H24/3406

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON..... 13 May 2003 .....
.....[redacted].....
Sec. Research Graduate School Committee

The human immune response to the latex allergen Hev b 5

Dr Michael F. Sutherland

M.B., B.S., F.R.A.C.P.

Department of Pathology and Immunology

Monash University

Submitted to Monash University in accordance with the requirements for the degree of Doctor of Philosophy February 2003

TABLE OF CONTENTS

Summary

Latex allergy is an immediate hypersensitivity to proteins from *Hevea brasiliensis* used in the manufacture of natural rubber latex (NRL). This disorder mainly affects health care workers (HCW). It can be associated with considerable morbidity, including urticaria, rhino-conjunctivitis, asthma and occasionally angioedema or anaphylaxis. Apart from allergen avoidance, no specific immunotherapy for latex allergy that is both safe and effective exists, and in rare cases, highly trained HCW may have to leave their profession because of this disorder. The latex allergen Hev b 5 was previously reported to react with IgE from 12/13 latex-allergic HCW, making it an important allergen to retain in future vaccine-based approaches to the immunotherapy of latex allergy. No information on T cell reactivity was previously described, information critical in producing novel, T cell-targeted immunotherapeutic approaches that improve safety but retain efficacy. In addition, previously reported monoclonal antibodies (mAbs) to Hev b 5 were not monospecific, hampering allergen-quantitation analysis of latex extracts, with important implications in both allergen avoidance and immunodiagnosis of latex allergy. This thesis describes the immune response to Hev b 5 in 32 latex-allergic subjects, predominantly HCW, and 19 control individuals. Examination of the humoral immune response to Hev b 5 within the study population confirmed Hev b 5 as a major latex allergen, with 17/32 (53%) of the latex-allergic subjects, but none of the control group, having Hev b 5-specific IgE. In addition, latex-allergic subjects with Hev b 5-specific IgE were often negative when assayed by currently available immunoassays for latex allergy, such as the Pharmacia UniCAP®, which utilises an unprocessed NRL as its capture antigen, suggesting that these assays may have reduced sensitivity because of lack of, or structurally different, Hev b 5. Characterisation of the human cellular response using overlapping, synthetic Hev b 5 peptides, indicated that 'ominant T cell determinants exist. More that 50% of the latex-allergic Hev b 5 peptide-responsive subjects showed reactivity to each of Hev b

5(1-20) and Hev b 5(46-65), indicating that these T cell determinants would be important to retain in any candidate vaccine. Moreover, in the case of Hev b 5(46-65), this reactivity was associated with strong IL-5 secretion. In addition to characterisation of the human cellular response to Hev b 5, murine Hev b 5-specific mAbs were prepared. These indicated that Hev b 5 is a relatively abundant component of NRL gloves compared with raw NRL extracts, suggesting a rational explanation for the frequency of sensitivity to this allergen among HCW. Taken together, the information presented in this thesis shows that Hev b 5 is a major latex allergen, relatively abundant in NRL gloves, and an allergen for which dominant T cell determinants exist, making it a rational target for novel immunotherapeutic approaches to the treatment of latex allergy.

Declaration

This thesis contains no material which has been accepted for the award of any 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.

Dr Michael F. Sutherland ....[redacted].....17/2/03

Acknowledgements

The work contained within this thesis would not have been possible without the contributions and support of many people.

Firstly, to my supervisors, Professor Robyn E. O'Hehir, Associate Professor Jennifer Rolland and Dr Cenk Suphioglu goes my sincere gratitude for their caring, vision, guidance and, above all, organisation.

To the scientists in Pathology and Immunology, and in particular those in the Allergy Research Group, who have cheerfully and unselfishly assisted me to learn the many practicalities and techniques of scientific enquiry, thankyou all. Special mention must go to Dr Harini de Silva and Ms Neeru Eusebius for showing me human tissue culture techniques and Dr Alec Drew for performing the cloning work for Hev b 5. Acknowledgement is also given to my collaborators, Dr Jay Slater, from the USA Food and Drug Administration who provided the Hev b 5/MBP construct and Drs Euan Tovey and Dr Teresa Mitakakis who performed the Halogen® assays for Hev b 5 aeroallergen analysis.

The National Health and Medical Research Council of Australia, the Alfred Research Trusts and the Co-operative Research Centres for Asthma are gratefully acknowledged for providing financial support.

To my family, wife Suzy, sons James and the PhD baby, William: thankyou always for your love, support, faith, and above all, patience.

To the patients with latex allergy who have taken part in this study: their participation and encouragement has been an inspiration and a privilege to behold, and it is to them that I dedicate this thesis.

1. Literature review

1.1. Introduction

Allergic diseases are among the most common causes of morbidity in the Western world, directly affecting more than 30% of young Australians (Robertson *et al.*, 1998). Their prevalence has increased dramatically during the late 20th Century in most Western countries (Beasley *et al.*, 2000), paralleling our increased wealth and improved general healthcare.

The concept of allergy was first proposed in 1906 by Von Pirquet, during his investigation of serum sickness reactions, where he defined it as "a change in the ability to react" of the body in response to foreign antigens that had previously caused no insult with the first inoculation (cited by Simons, 1994). Since then, the breadth of this definition has been somewhat narrowed to be generally defined as an inappropriate or harmful immune response to foreign substances that are otherwise not harmful to the body, mediated largely, though not exclusively by immunoglobulin (Ig) E (Arshad *et al.*, 2001). Such, however, is the modern emphasis on IgE-based mechanisms in allergy that the two are often used synonymously (Kay, 2001). Soon after the pioneering work of Von Pirquet, the first clinical trial of allergen specific immunotherapy (SIT) was performed (Noon, 1911). In many ways the clinical practice of SIT has changed little since then. In 1921, Prausnitz and Küstner discovered "reagin", a humoral factor that could mediate passive cutaneous anaphylaxis (cited by Simons, 1994). It was not until the late 1960s that this substance was isolated as IgE, a central discovery made independently by the Ishizakas and Johansson (cited by Simons, 1994). This allowed a rapid expansion of the understanding of the humoral immune response that leads to the end-effector response of allergy: the release of histamine and/or other inflammatory mediators by mast cells, eosinophils and basophils. With the discovery of monoclonal antibodies (Kohler and Milstein, 1975) and recombinant DNA techniques and the subsequent explosion in the

knowledge of cell surface receptors, cytokines and chemokines that this has allowed, the focus in allergy research has now moved to the role of the T cell and more recently the dendritic cell in the genesis and modulation of the allergic immune response. In addition, genetic engineering has allowed new insights *in vivo* in animal models into the roles of cytokines, chemokines and other mediators of allergic inflammation. More recently, this insight has been broadened with the advent of genetically engineered antibodies and recombinant cytokine receptors for human use that have seen use in trials of large numbers of patients. Supplementing this fundamental understanding has been large-scale epidemiological and genome based research that has provided critical information at a population level into the risk factors and causes of the "allergy epidemic".

Latex allergy provides an intriguing model of allergic disease. As will be discussed, it has appeared relatively late in the "allergy epidemic", not being reported in the Western medical literature until the late nineteen seventies (Nutter, 1979). It is a disease most prevalent among subjects with atopy, the genetic predisposition to form IgE to environmental allergens, with exposure to latex proteins pivotal. It has a propensity to cause the serious allergic syndromes of asthma and anaphylaxis. No safe SIT is available and it is a cause of significant morbidity and occasional mortality. Hev b 5 is a major latex allergen, but there were previously no published data on T cell responses to this allergen, information critical to the development of safe and effective SIT.

This chapter begins with a review of the immune response to allergens, with an emphasis on T cell studies in allergy. Following this, the risk factors, epidemiology and treatments of the clinical allergic syndromes are reviewed briefly. Finally, the latex allergy problem is addressed in detail and specifically the previous knowledge of the human immune response to Hev b 5.

1.2. The immune response to allergens

1.2.1. Allergens

To mount an immune response, the immune system must recognise a molecule as foreign. An allergen is an antigen; that is any substance capable of inducing an antibody response. In the case of an allergen, the response is allergic (IgE-based). Allergens are designated according to the accepted taxonomic name of their source as follows: the first three letters of the genus, then a space, followed by the first letter of the species, then a space, followed by an Arabic number. The numbers are assigned to the allergens in the order of their identification (King *et al.*, 1994). A major allergen is one defined as having IgE reactivity with more than 50% of a population of allergic individuals, whereas a minor allergen has IgE reactivity with less than 50% (King *et al.*, 1994). It is important to consider whether there are any intrinsic properties of allergens themselves that predispose to the development of allergic inflammation.

It is notable that allergens are often enzymes. For example, the major house dust mite (*Dermatophagoides spp*) allergen Der p 1 is a cysteine protease from mite faeces (Tovey *et al.*, 1981), that is able to disrupt epithelial cellular tight junctions increasing its availability to antigen presenting cells (APC) therefore increasing its immunogenicity (Wan *et al.*, 1999). It has also been shown to cleave the CD23 IgE receptor from B cells and the CD25 subunit of the T cell IL-2 receptor (Shakib *et al.*, 1998), resulting in a bias of T helper (TH) cell response toward TH2 (Ghaemmaghami *et al.*, 2001). Similarly, the major latex (*Hevea brasiliensis*) allergens Hev b 1, or rubber elongation factor, and Hev b 6.02, also known as hevein, are enzymes. Hev b 6 is of particular interest as it is a member of the plant enzyme family of defensins, the concentration of which is increased in latex sap by increased frequency of rubber tapping. This is one possible explanation for the increased allergenicity of rubber gloves during the late nineteen eighties, when demand for latex gloves outstripped supply (Subramaniam, 1995).

Alternatively, other allergens have physico-chemical characteristics, which make them preferentially available to the immune system for processing at mucous membranes (i.e. bioavailability). Examples of this include peanut (*Arachis hypogea*) allergens such as Ara h 2 which can survive the acid environment of the stomach with their IgE epitopes intact to be presented to the immune system in the small bowel (Maleki *et al.*, 2000) causing food allergy in susceptible individuals. Grass pollen allergens, contained within pollen grains of around 20 μm are delivered airborne and thus processed at the mucous membranes of the nasopharynx through inhalation. Because of their size, they are usually trapped by the nasal mucosa causing allergic rhinitis (Sutherland *et al.*, 1998), but in certain circumstances such as thunderstorms they can rupture to release allergenic starch granules of less than 2 μm that can penetrate to the lower airway bronchioles and cause asthma (Suphioglu *et al.*, 1992).

As will be discussed, latex allergens may be presented to the immune system through the skin (Belsito, 1990), adsorbed to glove donning powder and inhaled as aeroallergens (Lagier *et al.*, 1990), or ingested as glove protein-contaminated foods (Beezhold *et al.*, 2000) or cross-reactive fruit proteins (Blanco *et al.*, 1994).

In general however, it appears that host factors are more important in determining the TH1/TH2 bias in the immune response to allergens, rather than intrinsic properties of the allergens themselves. In addition to these host factors, low antigen dose (Sakai *et al.*, 1999) with repeated exposure often via the respiratory tract or skin (Nelde *et al.*, 2001), as seen in latex allergy, are important in the genesis of TH2 responses.

1.2.2. The generation of allergic inflammation

Allergic inflammation begins from an acquired immune response resulting in the generation of IgE. Particularly early in life, the innate immune response, via its recognition of environmental danger signals (Matzinger, 1994), is critical in influencing this acquired response,

and, together with genetic factors modulates the cytokine milieu that is responsible for the commitment of T helper cells to TH1 or TH2 (Kay, 2001). This commitment is central to the genesis of allergic inflammation.

Once a commitment to TH2 has occurred, IgE production is established to the allergen; thus on subsequent re-exposure and cross-linking of effector cell-bound IgE with pro-inflammatory mediator release, the clinical manifestations of allergy occur (Figure 1.1). The manifestations or syndromes of allergy themselves are dependent on the anatomical location of this inflammatory reaction, and include allergic asthma (airways), allergic rhinitis (nasal mucosa), allergic eczema or urticaria (skin) and anaphylaxis (systemic blood vessels).

Allergic inflammation can be divided into an early phase and a late phase response. The early phase response is seen in, for example, skin tests, and consists predominantly of mast cell-bound IgE molecules contacting their ligand allergen, cross-linking and initiating the intracellular signalling cascade from the high-affinity IgE receptor (FcεRI) that causes release from the mast cell of preformed inflammatory mediators. Histamine is the predominant agent that results in the increase in vascular permeability and the resultant characteristic wheal and flare reaction seen within the skin. Mast cells also release leukotrienes, prostaglandins, neutral proteases, neuropeptides and cytokines (Busse and Lemanske, 2001). These result in the recruitment of lymphocytes, eosinophils and basophils that are then responsible, through their mediator release, for the late phase response. The late phase allergic response is of particular importance in asthma, where it is associated with bronchoconstriction, airway oedema, mucus hyper-secretion and bronchial hyper-reactivity (Busse and Lemanske, 2001). This so-called late asthmatic response, has been found in animal models to be critically dependent on IL-5 and the influx of eosinophils (Foster *et al.*, 1996). It is likely that the situation in humans is more complex. In addition to the influx of eosinophils and

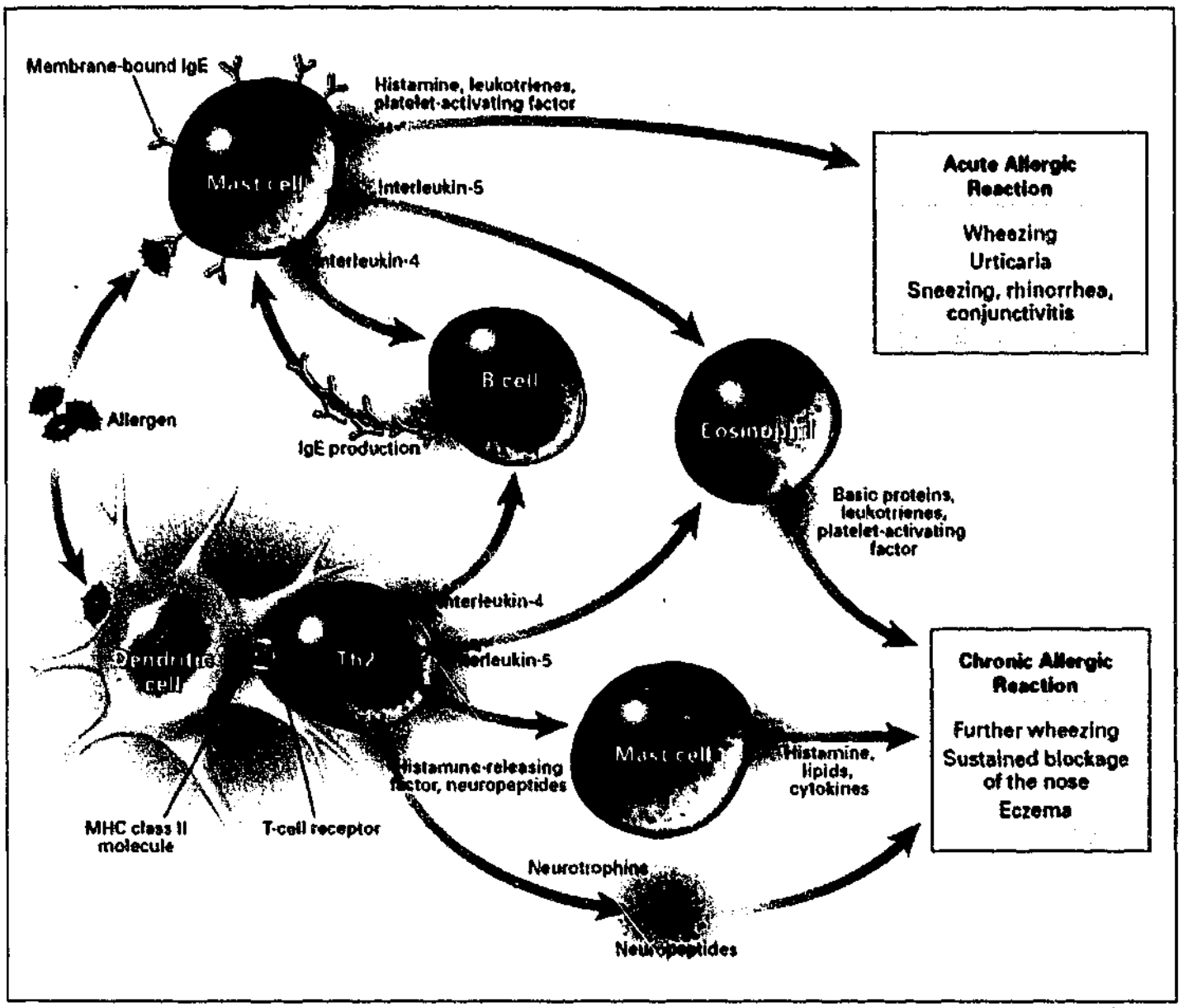


Figure 1.1 Overview of allergic inflammation (Kay, 2001)

basophils, T cells may produce a late phase response independent of IgE (Haselden *et al.*, 1999).

If there is continuing allergen or antigen exposure during the late phase response, allergic inflammation can become chronic, whereby in addition to the late phase changes discussed, there is associated tissue damage from such proteins as eosinophilic cationic protein. This is seen particularly in asthma, with airway epithelial denudation, smooth muscle hypertrophy and sub-epithelial fibrosis (Busse and Lemanske, 2001). It is in this chronic inflammatory state that the original TH2 bias of the antigen-specific TH cell may become less important and that in addition to allergen exposure, ongoing inflammatory processes may be exacerbated by non-specific stimuli such as cigarette smoke or by TH1 stimuli such as viral infections or endotoxin exposure, leading to exacerbation of clinical disease.

1.2.3. T lymphocytes

T cells represent a critical part of the adaptive immune response. Unlike B cells, T cells recognise antigen as peptide expressed with cell surface molecules. These cell surface molecules are the MHC, also known as the human leukocyte antigen (HLA) system in humans. These are glycoproteins which associate with the peptide antigens on the cell surfaces of APC for presentation to T cells. There are two classes of MHC molecules, Class I and II and these restrict two functional T cell subsets in the type of antigens they recognise. Class I MHC is present on most cells and is important for the presentation of foreign intracytoplasmic antigens such as viruses. Peptides generated from such organisms and presented with Class I MHC are recognised by CD8+ T cells. Class II MHC is present mainly on antigen presenting cells and other immune cells such as thymic epithelial cells and is important in presentation of extracellular proteins such as allergens and also intracellular bacteria and parasites. Peptides generated from the processing of such organisms are presented in association with Class II molecules and are recognised by CD4+ T cells. It is these cells

therefore that are central to the genesis of allergic inflammation. There is some recent evidence that antigen at high concentration can be "cross-presented" to CD8+ T cells via the MHC Class I pathway (Kita *et al.*, 2002). Whether such cross-presentation is important in SIT remains to be determined. This review will concentrate on MHC Class II presentation and the CD4+ T cell system.

1.2.3.1. Antigen presentation to T cells

MHC Class II molecules consist of two polypeptide chains, α and β, which form a peptide binding cleft. MHC Class I, however is associated with β_2 microglobulin and binds peptides of between eight to ten amino acids in length. MHC Class II binds longer peptides of at least 13, though usually not greater than 17, amino acids in length (Janeway *et al.*, 2001). The crystal structures of both MHC Class I (Bjorkman *et al.*, 1987) and Class II (Brown *et al.*, 1993) have been resolved.

Peptides presented by MHC Class II molecules are generated in intracellular vesicles (Janeway *et al.*, 2001). The three classes of professional APC, macrophages, dendritic cells (DC) and B cells take up extracellular proteins by phagocytosis or micropinocytosis and internalise them into these acidified intracellular vesicles. They are then cleaved into peptide fragments by proteases in the proteosome and bind to newly synthesised MHC Class II molecules. This complex is then presented on the cell surface for recognition by T lymphocytes.

Recently the MHC II–peptide-T cell receptor (TCR) interaction has been clarified (Figure 1.2), with the co-crystallisation of a murine MHC Class II molecule, TCR from the same mouse strain and peptide fragment of conalbumin (Reinherz *et al.*, 1999). This has shown that the peptide is bound within the MHC peptide groove at an orthogonal angle (80°) compared with the diagonal angle of peptide MHC Class I interactions. Additionally, the COOH terminus of the peptide extends beyond the end of the peptide groove and has no contact with MHC or TCR. Peptide binding occurs through the first 8 residues (p1 to p8) and

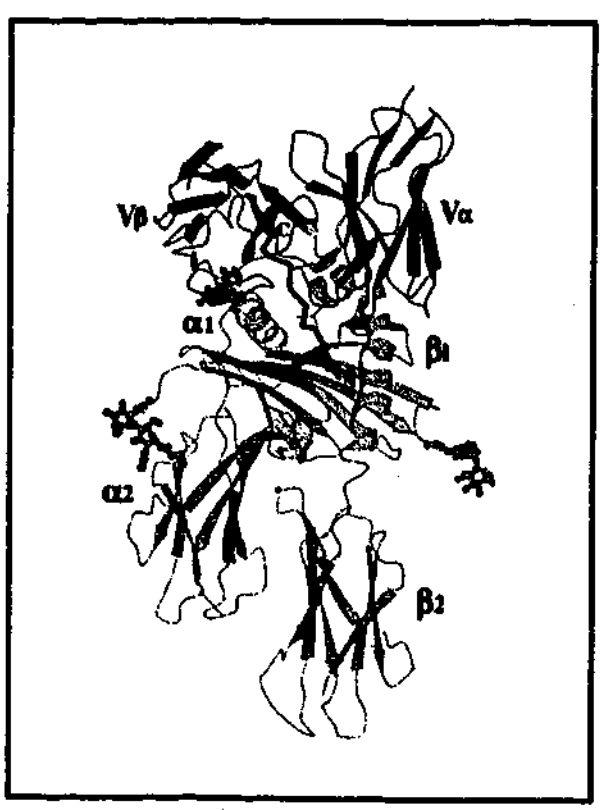


Figure 1.2 Ribbon diagram of TCR-peptide-MHC II interaction (Reinherz et al., 1999). MHC II is shown by yellow ribbons, TCR by green and blue ribbons. The peptide is shown as red.

is dominated by interaction with the Vα TCR domain and the β1 domain of the MHC Class II (Reinherz *et al.*, 1999).

The human MHC, the HLA, is a polygenic and highly polymorphic glycoprotein system pivotal to the successful generation of acquired immune responses.The HLA is encoded predominantly on Chromosome 6 (the β chain for Class I is encoded on chromosome 15). Its polygenic and polymorphic nature ensure that both at an individual and herd level there is enough variety of HLA molecules to associate with the endless number of possible peptides generated by processing the myriad potential pathogens (and allergens) faced by the immune system.

Certain HLA Class II genotypes have been associated with allergic disease. For example, HLA-DQB1*0301 has been associated with grass pollen allergy, HLA-DRB*08 with peanut allergy and HLA-DRB1*12 with carrot allergy (Boehncke *et al.*, 1998). In latex allergy, associations with serotypes DR4 and DQ8 have been found for IgE reactivity to the latex allergen Hev b 6 (Rihs *et al.*, 1997), while for IgE reactivity to Hev b 1, association with DR7 has been observed among spina bifida patients (Rihs *et al.*, 1998). It is generally accepted, however that these HLA associations with allergic disease are weak and that promiscuous peptide binding to different HLA haplotypes occurs (Counsell *et al.*, 1996). Large control populations are required to obtain meaningful results in studies of HLA specificity, because some HLA serotypes such as DPw4 occur in around 78% of Caucasians (Baselmans *et al.*, 2000).

1.2.3.2. Antigen recognition by T cells: the TCR

The TCR recognises the HLA molecule in association with its ligand peptide. In the case of CD4+ lymphocytes, antigen recognition occurs through HLA Class II molecules in association with peptide.

To ensure adequate diversity of acquired immune responses to external challenges, the TCR genes undergo rearrangement during T cell development, increasing the possible diversity of TCR to

approximately 10^6. Similarly to the HLA polymorphisms, the majority of the diversity in the TCR resides in the peptide binding region (Janeway *et al.*, 2001).

1.2.3.3. T cell activation

In addition to TCR engagement with the MHC/peptide complex, additional costimulatory signals are required for T cell activation. These include B7.1 (CD80) and B7.2 (CD86), which both interact with CD28 on T cells. These interactions result in effective transduction of the TCR receptor signal and generate T cell division and effector cell commitment. All of CTLA-4 (Keane-Myers *et al.*, 1997), CD80 (Harris *et al.*, 1997) and CD86 (Tsuyuki *et al.*, 1997) have been proven to be necessary for the successful generation of allergic inflammation in murine asthma models. In addition to the abovementioned, there are costimulatory signals and adhesion molecules such as LFA-1 (Faith *et al.*, 1995) and others which contribute to T cell activation.

Engagement by TCR of the MHC/peptide complex in the absence of costimulation results in anergy in human T cells (Gimmi *et al.*, 1993). It is possible that one of the means of efficacy of SIT is through the latter mechanism, where high dose, soluble antigen is delivered in the absence of the danger signals, meaning DC do not migrate to lymph nodes and up-regulate CD80, CD86 and MHC Class II, therefore resulting in anergy of allergen-specific T cells.

1.2.3.4. T cell effector cell subsets: the TH1/TH2 paradigm

Following activation of naïve T cells, their commitment to either TH1 or TH2 type is fundamental to our understanding of the genesis of allergic inflammation. These functional effector T cell subsets are defined by their cytokine profiles. TH1 refers to an IL-2 and IFN-γ dominant response, whereas TH2 refers to an IL-4, IL-5 and IL-13-dominant cytokine profile. These subsets were first described in mice (Mosmann *et al.*, 1986). They have also been demonstrated in humans (Parronchi *et al.*, 1991), but the clarity of the distinction between TH1

and TH2 has been less marked. Many clones have been found to secrete a mixed cytokine profile, and these cells have subsequently become known as TH0 (Liew, 2002). Interestingly, the commitment to TH1 or TH2 has been found to be reversible *in vitro* in studies of human TCC, but it is less sure whether this is the case *in vivo* where commitment appears long lasting. The major signals that cause commitment of naïve T cells to one subset or the other are the subject of intense research, and are crucially dependent on the signals received by the naïve TH cell from the APC on first exposure to antigen – that is the cytokine milieu (Figure 1.3). In favouring TH2 these include decreased sensitivity to, or levels of, IL-12, or increased sensitivity to, or levels of, IL-4; elevated PGE2 , histamine or cyclic AMP; dendritic cell type (DC2); low antigen dose (Sakai *et al.*, 1999); low affinity TCR interaction; MHC haplotype; costimulatory signal type; respiratory tract or epicutaneous sensitisation route (Nelde *et al.*, 2001) and IgE-mediated antigen presentation. Which of these stimuli is the most important *in vivo* in humans is as yet unknown. It is likely to be a multifactorial combination of genes and environment that create the cytokine mileu necessary for the generation of TH2 responses.

1.2.3.5. The TH3 paradigm: regulatory T cells

A recently proposed extension to the TH1/TH2 paradigm has been the concept of TH3. These are so-called "regulatory T cells" that secrete an IL-10 and transforming growth factor beta (TGF-β)-dominated cytokine profile, with little or no IL-4 secretion (Levings and Roncarolo, 2000). This regulatory T cell population is able to ablate established airway inflammation in a murine ova asthma model, a mechanism mediated in part by IL-10 (Cottrez *et al.*, 2000). TGF-β may also be an important regulatory cytokine secreted by these cells, as in murine asthma models, T cells transfected with this gene to express it constitutively can successfully ablate established TH2 inflammation (Hansen *et al.*, 2000). Additionally murine TGF-β knockouts die at a young age of uncontrolled inflammation (Kulkarni *et al.*, 1993), likely due to increased inducible nitric oxide synthase

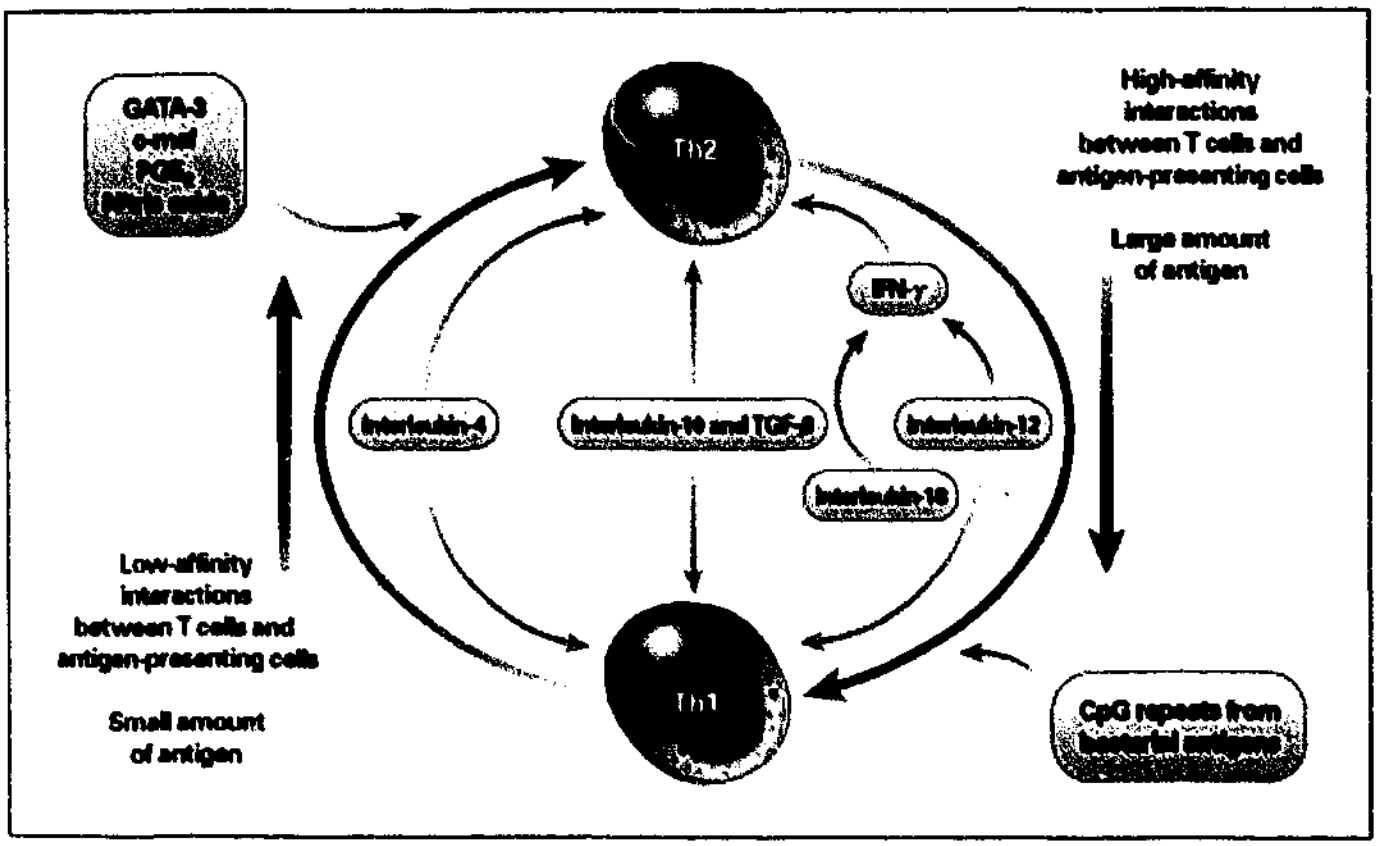


Figure 1.3 Factors influencing the commitment of naïve T cells to TH1 or TH2 (Kay, 2001).

