural derived Ara h 1. Attempts at endotoxin removal from recombinant Ara h 2 were unfortunately not successful. Other methods of purification such as acetone and ammonium sulphate precipitation lead to only partial purification, such that they could not be used individually, and did not remove proteins of the same molecular mass as contaminant proteins within the final preparations used in these experiments, making their addition to a multi-step protocol for protein purification unwarranted.

In conclusion, several methods of production of nAra h 1 and nAra h 2 from CPE using anion and cation exchange chromatography were explored. These fractions, while containing low quantities of potentially contaminating proteins, contained the major allergens in vastly greater relative abundance, were neither toxic nor mitogenic, and contained negligible quantities of endotoxin. These preparations were therefore suitable for use in the T cell experiments outlined in subsequent chapters.

CHAPTER 4 POLYCLONAL T CELL RESPONSES TO CRUDE PEANUT EXTRACT, ARA H 1 AND ARA H 2

4.1 INTRODUCTION

Peanut allergens have been well characterised with regards to their IgE reactivity. Studies to date have examined linear epitopes, and no data exist at present to confirm their relevance to conformational epitopes. On the basis of IgE reactivity, six peanut allergens have been described, of which two represent major allergens, recognised by serum IgE from greater than 50% of peanut allergic subjects (Burks *et al.* 1998). These allergens, Ara h 1 and Ara h 2, have been described in detail. Ara h 1 is a 65 kDa glycoprotein belonging to the vicilin seed storage family (Burks *et al.* 1991). On the basis of linear B cell epitope mapping, Ara h 1 contains 23 linear IgE epitopes, of which 4 are immunodominant (Burks *et al.* 1997). Ara h 1 has a trimeric structure, with its IgE binding epitopes concentrated around the linking areas, perhaps contributing to its intense allergenicity (Shin *et al.* 1998). Ara h 2 is a 17 kDa glycoprotein belonging to the conglutin seed storage family, and contains 10 linear IgE epitopes, of which 3 are immunodominant (Stanley *et al.* 1997).

Other significant peanut allergens remain less well characterised. These include Ara h 3, a 10 kDa glycoprotein belonging to the glycinin seed storage family and containing 4 linear IgE epitopes, with 1 of these being immunodominant (Rabjohn *et al.* 1999). The other peanut allergens are Ara h 4, Ara h 5 (profilin), and Ara h 6, and the panallergen lipid transfer protein (Kleber-Janke *et al.* 1999).

While the nature of IgE responses to the various peanut allergens has been described in detail, T cell responses to these allergens are relatively undefined, even for the major allergens Ara h 1 and Ara h 2. Polyclonal and monoclonal proliferative responses to CPE by T cells have been demonstrated to occur for both allergic and non-allergic donors, although some authors have demonstrated that these occur to a greater degree amongst peanut allergic donor T cells (Higgins *et al.* 1995; de Jong *et al.* 1996; Laan *et al.* 1998). Cytokine responses have previously been demonstrated to be of a T_H2 phenotype for allergic donor T cells stimulated with CPE, with both IL-4 and IL-5 production (Higgins *et al.* 1995; de Jong *et al.* 1996). In a recent paper, Turcanu et al. have shown that peanut specific T cells from peanut allergic children demonstrate a T_H2 phenotype, but similar cells from children who have outgrown their peanut allergy, or are non-peanut allergic, demonstrate a T_H1 phenotype (Turcanu *et al.* 2003). Variable data exist with regards to the quantity of IFN-γ produced in response to T cell stimulation with peanut antigens. Dorion et al. have found an inverse correlation between the quantity of IFN-γ production by T cells after Ara h 2 stimulation and

the quantity of serum Ara h 2 specific IgE (Dorion *et al.* 1994). While as a group, peanut allergic subjects produced less IFN-γ than controls following PBMC stimulation with Ara h 2, correlation existed for the degree of proliferation and quantity of IFN-γ secreted (Dorion *et al.* 1994). Other papers have suggested that peanut specific T cells from peanut allergic donors produce only low quantities of IFN-γ (Higgins *et al.* 1995; Turcanu *et al.* 2003). There is currently a paucity of literature reporting T cell recognition of the major allergens of peanut by CPE specific TCL. As such, in this chapter, analysis of the polyclonal T cell response to CPE, nAra h 1 and nAra h 2 is described. Using PBMC stimulated for seven days with each antigen, proliferative responses to each antigen are described by both peanut allergic and non-peanut allergic donors. Cytokine assays are also detailed, comparing responses on the basis of IL-5 and IFN-γ production.

4.2 RESULTS

4.2.1 Subject Characterisation

The clinical characteristics of donor peanut allergic subjects for this set of experiments are summarised in Table 4.1. Nine of the 15 subjects were female, and the mean age of the group was 31 (standard deviation (SD) ± 10). All 13 of the subjects who underwent skin prick testing were atopic, demonstrating sensitivity to at least one common aeroallergen. Twelve of the group described reactions to other nuts, hazelnut (8 out of 12 subjects) being the tree-nut most commonly reported by subjects as an allergen. All subjects described features of anaphylaxis on exposure to peanut, with facial angioedema and laryngeal oedema being the most common symptoms (12 of 15 subjects each). Nine subjects reported asthmatic symptoms, while only 2 subjects reported symptoms consistent with hypotension. The median age of onset of reactions was 3, and ranged between 6 months and 19 years. Despite life threatening anaphylaxis with nut exposure, serum IgE quantified via RAST scores did not predict the severity of such reactions, with RAST scores ranging from grade 1 to grade 5. The non-peanut allergic cohort included 2 atopic and 3 non-atopic subjects.

4.2.2 Peanut Allergens

Peanut allergens used in these experiments, including CPE, nAra h 1 and nAra h 2, were produced using protocols described in chapter 3. Each was filter sterilised to remove bacterial contamination, and underwent T cell mitogenicity and toxicity assays, as well as endotoxin level estimation, to exclude these confounding properties.

Table 4.1 Summary of peanut allergic subjects donating PBMC for proliferation and cytokine assays

Subject	Age	Sex	Allergic disease	Family history	Nature of reaction to peanut	Known nut allergens	Other food allergens	Age at first reaction (yrs)	Time since last reaction (mths)	Peanut RAST (IU)	Peanut RAST score	Atopic (via SPT)
1	50	F	nil	No	laryngeal oedema, generalised urticaria, facial angioedema	peanut		18.5	21	6.87	3	Yes
2	27	F	asthma, eczema, rhinitis	No	laryngeal oedema, generalised urticaria, facial angioedema	peanut, hazelnut, walnut	sesame seed, baked beans	10	2	2.07	2	Yes
3	33	M	asthma, eczema	No	asthma, laryngeal oedema, facial angioedema	peanut, almond, hazelnut, walnut		3	36	13.9	3	Not done
4	24	F	asthma, eczema, rhinitis	No	asthma, laryngeal oedema, facial angioedema	peanut, almond, cashew, walnut	fish, crustacea	1.5	24	63.5	5	Yes
5	20	F	asthma	No	laryngeal oedema, generalised urticaria	peanut, almond, Brazil nut, cashew, hazelnut, macadamia, walnut	sesame seed, crustacea	3	0	3.09	2	Yes
6	30	M	asthma	No	asthma, laryngeal oedema, facial angioedema	peanut, hazelnut		12	18	0.51	1	Yes
7	23	F	rhinitis	No	asthma, laryngeal oedema, facial angioedema	peanut, hazelnut, pistachio	sesame seed	8	9	0.39	1	Yes
8	21	F	asthma	No	laryngeal oedema, generalised urticaria, facial angioedema	peanut		0.5	0	16.3	3	Yes
9	27	F	eczema	No	asthma, laryngeal oedema, generalised urticaria, facial angioedema	peanut, almond		19	11	9.53	3	Not done

Table 4.1 (continued) Summary of peanut allergic subjects donating PBMC for proliferation and cytokine assays

Subject	Age	Sex	Allergic disease	Family history	Nature of reaction to peanut	Known nut allergens	Other food allergens	Age at first reaction (yrs)	Time since last reaction (mths)	Peanut RAST (kU (A)/ml)	Peanut RAST score	Atopic (via SPT)
10	32	F	nil	No	generalised urticaria	peanut, hazelnut, pine nuts		0.5	3	13.31	3	Yes
11	55	M	asthma, eczema	No	asthma, laryngeal oedema, facial angioedema	peanut, walnut	banana	1.5	120	2.01	2	Yes
12	33	M	asthma,eczema	No	asthma, laryngeal oedema, generalised urticaria, facial angioedema	peanut, almond, pecan, pine nuts, pistachio, walnut		3	1	2.42	2	Yes
13	30	M	asthma, rhinitis	No	facial angioedema	peanut	peach	19	96	2.92	2	Yes
14	26	M	asthma,eczema, rhinitis	Yes	asthma, laryngeal oedema, hypotension	peanut, almond, cashew, hazelnut, pine nuts, walnut	sesame seed	1.5	9	1.45	2	Yes
15	36	F	asthma, eczema	No	asthma, hypotension, facial angioedema	peanut, hazelnut	sesame seed	4	2	3.09	2	Yes

4.2.3 Western Blotting for IgE Reactivity to nAra h 1 and nAra h 2

To assess associations between specific IgE reactivity towards nAra h 1 and nAra h 2, and corresponding cellular responses to CPE, nAra h 1 and nAra h 2, western blotting of CPE was performed. Different protocols were used, as described earlier, to address the tendency of nAra h 1 to bind IgE non-specifically for both peanut allergic subjects and control subjects. These results are illustrated in Figure 4.1. nAra h 1 was recognised by IgE from 10 of the 15 peanut allergic subjects (67%). In comparison, of the 6 non-peanut allergic subjects, 2 recognised nAra h 1. nAra h 2 was recognised by serum IgE from 13 peanut allergic subjects (87%), with both upper and lower bands demonstrating intense IgE binding in the majority of subjects. Several of the non-peanut allergic subjects demonstrated weak IgE binding to nAra h 2, but this was far less intense than that demonstrated by peanut allergic subjects. As this chapter focuses on the T cell response to major peanut allergens nAra h 1 and nAra h 2, responses to other proteins are not illustrated, but clearly multiple proteins within peanut extract apart from these allergens were able to bind IgE.

4.2.4 Polyclonal T cell Proliferative Responses to Crude Peanut Extract, nAra h 1 and nArah 2

To determine the optimum concentration of CPE for use within PBMC proliferation assays, preliminary dose-response curves were performed using donor peanut allergic and non-peanut allergic PBMC. These assays established that all subjects from both peanut allergic and non-peanut allergic groups demonstrated proliferative responses to CPE, with proliferation typically having a SI > 2.5 from the lowest concentration of 12.5 μg/ml and maximal proliferative responses occurring between 100 and 400 μg/ml (illustrated in Figure 4.2). No difference existed between peanut allergic and non peanut allergic groups with regards proliferative responses to CPE. On the basis of these data, CPE at a concentration of 100 μg/ml was used as a control in subsequent assays for proliferation and cytokine responses to nAra h 1 and nAra h 2.

To examine PBMC responses to nAra h 1 and nAra h 2, 7 day proliferation assays were performed using a range of concentrations of each antigen. Dose-responses are illustrated in Figure 4.3, and peak stimulation indices summarised in Table 4.2. Both peanut allergic and non-peanut allergic donors demonstrated proliferative responses to each antigen. No difference could be demonstrated between peanut allergic and non-peanut allergic groups for peak polyclonal proliferative response, the concentration of nAra h 1 at which that response occurred, peak stimulation index, or the lowest concentration of nAra h 1 at which a stimulation index greater than 2.5 was detected. For nAra h 2, similar data were obtained, with no significant difference in concentration of antigen eliciting the peak proliferative

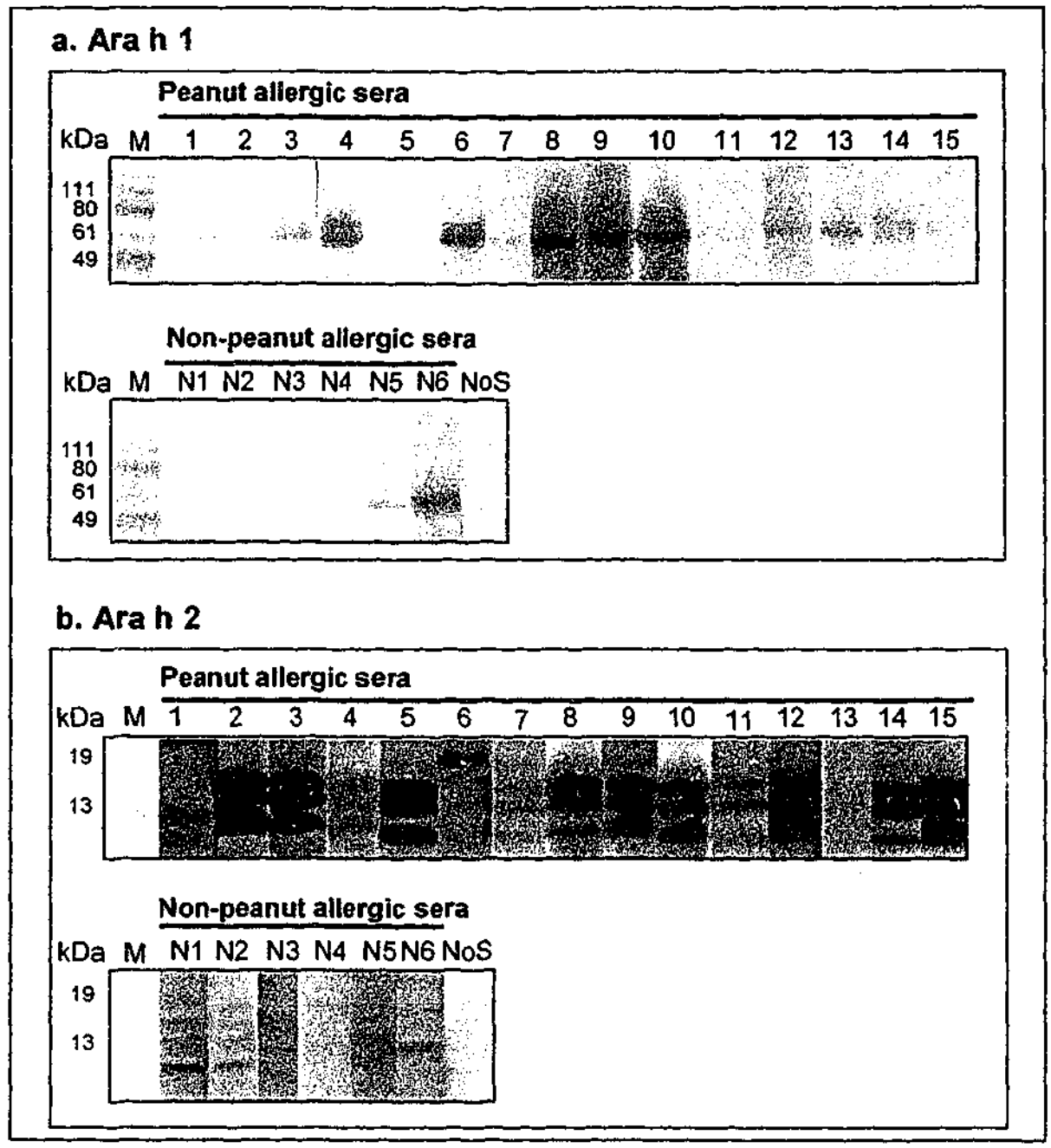


Figure 4.1 Western blotting of Ara h 1 and Ara h 2 by patient serum IgE
kDa: kiloDaltons; M: molecular mass markers; 1-15: peanut allergic subjects; N1-6: non-peanut allergic controls; NoS: no serum control

Following 14% SDS-PAGE separation, CPE was transferred to nitrocellulose and probed with sera from the study population, non-allergic controls and a no serum control.

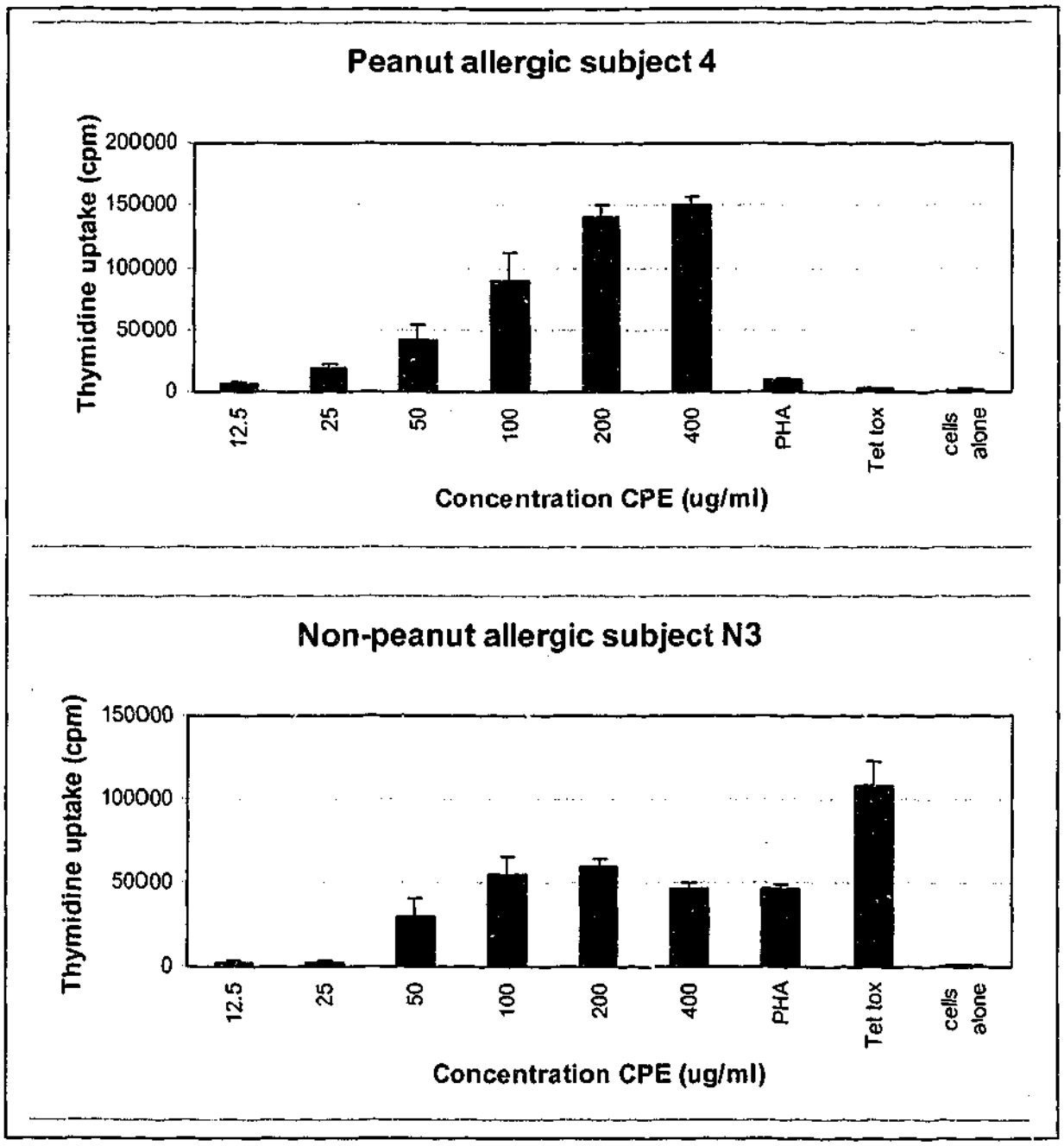


Figure 4.2 Representative polyclonal T cell responses to CPE in a peanut allergic and non-peanut allergic subject

CPE, crude peanut extract; PHA: phytohaemagglutinin; Tet tox: tetanus toxoid.

Triplicate cultures of PBMC (1 x 10^5 cells/well) were stimulated with CPE or controls for 7 days. For the last 12 hours, cells were pulsed with ^{3}H-thymidine before harvesting and counting. ^{3}H-thymidine incorporation was determined and results for each antigen concentration or control expressed as mean cpm + SEM.

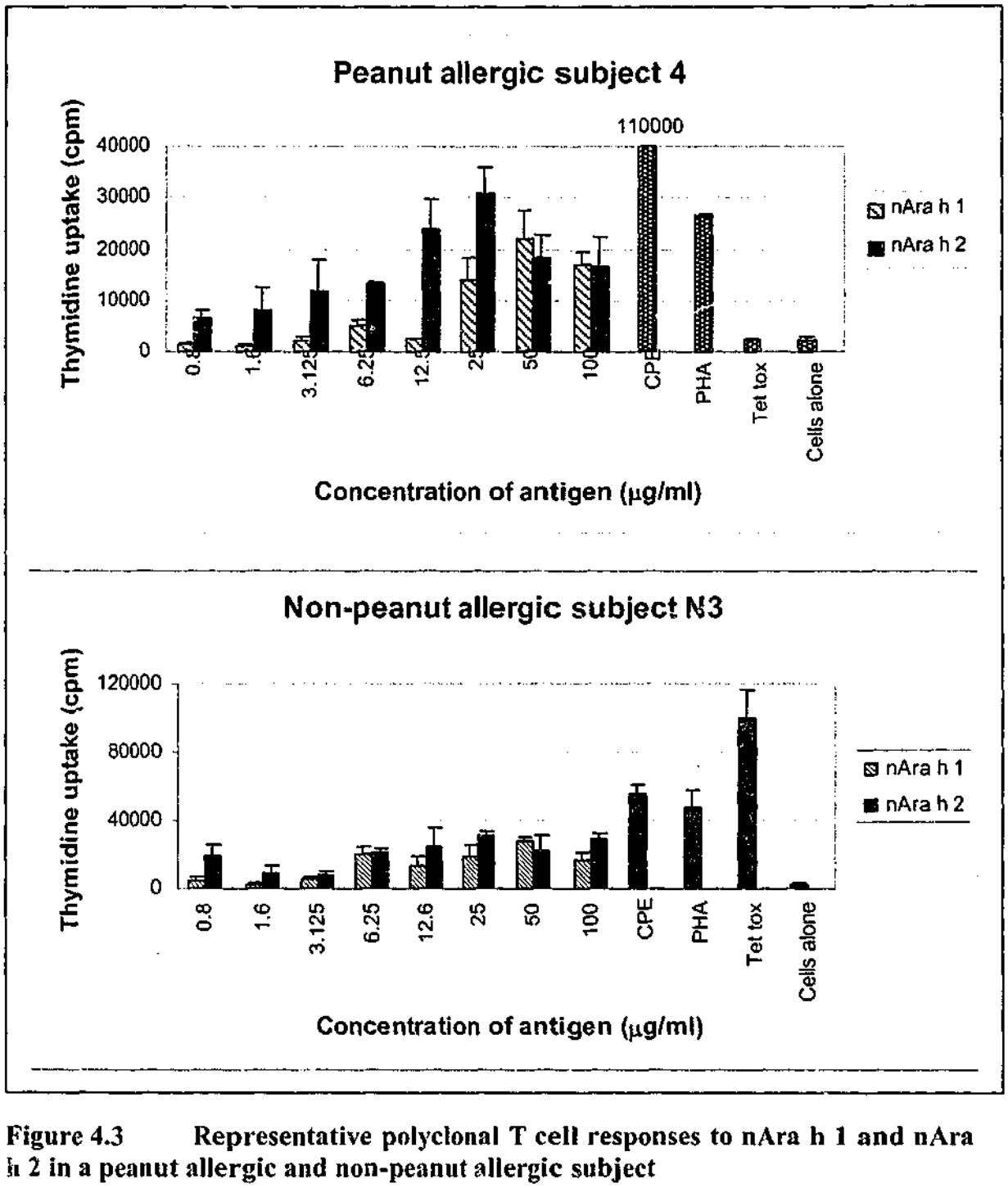


Figure 4.3 Representative polyclonal T cell responses to nAra h 1 and nAra h 2 in a peanut allergic and non-peanut allergic subject

CPE, crude peanut extract; PHA: phytohaemagglutinin; Tet tox: tetanus toxoid.

Triplicate cultures of PBMC (1 x 10^5 cells/well) and a range of concentrations of nAra h 1 or nAra h 2, for 7 days. For the last 12 hours cells were pulsed with ^{3}H-thymidine before harvesting and counting. ^{3}H-thymidine incorporation for each antigen or control was estimated and expressed as mean cpm + SEM.

Table 4.2 PBMC proliferative responses to nAra h 1 and nAra 2

	nAra h 1				nAra h 2			
	Peak proliferative response (cpm)	Antigen concentration for peak (µg/ml)	Peak stimulation index (SI)	Antigen concentration at which SI > 2.5	Peak proliferative response (cpm)	Antigen concentration for peak (µg/ml)	Peak stimulation index	Antigen concentration at which SI > 2.5
Non-peanut allergic subjects								
N1	2138	100	7.87	100	1500	100	5.52	25
N2	46040	100	18.32	6.25	33088	25	13 17	0.8
N3	28057	50	13.37	3.125	31376	25	14.96	0.8
N4	25264	6.25	2.86	6.25	25089	3.125	2.84	3.125
N5	25046	100	115.24	3.125	41737	50	192.04	3.125
N6	32938	100	15.59	3.125	29205	6.25	13.59	3.125
mean	26581	76.0	28.88	20.3	26999	34.9	40.35	6.0
SEM	14302	39.6	42.67	39.1	13654	36.0	74.47	9.4
Peanut allergic subjects								
1	2758	100	5.57	12.5	2070	50	4.18	6.25
2	38957	12.5	8.33	1.6	22804	1.6	4.87	1.6
3	10863	12.5	6.60	3.125	9836	12.5	5.98	6.25
4	22085	50	10.30	25	30937	25	14.43	0.8
5	19492	12.5	22.77	3.125	10532	50	12.30	0.8
6	6857	6.25	2.91	6.25	11552	12.5	4.90	0.8
7	26738	100	8.79	1.6	11630	12.5	3.82	3.125
8	22561	50	9.85	0.8	22491	0.8	9.82	0.8
9	31346	50	18.43	0.8	15268	100	8.98	0.8
10	33728	12.5	75.29	0.8	39843	6.25	88.94	1.6
11	5086	12.5	5.58	6.25	4739	25	5.20	6.25
13	3761	25	8.89	25	10150	25	23.98	3.125
12	11907	100	30.07	1.6	10763	100	27.18	0.8
14	2787	100	7.88	1.6	8592	25	24.28	3.125
15	33046	100	35.83	3.125	25855	12.5	28.03	1.6
mean	18131	49.6	17.14	6.2	15804	30.6	17.79	2.5
SEM	12684	39.7	18.73	8.2	10424	31.8	21.60	2.1

Triplicate cultures of PBMC (1 x 10^5 cells/well) and a range of concentrations of nAra h 1 or nAra h 2, for 7 days. For the last 12 hours cells were pulsed with ^{3}H-thymidine before harvesting and counting. ^{3}H-thymidine incorporation for each antigen was estimated and expressed as mean cpm + SEM.

response, peak stimulation index or lowest nAra h 2 concentration producing a stimulation index greater than 2.5. However, curiously there was a non-significant trend for peak polyclonal proliferative responses to nAra h 2 to be greater for non-peanut allergic subjects than peanut allergic counterparts, means for each group being 2.7×10^4 and 1.6×10^4 cpm, respectively ($p = 0.07$). Amongst peanut allergic polyclonal responses, no difference could be distinguished with regards to response to nAra h 1 or nAra h 2 in any of the four parameters listed previously. Similarly, non-peanut allergic donor responses were not different to either antigen.

4.2.5 Polyclonal T cell Cytokine Responses to Crude Peanut Extract, nAra h 1 and nAra h 2

To determine the nature of the cytokine response associated with polyclonal proliferative responses to nAra h 1 and nAra h 2, PBMC culture supernatants were collected after 48 hours and subjected to sandwich ELISA for the cytokines IL-5 and IFN-γ. These data are summarised in Tables 4.3 and 4.4. In comparison to peanut allergic donors, there was a non-significant trend for non-peanut allergic subjects to produce more IFN-γ in response to nAra h 1 stimulation ($p = 0.14$). For nAra h 2, this difference was statistically significant ($p = 0.006$). There was no significant difference in IFN-γ levels between peanut allergic and non-peanut allergic subjects with regards to response to CPE, PHA or for unstimulated cells although mean values for each were higher for the non-peanut allergic group. Amongst peanut allergic polyclonal responses, no significant difference occurred between responses to nAra h 1 and nAra h 2 with regards to IFN-γ secretion. However, in comparison to CPE, and PHA, nAra h 1 and nAra h 2 were associated with significantly less IFN-γ secretion ($p < 0.05$). Non-peanut allergic subjects demonstrated this difference for nAra h 1 only ($p < 0.05$).

IL-5 production in response to both nAra h 1 and nAra h 2 stimulation was significantly greater amongst peanut allergic donor PBMC than those from non-peanut allergic donors (for nAra h 1, mean 158 ± 87 pg/ml versus 7 ± 1 pg/ml; $p < 0.005$; and for nAra h 2, mean 186 ± 133 pg/ml versus 14 ± 7 pg/ml; $p = 0.036$). CPE stimulation was associated with a trend for increased IL-5 production among peanut allergic donor PBMC ($p = 0.062$), while PHA stimulated cells and unstimulated cells were associated with similar degrees of IL-5 secretion in both peanut allergic and non-peanut allergic groups. Similar findings emerged when the IL-5/IFN-γ ratio was examined, nAra h 1 and nAra h 2 being associated with a significantly higher ratio in peanut allergic subjects compared to non-peanut allergic controls ($p = 0.016$, and $p = 0.005$, respectively), and a non-statistically significant trend for such a finding being demonstrated for CPE stimulation ($p = 0.062$). Stepwise logistic regression analysis revealed that there was a high degree of association between the secretion of IL-5 in

Table 4.3 Peanut allergen stimulated PBMC IFN-γ secretion profiles

	nAra h 1	nAra h 2	CPE	PHA	Cells alone
Non-peanut allergic subjects					
N1	272	629	230	5000	516
N2	142	1148	1264	1566	46
N3	505	1218	2080	2965	125
N4	1288	2673	2434	829	71
N5	1241	2230	2032	886	6
N6	96	609	1486	4949	347
mean	591	1418	1587	2699	185
SEM	242	381	353	860	90
Peanut allergic subjects					
1	6	15	6	2630	396
2	129	140	249	1123	6
3	83	178	98	4086	140
4	14	32	184	829	180
5	1170	1112	1499	2514	282
6	119	467	508	1672	36
7	211	389	197	107	2
8	319	1586	3273	2008	10
9	404	455	1123	1554	15
10	1134	984	2255	3330	15
11	81	263	587	3835	9
12	161	467	696	280	2
13	82	275	59	232	2
14	378	438	859	2586	282
15	480	1009	2088	3031	8
mean	318	521	912	1988	92
SEM	95	117	250	336	34

PBMC (2.5 x 10^6/ml) were cultured in 2 ml aliquots with nAra h 1 and nAra h 2 at concentrations producing maximal proliferative responses, and with CPE and PHA as controls. Supernatants were harvested at 48 hours and assayed for IFN-γ by sandwich ELISA. Data are expressed as mean concentrations (pg/ml) of duplicate tests.

Table 4.4 Peanut allergen stimulated PBMC IL-5 secretion profiles

	nAra h 1	nAra h 2	CPE	PHA	Cells alone
Non-peanut allergic subjects					
N1	5	5	13	314	6
N2	4	9	111	542	6
N3	12	5	71	182	10
N4	7	44	57	27	15
N5	7	5	11	1234	11
N6	8	16	258	1063	9
mean	7	14	87	560	10
SEM	1	7	41	219	1
Peanut allergic subjects					
1	6	5	6	183	15
2	14	6	25	155	7
3	6	6	7	581	12
4	7	5	6	27	14
5	12	71	67	695	6
6	6	7	9	489	8
7	889	520	1991	157	12
8	160	69	313	116	4
9	9	9	155	1655	6
10	1061	1993	1950	1032	8
11	6	9	71	240	6
12	109	29	156	310	5
13	9	5	10	4554	5
14	63	56	172	233	10
15	4	3	83	1145	7
mean	158	186	335	772	8
SEM	87	133	173	295	1

PBMC (2.5 x 10^6/ml) were cultured in 2 ml aliquots with nAra h 1 and nAra h 2 at concentrations producing maximal proliferative responses, and with CPE and PHA as controls. Supernatants were harvested at 48 hours and assayed for IL-5 by sandwich ELISA. Data are expressed as mean concentrations (pg/ml) of duplicate tests.

response to nAra h 1 or nAra h 2 stimulation, and responses to the alternate allergen and CPE (for nAra h 1, $r^2 = 0.984$ for CPE and 0.995 for nAra h 2; for nAra h 2, $r^2 = 0.913$ for CPE and 0.784 for nAra h 1, $p < 0.005$). No correlation could be made between IL-5 secretion and the level of peanut-specific IgE binding, as quantitated by RAST class and score, or on the basis of the presence of specific IgE towards nAra h 1 and nAra h 2 observed on western blotting. Some subjects with very low levels of IgE binding produced large quantities of IL-5 in response to the same allergen, while others with strong IgE binding produced little or no detectable IL-5. Within the peanut allergic group, no significant difference in the quantity of IL-5 secretion produced in response to nAra h 1 could be demonstrated in comparison to nAra h 2 stimulation; a similar finding occurred within the non-peanut allergic cohort.

4.3 DISCUSSION

In this chapter, the proliferation and cytokine responses of PBMC from peanut allergic and non-peanut allergic donors to stimulation with CPE, as well as purified Ara h 1 and Ara h 2 are compared. Proliferative responses to each major allergen and CPE were detected in both subject groups with equal frequency and intensity. Cytokine responses were less frequently demonstrated, but where detected amongst peanut allergic subjects, were of a T_H2 phenotype. No difference could be demonstrated between nAra h 1 and nAra h 2 with regards to the frequency or intensity of proliferative or cytokine response within the peanut allergic population.

Within this study, no correlation was found between peanut specific IgE responsiveness, and the nature of the T cell response to each allergen. While IgE responsiveness to nAra h 1 was limited to 10 of the 15 peanut allergic subjects, proliferative responses were ubiquitous in both peanut allergic and non-peanut allergic groups. Additionally, subjects who had minor or no IgE responsiveness to nAra h 1 clearly produced IL-5 in response to T cell stimulation. For nAra h 2, IgE binding was more widespread, occurring in 13 out of 15 subjects, yet cytokine responses were limited to 6 subjects. Hourihane et al. have demonstrated previously for CPE that proliferative responses to CPE cannot be predicted by the degree of IgE reactivity (Hourihane *et al.* 1998). Such poor correlation between IgE binding and T cell responsiveness has been demonstrated in several other studies exploring the nature of T cell responses to purified allergens from the same source. Hales et al. have studied T cell responses to the major dust mite allergen Der p 1 and the minor dust mite allergen Der p 7 (Hales *et al.* 2000). Within that study, using PBMC in six day assays, similar results to those demonstrated within the present experiments were achieved, with no significant difference demonstrated between each allergen for T cell proliferative or iL-5

responses despite clear differences in the frequency of their IgE responsiveness (Hales *et al.* 2000). No correlation could be demonstrated between the degree of IgE binding to the mite group 7 allergen and T cell production of IL-5 in response to that allergen, and some subjects demonstrated IL-5 production, despite no apparent IgE binding. In earlier studies comparing T and B cell responses to the house dust mite *Dermatophagoides farinae*, O'Hehir et al. again demonstrated that IgE reactivity to allergens bore only partial correlation to T cell responsiveness (O'Hehir *et al.* 1987). Within that study, both IgE and T cell reactivity to the major *D. farinae* allergen Der f 2 could be demonstrated. However, while T cell responsiveness was limited to that allergen, IgE responsiveness occurred to several other *D. farinae* allergens (O'Hehir *et al.* 1987). Bohle et al. examined T cell responses to the major allergens of latex, Hev b 1 and Hev b 3 (Bohle *et al.* 2000). Within that study, using T cell clones specific to Hev b 3, no responses could be established to peptides based upon the Hev b 1 sequence, despite high degrees of sequence similarity and IgE cross-reactivity.