(Vodovotz *et al.*, 1996). These antigen-specific regulatory cells may be absent or reduced in allergic inflammation and represent a distinct population, particularly important within mucosal cell populations (Levings and Roncarolo, 2000). The role of these regulatory cell populations, such as CD4+CD25+ subset and other regulatory T cells, described for control of autoimmune and other inflammatory condiditions, is not yet clear in allergy.

1.2.3.6. T cell epitope mapping studies in allergy

An epitope is an antigenic determinant. As discussed above, for T cells these are linear peptide fragments processed from the whole antigen and presented with MHC Class II molecules on the surface of APC. In order to document the T cell response to various allergens as a prelude to developing vaccine approaches to therapy, T cell epitope mapping is a valuable experimental technique.

Numerous studies have identified T cell epitopes in diverse allergens. A selection of studies in common allergens is discussed below in some detail to highlight the range of methodology and results. The few studies of latex allergens are discussed in the section on latex allergy. The following review is not comprehensive, however some common themes emerge, which are summarised at the end of this section.

T cell epitope mapping studies have been performed for the major honeybee venom allergen phospholipase A_2 (PLA_2) since 1992 (Dhillon *et al.*, 1992). It is a well characterised allergen (King and Spangfort, 2000), and honeybee venom allergy is potentially fatal (Harvey *et al.*, 1984). In addition, anaphylaxis is often associated with honeybee venom SIT (Westall *et al.*, 2001). The latter two aspects are in common with latex allergy. In the first study of T cell epitope mapping in honeybee venom, Dhillon *et al* made short term T cell lines (TCL) against purified PLA_2 (0.2 μg/ml) from one honeybee venom allergic individual, showing 2 dominant epitopes (Dhillon *et al.*, 1992). In a larger epitope mapping study using 13 PLA_2-specific T cell clones (TCC), Carbadillo *et al* found that 8/13 clones each recognised 1 of 3

(from a total of 62) dodeca-peptides of PLA_2 and there was no difference between allergic and non-allergic (sensitised) individuals in the pattern of recognition (Carballido *et al.*, 1993). This was confirmed by then testing the dodeca-peptides in peripheral blood mononuclear cell (PBMC) culture, showing that the dominant peptides identified by the T cell cloning were able to cause the greatest proliferation amongst PBMC. Furthermore, no additional epitopes were found using the PBMC proliferation assays (Carballido *et al.*, 1993).

In contrast to this, Kammerer *et al* found that short peptides were less sensitive than long peptides in determining proliferation responses (Kammerer *et al.*, 1997). Using 40-60mer overlapping peptides they were able to elicit proliferation responses in patients that had been unresponsive to 12mer peptides. They did not discover a few dominant epitopes; rather this study showed T-cell responses to multiple epitopes in several patients.

The major cat allergen Fel d 1 has also been studied in T cell epitope mapping studies. Counsell et al used short term TCL stimulated with affinity purified Fel d 1 (10 μg/ml) to test response to overlapping peptides (15-17mers with 7 amino acid overlap) of the two chains of Fel d 1 (Counsell *et al.*, 1996). T cell reactive regions were found throughout the molecule, but particularly in the N-terminal region of chain 1 of the Fel d 1 protein. They found in addition that primary *in vitro* T-cell responses to Fel d 1 peptides were weak, necessitating the use of short term TCL. Importantly also, they found that there was a wide diversity of MHC class II types in this relatively large study of 53 patients. More recently, Haselden *et al* used PBMC assays to investigate the T-cell epitopes of chain 1 of Fel d 1, with particular reference to cytokine production (Haselden *et al.*, 2001). This study also found that T-cell epitopes were located throughout the molecule, and additionally, that cytokine production was dissociated from proliferation. One problem with this study was that proliferation responses were generally weak and therefore each assay had to be

performed in eight replicates with a resultant heavy reliance on statistics to show differences. In addition a relatively high dose of peptides (100 µg/ml) was used and this may have resulted in more non-specific stimulation of low-affinity TCC not directly involved in the allergic immune response *in vivo* and in addition a TH1-skewed cytokine response because of the high dose possibly eliciting TH2 cell anergy.

Grass pollen allergens are an important cause of seasonal allergic rhinitis and asthma and their use is well established for SIT of these conditions (Bousquet, 2001). In their study of PBMC responses to purified and crude rye grass extracts, Bungy et al found responses to the peptides of Lol p 1 to be localised to pool 17 which induced positive responses in 5/6 donors studied (Bungy *et al.*, 1994). The peptides used were 12mer peptides overlapping by 2 amino acids each (therefore each pool spanned 22 amino acids). This pool was then further "decoded" to determine the T cell epitope and two adjacent peptides of the pool were found to be reactive, spanning amino acids (193-206). The study used 12-24 replicates for each well and Poisson statistics for analysis of significance with any well greater than 3 standard deviations greater than control being assigned significance. Also of note in this study was that atopics had a greater response to all antigens utilised than non-atopics, while non-atopics responded only to the crude rye grass pollen antigen and not at all to purified Lol p 1 or the peptides. A second, larger study using TCL and TCC specific for Phl p 5 found that there were more (19) T cell epitopes in Phl p 5, with 81% of mapped T cells recognising three T cell reactive regions (Muller *et al.*, 1996). A recent Australian study using overlapping 20mer peptides of the major ryegrass allergens Lol p 1 and Lol p 5 again found reactivity spread throughout the whole molecule, with a few peptides showing dominance (Burton *et al.*, 2002).

Also of interest in grass pollen allergy is the notion of cross-reactivity, both between allergens and between grass species, and this

has been examined at a T cell epitope level with grass pollen-specific TCC. This has shown that TCC raised against the major Timothy grass pollen allergens Phl p 1 and 5 showed recognition of recombinant Phl p 5 isoallergens (van Neerven *et al.*, 1999; Wurtzen *et al.*, 1999). Further evaluation with HLA-DP, DQ and DR-specific inhibitory monoclonal antibodies showed that all these HLA types were involved in the recognition of these grass pollen allergens.

Further studies have confirmed the "promiscuous" binding characteristics of T cell epitope peptides from ryegrass pollen proteins. Lol p 5 peptides synthesised from predicted antigenic sites using the TEPITOPE program were able to stimulate TCC from subjects with diverse MHC haplotypes (de Lalla *et al.*, 1999). In addition, using both TCL and TCC to determine the T cell epitopes of Lol p 1 using synthetic overlapping peptides (20mers) 16/23 peptides were recognised by one or more of the nine donors and there was no correlation between epitope specificity, cytokine secretion or atopic status (Spiegelberg *et al.*, 1994).

House dust mite (HDM) allergens from *Dermatophagoides pteronyssinus* and *D. farinae* are an important cause of perennial rhinitis and have also been extensively studied for T cell reactivity. Using PBMC and TCC to evaluate the T cell response to Der p 2, O'Hehir *et al.* found that all peptides caused significant proliferation in one or more subjects but that 2 peptides (61-86; 78-104) were recognised by 16 of 18 individuals. Similar proliferative responses were seen in HDM-allergic and non-allergic subjects (O'Hehir *et al.*, 1993). In fact, some workers have found that T cells from most subjects, regardless of atopic state will proliferate to house dust mite antigens (Upham, 1997). This was also observed in another study, showing reactivity throughout the whole molecule of Der p 2 with heterogeneity seen between donors (van Neerven *et al.*, 1993). Echoing the observations in the T cell responses to grass pollen allergens, Der p 1-specific TCC can proliferate in response to its ligand peptide (Der p

1, 110-143) when presented by different HLA specificities (Yssel *et al.*, 1992; Verhoef *et al.*, 1993; Higgins *et al.*, 1994). In addition, peptide or HLA specificity does not influence cytokine secretion profile of TCC when stimulated by Der p 2 (van Neerven *et al.*, 1994).

Japanese cedar pollen is an important cause of seasonal allergic rhinitis in Japan. Its two major allergens Cry j 1 and Cry j 2 have been evaluated in T cell epitope studies. In a study of 18 subjects with Japanese cedar pollinosis, short term TCL were generated using purified Cry j 1 or Cry j 2. These lines were then stimulated with peptides (15mers with 1 amino acid overlap). They demonstrated 6 peptides which caused proliferation in 50% or more of donors. In all, 72% of the peptides were recognised as T cell epitopes by the TCL (Sone *et al.*, 1998). Also of interest, it has been shown that altered peptide ligands induce differential effects on Japanese cedar pollen specific TCC (Ikagawa *et al.*, 1996). A single amino acid substitution from ^{339}threonine to valine caused increased IFN-γ production whereas substitutions of glycine or glutamine for the same residue induced TCR antagonism without change in MHC binding affinity.

Finally, two recent studies looking at the major Trichophyton allergen Tri r 2 are of interest because they have found that T cell responses to this allergen are mediated by two distinct T cell repertoires. That is, subjects with immediate hypersensitivity responses to Trichophyton recognised predominantly different epitopes from subjects with delayed type hypersensitivity to trichophyton (Woodfolk *et al.*, 2000). A dominant T cell epitope was found in subjects with delayed type hypersensitivity reactions, and was associated with diverse MHC haplotypes (Woodfolk *et al.*, 2000).

In summary, T cell epitope mapping of allergens has revealed that most allergens have more than one epitope in the context of a population of allergic individuals. Usually, there are one or more dominant epitopes (defined as a peptide showing significant proliferation with more than 50% of subjects on a population basis) present within the allergen

molecule of interest. These epitopes are usually the same for allergic and non-allergic individuals (except in the tricophyton data). The concept of promiscuity of peptides (that is, peptides being able to associate with and be presented by more than one MHC molecule) illustrates the lesser rigidity defining peptide binding seen with MHC Class II presentation when compared with Class I. It also appears that longer peptides induce a greater proliferative response. This may well be because very long peptides may contain multiple T cell epitopes. In addition, the T cell epitope studies presented have generally shown that new epitopes are not found whether clones, TCL or PBMC are used. The former two methods raise the possibility of *in vitro* bias meaning that epitopes may be lost or alternatively artificially generated in their utilisation, but have the advantage of an enriched antigen specific population and thus a larger signal. The latter method avoids the *in vitro* bias but may have problems of its own, in particular the low signal: noise ratio necessitating multiple replicates and high antigen doses which may bias the observed cytokine responses.

1.2.4. Cytokines in allergic inflammation

Cytokines are proteins or glycoproteins secreted by a wide variety of cells both immune and non-immune, that signal between cells (paracrine function), stimulate the cell that secreted them (autocrine function) or signal to cells in distant body sites (endocrine function) such as the bone marrow (Chung and Barnes, 1999). There are many cytokines that play a role in allergic inflammation (see Table 1.1). The following cytokines presented here in detail have been selected for discussion because of their central importance in the genesis, maintenance or control of allergic inflammation. Additionally, however, it is critical to appreciate that cytokines act in a network, increasing the number of possible effects from interaction considerably. As will be shown, a common theme is that of redundancy; that is, different cytokines performing similar functions.

Table 1.1 Cytokines involved in allergic inflammation (adapted from Busse and Lemanske, 2002)

CYTOKINE	EFFECTS OR FUNCTION
BFGF	Stimulation of the production of fibroblasts, matrix
G-CSF	Maturation, differentiation of neutrophils
GM-CSF	Proliferation, differentiation, activation of eosinophils, neutrophils, macrophages
IFN-α	Inhibition of viral replication
IFN-β	Inhibition of viral replication
IFN-γ	See text
IL-1	Production of cytokines by CD4+ T cells, proliferation and differentiation of, and Ig production by, B cells
IL-2	Clonal expansion of, and cytokine secretion by, antigen specific CD4+ T cells
IL-3	Proliferation and differentiation of haemopoetic stem cells
IL-4	See text
IL-5	See text
IL-6	Maturation of B cells to plasma cells and class switching to IgA; production of IL-1 and TNF-α
IL-7	Proliferation of T and B cell progenitors and activated T cells
IL-8	Directed migration of neutrophils
IL-9	Enhancement of B cell response to IL-4
IL-10	See text
IL-11	Similar to IL-6
IL-12	See text
IL-13	See text
IL-14	Expansion of B cell clones and suppression of Ig secretion
IL-15	Enhancement of cytotoxicity of CD8+ T cells and NKT cells, expression of ITAM-3
IL-16	Chemoattraction and activation of CD4+ T cells
IL-17	Proliferation of CD4+ T cells
IL-18	Similar to IL-12
M-CSF	Differentiation of monocytes
PDGF	Proliferation of smooth muscle cells and fibroblasts
Stem-cell factor (c-kit ligand)	Chemoattraction and proliferation of, and histamine release by, mast cells

1.2.4.1. IL-4

IL-4 occupies a central role in the generation of TH2 responses that orchestrate allergic inflammation. IL-4 is generated by T cells, NK cells, mast cells, eosinophils and basophils. It commits naïve T cells to a TH2 phenotype whereby they secrete predominantly IL-4, IL-5, and IL-13 and have a decreased ability to secrete IFN-γ (Tepper *et al.*, 1990). IL-4 is critical for IgE production (Vercelli *et al.*, 1989) and initiates Ig class switching to IgE and promotes the survival of T lymphocytes by preventing apoptosis (Janeway *et al.*, 2001). It also upregulates both the high affinity IgE receptor (Gosset *et al.*, 2001) and CD 23 (Yokota *et al.*, 1988) and increases transcription of the adhesion molecule VCAM1 and the chemokine eotaxin (Mochizuki *et al.*, 1998) which is chemotactic for eosinophils. IL-4 also promotes eosinophil survival by inhibiting apoptosis and increases mucus hyper-secretion and bronchial hyper-reactivity and enhances mast cell mediator release (Ochi *et al.*, 2000). This cytokine shares many of these biological functions with IL-13, with which it shares significant homology. Transgenic mice over-expressing IL-4 develop elevated IgE and an inflammatory state similar to that in allergic inflammation (Tepper *et al.*, 1990). Murine IL-4 knockout models do not produce IgE (Grunewald *et al.*, 1998) and are largely unable to develop TH2 type T cell responses (Kopf *et al.*, 1993). Similarly, mice treated with anti-IL-4 antibodies have markedly attenuated responses to allergens and decreased eosinophilia, but retained bronchial hyper-reactivity (Hogan *et al.*, 1998). In human trials, IL-4 receptor antagonists have proven more effective than placebo in the treatment of allergic asthma but are unlikely to have a major clinical role in asthma treatment, largely because of their expense and the safety and efficacy of existing treatments such as inhaled corticosteroids (Borish *et al.*, 1999).

1.2.4.2. IL-5

IL-5 is produced predominantly by T cells and is an important cytokine in TH2 responses. It has endocrine function as it causes the division

and migration of eosinophils from the bone marrow. IL-5 deficient mice mount poor IgE responses that are characterised by a lack of eosinophilic inflammation and bronchial hyper-reactivity (Foster *et al.*, 1996). Human trials of anti-IL-5 antibodies have, however, so far been disappointing (Leckie *et al.*, 2000). Although there was complete abrogation of blood eosinophilia and marked reduction in BAL eosinophils, the early and late responses to allergen challenge among asthmatics were not ablated, possibly because of the persistence of some tissue based eosinophils within the lung compartment (Leckie *et al.*, 2000).

1.2.4.3. IL-13

IL-13 has significant homology to IL-4. Having many similar functions to IL-4 (such as IgG class switching to IgE and adhesion molecule up-regulation) it is an example of the redundancy that exists among cytokines (Chun and Barnes, 1999). There is also evidence however, of a synergistic effect between IL-4 and IL-13 in the generation of TH2 responses (McKenzie *et al.*, 1999). In addition, like IL-4, IL-13 has TH2 regulatory properties, through suppression of IL-12 secretion from PBMC *in vitro* (Minty *et al.*, 1993; D'Andrea *et al.*, 1995). One important difference from IL-4 is that IL-13 appears critical for the generation of bronchial hyper-reactivity in mouse models of asthma (Mattes *et al.*, 2001; Walter *et al.*, 2001).

1.2.4.4. IL-10

IL-10 is an important regulatory cytokine, produced by both TH1 and TH2 committed lymphocyte clones and able to down-regulate both established TH1 and TH2 mediated inflammation (Del Prete *et al.*, 1993). It interrupts the CD 28 costimulatory pathway and thus produces an anergic state in T cells it acts on (Joss *et al.*, 2000). Murine IL-10 knockouts develop an inflammatory bowel disease similar to human Crohn's disease (Madsen, 2001). Interestingly, this disease in the IL-10 deficient mouse does not develop when the animals are raised in germ-free conditions and can be modified by early antibiotic treatment or the

use of probiotics which favourably alter the bowel flora (O'Mahony *et al.*, 2001) both suggesting dysregulation of the immune response to normal bowel flora. Probiotics show promise in the prevention of allergy in humans (Kalliomaki *et al.*, 2001). A recent epidemiological study of 520 Gabonese schoolchildren found that chronic parasitic infection and resultant high levels of IL-10 were associated with a decrease in atopy, suggesting again an immune-regulatory role for this cytokine (van den Biggelaar *et al.*, 2000) The generation of regulatory T cells and increased IL-10 secretion are also believed to be critical in the operation of successful allergen immunotherapy (Akdis and Blaser, 2001). Tempering this anti-allergic promise of IL-10 however is the interesting observation that IL-10 is necessary for the development of bronchial hyper-reactivity, with IL-10 knockouts developing pulmonary inflammation but not airway hyper-reactivity in a murine ovalbumin-sensitisation model of asthma (Makela *et al.*, 2000) and there appear to be some differences in the effects of IL-10 between mice and humans (Akdis and Blaser, 2001).

1.2.4.5. IFN-γ

IFN-γ is an important cytokine in the development of TH1 mediated responses. There is compelling evidence from studies of PBMC from children, that a large part of the atopic diathesis is due to a defect in the signalling pathway leading to IFN-γ production (Tang *et al.*, 1993) especially in response to danger signals such as LPS (Pohl *et al.*, 1997). It has also been shown that children who have decreased IFN-γ at birth when followed prospectively are much more likely to be atopic on testing one year later (Tang *et al.*, 1994). There is also evidence, that at least in some subjects with reduced IFN-γ secretion, that this is due to defects in the IL-12-IFN-γ signalling pathway (Shikano *et al.*, 2001) such as mutations in the β chain of the IL-12 receptor (Kondo *et al.*, 2001).

In humans the IFN-γ gene is highly conserved (Hayden *et al.*, 1997) with polymorphisms not detected. Murine IFN-γ knockouts are

protected from endotoxic shock (Car *et al.*, 1994), but deficiency in humans usually leads to lethal cell-mediated immune deficiency. Recombinant IFN-γ (r IFN-γ) shows efficacy in the treatment of atopic dermatitis (Hanifin *et al.*, 1993) however in a murine model of asthma, local IFN-γ gene delivery is more effective than rIFN-γ treatment (Dow *et al.*, 1999).

1.2.4.6. IL-12

Along with IFN-γ, IL-12 is critical for the generation of TH1 responses (Magram *et al.*, 1996) and is thus an "anti-allergic" cytokine. Individuals with multiple sclerosis have a low incidence of allergic disease and enhanced IL-12 responses (Tang *et al.*, 1998). Therefore, administration of IL-12 to treat allergy was a logical step. In human trials, however, it has shown evidence of severe toxicity with the death of two patients in a phase II trial of its use in advanced malignancy. This was thought to be due to IFN-γ release (Leonard *et al.*, 1997). When used as systemic therapy in asthma, again toxicity was noted (cardiac arrhythmias and flu-like symptoms), and although it decreased eosinophilia and markers of airway inflammation, it did not abrogate bronchial hyper-reactivity or the late phase asthmatic response to allergen (Bryan *et al.*, 2000). Topical delivery may be necessary to avoid toxicity in humans, and such an approach has been successful in murine asthma models by abrogating established inflammation (Kips *et al.*, 1996).

1.2.5. Chemokines in allergic inflammation

Chemokines are a class of cell signalling molecules involved in the attraction and activation of cells to sites of inflammation. There are numerous chemokines believed to be important in allergic inflammatory disorders such as asthma (Busse and Lemanske, 2001). The receptors for some, such as CCR3, the receptor for eotaxin and RANTES are the subject of intense investigation for antagonists (Naya *et al.*, 2001) for the treatment of allergic diseases such as asthma. The chemokine CCR3 is attractive, because it is expressed on both TH2

cells and eosinophils, basophils and mast cells (de Paulis *et al.*, 2001) and so inhibitors may be capable of down-regulating allergic inflammation as a whole.

1.2.6. The epidemiology of allergic disease

Allergic disease has been increasing, particularly in the late 20th century, and nowhere more than in Australia. Allergic rhinitis, the allergic disease *par excellence*, used to be a disease of the landed gentry in the 19th century (Simons, 1994), but now afflicts 19.6% of 13-14 year old Australians (Robertson *et al.*, 1998). Similarly, wheeze (29.4%) and eczema (9.7%) are also highly prevalent among the same group (Robertson *et al.*, 1998). This trend has been noted throughout Western developed nations (ISAAC, 1998), and also in an older (20-45y) age-group within these countries (ECRHS, 1996), suggesting that factors peculiar to the Western lifestyle impact strongly on the generation of allergic disease. These are discussed below. Additionally, genetic factors are important, and a number of these have been identified as risk factors for allergic disease and it is this interplay between genes and environment that is crucial.

1.2.7. Genetic risk factors for allergic disease

Prospective twin studies using monozygotic and dizygotic twins have been useful in confirming the clinical observation that allergy "seems to run in families". Overall the estimated heritability of the allergic conditions of hay fever and asthma is estimated at between 60 and 70% (Duffy *et al.*, 1990). The risk of allergy for a newborn is 20% in the general population, 50% if one parent has allergy and 66% if both parents have allergy (Cogswell, 2000). There is also evidence that there is a greater risk of transmission of atopy if the mother is atopic and this risk goes beyond the in utero environment. This is felt to result from the poorly understood process of genomic imprinting, whereby alleles from one parent are differentially expressed from those

from the other parent. Genomic imprinting is thought to occur where "conflict of interest" between paternal and maternal genes has occurred, such as those that control foetal growth (Cookson, 1999).

A number of possible genetic linkages have been identified with atopy, asthma and allergic diseases. Because it is a multi-gene disorder, linkage analysis is difficult and consistency across numerous studies has been difficult to obtain. The regions of the human chromosome most consistently located on genome-wide screens have been on chromosomes 5, 6, 12 and 13 (Cookson and Moffat, 2000).

Chromosome 5 appears particularly important. The region 5q31 includes genes encoding IL-4, IL-5, IL-13, GM-CSF, and CD14. The gene for IL-13 appears one of the most important, polymorphisms being associated with allergic asthma (van der Pouw Kraan *et al.*, 1999) and elevated IgE in a large population study of Caucasian children (Graves *et al.*, 2000). Polymorphisms in the IL-4 promoter region (Suzuki *et al.*, 2000) or receptor (Takabayashi *et al.*, 2000) have also been associated with elevated IgE or allergic disease, but less strongly. In addition, polymorphisms in the promoter region of CD14, the endotoxin receptor, which cause a decrease (C/C homozygotes) or increase (T/T homozygotes), in soluble CD14 and thus endotoxin mediated APC signalling are associated with atopy (Koppelman *et al.*, 2001) or decreased IgE levels respectively (Baldini *et al.*, 1999). Chromosome 6 encodes the MHC and many other genes important in the innate immune response. As has been discussed, MHC associations have been demonstrated with asthma and atopy (Boehncke *et al.*, 1998).

Chromosome 13 encodes the high-affinity IgE receptor, polymorphisms in which, have been associated with asthma and bronchial hyper-reactivity even in the absence of atopy (van Herwerden *et al.*, 1995).

Other areas localised include chromosome 12 and chromosome 13q14 and positional cloning studies are currently underway to elucidate these unknown genes (Cookson and Moffat, 2000).

Novel genes in atopy and asthma may also be identified using high throughput, automated techniques such as micro-array technology (Chtanova *et al.*, 2001).

1.2.8. The role of environment in allergy

The speed of the rise in the prevalence of allergic disease over the last fifty years indicates that in addition to genetic factors, environmental influences must be impacting significantly on this increase. The epidemiological observation that children born first into a family had a greater likelihood of being atopic spawned the hygiene hypothesis (Strachan, 1989). The logical deduction was that environmental influences such as childhood infections were somehow protective against the development of atopy. Similar observations have now been confirmed in other studies that have shown a protective effect on atopy of being in childcare at an early age, growing up on a farm with high endotoxin exposure (Von Ehrenstein *et al.*, 2000) or not being exposed to antibiotics in childhood. These observations have also suggested that there is a critical window of time in early life when sensitisation to allergens is occurring and in particular the commitment of TH cells to TH1 or TH2 occurs (Holt and Macaubas, 1997; Yabuhara *et al.*, 1997). Immunological studies to clarify the mechanisms behind the hygiene hypothesis have shown that the immune system of the fetus in pregnancy is biased toward TH2 responses, and that sensitisations can occur to allergens in utero (Macaubas *et al.*, 2000). Following birth the TH2 bias in the infant is rapidly suppressed in non-atopic individuals within the first 6 months of life (Prescott *et al.*, 1998). In atopy and allergy, under the influence of genetic and environmental signals there is a failure in this maturation and the resultant TH2 bias in the immune response at 6 months has a similar profile to the adult TH2 response among atopics (Prescott *et al.*, 1999).

The hygiene hypothesis has recently been extended with the proposal of the counter-regulation hypothesis (Wills-Karp *et al.*, 2001). This suggests that the Western lifestyle with reduced infective and

endotoxin exposures, results in a decrease in the production of IL-10, resulting in over-expression of both allergic and autoimmune diseases within our society. Novel preventative approaches such as the use of probiotics are thought to act by increasing the expression of the anti-inflammatory cytokines IL-10 and TGF-β in exposure to gut flora and provide supportive evidence for this hypothesis (Kalliomaki *et al.*, 2001).

1.2.9. The treatment of allergic disease

The treatment of allergic diseases is based on non-allergen- specific and allergen-specific modalities. The non-allergen-specific modalities centre on pharmacotherapy and are beyond the scope of this thesis. Allergen-specific modalities are those of allergen avoidance and SIT. The role of allergen avoidance is controversial in the primary prevention of allergy, and carefully conducted longitudinal studies are underway to establish its role. Once allergy is established however, allergen avoidance may have more importance and its usefulness has been shown in allergic asthma (Carswell *et al.*, 1999) and atopic dermatitis (Tan *et al.*, 1996). As will be discussed, allergen avoidance is of particular importance in latex allergy.

1.2.9.1. Management of established allergy through the use of SIT

SIT consists of the administration of incremental doses of an allergen, to which a patient has clinical symptoms and specific IgE, to effect clinical tolerance to the allergen. It is indicated in the management of hymenoptera allergy, whilst in the management of inhalant allergy such as dust mite, cat and grass pollen allergy, SIT is indicated after the failure of medical therapy. It has no role in the management of food allergy and is experimental in latex allergy (see below). It is an effective treatment for the allergic syndromes of allergic rhinitis (Bousquet, 2001) and asthma (Abramson *et al.*, 2000). The other important feature of SIT that sets it apart from all other treatment modalities is its ability to modify the immune response and thus effect

long lasting clinical tolerance of allergens some 3 –5 years after it has been ceased (Durham *et al.*, 1999). Its efficacy however is limited by side effects, with 2-5 deaths/per year occurring in the USA through anaphylaxis following SIT administration (Adkinson, 2001). The major group at risk is asthmatics, with 75% of deaths occurring within this group (Adkinson, 2001).