In the present study, proliferative responses could not be used to distinguish between the two allergens, as proliferation was ubiquitous, both amongst peanut allergic and non-peanut allergic donors. Similarly, no difference could be demonstrated between nAra h 1 and nAra h 2 with regards to the quantity of IL-5 or IFN-γ secreted by stimulated PBMC. Hales et al. in their experiments exploring differences in the cytokine and proliferative responses induced by Der p 1 and Der p 7 demonstrated that the minor allergen Der p 7 was associated with significantly more IFN-γ production than Der p 1 (Hales *et al.* 2000). Hence the lack of a significant difference in IFN-γ production in the present study may reflect the fact that both nAra h 1 and nAra h 2 in larger population studies have been characterised as major allergens.

Cytokine responses generated towards nAra h 1 and nAra h 2 amongst peanut allergic donors demonstrated a high degree of correlation, despite the lack of correlation between their IgE reactivities. Stepwise logistic regression demonstrated that IL-5 production towards one allergen was significantly associated with an IL-5 response towards the other allergen, as well as to CPE. This finding is very similar to the findings of Hales et al. for Der p 1 and Der p 7, which in 7 day assays using PBMC, demonstrated significant correlation for IL-5 production (Hales *et al.* 2000).

Interestingly, despite their role as major peanut allergens, nAra h 1 and nAra h 2 stimulated significant degrees of IFN-γ production as well as IL-5. Dorion et al. have previously demonstrated that Ara h 2 is associated with IFN-γ production, in strong association with the degree of proliferation induced with PBMC assays (Dorion *et al.* 1994). While this is in distinction to data presented by others with regards peanut allergen induced cytokine responses (Higgins *et al.* 1995; Turcanu *et al.* 2003), such a finding has been demonstrated

for other food allergens. Using PBMC from milk and egg allergic donors in 6 day cultures, Ng et al. have demonstrated that both IL-5 and IFN-γ secretion are readily measurable upon stimulation with ovalbumin (Ng *et al.* 2002).

Within the study by Ng et al, donor PBMC from children who developed tolerance towards milk and egg having previously being allergic towards these foods, did not demonstrate significant IL-5 production upon stimulation with those food allergens (Ng *et al.* 2002). Turcanu et al. have demonstrated similar findings for peanut allergic children, with those children becoming tolerant of peanut demonstrating reduced IL-5 production (Turcanu *et al.* 2003). In the present study, an adult population is used, supporting the notion that persistence of food allergy into adulthood is associated with the ongoing presence of IL-5 production amongst peanut specific T cells.

No difference was demonstrated between peanut allergic and non-peanut allergic donor PBMC with regards to their proliferative responses to CPE, nAra h 1 or nAra h 2. While there are currently no published reports of data comparing PBMC responses to the major allergens of peanut, several authors have explored proliferative responses to CPE or Ara h 2 (Dorion *et al.* 1994; Higgins *et al.* 1995; de Jong *et al.* 1996; Hourihane *et al.* 1998; Laan *et al.* 1998). Higgins et al. compared proliferative responses amongst peanut allergic and non-peanut allergic donor PBMC following stimulation with CPE and failed to demonstrate any significant difference (Higgins *et al.* 1995). Laan et al. observed that for PBMC derived from peanut allergic subjects with atopic dermatitis, proliferation occurred to a greater extent than that seen amongst non-peanut allergic subjects (Laan *et al.* 1998). de Jong et al. demonstrated a similar finding for PBMC from a single peanut allergic donor (de Jong *et al.* 1996). Several authors have demonstrated that proliferative responses to peanut allergens are MHC class II restricted and due to expansion of the CD4+ T cell subset (Higgins *et al.* 1995; Laan *et al.* 1998).

The major difference between the peanut allergic and non-peanut allergic PBMC responses in the present experiments was within their cytokine response to CPE, nAra h 1 and nAra h 2. Peanut allergic subjects produced greater IL-5 and higher IL-5/IFN-γ ratios than their non-peanut allergic counterparts. IL-5 secretion is strongly associated with the T_H2 response, and is particularly important for eosinophil stimulation and expansion (Robinson 2000). This finding therefore suggests that both the crude extract and purified allergens are able to stimulate a T_H2 response amongst peanut allergic donor PBMC. Additionally, the present experiments, in demonstrating that Ara h 1 and Ara h 2 are recognised by the majority of peanut allergic donor T cells, is consistent with their designation as major T cell allergens as well as major B cell allergens. Interestingly, IL-5 production occurred only among 7 peanut allergic subjects for nAra h 1 and 6 peanut allergic subjects for nAra h 2.

This may reflect poor sensitivity of the ELISA methodology used for cytokine detection. Sensitivity may have been increased using intra-cellular cytokine staining or by assaying for IL-5 mRNA.

In summary, these experiments have demonstrated that CPE, nAra h 1 and nAra h 2 induce significant secretion of IL-5 and greater IL-5/IFN-γ ratios in peanut allergic donor PBMC compared with non-peanut allergic PBMC, suggesting that each antigen is likely to be associated with the allergic response to peanut. No significant difference could be demonstrated between the two allergens with regards to the degree of proliferation induced, or the nature of the cytokine response, despite differences in the frequency of IgE binding. Additionally, no difference between peanut allergic and non-peanut allergic cohorts could be demonstrated with regards to their proliferative responses or degree of IFN-γ production to each allergen. These data provide support for the notion that these allergens will provide useful templates for non-IgE reactive molecules for use within allergen immunotherapy for peanut allergy.

CHAPTER 5 CHARACTERISATION OF THE T CELL EPITOPES OF THE MAJOR PEANUT ALLERGEN, ARA H 2

5.1 INTRODUCTION

Peanut allergy is associated with a significant risk of mortality, while the need for vigilant avoidance of exposure produces considerable psychological morbidity in both sufferers and carers (Sampson *et al.* 1992; Primeau *et al.* 2000). As a consequence, there is an urgent need for a specific cure for this disorder. Despite the existence of efficacious specific immunotherapy for a variety of aeroallergens for approaching 100 years, to date, efforts at specific immunotherapy for peanut allergy have met with extremely limited success (Noon 1911). Nelson *et al.* have shown that tolerance to peanut can be induced using a rush immunotherapy protocol, but that tolerance is lost in approximately half of the subjects during maintenance dosing and additionally that injections are associated with frequent episodes of anaphylaxis in the majority of subjects during both the induction and maintenance phases (Nelson *et al.* 1997). Oppenheimer *et al.* demonstrated similar findings within their study, again showing that active therapy is associated with a high rate of systemic anaphylaxis. Data collection in that study was terminated after the administration of peanut extract to a placebo randomised subject resulted in their death, highlighting the dangerous nature of this condition (Oppenheimer *et al.* 1992).

Development of novel strategies to overcome the morbidity that may be associated with allergen immunotherapy depends on an accurate understanding of the immunological basis to successful immunotherapy, as well as its side-effects. It has long been established that morbidity due to allergen immunotherapy is due to the cross-linking of IgE on mast cells and basophils, and that this action is not required for such therapy to be efficacious (Litwin *et al.* 1988). It is also well known that one of the critical actions of immunotherapy in producing tolerance is its ability to change the predominant specific T cell functional phenotype from a T_H2 to a T_H1 phenotype (Robinson 2000). Increasing data suggest that effective immunotherapy induces T cell anergy, T regulatory cells secreting IL-10 and TGF-β, apoptosis and immune deviation. This is likely to occur via the suppression of the T_H2 phenotype by IL-10, then reconstitution of a normal immune response via the actions of IL-2 and IL-15 (Rolland *et al.* 1998; Akdis *et al.* 2000)

A key difference in B cell and T cell responses is in antigen recognition, B cells and antibodies recognising conformational epitopes dependent on molecular tertiary structure, while CD4+ T cells recognise short linear peptides from the primary structure. This difference in antigen recognition is the basis of many novel strategies for immunotherapy,

including those using peptides based upon dominant T cell epitopes, altered peptide ligands and B cell epitope mutants (Akdis *et al.* 2001). Such approaches depend on the alteration or absence of molecular tertiary structure, so that IgE cross-linking and effector cell activation is lost, but critical T cell recognition is retained. Of these new strategies, peptide immunotherapy is the method for which there is the strongest evidence of efficacy, being documented for both cat dander allergy and bee venom allergy. Muller *et al.* showed that, in the absence of systemic side-effects, tolerance could be achieved for the major bee venom allergen phospholipase A2 (PLA2) using sequences based on three major T cell epitopes, while several authors have demonstrated that peptides based on T cell epitopes of the major cat allergen Fel d 1 can induce diminished clinical responses (Norman *et al.* 1996; Marcotte *et al.* 1998; Muller *et al.* 1998; Pene *et al.* 1998; Maguire *et al.* 1999; Fellrath *et al.* 2003; Oldfield *et al.* 2002).

Crucial to the development of such strategies is the retention of T cell epitopes, so that T cell phenotypic change can be induced. Based upon western blotting data from published work and our own cohort, the major peanut allergens are Ara h 1 and Ara h 2, with the latter being recognised by at least 80% of the peanut allergic population (Burks *et al.* 1991; Burks *et al.* 1992). Data presented in the previous chapter are consistent with Ara h 1 and Ara h 2 possessing major T cell epitopes, but to date no peer reviewed data have been published reporting the T cell epitopes of peanut allergens. In this chapter, analysis of the T cell epitopes of the major peanut allergen Ara h 2 is described. Two different approaches to optimisation of generation of Ara h 2 specific T cell lines are used, and the results of proliferation assays and cytokine assays are detailed.

5.2 RESULTS

5.2.1 Subject Characterisation

Clinical characteristics of donor subjects used for these experiments are detailed in Table 5.1. A total of 22 peanut allergic subjects were used for Ara h 2 T cell epitope mapping, of whom 15 were female. The average age of subjects was 32 years (range 19-55 years). Nineteen of the 22 subjects suffered from other allergic diseases, including asthma, eczema or allergic rhinitis. Of the 19 subjects for whom skin prick testing had been performed, only one was non-atopic. Each subject described typical features of anaphylaxis on exposure to peanut, beginning within minutes of that exposure. Laryngeal oedema was the most common symptom, with 17 of the 22 subjects describing this symptom at the time of anaphylaxis. Other common symptoms were asthma, generalised urticaria, and facial angioedema. Five of the 22 subjects reported anaphylaxis occurring only on exposure to peanut, but only one of

Table 5.1 Clinical features of the 22 peanut allergic subjects used for T cell Ara h 2 epitope mapping.

Legend: GP, grass pollen; B, birch; HDM, house dust mite; C, cat; A, Alternaria.

Subject	Sex	Age	Allergic disease	Clinical features	Known nut allergens	Other food allergens	Age at first reaction (yrs)	Time since last reaction (mths)	Peanut RAST		Atopic status
									level ku(A)/ml	score	
1	F	27	asthma, rhinitis, eczema	laryngeal oedema, urticaria, facial angioedema	peanut, hazelnut, walnut	sesame seed, baked beans	10	2	2.07	2	GP, B, HDM, C, A
2	F	34	asthma	asthma, laryngeal oedema, urticaria, facial angioedema	peanut	peas, lentils	14	192	0.73	2	GP, B, HDM
3	F	40	asthma, eczema	asthma, laryngeal oedema, loss of consciousness, urticaria, facial angioedema	peanut, Brazil nut, cashew nut, hazelnut	nil	2	12	3.61	3	C
4	M	27	rhinitis	asthma, laryngeal oedema, urticaria	peanut, almond, brazilnut, hazelnut, macadamia, walnut	pine nuts, citrus seeds	2	4	mixed nut RAST: 3/4		
5	F	31	rhinitis, eczema	GIT upset, asthma, laryngeal oedema, urticaria	peanut, almond, hazelnut, pistachio		1.5	18	13.6	3	GP, HDM, C
6	F	33	nil	GIT upset, urticaria	peanut, hazelnut	pine nuts	0.5	3	13.3	3	GP, B, HDM, C
7	F	27	eczema	asthma, laryngeal oedema, urticaria, angioedema	peanut, almond		19	11	9.53	3	
8	F	19	asthma	laryngeal oedema, urticaria	peanut, almond, Brazil nut, cashew, hazelnut, walnut	crustacea	3	12	3.09	2	GP, HDM, C
9	M	29	asthma, rhinitis, eczema	GIT upset, laryngeal oedema, facial angioedema	peanut, almond, Brazil nut, cashew, hazelnut, walnut	pine nuts	1.5	36	1.22	2	GP, B, HDM, C

Subject	Sex	Age	Allergic disease	Clinical features	Known nut allergens	Other food allergens	Age at first reaction (yrs)	Time since last reaction (mths)	Peanut RAST		Atopic status
									level ku(A)/ml	score	
10	F	49	nil	GIT upset, asthma, laryngeal oedema, loss of consciousness, urticaria	peanut, cashew nut	peas	2	18	17.6	4	GP
11	M	30	asthma	GIT upset, asthma, laryngeal oedema, facial angioedema	peanut, hazelnut		12	18	0.51	1	
12	F	22	asthma	laryngeal oedema, urticaria, facial angioedema	peanut		0.5	24	16.3	3	GP, HDM
13	F	36	asthma, eczema	GIT upset, asthma, hypotension, facial angioedema	peanut, hazelnut		4	2	3.09	2	GP, HDM, C
14	F	50	nil	laryngeal oedema, urticaria, facial angioedema	peanut		18.5	21	6.87	3	GP, HDM, C
15	M	27	asthma, rhinitis	asthma, laryngeal oedema, urticaria, facial angioedema	peanut, almond, hazelnut	avocado	1.5	24	12.4	3	GP, B, HDM
16	F	37	rhinitis, eczema	GIT upset, asthma, laryngeal oedema, hypotension	peanut, hazelnut	pine nuts	10	36	0	0	GP, HDM, peanut 14mm
17	M	35	asthma, rhinitis, eczema	asthma, urticaria, facial angioedema	peanut, hazelnut		8	12	2.82	2	
18	F	22	rhinitis	GIT upset, asthma, laryngeal oedema, facial angioedema	peanut, hazelnut, pistachio		8	9	0.39	1	GP

Subject	Sex	Age	Allergic disease	Clinical features	Known nut allergens	Other food allergens	Age at first reaction (yrs)	Time since last reaction (mths)	Peanut RAST		Atopic status
									level ku(A)/ml	score	
19	M	55	asthma, eczema	asthma, laryngeal oedema, facial angioedema	peanut, walnut	banana	1.5	120	2.01	2	GP, HDM, A
20	F	30	asthma	asthma, uticaria, laryngeal oedema, facial angioedema	peanut, Brazil nut, almond		3	228		0	
21	M	28	asthma	GIT upset, laryngeal oedema, urticaria	peanut, hazelnut, egg, milk		5	1	100	6	HDM
22	F	32	asthma, eczema	asthma, urticaria, facial angioedema	peanut,		0	0		0	

those five was also RAST test negative to all other nuts tested. RAST data showed no correlation to the severity of reactions subjects experienced with exposure to peanut.

5.2.2 Peanut Allergen Preparation

The manufacture and characterisation of CPE and rAra h 2 is described in chapters 2 and 3. The sequences of the Ara h 2 peptides used for epitope mapping are illustrated in Figure 5.1 (peptides purchased from Mimotopes, Australia). Each peptide was 20 amino acids in length with an 11 amino acid shared sequence with adjacent peptides, except for those closest to the C-terminus, where the matching sequence was 18 amino acids in length. Characterisation of peptides with regards T cell mitogenicity and toxicity is displayed in Figure 5.2, and demonstrates that all peptides were free of these potential confounders.

5.2.3 Western Blotting for Serum IgE Reactivity to Ara h 2

To determine the frequency of Ara h 2 serum IgE reactivity amongst the study cohort, western blotting of CPE was carried out. These results are shown in Figure 5.3 and demonstrate that 20 out of the 22 peanut allergic subjects possessed serum IgE reactive to a doublet of approximately 14 kDa, representing Ara h 2. Interestingly, apart from Ara h 2, 19 of the 22 subjects recognised a band of approximately 11 kDa, likely to represent either Ara h 3, Ara h 5 or Ara h 6. Non-peanut allergic controls also showed binding to Ara h 2 and the 11 kDa protein, but this was weak and potentially non-specific in this highly sensitive assay.

5.2.4 Polyclonal T cell Responses to Crude Peanut Extract

To ensure that CPE-specific T cells were present within the T cell repertoire of the study subjects, PBMC proliferative responses to stimulation with CPE were analysed, demonstrating a dose response for both peanut allergic subjects and non-peanut allergic controls. PBMC responses to CPE are discussed in detail in chapter 4. For each subject, the lowest dose that produced maximal stimulation at 7 days was used to drive peanut specific T cell lines for use in peptide assays, and ranged between 50 and 200 μg/ml.

5.2.5 Mapping of Ara h 2 T Cell Epitopes

To determine the T cell epitopes of Ara h 2, oligoclonal CPE-specific TCL were generated from PBMC of 21 peanut-allergic donors and 5 non-peanut allergic donors, and stimulated with a set of synthetic overlapping peptides spanning the entire Ara h 2 sequence (Figure 5.1). A peptide concentration of 10 μg/ml was determined to be the optimal stimulating

1-20 [1] L T I L V A L A L F L L A A H A S A R Q [20]

10-29 [10] F L L A A H A S A R Q Q W E L Q G D R R [29]

19-38 [19] R Q Q W E L Q G D R R C Q S Q L E R A N [38]

28-47 [28] R R C Q S Q L E R A N L R P C E Q H L M [47]

37-56 [37] A N L R P C E Q H L M Q K I Q R D E D S [56]

46-65 [46] L M Q K I Q R D E D S Y E R D P Y S P S [65]

55-74 [55] D S Y E R D P Y S P S Q D P Y S P S P Y [74]

64-83 [64] P S Q D P Y S P S P Y D R R G A G S S Q [83]

73-92 [73] P Y D R R G A G S S Q H Q E R C C N E L [92]

82-101 [82] S Q H Q E R C C N E L N E F E N N Q R C [101]

91-110 [91] E L N E F E N N Q R C M C E A L Q Q I M [110]

100-119 [100] R C M C E A L Q Q I M E N Q S D R L Q G [119]

109-128 [109] I M E N Q S D R L Q G R Q Q E Q Q F K R [128]

118-137 [118] Q G R Q Q E Q Q F K R E L R N L P Q Q C [137]

127-146 [127] K R E L R N L P Q Q C G L R A P Q R C D [146]

136-155 [136] Q C G L R A P Q R C D L D V E S G G R D [155]

138-157 [138] G L R A P Q R C D L D V E S G G R D R Y [157]

Figure 5.1 Ara h 2 20-mer peptide series

Peptides of 20 amino acids in length, overlapping by 11 amino acids except for those at the C-terminus (18 amino acid overlap), representing the entire amino acid sequence of Ara h 2 were used. The residue numbers of each peptide is shown in the left hand column, while overlap with peptides located adjacent to each peptide is demonstrated by the extent to which each peptide is overlaid within the figure. Peptide sequences are based upon the Ara h 2 sequence described by Stanley et al (Stanley *et al.* 1997).

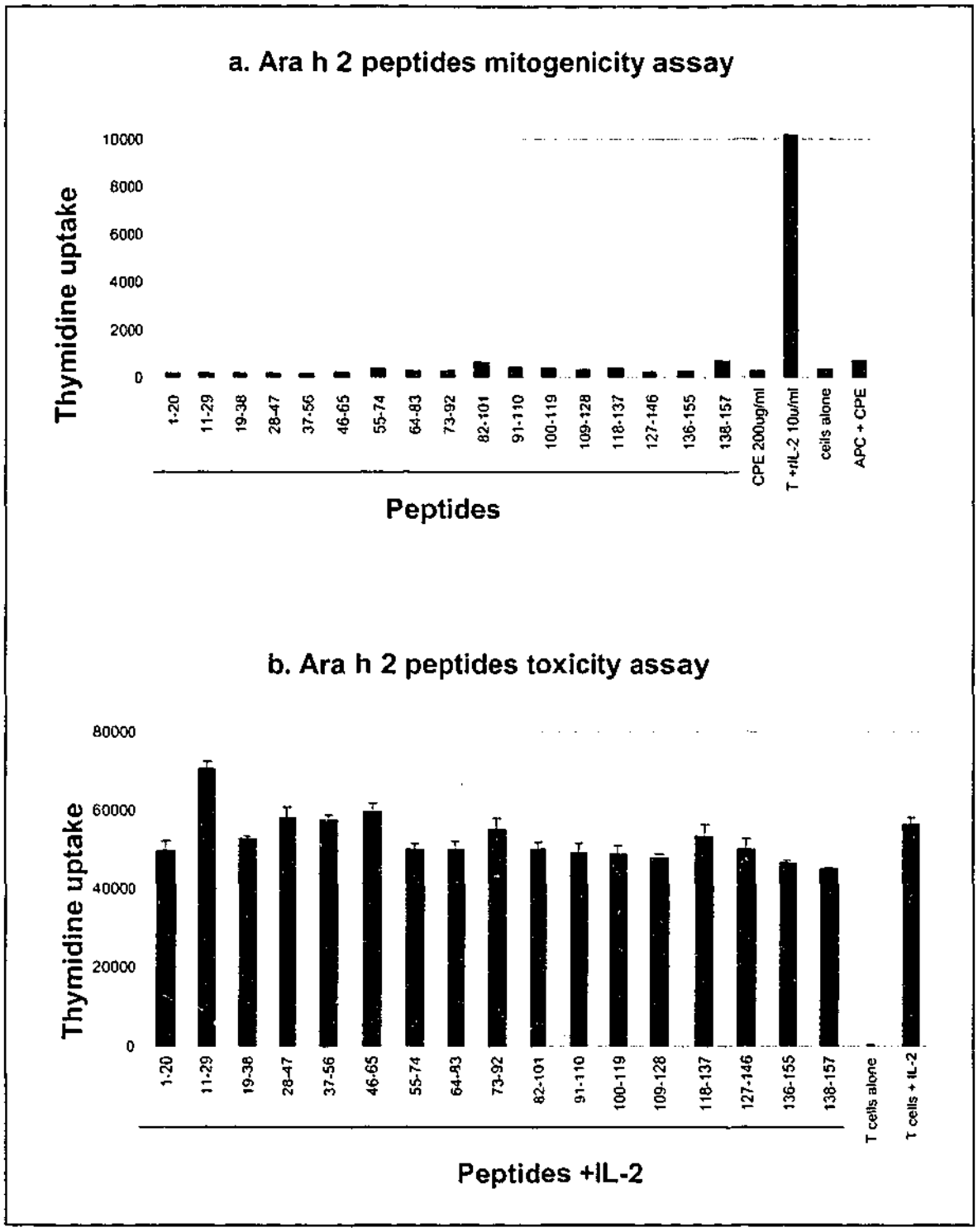


Figure 5.2 Evaluation of T cell mitogenic and cytotoxic potential of Ara h 2 peptides

a. Mitogenicity assay

Triplicate cultures of 5 x 10^4 cells/well of a 3 week oligoclonal HDM-specific TCL together with 5 x 10^4 cells/well of irradiated autologous PBMC and Ara h 2 peptides at a concentration of 10µg/ml for 72 hours. For the last 12 hours cells were pulsed with ^{3}H-thymidine before harvesting and counting. ^{3}H-thymidine incorporation for each peptide was estimated and expressed as mean cpm + SEM.

b. Toxicity assay

Triplicate cultures of 5 x 10^4 cells/well of a 3 week oligoclonal HDM-specific TCL stimulated with 50 IU/ml of recombinant human IL-2 and Ara h 2 peptides at a concentration of 10µg/ml for 72 hours. For the last 12 hours cells were pulsed with ^{3}H-thymidine before harvesting and counting. ^{3}H-thymidine incorporation for each peptide was estimated and expressed as mean cpm + SEM.

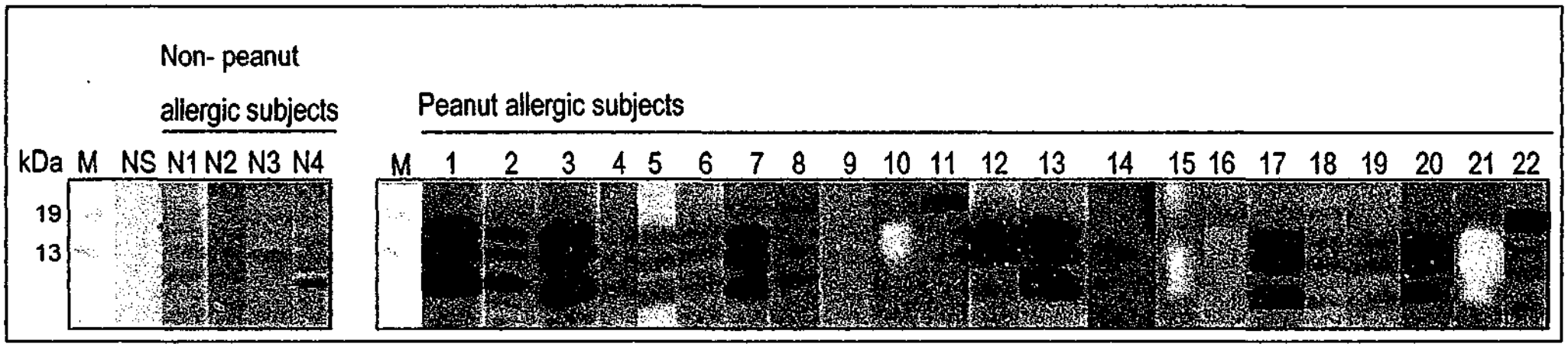


Figure 5.3 Western blot for serum IgE reactivity to Ara h 2 of peanut allergic and non-allergic subjects

Legend: M, molecular mass; NS, no serum.
Following resolution of CPE on a 14% polyacrylamide gel, proteins were transferred to nitrocellulose and probed with sera from the study population, and non-peanut allergic controls. IgE was detected using HRP conjugated mouse anti-human IgE and enhanced chemiluminescence.

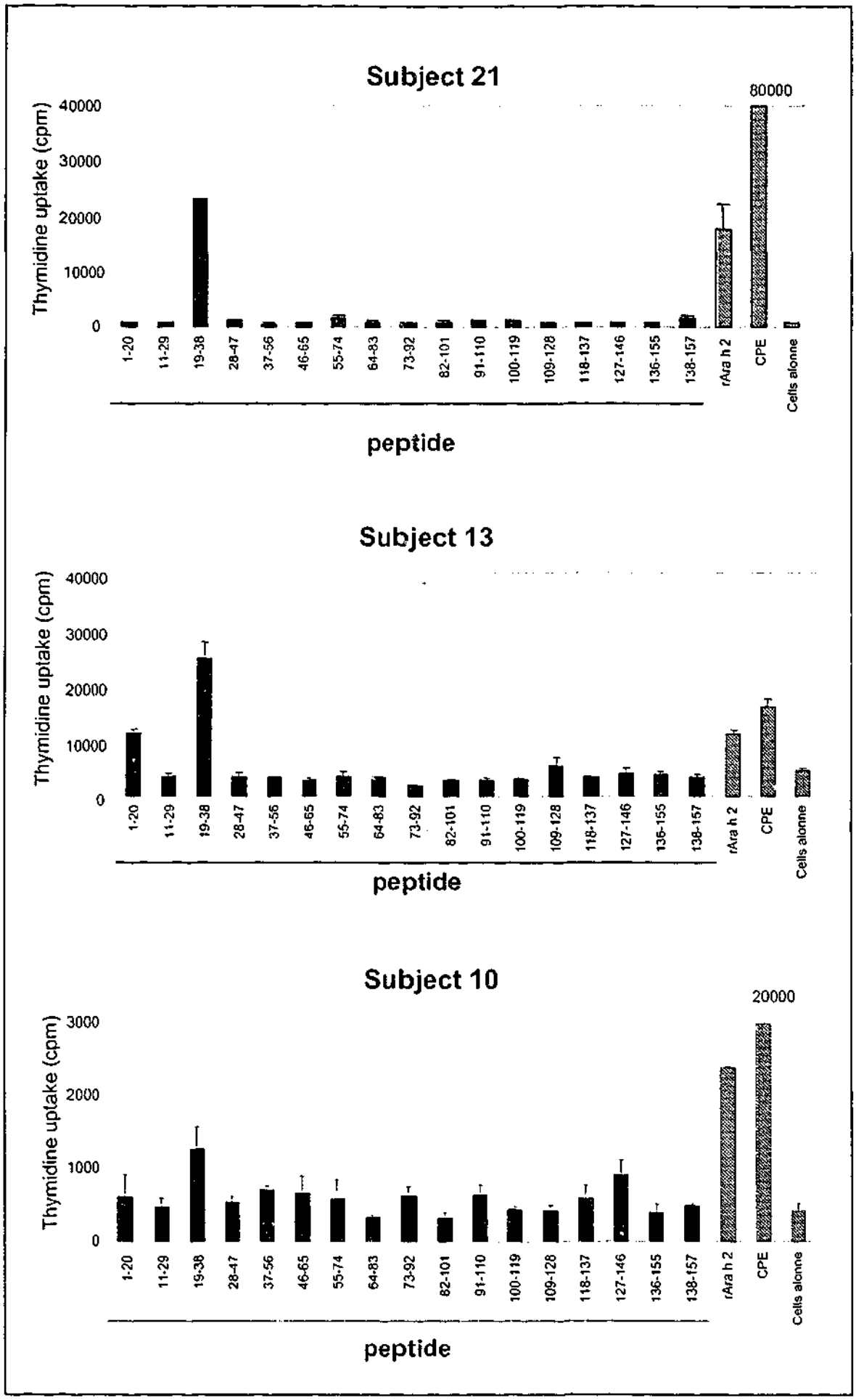
concentration within preliminary proliferation assays. Optimisation included assays at both 10 µg/ml and 30 µg/ml. Responses to each concentration proved similar, although some subjects who failed to respond significantly to any peptide at a concentration of 10 µg/ml demonstrated an indiscriminate low grade response to most peptides at the higher concentrations, that did not provide any differential signal. In this setting, and because of the limited availability of donor cells and the cost of commercially prepared peptides, the lower concentration was used.

Several approaches to the production of TCL were used, including stimulation of cells with two and three pulses of CPE separated by 7 days each, two pulses of CPE with a third pulse of rAra h 2, again separated by 7 days each, and three stimulations with rAra h 2. Responses by TCL receiving two CPE stimulations were in general lower than those receiving three stimulations, such that it was felt the sensitivity of the assay may be compromised. Responses of TCL receiving a third pulse with rAra h 2 were surprisingly infrequent on the basis of the frequent sensitivity demonstrated to Ara h 2 by western blotting, while responses by cells receiving three pulses with rAra h 2 were universally absent, suggesting that the rAra h 2 extract may have contained other antigens or substances blunting the T cell response to the allergen. For this reason a decision was made to use TCL stimulated with 3 pulses of CPE, each separated by 7 days for all subjects tested. Data from subjects demonstrating a peptide response to two pulses of CPE and a final pulse of rAra h 2 are also presented.

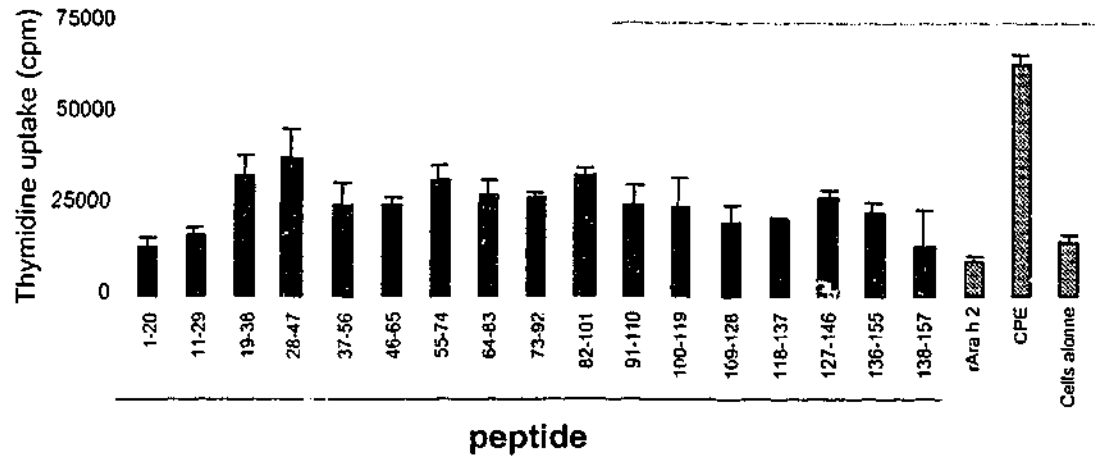
Individual responses to peptide, Ara h 2 and CPE are demonstrated in Figures 5.4 and 5.5 and summarised in Table 5.2. A total of 9 (41%) of the 22 peanut allergic subjects demonstrated a proliferative response to one or more of the Ara h 2 peptides, with 8 subjects responding using CPE driven TCL, two subjects responding using both methods of TCL generation, and one subject responding only when using a TCL driven with a pulse of rAra h 2. Of interest, PBMC from non-peanut allergic subjects could not be used to sustain TCL, cells becoming non-viable after two stimulations or demonstrating a non-discriminatory "high background" response to all antigens assayed. CPE specific TCL could be generated from 19 (86%) of the 22 peanut allergic subjects, with 6 (32%) of those 19 peanut specific TCL responding to rAra h 2. Proliferative responses to rAra h 2 showed best correlation with responses to Ara h 2 (19-38) and Ara h 2 (28-47), in that where a subject had a response to these peptides, 60% also had a proliferative response to Ara h 2. No other peptides were as clearly associated with a response to rAra h 2. Only 2 peptide non-responsive TCL demonstrated a proliferative response to rAra h 2. No subject without specific IgE towards Ara h 2 demonstrated a proliferative response to peptide stimulation, but the intensity of IgE reactivity could not otherwise be used to predict a peptide response.

Figure 5.4 Peanut allergic donor peanut specific TCL proliferative responses to Ara h 2 peptides, Ara h 2 and crude peanut extract

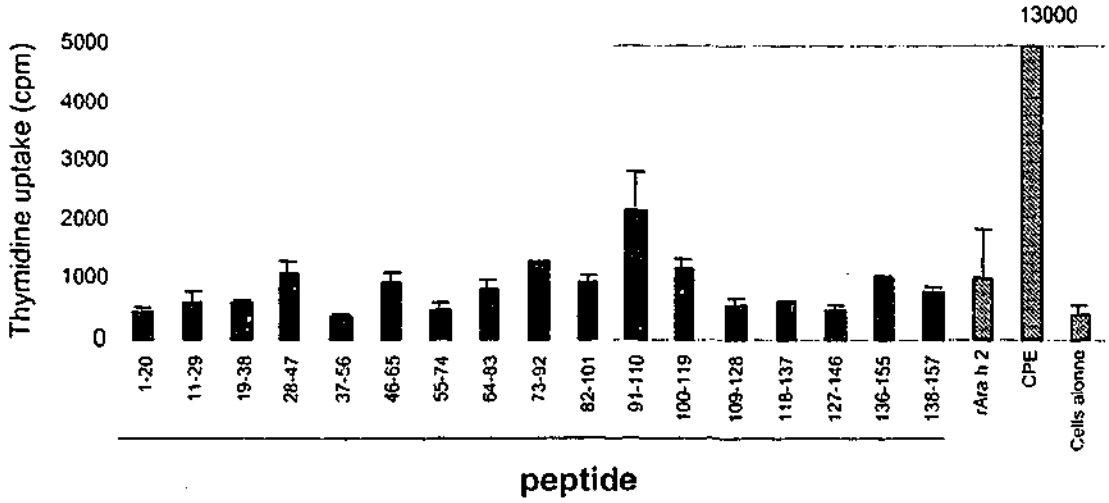
Peanut specific TCL (5 x 10^4 cells/ml) from individuals with peanut allergy generated using CPE were stimulated with Ara h 2 peptides at a concentration of 10 µg/ml in the presence of autologous irradiated PBMC as APC (5 x 10^4 cells/ml) in 3 day cultures. Proliferative responses were assessed by tritiated thymidine incorporation, and displayed as mean cpm + SEM of triplicate cultures.


Subject 21
Thymidine uptake (cpm)
40000
30000
20000
10000
0
80000
1-20
11-29
19-38
28-47
37-56
46-65
55-74
64-83
73-92
82-101
91-110
100-119
109-128
118-137
127-146
136-155
138-157
rAra h 2
CPE
Cells alonne
peptide
Subject 13
Thymidine uptake (cpm)
40000
30000
20000
10000
0
1-20
11-29
19-38
28-47
37-56
46-65
55-74
64-83
73-92
82-101
91-110
100-119
109-128
118-137
127-146
136-155
138-157
rAra h 2
CPE
Cells alonne
peptide
Subject 10
Thymidine uptake (cpm)
3000
2000
1000
0
20000
1-20
11-29
19-38
28-47
37-56
46-65
55-74
64-83
73-92
82-101
91-110
100-119
109-128
118-137
127-146
136-155
138-157
rAra h 2
CPE
Cells alonne
peptide

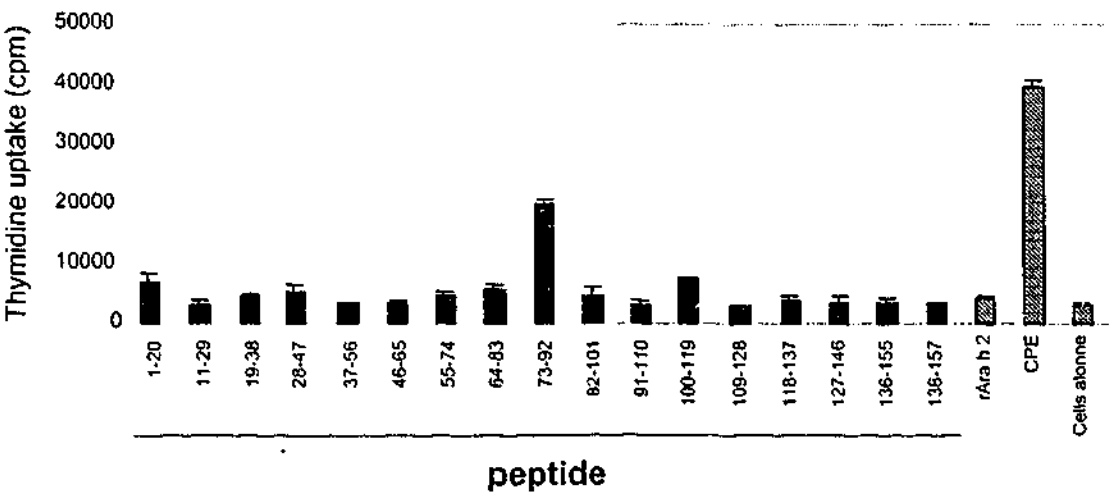
Subject 19



Subject 6



Subject 17



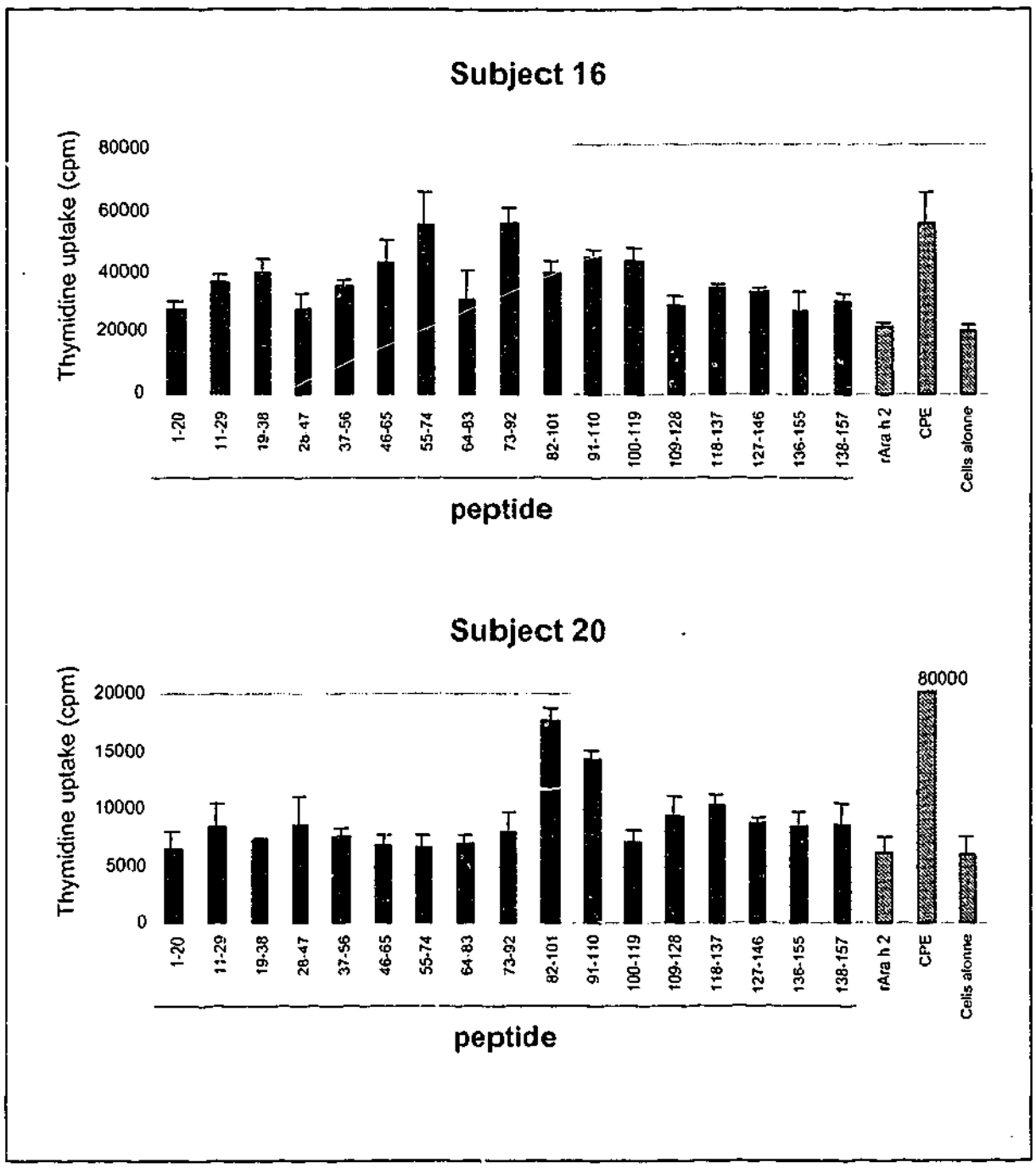

Subject 16
Thymidine uptake (cpm)
80000
60000
40000
20000
0
1-20
11-29
19-38
28-47
37-56
46-65
55-74
64-83
73-92
82-101
91-110
100-119
109-128
118-137
127-146
136-155
138-157
rAra h 2
CPE
Cells alonne
peptide
Subject 20
Thymidine uptake (cpm)
20000
15000
10000
5000
0
80000
1-20
11-29
19-38
28-47
37-56
46-65
55-74
64-83
73-92
82-101
91-110
100-119
109-128
118-137
127-146
136-155
138-157
rAra h 2
CPE
Cells alonne
peptide

Figure 5.5 Peanut allergic donor peanut specific Ara h 2 pulsed TCL proliferative responses Ara h 2 peptides, Ara h 2 and crude peanut extract
Peanut specific TCL (5 x 10^4 cells/ml) from individuals with peanut allergy were generated using CPE and rAra h 2, then stimulated with Ara h 2 peptides at a concentration of 10 μg/ml in the presence of autologous irradiated PBMC as APC (5 x 10^4 cells/ml) in 3 day cultures. Proliferative responses were assessed by tritiated thymidine incorporation, and displayed as mean cpm + SEM of triplicate cultures.

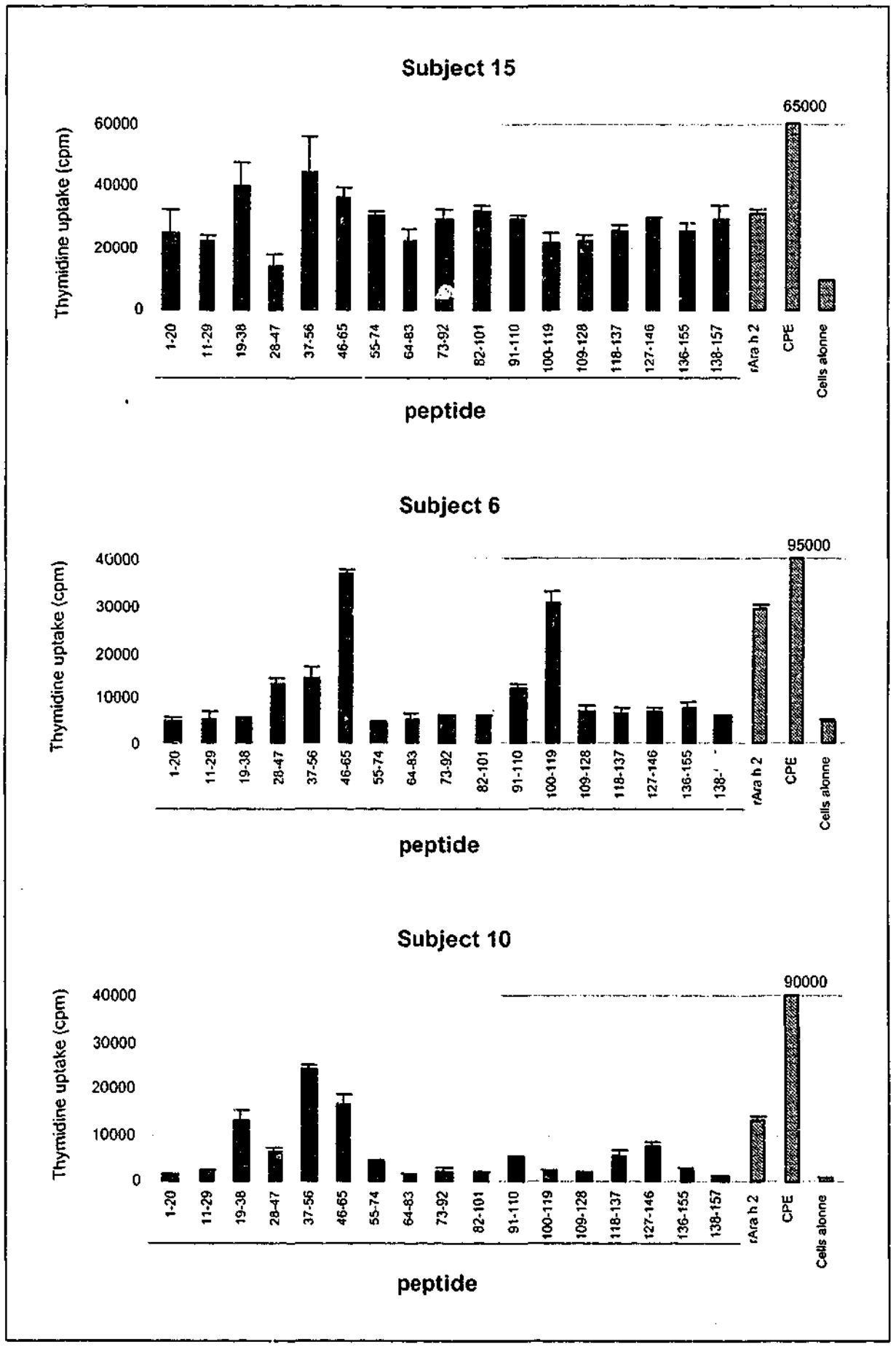

Subject 15
Thymidine uptake (cpm)
60000
40000
20000
0
65000
1-20
11-29
19-38
28-47
37-56
46-65
55-74
64-83
73-92
82-101
91-110
100-119
109-128
118-137
127-146
136-155
138-157
rAra h 2
CPE
Cells alonne
peptide
Subject 6
Thymidine uptake (cpm)
40000
30000
20000
10000
0
95000
peptide
Subject 10
Thymidine uptake (cpm)
40000
30000
20000
10000
0
90000
peptide

Table 5.2 Summary of TCL proliferative responses to Ara h 2 peptides, rAra h 2, and CPE.

Responses of TCL demonstrating a significant response to one or more rAra h 2 peptides are summarised according to stimulation index. Responses associated with a stimulation index ≥ 2.5 are shaded grey.

	1-20	10-29	19-38	28-47	37-56	46-65	55-74	64-83	73-92	82-101	91-110	100-119	109-128	118-137	127-146	136-155	138-157	Ara h 2	CPE
CPE driven lines																			
Subject 21	0.9	1.0	**26.8**	1.3	0.7	0.9	1.9	1.0	1.1	1.1	1.3	1.3	1.0	0.9	0.9	1.0	2.0	**20.9**	**89.9**
Subject 13	2.4	0.8	**5.2**	0.8	0.7	0.6	0.8	0.7	0.4	0.6	0.6	0.7	1.2	0.8	0.9	0.8	0.7	2.3	**4.8**
Subject 10	1.5	1.1	**3.1**	1.3	1.7	1.6	1.4	0.8	1.5	0.8	1.5	1.1	1.0	1.5	2.2	1.0	1.2	**5.8**	**52.0**
Subject 6	1.1	1.5	1.5	**2.8**	0.9	2.3	1.2	2.1	**3.3**	2.4	**5.3**	**2.9**	1.4	1.6	1.2	**2.6**	2.0	**5.7**	**34.2**
Subject 19	0.9	1.1	2.3	**2.6**	1.7	1.7	2.2	1.9	1.8	2.3	1.7	1.7	1.4	1.4	1.8	1.6	0.9	0.8	**4.4**
Subject 17	2.3	1.1	1.6	1.8	1.1	1.1	1.6	1.9	**6.7**	1.5	1.1	**2.5**	1.0	1.3	1.2	1.1	1.1	1.4	**13.9**
Subject 16	1.3	1.8	1.9	1.3	1.7	2.1	**2.7**	1.5	**2.7**	1.9	2.2	2.1	1.4	1.7	1.6	1.3	1.5	1.2	**3.2**
Subject 20	1.1	1.4	1.2	1.4	1.2	1.1	1.1	1.2	1.3	**2.9**	2.4	1.2	1.6	1.7	1.5	1.4	1.4	1.2	**15.7**
rAra h 2 pulse																			
Subject 10	1.7	**2.8**	**16.7**	**7.9**	**30.3**	**21.0**	**5.3**	1.8	**2.8**	2.3	**6.4**	**2.5**	2.1	**7.2**	**9.8**	**3.2**	1.4	**16.6**	**7.8**
Subject 6	1.0	1.1	1.0	**2.6**	**3.0**	**7.7**	0.9	1.1	1.1	1.2	2.5	**6.4**	1.4	1.4	1.4	1.6	1.2	**6.2**	**20.1**
Subject 15	**2.7**	2.4	**4.3**	1.5	**4.8**	**3.9**	**3.3**	2.4	**3.1**	**3.4**	**3.2**	2.3	2.4	**2.8**	**3.1**	**2.8**	**3.1**	**4.5**	**7.1**

Using CPE driven TCL, of the 17 peptides tested, 8 (47%) induced a proliferative response (Figure 5.6). Ara h 2 (19-38) and Ara h 2 (73-92) were associated with the greatest frequency of response, producing proliferative responses in 3 of the 8 responders. Other peptides inducing proliferative responses were located at Ara h 2 (28-47), Ara h 2 (55-74), Ara h 2 (82-101), Ara h 2 (91-110), Ara h 2 (100-119), and Ara h 2 (136-155). No peptide was associated with a response in over 25% of the 22 CPE driven TCL. Only 2 peptides produced responses in greater than 25% of CPE driven TCL that demonstrated a peptide response, these being Ara h 2 (19-38) and Ara h 2 (73-92). Comparison of the magnitude of response to each antigen demonstrates a great variation between subjects. When response magnitude was ranked for each subject, the greatest responses were towards Ara h 2 (19-38), with 3 subjects demonstrating their greatest response to this peptide.

Peptide responses by rAra h 2 pulsed TCL occurred in 3 of 20 TCL generated in this fashion. Peptide responses were widespread amongst those responders, and only two peptides, Ara h 2 (64-83) and Ara h 2 (109-128) did not produce a response in at least one subject. The magnitude of responses towards peptides by these TCL was greater than that demonstrated by CPE driven TCL, the greatest response being by subject 10 towards Ara h 2 (37-56), where the stimulation index was 30.3. Comparison of peptide responses demonstrated by the different types of TCL revealed some common peptide responses, but several differences. Only two subjects demonstrated significant peptide responses to both methods of TCL preparation. Subject 10 reacted to Ara h 2 (19-38) using both types of TCL preparation, but the magnitude of the response to this peptide in the rAra h 2 pulsed TCL was less than that generated to Ara h 2 (37-56) and Ara h 2 (46-65). The pattern of reactivity demonstrated by subject 6 was similar for both types of TCL, but did not include Ara h 2 (73-92) or Ara h 2 (136-155) for rAra h 2 pulsed TCL.

5.2.6 T cell Cytokine Responses to Ara h 2 Peptides Eliciting a Proliferative Response

To identify the phenotype of T cells associated with a peptide proliferative response, supernatants were collected from TCL cultures 48 hours after stimulation with peptides and assayed for the presence of IL-5 and IFN-γ. For each donor, peptides associated with a proliferative response and two peptides not associated with a proliferative response were tested. Of the 8 CPE driven TCL examined, 7 demonstrated detectable cytokine levels. Cytokine ratios for individual CPE driven TCL are demonstrated in Figure 5.7. The magnitude of cytokine responses associated with individual peptides varied greatly between subjects, from the lower limits of detection for both cytokines, up to 2054 pg/ml for IL-5, and 2966 pg/ml for IFN-γ. In general, cytokine responses were at the lower limits of

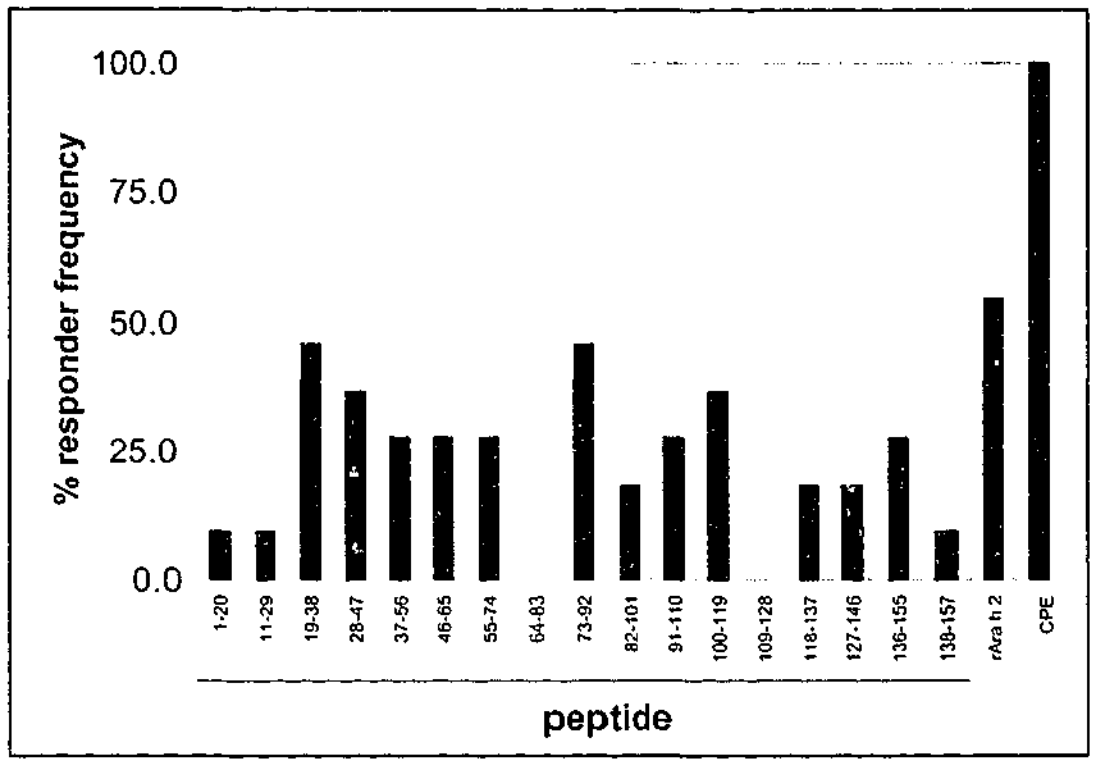
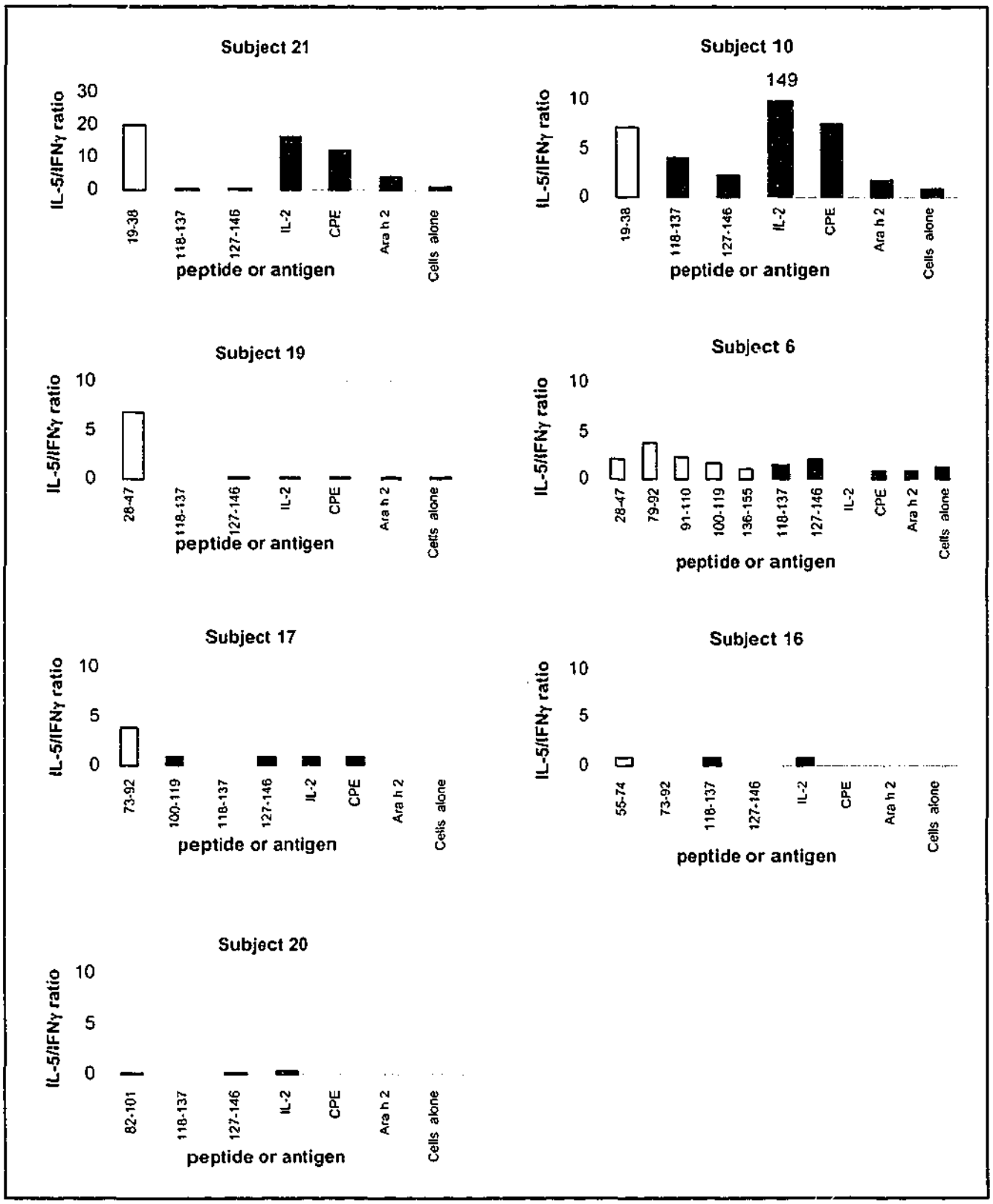


Figure 5.6 Percentage responder frequency to Ara h 2 peptides amongst peptide responsive TCL.

The percentage of peptide responsive TCLs demonstrating a stimulation index of ≥ 2.5 to individual peptides is demonstrated, along with responses to rAra h 2 and CPE.

Figure 5.7 Peanut allergic donor peanut specific Ara h 2 pulsed TCL cytokine responses to Ara h 2 peptides, Ara h 2 and crude peanut extract

Peanut specific TCLs (5 x 10^4/ml) from individuals with peanut allergy were generated using CPE, then stimulated with Ara h 2 peptides at a concentration of 10 ug/ml in the presence of autologous irradiated PBMC as APC (5 x 10^4/ml) in 3 day cultures. Supernatants were collected after 48 hours culture and assayed for cytokines by specific ELISA. Data are displayed graphically as IL-5:IFN-γ ratio. Peptides associated with a significant proliferative response are shaded grey.


Subject 21
IL-5/IFNγ ratio
30
20
10
0
19-38
118-137
127-146
IL-2
CPE
Ara h 2
Cells alone
peptide or antigen
Subject 10
IL-5/IFNγ ratio
10
5
0
149
19-38
118-137
127-146
IL-2
CPE
Ara h 2
Cells alone
peptide or antigen
Subject 19
IL-5/IFNγ ratio
10
5
0
28-47
118-137
127-146
IL-2
CPE
Ara h 2
Cells alone
peptide or antigen
Subject 6
IL-5/IFNγ ratio
10
5
0
28-47
79-92
91-110
100-119
136-155
118-137
127-146
IL-2
CPE
Ara h 2
Cells alone
peptide or antigen
Subject 17
IL-5/IFNγ ratio
10
5
0
73-92
100-119
118-137
127-146
IL-2
CPE
Ara h 2
Cells alone
peptide or antigen
Subject 16
IL-5/IFNγ ratio
10
5
0
55-74
73-92
118-137
127-146
IL-2
CPE
Ara h 2
Cells alone
peptide or antigen
Subject 20
IL-5/IFNγ ratio
10
5
0
82-101
118-137
127-146
IL-2
CPE
Ara h 2
Cells alone
peptide or antigen

detection. The greatest individual IL-5 response was to Ara h 2 (19-38) with subject 21 producing 2054 pg/ml of IL-5 and subject 10 producing 540 pg/ml of IL-5 towards this peptide. These responses were substantially greater than the next largest cytokine response demonstrated by other subjects.

IL-5/IFNγ ratios were skewed towards greater IL-5 production for peptide responses in 5 of the 7 subjects whose CPE driven TCL demonstrated a proliferative response. The greatest ratio occurred towards Ara h 2 (19-38), being approximately 20 for subject 21, and 7 in subject 10. Only these subjects demonstrated an IL-5/IFN-γ ratio of greater than 1 for rAra h 2 and CPE. For peptides not associated with a proliferative response, the IL-5/IFN-γ ratio was less than 1 for 5 of 7 subjects.

Cytokine responses by rAra h 2 pulsed TCL could only be demonstrated in two of the three TCL demonstrating a proliferative response to peptides and IL-5 levels are illustrated in Figure 5.8. The response of these TCL was predominantly characterised by IFN-γ production (data not shown), again suggesting that rAra h 2 pulsed TCL were of a different phenotype to CPE generated TCL, and may have been contaminated with either different antigens or other immunomodulatory substances. IL-5 production could only be detected in low levels or not at all for peptides not associated with a proliferative response, but was clearly detectable for those producing proliferation. This is in contrast to IFN-γ, for which detectable levels occurred for all peptides, although at increased levels for those peptides producing a proliferative response.

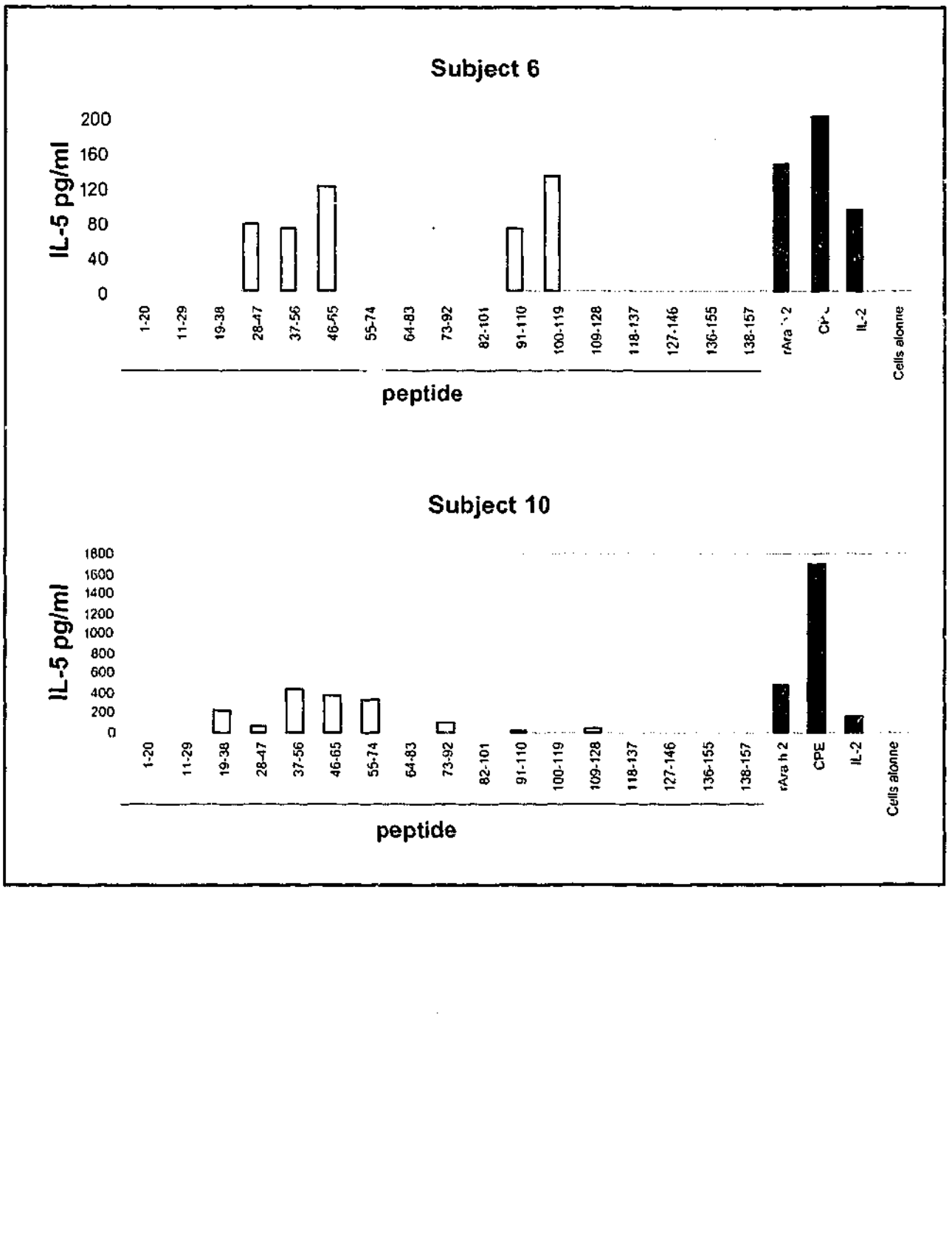
5.3 DISCUSSION

Within this chapter, oligoclonal CPE specific TCL have been stimulated with CPE, rAra h 2 and an overlapping set of 20-mer peptides representing the entire length of Ara h 2. A distinct pattern of proliferative responses to Ara h 2 peptides has been demonstrated amongst the TCL, with certain clusters of peptides being more frequently associated with proliferative responses and producing a predominantly IL-5 cytokine response, suggesting that those peptides contain epitopes relevant to the T cell immune response of peanut allergic subjects.

Of the 22 peanut allergic subjects whose PBMC were used, 19 (83%) could be utilised successfully to produce CPE driven TCL, the remainder not producing sufficient viable cells using the protocol established within optimisation experiments. Whether a viable CPE driven TCL could be produced from donor PBMC was not suggested by any particular clinical feature of the subjects, their serology or their polyclonal T cell proliferative response. Reactivity to rAra h 2 occurred infrequently amongst CPE driven TCL, being demonstrated in 32% of cases. Such responses were also of lower magnitude than those demonstrated

Figure 5.8 Peanut allergic donor Ara h 2 pulsed TCL cytokine responses to Ara h 2 peptides, Ara h 2 and crude peanut extract

Peanut specific TCL (5 x 10^4 cells/ml) from individuals with peanut allergy generated using CPE and pulsed with rAra h 2, were stimulated with Ara h 2 peptides at a concentration of 10 μg/ml in the presence of autologous irradiated PBMC as APC (5 x 10^4 cells/ml) in 3 day cultures. Supernatants were collected after 48 hours culture and assayed for cytokines by specific ELISA. Data are displayed graphically as IL-5 concentration (pg/ml). Peptides associated with a significant proliferative response are shaded grey.


Subject 6
IL-5 pg/ml
200
160
120
80
40
0
1-20
11-29
19-38
28-47
37-56
46-65
55-74
64-83
73-92
82-101
91-110
100-119
109-128
118-137
127-146
136-155
138-157
rAra h 2
CPE
IL-2
Cells alonne
peptide
Subject 10
IL-5 pg/ml
1800
1600
1400
1200
1000
800
600
400
200
0
1-20
11-29
19-38
28-47
37-56
46-65
55-74
64-83
73-92
82-101
91-110
100-119
109-128
118-137
127-146
136-155
138-157
rAra h 2
CPE
IL-2
Cells alonne
peptide

towards CPE, the mean maximal stimulation index to CPE being 17.7 while that towards rAra h 2 was 3.0. This difference may reflect the difference in concentration used for each extract, although the difference in concentration is similar to the fractional concentration of Ara h 2 within CPE, Ara h 2 being approximately 6-9% of total peanut protein content (Koppelman *et al.* 2001). It is thus more likely that the difference in response to the two extracts reflects the presence of more than one T cell reactive protein within CPE, producing expansion of multiple peanut reactive T cell lineages.

Structural features of the rAra h 2 molecule may have affected the T cell response. Clearly, the recombinant molecule represents only one isoform of Ara h 2. Others have previously demonstrated that differences in responses to isoallergens may be profound; Muller *et al.* demonstrated for the major Timothy grass allergen Phl p 1 that, of 82 T cell clones reactive to its natural form, only 22/73 (30%) tested proliferated to recombinant Phl p 5a and 42/80 (53%) proliferated to recombinant Phl p 5b with just 13 T cell clones recognising both isoforms (Muller *et al.* 1998). An additional structural feature of the rAra h 2 used in these experiments was its lack of carbohydrate side chains, being produced in a prokaryotic system. Carbohydrate has previously been shown to exert significant effects on the T_H2 immune response by several authors (Dudler *et al.* 1995; Okano *et al.* 2001). Okano has shown in a number of models exploring T_H2 responses - including those directed towards Japanese cedar major allergen Cry j 1, as well as towards *Schistosoma mansoni* - that the presence of carbohydrate is crucial for the generation of a T_H2 response (Okano *et al.* 1999; Okano *et al.* 2001). Finally, the T cell response may have been altered by the presence of endotoxin, within the rAra h 2 extract, as demonstrated in chapter 3. It is possible that the presence of endotoxin lead to diminished T cell proliferation; Wolk *et al.* have demonstrated that monocyte antigen presenting ability is diminished by endotoxin, leading to diminished T cell proliferative responses to recall antigens (Wolk *et al.* 2000).