1.2.9.1.1. Mechanisms of SIT

The mechanism of SIT remains unclear. Proposed mechanisms include T cell anergy, immune deviation or deletion (Rolland and O'Hehir, 1998). These three mechanisms recognise the pivotal role of the T cell in determining the nature of, or in modulating, the subsequent acquired immune response to allergens. In addition, allergen specific regulatory T cells may be important (Figure 1.4). Humoral factors such as blocking antibodies may play a role, however the rapid onset of the changes seen for example in rush immunotherapy makes this mechanism unlikely to be responsible.

1.3. Latex allergy

1.3.1. History of the latex allergy epidemic

An IgE-mediated reaction to latex was first reported in the German literature in 1927 (Granady and Slater, 1995). This was a case of angioedema with the use of a rubber dental prosthesis. It was not until 1979, however, when a case of contact urticaria was described in a 34-year-old housewife, that the condition appeared in the English literature (Nutter, 1979).

Between 1979 and 1988, there were numerous reports in the European literature of immediate allergic reactions to latex (Forstrom, 1980; Galinsky and Kleinhans, 1982; Meding and Fregert, 1984). Mostly these were related to contact urticaria. Intra-operative anaphylaxis was described among latex allergic HCW (Axelsson *et al.*, 1987), but it was not until 1989 that the first reports appeared in the North American literature. Slater described two children with spina bifida who

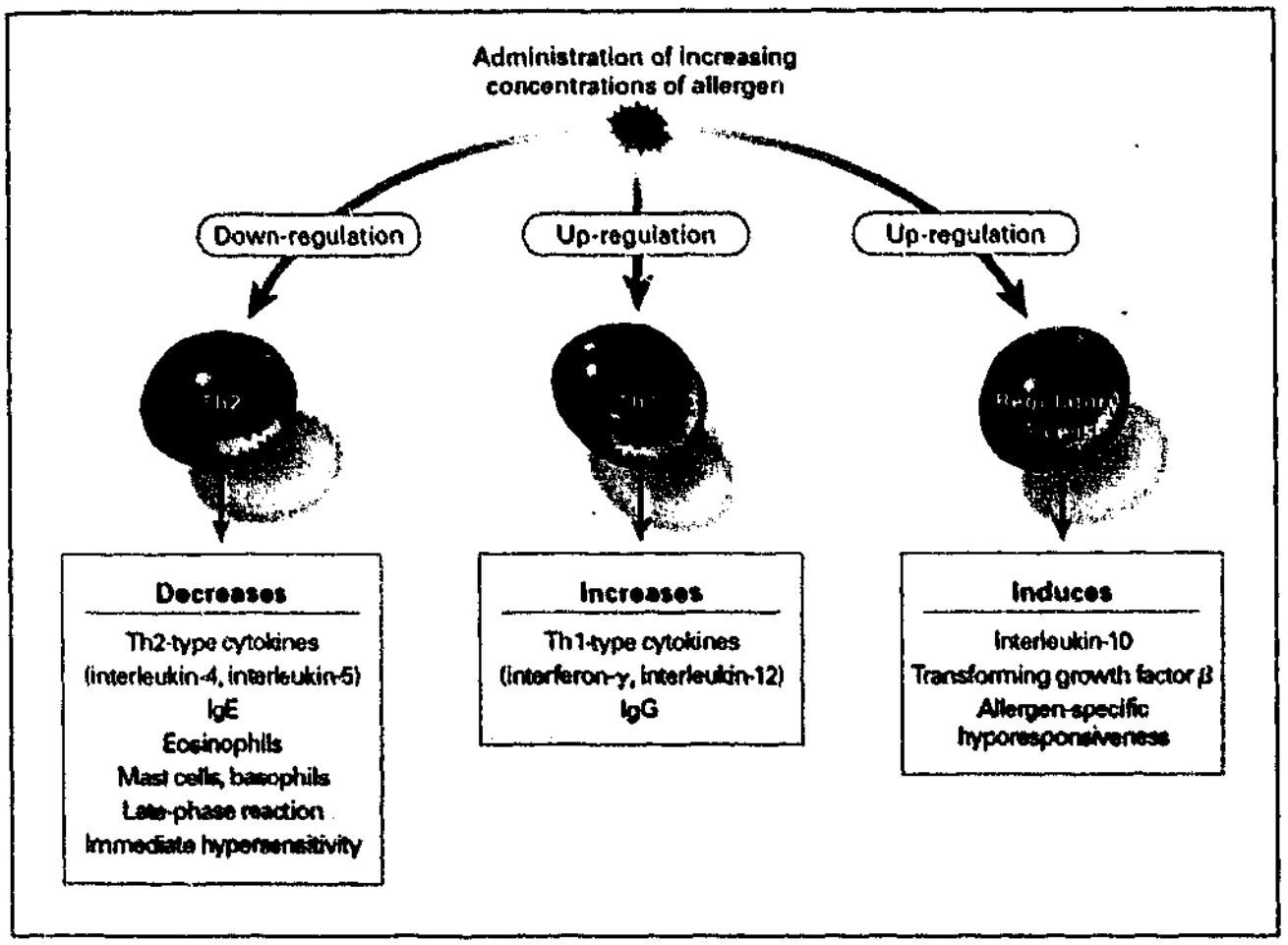


Figure 1.4 Proposed mechanisms of allergen-specific immunotherapy (SIT) through its effect on T cells (Kay, 2002).

experienced intra-operative anaphylaxis (Slater, 1989). Since that time the US Food and Drug Administration has received over 1100 reports describing allergic reactions experienced by patients or HCW associated with the use of latex gloves for medical products (Granady and Slater, 1995). The majority of cases have involved latex gloves and barium enema catheters. The 15 deaths associated with latex exposure were all in patients who received barium enemas (Ownby *et al.*, 1991). Interestingly, follow-up interviews did not reveal specific risks in these patients: there were no HCW in the group, and none of the patients reported significant occupational exposure to latex products (Ownby *et al.*, 1991). The important epidemiological fact identified on history, however, was that five of the six patients were atopic and three of the six patients had active atopic dermatitis. All of the patients with atopic dermatitis reported using latex gloves to protect their hands during house work. Although most cases of latex allergy involve direct mucosal contact, among HCW, aerosol transmission of latex proteins are particularly important (Tarlo *et al.*, 1994).

It is interesting to consider why the latex allergy epidemic has occurred now and not earlier. At least part of the explanation may be due to increasing familiarity with the general clinical picture and awareness of the problem. Coupled with this has been the HIV epidemic which has resulted in greater glove use and therefore in greater exposure to latex proteins. The increasing demand for latex gloves may have reduced the protein elution time and the glove shelf life both of which may have increased the extractable protein content (see below). As noted previously, these changes have coincided with a general increase in the incidence of atopic disorders within our community.

1.3.2. The manufacture of natural rubber latex

Many plants produce a latex from which natural rubber can be produced. *Hevea brasiliensis* is the main source of commercial rubber and is grown in plantations in South East Asia, India and Central and

South America. World rubber consumption is 5.5 million tons per year (Subramaniam, 1995). Tapping and collection of latex is performed by cutting the bark of the rubber tree at an angle, causing liquid latex to flow from the wound into a collection vessel. Immediately after collection and before transport, stabilisers such as ammonia are added to prevent premature coagulation of the latex, which is primarily due to bacterial action on the liquid latex. The rubber hydrocarbon is a cis-1, 4-poiyisoprene. Its molecular weight averages 200 to 600 kDa and shows a clonal variation. The protein content amount can depend on the environmental and seasonal conditions and may also be increased by frequent tapping, which may be an explanation for the increased allergenicity of gloves during the late nineteen eighties when demand outstripped supply (Subramaniam, 1995). Different methods are used for the manufacture of dry rubber products, such as tyres or solid rubber mouldings, compared with dipped rubber products such as gloves, balloons or condoms. The former process occurs at far higher temperatures, utilises acid dipping of rubber and prolonged high temperature leaching, and results in a product with reduced flexibility, less extractable protein and therefore less allergenicity (Yunginger *et al.*, 1994). The manufacture of dipped rubber products entails the addition of accelerators (chemicals to speed the cross linking of the poly-isoprene hydrocarbon units to form the elastic polymer in the finished rubber product) and other preservatives during the manufacturing process. The main accelerators are the thiurams, xanthates and thiazoles (Subramaniam, 1995). Thiurams in particular, are the most common cause of allergic contact dermatitis (Conde-Salazar *et al.*, 1993). Allergic contact dermatitis is a delayed type hypersensitivity reaction distinct from the IgE-mediated processes that form the subject of this thesis. Antioxidants and antiozonants may be added to the latex compounds depending on the service conditions required; the antioxidants used are the phenols and cresols and these can also cause contact hypersensitivity (Rich *et al.*, 1991). Finally, the compounding ingredients such as colouring agents (e.g. dyes), thickeners such as casein and whiteners such as titanium oxide may be

added. In *in vitro* studies, subjects with milk allergy can show specific IgE to these milk proteins, however the clinical importance of this is uncertain (Ylitalo *et al.*, 1999). Drying and vulcanisation (the chemical reaction usually mediated by sulphur to cause cross linking of the poly-isoprene hydrocarbon units of the rubber) of the latex dipped product is then done using high temperature air, initially at 90° C, and then finally at 120° C (Subramaniam, 1995).

1.3.2.1. Leaching and surface treatment

During the manufacture of most rubber products and in particular surgical gloves, leaching with water is an integral part of the final production, mainly in an effort to reduce protein content and therefore allergenicity (Subramaniam, 1995).

Surface treatment of latex products is carried out to decrease their "stickiness" during glove donning. Powders may be applied and there are two main types of these: talcum powder and bioabsorbable starch. In addition, the surface of the glove can be modified by chlorination and this reduces the friction and also the allergen protein content, however it must be carefully controlled to avoid damage to the latex product. Finally, the latex product may be dipped in a synthetic polymer to reduce friction and also the protein in contact with the patient as occurs in the Johnson and Johnson Biogel® process (Subramaniam, 1995).

1.3.2.2. Extractable protein content in latex products and allergy

Studies from operating theatres have found that latex gloves are the major cause of airborne latex antigen (Heilman *et al.*, 1996). Additionally, it has been shown that powdered latex gloves have a higher extractable protein content than non-powdered gloves (Yunginger *et al.*, 1994) and also that examination gloves have a higher extractable protein content than surgical gloves (Yunginger *et al.*, 1994). Other studies have shown that the latex allergen content of gloves has decreased from the early to the late nineteen nineties

(Palosuo *et al.*, 1998), likely due to changes in manufacturing processes and improvements in allergen leaching and surface treatment. Unfortunately from a scientific viewpoint, the precise manufacturing methods of most gloves are unavailable, being trade secrets.

Apart from latex gloves, the only other products with comparable extractable protein levels are toy balloons (Yunginger *et al.*, 1994). Products such as condoms and anaesthesia rebreathing bags have lower levels (Yunginger *et al.*, 1994). Baby's teats, and other solid rubber products have lower still extractable protein levels (Yunginger *et al.*, 1994). Whilst therefore, these solid rubber products are likely unimportant in sensitisation and have not been found to be associated with the development of latex allergy (Niggemann *et al.*, 1998), in severely allergic individuals, solid products may elicit reactions (Primeau *et al.*, 2001).

1.3.3. The epidemiology of latex allergy

Latex allergy prevalence differs between countries (Table 1.2). This is to be expected, given the huge range of international prevalence of other atopic diseases seen in studies such as the ISAAC study (ISAAC, 1998). In epidemiological studies of latex allergy, there has also been the additional problem of a wide variety of diagnostic methods used, from glove eluates to standardised skin test or serological assay reagents, making comparison between studies difficult. Exposure to latex proteins is critical and the established risk factors are occupational exposure (eg HCW), spina bifida, multiple surgical procedures, atopy, eczema and fruit allergy (Poley and Slater, 2000). HCW are the main risk group considered in detail within this thesis.

Well-conducted and large epidemiological studies from USA have shown a prevalence of latex-specific IgE of around 9% among HCW using the Pharmacia UniCAP® fluoro-enzymatic immuno-assay (FEIA) system (Grzybowski *et al.*, 1996). However, as shown in Table 1.2, there is a wide variation in reported rates of latex-specific IgE from different countries and even within countries. Serological prevalence

Table 1.2 International prevalence of latex sensitisation among HCW

Study	Site	Sample size	Positive SPT (%)	Positive RAST(%)
(Aichane et al., 1997)	Casablanca	600	5.3	0.7
(Brown et al., 1998)	USA	168		
(Camacho Ibarra et al., 1997)	Mexico	90	8	
(Douglas et al., 1997)	Australia	140	22	
(Grzybowski et al., 1996)	US	741		8.9
(Hack, 2001)	Australia	102		1
(Handfield-Jones, 1998)	UK	867	0.9	
(Heese et al., 1995)	Germany	206	8.7	
(Kaczmarek et al., 1996)	US	504		4.1
(Kibby and Akl, 1997)	US	132	8.2	6.1
(Konrad et al., 1997)	Switzerland	101	15.8	3
(Leung et al., 1997)	Hong Kong	1472	6.8	
(Safadi et al., 1996)	US	93	10	10
(Safadi et al., 1996)	US	34	12	
(Saraclar et al., 1998)	Turkey	61	11.4	
(Sener et al., 2000)	Turkey	206	9.22	
(Shahnaz et al., 1999)	Malaysia	130	3.1	
(Sussman et al., 1995)	Canada	50	8	
(Tarlo et al., 1997)	Canada	203	10	
(Vandenplas et al., 1995)	Belgium	273	4.7	
(Vila et al., 1999)	Spain	301	5	
(Wrangsjo et al., 1994)	Sweden	202	2	

studies however suffer from the limitation in specificity of these assays (see below), which for the Pharmacia UniCAP® latex specific FEIA has been reported at between 90.2% (Ownby *et al.*, 2000) and 96.7% (Hamilton *et al.*, 1999), giving false positive rates of 9.8% and 3.3% respectively. The other important fact in interpretation of the epidemiological data is the difference between allergic sensitisation (allergen-specific IgE antibodies without symptoms on exposure) and the manifestation of an allergic disease (allergen-specific IgE antibodies together with immediate type symptoms upon allergen exposure). The manifestation of an allergic disease occurs in between 20% (Brown *et al.*, 1998) and 53% (Vandenplas *et al.*, 1995) of those who are sensitised and again depends on the level of antigen exposure that an individual experiences.

The three local prevalence studies deserve special mention (Katelaris *et al.*, 1996; Douglas *et al.*, 1997; Hack, 2001) because they pertain to the Australian population but also illustrate the difficulty in comparison between prevalence studies occurring at different time-points and using different diagnostic methods. In a study performed among nurses at the Alfred Hospital, a prevalence of 22% of latex sensitisation was reported, the highest figure reported in the world literature (Douglas *et al.*, 1997). There may be a number of reasons for this. Firstly, the participants were volunteers, which may have biased the sample. Secondly, eluates from 5 different gloves were used in the evaluation of all subjects which may have increased the diagnostic sensitivity. Despite these possible biases however, the study remains important and likely reflected a real clinical problem within the wards at the Alfred which has been since addressed with the introduction of powder-free low allergen gloves to the wards. Additionally, the study showed the lack of specificity for latex allergy of hand itching and erythema, these being equally common among allergic and non-allergic individuals (Douglas *et al.*, 1997). Another study evaluated the prevalence of latex allergy within theatre personnel at a Brisbane teaching hospital. The Sanofi-Pasteur Diagnostics Enzyme Allergosorbent Test (EAST) was

used and subjects underwent spirometry before and following their shifts. Only one individual was EAST positive giving a prevalence of only 1%, though this individual did exhibit decreases in Forced Expiratory Volume in one second (FEV1) following his shift consistent with occupational asthma due to latex and therefore significant latex allergy (Hack, 2001). This study again may have been biased by a volunteer effect and the use of a serological assay, which may have reduced sensitivity, though no published data are available on the assay performance. In addition the sample size was relatively small so it may have suffered from a Type II error. Again, however, this more recent study may reflect a real reduction in sensitisation due to improved glove technology and decreased extractable protein content. Finally, there has also been one questionnaire-based study of latex allergy prevalence among Australian dental hospital personnel, which suggested a probable prevalence of 9% (Katelaris *et al.*, 1996). As noted by the authors however, it did not utilise latex-specific IgE assays or skin prick tests, so could not make a definitive assessment of true latex allergy prevalence (Katelaris *et al.*, 1996).

Spina bifida is another high-risk group for latex allergy, with a reported prevalence of latex sensitisation in these patients ranging from 4.3% in Venezuela to 40% and 60% in Europe (Mazon *et al.*, 2000) and USA (Ellsworth *et al.*, 1993) respectively. Australian data have shown a prevalence of 36.9% of latex sensitisation among spina bifida patients (Valentine *et al.*, 1999). Spina bifida patients, through their different route and type of latex allergen exposure, appear to be sensitised to different allergens when compared with a HCW population.

Multiple surgical procedures are also a risk factor in the development of latex allergy. Studies have shown an increased number of surgeries among latex sensitised children with spina bifida than similar, non-sensitised children (Ylitalo *et al.*, 1997). The risk of latex allergy increases with surgery number (Bode *et al.*, 1996). The exact number

of operations that puts a child at increased risk is unknown, but it is currently believed that 5 or more operations with general anaesthetic increase a child's risk of developing latex allergy (Porri *et al.*, 1997). In adults, similar results have been shown, with more than 10 operations significantly associated with a risk of latex allergy (Rueff *et al.*, 2001).

Atopy, as might be expected, is a risk factor for latex allergy (Grzybowski *et al.*, 1996; Douglas *et al.*, 1997; Leung *et al.*, 1997). Despite this, it is possible to have latex allergy and be non-atopic, with up to 19% of individuals with latex allergy reported to be non-atopic (Aichane *et al.*, 1997).

The presence of hand dermatitis is also an accepted risk for latex allergy, likely through the greater penetrance of latex allergens through the epidermis (Boxer, 1996). In addition, it has been shown that Type IV responses (allergic contact dermatitis) to rubber gloves can co-exist with Type I or immediate latex allergy.

Studies of the risk of latex allergy among the normal population using skin tests have generally shown a prevalence in the community of less than 1% (Liss and Sussman, 1999). Studies of blood donors relying on serological assays only have shown rates of latex sensitisation as high as 6.4%, which may be an overestimate of the true prevalence of latex allergy because of the limitations in specificity of serum-IgE assays (Yeang, 2000).

1.3.3.1. Fruit allergy in latex allergy

Up to 52% of latex allergy sufferers have sensitivity to various fruits and vegetables (Blanco *et al.*, 1994). Also, latex allergic individuals have four times the risk of the general population of food allergy. Food allergy amongst latex-sensitive subjects manifests as anaphylaxis in up to 36% of cases (Blanco *et al.*, 1994). Therefore, the latex-fruit syndrome is of considerable clinical importance. A Medline search identified more than 20 foods, fruits or plants that have been reported

Figure 1.5 Taxonomic classification of fruits, vegetables and plant tissues reported to cross-react with latex. References are cited in brackets following common names.

Kingdom	Sub-kingdom	Super-division	Division	Class	Sub-class	Order	Family	Genus, species (Common name) (Reference)
Plantae (Plants)	Tracheobionta (Vascular plants)	Spermatophyta (Seed plants)	Magnoliophyta (Flowering plants)	Magnoliopsida (Dicotyledons)	Hamamelidae	Apiales	Apiaceae (Carrot family)	*Apium* L. (**Celery**) (Levy *et al.*, 2000)
						Euphorbiales	Euphorbiaceae (Spurge family)	***Hevea brasiliensis*** (Willd. Ex Adr. Juss.) Muell. Arg. (**Rubber tree**)
						Rosales	Rosaceae (Rose family)	***Malnus* P. Mill.** (**Apple**) (Levy *et al.*, 2000) *Prunus* (L.) (**Plum**) (Weiss and Halsey, 1996) ***Prunus avium* (L.) L.** (**Sweet cherry**) (Kim and Hussain, 1999) *Prunus persica* (L.) Batsch (**Peach**) (Garcia Ortiz *et al.*, 1998) *Prunus persica* (L.) Batsch var. *nucipersica* (Suckow) C. Schneider (**Nectarine**) (Weiss and Halsey, 1996)
						Sapindales	Anacardiaceae (Sumac family)	*Mangifera indica* L. (**Mango**) (Duque *et al.*, 1999)
					Magnoliidae	Lauraceae	Lauraceae (Laurel family)	*Persea americana* P. Mill. (**Avocado**) (Blanco *et al.*, 1994)
						Magnoliales	Annonaceae (Custard-apple family)	*Annona cherimola* P. Mill. (**Cherimoya**) (Sanchez-Guerrero *et al.*, 2000) *Asimina triloba* L. (**Pawpaw**) (Blanco *et al.*, 1994)
					Hamamelidae	Fagales	Fagaceae (Beech family)	***Castanea sativa*** P. Mill. (**European chestnut**) (Blanco *et al.*, 1994)
						Urticales	Moraceae (Mulberry family)	***Artocarpus altilis* (Parkinson) Fosberg** (**Breadfruit**) (De Greef *et al.*, 2001) ***Ficus benjamina* L.** (**Weeping fig**) (De Greef *et al.*, 2001)
					Dilleniidae	Ebenales	Ebenaceae (Ebony family)	*Diospyros virginiana* L. (**Persimmon**) (Anliker *et al.*, 2001)
						Theales	Actinidiaceae (Chinese gooseberry family)	*Actinidia chinensis* Planchon. (**Kiwi fruit**) (Blanco *et al.*, 1994)
						Violales	Cucurbitaceae (Cucumber family)	*Cucumis* L. (**Melon**) (Garcia Ortiz *et al.*, 1998) *Cucurbita pepo* L. (**Zucchini**) (Reindl *et al.*, 2000)
					Caryophyllidae	Polygonales	Polygonaceae (Buckwheat family)	*Eriogonum* Michx. (**Buckwheat**) (Abeck *et al.*, 1994)
						Solanales	Solanaceae (Potato family)	***Solanum lycopersicum* L.** (**Tomato**) (Abeck *et al.*, 1994) ***Solanum tuberosum* L.** (**Potato**) (Seppala *et al.*, 2000)
				Liliopsida (Monocotyledon)	Zingiberidae	Bromeliales	Bromeliaceae (Bromeliad family)	***Ananas comosus* (L.) Merr.** (**Pineapple**) (Levy *et al.*, 2000)
						Zingiberales	Musaceae (Banana family)	***Musa acuminata* Colla** (**Banana**) (Alenius *et al.*, 1996)
					Commelinidae	Cyperales	Poaceae (Grass family)	***Phleum pratense* L.** (**Timothy grass**) (Fuchs *et al.*, 1997)

to be immunologically cross-reactive with latex (Abeck *et al.*, 1994; Blanco *et al.*, 1994; Blanco *et al.*, 1994; Alenius *et al.*, 1996; Antico, 1996; Weiss and Halsey, 1996; Fuchs *et al.*, 1997; Garcia Ortiz *et al.*, 1998; Duque *et al.*, 1999; Kim and Hussain, 1999; Levy *et al.*, 2000; Reindl *et al.*, 2000; Sanchez-Guerrero *et al.*, 2000; Seppala *et al.*, 2000; Anliker *et al.*, 2001; De Greef *et al.*, 2001). The most commonly described are banana, avocado, kiwifruit and chestnut. Table 1.4, however, shows the fruits and vegetables reported cover a wide proportion of the plant kingdom. This is indicative of two important issues: firstly, that a pan-allergen (or allergens) is likely important, and secondly, the breadth of dietary components that may put severely latex-allergic individuals at risk of food allergy symptoms. Latex allergic patients are not currently advised to avoid all such foods unless they have specific symptoms. However, it is incumbent upon clinicians to warn patients of the potential for these reactions. The major latex allergen Hev b 6 has been identified as being the principal latex allergen responsible for this cross-reactivity (Mikkola *et al.*, 1998). The hevein molecule (Hev b 6.02) has striking homology with Class 1 chitinases (Blanco *et al.*, 1999), which have a hevein-like domain and occur widely in plants, thus fitting the description of a "pan-allergen". RAST inhibition and skin testing studies have provided further correlation of this molecular observation. Although primary sensitisation via fruit exposure has been reported and is a risk factor for latex allergy (Garcia Ortiz *et al.*, 1998), current evidence indicates that primary sensitisation to latex accounts for the vast bulk of the latex-fruit syndrome. Whether other latex allergens are responsible for food cross-reactivity is not so clear. Hev b 5 has sequence homology to an acidic protein in kiwi fruit (Ledger and Gardner, 1994) and also in sugar beet (Fowler *et al.*, 2000) but no evidence for cross-reactivity has been found. Hev b 7 has sequence homology to patatin, which can cause positive skin test results and inhibition of IgE binding to Hev b 7 among Hev b 7-sensitised individuals (Seppala *et al.*, 2000). Hev b 8 is a profilin; homologues of which are identified as allergens in many fruits and vegetables. Clear evidence of primary sensitisation to Hev b

8 via latex exposure with subsequent food allergy has not been demonstrated and, therefore, for Hev b 8-sensitive individuals, primary sensitisation to grass pollen profilins is the likely cause (Rihs *et al.*, 2000).

1.3.4. Current management of latex allergy

1.3.4.1. The diagnosis of latex allergy

As in other allergies, the diagnosis of latex allergy rests upon an appropriate clinical history and the demonstration of latex-specific IgE by serologic, skin or challenge testing.

1.3.4.1.1. Clinical features and history

Latex allergy is dependent on exposure to latex proteins. Thus an appropriate and reliable temporal relationship to latex exposure and the *immediate* (within minutes) development of symptoms is critical. A risk group as discussed above is often present as is associated food allergy (Blanco *et al.*, 1994). The symptom constellation as with other allergic diseases depends on the route of exposure. Latex allergy is commonly manifest as hand urticaria (Valsecchi *et al.*, 2000). Hand itching by itself is a poor predictor of latex allergy (Douglas *et al.*, 1997), as it can be also consistent with irritant or allergic contact dermatoses, which occur in up to 37.6 % of glove users (Uveges *et al.*, 1995). Irritant is by far the most common of these, accounting for 98.25 % of the dermatoses diagnosed in one study (Uveges *et al.*, 1995).

Latex allergy is also a common cause of allergic rhino-conjunctivitis (Archambault *et al.*, 2001) and occupational asthma (McDonald *et al.*, 2000; Hnizdo *et al.*, 2001).

The onset between commencement of exposure to rubber proteins (sensitisation) and symptoms was found in one study to average 5 years (Allmers *et al.*, 1996).

Another interesting aspect to latex allergy relevant to the clinical history is the propensity of latex allergic individuals to develop anaphylaxis on exposure to latex proteins. Latex is one of the main causes of intra-operative anaphylaxis (Kelly *et al.*, 1994) and is also the commonest reagent (including nuts, bee venoms and antibiotics) to induce anaphylactic reactions on skin testing (Valyasevi *et al.*, 1999). In addition, there are numerous reports in the literature of anaphylaxis in latex allergic individuals on exposure to tiny amounts of latex protein in seemingly innocuous everyday activities such as licking a postage stamp (Pumphrey *et al.*, 2001) or eating food prepared by food handlers using latex gloves (Nixon and Lee, 2001). The potency of latex allergens is also illustrated by the recent finding that eluates from solid rubber medication vials are sufficient to induce wheal and flare reactions on skin testing of latex allergic individuals (Primeau *et al.*, 2001) and also that positive skin tests have been reported down to a LAL concentration of 70 pg/ml in highly sensitive individuals (Yip *et al.*, 2000).

1.3.4.1.2. Occupational asthma due to latex allergy

Latex is an important cause of occupational asthma. In a recent South African study it was the commonest agent responsible, followed by isocyanates and platinum salts (Hnizdo *et al.*, 2001). In the United Kingdom a similar recent study found latex to be a common causative agent for occupational asthma, and one that had increased dramatically in its prevalence compared to previous studies (Ross *et al.*, 1998). In apprentices starting as dental technicians, the cumulative incidence of new cases of occupational asthma due to latex over a 32-month period was 4.5% (Archambault *et al.*, 2001). In a well-conducted Dutch study with latex glove powder inhalation challenge, occupational asthma to latex occurred with a prevalence of 2.5%, approximately one half of subjects with a positive skin test to latex (Vandenplas *et al.*, 1995).

1.3.4.1.3. *In vitro* latex-specific IgE assays

The three *in vitro* IgE assays for which there is published data on diagnostic efficiency are the Pharmacia UniCAP® FEIA, microplate AlaSTAT and Hycor HY-TECH EIA system. The latter two assays are not in widespread use in Australia. The Pharmacia UniCAP® is the most widely used system in the world and is the second most commonly used in Australia. The most common system in Australia is the Sanofi-Pasteur Diagnostics enzyme allergosorbent test (EAST). There are no published data on the diagnostic efficiency of the Sanofi-Pasteur Diagnostics latex-specific EAST. The solid phase antigen in this test is a ground latex glove (Lex Lancaster, Regional Manager Sanofi-Pasteur Diagnostics Australia, personal communication). Both the Pharmacia UniCAP® FEIA and AlaSTAT utilise a NAL reagent (clone 600 RRIM) in the solid phase for the detection of latex-specific IgE. As will be discussed, this may limit diagnostic efficiency because the relative abundance of some antigens (Hev b 5 in particular) may be reduced (Chen *et al.*, 2000) compared with that in processed rubber products such as gloves that are the mainstay of exposure to humans.