Within these experiments, 9 of the 22 peanut allergic subjects tested demonstrated a peptide response. Examination of different methods of TCL generation, showed that TCL generated purely with CPE produced peptide responses in 8 of 22 subjects, and lines receiving a rAra h 2 pulse produced responses in 3 of 21 subjects. The observation that more peptide responsive rAra h 2 pulsed TCL could not be generated is likely to reflect those confounding features of the rAra h 2 extract that lead CPE generated TCL to show such limited responsiveness to that extract. The majority of TCL generated in this fashion displayed no peptide response, or proliferated non-specifically without antigen stimulation, presumably due to enhanced background proliferation. Each phenomenon has been described within the spectrum of effects that endotoxin has been reported to have on T cell activity (Tough *et al.* 1997; Wolk *et al.* 2000). The ability of endotoxin to produce a diminished T cell response has been described above. Tough *et al.* have shown that, although endotoxin does not appear to

produce expansion of CD4 T cell populations. CD8 T cell and B cell populations proliferate at even low concentrations of endotoxin (Tough *et al.* 1997). Hence, pulsing with the endotoxin rich rAra h 2 extract may have lead to expansion of these subsets within the TCL, particularly as this occurred when cell lines were at a more immature stage and hence likely to contain populations of both B cells and CD8 cells. Whether this proliferation would be carried forward beyond 7 days and after washing the cells is unclear. Finally, within this set of experiments, where TCL received a rAra h 2 pulse and demonstrated a peptide response, this was widespread, suggesting that the endotoxin rich rAra h 2 extract lead to a general augmentation of T cell responses. Such a finding is consistent with findings reported by Goodier *et al.*, who showed that PBMC when stimulated with tetanus toxoid peptides and cocultured with endotoxin, demonstrated enhanced proliferative responses (Goodier *et al.* 2001).

Despite Ara h 2 being recognised by serum IgE in 91% of peanut allergic subjects used in this study, CPE driven TCL did not exhibit frequent Ara h 2 peptide responses. This was despite significant quantities of Ara h 2 being clearly demonstrable upon resolution of CPE on a polyacrylamide gel. The basis to this has been discussed above in relation to responses to rAra h 2: stimulation with CPE leads to responses to multiple allergens, with some of these proliferative responses potentially as vigorous as the response to Ara h 2 so that T cells specific to this allergen remain present in relatively small numbers. Additionally, just as rAra h 2 represents only one isoform of the allergen, so too are the peptide sequences based upon that isoform.

Examination of both methods of TCL production reveals that 15 of the 17 peptides were associated with a stimulation index of greater than or equal to 2.5, with the only peptides not associated with proliferative responses being Ara h 2 (64-83) and Ara h 2 (109-128). Although a large number of peptides were associated with a response when cells received a pulse with rAra h 2, CPE driven TCL showed significant proliferation ($SI \geq 2.5$) to just 8 of the 17 peptides. Examination of the pattern of responses suggests that these responses were clustered within two regions of the Ara h 2 molecule, located between Ara h 2 (19-38) and Ara h 2 (28-47), with a second region of significant proliferation between Ara h 2 (73-92) and Ara h 2 (100-119). These regions produced stimulation in 5 and 4 of the 8 subjects respectively, with only one subject demonstrating a $SI \geq 2.5$ at both sites. Interestingly, only subjects with responses in the region containing Ara h 2 (19-38) to Ara h 2 (28-47) had responses to rAra h 2. Because the responses associated with the region between Ara h 2 (73-92) and Ara h 2 (100-119) involved more than three adjacent peptides, it is likely that these regions contain more than one T cell epitope. In addition, subjects responded to either Ara h 2 (19-38) or Ara h 2 (28-47), suggesting the presence of greater than one T cell epitope in

that region. Further delineation of the number of T cell epitopes and their sequence requires stimulation with truncated peptide series.

Within this study, an arbitrary stimulation index of 2.5 was pre-defined as being significant, this figure having previously been used in other studies of T cell epitopes carried out within our laboratory (de Silva *et al.* 2000; Burton *et al.* 2002; Eusebius *et al.* 2002). However, because peptide proliferative responses were generally associated with low stimulation indices, it is possible that a stimulation index threshold value below 2.5 should be used as that representing a significant response. Using a stimulation index >2.0 provides interesting and supportive data for the impression that two regions of the Ara h 2 molecule contain T cell epitopes. The total number of CPE driven TCL with a peptide response in this setting is 13. The number of subjects that react to both regions increases from 1 to 2, while the number of subjects responding to either region increases to 9. Thus, the regional pattern of reactivity is retained. Two other regions also become apparent with the lower threshold for stimulation: 4 subjects showed an SI>2.0 at Ara h 2 (46-65) to (55-74) and 4 subjects showed such reactions at Ara h 2 (127-146) to Ara h 2 (136-155).

Examination of those peptides associated with stimulation indices of the greatest magnitude also provides support for there being two regions within Ara h 2 that contain the major T cell epitopes. Using a stimulation index of > 5.0 as a threshold for significance, reveals that only three peptides produced responses of this magnitude. Ara h 2 (19-38) produced the largest response, with an SI of 26.8 in subject 21, as well as 5.2 in subject 13. Two other peptides, Ara h 2 (73-92) and Ara h 2 (91-110) produced an SI > 5.0 in one subject each. No peptides outside of the two regions described produced a stimulation index of this magnitude.

A further method of corroboration of these data is to examine the cytokine response associated with each peptide. Peptides that contain T cell epitopes relevant to the allergic response would be expected to cause secretion of cytokines associated with a T_H2 response, such as IL-5. Data with regards cytokine secretion was available for 7 subjects, and revealed that the IL-5:IFN-γ ratio was significantly skewed towards IL-5 production for peptides associated with proliferative responses in CPE driven lines from 4 of those subjects. The largest responses again occurred for Ara h 2 (19-38), both on the basis of absolute concentration of IL-5 and the IL-5/IFN-γ ratio, with one subject recording an IL-5 concentration of 2054 pg/ml and an IL-5:IFN-γ ratio of 20. Interestingly, those subjects with proliferative responses to this peptide also produced large quantities of IL-5 with non-specific stimulation with IL-2, suggesting that, after 3 stimulations with CPE, the dominant cell type was of a T_H2 phenotype in these subjects. Stimulation with peptides not associated with a proliferative response in general did not cause cytokine secretion, although did to a lesser degree in two subjects. Thus, these data in general provide further support for those

peptides producing proliferative responses being those associated with the allergic response. Examination of the intracellular cytokines by flow cytometry would be desirable to confirm the T_H2 phenotype of these cell lines, although TCL produced in this manner for hazelnut (chapter 6) have been demonstrated to be of this phenotype.

Within this set of experiments, sustainable peanut specific TCL could not be generated from non-peanut allergic subjects using the same protocol as that used for peanut allergic subjects. Disparate proliferation between PBMC and oligoclonal TCL is an unexpected finding on the basis of PBMC proliferative data, whereby stimulation with peanut extract lead to identical magnitudes of proliferation amongst both peanut allergic and non-allergic subjects (chapter 4), suggesting precursor frequency is similar in both groups. Such a phenomenon has also been found within our laboratory for HDM specific TCL and donor PBMC from HDM allergic individuals (Leanne Gardner, personal communication). The protocol used has previously been shown to favour the production of cells of a T_H2 phenotype, while on the basis of cytokine data, it appeared that the predominant cell type amongst proliferating non-peanut allergic T cells was of a T_H1 phenotype. It may be that as non-peanut allergic donor cells are predominantly of a T_H1 phenotype, these cells are simply unable to survive this method of TCL generation. One method that reportedly enhances non-atopic T cell responses is the use of serum free medium (Upham *et al.* 1995). However, in our laboratory, this observation has not been reproducible.

The data presented on the basis of these experiments would clearly be stronger were corroborative data available within the available literature. To date, there is only one reference available with regards to the mapping of Ara h 2 T cell epitopes (Burks *et al.* 1998). In that review article, no references are cited for the basis to their T cell epitope data. Nevertheless, the amino acid sequences cited correspond reasonably neatly with those regions found to produce responses within our experiments. Four T cell immunodominant regions were suggested: within amino acid residues 19-28 (corresponding to Ara h 2 (19-38) in these experiments), 45-53 (Ara h 2 (46-65)), 96-114 (Ara h 2 (91-110), and 131-139 (Ara h 2 (127-146)). Two of the four regions, amino acid residues 19-28 and 96-114, correspond with regions producing maximal responses in the cohort in the present study, while the other two correspond with regions that are only significant if one uses a threshold stimulation index of 2.0.

The major problem with the data from the experiments described in this chapter is the lack of responses for the entire peanut allergic cohort. This occurred despite the investigation of several methods for TCL and the use of a range of peptide concentrations. It is possible that greater sensitivity could be achieved with the use of alternative methods of assessing T cell stimulation, such as ELISPOT, flow cytometry for markers of T cell activation or via

bioinformatic approaches (Anthony *et al.* 2003; Bian *et al.* 2003; Hoffmeister *et al.* 2003). Alternatively, MHC class II tetramers could have been generated to explore T cell epitopes (Reijonen *et al.* 2003). These have been used successfully for the assaying of T cell antigens in a variety of models, but have proved difficult to generate for MHC class II molecules (Nepom *et al.* 2002; Danke *et al.* 2003; Day *et al.* 2003). Similarly cytokine assay sensitivity may have been improved by the use of flow cytometric methods using intracellular cytokine staining.

Before this data was applied to the preparation of allergen immunotherapy for the general population, the MHC haplotype of responding subjects should be determined to ensure applicability to the broad population. However, it has previously been shown that individual allergenic proteins retain their immunodominant regions despite presentation by MHC molecules of different haplotypes due to promiscuity of peptide binding to a range of MHC class II alleles. An example of such a phenomenon exists for the major cat dander allergen Fel d 1, where the dominant region is presented by cells possessing HLA DR1, DR4 or DR13 (Haselden *et al.* 1999). It is likely that, despite the relatively small numbers of peptide responses demonstrated in the current study, these findings will be applicable to the general population.

An additional precondition to the use of peptide immunotherapy is that those peptides are unable to elicit significant IgE induced effector cell reactivity, as this may lead to systemic allergic reactions (Litwin *et al.* 1988). IgE binding regions of Ara h 2 have previously been mapped in linear epitope studies to three regions, representing amino acid residues 17-39, 41-80, and 114-157 (Stanley *et al.* 1997). Immunodominant regions for serum IgE within those regions have been determined to exist at Ara h 2 (27-36), Ara h 2 (57-66), and Ara h 2 (65-74) (Stanley *et al.* 1997). The interpretation of linear epitope studies with regards to IgE binding is limited by the fact that these peptide structures represent only a fraction of the conformational epitopes that elicit IgE binding (Aalberse 2000). However, the presence of an IgE immunodominant region within Ara h 2 (19-38) - one of the T cell binding epitopes suggested within this study - mandates the need for further clarification of the T cell binding region. In the first instance, the precise T cell epitope needs to be determined, to assess the degree of overlap with the IgE immunodominant epitope, and functional assays of effector cell activation performed to assess the relevance of IgE binding.

Data presented within this chapter provides a platform for the preparation of novel allergen immunotherapy. One such approach could be via the production of a B cell epitope mutant whereby IgE conformational epitopes are destroyed. Those peptides producing responses in the current study are likely to contain those sequences that should be retained to maintain efficacy of the mutant Ara h 2 structure, treatment with immunodominant T cell epitopes

being those maximally suppressive of allergic responses on subsequent allergen challenge (Hoyne *et al.* 1997). Such an approach has been used for the major birch pollen allergen Bet v 1, and the grass pollen Phl p 5 (Ferreira *et al.* 1998; Swoboda *et al.* 2002). Alternatively, with the identification of the precise T cell epitope using truncated peptide sequences, this information could be used to generate peptide immunotherapy, as has been performed successfully for bee venom and cat dander (Oldfield *et al.* 2002). It is possible that not all T cell epitopes would be required for peptide immunotherapy, such that the use of dominant epitopes might be sufficient for efficacious immunotherapy. Hoyne *et al.* demonstrated that intra-molecular suppression of responses to T cell epitopes within the same molecule occurs when using peptides based upon the structure of the major dust mite allergen in a murine model of intra-nasal immunotherapy (Hoyne *et al.* 1999).

In conclusion, data in this study have demonstrated that up to four regions are likely to contain T cell epitopes for Ara h 2. The most significant responses occurring for the region between Ara h 2 (19-38) and Ara h 2 (28-47), and for the region between Ara h 2 (73-92) and Ara h 2 (100-119). The most likely epitope containing peptide, based upon the observed pattern of peptide responses across the cohort, the magnitude of the peptide responses, the corroborative cytokine data and corroborative data within the medical literature, is located within Ara h 2 (19-38). It is probable that other peptides contain T cell epitopes, but only Ara h 2 (19-38) provides significant data within all of these areas. Three of the 4 regions were associated with corroborative cytokine responses, and each was included in those regions described by Burks *et al.* as containing T cell epitopes. While additional supportive data are required before this research can be applied to novel allergen immunotherapy methods, these data provide a starting point for those studies.

CHAPTER 6 CROSS-REACTIVITY OF THE ALLERGIC IMMUNE RESPONSE TO PEANUTS, TREE NUTS AND SEEDS

6.1 INTRODUCTION

Peanut and tree-nut allergy are characterised by the immediate onset of anaphylaxis in sensitised patients on exposure to minute quantities of each food. Peanut allergy occurs in approximately 0.6% of the adult Western population, while tree-nut allergy occurs in approximately 0.5% (Sicherer *et al.* 1999). The extent to which each condition is clinically associated with the other so as to produce multiple nut sensitivity remains unclear. Researchers using subjects presenting to allergy clinics as their study group have reported the prevalence of sensitivity to both tree-nuts and peanuts to be between 30 and 40% (Ewan 1996; Sicherer *et al.* 1998). However, when Sicherer et al. studied a randomly sampled selection of the general population, the prevalence of such sensitivity appeared to be much lower, at only 1.6% (Sicherer *et al.* 1999).

Just as the evidence with regards the prevalence of combined peanut and tree-nut allergy remains conflicting, so too does the basis of its genesis. Several models of allergy occurring to multiple foods exist upon which current theories are based and include that seen as part of the oral-allergy syndrome, between latex and certain fruits, between fish or shellfish, and between milk of different species (Daul *et al.* 1994; Valenta *et al.* 1996; Hansen *et al.* 1997; Nel *et al.* 1998; Restani *et al.* 1999). Such models have suggested that the epidemiological association of certain food allergies or between certain foods and other allergens is due to the presence of homologous proteins within each food, shared epitopes being represented by either their primary or tertiary sequence, and recognised by either IgE or T cell receptors.

For IgE cross-reactivity to occur, protein tertiary structure needs to be similar, as IgE epitopes are conformational rather than linear protein sequences (Aalberse 2000). As such, proteins belonging to the same protein family have most frequently been described as possessing such cross-reactivity. Many examples of such proteins exist. For foods of plant origin, they include the pathogenesis related proteins including Bet v 1 (the major allergen of birch pollen) homologous proteins and lipid transfer protein, profilins, seed storage proteins, as well as several other protein species (Breiteneder *et al.* 2000). These proteins may be amongst the major allergens of those substances such as Bet v 1, or simply a ubiquitous protein such as profilin.

The alternative method through which multiple food sensitivities may be linked is via sequence homology or similarity between protein primary structures. Although similarities in

linear structure need to be extensive for IgE cross-reactivity to occur, such concerns do not exist for T cell cross-reactivity, whereby it appears that sequence homology may only exist at several key amino acid residues for a shared T cell response to occur. Examples whereby T cell cross-reactivity has been demonstrated between allergenic proteins include between both the major allergens - Der p 1 and Der f 1 - and minor allergens – Der p 7 and Der f 7 - of the dust mite species *D. pteronyssinus* and *D. farinae*, and between the major allergens of birch pollen and apple, Bet v 1 and Mal d 1 (Hales *et al.* 1997; Fritsch *et al.* 1998). Bet v 1 and Mal d 1 share approximately 65% sequence identity, but cross-reacting peptide fragments shared only 50% sequence identity in that study (Fritsch *et al.* 1998). Other studies have not demonstrated cross-reactivity with greater degrees of sequence identity. For the major allergens of latex, Hev b 1 and Hev b 3, no cross-reactivity exists despite 60% sequence similarity (Bohle *et al.* 2000). Further data have demonstrated that single amino acid substitutions, or change in allergen isoform, can lead to loss of T cell reactivity confirming the notion that shared key amino acids are likely to be particularly important for cross-reactivity to occur (Sparholt *et al.* 1997; Muller *et al.* 1998; Wurtzen *et al.* 1999).

Finally, it has been suggested that cross-reactivity may occur via non-protein structures such as carbohydrate side chains (Carbone *et al.* 1997). Cross-reactivity between peanut and grass pollen has been demonstrated to occur via such a mechanism, although the clinical relevance of this finding is likely to be minimal as, within that study, grass pollen allergic subjects with a positive RAST to peanut did not demonstrate clinical sensitivity to peanut (van der Veen *et al.* 1997). Whether this mechanism is similarly unimportant clinically for glycoproteins unique to nut species remains to be elucidated, although recently it has been highlighted that carbohydrate side chains are important in the generation of T_H2 responses, in models using *Schistosoma mansoni* and Japanese cedar pollen (Okano *et al.* 1999; Okano *et al.* 2001). This may be particularly relevant for nut allergy, where allergenic proteins are without exception glycoproteins.

The literature detailing examples of cross-reactive immune responses towards peanut and tree-nuts remains relatively sparse, and to date the relevant proteins inducing cross-reactivity have not been clearly characterised. de Leon *et al.* have used ELISA with unfractionated nut extracts to demonstrate that IgE cross-reactivity exists between peanut and the tree nuts hazelnut, almond, and Brazil nut, but not cashew (de Leon *et al.* 2003). Peanut extract possesses near total IgE cross-reactivity with walnut extract (Teuber *et al.* 1999). A larger body of literature exists demonstrating cross-reactivity between various tree nuts. Sutherland *et al.* have demonstrated partial IgE cross-reactivity between hazelnut and macadamia nut, with subtotal diminution of binding to a 17.4 kDa protein within hazelnut by preincubation with macadamia nut (Sutherland *et al.* 1999). Pistachio nut and cashew nut have demonstrated IgE cross-reactivity in RAST inhibition studies (Fernandez *et al.* 1995).

Walnut and hazelnut albumin fractions have shown cross-reactivity with almond conglutin γ (Poltronieri *et al.* 2002). Only one example of T cell cross-reactivity exists; Higgins *et al.* reported that peanut specific T cell clones demonstrate a proliferative response to both hazelnut and peanut, but a T_H2 functional phenotype was shown only to those nuts to which the donor subject was sensitised (Higgins *et al.* 1995).

In the set of experiments presented in this Chapter, the nature of both B and T cell cross-reactive responses between peanut and tree nuts and seeds are explored. The nature of the T cell cross-reactive immune response between peanut and hazelnut is investigated and the role of the major peanut allergens Ara h 1 and Ara h 2 in that response is determined, using oligoclonal hazelnut specific T cell lines. To further characterise IgE cross-reactive responses, a novel cross-reactive immune response between citrus seeds and peanut is demonstrated. To determine whether these responses can be ascribed to a single protein, donor serum from a subject demonstrating clinical sensitivity to each food is used for inhibition immunoblotting of orange seed extract.

6.2 T CELL CROSS-REACTIVITY IN THE ALLERGIC RESPONSE TO PEANUT AND HAZELNUT

6.2.1 Extract Characterisation

To examine the molecular mass profile of proteins contained within peanut and hazelnut extracts used within these experiments, each extract was resolved on 14% SDS-polyacrylamide gels, as illustrated in Figure 6.1. The molecular masses of proteins contained within the hazelnut extract ranged from 8 kDa to 220 kDa, (higher molecular mass proteins were only faintly visible and are below the resolution of the accompanying figure). The most abundant protein had a molecular mass of 8 kDa. Other significant bands were present at 11 kDa, 14 kDa, 15 kDa, 17 kDa, 29 kDa, and 43 kDa. Any of the five protein bands between 8 and 15 kDa could represent the pan-allergens profilin and lipid transfer proteins, which have molecular masses of between 12-15 kDa and 9 kDa respectively. Six protein bands carried molecular masses of similar order to proteins present within crude peanut extract, including several likely to represent pan-allergens.

To ensure that extracts used in these experiments were not mitogenic or toxic towards PBMC used to establish T cell lines, mitogenicity and toxicity assays were carried out, the results of which are illustrated in Figure 6.2. Across a range of concentrations representative of those typically used for T cell line generation and stimulation, no significant mitogenic or

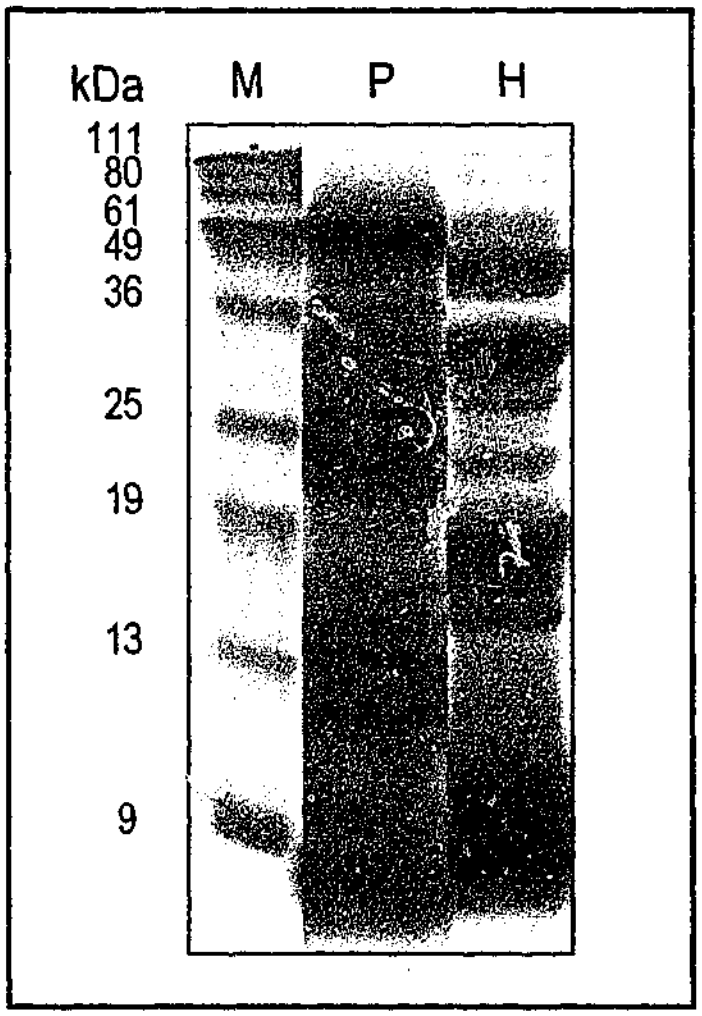


Figure 6.1: SDS-PAGE profile of peanut and hazelnut extract.
kDa: relative molecular mass; M: molecular mass markers; P: crude peanut extract; H: crude hazelnut extract. Crude nut extracts are resolved by 14% SDS-PAGE and total proteins visualised with CBB staining.

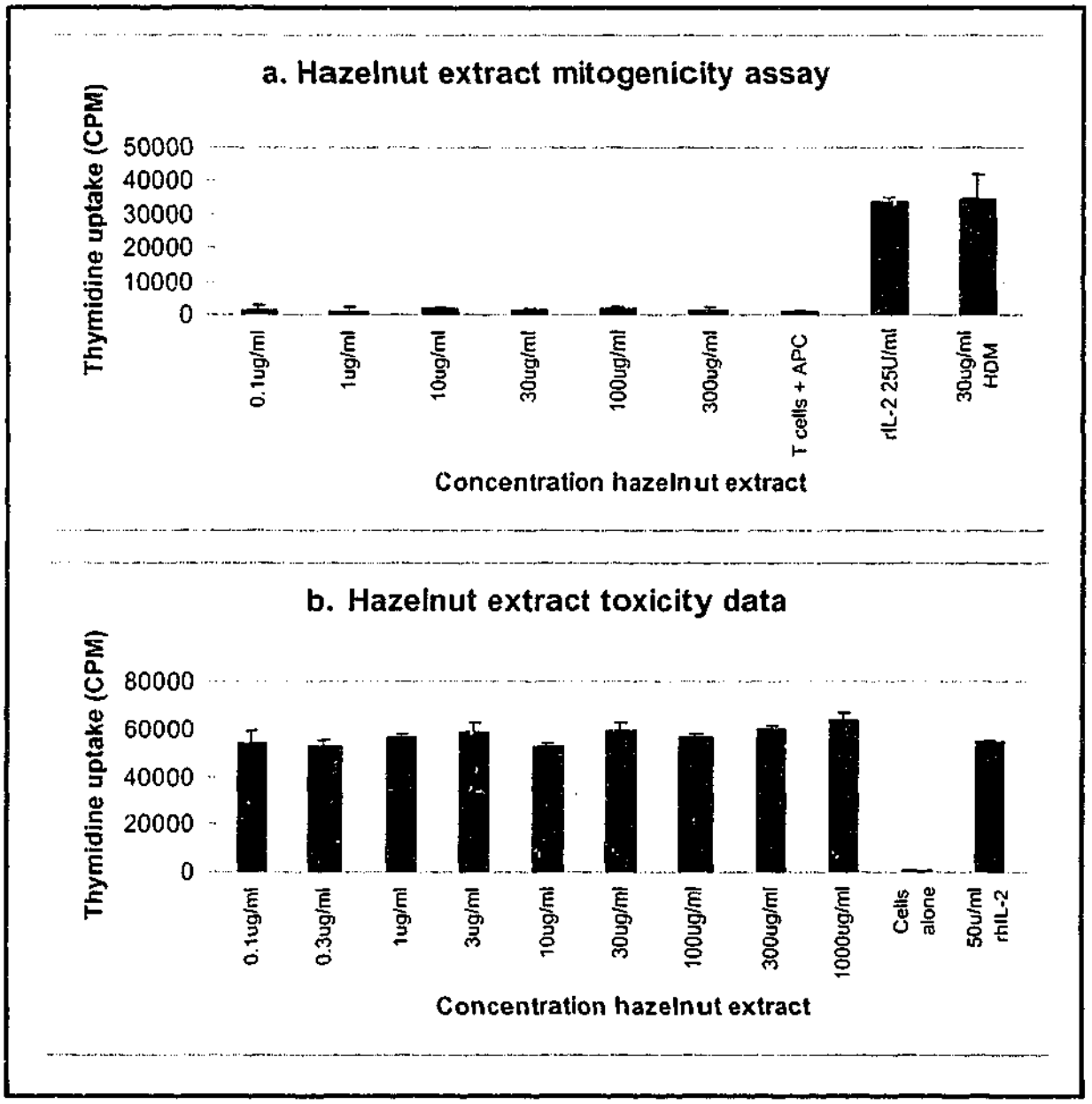


Figure 6.2: Hazelnut extract T cell mitogenicity and toxicity assays.

a. **mitogenicity assay:** Triplicate cultures of 5×10^4 cells/well of a 3 week HDM specific TCL (kind gift of Ms Leanne Gardner) plus APC, stimulated with a range of concentrations of hazelnut extract for 72 hours.

b. **toxicity assay:** Triplicate cultures of 5×10^4 cells/well of a 3 week HDM specific TCL plus APC, stimulated with a range of concentrations of hazelnut extract in the presence of 25U/ml of rIL-2 for 72 hours.

Proliferation was assessed by tritiated thymidine (^{3}H-TdR) incorporation over the last 18 hours of culture. ^{3}H-thymidine incorporation for each antigen concentration and control was estimated and expressed as mean cpm + SEM.

toxic potential was demonstrated. Similar assays for the characterisation of peanut extract have already been described in chapter 3.

6.2.2 Subject Characterisation

The clinical characteristics of 5 atopic nut allergic subjects who donated blood for this set of experiments are summarised in Table 6.1. Three of the five subjects used were female, and the mean age of the group was 32 (range 24 to 50). Only one of the subjects did not suffer from other forms of atopic disease. While the entire group described reactions to both peanut and hazelnut, three of the five also described reactions to other nuts including almond, walnut and cashew. Each subject described features of anaphylaxis on exposure to peanut and hazelnut Reactions had begun in childhood, although later in life than might be generally expected, reflecting the fact that subjects were drawn from an adult allergy clinic. Each had suffered reactions in the previous 3 years, reflecting the difficulty in avoiding nut exposure over a lifetime. Despite life threatening anaphylaxis with nut exposure, serum IgE quantitated via RAST scores did not predict the severity of such reactions, with RAST ranging in score from grade 1 to grade 5. All subjects demonstrated skin prick test reactivity to house dust mite, allowing the use of this antigen as an irrelevant control within T cell assays.

6.2.3 Western Blotting Data

To examine for associations between multiple nut sensitivity and the presence of specific IgE reactivity towards proteins contained within hazelnut and peanut extracts, western blotting of crude hazelnut (Figure 6.3) and peanut extracts (Figure 6.4 and 6.5) was performed. Upon Western blotting of crude hazelnut extract, using sera from hazelnut allergic subjects as well as five non-nut allergic controls (2 atopic, 3 non-atopic), numerous protein bands were associated with hazelnut allergy, ranging in molecular mass from 10 kDa to 80 kDa. Interestingly, in this small population, no single band was consistently associated with hazelnut allergy, although a faintly reactive band at 41 kDa was recognised by IgE from 4 out of the 5 subjects. Proteins of 61 kDa, 22 kDa, and 15 kDa were the next most frequently associated with hazelnut allergy being present in 3 of the 5 subjects. Other proteins binding IgE in 2 of the 5 subjects were 45 kDa, 27 kDa, and 9 kDa in molecular mass. Notably, while IgE to the 15 kDa band was detectable in 3 out of 5 subjects, this band was also recognised by 3 non-atopic subjects, suggesting a degree of non-specific IgE binding.

Western blotting of crude peanut extract to examine for the presence of specific IgE towards Ara h 1 and Ara h 2 was carried out using different protocols to address the tendency of Ara h 1 to cause non-specific IgE binding in both nut allergic subjects and control subjects (see

Table 6.1 Summary of subject characteristics used for hazelnut-peanut cross-reactivity experiments.

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Sex	F	M	F	F	M
Age	50	30	24	27	33
Atopic disease	nil	asthma	asthma, rhinitis, eczema	asthma, rhinitis, eczema	asthma, rhinitis
Family history	nil	nil	nil	nil	nil
Known nut sensitivities	peanut, hazelnut	peanut, hazelnut	peanut, hazelnut, almond, walnut, cashew	peanut, hazelnut, walnut	peanut, hazelnut, almond, walnut
Other food sensitivities	nil	nil	fish and crustacea, childhood egg allergy	sesame seeds, navy beans	nil
Clinical features	laryngeal oedema, generalised urticaria, facial angioedema	laryngeal oedema, facial angioedema, asthma, GIT upset	laryngeal oedema, facial angioedema, asthma	laryngeal oedema, generalised urticaria, facial angioedema	laryngeal oedema, facial angioedema, asthma
Age at first reaction (yrs)	18	12	1	10	3
Time since last reaction (mths)	21	18	24	2	36
Peanut RAST and score	6.87 (3)	0.51 (1)	63.5 (5)	2.07 (2)	13.9 (3)
Hazelnut RAST and score	0.4 (1)	0.56 (1)	2.31 (2)	17.5 (4)	13.52 (3)
Skin prick test reactivity	GP, HDM, C	HDM	GP, HDM, C, D, M	GP, HDM, C, D. M	HDM

GP, grass pollen; HDM, house dust mite; C, cat; D, dog; M, mould.

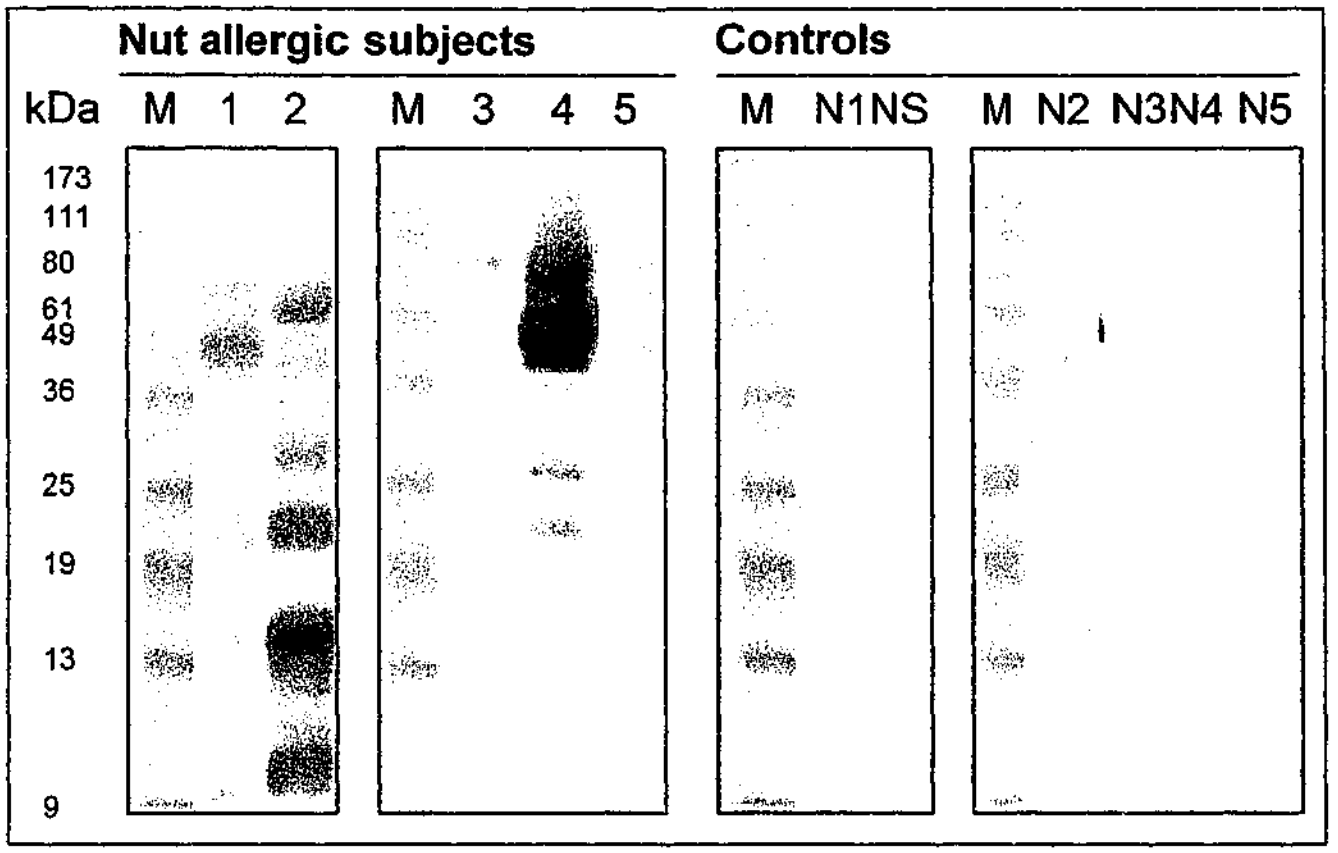


Figure 6.3: Western blot of hazelnut extract by serum IgE
kDa, relative molecular mass; M, molecular mass markers; NS, no serum, N1-N5, non nut allergic controls. Following 14% SDS-PAGE separation of crude hazelnut extract under reducing conditions, proteins were transferred to nitrocellulose and probed with allergic and non-allergic serum, along with a no serum control.

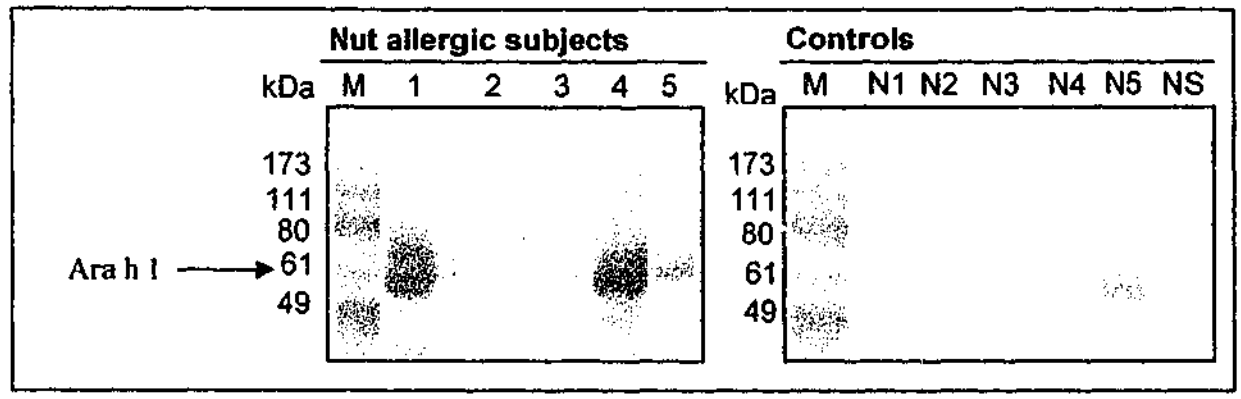


Figure 6.4 Identification of sera demonstrating IgE specific to Ara h 1 by immunoblotting of crude peanut extract.
kDa, relative molecular mass; M, molecular mass markers; NS, no serum. Following 14% SDS-PAGE separation of crude peanut extract, proteins were transferred to nitrocellulose and probed with sera from the study population, non-allergic controls and a no serum control.

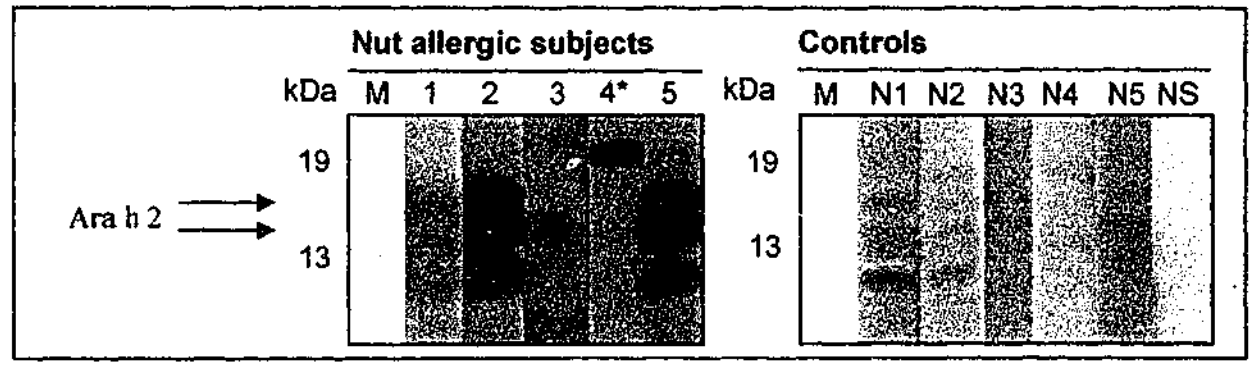


Figure 6.5 Identification of sera demonstrating IgE specific to Ara h 2 by immunoblotting of crude peanut extract.
kDa, relative molecular mass; M, molecular mass markers; NS, no serum. Following 14% SDS-PAGE separation of crude peanut extract, proteins were transferred to nitrocellulose and probed with sera from the study population, non-allergic controls and a no serum control. *Subject 4 was performed with serum diluted 1:20 due to excessive signal at 1:5 dilution.

Chapter 4). These results are illustrated in Figures 6.4 and 6.5. Ara h 2 was recognised by the serum of each of the 5 nut allergic subjects, although subject 1 appeared to recognise only its lower molecular mass isoform, and subject 5 demonstrated only weakly reactive IgE. Ara h 1 was recognised by IgE from 4 out of 5 of the subjects.

6.2.4 Polyclonal Proliferative Responses to Peanut, Hazelnut and House Dust Mite Extracts

To ensure that the five subjects included within the study possessed T cells reactive towards peanut, hazelnut and house dust mite, PBMC proliferative responses to the three antigens were assessed in 7 day assays. Mitogenicity of each extract had previously been excluded. As is demonstrated in Figure 6.6, PBMC from all 5 subjects demonstrated a dose-dependent proliferative response to crude peanut extract, crude hazelnut extract, and to house dust mite extract. Maximum proliferative responses ranged from 4.5×10^4 cpm to 1.4×10^5 cpm for hazelnut, 4.0×10^4 cpm to 1.5×10^5 cpm for peanut, and 2.9×10^4 cpm to 9.2×10^4 cpm for house dust mite. Each response was significantly higher than that demonstrated by cells cultured without antigen ($p < 0.05$). Maximal proliferative responses and responses relative to cells cultured without antigen (stimulation index, SI) are summarised in Table 6.2. No statistically significant difference existed for any group with regards to their stimulation indices ($p < 0.05$).

6.2.5 T Cell Proliferative Responses

To determine whether T cell proliferative responses to peanut and hazelnut were due to expansion of T cell populations specific to antigens common to each nut, five short-term hazelnut specific oligoclonal T cell lines were prepared and stimulated with peanut, hazelnut and house dust mite extracts. Data from these experiments are shown in Figure 6.7, and demonstrate that a dose-response to hazelnut and peanut was maintained in all of the 5 cell lines but was significantly abrogated or lost to house dust mite in all lines. Maximum proliferative responses and stimulation indices are summarised in Table 6.3. These data demonstrate that the mean maximum stimulation index for both hazelnut and peanut is > 10, and is significantly greater than those levels for house dust mite extract ($p < 0.05$). Of note, the mean maximum proliferation cpm and stimulation index for hazelnut, 6.2×10^4 and 20.9 respectively, appear to be significantly greater than those for peanut, being 3.0×10^4 and 10.1 respectively, but only reach statistical significance for total proliferation count ($p < 0.05$). This reduced response to peanut suggests that peanut contains only a portion of those antigens within hazelnut extract leading to proliferation in these hazelnut specific lines.

Figure 6.6 Proliferation of PBMC to (a) crude hazelnut extract (b) crude peanut extract and (c) crude house dust mite extract

PBMC (1×10^6/ml) were cultured with a range of concentrations of crude hazelnut extract, crude peanut extract or house dust mite extract in 7 day cultures. Proliferation was assessed by ^{3}H-TdR incorporation over the last 18 hours of culture. Results are given as mean counts per minute (cpm) plus standard error of the mean for triplicate cultures.

Figure 6.6 (a) Crude hazelnut (HZN) extract.

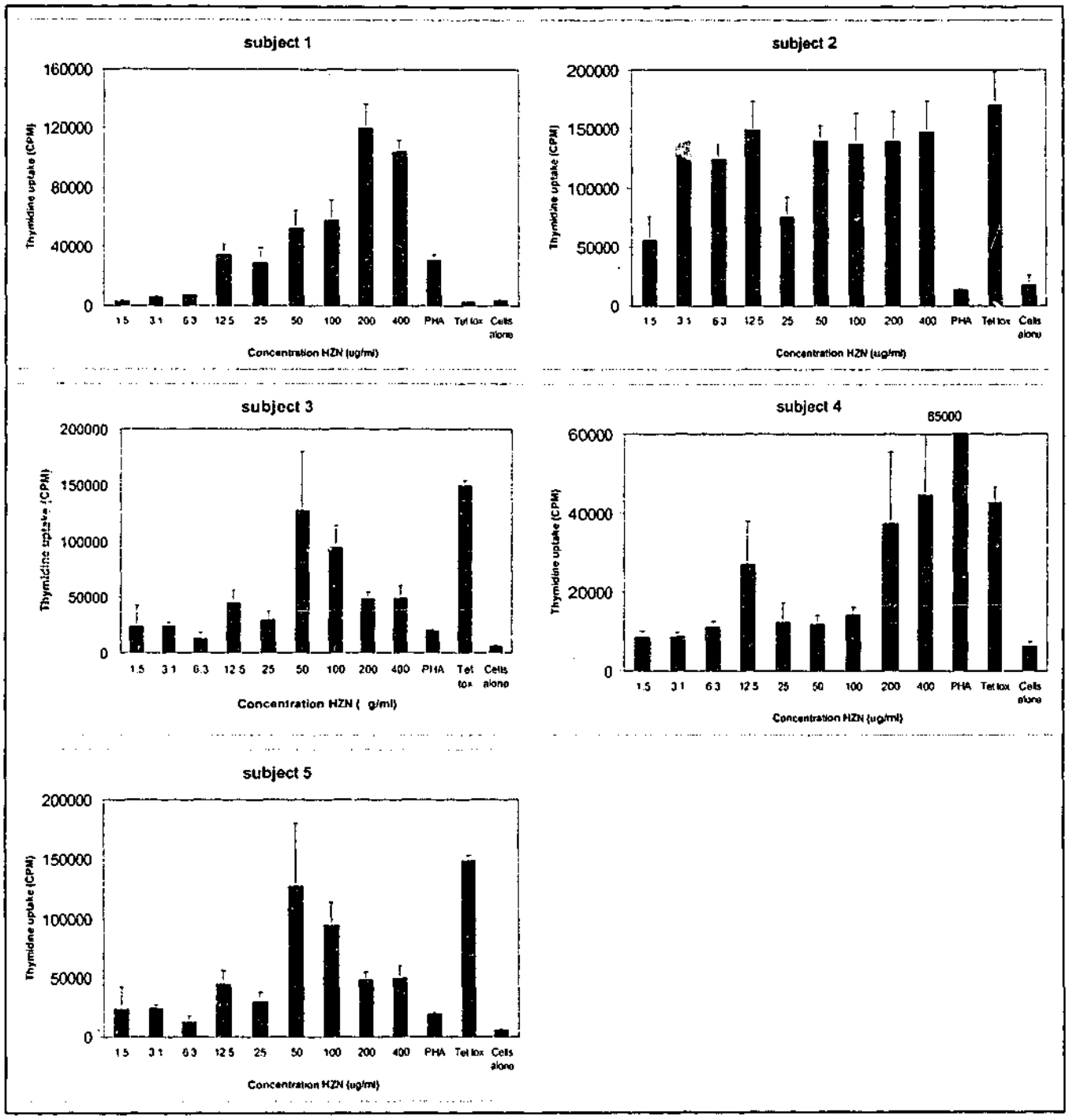


Figure 6.6 (b) Crude peanut extract (CPE).

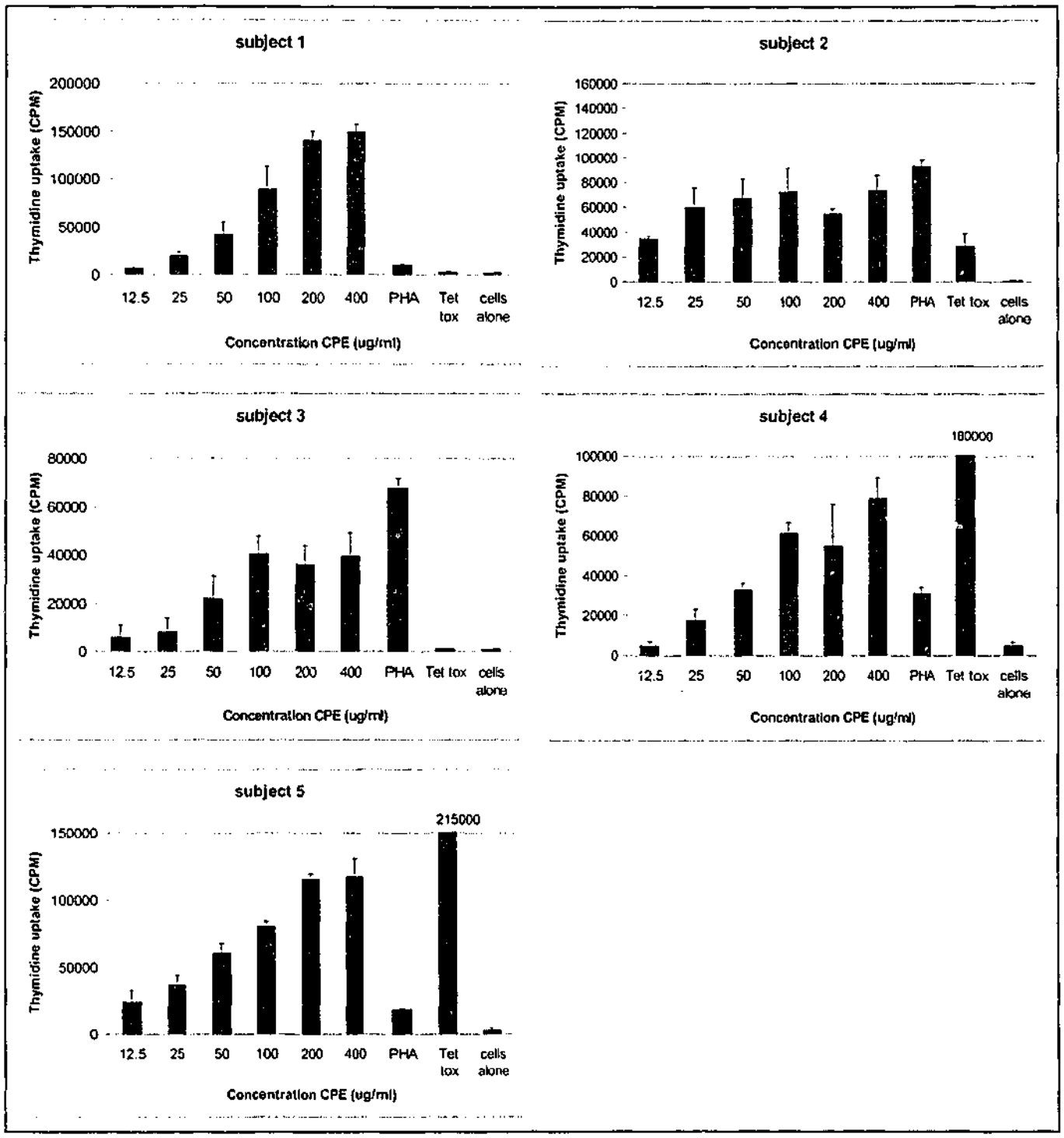


Figure 6.6 (c) House dust mite (HDM) extract.

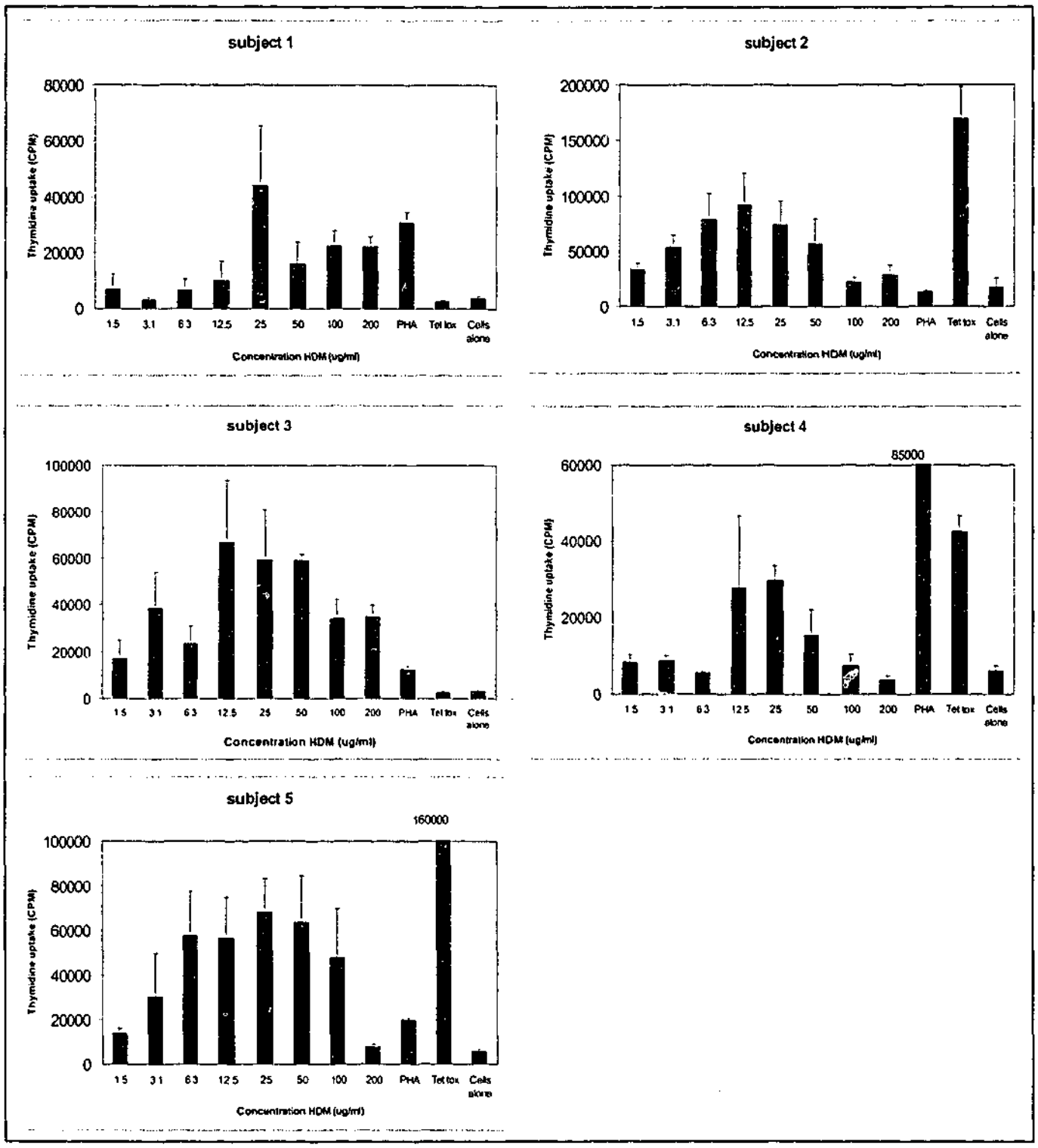


Table 6.2 Polyclonal proliferative responses to crude extracts

Subject	Maximum proliferation cpm and (stimulation index)			
	HZN	CPE	HDM	PHA
1	120241 (33.4)	149739 (75.6)	44043 (12.2)	30762 (8.5)
2	149132 (8.5)	73594 (89.6)	91851 (5.2)	170022 (9.7)
3	57440 (19.4)	40280 (45.9)	66759 (22.5)	12346 (4.2)
4	44605 (7.3)	78782 (16.5)	29763 (4.9)	88596 (14.5)
5	90637 (22.0)	117501 (35.1)	68256 (11.8)	19852 (3.4)

PBMC (1.0 x 10^6/ml) from nut allergic individuals were cultured with crude extracts over a range of concentrations in 7 day cultures. Maximum proliferative responses for each subject as assessed by ^{3}H-TdR incorporation (mean counts per minute of triplicate cultures) are shown, with the stimulation index in brackets.

Figure 6.7 Nut allergic donor hazelnut TCL proliferative responses to crude peanut, hazelnut and dust mite extract

TCL (5 x 10^5/ml) specific to crude hazelnut extract were generated from individuals with peanut and hazelnut allergy and stimulated with crude hazelnut extract, crude peanut extract and house dust mite extract over a range of concentrations in the presence of irradiated autologous PBMC as APC (5 x 10^5/ml) in 3 day cultures. Proliferative responses for each concentration were assessed by tritiated thymidine incorporation (mean counts per minute [cpm] of triplicate cultures).

Figure 6.7 Nut allergic donor TCL proliferative responses to crude peanut, hazelnut, and dust mite extract

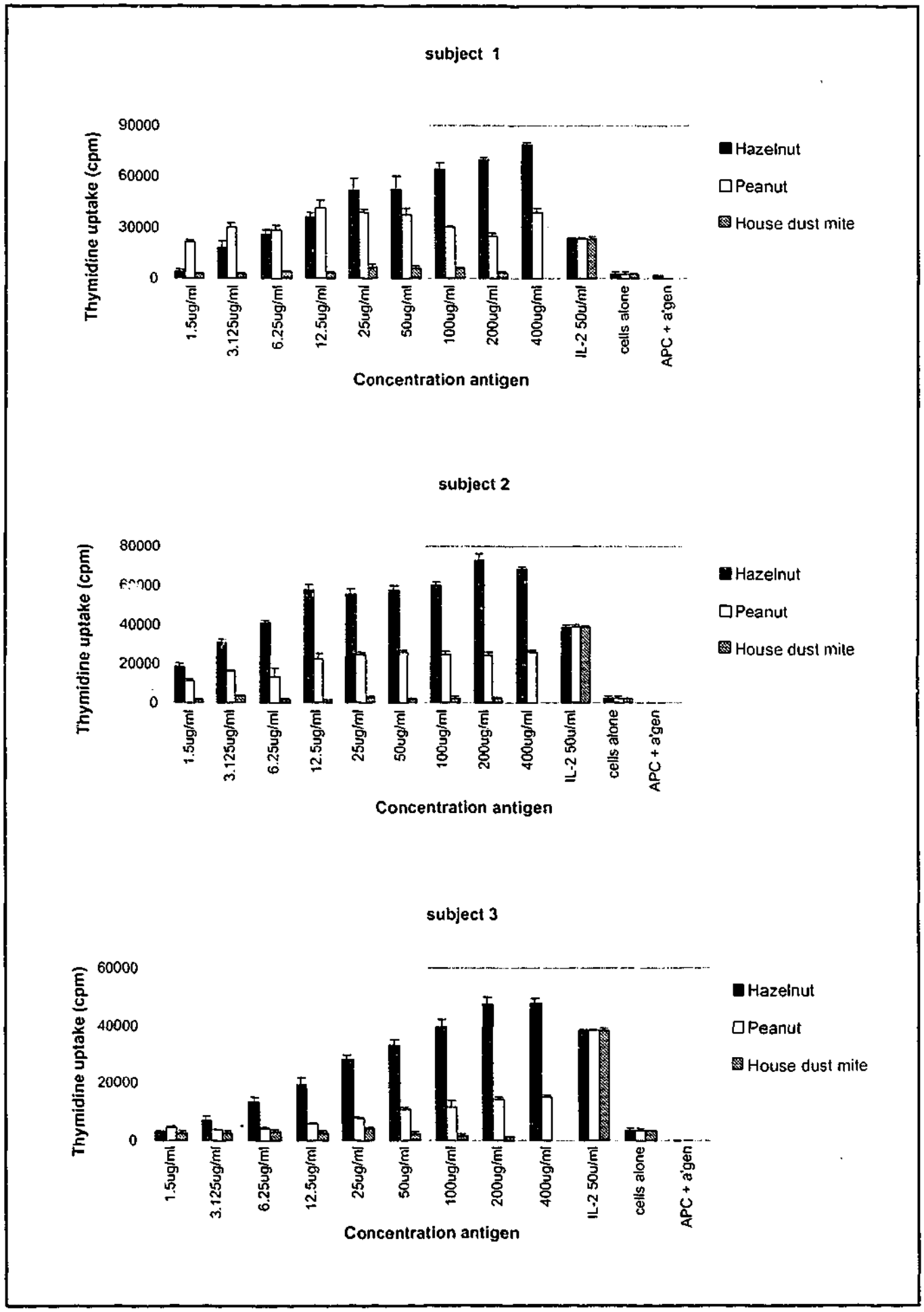


Figure 6.7 (cont.) Nut allergic donor TCL proliferative responses to crude peanut, hazelnut, and dust mite extract

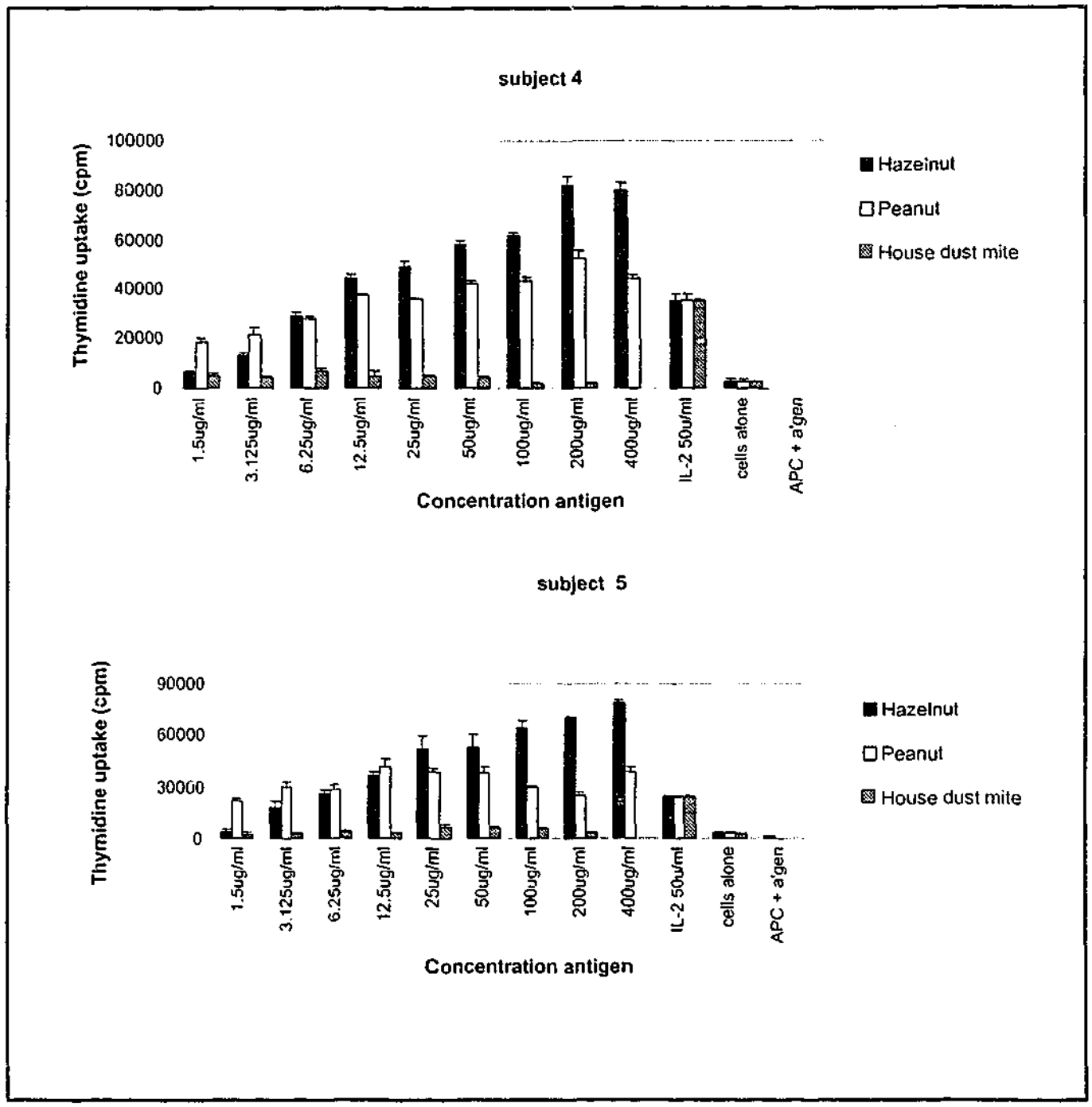


Table 6.3 Nut allergic donor hazelnut TCL proliferative responses to crude peanut, hazelnut and dust mite extracts.

	Maximum proliferation cpm and (stimulation index)			
Subject	HZN	CPE	HDM	IL-2
1	78871 (27.2)	41471 (14.3)	6475 (2.2)	23459 (8.1)
2	72704 (30.2)	25801 (10.7)	2658 (1.1)	38452 (16.0)
3	47951 (13.5)	14931 (4.2)	4104 (1.2)	38156 (10.7)
4	81768 (29.2)	52384 (18.7)	7012 (2.5)	35464 (12.7)
5	29554 (4.4)	17440 (2.6)	14024 (2.1)	55299 (8.2)
mean [SEM]	62170[22583] (20.9[11.4])	30405[16080], (10.1[6.8])	6855[4380] (1.8[0.6])	38166[11371] (11.1[3.3])

TCL (5 x 10^5/ml) specific to crude hazelnut extract were generated from individuals with peanut and hazelnut allergy and stimulated with crude hazelnut extract, crude peanut extract and house dust mite extract over a range of concentrations in the presence of irradiated autologous PBMC as APC (5 x 10^5/ml) in 3 day cultures. Proliferative responses for each concentration were assessed by ^{3}H-TdR incorporation (mean counts per minute of triplicate cultures).

To examine whether cross-reactive T cell responses were attributable to reactivity to the major peanut allergens Ara h 1 and Ara h 2, hazelnut specific oligoclonal T cell line proliferative responses were measured after 3 days culture with each allergen. These data are shown in Figure 6.8. Clear-cut dose responses occurred using purified Ara h 2 in 3 of the 5 subjects. In a fourth subject, despite demonstrating proliferation to crude hazelnut and peanut extracts, a dose response to either Ara h 1 or Ara h 2 could not be demonstrated. In the fifth subject, whose PBMC response to peanut was less clear, no response to either Ara h 1 or Ara h 2 could be found. Maximal stimulation indices are demonstrated in Table 6.4. These show that responses to Ara h 2 were greater than for Ara h 1 ($p < 0.05$). Two subjects reached a stimulation index of > 2.5 for Ara h 1, but only one demonstrated a clear dose response. In contrast, the 3 subjects with dose responses to Ara h 2 reached stimulation indices > 5. All dose responses to Ara h 2 were lower than that demonstrated to peanut extract except at equivalent concentrations of Ara h 2 and peanut in 1 subject.

6.2.6 Phenotype of Cross-Reactive T Cells

To examine the surface marker phenotype of cells contained within oligoclonal hazelnut specific T cell lines, flow cytometry was performed and analysis made of cells within the lymphocyte subset. Phenotypic data from 1 subject, representing the typical response of all subjects within the study group, are displayed in Table 6.5 and demonstrate that the predominant cell type contained within these lines was $CD4^+$, while $CD8^+$, $CD14^+$ and $CD19^+$ cells were present in either low numbers or absent. This suggests the predominant proliferating cell type was the $CD4^+$ T lymphocyte.

To determine the cytokine profiles of these cells, IL-5 and IFN-γ levels were measured by ELISA in the supernatants of hazelnut specific oligoclonal T cell lines, recovered after 48 hours of coculture with hazelnut, peanut, Ara h 1 and Ara h 2 extracts. Results of these assays are summarised in Table 6.6. Low to moderate levels of IFN-γ were detected in all cell lines in response to all antigens tested, but importantly, IL-5 was clearly present in association with T cell proliferative responses to both hazelnut and peanut extract. Proliferative responses to Ara h 2 were associated with IL-5 production in all 3 subjects demonstrating a T cell proliferative response, and varied significantly in magnitude (7-102 pg/ml). Of note, only 1 subject showing a proliferative response to Ara h 1 demonstrated IL-5 production, and this was in minimal amounts (9 pg/ml). One other subject produced low levels of IL-5 on Ara h 1 stimulation, in the absence of a significant proliferative response. Interestingly, IL-2-stimulation induced IL-5 production in all cell lines. These data suggest that T_H2-type responses were associated with T cell proliferative responses to the cross-reactive allergens.

Figure 6.8 Nut allergic donor hazelnut TCL proliferative responses to Ara h 1 and Ara h 2

TCL (5 x 10^5/ml) specific to crude hazelnut extract were generated from individuals with peanut and hazelnut allergy and stimulated with purified Ara h 1 and Ara h 2 extract over a range of concentrations in the presence of irradiated autologous PBMC as APC (5 x 10^5/ml) in 3 day cultures. Proliferative responses for each concentration were assessed by ^{3}H-TdR incorporation (mean counts per minute [cpm] of triplicate cultures).