There have been two large studies that evaluated the Pharmacia UniCAP® latex-specific IgE assay for the diagnosis of latex allergy. Both studies used a physician-administered questionnaire and latex skin prick test as the combined gold standard for latex allergy. The first study in a large sample of 195 allergic individuals, predominantly HCW, found a sensitivity of 76.3% and a specificity of 96.7% (Hamilton *et al.*, 1999). The second study in a population of 60 latex allergic individuals found a sensitivity of 79.5% and a specificity of 90.2% (Ownby *et al.*, 2000).

1.3.4.1.4. Other *in vitro* assays for latex allergy

The utility of PBMC proliferation assays to crude latex glove extracts in the diagnosis of latex allergy, has been evaluated by Turjanmaa and colleagues. It found that proliferation was an insensitive diagnostic

tool, with positive responses in only 20% of latex allergic subjects (Turjanmaa *et al.*, 1989).

In a similar study to Turjanmaa *et al*, a Dutch group compared two latex specific IgE assays (the Pharmacia UniCAP® FEIA and the AlaSTAT EIA) together with skin testing and PBMC proliferation assays to investigate the utility of these assays for diagnostics. For the proliferation assays they used a crude NAL extract at a concentration of 20 µg/ml. When compared with a control latex non-allergic group, PBMC proliferation was again found to be insensitive, with only 35% of latex allergic subjects showing significant proliferation compared with the control group (Ebo *et al.*, 1997). This study was notable for its large number of both atopic and non-atopic control subjects (n=44) (Ebo *et al.*, 1997).

1.3.4.1.5. Skin prick testing assays

Skin prick testing (SPT) assays are generally considered the most sensitive diagnostic assays for the diagnosis of latex allergy and, in the absence of standardised challenge protocols, when combined with an appropriate clinical history, skin prick testing assays are generally accepted as the gold standard for the diagnosis of latex allergy.

There is no licensed skin-testing reagent available for general use in Australia or USA. In Australia, the Stallergenes LAL extract is available on an individual patient basis. This is a standardised reagent, with a reported 100% specificity and 93% sensitivity when used at the biological potency of 100 Index of Reactivity (IR) Units or 22 µg/ml protein concentration in a population of 46 latex allergic subjects and 76 non-allergic controls (Turjanmaa *et al.*, 1997).

In USA a NAL reagent (Greer) has been undergoing trials seeking Food and Drug Administration approval as a skin testing preparation. This reagent has a higher protein concentration (1 mg/ml) and has a reported sensitivity of 99% and specificity of 96% at 1 mg/ml and 95% and 100% at 100 µg/ml (Hamilton and Adkinson, 1998). The major

drawback to the skin test is systemic reactions ("mild" reactions such as pruritus and urticaria occurred at a rate of 16.1% using the Greer NAL reagent). Anaphylaxis has also been reported with the Stallergenes LAL reagent (Nettis *et al.*, 2001) and appears more likely still when latex glove eluates are used in skin testing (Kelly *et al.*, 1993).

The appropriate diagnostic algorithm therefore is to take a clinical history and then proceed to *in vitro* testing first unless there is a low likelihood of latex allergy (and therefore high likelihood of a negative skin-test) when it is safe to proceed directly to skin-testing. In the event of negative *in vitro* and skin tests but a positive clinical history it is appropriate to proceed to challenge testing.

1.3.4.1.6. Challenge testing for latex allergy

Although considered the "gold standard" by many, challenge testing is poorly standardised at present because of the variability of protein contents in latex gloves. It is usually carried out according to the method of Turjanmaa (Turjanmaa, 1995), where a powdered latex glove is placed on the wetted finger initially, and then hand of the blinded subject, with a non-latex glove as a control. Two or more patches of urticaria are considered positive (Turjanmaa, 1995).

Standardised inhalation challenge tests using latex glove powder are currently under development (Kurtz *et al.*, 2001), but can only be considered research tools at present.

1.3.4.2. Current treatment of latex allergy

The current treatment of latex allergy is heavily biased toward allergen avoidance. SIT for latex allergy, with its high level of systemic side effects, can only be considered experimental at this time.

1.3.4.2.1. Allergen avoidance in latex allergy

Because the latex allergy problem is a recent one, and there is a lack of long term prospective studies to determine if allergen avoidance is

effective, much of the evidence for allergen avoidance is anecdotal. As far as secondary prevention is concerned there is evidence that latex-allergic individuals can safely undergo operations in latex-free theatres (Birmingham *et al.*, 1996) and that the latex specific IgE falls with time in latex allergic spina bifida patients (Niggemann *et al.*, 2000) and HCW with strict allergen avoidance (Allmers *et al.*, 1998). There is also evidence that almost all latex allergic HCW can return to work in an environment where they are provided with non-latex gloves and any latex gloves worn by others are of low protein and powder free (Turjanmaa *et al.*, 2002). Tempering this however is the information from a study of sensitised anaesthetists showing that if powdered latex gloves are still used by surgical staff in theatre despite personal allergen avoidance by anaesthetists, latex-specific IgE rose in those who had been sensitised for 18 months or less (Hamilton and Brown, 2000).

The evidence for allergen avoidance in primary prevention is variable. A large prospective study from Canada failed to demonstrate any difference in the rate of new sensitisation (1% of HCW/ year) between the group randomised to powder-free gloves or continued use of powdered gloves, but this may have been due to lack of numbers despite the study's large sample size of 1351 (Liss *et al.*, 1997; Sussman *et al.*, 1998). In a retrospective study by the same group it was found that in a large teaching hospital in Ontario with 8,000 employees, following the move to low-protein, powder-free, non-sterile examination gloves in 1997, only 1 new diagnosis of latex allergy was made up until 1997 (Tarlo *et al.*, 2001). Additionally, the same group found a reduction in new sensitisations among dental students since the adoption of low protein powder-free gloves (Saary *et al.*, 2002). A recent German study found that the incidence of reported latex allergy was on the increase in that country until the majority of gloves were non-powdered and since that time have steadily declined (Allmers *et al.*, 2002). Further prospective studies are underway but current guidelines in both USA and Australia reflect the general consensus that

the use of powder-free and probably more importantly low-protein latex gloves will reduce the incidence of sensitisation and symptoms among HCW. German researchers have suggested a proposed threshold limit for sensitisation and symptoms for latex allergens of 0.6 ng/m^3 (Baur *et al.*, 1998). What is not entirely clear is whether low protein gloves will be enough and whether total avoidance of latex (as now happens with spina bidifa children) will be necessary to prevent ongoing sensitisation.

1.3.4.2.2. SIT in latex allergy

Despite the effectiveness of allergen avoidance in latex allergy, there remain a core of latex allergic individuals, often with severe allergy and associated food allergy for whom allergen avoidance is insufficient and for whom safe SIT would be desirable.

Three studies have evaluated SIT in latex allergy, and all have used crude ammoniated latex preparations. The most important of these studies was a randomised, placebo-controlled trial using the Stallergenes LAL preparation (Leynadier *et al.*, 2000). This is the standardised extract used widely in Europe and on a restricted basis in Australia for skin testing in the diagnosis of latex allergy (Turjanmaa, 1995). The preparation was tested in a multi-centre study in which 17 HCW with latex allergy were randomised to SIT or placebo (Leynadier *et al.*, 2000). The SIT regimen involved a two day rush protocol where patients were up-dosed to their maximum tolerated dose with the 10 Index of Reactivity (IR) or 100 IR vial of LAL, depending on sensitivity. This was followed by a 12-month maintenance phase of injections at the maximum tolerated dose, initially fortnightly and then monthly. The active treatment group reported a statistically significant decrease in symptoms of rhinoconjunctivitis and urticaria but not of asthma, though it was noteworthy that the placebo group had an increased incidence of asthma at the commencement of the study compared with the active group (Leynadier *et al.*, 2000). Although efficacy for the treatment was shown, side-effects were noted. These

included a local reaction rate of over 40% in the active treatment group compared with 15% in the placebo group. In addition, 15% of injections in the active group induced episodes of rhinitis, 2.7% induced asthma, 0.6% angioedema and 0.3% systemic anaphylaxis (Leynadier *et al.*, 2000). Most importantly, these reactions occurred just as frequently in the maintenance phase of injections as compared with the up-dosing phase of treatment.

The two other reports of SIT in latex allergy are case reports. In the first, Pereira *et al.* described the desensitisation of a 31-year-old radiology technician using a low ammoniated latex extract manufactured by ALK-Abello, Denmark (Pereira *et al.*, 1999). During the up-dosing phase of SIT she had a systemic reaction with hypotension and voice hoarseness requiring adrenaline. Again, once in the maintenance phase, symptoms upon latex exposure were reported by the patient to be much reduced and this was validated by a reduction in reactivity on latex provocation testing and skin testing. Interestingly, there were also decreases in *in vitro* reactivity to banana, kiwi and chestnut. The second of the case reports described the sublingual desensitisation of a latex allergic medical student using the ALK-Abello LAL extract (Nucera *et al.*, 2001). There were no systemic side effects reported up to a cumulative dose of 500 µg of the extract, while there was a reduction in symptoms and reactivity to latex-specific provocation of the subject.

These studies indicate some of the inherent difficulties with SIT for latex allergy. Firstly, paradoxically, those patients who are the most latex-sensitive, and thus have the greatest potential benefit from desensitisation, are most at risk from therapy. Patients with mild or moderate latex allergy may be managed successfully with allergen avoidance measures alone (Bubak, 2000). SIT for latex allergy, to be of real benefit, must safely help subjects with severe allergy for whom allergen avoidance is more difficult because of their propensity to develop symptoms on exposure to even minute quantities of latex

protein. Latex is a ubiquitous material within our modern day environment and these severely affected individuals may experience symptoms where mildly affected individuals have no difficulty. In fact, they may be put at risk of anaphylaxis through everyday activities in the community (Fiocchi *et al.*, 2001). The studies to date have indicated that there is a high risk of anaphylaxis, or at the least severe local side effects with SIT using crude latex extracts. Thus, highly latex-sensitive individuals may be exposed to considerable risk by immunotherapy using crude extracts. At present, SIT with crude extracts can only be considered experimental and must be performed by an experienced allergist in a hospital setting where intensive care backup is readily available. Further trials of SIT with crude extracts are awaited with interest and are underway in Europe with the Stallergenes extract and in the USA with the Greer non-ammoniated latex (NAL) extract.

The difficulty of SIT for latex allergy using crude extracts means that novel approaches using peptides, hypoallergenic mutants or DNA vaccines are desirable. As a prelude to these approaches, it is necessary to characterise the immune response to the relevant allergens.

1.3.5. Latex allergens

So far, 11 latex allergens have been cloned and sequenced (WHO-IUIS, 2002), as shown in Table 1.3. There have been a number of studies of prevalence of IgE-reactivity to these allergens (Alenius *et al.*, 1996; Slater *et al.*, 1996; Yeang *et al.*, 1996; Chen *et al.*, 1997; Fuchs *et al.*, 1997; Kurup *et al.*, 2000; Seppala *et al.*, 2000; O'Riordain *et al.*, 2001; Wagner *et al.*, 2001). These used differing methodologies, including Western blotting, ELISA and RAST (Table 1.4). These have shown that Hev b 1 and 3 are major allergens (>50% reactivity) in the spina bifida population, but generally minor allergens (<50% reactivity) among HCW apart from one study which found Hev b 1-specific IgE was present in 52.3% of HCW (Chen *et al.*, 1997). Conversely, Hev b 4, 5, 6 are major allergens (and Hev b 7 is borderline) among the HCW

Table 1.3 Latex allergens (WHO-IUIS, 2002)

Allergen	Common Name
Hev b 1	Rubber elongation factor
Hev b 2	Beta-1, 3-glucanases
Hev b 3	Small rubber-particle protein
Hev b 4	Micro helix component
Hev b 5	Acidic latex protein
Hev b 6.01	Prohevein, hevein preprotein
Hev b 6.02	hevein
Hev b 6.03	Prohevein C-terminal domain
Hev b 7	Patatin-like proteins
Hev b 8	Latex profilin
Hev b 9	Latex enolase
Hev b 10	Mn-superoxide dismutase
Hev b 11w	Class I endochitinase

Table 1.4 Sero-prevalence of specific IgE to individual latex allergens among latex allergic subjects

Allergen	Spina bifida	HCW	Reference
Hev b 1	81	52.3	(Chen, 1997)
	54	13	(Kurup, 2000)
Hev b 2	38	48	(Kurup, 2000)
Hev b 3	100	0	(Yeang, 1996)
	85	19	(Kurup, 2000)
Hev b 4	46	61	(Kurup, 2000)
Hev b 5	56	92	(Slater, 1996)
Hev b 6.01	38	45	(Kurup, 2000)
		69	(Alenius, 1996)
Hev b 6.02		48	(Alenius, 1996)
Hev b 6.03		21	(Alenius, 1996)
Hev b 7	23	23	(Kurup, 2000)
	3	49	(Seppala, 2000)
	40		(Wagner, 2001)
Hev b 8		35	(Fuchs, 1997)
Hev b 9		15	(Wagner, 2000)
Hev b 10		33	(Wagner, 2001)
Hev b 11w		23	(O'Riordain, 2001)

population using *in vitro* IgE binding assays. Such *in vitro* IgE assays are limited in their usefulness however, as they are dependent on such variables as total serum IgE and do not always indicate whether a patient will be reactive *in vivo*. A study using purified component latex antigens in skin testing has provided valuable information *in vivo* indicating which are the most clinically relevant latex allergens (Yip *et al.*, 2000). The skin testing study performed by Yip *et al.* used recombinant and purified latex allergens in skin testing of latex allergic patients. This was novel since it employed an *in vivo* method and the allergens were systematically evaluated in serial dilutions. The allergens tested were Hev b 2, 5, 6, 8 (recombinant) and Hev b 3 and 7 (purified natural protein). All allergens were skin tested at 10-fold serial dilutions in 31 latex allergic individuals (predominantly HCWs), two of whom were excluded from the analysis (one due to dermographism, the other due to non-reactivity to the LAL positive control), and 10 non-latex allergic controls. The important findings of the study were that 93% of subjects with a positive skin test to the raw latex extract (Bencard, LAL) reacted to one or more of the recombinant or purified latex allergens. Furthermore, the most frequent reactivity was to Hev b 6 (66%), Hev b 5 (62%) or Hev b 7 (41%) and all subjects who reacted to one or more recombinant or purified latex allergens reacted to one or more of Hev b 5, 6 and 7, giving a 93% diagnostic sensitivity by using these three recombinant allergens alone. None of the 10 control individuals reacted to the recombinant or purified allergens, giving 100% specificity. More importantly, no adverse reactions were reported on skin testing with the recombinant allergens, though detailed analysis of adverse events was not given. Another interesting finding of the study was the presence of mono-sensitisation, particularly to Hev b 5 (17%), 6 (10%) and 7 (10%), again making SIT with a combination of these allergens more attractive. One weakness of the study was that not all the cloned and sequenced latex allergens were used; particularly Hev b 1 and Hev b 4. Hev b 1 appears to be more important among the *spina bifida* population and shows cross-reactivity with Hev b 3 (which was used) while Hev b 4 may be an

important allergen among HCW (Kurup *et al.*, 2000), but further skin testing studies are needed to establish its importance *in vivo*. Additionally, only a small number of controls were used in the Yip study, and larger studies will be needed to establish the sensitivity and safety of these recombinant and natural latex antigens for future use in the *in vivo* diagnosis and therapy of latex allergy. This study has suggested that Hev b 5, 6 and 7 are the most clinically important allergens among HCW. Some controversy remains surrounding the importance of Hev b 7, as some investigators have found a low prevalence of IgE reactivity (23%) to this allergen (Kurup *et al.*, 2000), questioning its clinical relevance.

1.3.5.1. PBMC and T cell studies of latex allergens

There are limited reports of PBMC and T cell responses to latex allergens. Responses to crude latex antigens have already been alluded to above (Turjanmaa *et al.*, 1989; Ebo *et al.*, 1997).

1.3.5.1.1. PBMC studies

Raulf-Heimsoth and colleagues evaluated PBMC responses among occupationally exposed latex-allergic subjects and a control population of exposed, but non-allergic, individuals (Raulf-Heimsoth *et al.*, 1998). They found that Hev b 1 induced lymphocyte proliferation in 52% of the latex sensitised individuals and 25% of the latex exposed but non-allergic controls (Raulf-Heimsoth *et al.*, 1998). They showed that proliferation was related to latex exposure but did not correlate with allergen-specific IgE levels.

A further study evaluated the response to multiple purified latex antigens in eliciting PBMC responses (Johnson *et al.*, 1999). The antigens used were Hev b 1, 2, 3, 4 (natural) and Hev b 6 and 7 (recombinant). In contrast to Raulf-Heimsoth and colleagues, Johnson and co-workers showed that non-allergics did not have any significant proliferation (defined as SI>3 and standard deviation not overlapping with no antigen control) to latex antigens . They showed that in their

group of 28 latex allergic HCW and 6 non-allergic HCW controls, the greatest frequency of reactivity in the allergic group was shown to Hev b 2 (16/28; 57%) followed by Hev b 6 (12/28; 43%) then Hev b 1, 3 and 7 (each 7/28; 25%) and finally the least reactivity was to Hev b 4 (4/28; 14%). Interestingly, there was no significant correlation between the specific IgE levels and the PBMC proliferation to these antigens, however there appeared to be a trend with Hev b 2 and Hev b 6, whereby 35% and 39% respectively of the patients with specific IgE to these antigens showed PBMC proliferation upon stimulation with the same antigen. Cytokines were not assayed in this study.

1.3.5.1.2. T cell epitope mapping studies on latex allergens

Only two T cell epitope mapping studies have been performed in humans with latex allergy.

Raulf-Heimsoth and colleagues investigated the T cell epitopes of Hev b 1, using 19mer peptides with 3 amino acid overlap in PBMC proliferation assays (Raulf-Heimsoth *et al.*, 1998). Replicates of 6 wells for each peptide or antigen were used and the cut-off SI set at 2.5. The cultures were for 5 days. Reactivity was shown throughout the whole molecule of Hev b 1, with subjects reacting to one or more of the Hev b 1 peptides. The highest responder frequencies were observed against Hev b 1(31-49) and Hev b 1(91-109), both at 61% of subjects. Again, responses were noted to be correlated with exposure but not with antigen specific IgE. The authors also noted that specific peptide responses were generally low when compared with epitope mapping studies using other allergens (Raulf-Heimsoth *et al.*, 1998). In addition, no cytokine responses were assayed, so the T helper cell type could not be analysed. They did, however, tissue type all subjects and noted that 50% of the Hev b 1 peptide responsive subjects tested (6/12) were HLA DR4 positive, though with the small numbers of both subjects and controls the significance of this is uncertain. They did not perform HLA blocking experiments to validate this, but did monitor

CD25 up-regulation on CD4 cells, confirming antigen specific T cell activation after peptide stimulation.

The T cell responses to Hev b 3 have also been evaluated (Bohle *et al.*, 2000). Using oligoclonal TCL and TCC, the authors demonstrated Hev b 3 (103-114) was a dominant peptide causing significant proliferation in 4/8 or 50% of subjects. This study used 12mer peptides with 3 amino acid overlap, which may have meant some T cell determinants were missed. It also had a stringent cut-off for significant proliferation of SI>5 for oligoclonal TCL and SI>10 for TCC and was only able to obtain peptide specific T cell responses in 8 of 11 (73%) latex allergic spina bifida subjects with Hev b 3 specific IgE. Using cytokine analysis, the majority (57%) of the 21 TCC were of TH2 type (defined as IL-5:IFN-γ ratio >5), with 5/6 of the TCC recognising the dominant peptide being of TH2 type. Blocking and cross-presentation experiments using these TCC directed against the dominant peptide indicated HLA-DR restriction, with no evidence of the promiscuity seen with some other allergens such as house dust mite and cat (Verhoef *et al.*, 1993; Counsell *et al.*, 1996) noted above. The authors also investigated whether the cross-reactivity seen with Hev b 3 and Hev b 1 in IgE binding studies could exist at a T cell level given their significant sequence homology. They incubated all the oligoclonal TCL and TCC with peptides manufactured to the sequence of Hev b 1 but failed to demonstrate any reactivity with any Hev b 1 peptides, suggesting cross-reactivity is predominantly at the B cell level (Bohle *et al.*, 2000).

Apart from these studies, no other human T cell epitope mapping studies in latex allergy were published prior to the studies presented in this thesis.

1.3.5.2. Hev b 5 in latex allergy

Slater and Akasawa cloned the latex allergen Hev b 5 simultaneously in 1996 (Akasawa *et al.*, 1996; Slater *et al.*, 1996). The Slater group cloned Hev b 5 from a cDNA library using the serum of a highly latex

allergic HCW as a probe. The cDNA encoded a recombinant protein of 163 amino acids with an unusual amino acid composition (46% glutamic acid, 18% alanine, 14% proline and 13% threonine), with a predicted molecular mass of 17.455 kDa, a predicted pI of 3.894 and 46 % homology with an acidic protein from Kiwi fruit (Slater *et al.*, 1996). The Hev b 5 cDNA was then expressed in *E.coli* as a fusion protein with MBP. The fusion protein reacted by ELISA with the serum IgE of 12/13 latex allergic HCW with a positive RAST for NAL and 2/10 subjects with a positive history of latex allergy but a negative RAST. No immune reactivity was reported to the MBP alone.

Despite this, several uncertainties remained. Firstly, digestion of the rHev b 5/MBP fusion protein was incomplete and resulted in a digestion product of 36 kDa, clearly a different size from the predicted mass. In addition, no Western blot analysis of the digest was made to determine whether this digestion product was immunoreactive (Slater *et al.*, 1996). Secondly, a monoclonal antibody (mAb) was raised in BALB/c mice against the rHev b 5/MBP fusion protein. Reactivity of the antibody was reported against rHev b 5/MBP, MBP and NAL. This suggested that the mAbs were not mono-specific for Hev b 5. In addition, the mAb reacted with multiple bands within NAL and was of the IgM isotype again suggesting it may not have been mono-specific (Slater *et al.*, 1996).

Akasawa and colleagues reported the cloning of the Hev b 5 protein in the same issue as Slater's report, of the Journal of Biological Chemistry (Akasawa *et al.*, 1996). This group used a different approach to Slater *et al.* They purified the natural Hev b 5 (nHev b 5) using cation exchange chromatography, followed by high performance liquid phase chromatography (HPLC). The preparation was then analysed using SDS-PAGE, Western blotting and mass spectrometry. Mass spectrometry showed a molecular mass of 16 kDa for nHev b 5. On SDS-PAGE, however, Akasawa *et al* demonstrated that the protein showed aberrant migration, with a relative mobility of 24 kDa. The Hev

b 5 protein also stained poorly with Coomassie Brilliant Blue (CBB) but was visualised on silver staining. Akasawa did not perform Western blotting of normal SDS-PAGE gels but instead used IEF gels and used passive rather than electrophoretic transfer of proteins to nitrocellulose (NC) membranes. They demonstrated Hev b 5 to have a pI of 3.5, a blocked N-terminus and to be resistant to autoclaving (Akasawa *et al.*, 1996). The nHev b 5 was also able to cause histamine release from basophils of latex-allergic HCW, consistent with it being a true allergen (Akasawa *et al.*, 1996). Also of note in this paper was that there was some minor variation in DNA sequence between the 2 Hev b 5 cDNA clones that were sequenced from an *H. brasiliensis* cDNA library and in the amino acid sequences obtained from the natural protein. This suggests that there may be isoforms of Hev b 5, and also provides a possible explanation for the slight differences in sequence reported by Slater and Akasawa (Akasawa *et al.*, 1996; Slater *et al.*, 1996).

Since these studies, there was the skin testing study of Yip and co-workers, that used rHev b 5/MBP in skin testing of latex allergic adults who were predominantly HCW (Yip *et al.*, 2000). This study demonstrated that Hev b 5 is a major allergen, with reactivity reported among 62% of latex allergic adults, making it the second most frequent sensitiser amongst the latex allergens tested in this study (Hev b 2, 3, 5, 6, 7 and 8). Also of note was that Hev b 5 was the most common allergen to be the sole sensitiser at 17% (Yip *et al.*, 2000).

Chen and co-workers have presented in abstract form that there is no immunoreactive Hev b 5 detectable using inhibition ELISA in the k82 NAL reagent of the Pharmacia CAP FEIA for latex-specific IgE (Chen *et al.*, 2000). Moreover, when rHev b 5/MBP is used to enrich or "spike" the k82 NAL reagent used in the Pharmacia UniCAP® FEIA, the sensitivity of the resulting assay is enhanced in some patients with positive clinical histories for latex allergy, but negative latex

Pharmacia UniCAP® FEIA assay results, also suggesting that the NAL contains little immunoreactive Hev b 5 (Lundberg *et al.*, 2001).

The rHev b 5/MBP has also been used in a mouse model to determine T cell and B cell epitopes (Slater *et al.*, 1999) and the cDNA was used in DNA vaccination of BALB/c mice and was able to elicit a strong antigen-specific immune response (Slater and Colberg-Poley, 1997).

1.4. Conclusion, hypothesis and aims of thesis

Latex allergy is an important occupational hazard for HCW for which no safe and specific therapy apart from allergen avoidance exists. Hev b 5 is a major latex allergen as determined by previous studies using rHev b 5/MBP fusion protein. Several uncertainties regarding Hev b 5 remain however, which are addressed within this thesis. Firstly, Hev b 5-specific mAbs were not previously reported, making definitive study and quantification of Hev b 5 within natural latex extracts difficult. Secondly, rHev b 5 has only been expressed previously with MBP, itself an immunogenic molecule. Expression of rHev b 5 without the MBP is important for two reasons: firstly to confirm previous studies showing rHev b 5/MBP to be a major allergen were due to specific Hev b 5 binding, and, secondly, if SIT with rHev b 5 is contemplated, removing MBP is desirable. Finally, no information regarding T cell responses to Hev b 5 exists; information critical to the successful development of successful T cell targeted immunotherapeutic approaches to latex allergy.

The hypothesis of this thesis is that Hev b 5 is a major latex allergen and that dominant T cell reactive regions will be identified through T cell epitope mapping.

This thesis aims therefore, to describe the human humoral and cellular immune response to Hev b 5 among a group of Australian latex allergic and non latex-allergic individuals who are predominantly HCW. In addition, the thesis will describe the production, purification and characterisation of specific mAbs directed against Hev b 5, and their

application in the analysis of latex extracts and aeroallergen samples. These mAbs also aid the achievement of the first aim in providing probes which allow accurate description of the human humoral immune response to Hev b 5 and by demonstrating Hev b 5 in the latex products and air samples that lead to clinical latex allergy.

In fulfilling these aims, this thesis will demonstrate the central importance of the latex allergen Hev b 5 in the diagnosis, current management, and potential future SIT, of latex allergy.

2. Materials and methods

2.1. Materials

2.1.1. Animals

Inbred BALB/c mice were obtained from Monash University central animal facility and maintained in the Monash Medical School animal house in accordance with National Health and Medical Research Council (NH&MRC) guidelines.