Figure 6.8 Nut allergic donor hazelnut TCL proliferative responses to Ara h 1 and Ara h 2

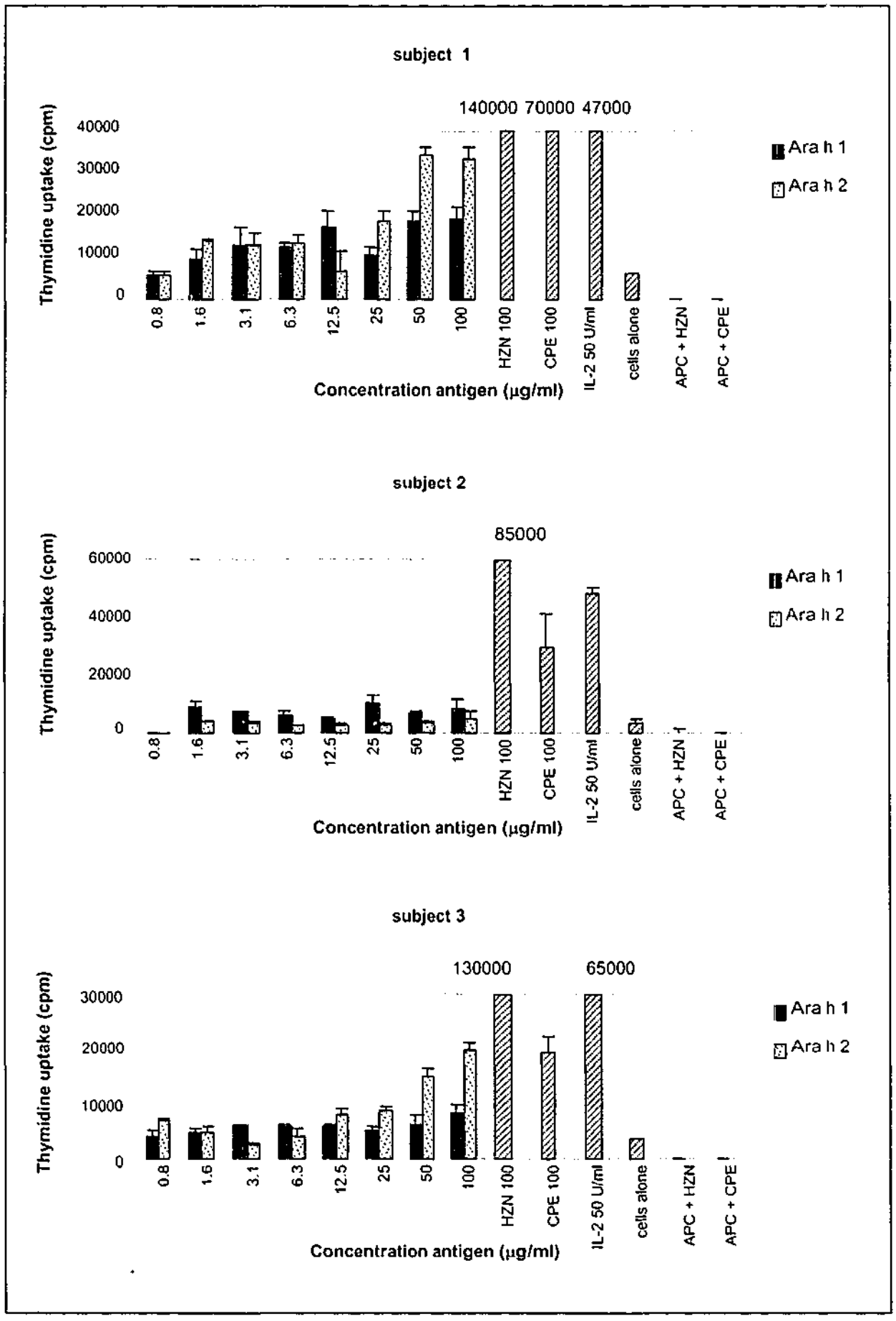


Figure 6.8 (cont.) Nut allergic donor hazelnut TCL proliferative responses to Ara h 1 and Ara h 2

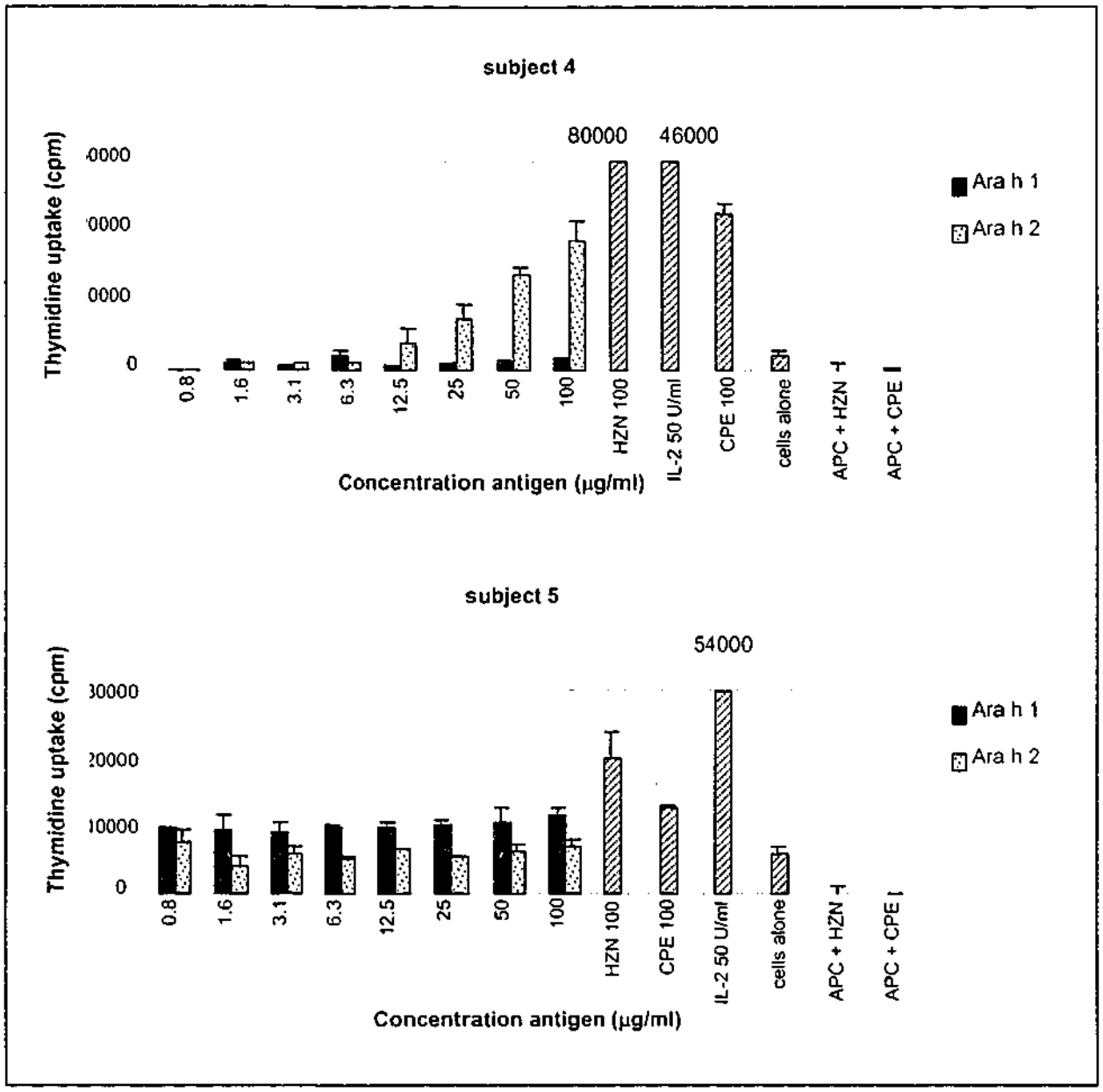


Table 6.4 Nut allergic donor hazelnut TCL proliferative responses to purified Ara h 1 and Ara h 2

TCL (5 x 10^5/ml) specific to crude hazelnut extract were generated from individuals with peanut and hazelnut allergy and stimulated with purified Ara h 1 and Ara h 2 over a range of concentrations in the presence of irradiated autologous PBMC as APC (5 x 10^5/ml) in 3 day cultures. Proliferative responses for each concentration were assessed by ^{3}H-TdR incorporation (mean counts per minute of triplicate cultures).

	Maximum proliferation cpm and (stimulation index)				Ratio to hazelnut response		
Subject	Ara h 1	Ara h 2	HZN	CPE	Ara h 1	Ara h 2	CPE
1	18880 (3.1)	34245 (5.7)	136399 (22.6)	69645 (11.6)	0.14	0.25	0.51
2	10175 (2.9)	5078 (1.5)	81051 (23.4)	29529 (8.5)	0.13	0.06	0.36
3	8415 (2.4)	19645 (5.5)	131811 (37.0)	19264 (5.4)	0.06	0.15	0.15
4	2085 (1.0)	18862 (9.4)	82203 (40.8)	46176 (22.9)	0.03	0.23	0.56
5	11492 (2.0)	7615 (1.3)	19951 (3.4)	12647 (2.2)	0.58	0.38	0.63
mean [+/-SEM]	10209[6042] (2.3[0.8])	17089[11598] (4.7[3.4])	90283[47298] (25.4[14.7])	35452[22920] (10.1[8.0])			

Table 6.5 Typical cell surface molecule expression of a hazelnut specific TCL

Marker	subject 4
CD4	77%
CD8	10%
CD14	<1%
CD19	<1%

A three week hazelnut-specific TCL was generated by culture with crude hazelnut extract, then stained for the presence of each of the cell surface molecules listed. Cells analysed were those within the lymphocyte gate on flow cytometry.

Table 6.6 Summary of nut allergic donor hazelnut specific TCL cytokine responses to stimulation with nut extracts.

	HZN			CPE			HDM		
subject	IL5	IFNγ	ratio	IL5	IFNγ	ratio	IL5	IFNγ	ratio
1	938	1770	0.53	60	553	0.11	6	154	0.04
2	1957	1291	1.52	305	288	1.06	14	65	0.21
3	1152	379	3.04	65	118	0.55	10	5	2.07
4	2555	1191	2.15	1168	833	1.40	8	18	0.44
5	79	360	0.22	70	271	0.26	0	174	0

	Ara h 1			Ara h 2		
subject	IL5	IFNγ	ratio	IL5	IFNγ	ratio
1	0	109	0.00	7	213	0.03
2	9	72	0.13	3	20	0.14
3	7	62	0.11	40	138	0.29
4	0	2	0.00	102	87	1.18
5	0	131	0.00	0	9	0

	IL-2			cells alone		
subject	IL5	IFNγ	ratio	IL5	IFNγ	ratio
1	12	107	0.11	6	40	0.15
2	157	157	1.00	0	2	0
3	273	62	4.39	8	11	0.75
4	313	49	6.38	6	11	0.56
5	141	106	1.33	0	34	0

TCL (5 x 10^5/ml) specific to crude hazelnut extract were generated from individuals with peanut and hazelnut allergy and stimulated with crude nut extracts, house dust mite and purified Ara h 1 and Ara h 2 extract over a range of concentrations in the presence autologous irradiated PBMC as APC (5 x 10^5/ml) in 3 day cultures. Supernatants were collected after 48 hours culture and assayed by specific ELISA for concentration of IFN-γ and IL-5. IL-2 and cells alone cultures were used as positive and negative controls.

6.3 ANALYSIS OF B CELL RESPONSES IN A SUBJECT ALLERGIC TO PEANUT AND CITRUS SEEDS

6.3.1 Extract Characterisation

To examine the qualities of *Citrus sinensis cultivar Valencia* (orange), *Citrus reticulata cultivar Kara* (mandarin), and *Citrus limon cultivar Lisbon* (lemon) seed extracts prepared for use in these experiments, each extract was resolved by 14% SDS-PAGE. This result is illustrated in Figure 6.9. Each seed extract displayed a similar profile, highlighting their phylogenetic similarities. Protein bands were visible between 9 and 61 kDa for each extract. Strong bands were visible at 27 kDa, 15 kDa, 14 kDa and 9 kDa, representing the most abundant proteins in these extracts. Bands present between 9 and 15 kDa were present in all three extracts, and again may represent the pan-allergens profilin and lipid transfer protein, these proteins having similar molecular masses. The major protein components of citrus fruit seeds are the globulin seed storage proteins known as citrins (Koltunow *et al.* 1996). These proteins have been shown to form 33 kDa and 22 kDa subunits on SDS-PAGE under reducing conditions and are well visualised in all 3 citrus extracts along with a 57 kDa band, likely to represent the intact protein. Albumin seed storage proteins are also described with a prominent band at 41 and 42 kDa on SDS-PAGE – this fraction is only faintly visible in our extract, probably due to differences in protein extraction technique (Koltunow *et al.* 1996). In comparison to peanut extract, multiple protein bands in the citrus extract gels could be seen at similar molecular masses, but whether these represent similar protein structures is unclear in the absence of further characterisation.

Because peanut extract is used as an inhibitor in these experiments, non-specific inhibition of IgE binding was excluded in western blotting experiments using an unrelated antigen, illustrated in Figure 6.10. These results demonstrate that over a range of concentrations representative of those used in subsequent inhibition experiments, peanut was unable to prevent the binding of serum IgE to the recombinant latex allergen Hev b 5.

6.3.2 Subject Characterisation

Important characteristics of the study subject are detailed in Table 6.7. The patient described typical features of anaphylaxis, occurring within seconds of exposure to a variety of nuts as well as citrus seeds. Reactions to foods were present since childhood at which time they had also included cow's milk allergy, which resolved subsequently. RAST to peanut, orange and lemon (Pharmacia CAP, Sweden) confirmed the presence of specific IgE to each food

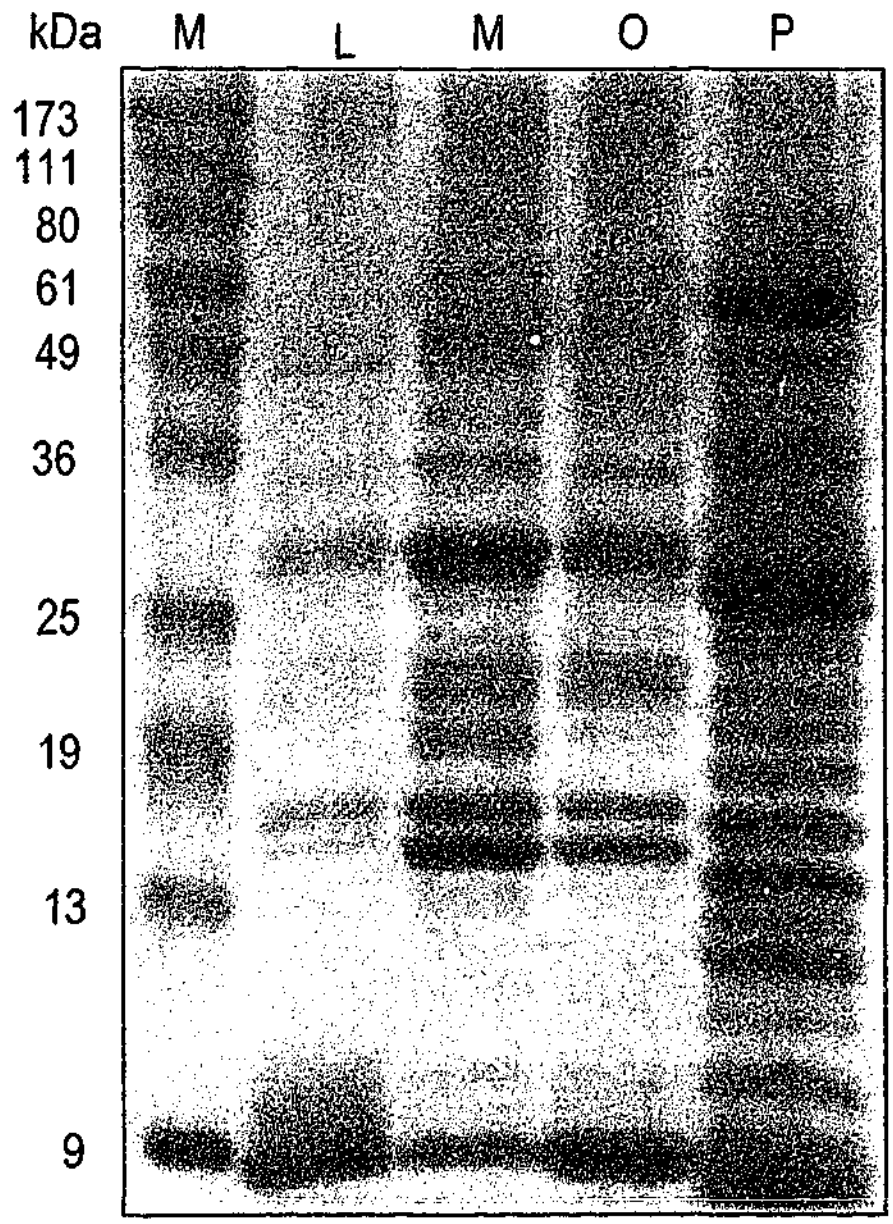


Figure 6.9 Analysis of crude citrus seed extracts and crude peanut extract
M, molecular mass markers; L, crude lemon extract; M, crude mandarin extract; O, crude orange extract; P, crude peanut extract.
Crude orange, lemon and mandarin extracts resolved under reducing conditions by 14% SDS-PAGE and stained with CBB.

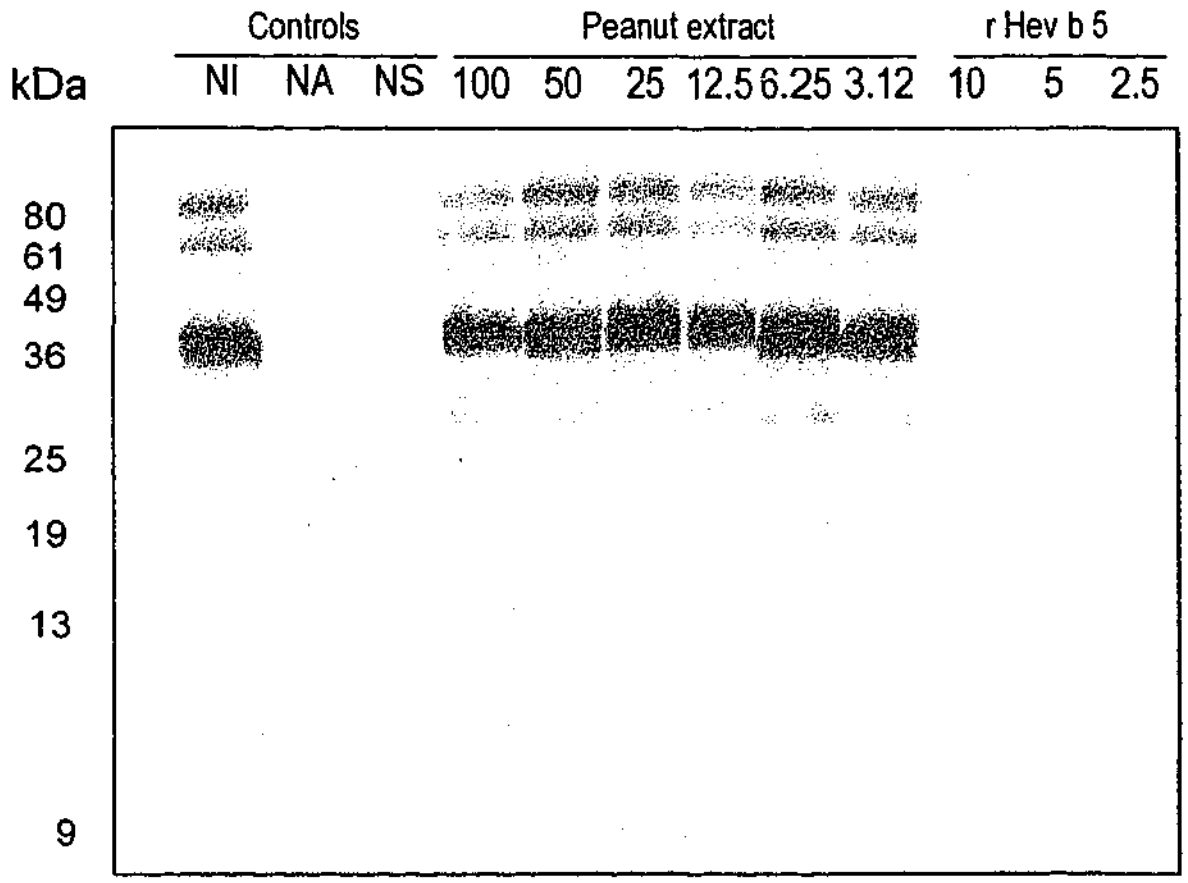


Figure 6.10 Inhibition immunoblotting of recombinant Hev b 5 using crude peanut extract as an inhibitor

M, **molecular mass** markers; NI, no inhibitor; NS, no serum; NA, non allergic atopic serum. All concentrations are measured in μg/ml. Recombinant Hev b 5 was resolved by 14% SDS-PAGE under reducing conditions then transferred to nitrocellulose and probed with latex allergic donor serum. Serum was preincubated with a range of concentrations of crude peanut extract (3-100 μg/ml) and recombinant Hev b 5 (2.5-10 μg/ml) as a positive control.

Table 6.7 Clinical characteristics of subject used for peanut-citrus seed cross-reactivity experiments.

Characteristic	Study Subject
Age	26
Sex	M
Allergic disease	asthma, allergic rhinitis
Family history	nil
Time to onset of reaction	< 1 minute
Clinical features of reaction	asthma, laryngeal oedema, generalised urticaria
Known nut allergens	peanut, Brazil nut, almond, hazelnut, macadamia, pine nuts
Other known allergens	citrus seeds, childhood cow's milk allergy
Age at first reaction	childhood
Time since last reaction	4 months
Peanut RAST class	3
Orange RAST (kU/ml)	4.0
Orange RAST class	3
Lemon RAST (kU/ml)	1.1
Lemon RAST class	2
Skin prick testing results	non-atopic

allergen; RAST to mandarin was unavailable. Surprisingly, skin prick testing did not demonstrate associated atopy towards common aeroallergens.

6.3.3 Optimisation of Western Blotting

To ensure adequate visualisation of binding of specific IgE to allergens contained within each extract, western blotting experiments were carried out over a range of serum dilutions and secondary antibody concentrations as illustrated in Figure 6.11. Serum was diluted 1:10 and the secondary antibody concentration was 1:2000 for control strips within these experiments. Serum concentrations of 1:5 and 1:10 were equivalent in demonstrating IgE binding to individual protein bands. Secondary antibody concentrations below 1:1000 did not clearly detect IgE binding to all bands even with prolonged development times; as such a secondary antibody concentration of 1:500 was used.

6.3.4 Western Blotting of Citrus Seed and Peanut Extracts

To examine the profile of proteins recognised by IgE in orange and peanut extract, western blotting was performed. Results from these experiments are shown in Figure 6.12. Within orange extract, IgE was demonstrated to bind proteins with molecular masses ranging from 48 kDa to 9 kDa. A total of 10 bands were recognised, with the most intense at 48 kDa, 43 kDa, 27 kDa, 22 kDa and 9 kDa. No IgE binding was demonstrated by the non-allergic atopic control serum (data not shown). Within peanut extract, IgE was shown to bind protein bands representing the major allergens Ara h 1, Ara h 2 and Ara h 3, along with several other bands of unknown identity.

6.3.5 Inhibition Immunoblotting

To examine whether IgE specific to allergenic proteins in orange seed extract was cross-reactive with allergenic proteins in other citrus seed extracts as well as peanut, inhibition immunoblotting experiments were carried out. Results from these experiments are illustrated in Figure 6.13. The control protein, keyhole limpet haemocyanin, did not produce inhibition at any concentration. Preincubation of serum with orange extract as well as the other citrus extracts, mandarin and lemon, lead to near total abrogation of IgE binding to all allergenic proteins within orange extract. Non-specific binding of the secondary antibody took place towards a 9 kDa protein, but clearly less signal is emitted than for strips incubated with serum alone. Preincubation with peanut extract lead to loss of signal from all protein bands except those at 27 kDa and 9 kDa. This suggests complete IgE cross-reactivity occurs for proteins contained within orange, lemon and mandarin extracts, but only partial IgE cross-

Figure 6.11 Optimisation of Western blotting technique for citrus seed extracts
Crude orange extract was resolved by 14% SDS-PAGE under reducing conditions, transferred to nitrocellulose, then probed with donor serum at a dilution of either (a) 1:5 or (b) 1:10. After washing in PBS-Tween 0.05%, each strip was incubated in rabbit anti-human IgE–HRP at a range of concentrations, then developed using enhanced chemiluminescence.

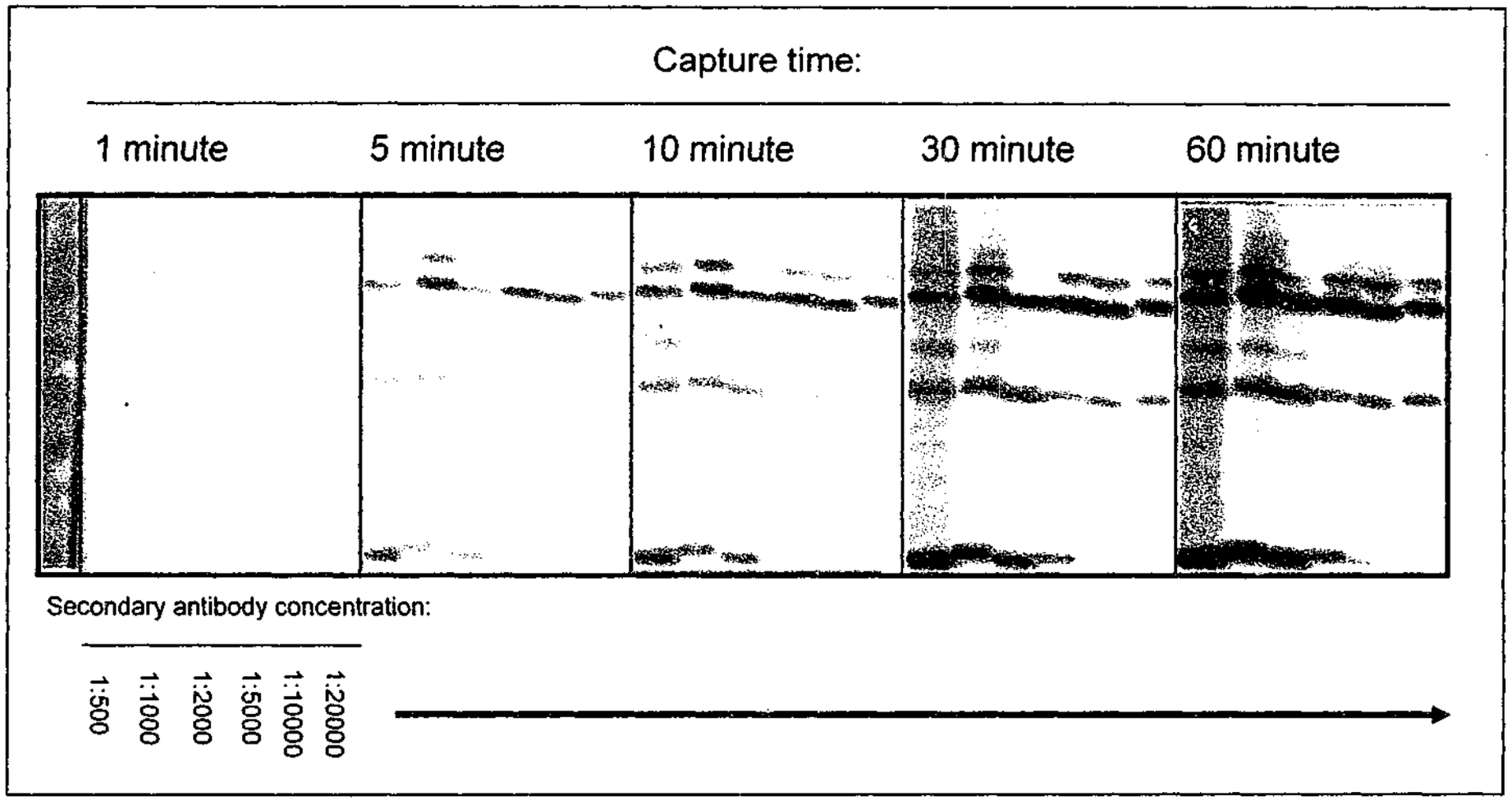

Capture time:
1 minute
5 minute
10 minute
30 minute
60 minute
Secondary antibody concentration:
1:500
1:1000
1:2000
1:5000
1:10000
1:20000

Figure 6.11 (a)

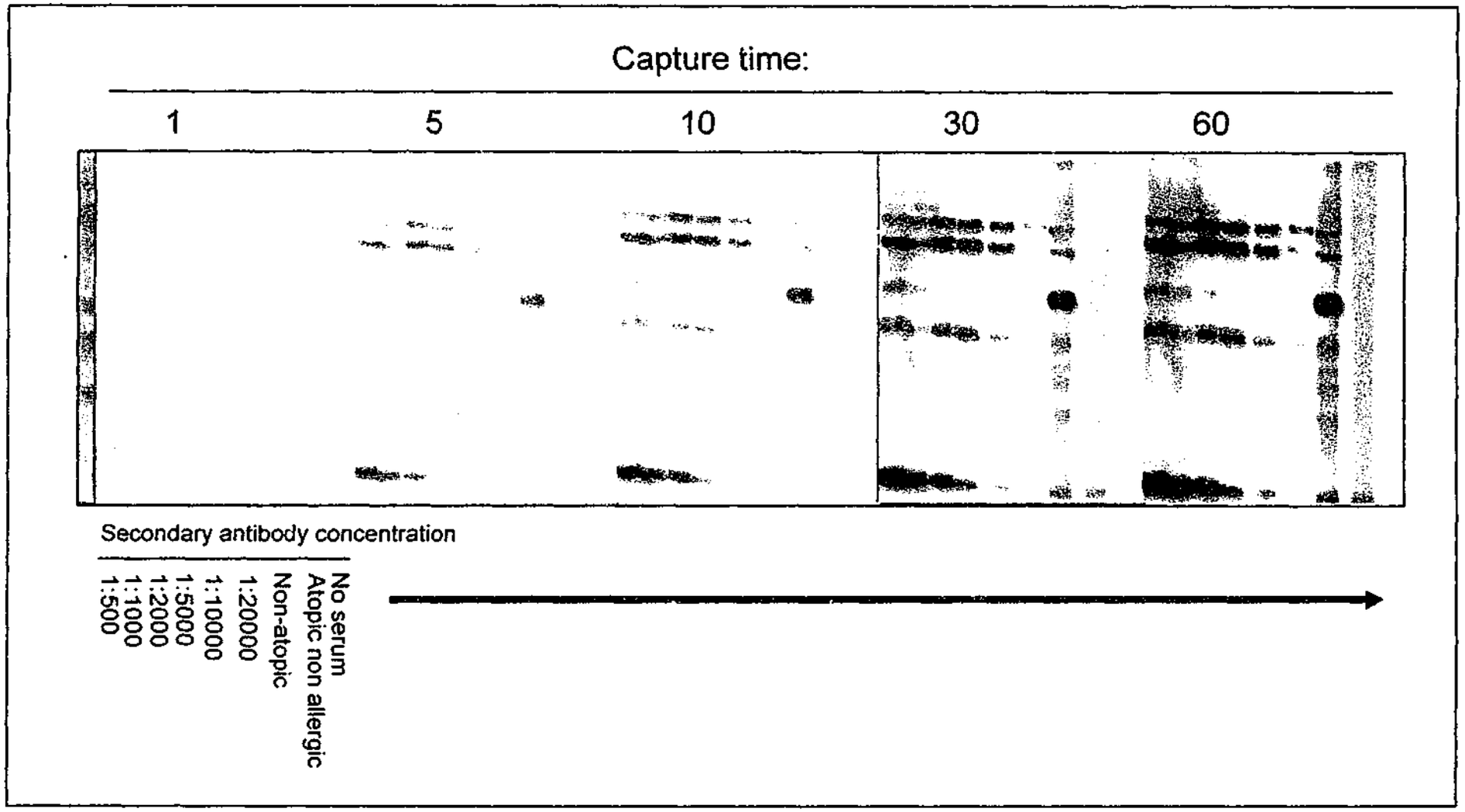
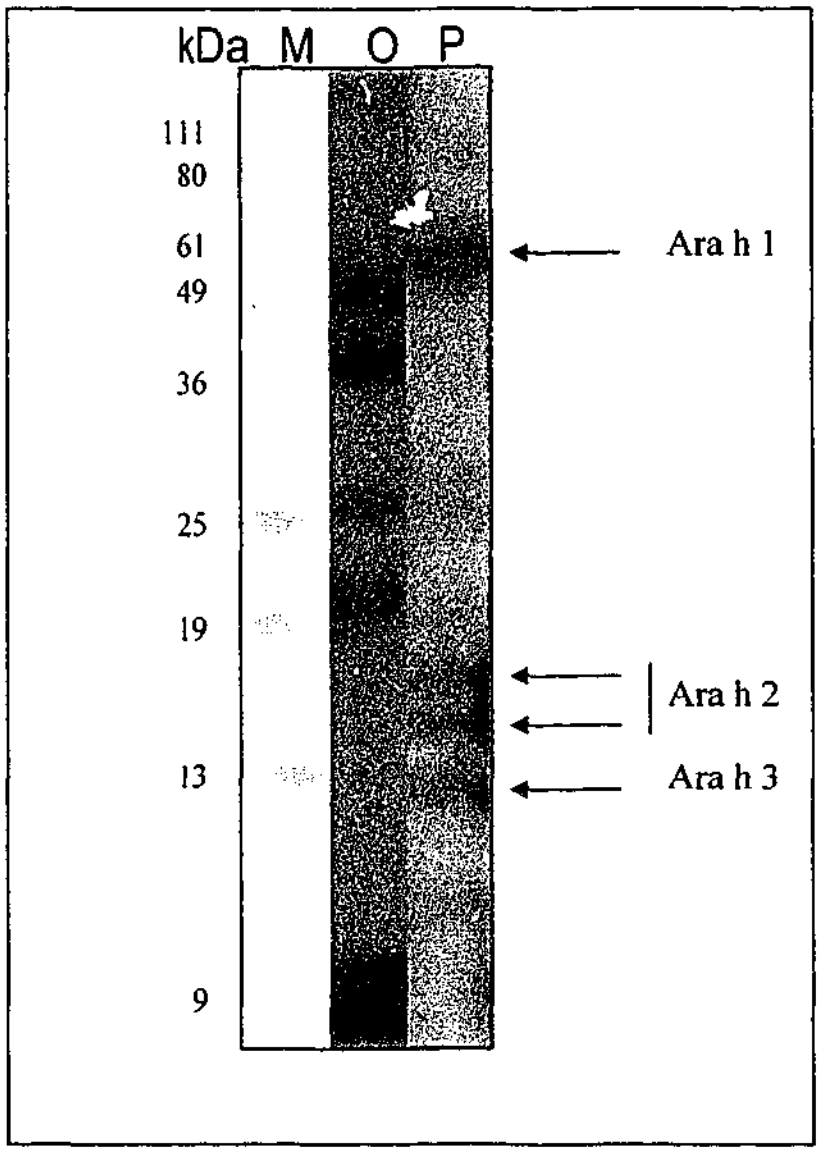

Capture time:
1
5
10
30
60
Secondary antibody concentration
No serum
Atopic non allergic
Non-atopic
1:20000
1:10000
1:5000
1:2000
1:1000
1:500

Figure 6.11 (b)

Figure 6.12 Western blotting of peanut and orange extract.



M, molecular mass markers; P, crude peanut extract, O, orange extract. Crude peanut extract and crude orange extract were resolved by 14% SDS-PAGE under reducing conditions then transferred to nitrocellulose and probed with donor allergic serum at a 1:5 dilution.

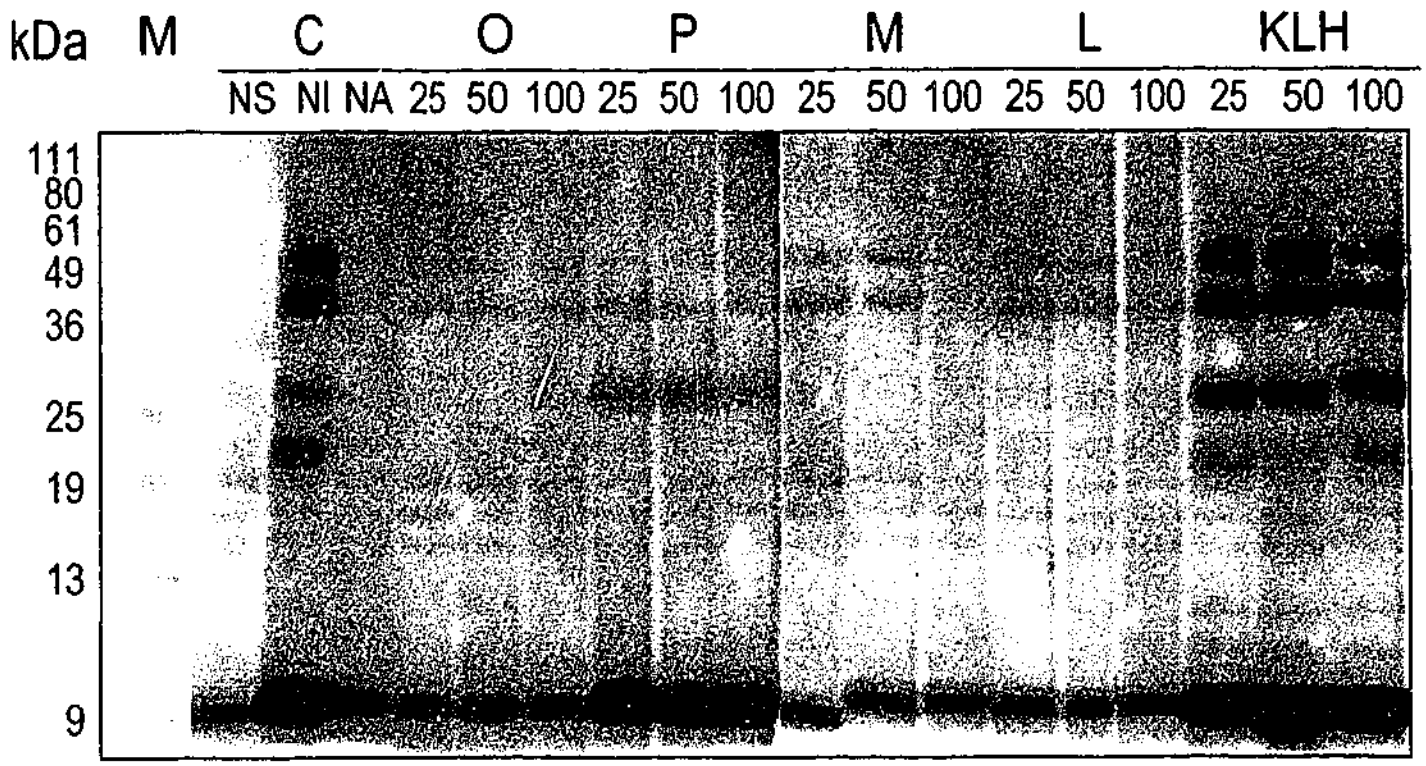


Figure 6.13 Inhibition immunoblotting of orange extract

M, molecular mass markers; C, control strips; NS, no serum; NI, no inhibitor; NA, non-allergic atopic serum; inhibitors at different concentrations (μg/ml): O, crude orange extract; P, crude peanut extact; M, crude mandarin extract; L, crude lemon extract; KLH, keyhole limpet haemocyanin.

Crude orange extract was resolved by 14% SDS-PAGE under reducing conditions then transferred to nitrocellulose and probed with donor allergic serum. Serum was preincubated with either lemon or mandarin extract, peanut extract, or orange and keyhole limpet haemocyanin as positive and negative controls, respectively.

reactivity occurs between orange seed and peanut.

6.3.6 Sequencing

To examine the identity of the non-cross-reactive peanut protein with a molecular mass of 27 kDa, N-terminal sequencing was performed. The first five residues were analysed and revealed a sequence containing AP/QREQ. Homologous protein sequences were sought using the National Centre for Biotechnology Information BLAST program. Restricting the search to seed or citrus proteins, no sequence of similar structure was identified. This may reflect difficulty in the sequencing method as only very limited signal was detected despite abundant protein available on the nitrocellulose strip.

6.4 DISCUSSION

The experiments described above, in providing novel information about hazelnut and peanut cross-reactivity and citrus seed and peanut cross-reactivity, illustrate the different mechanisms through which cross-reactivity may occur within the allergic immune response. Sensitivity to multiple foods is frequently associated with IgE cross-reactivity to numerous proteins within each food type, and this is demonstrated within the case report describing citrus seed and peanut IgE cross-reactivity. T cell cross-reactive responses may also occur within subjects sensitive to multiple foods, and this is demonstrated by the shared T cell responses between hazelnut, peanut, and its major allergen Ara h 2 *in* subjects sensitive to peanut and hazelnut.

Each experiment described above provides separate information about the nature of IgE reactivity within the cross-reactive immune response. Within the case report describing citrus seed and peanut cross-reactivity, it is clear that IgE cross-reactivity lies at the level of many of the proteins contained within the orange seed extract. Of the twelve protein bands demonstrating IgE binding on western blotting, all demonstrated cross-reactivity for the other citrus seed extracts and only two – those bands at 27 kDa and 9 kDa - did not demonstrate abrogated binding with preincubation of serum with peanut extract. This suggests near total IgE cross-reactivity between proteins contained within the citrus extract and proteins contained within the peanut extract. Previous studies exploring cross-reactivity between hazelnuts and a variety of foods or pollens using inhibition immunoblotting have also demonstrated this characteristic. Sutherland *et al.* demonstrated that preincubation with hazelnut nut extract produced near total loss of serum IgE binding to macadamia proteins resolved onto nitrocellulose (Sutherland *et al.* 1999). Hirschwehr *et al.* detected less IgE binding proteins in hazelnut extract than were detected in our group and showed binding to

those proteins were significantly abrogated by hazel pollen proteins (Hirschwehr *et al.* 1992).

The data presented above suggest that cross-reactive immune responses may be due to shared allergy to a number of proteins within each food or aeroallergen. While numerous common proteins have been described as being potential pan-allergens, it is somewhat surprising that so many proteins may contribute to cross-reactivity. Indeed, studies performed examining legume cross-reactive responses have clearly demonstrated that the majority of such cross-reactive proteins do not appear to have any clinical impact, as despite cross-reactivity being clearly demonstrated for IgE responses to legume proteins, allergic reactions to other legumes by peanut allergic subjects occur in only 5% of cases (Bernhisel-Broadbent *et al.* 1989). The target of this irrelevant IgE binding may be carbohydrate side chains of the many glycoproteins contained within nuts and seeds. van der Veen *et al.* showed that grass pollen allergic subjects lacking any history of food allergy and negative skin tests to peanut had positive RAST for peanut, which became negative on pre-incubation of sera with pure carbohydrate cleaved from grass pollen (van der Veen *et al.* 1997).

Shared sensitivity to citrus seed and peanut is an unusual clinical phenomenon. For this subject, this could not be predicted on the basis of serum IgE reactivity to peanut proteins on western blotting, the intensity of binding and number of bands recognised being similar to other peanut reactive sera (data not shown). This suggests that it is the citrus seed reactive antibodies that have lead to this cross-reactive immune response. Alternatively, at a micro-molecular level, this subject may recognise unusual peanut IgE epitopes that are cross-reactive with citrus seed allergens, but are not shared by the majority of nut allergic subjects.

This research provides valuable information about the pattern of IgE binding to citrus seed proteins. Within the orange seed extract used in these experiments, 12 separate protein bands with molecular masses between 9 and 61 kDa were visible, 4 of which ran as doublets, suggesting they may be isoforms of the same protein. Of the 10 IgE reactive bands visible on western blotting, binding of maximal intensity was towards 5 proteins with molecular masses of 48 kDa, 43 kDa, 27 kDa, 22 kDa and 9 kDa. Binding of IgE did not correlate with the relative abundance of each protein within the extract as the 48 kDa and 43 kDa proteins were only poorly visualised with Coomassie brilliant blue staining. A single paper from China has speculated, on the basis of skin testing with the individual components of orange, that its seeds contain the allergenic structures, without exploring further the allergenic proteins within the seeds (Zhu *et al.* 1989). The major component of citrus seeds is the globulin protein citrin: only the 22 kDa subunit of this protein was among the IgE reactive protein bands within the citrus extract within this subject (Koltunow *et al.* 1996). The major albumin fraction, with a previously stated molecular mass of 41 kDa, is likely to be represented by

the 43 kDa immunoreactive band in these blots (Koltunow *et al.* 1996). Whether these proteins or other less well characterised proteins are major allergens will only become clear with a population study.

Western blotting data from the cohort used within the hazelnut-peanut T cell cross-reactivity experiments also provide insights into the cross-reactive immune response. Within our group, no hazelnut protein demonstrated IgE reactivity for all subjects tested. Twelve hazelnut proteins were IgE reactive, with molecular masses ranging from 9 kDa to 70 kDa. The most frequently recognised protein in our cohort was approximately 41 kDa, with 4 out of 5 subjects possessing IgE reactive to that band. This is remarkably close in size to the hazelnut allergen Cor a 9, an allergen described by Beyer et al. as being associated with hazelnut anaphylaxis (Beyer *et al.* 2002). The pattern of reactivity did not match the pattern shown in the paper by Pastorello et al. in which sera from a cohort of hazelnut allergic subjects were used for immunoblotting. Within that group, the molecular masses of the major allergens were 47 kDa, 18 kDa, 32 kDa and 35 kDa. This may reflect the reactivity of that cohort, the subjects predominantly suffering oral allergy syndrome and being sensitised presumably via cross-reactive birch or hazel pollen.

Cor a 9 is an 11S-globulin and shares 67% sequence homology to Ara h 3 at 1 IgE binding epitope (Beyer *et al.* 2002). Three out of the 4 subjects demonstrating cross-reactive T cell responses possessed specific IgE towards this protein, including the 3 subjects who reacted to the Ara h 2 extract. As this extract contained small quantities of Ara h 3, it is possible that this is the basis for the cross-reactive T cell response However, 1 subject who did not have cross-reactive T cell responses also demonstrated IgE binding to Cor a 9. Additionally, 2 subjects with IgE specific for the 11 kDa band likely to represent Ara h 3 did not demonstrate cross-reactive responses to Ara h 2 and 1 subject who did demonstrate cross-reactive responses to Ara h 2 had minimal IgE reactivity to Ara h 3.

As such, it is possible that similarities in structure between Ara h 2 and Cor a 9 form the basis to cross-reactive immune responses. Ara h 2 is a member of the conglutin seed storage family, and hence belongs to a different seed storage protein family to Cor a 9. However, while Ara h 2 IgE reactivity was ubiquitous amongst the study cohort, Ara h 2 cross-reactive T cell responses occurred in only 3 of 5 subjects. It is possible that IgE binding to specific Ara h 2 epitopes, rather than the pattern of binding to the entire molecule as demonstrated on western blotting, is predictive of T cell cross-reactivity; such a phenomenon would require exploration of the pattern of reactivity to individual Ara h 2 conformational epitopes.

Ara h 1 produced cross-reactive T cell responses to a minor degree in 2 subjects, yet IgE reactivity occurred in 4 subjects. Analysis of the IgE reactivity to other peanut allergens of the study cohort in comparison to a larger peanut reactive cohort did not show any uniquely

IgE reactive proteins within this cohort (data not shown). As for Ara h 2, it remains possible that it is the presence of reactivity to an individual IgE epitope rather than reactivity to whole protein structures that can be used to predict cross-reactive clinical and T cell responses, but western blotting of crude peanut extracts appears to show little correlation for these phenomena.

This research has also provided information with regards to T cell cross-reactivity in the immune response to food allergens. In these experiments, it has been demonstrated that in a cohort of 5 subjects with dual sensitivity to hazelnut and peanut, 4 of those 5 subjects had cross-reactive T cell responses when oligoclonal hazelnut specific T cell lines were stimulated with each antigen. The lack of cross-reactive T cell responses amongst all 5 subjects with multiple nut sensitivity, suggests that while cross-reactivity between the peanut and hazelnut occurs at the level of the T cell, the T cell response to hazelnut is both to unique and shared epitopes.

This study has provided evidence that Ara h 2 may be important in the causation of cross-reactive T cell responses. Of the 4 subjects demonstrating a cross-reactive T cell response for hazelnut and peanut, 3 demonstrated a significant dose response when oligoclonal hazelnut specific T cell lines were stimulated with Ara h 2, with the maximal stimulation index being > 5 for each. Upon *N*-terminal sequencing of the major allergens of hazelnut, listed above, it has been shown that the 32 kDa protein represents a 2S albumin, a group of seed storage proteins that also contains Ara h 2, Ara h 5 and Ara h 6 (Kleber-Janke *et al.* 1999). Other protein families represented in both hazelnut and peanut include the 11S globulins described above (Ara h 3 and Cor a 9), and the vicilins (Ara h 1 and the 47 kDa hazelnut protein). As these proteins from similar protein families are likely to share significant sequence homology, T cell cross-reactivity could occur for multiple proteins within each extract. The results of this study suggest that this is likely as Ara h 2 was unable to induce the same degree of proliferation as the crude peanut extract unless used at a much higher concentration.

The apparent correlation of IgE binding to Cor a 9 and cross-reactive T cell responses to Ara h 2 has an interesting parallel amongst potential T cell cross-reactive linear epitopes. T cell epitopes for the major allergens of peanut and hazelnut are at present not reported within the literature. In experiments detailed in chapter 5, 2 Ara h 2 peptides are described that are most strongly associated with proliferative and cytokine responses. Of note, in comparison with protein structures present within hazelnut, the first 18 amino acids of the dominant T cell reactive peptide Ara h 2 (19-38) demonstrate 50% identity and 70% similarity with a region of Cor a 9. This represented the greatest degree of similarity between this sequence and any hazelnut protein. Whether this degree of similarity is sufficient to produce cross-reactive T

cell responses requires further experiments using this peptide as well as the corresponding sequence in Cor a 9.

In general, cross-reactivity at both T and B cell levels has been thought to occur between proteins of similar structure. Despite its status as a major hazelnut allergen and belonging to the same seed storage protein family as Ara h 2, the 32 kDa hazelnut protein did not clearly bind IgE in our cohort. Given this protein is most likely on structural grounds to be the basis for both B and T cell cross-reactivity between hazelnut and Ara h 2, this phenomenon is surprising and again suggests that western blotting is not a useful predictor of T cell cross-reactivity amongst nuts and seeds.

Within the study cohort, a clear proliferative response by hazelnut specific T cells to Ara h 1 occurred for only 1 subject. This adds to the established evidence with regards vicilin seed storage proteins and their lack of cross-reactivity and provides novel data with regards to T cell responses. The vicilin seed storage family includes Ara h 1 and several family members in other nuts, including a 47 kDa protein within hazelnut, said to be recognised by IgE from over 95% of subjects with hazelnut allergy (Pastorello *et al.* 2002). Several groups have demonstrated that IgE cross-reactivity does not appear to occur between vicilins within different nut species. Teuber et al. have demonstrated that crude peanut extract does not abrogate binding to the major vicilin allergen of walnut, Jug r 2 (Teuber *et al.* 1999). Similarly the major cashew vicilin allergen Ana o 1, also displays no homologous IgE epitopes on linear epitope studies (Wang *et al.* 2002). Data produced in this set of experiments, within which only minimal cross-reactive responses to Ara h 1 occurred, therefore suggest that T cell responses parallel IgE responses towards vicilin proteins.

These data confirm the data of Higgins *et al.* that T cell cross-reactivity occurs between hazelnut and peanut and extends that data by providing information as to the phenotype of cross-reactive T cells in subjects allergic to both nuts (Higgins *et al.* 1995). Within this set of experiments, the predominant cell type within the oligoclonal hazelnut specific T cell line was the CD4+ T lymphocyte. Analysis of the cytokines produced by these cells upon stimulation with each of hazelnut, peanut and the major allergens Ara h 1 and Ara h 2 where cross-reactive immune responses occurred, suggested that these cells demonstrated a predominant T_H2 phenotype, although both IL-5 and IFN-γ could be demonstrated. This finding confirms that cross-reactive immune responses share a T_H2 phenotype and suggests that the observed cross-reactive proliferative response is of relevance in the pathogenesis of shared peanut and hazelnut sensitivity. The presence of IFN-γ in this response is not surprising, having also been described in T cell responses to other food allergens, including milk and egg (Ng *et al.* 2002).

In conclusion, this research has demonstrated aspects of the cross-reactive immune response for foods using two different clinical models. Using a subject allergic to both peanut and citrus seeds, it has been shown that IgE cross-reactivity may occur at the level of many proteins within cross reactive foods, with this being universal for all proteins contained within citrus seeds, and widespread between peanuts and citrus seeds except for two non-cross-reactive proteins. Which of these cross-reactive proteins is clinically relevant remains to be elucidated, and requires analysis of individual IgE epitopes within each protein. A need for such studies has also been demonstrated using a cohort of subjects allergic to both peanut and hazelnut as a model for the exploration of shared T cell responses, as in the study cohort, the pattern of IgE reactivity on western blotting in general could not be used to predict T cell cross-reactivity amongst responses to peanut and hazelnut. An interesting correlation between T cell and B cell data has been demonstrated with regards to Cor a 9 and Ara h 2 that provides a platform for further studies. Finally these studies have confirmed that T cell proliferative responses are cross-reactive for each nut, and that this may be due to reactivity towards Ara h 2. Such cross-reactive T cell responses shared a T_H2 phenotype amongst individuals allergic to both peanut and hazelnut, highlighting their clinical relevance.

CHAPTER 7 GENERAL DISCUSSION

7.1 INTRODUCTION

Peanut and tree-nut allergy are currently incurable, frequently life-threatening conditions, affecting between 1 and 2 percent of the population (Kagan *et al.* 2003). Evidence exists that the prevalence of this condition has doubled in the last 5 years, in concert with other allergic conditions (Sicherer *et al.* 2003). Within the paediatric population, reactions may be characterised by both an exacerbation of dermatitis or anaphylaxis, while the latter is the typical reaction among adults (Sampson 2002). Exposure to minute quantities of peanut and tree-nuts is sufficient to produce anaphylaxis in sensitised individuals and, when compared to other food allergens, anaphylaxis due to these allergens is more likely to be of a life-threatening nature or associated with death (Sampson 1992; Hourihane *et al.* 1997). Exposures may occur in a variety of forms, including topical preparations, inhalational exposures, as well as common subtle ingestible forms, making avoidance of peanut and tree-nuts difficult for the majority of nut allergic individuals over a prolonged period (Sicherer *et al.* 1998; Sicherer *et al.* 1999; Lack *et al.* 2003). Multiple nut sensitivity, the coexistence within the same individual of allergy to multiple tree-nuts or tree-nut and peanut, is a frequent finding and increases further the variety of exposures that might produce anaphylaxis in sensitised subjects (Ewan 1996). Nut allergy is therefore a double-edged sword carrying both acute morbidity and mortality upon exposure, and chronic psychological morbidity for patient and carer due to the need for constant vigilant avoidance (Primeau *et al.* 2000).

At present, no cure exists for peanut and tree nut allergy, with current strategies for the prevention of reactions limited to avoidance of exposure and the provision of adrenaline based first aid kits to sufferers. Such strategies have proven at best partially effective. Two studies have provided proof of concept that immunological desensitisation might be possible for peanut allergy but neither study was able to maintain desensitisation, and each was associated with a high prevalence of anaphylactic reactions (Oppenheimer *et al.* 1992; Nelson *et al.* 1997). No safe method of desensitisation for nut allergy currently exists.

Seven allergenic proteins have been described within peanut, with its major allergens being Ara h 1 and Ara h 2 (Burks *et al.* 1991; Burks *et al.* 1992; Kleber-Janke *et al.* 1999; Rabjohn *et al.* 1999). Peanut allergens are glycoproteins and in general belong to the seed storage families of proteins. Ara h 1, which is a member of the vicilin seed storage family, is relatively abundant within peanut and forms a trimeric structure in natural conditions which concentrates its IgE binding regions, perhaps explaining the potency of peanut induced

allergic reactions (Shin *et al.* 1998). Ara h 2, a conglutin seed storage protein, is smaller and less abundant than Ara h 1, and runs as a doublet on SDS-PAGE (Clarke *et al.* 1998). The larger of its two isoforms is perhaps more allergenic, carrying an extra copy of one its major linear IgE binding epitopes, although stronger IgE binding was not demonstrated on western blotting using sera from the study cohort (Chatel *et al.* 2003).

An increasing volume of data exists for tree-nut allergens, within which it has been demonstrated that the major allergens also belong in general to the seed storage protein families (Teuber *et al.* 1999; Pastorello *et al.* 2002; Wang *et al.* 2002). Additionally, it has been demonstrated that pan-allergens such as lipid transfer protein and profilin are frequently recognised by tree-nut specific IgE in sensitised individuals (Pastorello *et al.* 2002). B cell data suggest that it is via these proteins of similar structure that cross-reactive immune responses and hence multiple nut sensitivity might occur (Breiteneder *et al.* 2000).

While a relatively large quantity of data exists for peanut and tree-nut allergens with regards to the elicited B cell humoral response, only limited T cell data are available (Higgins *et al.* 1995; de Jong *et al.* 1996; Hourihane *et al.* 1998; Laan *et al.* 1998; Turcanu *et al.* 2003). The presence of the dominant T cell epitopes within allergen immunotherapy extracts is critical for the efficacy of clinical and immunological tolerance induction. Therefore the availability of such data for peanut and tree-nut allergens is crucial to facilitate the development of novel forms of immunotherapy for these allergens (Rolland *et al.* 2000). The major contribution of this thesis is to provide data with regards the T cell response to peanut and tree-nuts. A large cohort of peanut and tree-nut allergic subjects has been gathered, providing novel information about this disease in its adult form. Following purification of nAra h 1 and nAra h 2 from unfractionated natural peanut extract, each has been demonstrated to be a major T cell allergen. The regions of Ara h 2 containing the major T cell epitopes are described, providing essential information to facilitate the future development of peanut specific vaccines. Exploring potential T cell cross-reactive proteins within peanut and hazelnut, it is demonstrated that Ara h 2 is able to induce cross-reactive T cell responses in hazelnut specific T cell lines, providing information relevant to both the development of multiple nut sensitivity as well as its potential therapy.

7.2 UNIQUE FEATURES OF NUT ALLERGIC SUBJECTS

7.2.1 Multiple Nut Sensitivity

The cohort of peanut and tree nut allergic subjects used to carry out research within this thesis was drawn from the Alfred Hospital Allergy and Asthma Outpatient Clinic. This is an adult clinic and the mean age of subjects was 30 years old, ranging between 17 and 52 years

old. Previous studies of peanut and tree-nut allergy have used predominantly paediatric cohorts, such that the present study provides a unique insight into this illness in its adult form (Ewan 1996; Clarke *et al.* 1998; Sicherer *et al.* 1998).

The principle difference demonstrated by subjects within the present study from those described by others previously was in the high frequency of subjects demonstrating multiple nut sensitivity. Previous studies have described this feature as occurring in vastly disparate frequencies, depending on how the study was carried out. Studies based upon ambulatory clinic cohorts have suggested a frequency of multiple nut sensitivity of between 30 and 40%, but in a telephone survey of the general population, only 2% of nut allergic subjects described that phenomenon (Ewan 1996; Sicherer *et al.* 1999). In the present study, multiple nut sensitivity was ubiquitous. Eighty seven percent (26/30 subjects) described reactions to greater than one type of tree-nut or peanut, while among those undergoing RAST to more than one tree-nut or peanut, 97% (29/30) were poly-sensitised. Indeed, only one of the thirty subjects enrolled in the study demonstrated neither clinical nor immunological evidence of sensitivity to more than one nut (peanut, in that individual).

Previous studies based on younger cohorts have suggested that tree-nut allergy occurs later in life than peanut allergy (Sicherer *et al.* 1998). Standard management of nut allergy is to mandate the avoidance of all tree-nuts and peanut from the time of diagnosis (Sampson 2002). Given that diagnosis of nut allergy occurs generally in early childhood, it is therefore possible that exposure to other nuts and discovery of multiple nut sensitivity will happen only infrequently or not at all, with the only evidence being serological. It is therefore likely that previous studies underestimate the prevalence of multiple nut sensitivity amongst adults.

7.2.2 Other Significant Clinical Features

The cohort used in the present study has demonstrated a number of other interesting findings. Subjects reported sensitivity to a number of different foods, including sesame seed and pine nuts. Of note was the high frequency of reported sensitivity towards sesame seed, with 40% (12/30 subjects) describing this phenomenon. Such a finding has been described by Sporik *et al.* in a paediatric study published in 1996, and the present study confirms their finding while suggesting that sesame seed allergy has increased in frequency since then, presumably in the setting of ongoing increased exposure to sesame seed through the latter part of the last century (Sporik *et al.* 1996).

The present study confirmed the severity of peanut and tree-nut allergy, the majority of sufferers describing life-threatening symptoms, developing within seconds of exposure. Previous studies have demonstrated that of subjects suffering fatal or near fatal anaphylaxis 31% were allergic to peanut and 46% were allergic to tree-nut (Sampson 1992). Within the

present study, 23% (7/30 subjects) required a hospital admission for their condition, 47% (14/30 subjects) required adrenaline, and the mean time since last exposure was a matter of months rather than years. Disappointingly, the study confirmed the previously reported lack of adherence of a significant number of subjects to current preventive measures, with only 40% (12/30 subjects) carrying their self-injectable adrenaline with them at the time of recruitment (Sicherer *et al.* 2000).

7.3 THE IMMUNOLOGY OF THE ALLERGIC RESPONSE TO PEANUT

Within the present study, it has been confirmed that Ara h 1 and Ara h 2 are major B cell antigens, being recognised by peanut specific IgE in 67% and 87% of cases (10 and 13 of 15 subjects, respectively), respectively. This correlates neatly with data provided by Clarke *et al.* but, for Ara h 1, represents a lower frequency of recognition than the 100% described by Burks *et al.* (Burks *et al.* 1991; Clarke *et al.* 1998). Within that paper, only 6 peanut-allergic and 2 non-allergic controls are used such that the extent that Ara h 1 is recognised by peanut allergic subjects may be over represented due to the small sample size or over sensitivity of the assay technique.

Within the present study as well as several previous studies, ubiquitous binding of peanut specific IgE to these major allergens was not demonstrated, suggesting that other allergens within peanut carry a role in the induction and maintenance of sensitisation in some subjects (Clarke *et al.* 1998; de Jong *et al.* 1998). Although a number of varieties of peanut exist, it is unlikely that exposure to different forms of peanut is responsible for differing patterns of IgE reactivity, peanut allergic individuals recognising proteins within different varieties of peanut with the same frequency (Koppelman *et al.* 2001). It is probable that differing patterns of sensitisation reflect the HLA type of individuals and the dose and route of antigen delivery, each of which may impact on the manner of antigen processing and presentation (Secrist *et al.* 1995; Howell *et al.* 1998; Alpan *et al.* 2001). It is also possible that sensitisation may occur via a cross-reacting tree-nut in the first instance, which may again alter the pattern of IgE reactivity, although within the present study no clear difference existed for those subjects poly-sensitised to peanut and hazelnut, as compared to those lacking hazelnut sensitivity.

Of interest within the present study, Ara h 1 bound IgE amongst peanut allergic and non-allergic sera using the standard laboratory protocol for IgE specific western blotting with similar frequency. Previous studies demonstrated that among a grass pollen allergic cohort, clinically irrelevant IgE binding to an unfractionated peanut extract on RAST occurred with a frequency of approximately 1:3 and that this binding was directed towards the

carbohydrate component of the extract (van der Veen *et al.* 1997). Ara h 1 is a glycoprotein, containing 4 N-linked oligosaccharide sidechains of different structure that have previously been demonstrated to bind IgE (van Ree *et al.* 2000). Given the clinically irrelevant IgE binding to Ara h 1 demonstrated within the non-peanut allergic cohort on western blotting, it is probable that this was directed towards the carbohydrate sidechains. Cleavage of these carbohydrates from Ara h 1 and repeat western blotting would aid in confirmation of this impression.

Although Ara h 1 and Ara h 2 bound IgE with a greater frequency than any other protein, a large number of other proteins were recognised by peanut specific IgE. Such a finding has been described by other groups, Clarke *et al.* describing 3 proteins and de Jong *et al.* describing 6 proteins as being recognised by > 50% of peanut allergic subjects (Clarke *et al.* 1998; de Jong *et al.* 1998). Of particular note within the present study were bands of 36 kDa and 11 kDa, each recognised by 83% of subjects. The 36 kDa band is the same mass as that reported for Ara h 4, while the 11 kDa protein is likely to contain a number of proteins including Ara h 3, Ara h 5 and Ara h 6 (Kleber-Janke *et al.* 1999; Rabjohn *et al.* 1999). Research using purified peanut allergens as part of functional assays of IgE binding, such as basophil histamine release assays, would aid greatly in the determination of the significance of the extensive IgE binding demonstrated by the majority of peanut allergic subjects. Hazelnut allergic subjects at risk of anaphylaxis have been shown to be sensitised to Cor a 8 and Cor a 9, but no particular pattern of sensitisation on western blotting, nor the extent of IgE reactivity on RAST, could be used to predict the severity of reaction in this peanut allergic cohort (Beyer *et al.* 2002; Schocker *et al.* 2004).

Within this study, it has been demonstrated that Ara h 1 and Ara h 2 behave in a manner consistent with their designation as major T cell allergens. In comparison to PBMC belonging to a group of non-peanut allergic controls, PBMC from peanut allergic subjects produced significantly more IL-5 and a greater IL-5:IFN-γ ratio upon stimulation with nAra h 1 and nAra h 2. Previous studies demonstrated that stimulation with Ara h 2 produced a T_H2 functional phenotype on flow cytometric analysis, but this is the first study to demonstrate that each major allergen demonstrates significant T cell responses (Turcanu *et al.* 2003). No significant difference could be detected between the two major allergens in the magnitude of cytokine responses, highlighting that each is likely to be important in the T cell response to peanut. Whether other proteins are also of significance to the T cell response requires further study, but seems likely in the setting of the high number of peanut proteins recognised by peanut specific IgE on western blotting.

Of note within this study was the lack of difference between peanut allergic and non-allergic subjects in their proliferative responses to unfractionated peanut extract, nAra h 1 and nAra h

2. Previous studies demonstrated that on an individual level, polyclonal proliferative responses are detectable from both non-peanut allergic and peanut allergic subjects upon stimulation with crude peanut extract, but when a panel of peanut allergic and non-peanut allergic subjects is compared, these responses occur at a lower concentration of peanut extract and to a greater level in the peanut allergic subjects (Laan *et al.* 1998). That this finding could not be repeated in the present study was not due to T cell mitogenicity of the extracts used, but may reflect differences in the methodology used for PBMC stimulation, while the larger number of subjects within the present study diminishes the chance of spurious statistical differences.

In light of the above findings, the present study has confirmed that both Ara h 1 and Ara h 2 are major peanut T cell and B cell allergens, raising the prospect that they form suitable targets for manoeuvres aimed at improving the safety of peanut immunotherapy products. The production of recombinant forms of Ara h 1 and Ara h 2 with single amino acid substitutions has demonstrated that IgE binding can be greatly abrogated in this manner (Stanley *et al.* 1997; Shin *et al.* 1998). Studies using altered peptide ligands have demonstrated that single amino acid substitutions may lead to loss of T cell responsiveness or to the induction of T cell responses of a T_H1 phenotype (Pfeiffer *et al.* 1995). This suggests that a genetically modified peanut crop containing these modified forms of peanuts major allergens, could feasibly be associated with reduced potency for the induction of anaphylaxis in sensitised individuals as well as diminished propensity to peanut sensitisation in peanut naïve individuals. Whether a genetically modified peanut containing these modified forms of protein would produce a viable crop is unclear, as is the need to manipulate the structure of the many other proteins recognised by peanut specific IgE within peanut. It is possible however that fewer proteins would need to be modified to avoid peanut sensitisation if intermolecular help were important in the generation of IgE directed towards other peanut proteins. Research directed at the recognition of proteins important in the sensitisation of individuals towards peanut would provide important information in this area.

7.4 THE HUMAN T CELL RESPONSE TO ARA H 2

Critical to the generation of safe immunotherapy for peanut allergy is the retention within future vaccines of dominant T cell epitopes of the major allergens of peanut (Larche 2000). Within the present study, it has been shown that two dominant regions exist within the major peanut allergen Ara h 2 that contain T cell epitopes including Ara h 2 (19-47), with a second region at Ara h 2 (73-119). Of particular interest is the peptide Ara h 2 (19-38), which produced the most frequent response and the greatest magnitude of proliferative response together with induction of IL-5 production. These data provide the first evidence that this

region contains a major T cell epitope. Future studies using truncated peptides to delineate the minimal T cell epitope are now required. Additionally, although this region is a short linear peptide and unlikely to bind significant IgE, it is remarkably close to the site of a dominant linear IgE epitope, described by Stanley et al. as lying within Ara h 2 (27-36) (Stanley *et al.* 1997). Therefore further studies including functional assays of IgE binding such as basophil histamine release assays or flow cytometric basophil activation tests of upregulated CD63 expression are required to ensure the safety of this molecule for immunotherapy (Crockard *et al.* 2001; Sanz *et al.* 2001).

7.5 THE IMMUNOLOGY OF MULTIPLE NUT SENSITIVITY

As discussed above, it was an almost ubiquitous finding within the subject cohort used for the present study that there was clinical and serological evidence of multiple nut sensitivity, occurring to a far greater level than that suggested by the previous, predominantly paediatric literature (Ewan 1996). As a consequence, any approach to cure or control of peanut allergy needs to encapsulate a thorough understanding of multiple nut sensitivity.

It remains unclear whether multiple nut sensitivity arises as a result of sensitisation to a single food allergen, with this being sufficient to sensitise an individual to multiple similar foods. On epidemiological grounds, this seems likely as only 12% of children allergic to cashew describe having had prior exposure, suggesting the initial sensitising exposure was unrecognised, perhaps due to sensitisation via a different food (Rance *et al.* 2003). Oral allergy syndrome gives the best evidence for sensitisation being via a single allergen, in that instance sensitisation occurring initially to the major birch pollen allergens Bet v 1 and Bet v 2 with subsequent cross-reactivity being generated towards proteins of the same family in foods, such as those of the prunoideae and rosaceae families (Breiteneder *et al.* 2000). T cell cross-reactivity between birch pollen and apple has been suggested to occur via six peptides sharing 50% sequence identity within their respective major allergens Bet v 1 and Mal d 1 (Fritsch *et al.* 1998).

In the present study, stimulation of hazelnut specific T cell lines with peanut was able to elicit proliferative responses from most but not all lines, suggesting that although some individuals demonstrate T cell cross-reactivity as part of their multiple nut sensitivity, others were sensitised to unique T cell epitopes. In those individuals lacking responses to both nuts, it seems likely that sensitisation to hazelnut did not occur via initial sensitisation to peanut, but as a separate phenomenon. Initial sensitisation to a single cross-reactive protein seems unlikely to be the basis to multiple nut sensitivity in every subject.

An increasing literature has sought to determine which proteins are important in the generation of cross-reactive immune responses towards food (Breiteneder *et al.* 2000).

Within the present study, it has been demonstrated for citrus seed proteins, that B cell cross-reactivity is essentially ubiquitous. Surprisingly, however, cross-reactivity appeared to occur for the majority of peanut proteins as well, in a subject demonstrating allergy to both foods. This finding highlights the difficulty in interpretation of the cross-reactivity literature, the majority of which to date has sought to demonstrate B cell cross-reactivity. The principle criticism of these data has been the lack of correlation between immunological cross-reactivity and clinical responses, raising questions about the relevance of the laboratory findings. A clear example of such difficulties is the finding that cross-reactive IgE is readily demonstrable within sera from the majority of peanut allergic subjects for other legumes such as soybean, yet the incidence of sensitivity to both is less than 5% of peanut allergic individuals, providing clear evidence that such a finding has limited clinical or pathological relevance (Bernhisel-Broadbent *et al.* 1989).

For peanut and tree-nuts, multiple protein families have been described for which immune responses may be shared, including seed storage proteins such as the 2S-albumins, 11S-globulins, vicilins, and conglutins as well as other panallergens such as lipid transfer proteins and profilins (Breiteneder *et al.* 2000). However, it appears the presence of structurally similar proteins within different types of nuts is no guarantee that IgE cross-reactivity will occur. An example of such a phenomenon occurs most clearly for the vicilin seed storage proteins. While clinically relevant cross-reactivity between peanut and pea has been suggested to occur between the vicilin Ara h 1 and proteins belonging to the same seed storage family in pea, for peanut and vicilin containing tree-nuts such as walnut and cashew, cross reactivity with Ara h 1 has not been demonstrated (Teuber *et al.* 1999; Wang *et al.* 2002).

Within the present study it has been demonstrated that cross-reactive T cell responses exist between Ara h 2 and unfractionated hazelnut extract. Of interest is the observation that the region described within the present study as containing a major Ara h 2 T cell epitope shares 70% sequence similarity and 50% sequence identity with a segment of Cor a 9, one of the major allergens of hazelnut associated with anaphylaxis (Beyer *et al.* 2002). This finding is interesting in several aspects. Firstly, it suggests that the assignment of probability of cross-reactivity between proteins based upon their protein family is less relevant to the analysis of T cell cross-reactivity, where only short linear peptides are recognised. Secondly, as each of these proteins is a major allergen associated with anaphylaxis, it suggests that major nut allergens rather than proteins of similar structure should be used as the basis for future studies exploring cross-reactivity. Further research, mapping the T cell epitopes of Cor a 9 would provide useful information to confirm whether this region is the site for Ara h 2 cross-reactivity, and confirm the importance of this region for inclusion in future immunotherapy.

The presence of immunologically demonstrable but clinically irrelevant cross-reactivity poses issues for diagnosis and appropriate counselling of subjects at risk of multiple nut allergies. Advice upon taxonomic grounds is now well recognised as being of little use, in contrast to grass pollen allergy (de Leon *et al.* 2003). Current recommendations are that subjects allergic to one tree-nut or peanut should avoid all nuts, with the associated impact this makes with regard to the need for vigilant avoidance and quality of life (Sampson 2002). It may be that correlation with T cell proliferative and cytokine responses would prove useful. Subjects in the present study demonstrated a T_H2 phenotype in their cross-reactive response, whereas in a paper exploring monoclonal responses, T cells from a non-hazelnut allergic donor produced a T_H1 response (Higgins *et al.* 1995). Assays that so might provide greater relevance to in vivo responses could include basophil histamine responses or flow cytometric basophil activation tests showing upregulation of CD63 expression which are assays that better preserve protein tertiary structure and as a consequence allow detection of conformational IgE epitopes (Crockard *et al.* 2001; Sanz *et al.* 2001) (Lehmann *et al.* 2003).