2.1.2. Antibodies

2.1.2.1. Monoclonal antibodies

mAb A1(mouse anti-Lol p 1)	Laboratory Stocks (Smart *et al.*, 1983)
mAb isotyping kit	Becton Dickinson, USA
Mouse anti-human IgE	Dako, USA
Mouse anti-human IgG_4	Becton Dickson, USA
Rat anti-human IL-5	Becton Dickinson, USA
Rat anti-human IL-5 (biotinylated)	Becton Dickinson, USA
Rat anti-human IFN-γ	Endogen, USA
Rat anti-human IFN-γ (biotinylated)	Endogen, USA

2.1.2.2. Polyclonal antibodies

Rabbit anti-human IgE (horse radish peroxidase {HRP} conjugated)	Dako, USA

Rabbit anti-human IgE Ig	Dako, USA
Rabbit anti-human Ig-HRP	Dako, USA
Rabbit anti-rHev b 5/MBP polyclonal hyperimmune Ig	Laboratory stocks, kind gift of Dr Harini de Silva
Rabbit Ig (non-immune)	Sigma, USA
Sheep anti-mouse Ig – HRP	Silenus, USA
Pig anti-rabbit Ig – HRP	Dako, USA

2.1.3. Antigens

Hev b 5 20mer peptides (Fig 2.1)	Mimotopes, Australia
House dust mite (HDM; Dermatophagoides pteronyssinus)	CSL, Australia
Ryegrass pollen extract	kind gift of Dr Cenk Suphioglu
Tetanus toxoid	CSL, Australia

2.1.4. Buffers and solutions

Coomassie Brilliant Blue (CBB) stain	50% w/v tri-chloro-acetic acid, 0.1% w/v CBB R-250.
Phosphate buffered saline (PBS)	8.5g NaCl, 0.39g $NaH_2PO_4.2H_20$, 1.07g $NaHPO_4$ made up to 1l with H_2O.
Ponceau S stain	1% Ponceau S stain, 5% acetic

Figure 2.1 Hev b 5 peptides

p(1-20) MASVEVESAATALPKNETPE
p(10-29) ATALPKNETPEVTKAEETKT
p(19-38) PEVTKAEETKTEEPAAPPAS
p(28-47) KTEEPAAPPASEQETADATP
p(37-56) ASEQETADATPEKEEPTAAP
p(46-65) TPEKEEPTAAPAEPEAPAPE
p(55-74) APAEPEAPAPETEKAEEVEK
p(64-83) PETEKAEEVEKIEKTEEPAP
p(73-92) EKIEKTEEPAPEADQTTPEE
p(82-101) APEADQTTPEEKPAEPEPVA
p(91-110) EEKPAEPEPVAEEEPKHETK
p(100-119) VAEEEPKHETKETETEAPAA
p(109-128) ΓKETETEAPAAPAEGEKPAE
p(118-137) AAPAEGEKPAEEEKPITEAA
p(127-146) AEEEKPITEAAETATTEVPV
p(132-151) PITEAAETATTEVPVEKTEE

	acid
Reducing sample buffer	10% SDS 4ml, di-thiothreitol (DTT) 78mg, glycerol 2ml, bromphenol blue 5ml, SDS-PAGE running buffer 2.5ml.
Rich broth medium (RBM)	Tryptone 10g, yeast extract 5g, NaCl 5g, glucose 2g, Ampicillin 100mg in 1 litre MilliQ H_20.
SDS-PAGE gel CBB destain solution	20% methanol, 7% acetic acid, 3% methanol.
SDS-PAGE running buffer	25mM Tris, 192mM glycine, 0.1% SDS pH 7.3
SDS-PAGE transfer buffer	20% methanol, 12 mM Tris HCl, 96mM Glycine

2.1.5. Cell lines and bacterial transfectants

Bermuda grass pollen-specific T cell line (TCL)	Kind gift of Ms Neeru Eusebius
D.pteronyssinus-specific TCL	Kind gift of Dr Harini [illegible] ilva
Murine myeloma cell line X63-Ag8.623	Kind gift of Professor Jim Goding
rHev b 5/MBP transfected DH5α *E.coli*	Kind gift of Dr Jay Slater, US Food and Drug Administration (FDA)

2.1.6. General reagents

Acetic acid	Merck, Australia
Acetone	Merck, Australia
Acrylamide – bis powder 29:1	Bio-Rad Laboratories, USA
Agarose	Progen, USA
Ammonium persulphate (AP)	Bio-Rad Laboratories, USA
Ampicillin	Sigma, USA
Bovine serum albumin	Sigma, USA
Bovine gamma globulin	Sigma, USA
Bromphenol blue	Sigma, USA
Calcium chloride di-hydrate	Merck, Germany
CBB-R250	Merck, Germany
CHAPS	BDH, Australia
Complete Freund's Adjuvant (CFA)	Sigma, USA
Citric acid	Sigma, USA
Dimethylsulphoxide (DMSO)	Sigma, USA
Disodium citrate 1.5 hydrate	BDH, England
Dithiothreitol (DTT)	Sigma, USA
Enhanced chemiluminescence reagent (ECL)	NEN Life Sciences, USA
Ethanol	Ajax Chemicals, Australia
Ethylenediamine tetra acetic acid	Merck, Australia

(EDTA)	
Gel drying solution	Invitrogen, USA
Glucose	Sigma, USA
Glycerol	Ajax Chemicals, Australia
Glycine	BDH, Australia
Hydrochloric acid (HCl)	BDH, Australia
Hydrogen peroxide (H_2O_2; 30%)	Ajax Chemicals, Australia
Hypoxanthine, aminopterin, thymidine (HAT) medium	Sigma, USA
Incomplete Freund's Adjuvant (IFA)	Sigma, USA
Methanol	Merck, Australia
Nitrocellulose (NC) membrane	Schleicher and Schuell, Germany
Phosphate citrate buffer with perborate capsules	Sigma, USA
Propan-2-ol (iso-propylalcohol)	Merck, Australia
Silver staining kit (SilverExpress®)	Invitrogen, USA
Skim milk powder	Diploma, Australia
Sodium azide	Merck, Australia
Sodium carbonate	BDH, England
Sodium dodecyl sulphate (SDS)	BDH, England
Sodium hydrogen carbonate	BDH, England

Sodium hydroxide	Merck, Australia
Sodium phosphate	Sigma, USA
Sulphosalacylic acid	BDH, England
Sulphuric acid	Merck, Australia
Syringe Filter (0.2 μm)	Gelman Life Sciences, USA
Trypan Blue	Sigma, USA
Tris	Merck, Australia
Triton X-100	Merck, Germany
Tryptone	BBL Trypticase
Tween 20	BDH, Australia
Urea	BDH, Australia
Yeast extract	Sigma, USA

2.1.7. Human Subjects

Human subjects were recruited from the Asthma and Allergy Clinic at the Alfred Hospital, Commercial Road, Prahran, Victoria, Australia (Appendix 1). All subjects gave informed consent and the study was authorised by the Alfred Hospital Ethics Committee in accordance with NH&MRC guidelines.

2.1.8. Tissue Culture Reagents

Ficoll-Paque	Pharmacia, Sweden
Foetal Bovine Serum (FBS)	CSL, Australia
Gentamicin	David Bull Laboratories, Australia

Human AB+ serum	Sigma, USA
Oxalate-pyruvate-insulin (OPI)	Sigma, USA
Penicillin-streptomycin-glutamate (PSG) 10 000 U/ml	Life Technologies, USA
Phytohaemagglutinin (PHA)	Sigma, Australia
Recombinant IL-2 (rIL-2)	Cetus Corporation, USA
RPMI 1640	Life Technologies
Sodium heparin 5000 IU/ml (preservative free)	David Bull Laboratories, Australia
Sterile water for irrigation	Baxter, Australia

2.2. Methods

2.2.1. Antigens

2.2.1.1. Preparation of NAL extract

NAL was obtained snap frozen from the Rubber Research Institute of Malaysia (RRIM), from rubber trees of *H. brasiliensis* RRIM Clone 600. It was thawed in a water bath at 20°C. The whitish, sweet smelling solid coagulum was removed and the remaining serum (250 ml) centrifuged at 50 000 *g* for 60 min at 4°C. The clear C-serum (the middle aqueous layer between the solid particles and the floating material) was then carefully removed using a glass Pasteur pipette and filter sterilised through a 0.2 µm syringe filter before protein estimation and aliquotting at -20°C. It was then further analysed by SDS-PAGE and immunoblotting as discussed below. For tissue culture, the extract was dialysed for 24 hr against a 3.5 kDa cutoff membrane (Peirce, USA) at 4°C using 3 changes of 5 l of PBS before being filter

sterilised with a 0.2 µm syringe filter and aliquotted under sterile conditions for storage at –20°C.

2.2.1.2. Preparation of LAL extract

LAL (0.7% ammonia) was obtained from Ansell Corporation (Pacific Dunlop, Australia). It was obtained at room temperature and was then processed as for NAL.

2.2.1.3. Preparation of latex glove extracts

Gloves (Table 2.1) were weighed and then sterile PBS (Life Technologies, USA; 1ml/g of glove) was pipetted into the interior of the glove the neck of the glove tightly sealed with masking tape around the exterior of the glove neck to prevent leakage. The gloves were then placed on an orbital shaker and agitated at 120 RPM for 60 min at RT. The fluid in the interior of the glove was then removed by expressing it into a sterile 50 ml tube without exposure to the outside of the glove or other materials. The gloves were then centrifuged at 5000 *g* for 10 min to remove particulate matter and then filter sterilised with a 0.2 µm filter. Protein content was then assayed using the BCA assay and the extracts analysed using SDS-PAGE, ELISA or immunoblotting and also in the case of the Uniglove extract aliquotted at –20 °C for later use in human tissue culture assays.

2.2.1.4. Recombinant protein antigens

2.2.1.4.1. Preparation of recombinant Hev b 5/MBP

The plasmids encoding Hev b 5 were obtained from Dr Jay Slater (U.S. Food and Drug Administration). The construct was ligated into *E.coli* DH5-α by Dr Harini de Silva as described previously (Slater *et al.*, 1996) and then expressed by the author in the pMAL bacterial expression system and purified as follows:

Firstly, 5 x 10 µl aliquots of a culture of DH5-α rHev b 5/MBP *E.coli* were inoculated into 10 ml aliquots of RBM under sterile conditions, together with 50 µl of 0.1g/ml Ampicillin. The sixth tube was RB alone

Table 2.1 Latex and non-latex gloves utilised for extract preparation

Glove Brand Name (Manufacturer)	Lot Number	Glove Type
Nutex (Ansell, Australia)	812450203	L, NP, S
Conform (Ansell, Australia)	810427203	L, P, S
Profeel (Unimex, Malaysia)	880711	L, P, S
Uniglove (Uniglove, Malaysia)	702022	L, P, NMUG
Handiglove (Ansell, Australia)	Not specified by manufacturer	L, P, NMUG
Progaurd	030199	NL, NP, NMUG

Key: Latex (L), Non-Latex (NL), Powdered (P), Non-powdered (NP), Examination glove (EG), Non-medical utility glove (NMUG), Sterile surgical (S)

as a negative control. These were then incubated overnight at 37°C with agitation at 240 RPM.

The following morning, the five cultures were pooled and then expanded by mixing with 450 ml of RBM + 100 µg/ml ampicillin to make two cultures of 250 ml. After 4.5 hr at 37°C with agitation at 240 RPM, 250 µl of 0.6mM IPTG was added to each 250 ml culture and the cultures grown for a further 2hrs. The cultures were then removed, and pooled before cooling to 4°C. Once cooled, the culture was mixed well and then centrifuged at 5000 *g* for 15 min. The supernatant was then discarded and the pellets resuspended in column buffer (20mM Tris HCl, 100mM NaCl, 1 mM EDTA) and pooled. The suspension was then frozen O/N at -20°C. It was then thawed and sonicated on ice using a Branson sonifier on duty cycle 90%, output control 5, in bursts of 1 min until it changed from white to a light brown colour (6 min). The bacterial sonicate was then centrifuged for 30 min at 4°C at 39 000 *g*. The supernatant was then carefully removed.

The amylose column (New England Biolabs, USA) was equilibrated with 5 column volumes of column buffer. Ten ml of the bacterial lysate supernatant (BLS) was loaded on the column. After allowing passage of the BLS, it was eluted using elution buffer (column buffer + 10 mM maltose). Fractions of 1.5 ml were collected in microfuge tubes and then analysed for protein concentration (Bradford assay) and purity (SDS-PAGE).

2.2.1.4.2. Cleavage of rHev b 5 from Hev b 5/MBP

Cleavage of rHev b 5/MBP was carried out using Factor Xa at 10 IU/mg of fusion protein as according to the manufacturer's instructions (New England Biolabs, USA). Digestion however was incomplete, and in an effort to improve digestion levels various detergents and denaturing conditions were evaluated. These included SDS 0.5%, Triton X-100 0.5%, Tween 20 0.5%, CHAPS 0.5%. and Urea 0.1M comparing for each with and without Factor Xa and a timecourse of 0,

2, 4, 8 and 24 hr. These were then analysed for cleavage efficiency using SDS-PAGE.

2.2.1.4.3. Preparation of rHev b 5 in pProX-HTa bacterial expression vector

Because of the difficulties of incomplete digestion of the rHev b 5/MBP construct and the immunoreactivity of MBP itself, it was necessary to express the plasmid encoding the cDNA for Hev b 5 in an expression system that did not use a large fusion protein system. The pProX-HTa system was selected as it expresses target proteins with a small hexa-histidine tag which allows purification on a nickel column. This cloning work was performed by Dr Alec Drew from the Allergy Research Group, Monash University. After optimisation by Dr Drew, the expression, purification and analysis steps were performed by the author as follows:

The sequence encoding the Hev b 5 protein was amplified by the polymerase chain reaction (PCR) using plasmid pMAL/Hev b 5 DNA as the template (Slater *et al.*, 1996). In detail, 20 ng of plasmid DNA was amplified using the primers: HEVB5F (5'–GCGGAATTCATGGCCAGTGTTGAGGTTG-3') and HEVB5R (5'-GCGGTCGACTTATTCCTCTGTTTTTTCCACC-3'). Thirty cycles of amplification were conducted with each cycle consisting of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C, followed by incubation at 72 °C for 10 min. The pProX-HTa vector (Life Technologies; possessing a hexahistidine tag to facilitate purification) and the Hev b 5 PCR product were cleaved with *Eco*RI and *Sal*I and purified by agarose gel electrophoresis followed by extraction of the DNA from the gel (QIAGEN Gel purification kit, Qiagen Corporation, USA). Vector DNA and the PCR product were ligated using T4 DNA ligase and transformed into DH5α competent cells. *E. coli* DH5α cells were grown to logarithmic stage and expression of rHev b 5 induced by the addition of isopropyl-1-thio-β-D-galactoside to a final concentration of 0.6 mmol/L. Following induction of expression the cells were grown

for 4 hr at 37 °C. The bacteria were harvested by centrifugation for 15 min/ 4000 *g* at 4 °C and the pellets frozen overnight at –20 °C. The pellets were resuspended in 20 ml of native lysis buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 10 mM imidazole) per litre of original culture. The cell suspension was sonicated 6 × 20 sec on ice, using a Branson sonifier model 250 and then centrifuged at 4 °C for 30 min at 10 000 *g*. The supernatant was applied to a column containing Ni-NTA agarose (Qiagen Corporation, USA) with a bed volume of 5 ml. The column was washed with native lysis buffer and then eluted (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 250 mM imidazole). Recombinant Hev b 5 was soluble with a yield of 10 mg per litre of broth culture. The concentration of rHev b 5 was determined by the bicinchoninic acid (BCA) assay (Pierce, USA) with bovine gamma globulin as the standard. SDS-PAGE (under reducing and denaturing conditions) and Western analysis was used to assess purity and integrity of rHev b 5.

2.2.2. Human subjects: clinical and laboratory assessment

Subjects were recruited by the author from the Asthma and Allergy Clinic at the Alfred Hospital, Commercial Road, Prahran, during the course of his medical work as a consultant physician. Subjects were predominantly HCW, however there was no selective bias to recruit HCW (Appendix I). Subjects gave informed consent to be venesected and have 120 ml of whole blood taken for both cellular (100 ml whole blood) and serological (20 ml of whole blood) assays. The study was conducted with the ethical approval of the Alfred Hospital Ethics Committee according to National Health and Medical Research Council (NH&MRC) guidelines.

2.2.2.1. Latex allergy questionnaire

A self administered questionnaire (SAQ) to collect clinical information was used as part of general clinical assessment and was based on the questionnaire used in the Alfred Hospital nurses prevalence study (Douglas *et al.*, 1997). The former questionnaire was 4 pages, so the

SAQ developed for the current study was shortened to be one page in length for ease of completion and convenience (Figure 2.2).

2.2.2.2. Latex, banana, avocado, kiwi EAST

This was performed by the Alfred Hospital Pathology Service according to the manufacturer's instruction (Sanofi-Pasteur Diagnostics, USA). The results are expressed in Allercoat EAST Units/ml (AEU/ml), which is the bio-equivalent of 0.5 IU/ml of IgE. For the purposes of log transformation and statistical analysis, results of <0.18 AEU/ml were assigned a value half way between this and zero, i.e. 0.09 AEU/ml.

2.2.2.3. Pharmacia UniCAP® FEIA for latex-specific IgE

This was performed by the Alfred Hospital Pathology Service according to the manufacturer's instruction (Pharmacia, Sweden). These were performed in a single batch, by a single, blinded path technician. Results are expressed as IU/ml. Again, for the purposes of log transformation and statistical analysis, results of <0.35 IU/ml were assigned a value half way between this and zero, i.e. 0.18 AEU/ml. UniCAP® class was assigned according to the manufacturer's instructions.

2.2.2.4. Total IgE

This was performed by the Alfred Hospital Pathology Service according to the manufacturer's instructions (Pharmacia, Sweden). Results are expressed as IU/ml.

2.2.3. Human tissue culture

2.2.3.1. Separation of PBMC from whole blood

This was carried out according to the method of O'Hehir and colleagues (O'Hehir *et al.*, 1993). Whole blood (100 ml) was obtained through venesection of volunteers into 2 heparinised (John Bull, Australia) 60 ml latex-free syringes (Becton Dickinson, USA; 1000 Units/syringe approximately) and then agitated gently at RT on a

Figure 2.2

Latex Allergy Questionnaire

Thankyou for taking to time to fill in this one page survey. You are not identified by name and all responses remain strictly confidential.

ID NUMBER ☐ Initials ☐ Date ☐

1. Age ☐ yrs Sex M ☐ F ☐

2.Occupation (be specific eg theatre nurse) ☐

3.How long have you worked in your current occupation ☐ years

4.How many operations with a general anaesthetic have you had? ☐ ☐

5.Do you have spina bifida? yes ☐ no ☐

6.Have you ever been diagnosed with latex allergy by a doctor? yes ☐ no ☐

If yes, when ☐

7.What medications have you taken in the last week(include skin creams,natural meds, contraceptives)

☐

8.Have you ever suffered from (tick if yes)

Hay fever ☐ Asthma ☐ Eczema ☐ Food Allergies ☐

9.Are you currently allergic to any foods ? yes ☐ no ☐

If yes please list ☐

10.Are you allergic to (tick if yes) banana ☐ avocado ☐ kiwi fruit ☐

11.Does contact with natural rubber/latex products produce....?(tick if yes)

- ☐ Skin itching, redness and irritation localised to the point of contact
- ☐ Skin hives (red itchy lumps like mosquito bites)
- ☐ Redness of the skin all over the body
- ☐ Sneezing, itchy eyes or running nose
- ☐ Facial swelling
- ☐ Throat or tongue swelling
- ☐ Shortness of breath, wheezing or asthma
- ☐ Faintness, collapse or anaphylaxis

12. How many times have you had reactions to latex?

none ☐ 1-5 ☐ more than 5 ☐

13.Which latex product(s) cause problems for you?

powdered latex gloves ☐ non-powdered latex gloves ☐ balloons ☐

condoms ☐ other(please list) ☐

14.Do you wear latex gloves at your place of work? yes ☐ no ☐

If yes, how many changes of powdered ☐ or non-powdered gloves ☐ do you have per day?

15.If you don't wear latex gloves at work, what gloves do you wear?

Dont wear gloves ☐ vinyl ☐ nitrile ☐ neoprene ☐

16.Do others wear powdered latex gloves in your working environment?

yes ☐ no ☐ don't know ☐

Again, thankyou very much for your assisstance with filling out this questionnaire

rocker shaker to prevent coagulation until processing (always less than 6 hours following venesection).

Whole blood was then diluted 1:1 in pre-warmed RPMI with Heparin 50 Units/ml and PSG (RPMI-heparin) in sterile 50 ml tubes (Becton Dickinson, USA). The mixture (25 ml per tube) was then layered gently over 15 ml of Ficoll (Pharmacia, Sweden) at RT in sterile 50 ml tubes. These were then centrifuged at 652 *g* for 25 min with the brake off. The resultant Buffy coat was then gently removed using a sterile plastic Pasteur pipette and diluted 1:1 into RPMI with PSG (RPMI-wash medium; Invitrogen, USA). These cells were then centrifuged for 15 minutes at 452 *g* with brake on, then the supernatant discarded, resuspended in 50 ml of RPMI-wash medium and centrifuged once more for 10 min at 340 *g* (brake on). The supernatant was then discarded and the cells were resuspended in 5 ml RPMI with 5% Pooled Human AB + serum and PSG (Complete Medium) and the cells counted using a haemocytometer. The excess cells were then processed for freezing and storage under liquid nitrogen as described below or diluted to the appropriate concentration for use in cellular assays.

2.2.3.2. Freezing of cells and cell lines

Cells were first resuspended in Complete Medium at 20 x 10^6/ml for PBMC and lesser densities for T cell lines. The cell suspension was then cooled on crushed ice. An equal volume of ice-cold 12% DMSO in FBS was then added dropwise to the cell suspension. One ml of the cell suspension was then added per cryovial (Nunc, Sweden) and then the cryovials frozen overnight in a Mr Frosty® at -70°C before transfer to LN tanks.

2.2.3.3. Thawing of cells and cell lines

Cells were recovered from LN storage and transported to the lab on dry ice. They were then thawed rapidly in a water bath at 37°C. The cells were then removed from the cryovial using a sterile Pasteur pipette and RPMI wash medium added slowly drop wise. The cells were then

washed twice as described below before resuspension in complete medium at the desired cell concentration.

2.2.3.4. Washing of cells

This was performed by diluting the cell suspension in RPMI wash medium to 20 ml in a 20 ml centrifuge tube. The cells were then centrifuged at 340 *g* for 10 min, before discarding the supernatant and repeating the wash cycle, or resuspending in complete medium.

2.2.3.5. Irradiation of cells

After thawing, washing twice and resuspension in complete medium, cells were irradiated, where required, with 3000 rad, in a Gammacell irradiator (Nordion International, USA).

2.2.3.6. PBMC primary proliferation assays

PBMC (1 x 10^5/well) were added to 96 well round bottomed plates (ICN Flow, USA) with or without antigen stimulation and cultured in Complete Medium. All assays were performed in triplicate with a triplicate no-antigen control. Antigens tested included PHA (3 μg/ml), tetanus toxoid (0.3 LF/ml), LAL (3 and 30 μg/ml), Uniglove GE (3 and 30 μg/ml) and rHev b 5 (1 and 10 μg/ml). These antigen doses were chosen after considering studies using other latex antigens (Johnson *et al.*, 1999) and in-house experience (de Silva *et al.*, 2000) and also with a view to considering the number of concentrations that could physically be achieved in examining a group of 51 patients. Plates were then cultured in 5% CO_2 at 37°C for 7 days. On day 6 in the evening prior to harvest, to assay cellular proliferation, tritiated thymidine (^{3}H-thymidine), 1 μCi/well was added and the plates harvested using a 96 well automatic cell harvester (Skatron, U.K.) 12-18 hours later to fibrous paper mats (Wallac, U.K.). After drying at 37°C for 4 hours, scintillant was then added and ^{3}H-thymidine incorporation measured by liquid scintillation spectroscopy (Wallac 1205 β-counter; Wallac, UK). Results were expressed as counts per minute (CPM).

2.2.3.7. PBMC primary assays for cytokine measurement

In a subset of the study group, where sufficient cells were available, PBMC cultures were set up in 24 well plates, 2ml per well at a density of 1 x 10^6/ml complete medium. Antigens tested were PHA (3 µg/ml), GE (10 µg/ml), rHev b 5 (10 µg/ml) and no antigen control. Supernatants (500 µl) were taken, and replaced with fresh Complete Medium, at 72 hr and at day 6 for cytokine level determination, for IL-5 and IFN-γ, as discussed below.

2.2.3.8. Generation of oligoclonal TCL

Oligoclonal TCL were generated as follows after 2 rounds of PMBC proliferation. Cells were cultured in 24 well plates, as for the PBMC assays for cytokine measurement, with GE at 10 µg/ml. Generally, 4 wells for each patient were set up, where sufficient cells were available. At day 7, cells were recovered, washed once in RPMI wash (340 *g*/10 min) and then restimulated with GE at 10 µg/ml, together with the addition of autologous PMBC, thawed and irradiated as described, at a one to one ratio. The total number of cells per well was 2×10^6. At day 8 and 10, 500 µl rIL-2 was added, for a concentration of 25 IU/ml. At day 14, cells were recovered once more, washed once and restimulated with Hev b 5 peptides as described below.

2.2.3.9. T cell proliferation assays for Hev b 5 peptides

Two week oligoclonal TCL (5×10^4/well) were added to 96 well plates together with thawed, washed and irradiated autologous PBMC in a 1:1 ratio. They were stimulated with rHev b 5 (0.1, 1 and 10 µg/ml), GE (0.3, 3, 30 µg/ml) and Hev b 5 peptides (10 µg/ml). Initial optimisation experiments in reactive subjects were performed stimulating at 10 and 30 µg/ml. Subsequent to these, 10 µg/ml only was used. In addition, rIL-2 at 50 IU/ml and no antigen were used as positive and negative controls respectively. Cells were cultured for 72 hours total before harvesting, with ^{3}H-thymidine added in the last 12-18 hr, and proliferation assayed as described above. In addition, at 48 hr, 70 µl/ml

supernatant was removed and replaced with the same volume of CRPMI for the measurement of IL-5 and IFN-γ as described below.

2.2.3.10. Cytokine ELISA

2.2.3.10.1. IL-5

This was measured by capture ELISA. Opaque (white) ELISA plates (Costar, USA) were coated with capture antibody (anti-IL5, 1 µg/ml; 50 µl/well) diluted in ELISA coating buffer overnight at 4°C. Plates were then washed twice in 0.05% PBST and then blocked with 1% BSA-PBST. They were then washed 3 times in 0.05% PBST and then standards (recombinant IL-5 in doubling dilutions from 5000 to 1.2 pg/ml diluted in 1% BSA-PBST) or samples (T cell or PBMC supernatants pooled from triplicate cultures) were added in duplicate (30 µl/well) and incubated overnight at 4°C. Plates were then washed 5 times in 0.05% PBST before adding the detection antibody (biotinylated anti-IL5 1 µg/ml; 50 µl/well) for 1 hour at RT. Plates were then washed 5 times in 0.05% PBST before adding streptavidin peroxidase 1:2000 in 1% BSA and incubating at RT for 1 hour. The plates were then washed 4 times PBST% and 4 times in PBS. Fifty microlitres per well of ECL reagent (oxidising reagent: luminol reagent, 1:1) was then added and the plates read in a Lumicount microplate glow luminometer (Packard Instrument Company, USA), 0.5 seconds/ well, automatic sensitivity setting. Standard curve generation and interpolation of unknown cytokine levels was performed using Packard I-Smart software (Packard, USA).

2.2.3.10.2. IFN-γ

This was also measured by capture ELISA. The method was similar to that for IL-5, however the capture antibody (anti-IFN-γ) was coated at 1 µg/ml, the standard was recombinant IFN-γ and the detection antibody, biotinylated anti-IFN-γ was added at 0.5 µg/ml.

2.2.4. Monoclonal antibodies to rHev b 5

2.2.4.1. Immunisation schedule

Six week old female BALB/c mice were obtained from the Monash University central animal facility and maintained in the Monash Medical School animal house in accordance with National Health and Medical Research Council (NH&MRC) guidelines. The study was approved by the Monash University Animal Ethics Committee.

The mice were injected according to the following schedule with varying amounts of rHev b 5/MBP with or without boosting doses of NAL. The variations were used because there was no published schedule of immunisation doses for previous attempts to make Hev b 5 specific mAbs and therefore different doses and antigen boosting regimens (rHev b 5 with or without NAL) were used to achieve optimisation. General principles of immunisation schedules and doses were based on those of Goding (Goding, 1996) and Harlow and Lane (Harlow and Lane, 1988). Mice were ear tagged for identification and numbered #1-#6. The immunisation doses of Complete Freund's Adjuvant (CFA) or Incomplete Freund's Adjuvant (IFA) together with PBS and rHev b 5/MBP were prepared under sterile conditions and vortexed for 30 sec in sterile microfuge tubes immediately prior to injection to achieve a satisfactory emulsion.

23/8/99 (Day 0) 50 µg rHev b 5/MBP in 125 µl PBS with 125 µl CFA (#1-6).

6/9/99 (Day 14) 50 µg rHev b 5/MBP in 125 µl PBS with 125 µl IFA (#1-3) or 125 µl PBS(#4-6)

#1 – humanely killed after it developed ascites

22/9/99 (Day 30) 50 µg rHev b 5/MBP in 250 µl PBS (#2-6)

17/10/99 (Day 55) 50 µg rHev b 5/MBP in 250 µl PBS (#5 only)

1/11/99 (Day 60) 50 µg rHev b 5/MBP in 250 µl PBS (#4 only)

8/12/99 (Day 97) 100 µg LAL in 250 µl PBS (#4 & 5 only)

3/2/00 (Day 154) 20 µg rHev b 5/MBP in 250 µl PBS (#3-6), 10 µg rHev b 5/MBP #2.

7/4/00 (Day 213) 10 µg rHev b 5/MBP in 250 µl PBS (#2) - fusion

5/5/00 (Day 243) 20 µg rHev b 5/MBP in 250 µl PBS (#3) – fusion.

2.2.4.2. Screening for polyclonal immune response

Screening for the polyclonal immune response was performed using mouse serum. Whole blood (20 µl) was obtained by gently placing a glass tube into the retrobulbar plexus of the mouse (eye bleed technique). The whole blood was then allowed to clot for 1 hour at room temperature in sterile microfuge tubes, before being centrifuged for 2 minutes at 1000 *g*.

Samples were obtained 4 days prior to the first immunisation and then 4 days prior to each subsequent immunisation and the specific immune response measured by ELISA and Western blotting techniques (see below).

2.2.4.2.1. Optimisation of screening assays

The ELISA was optimised for antigen concentration by titrating antigen dose against a constant serum dilution of the hyper-immune serum at 1:2000. This was done for rHev b 5/MBP, MBP and NAL. ELISA plates (96 well polyvinyl high binding; Costar®; Corning, USA) were coated with antigen diluted in ELISA coating buffer overnight at 4°C. Plates were then washed 8 times with PBST, before blocking overnight with 5% SMP-PBST. Plates were then washed 8 times with 0.05% SMP-PBST then the serum dilutions (1:2000 in 0.05%PBST) were added, 50 µl/well and incubated at 37°C for 1 hour. Plates were then washed once more 8 times 0.05% SMP-PBST. The secondary antibody, was used at a dilution of 1:5000 in 1% SMP-PBST. This dilution was chosen following titration experiments using hyper-immune serum from mouse #4 at 4 dilutions together with sheep anti-mouse HRP at dilutions of

1:1000, 1:2000 and 1:5000 in 1% SMP-PBST. The incubation of the secondary antibody was for 1 hour at 37°C. The plates were then washed 4 times with 1% SMP-PBST and 4 times with PBS alone. The substrate, 50mg OPD dissolved in 25 ml of phosphate-citrate buffer with perborate was added (50 µl/well) for 20 minutes, in the dark, at 37°C. The reaction was stopped with 50 µl/well of 4M HCl and colour development quantified by measurement of absorbance at 490 nm on an optical plate reader (BioRad, USA).

2.2.4.2.2. Western blotting assays of polyclonal immune response

To examine reactivity of immunised mouse to natural latex proteins, Western blots were performed against NAL using polyclonal immune sera at a concentration of 1:5000 in 1% SMP-PBST and secondary antibody at 1:5000 in 1% SMP-PBST as described for Western blotting using ECL development. Inhibition immunoblots were also performed using rHev b 5/MBP and rMBP as inhibitors.

2.2.4.3. Hybridoma fusion

Mice were humanely killed by cervical dislocation. They were then bled from the heart (400 µl approximately) to obtain whole blood for polyclonal hyper-immune serum. This was processed as for the other mouse sera and aliquotted in 50 µl lots before storage at -20°C for later use as positive controls in hybridoma screening assays.

The splenocyte fusion partner, murine myeloma cell line X63-Ag8.623 (kind gift of Professor Jim Goding) was thawed rapidly and washed twice as described and cultured in RPMI with 10% fetal calf serum (FCS; CSL, Australia). The cells were passaged for several cycles to ensure health and also a small number were sub-cultured in HAT medium (they died as expected within 48 hr confirming HAT sensitivity). The cells that had been passaged in RPMI+10%FCS+PSG were then grown to a density of 2×10^5/ml in a volume of 100 ml of RPMI+10%FCS+PSG for a total of 20×10^6 cells. They were checked by light microscopy for health (round, no blebs or crenellations) and

trypan blue exclusion for viability (>99% viable). The cells were then centrifuged at 340 *g* for 10 minutes at RT before resuspension in 5 ml of RPMI+10% FCS+PSG.

Murine splenocytes were prepared as follows: The spleen from the hyper-immune animal was removed using aseptic technique and then placed in a sterile petri dish. It was then gently macerated using two nineteen gauge sterile needles. The macerated spleen was then suspended in 20 ml of RPMI without serum in a sterile 50 ml tube. The solid, fibrous material within the spleen was then allowed to settle by gravity for 2 minutes and the supernatant was then carefully poured off into another sterile 50 ml tube. The cells were then washed once (centrifuged at 340 *g* for 10 minutes) before resuspension of the pellet in 20 ml RPMI+PSG, and centrifugation once more at 340 *g* for 10 minutes. The splenocyte cell pellet was then resuspended in 5 ml of RPMI+10% FCS+PSG and then gently mixed with the 5 ml suspension of the myeloma fusion partner. The combined 10 ml cell suspension was then centrifuged at 340 *g* for a further 5 minutes.

One ml of 50% PEG in RPMI+PSG was heated to 80° C on a heatblock to melt the PEG before being cooled to 37 °C in a sterile 10 ml tube with gentle agitation to keep it in solution. The 50% PEG was then added slowly to the cell pellet drop by drop with a sterile Pasteur pipette over one minute and the cell pellet itself gently mixed with the tip of the Pasteur pipette. Immediately following this, a further 1 ml of RPMI+PSG was added over 1 min then another 9 ml over the next 2 min, stirring gently at all times with the Pasteur pipette tip. The cells were then centrifuged for 5 min at 340 g. The cells were then resupended in 60 ml of RPMI+20% FCS, 1×OPI, 1×HAT and PSG. The cell suspension was then plated into 6 sterile flat bottom tissue culture plates (100 µl/well) using a multi-channel pipette (Gilson, France).

2.2.4.4. Culture, subculture, screening and cloning of hybridomas

The hybridoma cells were cultured in an incubator at 37°C with 7% CO_2. Cultures were "fed" with a further 100 µl/well of RPMI+20% FCS, 1×OPI, 1×HAT and PSG at day 4. The first screening assay was carried out on day 7, by which time the larger hybridoma colonies were visible by eye. A further screening assay was performed on day 10 and where there were slow-growing colonies on day 14. Screening assays were performed by ELISA against rHev b 5/MBP and rMBP for all wells. Briefly, 100 µl/well of supernatant was removed from each well. Duplicate ELISA were performed for rHev b 5/MBP (10 ng/well) and MBP (10 ng/well), with 50 µl/well of hybridoma supernatant per well and further steps as described above. Wells that were positive for rHev b 5/MBP but negative for MBP alone were then removed from 96 well plates and plated into 24 well plates with 200 µl RPMI+10% FCS + 1×HAT medium. They were then observed daily and additional medium added as required. Once covering the whole base of a 24 well plate well, they were split and some cells taken for cloning by limiting dilution. This was carried out according to established methods (Goding, 1996). Briefly, 200 µl of a suspension of the hybridoma cells at 1 × 10^5/ml were placed in well A1 of a 96 well plate. Doubling dilutions were then begun down column 1 (B1, C1 and so on) diluting into 100 µl of a non-immune splenocyte suspension from a female BALB/c mouse prepared in the same way as the immune splenocytes for a fusion. Once column 1 was completed, a further 100 µl of splenocyte suspension was added to each well of column 1 and then doubling dilutions of 100 µl performed across the plate from column 1 to column 12 using a multi-channel pippette. The plate was then left for 10 to 14 days until the most distal hybridomas derived from a single cell had grown to sufficient size to allow screening. If all wells containing hybridomas were positive, the process was repeated using the distal hybridomas derived from single cells and on the second

cloning the most distal cells were expanded for larger scale purification and storage.

2.2.4.5. Purification of monoclonal antibodies

MAbs 6A10 and 3G3 were purified on a protein G column (Pharmacia, Sweden). MAb 6F6 was purified using a Biosepra® protein A column (Life Technologies, USA). Briefly, hybridoma 6F6 was grown to maximal density in roller bottles (Sigma, USA), rotating at 2 RPM in a warm room at 37° C. The supernatant was then removed and then centrifuged at 5000 *g* for 10 min. The supernatant was then filter sterilised and loaded directly onto the column (either protein G or protein A) and run by gravity feed at 15 ml/hr. Following loading of the hybridoma supernatant onto the column, the column was then washed with 10 column volumes of 0.5M NaCl 0.1M PO4 buffer pH 7.5. Elution was then carried out using 0.1M citric acid pH 3 in 2.5 ml aliquots. Each aliquot was immediately neutralised with 1M Tris HCl pH8 (500 µl / aliquot). Aliquots were then assayed for protein by the BCA protein assay using a solution of 2.5ml PBS with 500 µl 1M Tris HCl pH 8 as a blank, and for immunologic activity by rHev b 5-specific ELISA.

2.2.4.6. Characterisation of rHev b 5-specific mAbs

2.2.4.6.1. Isotyping of monoclonal antibodies

Isotyping was carried out by ELISA using a commercial kit (Becton Dickinson, USA) according to the manufacturer's instructions.

2.2.4.6.2. Western blotting of latex extracts using mAbs

Western blotting was carried out according to the method described below, with dilutions of purified mAbs of 1:500 for 4-chloro-1-naphthol development or 1:5000 for ECL development.

2.2.4.6.3. Direct ELISA of latex and protein extracts using mAbs

Purified mAbs were used in ELISA assays as described below against rHev b 5, NAL, LAL, GE (Uniglove), RGPE and kiwi. To ensure specificity, inhibition ELISA was performed using varying concentrations of rHev b 5 pre-incubated with the mAb dilution for 1 hr at 37°C.

2.2.4.6.4. Direct ELISA of Hev b 5 peptides using mAbs & human IgE

To map linear epitopes in Hev b 5 recognised by the mAbs and human IgE, rHev b 5 peptides were used to coat ELISA plates at 10 µg / ml overnight at 4° C. The three mAbs purified, 6A10, 6F6 and 3G3 (1: 1000 in 1% SMP-PBST), together with latex allergic subject 17 (1:20 dilution in 1% SMP-PBST) were then used in ELISA assays as described below.

2.2.4.6.5. Testing for sandwich formation using rHev b 5 specific mAbs and human IgE

To determine whether sandwich formation occurred between the rHev b 5 specific mAbs, mAb 6F6 was used to coat ELISA plates at a concentration of 1 µg/ml. Varying concentrations of rHev b 5 and NAL were added, then mAbs 6A10 and 3G3 at concentrations of 1:1000 in 1%SMP-PBST. The ELISA was then developed as below except that the detection Ab was antimouse IgG1. In addition to the mAbs, serum from latex-allergic human subject 17 (1:10 dilution in 1% SMP-PBST) was used in the assay and developed as below.

2.2.5. Protein analysis

2.2.5.1.1. Protein concentration estimation using the Bichinchoninic assay

The BCA protein assay (Pierce, USA) was performed according to the manufacturer's instructions. Briefly, Reagent A (Sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.2 M sodium hydroxide) was mixed with Reagent B (4% cupric sulphate) in a

ratio of 50:1 to make the Working Reagent (WR). Two hundred microlitre per well of WR were added to a 96 well microtitre tray (Becton-Dickinson, USA). Fifty microlitre of diluted protein standard (Bovine gamma globulin, BioRad, USA), unknown sample or blank (PBS) were then added to each well and mixed by gentle agitation. They were then incubated in the dark at 37°C for 30 min before being read on a plate reader at 595 nm as for the Bradford assay above.

2.2.5.2. SDS-PAGE

SDS-PAGE was carried out according to the method of Laemmli (Laemmli, 1970) as described below.

Minigel® (Invitrogen, USA) gel cassettes were used for all one dimensional SDS-PAGE gels. Gels were made according to the manufacturer's instructions.

Briefly, polyacrylamide gels were made according to the following recipe for a 16% SDS-PAGE gel:

Separating Gel

6 ml of 50% Acrylamide/BIS 29:1 (48.3g Acrylamide, 1.7g BIS brought up to 100 ml with H_2O)

9.4 ml of IM Tris HCl pH 8.8 (30.3g tris in 150ml H_2O)

250 µl 10% SDS

8.8 ml of H_20

6.25 µl TEMED

625 µl 50 mg/ml ammonium persulphate

The ingredients were mixed and then carefully pippetted into the Minigel® cassette without introducing air before being overlayed with MilliQ H_2O. The gels were then allowed to set at RT for 1hr. The percentage of polyacrylamide that was added to the original mixture

was varied as required for gels from 12-20%. After the separating gel had set, the water was removed and the stacking gel added. This had the following recipe:

1 ml of 50% Acrylamide/Bis 29:1

4.2 ml 0.375M Tris HCl pH 6.8

125 µl 10% SDS

6.3 ml H_2O

5 µl TEMED

1.0 ml of ammonium persulphate 50 mg/ml.

2.2.5.3. Immunoblotting

2.2.5.3.1. Dot immunoblotting

Dot blotting was performed by applying the protein of interest diluted in 5 µl of PBS to the centre of a 1 cm^2 area of NC membrane. The membrane was then processed as described in Western blotting.

2.2.5.3.2. Electrophoretic protein transfer

Electrophoretic protein transfer was carried out from the SDS-PAGE gels to NC membrane in transfer buffer with 25V DC for 60 min at RT. Following this, the NC membranes were removed and processed as described in Western blotting.

2.2.5.3.3. Western blotting with human IgE

NC membranes were blocked in 5% SMP-0.05% PBST for 1 hr at RT, washed 2 times in 0.05% PBST then incubated with rabbit anti-human Ab (Dako, Denmark) diluted 1:500 in 1% SMP-0.05%PBST for 1 hr at RT. They were then washed 3 times for 5 minutes each in PBST and then incubated with pig anti-rabbit HRP conjugate (Dako, Denmark) at 1:1000 for 1 hour at RT. They were then washed twice in 0.05% PBST and once in PBS alone before development in 4-chloro-1-napthol as

described below. Where ECL reagent was used in development, the human serum was diluted 1:10 in 1%SMP-0.05%PBST and a directly conjugated secondary antibody, rabbit anti-human IgE-HRP at 1:1000 in 1% SMP- 0.05% PBST was used because of the development method's greater sensitivity. In addition, washing steps were increased to five washes of 5 min each in 0.05% PBST.

2.2.5.4. Western blotting with Hev b 5-specific mAbs

For Western blotting murine mAbs, the procedure was similar to the above, except the primary antibodies were used as hybridoma supernatants at 1:10 dilution in 1% SMP-PBST and purified antibodies at 1:1000 in 1% SMP-PBST where 4-chloro-1-naphthol development was used. The secondary detection antibody was sheep anti-mouse HRP conjugate (Silenius) diluted 1:1000 in 1% SMP-0.05% PBST. Again, where ECL development was used, primary and secondary dilutions were both increased to 1:5000 in 1% SMP-0.05% PBST and washing steps also increased as above.

2.2.5.4.1. 4-Chloro-1-naphthol Western blot development

Following the washing steps described above, the Western blots were developed in 38 mg of 4-chloro-1-naphthol dissolved in 40 ml of MQH_2O heated to 37°C and 10 ml of methanol together with 50 µl of 30% hydrogen peroxide. Colour development was observed and then the reaction stopped with 3 changes of MQH_20 at RT.

2.2.5.4.2. ECL reagent Western blot development

This was carried out using a ECL reagent kit according to the manufacturer's instructions (NEN Life Sciences, USA). Briefly 0.125 ml total/cm^2 of NC membrane of a 1:1 mixture of the luminol reagent and the oxidising reagent were added and incubated with the NC membrane for 1 min at RT on the orbital shaker at 60 RPM. The excess ECL reagent was then removed and the wetted NC membrane placed between two plastic sheets and exposed to auto-radiography film

(Kodak, Australia) in a darkroom until the desired signal intensity was achieved.

2.2.6. ELISA

2.2.6.1. rHev b 5 ELISA for human IgE

ELISA plates (Costar High Binding, Corning, USA) were coated onvernight with rHev b 5 0.5 µg/ml in ELISA coating buffer. The coating buffer was then shaken out and the plates blocked with 5% SMP-PBST 200 µl/well overnight at 4°C. The plates were then washed 8 times with PBST. Human sera were then added at a 1:10 dilution in 1% SMP PBST for 2 hours at 37°C. The plates were then washed 8 times with PBST. Following this, the detection antibody, anti-Human IgE-HRP conjugate 1:500 in 1% SMP-PBST was added 50 µl/well for 2 hours at RT. The plates were then washed 4 times with PBST and 4 times PBS. The plates were then developed with OPD as described for the mAb ELISA. Assays were done in triplicate with a triplicate no antigen control. An arbitrary constant of 0.1 was added to all values to prevent negative values.

2.2.6.2. rHev b 5-specific IgG_4 ELISA

This was performed as for the IgE ELISA, however the detection antibody was biotinylated anti-human IgG_4 and this was then detected with streptavidin peroxidase conjugate as described below.

2.2.6.2.1. Optimisation of IgG_4 ELISA

In order to optimise the concentration of anti-human IgG_4 and streptavidin peroxidase for optimum signal/background ratio, the antibody and streptavidin conjugate were each titrated in a "checkerboard" fashion with dilutions of anti-human IgG_4 of 1:500, 1:1000 and 1:2000 in 1% SMP-PBST and of streptavidin peroxidase. The sera chosen included subject 27 who had a high rHev b 5 specific IgE and subject 37 who had no detectable rHev b 5 specific IgE as a negative control. Each were used at the constant dilution of 1:10 in 1% SMP-PBST. The anti-human IgG_4 antibodies were added at the above

dilutions (50 µl per well) and incubated for 1 hour at 37°C. The plates were then washed 8 times in PBST and then the streptavidin peroxidase conjugate added at the above dilutions for 1 hour at 37°C. The plates were then washed 8 times in PBST and the remainder of development and plate reading was carried out as for the IgE ELISA.

2.2.6.3. rHev b 5 specific IgG_4 in human subjects

Following optimisation, the rHev b 5 specific IgG_4 ELISA was carried out on the study population using a dilution of human serum of 1:10 in 1% SMP in PBST. Processing was as described above, with the optimal concentration of anti-human IgG_4 being 1:500 and the optimal concentration of the streptavidin peroxidase conjugate being 1:1000. Assays were performed in triplicate with a triplicate no antigen control. An arbitrary constant of 0.1 was added to all values to eliminate negative values.

2.2.7. Examination of aero-allergen samples using rHev b 5 specific mAbs in Halogen® assays

This was carried out at the Institute of Respiratory Medicine (IRM, Sydney, Australia) by Dr Theresa Mitakakis according to their previously published protocols (Graham *et al.*, 2000). MAbs 6A10 and 6F6 were used in these studies at a dilution of 1:2000 in 1% BSA-PBS (Mitakakis *et al.*, 2002).

2.2.8. Statistical Analysis

Statistical analysis was carried out using SPSS® for Windows® version 10. Categorical variables were examined using the Chi Squared test with a Yates correction for continuity or the Fisher's exact test where the expected cell frequency was less than 2. Continuous variables were examined using Wilcoxon rank sum test for non-parametric distributions and a Student's T test for normally distributed variables. The level of alpha was set at 0.05 with $p<0.05$ being considered statistically significant.

3. Monoclonal Antibodies to Hev b 5

3.1. Introduction

The production of monoclonal antibodies (mAbs) was first reported in a landmark paper by Kohler and Milstein in Nature (Kohler and Milstein, 1975). This important technique has led to the widespread use of mAbs as specific probes to elucidate mechanisms of immune function and more recently seen their *in vivo* use as novel, targeted immune therapies such as anti- human IgE (Milgrom *et al.*, 1999).

MAbs have also been used as probes against specific allergens, and mAb-based assays have been developed, for example, to quantify Der p 1 (Luczynska *et al.*, 1989), Fel d 1 (Chapman *et al.*, 1988) and Group V grass pollen (Schappi *et al.*, 1999) allergen levels. Similarly, Der p 1 and Fel d 1 mAbs have been used as specific probes in Halogen® assays of aeroallergen samples (Graham *et al.*, 2000).

Previously, mAbs were generated by Slater and colleagues, by immunising mice with rHev b 5/MBP (Slater *et al.*, 1996). As has been detailed (see section 1.3.5.2), these mAbs were not monospecific, with reactivity reported against rHev b 5/MBP, MBP and NAL (Slater *et al.*, 1996). They were also of IgM isotype, an isotype that is known for frequently being non-specific, because of its tendency to form large, "sticky", pentameric complexes (Goding, 1996). Specific mAbs directed against Hev b 5 would be useful, as they would allow the development of improved diagnostic assays and the quantification of immunoreactive Hev b 5 within latex extracts and aero-allergen samples, something previously lacking.

This chapter documents the generation and characterisation of Hev b 5-specific mAbs. In addition, Chapter 6 describes the application of these mAbs in the quantification of immunoreactive Hev b 5 levels within latex extracts and their application as probes for the measurement of latex aeroallergen levels.

3.2. Materials and methods

The antigen used as immunogen for mAb generation was the rHev b 5/MBP fusion protein. This was prepared as described (see section 2.2.1.4.1). Cleavage of the rHev b 5/MBP construct was previously reported as incomplete (Slater *et al.*, 1996), so optimisation experiments were performed to increase cleavage efficiency with the aim of producing larger amounts of Hev b 5 cleavage product, with the aim of immunising the mice without the MBP portion of the rHev b 5/MBP fusion protein (see section 2.2.1.4.2). As will be described, this was ultimately unsuccessful. Therefore, for the generation of the Hev b 5-specific mAbs, six-week-old BALB/c mice were immunised with rHev b 5/MBP (see section 2.2.4.1). A double screening assay was developed for hybridomas using an ELISA system (see section 2.2.4.2.1), as desirable clones were those that were positive for Hev b 5/MBP, but negative for MBP alone. The successful hybridomas were then cloned by limiting dilution, before expansion, purification of the mAbs and characterisation by isotyping, ELISA and Western blotting. In addition, assays to assess sandwich formation between the mAbs were perfomed. Finally, non-fusion Hev b 5 was produced in the pProX-HTa bacterial expression system (see section 2.2.1.4.3), and characterised by the Hev b 5-specific mAbs.

3.3. Results

3.3.1. Expression of rHev b 5/MBP

The expression of rHev b 5/MBP resulted in a high protein yield of 16 mg/ 500 ml rich broth medium, as calculated by the area under the elution curve (Figure 3.1 A). The purified rHev b 5/MBP migrated, as expected at 75 kDa and appeared stable under reducing 16 % SDS-PAGE (Figure 3.1 B). Cleavage as reported by Slater using 0.1 U/ml Factor Xa and 0.05% SDS (Slater *et al.*, 1996) was incomplete but cleavage products at 42 kDa (MBP) and 30 kDa (Hev b 5) were seen on 16 % reducing SDS-PAGE (Figure 3.2).

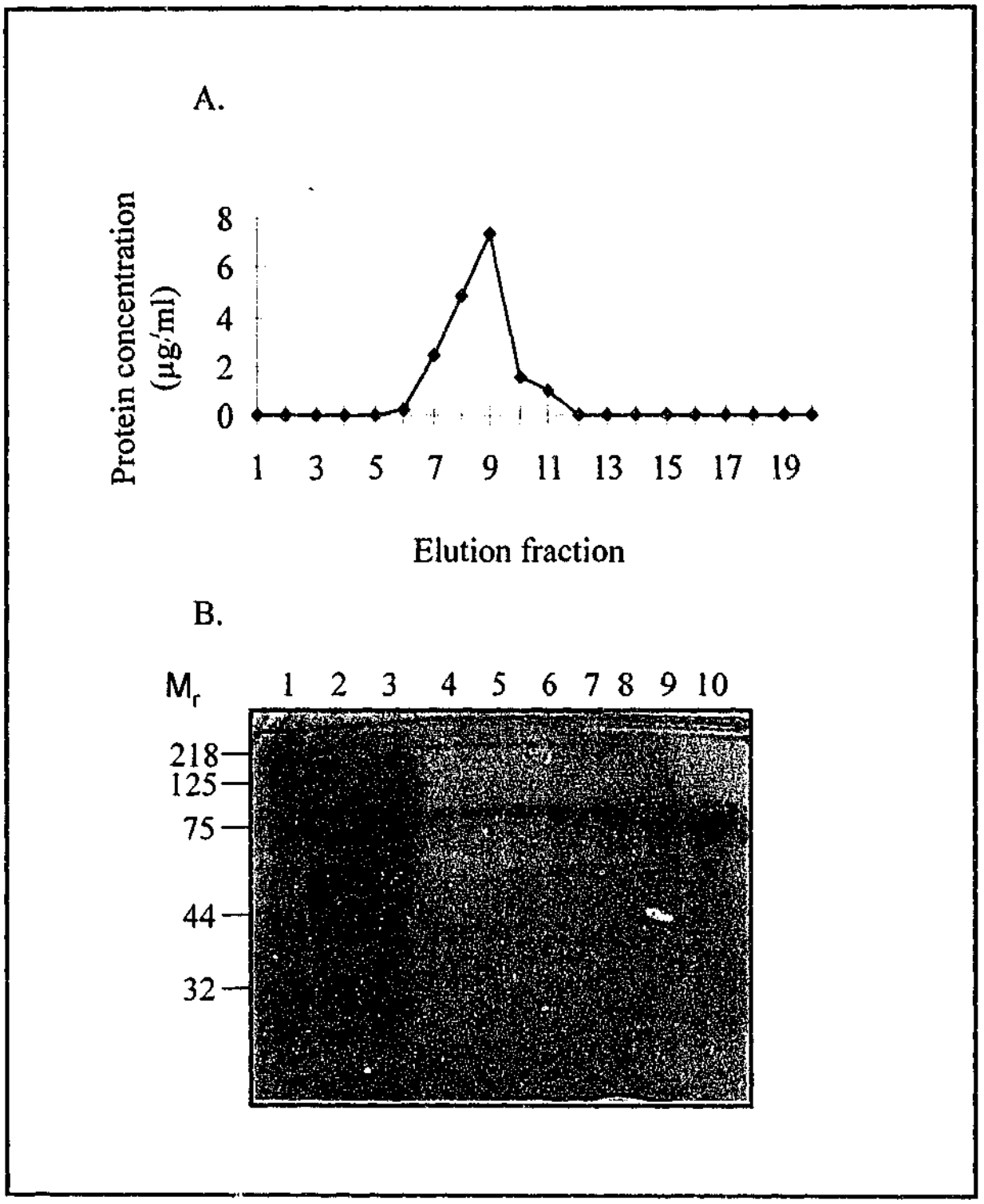


Figure 3.1 Purification and SDS-PAGE analysis of rHev b 5/MBP

Hev b 5 transfected DH5α *E.coli* were grown for 4 hours in LB + 100µg/ml ampicillin for 4 hours at 37°C, before induction with IPTG for a further 2 hours. After centrifugation the bacterial pellet was resuspended, sonicated and then purified on an amylose column. Elution fractions of 1.5 ml were then assayed for protein using the Bradford assay **(A)** . SDS-PAGE analysis (CBB stained gel) of bacterial sonicates and elution fractions was then perfomed **(B)**. Lane 1 – molecular mass markers, lane 2 – pMal Hev b 5 flow through, lane 3 – pMal Hev b 5 sonicate, lanes 4,5,6,7,8,9,10 correspond to elution fractions 14, 13, 12, 11, 10, 9, and 8 respectively from A.

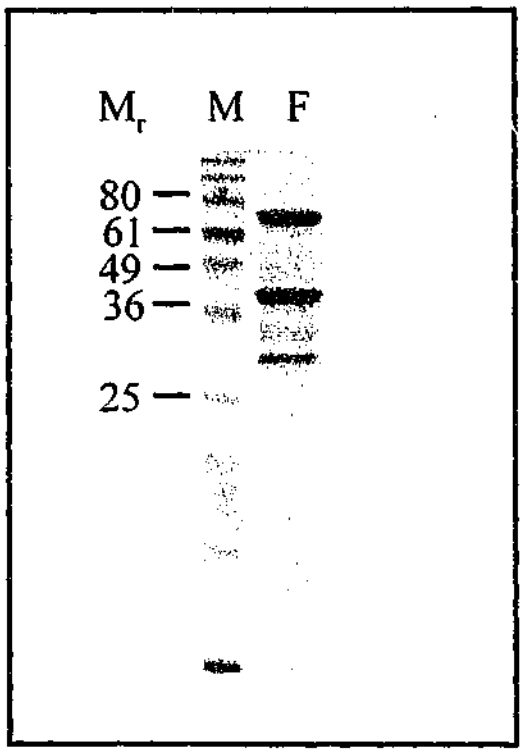


Figure 3.2 Factor Xa digest of rHev b 5/MBP fusion protein

CBB stain of 16% gel (reducing SDS-PAGE) of rHev b 5 fusion protein digest. The rHev b 5/MBP fusion protein (lane F) was incubated with 0.1 U/ml Factor Xa and 0.05% SDS for 4 hours at 37°C after the method of Slater et al (Slater *et al.*, 1996). Markers were Benchmark® prestained molecular mass markers (M) and relative mobility (M_r) is indicated.

3.3.2. Optimisation of fusion protein cleavage conditions

The availability of the purified cleavage product (rHev b 5) would have been desirable for both the generation of Hev b 5 specific mAbs and in human Hev b 5-specific IgE ELISA. To optimise cleavage conditions, in an attempt to increase Hev b 5 cleavage product production, different detergents were used at higher concentration (0.5%) and also 0.1M urea, all with or without Factor Xa, and compared with no detergent as a control (Figure 3.3). At the higher concentration of SDS, activity of the Factor Xa was abolished, resulting in a cleavage rate the same as that with no Factor Xa. The other three detergents tested, Tween 20, CHAPS, Triton X-100 and the denaturing agent urea all resulted in similar partial cleavage activity, not significantly superior to no detergent (Figure 3.3). As a result, further optimisation was abandoned and immunisations and screening assays proceeded with the whole rHev b 5/MBP fusion protein.

3.3.3. Polyclonal immune response of mice to latex antigens

Following immunisation with rHev b 5/MBP, the mice all mounted a strong antigen-specific immune response to rHev b 5/MBP, MBP alone and NAL as assayed by ELISA. A representative time course of the antigen-specific immune response following immunisation with rHev b 5/MBP is shown using mouse #3 (Figure 3.4). An antigen-specific immune response was apparent even following the first dose of antigen. The response was maximal after the 4^{th} immunisation.