A final issue with regards to multiple nut sensitivity is whether desensitisation to a single nut would be sufficient to produce a cure for allergy to cross-reacting nuts. There is clear evidence that such an approach is effective with birch pollen immunotherapy with regards to coexisting apple allergy, and this occurs in concert with well-established sequence homology in Bet v 1 and Mal d 1 T cell epitopes (Fritsch *et al.* 1998; Asero 2003). While there would appear to be shared T cell epitopes between Ara h 2 and Cor a 9, T cell responses to hazelnut clearly occurred in the absence of peanut cross-reactivity, suggesting unique T cell epitopes in addition to shared epitopes. Hoyne et al. established that not all T cell epitopes needed to be present within an immunotherapy extract for efficacy, dominant T cell epitopes producing linked suppression for responses to other epitopes (Hoyne *et al.* 1997). Whether this phenomenon would hold true for cross-reacting nuts remains undefined and is likely to depend on the number of dominant T cell epitopes present within each nut and the extent to which there is sharing of these determinants.

7.6 FUTURE APPROACHES TO IMMUNOTHERAPY FOR NUT ALLERGY

The principle focus of the present study has been to establish a better appreciation of the T cell response to peanut and tree-nuts, such an understanding being crucial for the development of new approaches to immunotherapy. Proof of concept that immunotherapy is possible for peanut and tree nut allergy was provided in two studies, each demonstrating that, following immunotherapy, increased tolerance to peanut occurred on oral challenge and skin prick test (Oppenheimer *et al.* 1992; Nelson *et al.* 1997). Unfortunately, each was associated

with a high frequency of systemic reactions and in one study accidental death was induced in a subject receiving placebo therapy via a dosing error (Oppenheimer *et al.* 1992). Such adverse reactions are precipitated via allergen specific IgE binding and effector cell activation, a mechanism that is not required for efficacy of immunotherapy (Larche 2001). In that context, methods of immunotherapy that circumvent IgE binding, including those using hypoallergenic B cell mutant proteins and/or T cell epitope peptide fragments are attractive models for future approaches to treatment of nut allergy.

That peptide immunotherapy may prove an efficacious approach to immunotherapy has been demonstrated in a number of models. Cat dander allergic subjects receiving desensitisation with two large peptides or, more recently, short overlapping peptides, based upon the structure of the major cat dander allergen Fel d 1 have demonstrated a number of features of efficacy (Norman *et al.* 1996; Oldfield *et al.* 2002). Desensitisation using peptides based upon the three major T cell epitopes of the major bee venom allergen phospholipase A2 has lead to tolerance of bee stings in 3 of the 5 subjects receiving therapy (Muller *et al.* 1998).

Several issues exist with regards to the application of peptide immunotherapy to nut allergy. By their nature, research such as the present study involves small subject numbers that may not be representative of the broader population. However, although concern has been raised that peptide immunotherapy based upon such research may lack the power to detect important T cell epitopes for a broad population, it appears that for differing HLA types, the same T cell epitope may be recognised as dominant (Haselden *et al.* 1999) due to promiscuity of peptide binding to the MHC class II molecules of different HLA class II alleles. A second matter for attention, demonstrated within the present study with the finding of at least two regions containing Ara h 2 T cell epitopes, is that multiple T cell epitopes exist for peanut and tree-nut allergens and each may need to be included within immunotherapy vaccines. Such an approach was addressed by Fellrath et al. who successfully used equimolar quantities of long peptides based upon the entire sequence of PLA_2 for bee venom desensitisation to induce T cell hyporesponsiveness and immune deviation (Fellrath *et al.* 2003). The successful study of cat peptide immunotherapy by Oldfield et al. included peptides representing the entire sequence of Fel d 1 (Oldfield *et al.* 2002). Within the current study, multiple proteins beyond Ara h 2 demonstrated significant IgE reactivity, making it likely that peptides based upon those additional proteins might need inclusion in a successful immunotherapy vaccine. For individual proteins such as Ara h 2, however, as discussed above, it is possible that only the dominant T cell epitopes would need inclusion (Hoyne *et al.* 1997).

B cell epitope mutants may be prepared within which IgE binding epitopes have been altered to abolish IgE binding while maintaining T cell reactivity. Such an approach has been used

to create mutant proteins based upon Ara h 3, each of which demonstrated in vitro evidence of retained T cell proliferative responses but loss of IgE binding (Rabjohn *et al.* 2002). Ara h 2 has 8 cysteine residues and therefore up to 4 disulphide bonds. The reduced form demonstrates significant alteration in secondary and tertiary structure, suggesting that these sites may be suitable targets for the creation of mutant proteins (Sen *et al.* 2002). No in vivo studies have yet been performed to suggest this approach will prove successful and the issues described above with regards the number of peanut and tree-nut allergens demonstrated in the present study hold true for this potential method.

While the present study provides significant information useful in the development of peptide immunotherapy, it may be that other methods will provide the ultimate cure for peanut and tree-nut allergy. It is unlikely that gene therapy will prove acceptable to sufferers of a condition only rarely associated with death. Nevertheless, Roy *et al.* were able to prevent anaphylaxis in mice sensitised to peanut, by creating a DNA vaccine containing a gene encoding Ara h 2 and transducing that gene into intestinal epithelium (Roy *et al.* 1999). An alternative approach is to prevent peanut or tree-nut specific IgE binding with the FcεRI receptor, thereby preventing effector cell activation and anaphylaxis. Such an approach has been used by Leung *et al.*, who demonstrated that subjects receiving 16 weeks subcutaneous therapy with humanised mouse IgG_1 anti-human IgE antibodies were able to tolerate significantly higher quantities of peanut protein without experiencing anaphylaxis (Leung *et al.* 2003). Whether this approach would prove successful in preventing anaphylaxis during conventional immunotherapy is unclear. Finally, the administration of peanut proteins with adjuvants that induce a T_H1 response may enhance the efficacy of immunotherapy, this approach demonstrating efficacy in diminishing anaphylaxis in a mouse model of peanut allergy, using rectally administered heat-killed *Escherichia coli* producing mutated Ara h 1, 2, and 3 (Li *et al.* 2003).

7.7 CONCLUSION

The present study confirmed that the major peanut allergens, Ara h 1 and Ara h 2, are both major B and T cell allergens in an adult population of peanut allergic subjects. These allergens are likely to prove important targets for future approaches to immunotherapy. Information useful in the development of that therapy has been provided in the present study through the demonstration of regions of Ara h 2 containing major T cell epitopes. Caveats exist to the likely success of peptide immunotherapy, in part because of the high frequency of multiple nut sensitivity in the adult population. However, this research has shown that for hazelnut and peanut allergy, shared sensitivity is associated with T cell cross-reactive proliferative and cytokine responses, raising the prospect that immunotherapy containing

cross-reactive T cell epitopes may prove efficacious for both sensitivities. Those epitopes are likely to include a region of Ara h 2, which has been shown in the present study to be likely to contribute to that phenomenon, potentially through a shared sequence with the major hazelnut allergen associated with anaphylaxis, Cor a 9.

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Characterisation of the T cell epitopes of a major peanut allergen, Ara h 2

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ABSTRACT

Background: The development of safe and effective immunotherapy for peanut allergy has been complicated by the high anaphylactic potential of native peanut extracts. We sought to map the T cell epitopes of the major peanut allergen Ara h 2 in order to develop T cell targeted vaccines. **Methods:** A panel of 8 peanut-specific CD4+ T cell lines (TCL) was derived from 8 peanut-allergic subjects and proliferative and cytokine responses to stimulation with a set of overlapping 20-mer peptides representing the entire sequence of Ara h 2 determined. Proliferation was assessed in 72 hour assays via tritiated thymidine incorporation, while IL-5 and IFN-γ production were assessed via sandwich ELISA of cell culture supernatants. **Results:** Eight of the 17 Ara h 2 peptides were recognised by 1 or more subjects, with the two peptides showing highest reactivity (Ara h 2 (19-38) and Ara h 2 (73-92)) being recognised by 3 subjects each. Adjoining peptides Ara h 2 (28-47) and Ara h 2 (100-119) induced proliferative responses in two subjects. Each of these peptides was associated with a Th2-type cytokine response. **Conclusion:** Two highly immunogenic T cell reactive regions of Ara h 2 have been identified, Ara h 2 (19-47) and Ara h 2 (73-119), providing scope for the development of safe form of immunotherapy for peanut allergy.

Running title: T cell epitopes of Ara h 2

Key words: allergens, peanut, Ara h 2, T cell epitopes

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INTRODUCTION

Peanut allergy is associated with a significant risk of mortality, while the need for vigilant avoidance of exposure produces considerable psychological morbidity in both sufferers and carers (1, 2). As a consequence, there is an urgent need for a treatment for this disorder. Despite the existence of efficacious specific immunotherapy for a variety of clinically important allergens for almost a century, to date, efforts at specific immunotherapy for peanut allergy have met with limited success (3). Two studies have provided proof of concept that desensitisation is possible for peanut allergy (4, 5). Each has shown that tolerance of peanut can be induced using a rush immunotherapy protocol, but that tolerance is lost in approximately half of the subjects during maintenance dosing. Additionally, injections with unmodified crude peanut extract were associated with frequent episodes of anaphylaxis in the majority of subjects during both the induction and maintenance phases.

It has long been established that the morbidity associated with allergen immunotherapy is due to the cross-linking of IgE on mast cells and basophils, and that this action is not required for such therapy to be efficacious (6). Because B cells and antibodies recognise conformational epitopes dependent on molecular tertiary structure, in contrast to CD4+ T cells which recognise short linear peptides from the primary structure, approaches that alter or bypass the need for allergenic tertiary structures within vaccines have provided a route to the development of novel immunotherapy strategies. Of these strategies, T cell epitope based peptide immunotherapy is the method for which the best evidence of efficacy exists, being documented for both bee venom allergy (7) and cat dander allergy (8).

Crucial to the development of T cell targeted strategies is the identification and retention of critical T cell epitopes in hypoallergenic immunotherapy preparations (9). To date, no primary data have been published identifying the T cell epitopes of peanut allergens. We present here an analysis of the T cell epitopes of the major peanut allergen Ara h 2. Peanut specific oligoclonal TCL are stimulated with a set of overlapping Ara h 2 peptides representing the entire sequence of Ara h 2, and proliferation and cytokine production are measured.

METHODS

Subjects

Eight peanut-allergic subjects were recruited from the Alfred Hospital Asthma and Allergy Clinic, Melbourne, Australia. Donors were selected on the basis of a history of anaphylaxis following peanut ingestion, and either a positive serum peanut-specific IgE immunoassay (Pharmacia CAP score ≥ 1), or a positive skin prick test (wheal diameter > 3 mm, and > 50% of histamine 10 mg/ml). Four non-peanut allergic controls were also recruited, three of whom were also non-atopic (as defined by the lack of symptoms of allergic disease and negative skin prick testing to common environmental aeroallergens). The study was approved by the Alfred Hospital Ethics Committee, and informed written consent was obtained from all donors.

Antigens

Crude peanut extract (CPE) was prepared as described previously (10) and dialysed against PBS at 4°C, using a 3.5 kDa cut-off dialysis membrane (Pierce, USA). For cell culture, the supernatant was filter sterilised through a 0.2 µm filter.

Peanut cDNA was synthesised from peanut mRNA using the Timesaver cDNA synthesis kit (Amersham Biosciences, USA). The cDNA encoding Ara h 2 was then amplified by PCR following our standardised methods (11) using primers based on the published sequence of Ara h 2 ((12); Genbank accession no. L77197) as outlined below:

Forward – (5' – GCGGAATTCCTCACCATACTAGTAGCC – 3')

Reverse – (5' – CGCCTGCAGTTAGTATCTGTCTCTGCC – 3')

Sequence comparisons showed 100% identity with the published sequence. The Ara h 2 cDNA was subcloned into the pPROEX-HTa vector (Invitrogen, USA) as previously described (11) and the plasmid construct was transformed into *Escherichia coli* strain ER1793 (New England Biolabs, USA) using the heat shock procedure. rAra h 2 expression was performed as outlined previously (11) and affinity purification using Ni-NTA agarose (Qiagen, Australia) was performed as described by the manufacturer but using modified denaturing wash (50 mM NaH_2PO_4, 300 mM NaCl, 50 mM imidazole and 8 M urea, pH 8.0) and elution (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole and 8 M urea, pH 8.0) buffers.

Ara h 2 peptides (20-mers, 11 amino acid overlap) were synthesised by Mimotopes, (Victoria, Australia) according to the Ara h 2 sequence published by Stanley *et al.*, (12).

Electrophoresis and Western immunoblotting

Crude peanut extract proteins were resolved by SDS-PAGE on 14% gels under reducing conditions, as described in de Leon *et al.* using the Xcell II Mini-Cell apparatus (Invitrogen, USA) (10). Proteins were then stained with Coomassie brilliant blue (Sigma, USA).

For Western immunoblotting, peanut proteins separated by 14% SDS-PAGE were transferred onto nitrocellulose membranes as outlined previously (10) using an Xcell II blotting apparatus (Novex, USA). Membranes were blocked in 10% milk powder-PBS for 1 hour. After washing in PBS, membranes were incubated overnight with sera diluted 1:5 with 1% milk powder-PBS-0.05% Tween. Following washing, rabbit anti-human IgE HRPO conjugate (Dako, USA) diluted 1:500 in 1% milk powder-PBS-0.05% Tween was applied and incubated 1-2 hours at room temperature. Following washing, membranes were incubated for 1 minute with chemiluminescence substrate (Du Pont, USA) then analysed using Labworks image acquisition software (UVP Laboratory Products, UK).

Generation of short-term peanut-specific TCL

PBMC proliferative responses to stimulation with CPE demonstrated a dose response for all peanut-allergic subjects and non-peanut allergic subjects. For each subject, the lowest dose that produced maximal stimulation at 7 days was used to drive peanut specific T cell lines for use in peptide assays; this dose ranged between 50 and 200 μg/ml. TCL generation was performed using our standard methodology (13). Briefly, PBMC were cultured in 24-well plates (Greiner Biotechnik, Germany) at 2.5 x 10^6 cells per well (2 ml volume) in complete medium with CPE at an individually optimised concentration for 7 days at 37°C in a 5% CO_2 humidified incubator. At day 7 and day 14, cells were washed and resuspended at 1 to 1.5 x 10^6 cells/ml and added together with 1 x 10^6/ml washed irradiated (3000 rads; Gammacell 1000 Elite, Nordion International, Inc.) autologous PBMC and antigen into fresh 24-well plates. At day 2 following restimulation, 25 U/ml of recombinant human interleukin-2 (rIL-2) was added and at day 4, 1 ml of culture medium was removed and replaced with fresh medium and 25 U/ml rIL-2. In all experiments, T cells were rested for 6 to 7 days after the last addition of antigen and APC.

TCL proliferation assays

Oligoclonal T cells (5.0 x 10^4/well) were cultured in triplicate in 96-well U bottom plates (Linbro ICN Biomedicals, USA) with 10 μg/ml Ara h 2 peptides, along with CPE in concentrations between 25 μg/ml and 100 μg/ml, and rAra h 2 in concentrations between 1 μg/ml and 10 μg/ml, in the presence of autologous irradiated (3000 rads) PBMC (5.0 x 10^4/well). Cell culture medium alone and rIL-2 (50 U/ml) were used as negative and positive controls respectively. Cultures were incubated for 3 days. In the last 16 hours, wells were pulsed with ^{3}H-thymidine (1 μCi/well), then harvested onto glass fibre filters with a 96-well automatic cell harvester. ^{3}H-thymidine incorporation was measured by liquid scintillation spectroscopy and proliferation determined according to the mean counts per minute (cpm) for triplicate cultures. Responses were considered positive if the stimulation index (SI, cpm of antigen-stimulated T cells divided by cpm of unstimulated T cells) was ≥ 2.5.

Cytokine detection in culture supernatants

IL-5 and IFN-γ levels in 48 hour culture supernatants were measured by sandwich ELISA. White 96 well ELISA plates (Corning, USA) were coated with capture mAb (IL-5 and IFN-γ, 2 μg/ml; 30μl/well) diluted in 0.1 M bicarbonate buffer overnight at 4°C. After washing in PBS/0.05% Tween (wash buffer), wells were blocked with 100 μl/well of 1% bovine serum albumin (BSA)/PBS (blocking buffer). Following washing, 30μl/well of serial dilutions of recombinant human IL-5 or IFN-γ (5000-0.15 pg/ml) in blocking buffer plus 0.05% Tween, or culture supernatants were incubated overnight at 4°C. After washing, plates were incubated with biotinylated detection mAb (IL-5, 1 μg/ml; IFN-γ, 0.5 μg/ml; 50 μl/well) for 1 hour then incubated with streptavidin-peroxidase diluted 1 in 2000 in blocking buffer (50 μl/well) for 45 minutes. Following washing, 100 μl/well of freshly prepared chemiluminescent substrate (Perkin-Elmer, USA) was added and plates read in a Lumicount microplate glow luminometer (Packard Instrument Company, USA). The lower limit of detection for IL-5 and IFN-γ ELISAs were 2 pg/ml and 4 pg/ml, respectively.

RESULTS

Subject characterisation

Clinical characteristics of the 8 peanut-allergic subjects are detailed in Table 1. The mean age of subjects was 38 years (range 28-55 years). Each subject described typical features of anaphylaxis on exposure to peanut, beginning within minutes of that exposure. There was no correlation between the level of serum specific IgE and the severity of reactions subjects experienced with exposure to peanut.

Western immunoblotting for serum IgE reactivity to Ara h 2

Seven of the 8 peanut-allergic subjects demonstrated specific IgE reactivity for a double band of approximately 15 kDa corresponding to Ara h 2 (Figure 1), as described by Burks et al, (14). Interestingly, the same 7 subjects also recognised a band of approximately 11 kDa, likely to represent either Ara h 3, Ara h 5 or Ara h 6 (15, 16). Subject 8, despite an excellent history of peanut induced anaphylaxis, demonstrated IgE reactivity to CPE on SPT, but not by Pharmacia CAP or Western immunoblotting (Figure 1). Two of the four non-peanut allergic controls also showed binding to the lower of the two Ara h 2 bands and three showed reactivity to the 11 kDa protein, but these reactions were weak and used as the cut-off for positive IgE binding to CPE in the peanut-allergic subjects.

Mapping of Ara h 2 T cell epitopes

Oligoclonal CPE-specific TCL were generated from PBMC of the 8 peanut-allergic donors. TCL could not be generated from any of the non peanut-allergic donors, cells becoming non-viable after two stimulations or demonstrating a non-discriminatory "high background" response to all antigens assayed (data not shown). Individual responses of peanut-allergic donor TCL to peptide, Ara h 2 and CPE are summarised in Table 2 and Figure 2. Of the 17 peptides tested, 8 (47%) induced a proliferative response. Proliferative responses to rAra h 2 showed best correlation with responses to Ara h 2 (19-38) and Ara h 2 (28-47), in that where a subject had a response to these peptides, 60% also had a proliferative response to Ara h 2.

Ara h 2 (19-38) and Ara h 2 (73-92) were associated with the greatest frequency of response, each producing proliferative responses in 3 of the 8 peanut-allergic subjects although not the same 3 subjects. Other peptides inducing proliferative responses in 2

subjects each were located at Ara h 2 (28-47) and Ara h 2 (100-119), while Ara h 2 (55-74), Ara h 2 (82-101), Ara h 2 (91-110), and Ara h 2 (136-155) induced proliferation in one subject each. A range of magnitudes of response to each peptide was seen but when response magnitude was ranked for each subject, Ara h 2 (19-38) responses ranked highest, with 3 subjects demonstrating their greatest response to this peptide.

Cytokine responses to peptides associated with a proliferative response

To identify the phenotype of T cells associated with a peptide proliferative response, supernatants were collected from TCL cultures 48 hours after stimulation with these peptides or with CPE and assayed for the presence of IL-5 and IFN-γ. All TCL demonstrated detectable cytokine levels upon stimulation with CPE and six of the 8 TCL produced cytokines on stimulation with proliferation-inducing peptides (Table 3). The magnitude of cytokine responses associated with individual peptides varied greatly between subjects, from the lower limits of detection for both cytokines, up to 2053 pg/ml for IL-5, and 937 pg/ml for IFN-γ. The greatest individual IL-5 response to a peptide was to Ara h 2 (19-38) with subject 1 producing 2053 pg/ml of IL-5 and subject 3 producing 506 pg/ml of IL-5 towards this peptide. Similar high IL-5 responses were also produced to CPE by these subjects. IL-5/IFNγ ratios were skewed towards greater IL-5 production for all peptide responses in the 6 subjects for whom cytokines could be detected, except for one peptide, Ara h 2 (73-92), which induced greater IFN-γ production in one subject. The greatest ratio occurred for Ara h 2 (19-38), being approximately 20 for subject 1, and 7 for subject 3.

DISCUSSION

Ara h 2 is a major peanut allergen, recognised by serum specific IgE of at least 80% of peanut allergic subjects. In order to develop a T cell targeted vaccine for use in peanut allergic patients it is crucial to identify dominant T cell epitopes of Ara h 2. In this study, oligoclonal CPE specific TCL were stimulated with CPE, rAra h 2 and a set of 20-mer peptides representing the entire length of Ara h 2. A distinct pattern of proliferative responses to Ara h 2 peptides was demonstrated amongst the TCL, with certain clusters of peptides being more frequently associated with proliferative responses and producing a predominantly IL-5 cytokine response, suggesting that those peptides contain epitopes relevant to the T-cell immune response of peanut-allergic subjects.

In this study, CPE driven TCL showed significant proliferation (SI ≥ 2.5) to 8 of the 17 peptides. Examination of the pattern of response suggests that T cell reactivity is

clustered within two regions of the Ara h 2 molecule, located at Ara h 2 (19-47) and Ara h 2 (73-119). These regions produced stimulation in 5 and 4 of the 8 subjects respectively; one subject demonstrated a SI ≥ 2.5 at both sites. Interestingly, only subjects with responses in the region Ara h 2 (19-47) had responses to rAra h 2. T cell reactivity to Ara h 2 was discussed previously in a review but no primary data was presented (17). The regions cited as showing T cell responses were amino acid residues 19-28, 45-53, 96-114, and 131-139. The dominant T cell reactive sites identified in the current study corresponded to two of these regions, providing further support for their clinical importance. Since individual reactivity to the two dominant regions of Ara h 2 generally showed responses to only one of the peptides spanning that region, it seems likely that each region contains more than one T cell epitope. Further delineation of the precise T cell epitopes and their core sequence requires stimulation with truncated peptide series.

Examination of the magnitude of proliferative responses to peptides also provides support for there being two dominant T cell reactive regions within Ara h 2 located at Ara h 2 (19-47) and Ara h 2 (73-119). Of the three peptides that produced responses > 5.0 SI, two were associated with these regions. Ara h 2 (19-38) produced the largest responses, with an SI of 26.8 in subject 1, as well as 5.2 in subject 3. No peptides outside of the two identified regions produced a SI of this magnitude.

The clinical significance of T cell peptide reactivity was further examined by assessing the cytokine response associated with each peptide. Peptides that contain T cell epitopes relevant to the allergic response would be expected to cause secretion of cytokines associated with a T_H2 response, such as IL-5. Data with regards to cytokine secretion were available for 6 subjects, and revealed that the IL-5:IFN-γ ratio was significantly skewed towards IL-5 production for peptides associated with proliferative responses. The largest responses again occurred for Ara h 2 (19-38), both on the basis of absolute concentration of IL-5 and the IL-5/IFN-γ ratio, with one subject recording an IL-5 concentration of 2053 ng/ml and an IL-5:IFN-γ ratio of 20. Thus, these data provide further support for those Ara h 2 peptides producing proliferative responses being those associated with the allergic response to this allergen. Examination of cytokine production on a single cell basis via flow cytometry would be helpful in confirming the T_H2-type phenotype of these cell lines (18).

A caveat to the use of peptide immunotherapy for allergy is that peptides are unable to elicit significant IgE induced effector cell reactivity, thus reducing the risk of systemic allergic reactions (6). Immunodominant regions for serum IgE binding for Ara h 2 have been identified at Ara h 2 (27-36), Ara h 2 (57-66) and Ara h 2 (65-74) (12). The

presence of an IgE immunodominant region within the T cell reactive peptide Ara h 2 (19-38) mandates the need for further clarification of the precise T cell epitope to assess the degree of overlap with the IgE immunodominant epitope, along with functional assays of effector cell activation by that region.

The data presented within this study provide a platform for the development of novel allergen immunotherapy. One such approach could be via the production of a hypoallergenic mutant whereby IgE conformational epitopes are destroyed as has been performed for Ara h 3 (19). Because Ara h 2 in its reduced form demonstrates significant alteration in secondary and tertiary structure, it is likely to prove suitable for similar strategies (20). Alternatively, with the identification of the precise T cell epitope using truncated peptide sequences, peptide based immunotherapy approaches could be generated, as has been performed successfully for bee venom and cat dander (7, 8). Although the MHC haplotype of responding subjects has not been determined in this study, it is likely that our findings will be applicable to the general population. It has been shown previously that individual allergenic proteins retain their immunodominance despite presentation by MHC molecules of different haplotypes (13, 21).

In conclusion, this study has identified two highly immunogenic T cell reactive regions of Ara h 2, namely Ara h 2 (19-47) and Ara h 2 (73-119). Within these regions, the peptide Ara h 2 (19-38) induced the greatest T cell proliferative responses, with corroboration by cytokine data that this region is clinically relevant. Our results provide the basis for the development of safe and effective T cell targeted immunotherapy.

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Table 1 Clinical features of peanut allergic subjects.

Subject	Sex	Age (yrs)	Allergic disease	Peanut induced symptoms	Known nut allergens	Other food allergens	Age at first reaction (yrs)	Time since last reaction (mths)	level (kU$_A$/l)	score	Atopic status
1	M	28	asthma	GIT upset, laryngeal oedema, urticaria	peanut, hazelnut, egg, milk		5	1	100	6	HDM
2	F	36	asthma, eczema	GIT upset, asthma, hypotension, facial angioedema	peanut, hazelnut		4	2	3.09	2	GP, HDM, C
3	F	49	nil	GIT upset, asthma, laryngeal oedema, loss of consciousness, urticaria	peanut, cashew nut	peas	2	18	17.6	4	GP
4	F	33	nil	GIT upset, urticaria	peanut, hazelnut, pine nuts		0.5	3	13.3	3	GP, B, HDM, C
5	M	55	asthma, eczema	asthma, laryngeal oedema, facial angioedema	peanut, walnut	banana	1.5	120	2.01	2	GP, HDM, A
6	M	35	asthma, rhinitis, eczema	asthma, urticaria, facial angioedema	peanut, hazelnut		8	12	2.82	2	NA
7	F	37	rhinitis, eczema	GIT upset, asthma, laryngeal oedema, hypotension	peanut, hazelnut, pine nuts		10	36	peanut SPT: 14mm		GP, HDM
8	F	30	asthma	asthma, uticaria, laryngeal oedema, facial angioedema	peanut, Brazil nut, almond		3	228	0.93	2	NA

Key: GP, grass pollen; B, birch; HDM, house dust mite; C, cat; A, alternaria.

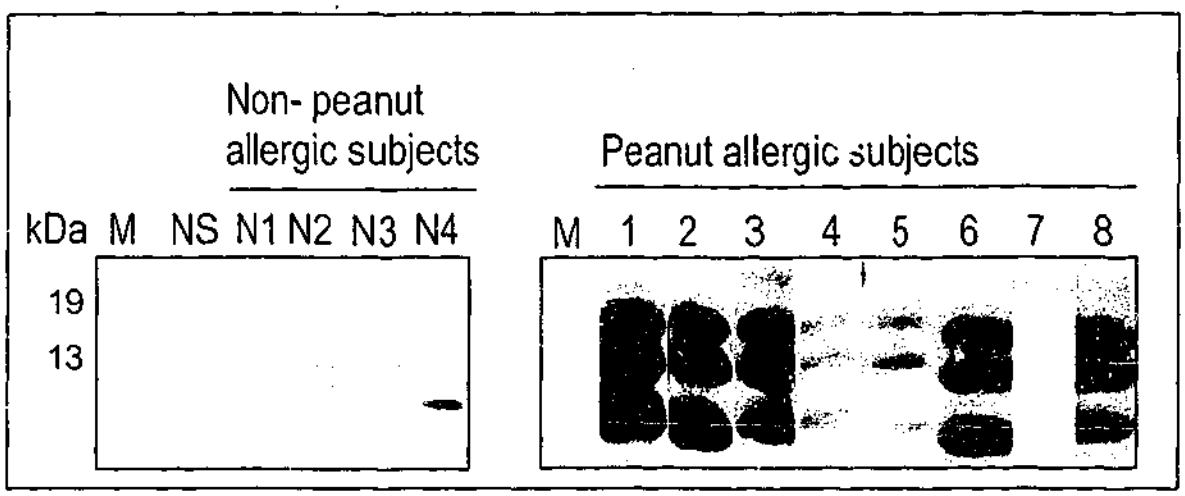

Figure 1 Western blot for serum IgE reactivity to Ara h 2 of peanut allergic and non-allergic subjects

Table 2 Oligoclonal T cell proliferative responses of peanut allergic subjects to Ara h 2 peptides.

	Ara h 2 peptides																	Ara h 2	CPE
Subjects	1-20	10-29	19-38	28-47	37-56	46-65	55-74	64-83	73-92	82-101	91-110	100-119	109-128	118-137	127-146	136-155	138-157		
1	0.9	1.0	**26.8**	1.3	0.7	0.9	1.9	1.0	1.1	1.1	1.3	1.3	1.0	0.9	0.9	1.0	2.0	**20.9**	**89.9**
2	2.4	0.8	5.2	0.8	0.7	0.6	0.8	0.7	0.4	0.6	0.6	0.7	1.2	0.8	0.9	0.8	0.7	2.3	**4.6**
3	1.5	1.1	3.1	1.3	1.7	1.6	1.4	0.8	1.5	0.8	1.5	1.1	1.0	1.5	2.2	1.0	1.2	**5.8**	**52.0**
4	1.1	1.5	1.5	**2.8**	0.9	2.3	1.2	2.1	**3.3**	2.4	**5.3**	**2.9**	1.4	1.6	1.2	**2.6**	2.0	**5.7**	**34.2**
5	0.9	1.1	2.3	**2.6**	1.7	1.7	2.2	1.9	1.8	2.3	1.7	1.7	1.4	1.4	1.8	1.6	0.9	0.8	**4.4**
6	2.3	1.1	1.6	1.8	1.1	1.1	1.6	1.9	**6.7**	1.5	1.1	**2.5**	1.0	1.3	1.2	1.1	1.1	1.4	**13.9**
7	1.3	1.8	1.9	1.3	1.7	2.1	**2.7**	1.5	**2.7**	1.9	2.2	2.1	1.4	1.7	1.6	1.3	1.5	1.2	**3.2**
8	1.1	1.4	1.2	1.4	1.2	1.1	1.1	1.2	1.3	**2.9**	2.4	1.2	1.6	1.7	1.5	1.4	1.4	1.2	**15.7**

Proliferative responses for Ara h 2 peptide-responsive TCL are shown as stimulation indices and values ≥ 2.5 are shaded grey. Background responses of T cells cultured with irradiated APC in the absence of antigen were 6269 ± 2579 cpm (mean ± SEM). The maximal CPE and rAra h 2 response is shown for each donor.

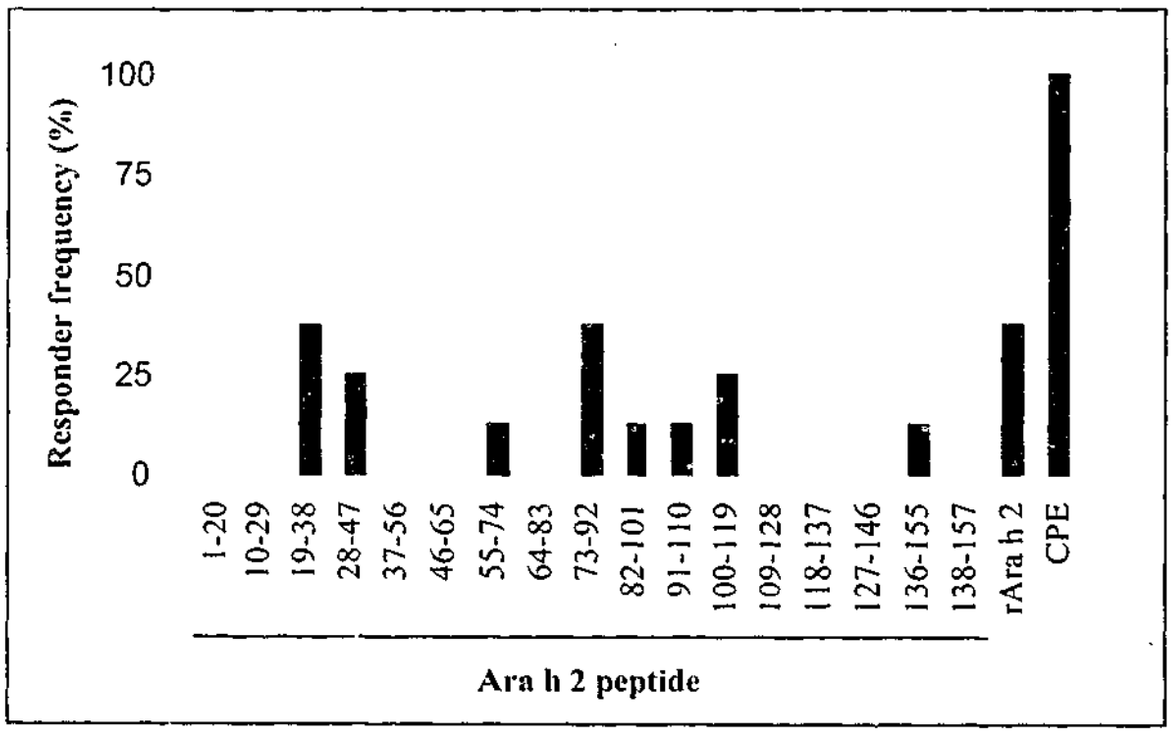

Figure 2 Percentage responder frequency to Ara h 2 peptides.

Table 3 IL-5 and IFN-γ production by CPE-specific TCL stimulated with Ara h 2 peptides

	Proliferation inducing peptides			Non-proliferation inducing peptides		CPE	
Subject	Peptide	IL-5	IFN-γ	IL-5	IFN-γ	IL-5	IFN-γ
1	19-38	2053	92	9	5	2004	157
2	19-38	3	UD	2	UD	4	UD
3	19-38	506	32	211	34	921	86
4	28-47	6	UD	3	UD	36	64
	73-92	UD	UD				
	91-110	2	UD				
	100-119	UD	UD				
	136-155	UD	UD				
5	28-47	UD	UD	UD	384	97	937
	118-137	UD	UD				
6	73-92	2	UD	UD	UD	6	UD
	100-119	UD	UD				
7	55-74	6	UD	3	36	3	91
	73-92	2	28				
8	82-101	UD	UD	UD	UD	UD	20

Supernatants were harvested at 48 h from T cell cultures and tested by ELISA for the presence of IL-5 and IFN-γ. Cytokine levels (pg/ml) induced by immunoreactive peptides, mean responses to the two unreactive peptides, and CPE are demonstrated. UD, undetectable.

Legends to Figures

Figure 1 Western blot for serum IgE reactivity to Ara h 2 of peanut allergic and non-peanut allergic subjects

M, molecular mass; NS, no serum.
Following resolution of CPE on a 14% polyacrylamide gel, proteins were transferred to nitrocellulose and probed with sera from the 8 peanut allergic subjects and 4 non-peanut allergic controls. IgE was detected using HRP-conjugated mouse anti-human IgE and enhanced chemiluminescence.

Figure 2 Percentage responder frequency to Ara h 2 peptides

Responder frequency of TCL from a panel of 8 peanut-allergic donors to individual Ara h 2 peptides. A stimulation index of ≥ 2.5 was considered positive and the number of donor TCL responding to each peptide is shown as a percentage.