The optimisation experiments titrating the dose of antigen coating the ELISA plates performed with immune and pre-immune serum from mouse #4 also indicated that a very strong reaction was mounted against rHev b 5/MBP. Significant reactivity was detected down to an antigen amount of 0.0001 µg/well (Figure 3.5 A). There was a marked reduction in reactivity from concentrations below 0.01 µg/well and therefore this was chosen as the coating antigen amount for rHev b 5/MBP. Higher antigen doses than this may have had a greater chance

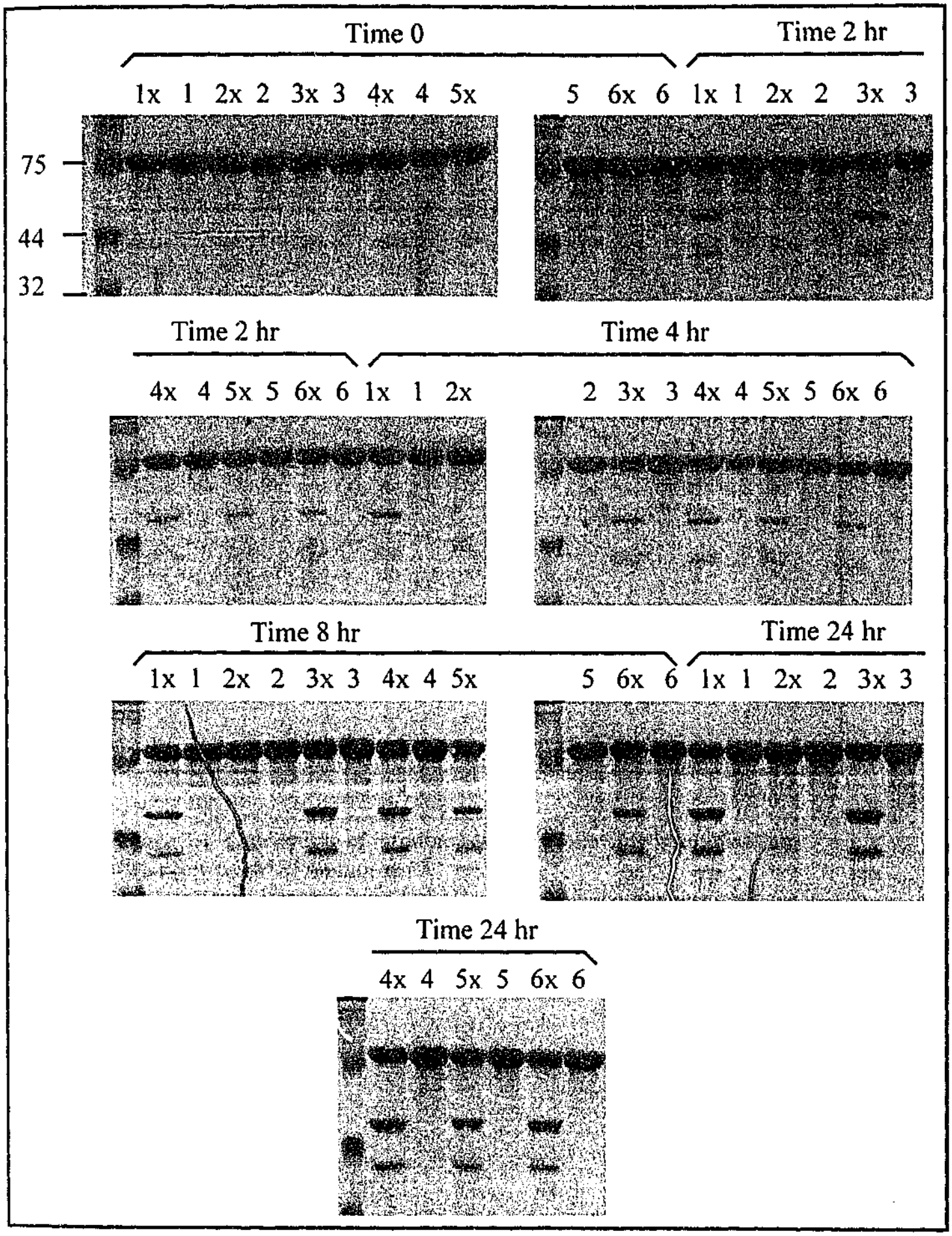


Figure 3.3 Timecourse and variation in detergent type to optimise cleavage conditions for rHev b 5/MBP

Recombinant Hev b 5/MBP was incubated at 37°C with (indicated as x) and without Factor Xa at 0, 4, 8 and 24 hours together with various detergents or reducing agents. Detergents used were no detergent control (1), SDS 0.5% (2), Triton X-100 0.5% (3), Tween 20 0.5% (4), CHAPS 0.5% (5), or the reducing agent Urea 0.1M (6). Samples were analysed on a reducing 12% SDS-PAGE gel and stained with CBB for visualisation of protein.

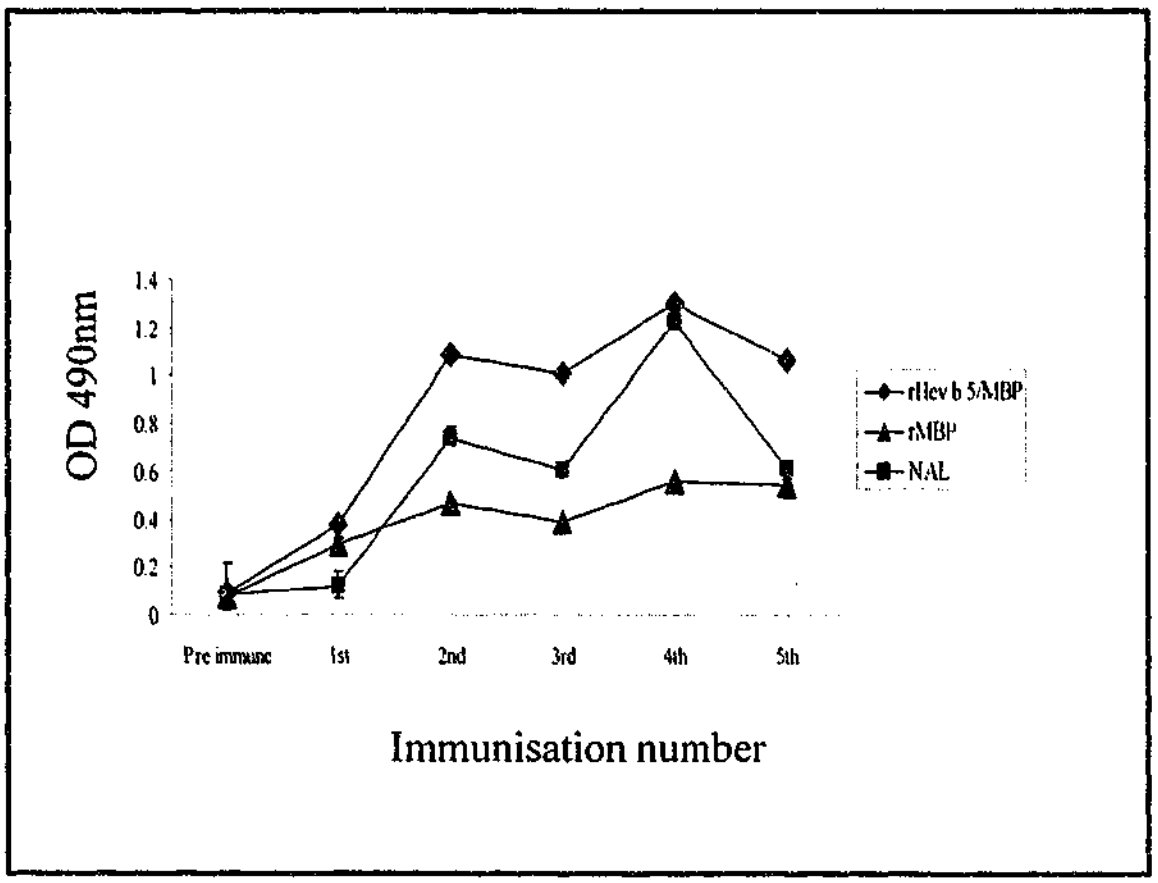


Figure 3.4 Timecourse of immune response following immunisation with rHev b 5/MBP in mouse #3

Following each immunisation with rHev b 5/MBP, the immune response in mouse #3 was measured by ELISA against rHev b 5/MBP, rMBP and NAL. Results are means +/- SD of triplicates.

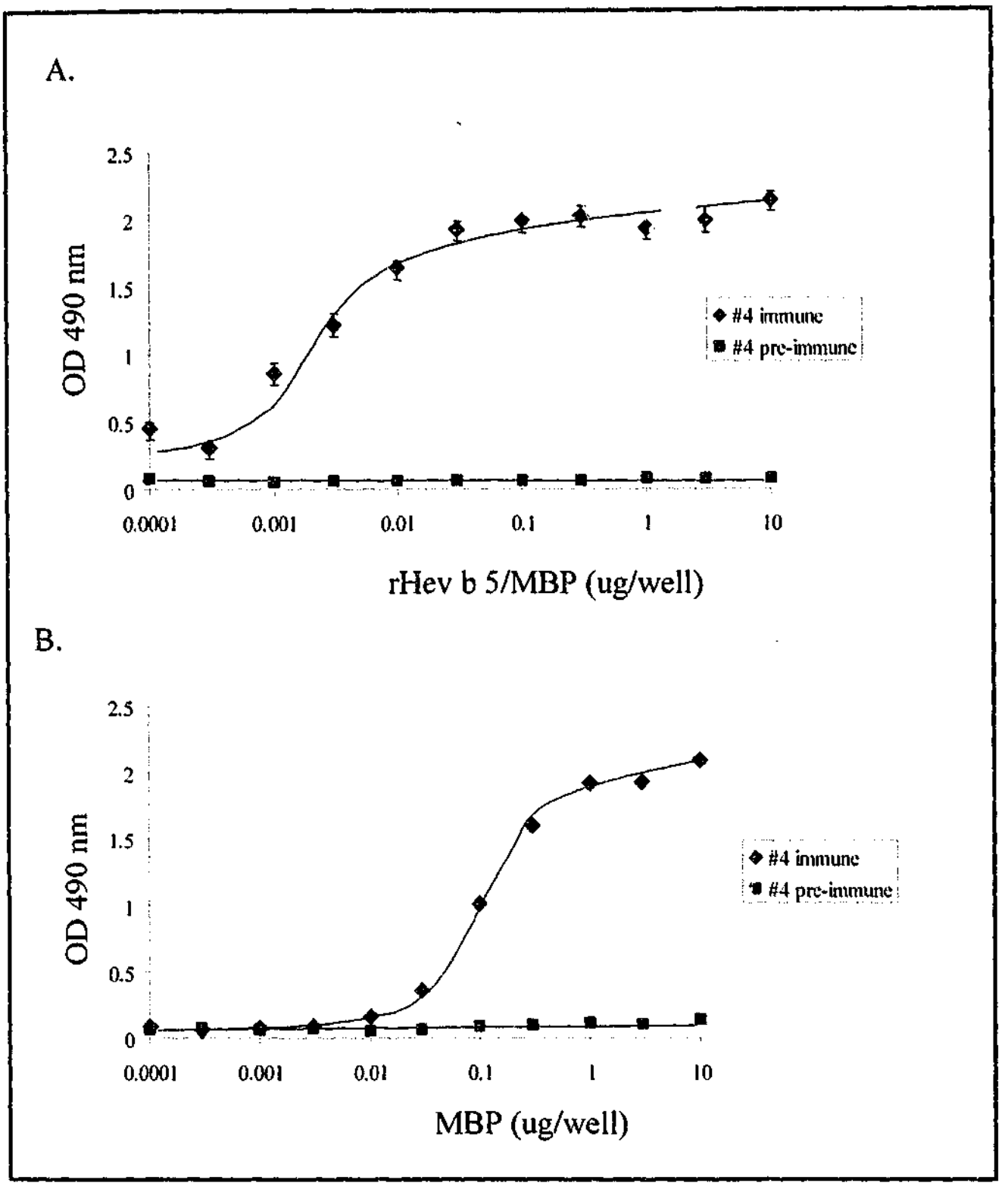


Figure 3.5 Optimisation of ELISA screening assay for rHev b 5/MBP and rMBP

Antigens (rHev b 5/MBP; **A.** and rMBP; **B.**) were coated onto polyvinyl microtitre plates in the amounts shown and the antigen-specific immune response assayed by ELISA using pre-immune and hyper-immune sera from mouse #4. Data points are means +/- SD of triplicates.

of selecting hybridomas specific for impurities, such as *E.coli* proteins, that often contaminate recombinant proteins in small amounts. This antigen amount is the same level as selected by Slater in his human RAST against rHev b 5/MBP (Slater *et al.*, 1996). The antibody response to MBP alone was less than that seen against Hev b 5 (Figure 3.5 B). In addition the antigen amount for NAL-specific ELISA was optimised (Figure 3.6 A) indicating that 6.25 µg/ml gave the highest signal strength. At higher concentrations, the signal actually decreased, possibly because of steric hindrance. The titration of hyper-immune serum against this antigen amount indicated a strong antigen-specific immune response against NAL was mounted by the rHev b 5/MBP immunised animals, with an antigen-specific response observed down to a serum dilution of 1: 64, 000 (Figure 3.6B). Finally, titration of the detection antibody (sheep anti-mouse HRP conjugate) was performed, indicating that although 1:1000 dilution was the most sensitive, 1: 5000 still had a high degree of sensitivity and therefore this dilution was used in ELISA to minimise costs (Figure 3.7).

3.3.4. Initial fusion using NAL as a final boost

This technique was initially used with the aim of obtaining antibodies directed against natural Hev b 5 (nHev b 5). In addition, in an attempt to reduce the number of assays needed for screening hybridomas, NAL alone was used as the screening method by ELISA. Mouse #4 was humanely killed and the fusion performed as described (see section 2.2.4.3), except dilution of the fusion pellet was into 200 ml of RPMI + 20% FCS + OPI + PSG. Hybridoma supernatants were screened by ELISA against NAL (coating concentration 6.25 µg/ml ELISA coating buffer) alone as described. Two hundred and six wells (from a possible 1920) had one or more hybridoma colonies. Following screening of hybridoma supernatants by NAL-specific ELISA, only one well was strongly positive, 1 moderately positive and 4 weakly positive by initial visual inspection when compared with the positive control (#4 terminal bleed, 1:5000 in 1%SMP-PBST). These hybridoma colonies were removed, subcultured and rescreened as described, however the

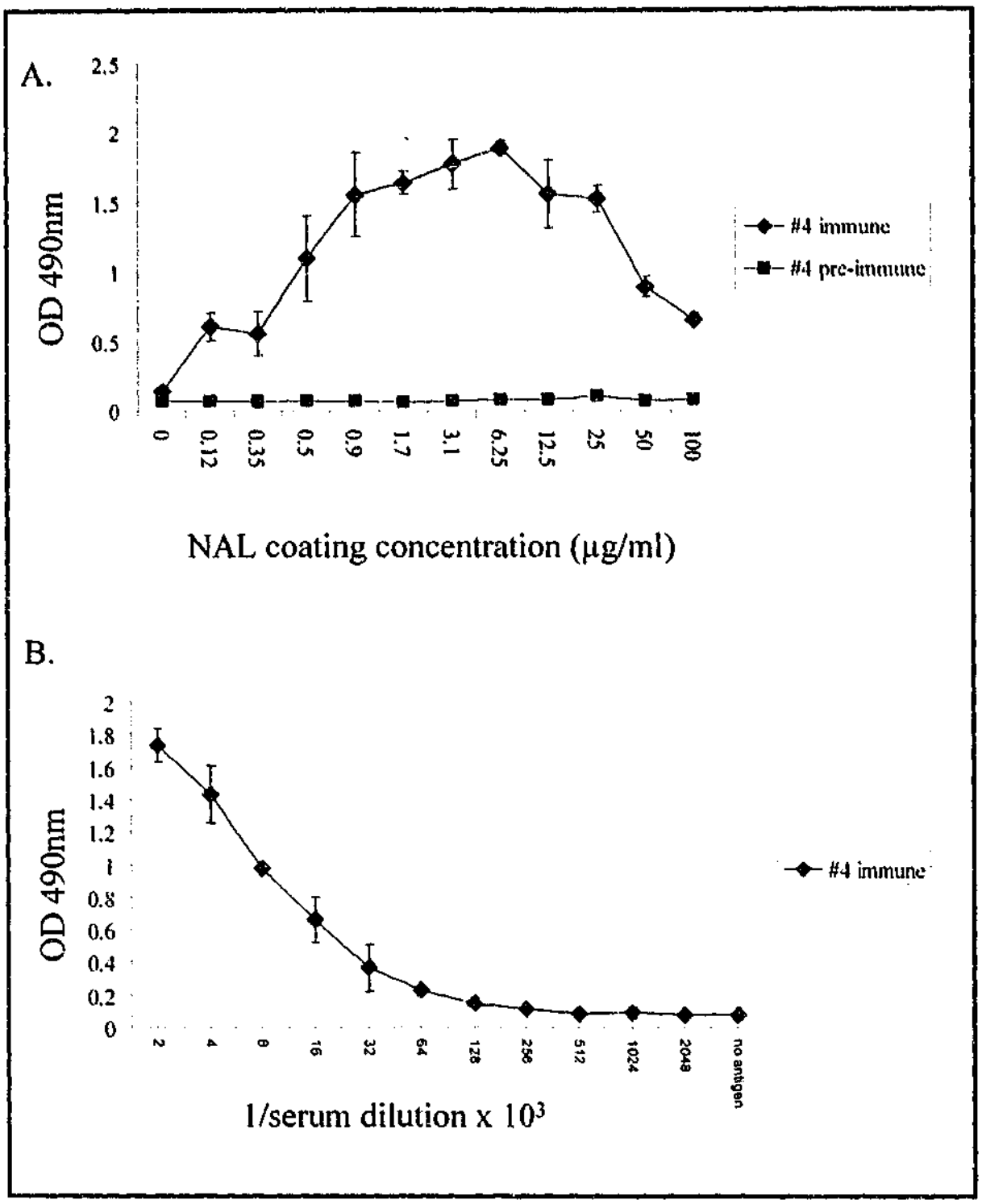


Figure 3.6 Optimisation of NAL screening ELISA

NAL was added to polyvinyl microtitre trays at the amounts indicated and the antigen-specific immune response assayed by ELISA (A) using sera from mouse #4 (hyper-immune and pre-immune). Once optimal coating amount was determined (6.25 µg/well), titration of hyper-immune serum against NAL was performed (B). Data points shown are means +/- SD of triplicates.

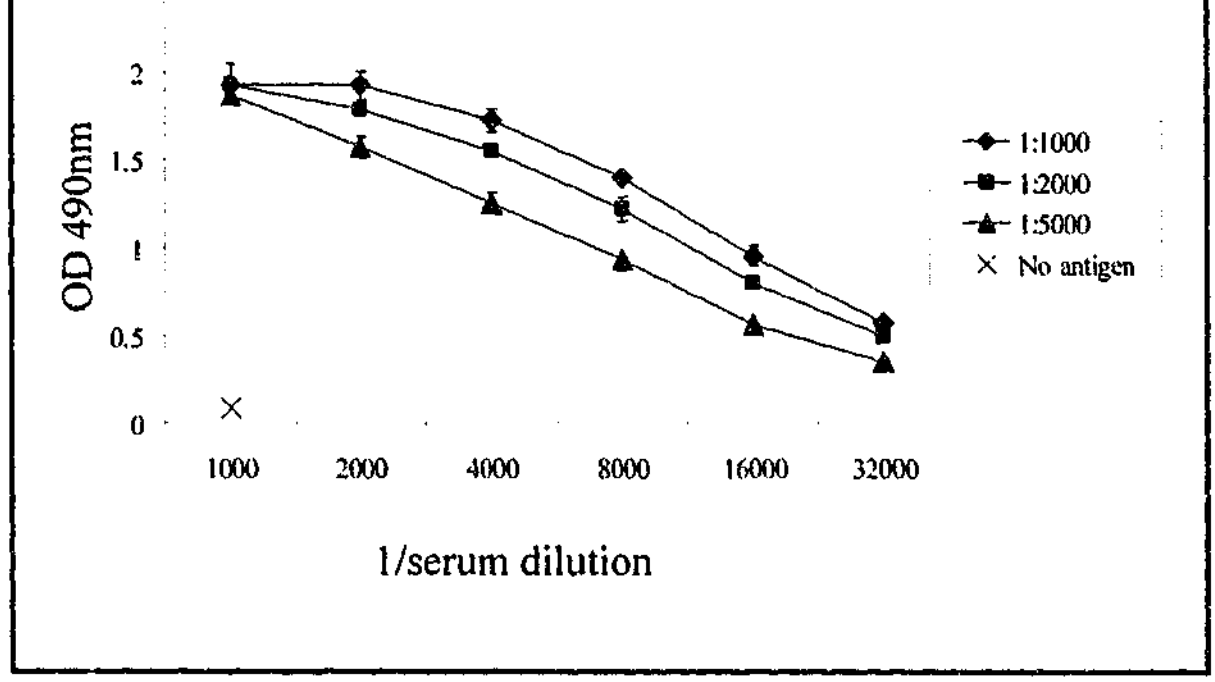


Figure 3.7 Titration of secondary antibody for murine ELISA

Using rHev b 5/MBP as antigen and hyper-immune serum from mouse #4 as primary antibody, the secondary antibody, sheep anti-mouse HRP conjugate (Silenius, Australia) was titrated by ELISA at the dilutions shown. Results expressed are means of triplicates +/-SD.

only surviving clone yielded antibodies specific for NAL and not rHev b 5/MBP or MBP.

3.3.5. Second fusion using rHev b 5/MBP boosting

The second fusion used mouse #2. This time, the fusion procedure was as for the first fusion, but screening was with rHev b 5 and MBP by ELISA. One hundred and twenty wells were positive by visual inspection for one or more hybridoma colonies. Of these, initially 7 wells were positive for Hev b 5/MBP but negative for MBP by ELISA. However, following subculture and cloning, none were found to be still positive.

3.3.6. Third fusion using rHev b 5/MBP boosting

The fusion was carried out as described (see section 2.2.4.3) using mouse #3. Because the fusion was plated out into only 6 plates, the majority of wells had one or more hybridoma colonies. Clones of interest were taken as those with OD rHev b 5/MBP > 0.15 and OD MBP <0.8. Using these criteria, there were 20 positive clones that were removed for further subculture, cloning and characterisation. After transfer to 24 well plates, many clones failed to keep growing, or lost reactivity, leaving 5 clones with good growth and continued reactivity: 6F6, 6A10, 3G3, 1C10 and 3E11.

3.3.7. ELISA of 5 successful clones

These hybridoma supernatants were tested by ELISA against Hev b 5/MBP, MBP and NAL, with the terminal bleed of mouse #3 as positive control, and pre-immune serum from mouse #3 as negative control (Figure 3.8). This showed that crude hybridoma supernatants from 3G3, 1C10 and 6F6 had the strongest reactivity to rHev b 5/MBP by ELISA, minimal or no reactivity with MBP alone and strong reactivity with NAL. Hybridoma 3E11 had weak reactivity with rHev b 5/MBP and hybridoma 6A10 intermediate reactivity.

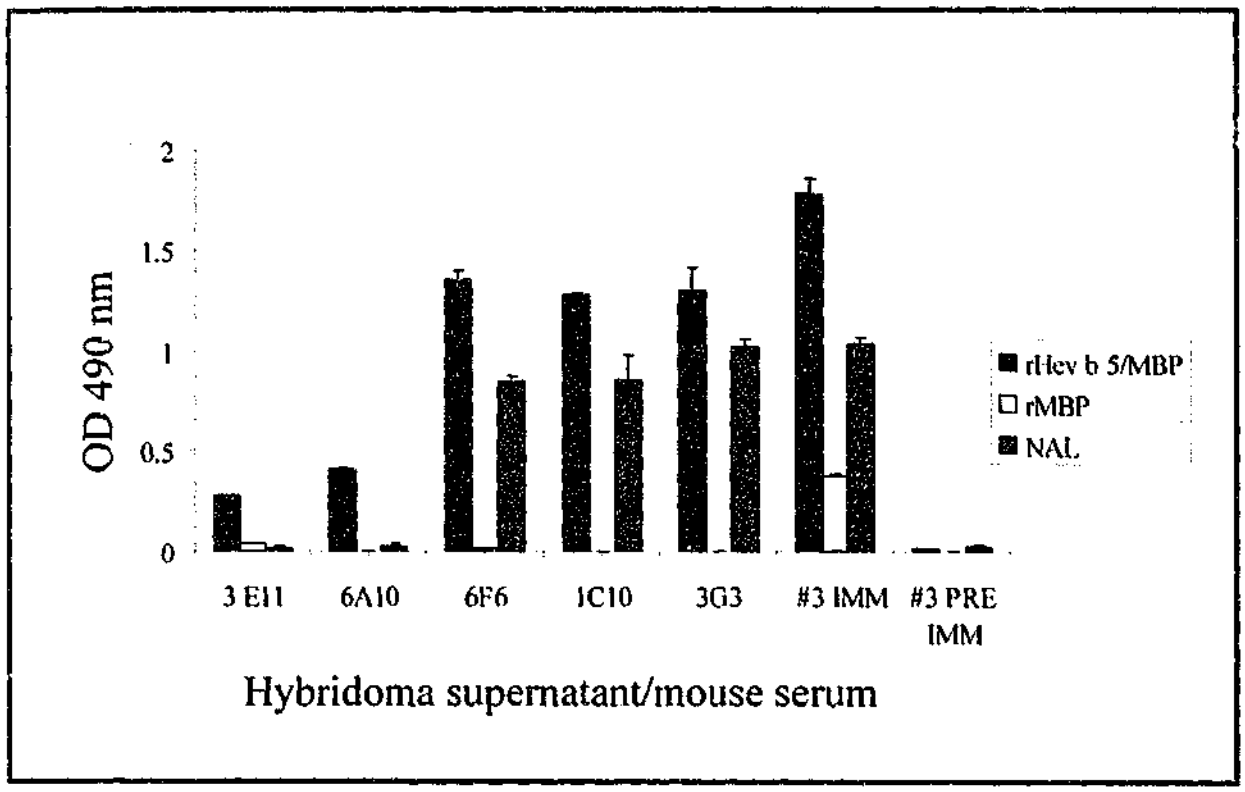


Figure 3.8 ELISA of hybridoma supernatants from fusion of mouse #3

Successful hybridomas from fusion #3 are shown. Hybridoma supernatants were used undiluted, while positive (#3 terminal bleed) and negative (#3 pre-immune) murine control sera were used at 1:1000 dilution.

3.3.8. Cloning by limiting dilution

This was performed twice for 6F6, 3G3, 1C10 and 6A10. During cloning, 1C10 lost reactivity, and so was not characterised beyond the hybridoma supernatant stage. Stocks of the bulk culture however are available for further attempts at cloning in the future. Because of its relatively weak reactivity at initial screening and the availability of the other rHev b 5-specific hybridomas, hybridoma 3E11 was not cloned further but frozen down from expanded bulk cultures in a T flask for further analysis later if ever required.

3.3.9. Isotyping

This showed that mAbs 6A10, 1C10, and 3G3 were isotype IgG_1 kappa, whilst mAb 6F6 was IgG_{2b} kappa (Figure 3.9). The other positive clone mAb3E11 was not tested.

3.3.10. Purification

The purification of mAb 6F6 being of isotype IgG2b was performed on a protein A column (Biosepra©, Life Technologies, USA). Fractions were assayed for protein content (Fig 3.10 A) and also analysed on reducing SDS-PAGE, where the antibody components migrated at the expected molecular mass for murine antibodies, with a high apparent purity (Figure 3.10 B). The yield from a one litre roller bottle culture supernatant was approximately 10 mg of purified antibody. Monoclonal antibodies 6A10 and 3G3 were purified from a protein G column (Pharmacia, Sweden), with similar yields to mAb 6F6 from one litre roller bottle culture.

3.3.11. Western blotting

Dot immunoblots were performed with mAb 6F6 crude hybridoma supernatant (Figure 3.11). This confirmed that this mAb showed reactivity to rHev b 5/MBP, NAL, GE, LAL, but not to rMBP. This reactivity could be inhibited by rHev b 5/MBP and partially by NAL but not by rMBP, confirming the specificity of the observed reactivity.

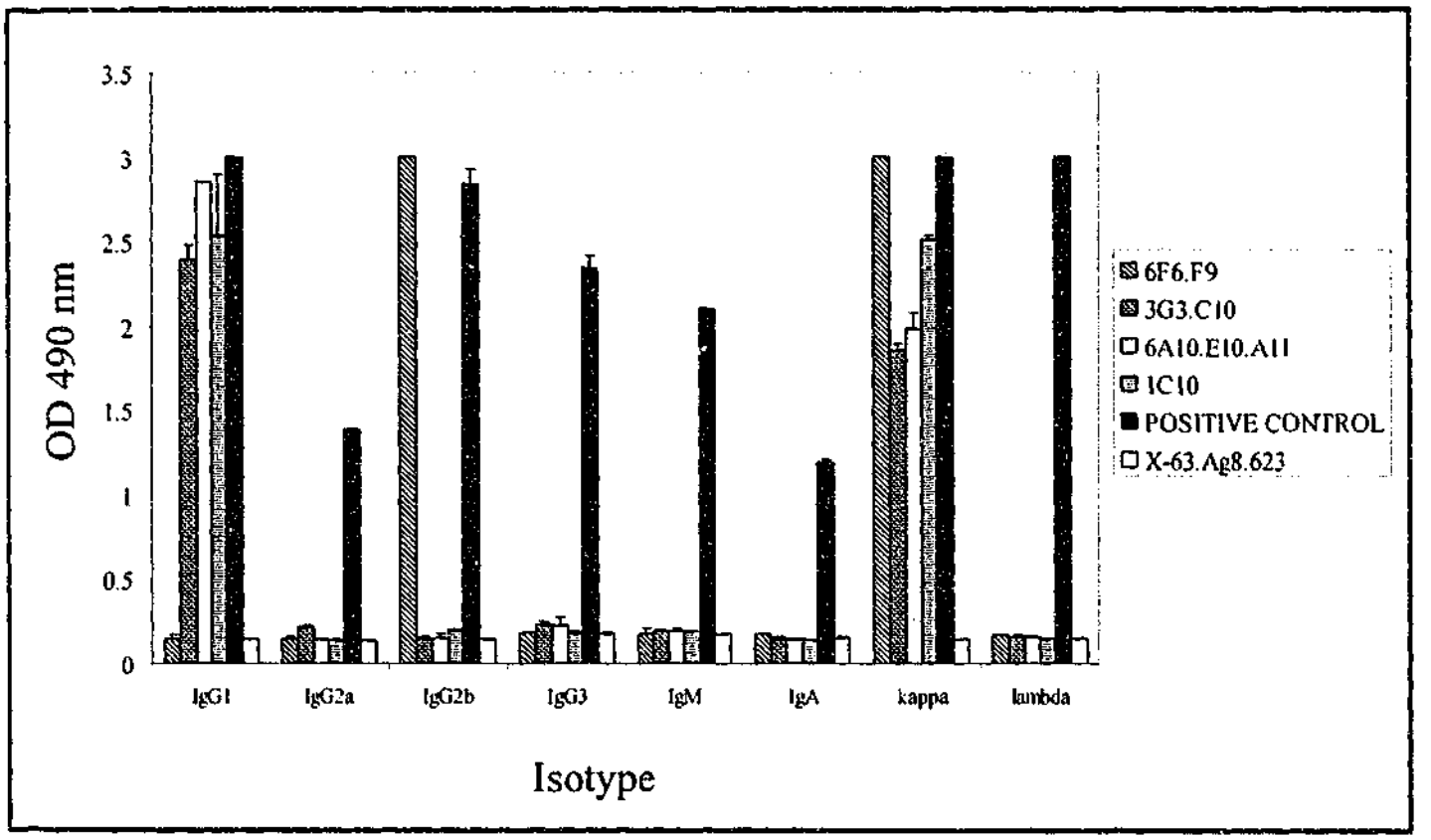


Figure 3.9 Isotype assay of rHev b 5-specific mAbs

Hybridoma supernatants were isotyped for immunoglobulin class & subclass using a commercial kit (Becton Dickinson, USA) according to the manufacturer's instructions.

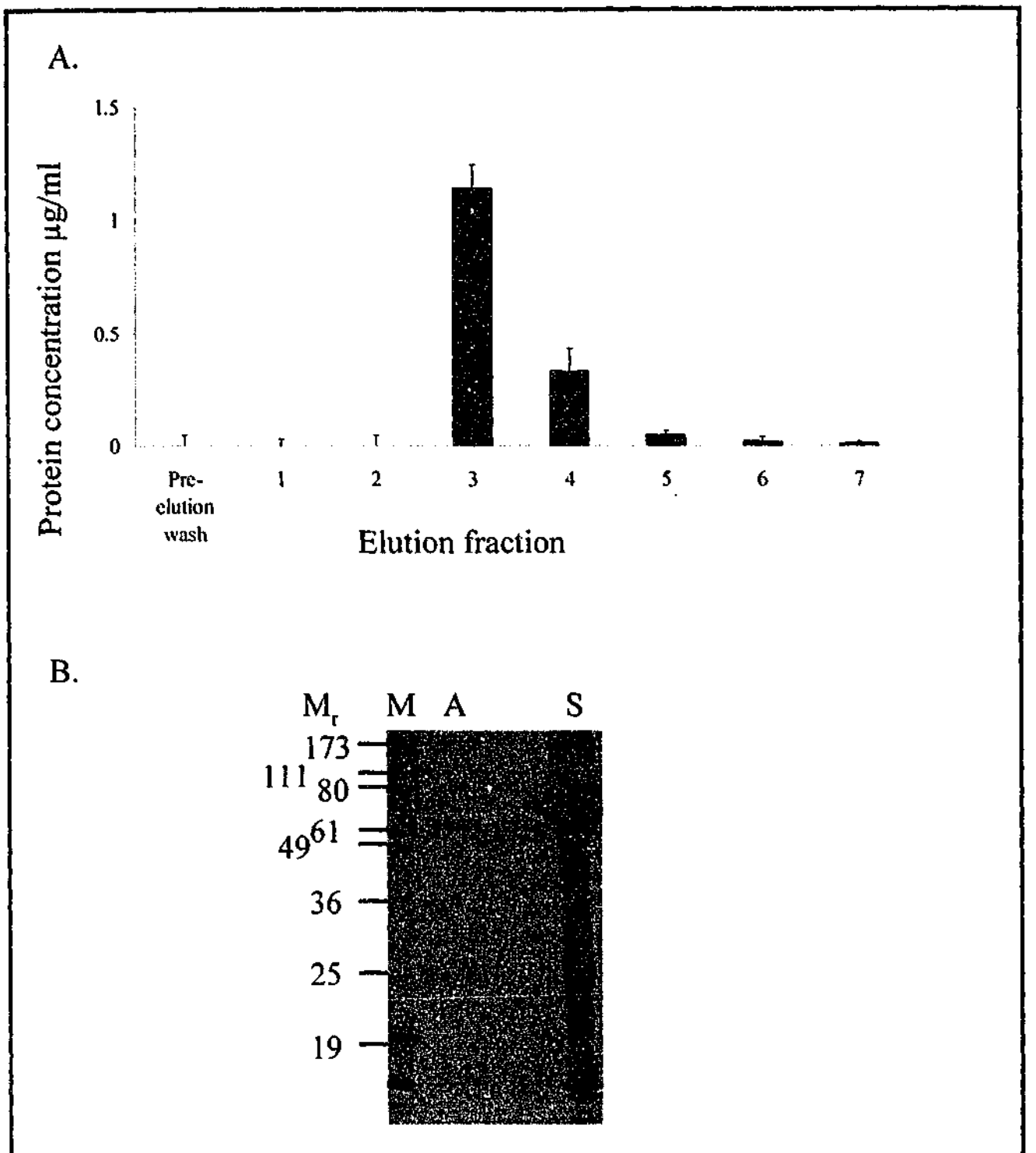


Figure 3.10 Purification of mAb 6F6

Rollerbottle supernatant (500ml) from hybridoma 6F6 was loaded onto a Biosepra Ceramic Hyper-D® Protein A column and then eluted in 3 ml fractions with 1M Citric Acid pH 3. Following neutralisation with 1M Tris HCl pH 8, fractions were assayed for protein content (A) and then the major protein containing fraction was separated on a reducing 16% SDS-PAGE gel before silver staining (B). Markers (M) are Benchmark protein standard, A is purified antibody 6F6, S is crude hybridoma supernatant, relative mobility (M_r) is indicated.

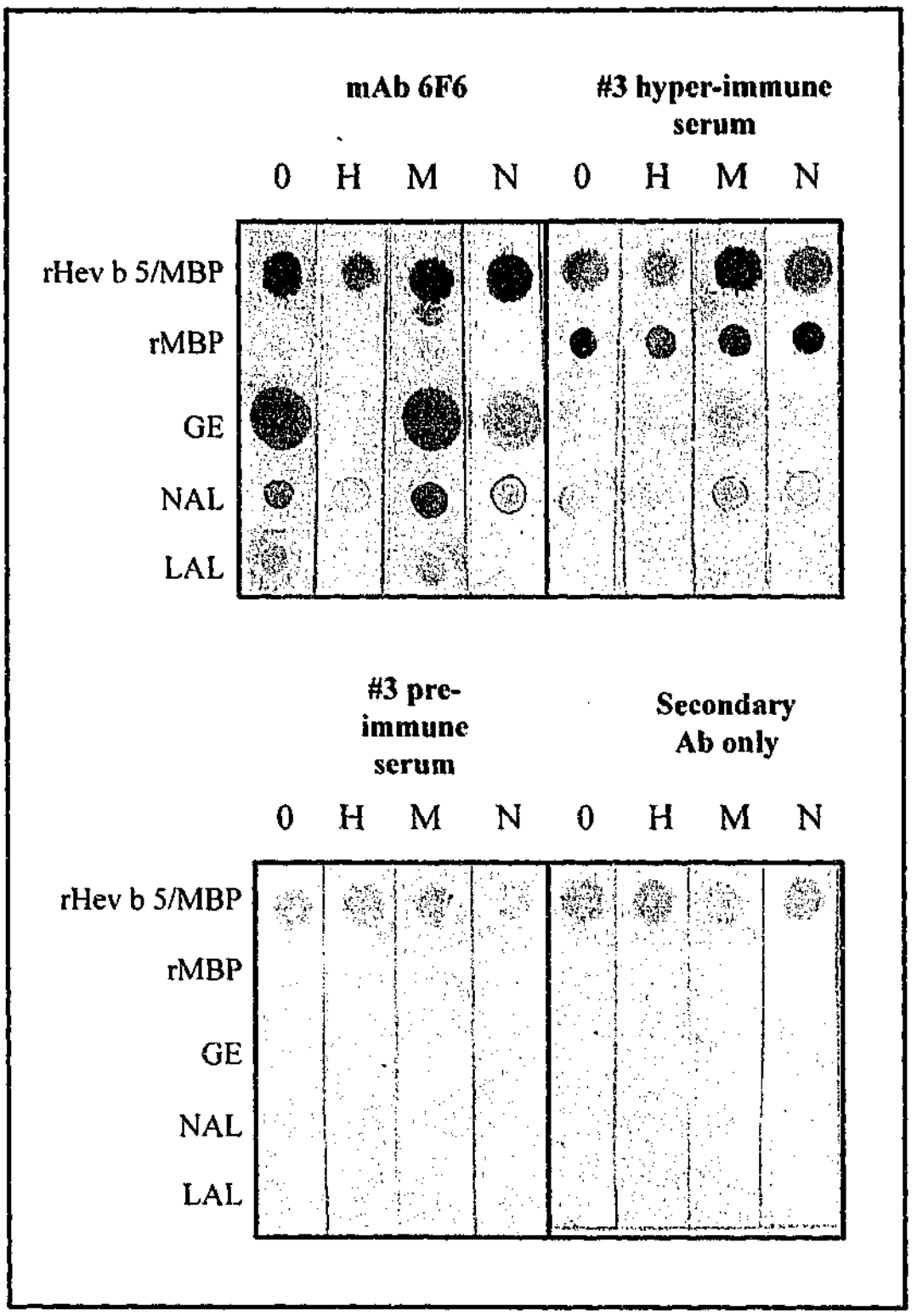


Figure 3.11 Inhibition dot immunoblot of mAb 6F6 with latex antigens

Antigens were dotted onto NC membrane (5 µl/dot) in the following amounts: rHev b 5/MBP and rMBP = 2.5 µg/dot, GE, NAL and LAL = 5 µg/dot. The membranes were allowed to dry, were blocked with 5% SMP-0.05%PBST and then probed with the mAb or sera as indicated above. Prior to incubation with the NC membrane, the mAb or sera were pre-incubated for one hour at 37°C with the inhibitors indicated above the dot blots: 0 = no inhibitor, H = rHev b 5/MBP 5 µg, M = rMBP 5 µg and N = NAL 146 µg.

In contrast to the mAb, as expected, the polyclonal hyperimmune serum detected both rHev b 5/MBP and rMBP.

Western blotting with the purified mAbs against rHev b 5/MBP showed reactivity with rHev b 5 but not MBP alone (Figure 3.12). When the mAbs were used to probe Western blots of NAL, they detected a band at around 26 kDa on a 12% SDS-PAGE gel (Figure 3.13).

3.3.12. Expression and purification of rHev b 5 in the pProX-HTa bacterial expression system.

In order to obtain rHev b 5 in the absence of MBP, Hev b 5 was expressed within the pProX-HTa system by Dr Alec Drew (Sutherland *et al.*, 2002). The protein was expressed and purified by nickel chromatography and the resultant protein analysed on SDS-PAGE. The protein was observed as 3 principal bands and a number of other fainter bands (Figure 3.14). These principal bands migrated in an aberrant fashion when separated on gels of varying density from 8-20 % when compared with both the markers and a prominent 25 kDa band within a control latex extract.

3.3.13. Western blotting of rHev b 5 with rHev b 5-specific mAbs

The rHev b 5 specific mAbs detected rHev b 5 when it was expressed without the MBP fusion protein as a hexa-his tagged protein in the pProX-HTa expression system. In addition to a prominent immunoreactive band at 35 kDa, this preparation also had immunoreactive components at 30 and 26 kDa (Figure 3.15). In addition, when ECL development was used, resulting in a higher sensitivity, immunoreactive aggregates were apparent in the higher molecular mass regions in multiple bands between 49 and 111 kDa (Figure 4.4); a similar pattern to that observed with a pooled serum of latex allergic HCW.

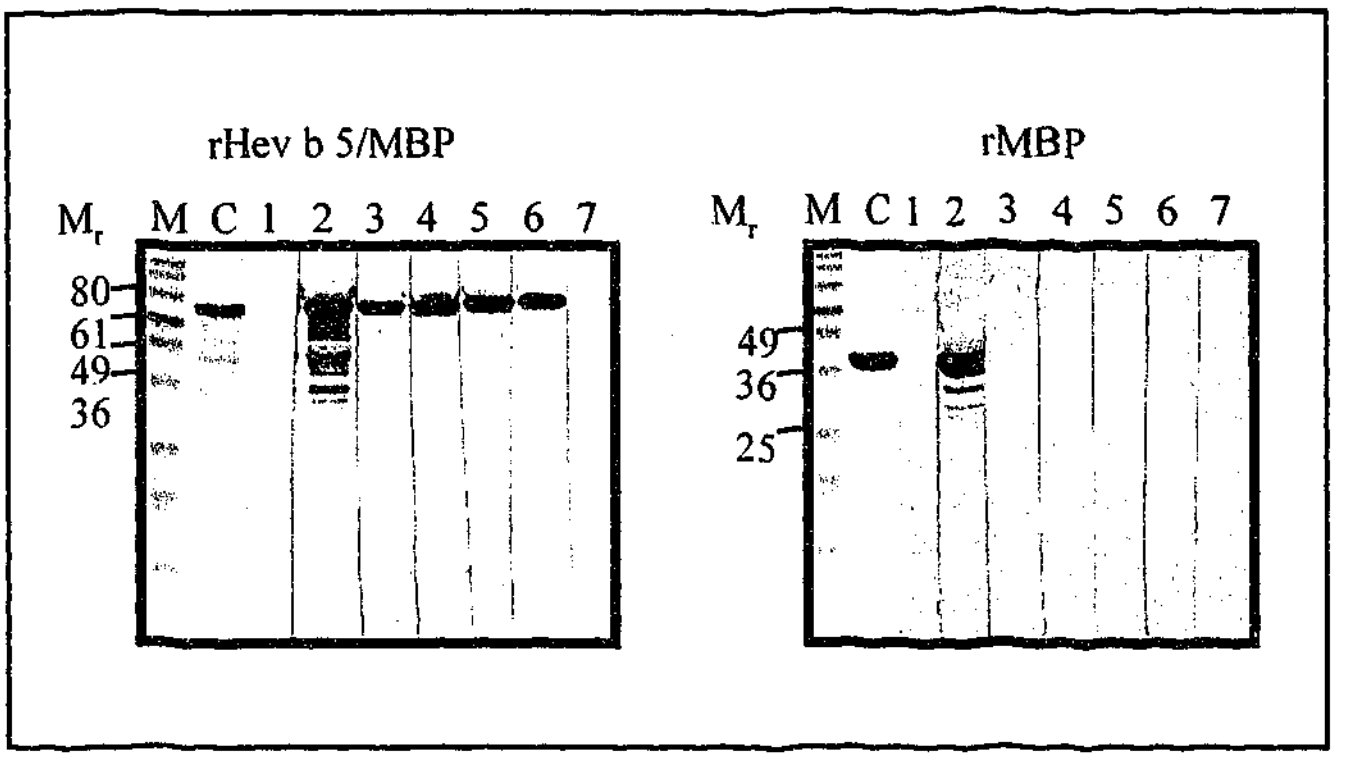


Figure 3.12 Western blotting of rHev b 5/MBP and rMBP with Hev b 5-specific mAbs

Recombinant proteins were separated on 12 % SDS PAGE gels before transfer to NC membranes and probing with mAbs (1:1000), hybridoma supernatant (1:10) or mouse sera (1:1000). Immunoblots were developed using 4-chloro-1-naphthol. M=Benchmark® prestained molecular mass markers, C=CBB stained gel, 1=pre-immune mouse serum, 2 = hyper-immune mouse serum, 3 = mAb6F6, 4 = mAb6A10, 5 = mAb3G3, 6 = mAb1C10 supernatant, 7 = mAbA1(isotype control). Relative mobility (M_r) is indicated.

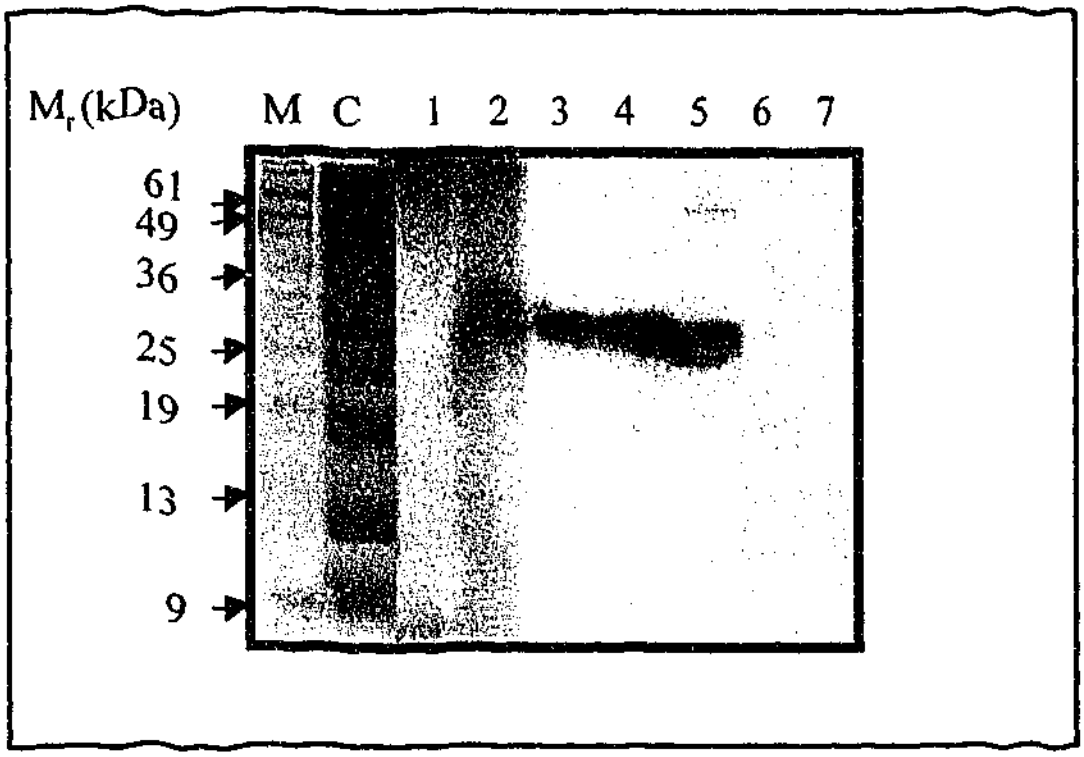


Figure 3.13 Western blot of NAL with Hev b 5- specific mAbs

NAL (75 µg/ lane) was separated on 12% SDS PAGE. Protein was then stained with CBB or electroblotted to NC for 1 hour at 25V DC. NC membranes were then blocked before diluted mAbs (1:5000); polyclonal sera (1:5000) or hybridoma supernatant (1:10) were incubated with NC for 1 hour at 37°C . Following washing, blots were then incubated with sheep anti-mouse HRP conjugate (1:5000) at 37°C for 1 hour. Blots were then washed and incubated with ECL reagent and exposed to autoradiography film. Lanes: M=Benchmark prestained protein ladder, C = CBB stain, 1 = pre-immune serum, 2 = hyper-immune serum, 3 = mAb6F6, 4 = mAb3G3, 5 = mAb6A10, 6 = mAb1C10 (hybridoma supernatant), 7 = mAbA1 (hybridoma supernatant). Relative mobility (M_r) is indicated.

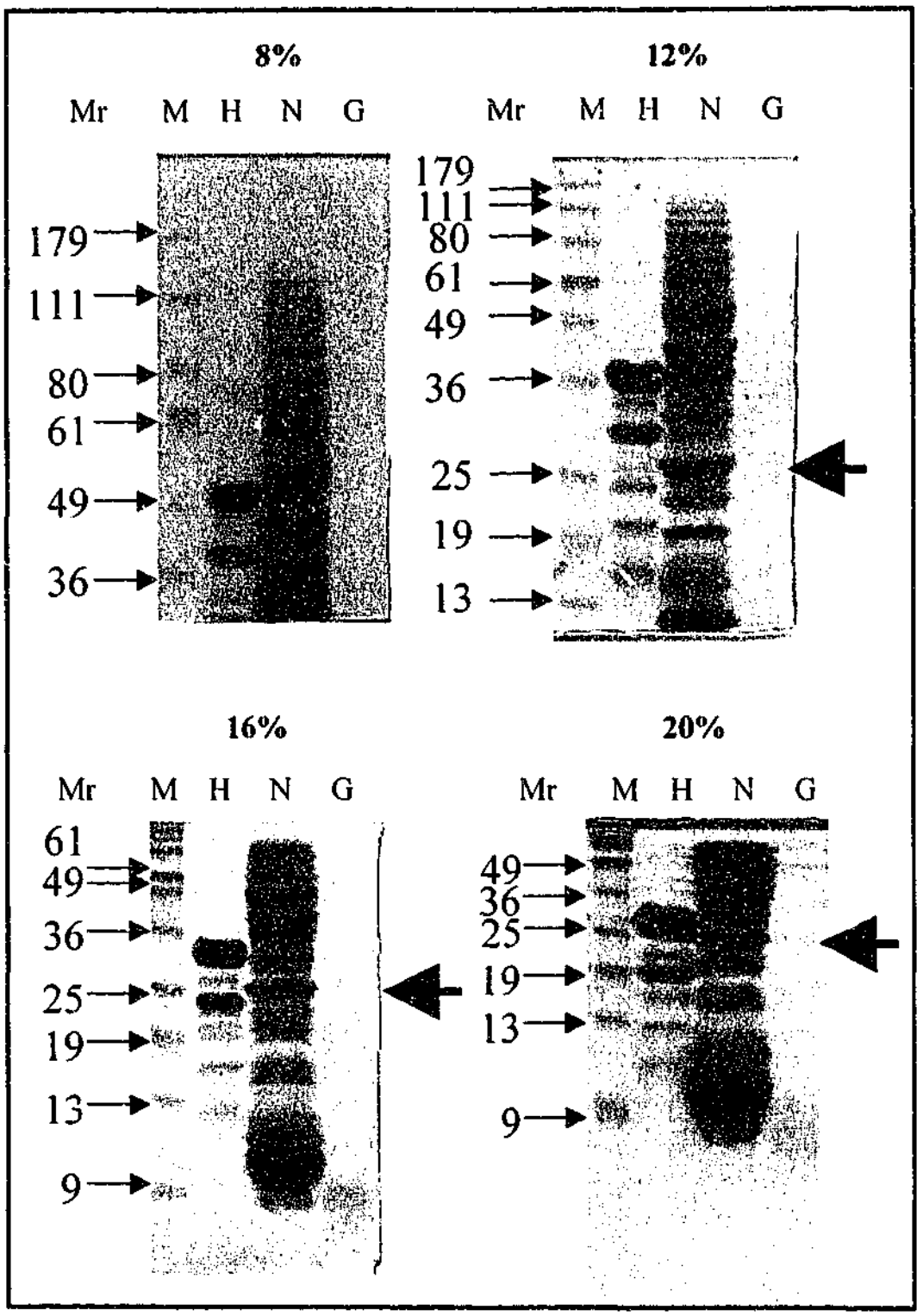


Figure 3.14 Aberrant migration of rHev b 5 on SDS-PAGE

Recombinant Hev b 5 (H), NAL (N) and GE (G) were separated on reducing SDS-PAGE gels of varying concentration (8-20%) and stained with CBB for protein visualisation. Benchmark® pre-stained protein ladder was used as a reference standard for relative mobility (M_r). The black arrows indicate a prominent band within NAL that migrated consistently at a similar position to the 25kDa marker irrespective of gel density, compared with rHev b 5 which displayed an increasing apparent molecular mass with decreasing gel concentration.

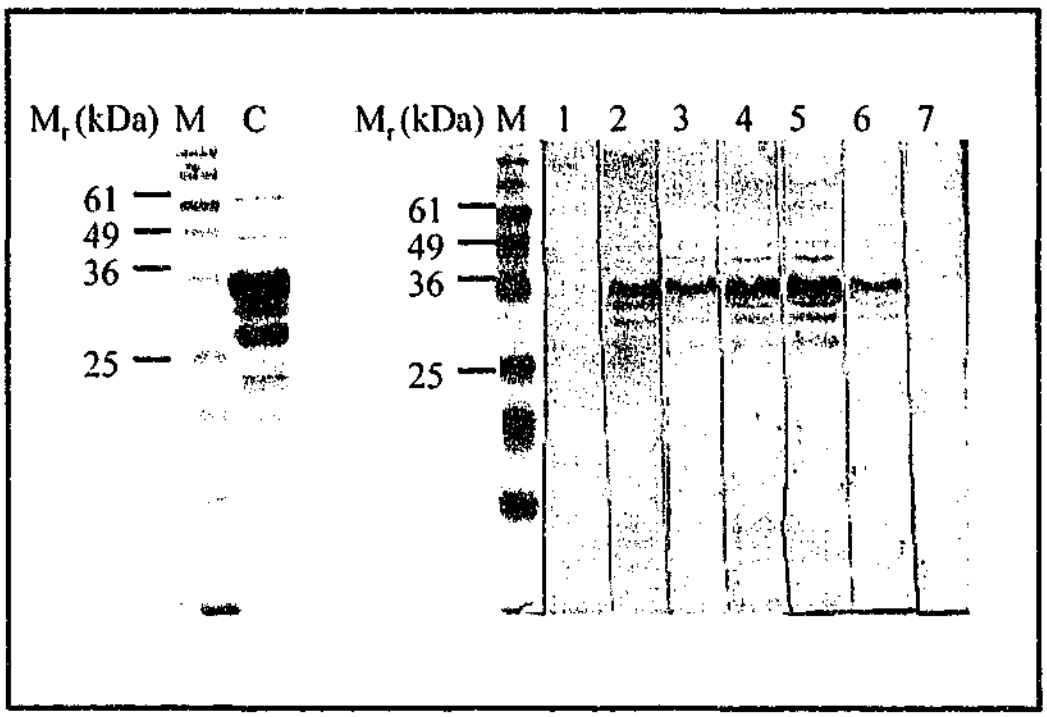


Figure 3.15 Western blotting of rHev b 5 with Hev b 5-specific mAbs

Recombinant Hev b 5 was expressed in the pProEx-HTa bacterial expression system and purified using nickel affinity chromatography. The resultant protein was analysed on 14% SDS-PAGE and stained with CBB(C) or transferred to NC membrane and immunoblotted using polyclonal mouse sera (1:1000), mAbs (1:1000) or hybridoma supernatant (1:10). Colour development was observed using 4-chloro-1-naphthol. Lanes: M = Benchmark © prestained protein ladder, C = CBB stain, 1 = pre-immune serum, 2 = hyper-immune serum, 3 = mAb6F6, 4 = mAb3G3, 5 = mAb6A10, 6 = mAb1C10 (hybridoma supernatant), 7 = mAbA1 (hybridoma supernatant). Relative mobility (M_r) is indicated.

3.3.14. Direct ELISA: rHev b 5-specific mAbs against rHev b 5

By direct ELISA, the purified mAbs strongly detected rHev b 5 in a classic sigmoidal dose response curve to a dilution of $1:1\times10^5$; a representative graph of mAb 6A10 is shown (figure 3.16A). There was no reactivity when ryegrass pollen extract (RGPE) was used as the coating antigen when compared with the RGPE-specific isotype control mAb A1 (Figure 3.16B).

3.3.15. ELISA of mAbs and human IgE against Hev b 5 peptides

To compare the linear IgE epitopes recognised by the Hev b 5 specific mAbs and human IgE, direct ELISA was performed against Hev b 5 peptides (Figure 3.17). The ELISA of Hev b 5-specific mAbs directed against overlapping Hev b 5 20mer peptides showed slight reactivity clustered around the N-terminus of the Hev b 5 molecule. Peptides showing weak reactivity were peptides Hev b 5 p(109-128), Hev b 5 p(118-137) and Hev b 5 p(132-151) for mAbs (recognised by all purified mAbs tested – 6F6, 6A10 and 3G3), while the latex allergic, rHev b 5-responsive subject 17 showed weak reactivity to Hev b 5 p(46-65) and Hev b 5 p(73-92). Negative controls (mAb A1 isotype control and non latex allergic, non-Hev b 5 responsive subject 50) showed no reactivity. Using the whole Hev b 5 molecule as a coating protein gave much higher reactivity with both the rHev b 5-specific mAbs and the latex allergic human IgE when compared with the Hev b 5 peptides.

3.3.16. Sandwich ELISA: rHev b 5-specific mAbs

In an effort to develop a two site binding assay for quantification of Hev b 5 within latex extracts, experiments to detect sandwich formation between the Hev b 5-specific mAbs were performed as described (see section 2.2.4.6.5). Using the isotype specific mAb detection antibody for IgG_1, some sandwich formation was observed at a high antigen concentration of 100 µg/ml (Figure 3.18A). The signal

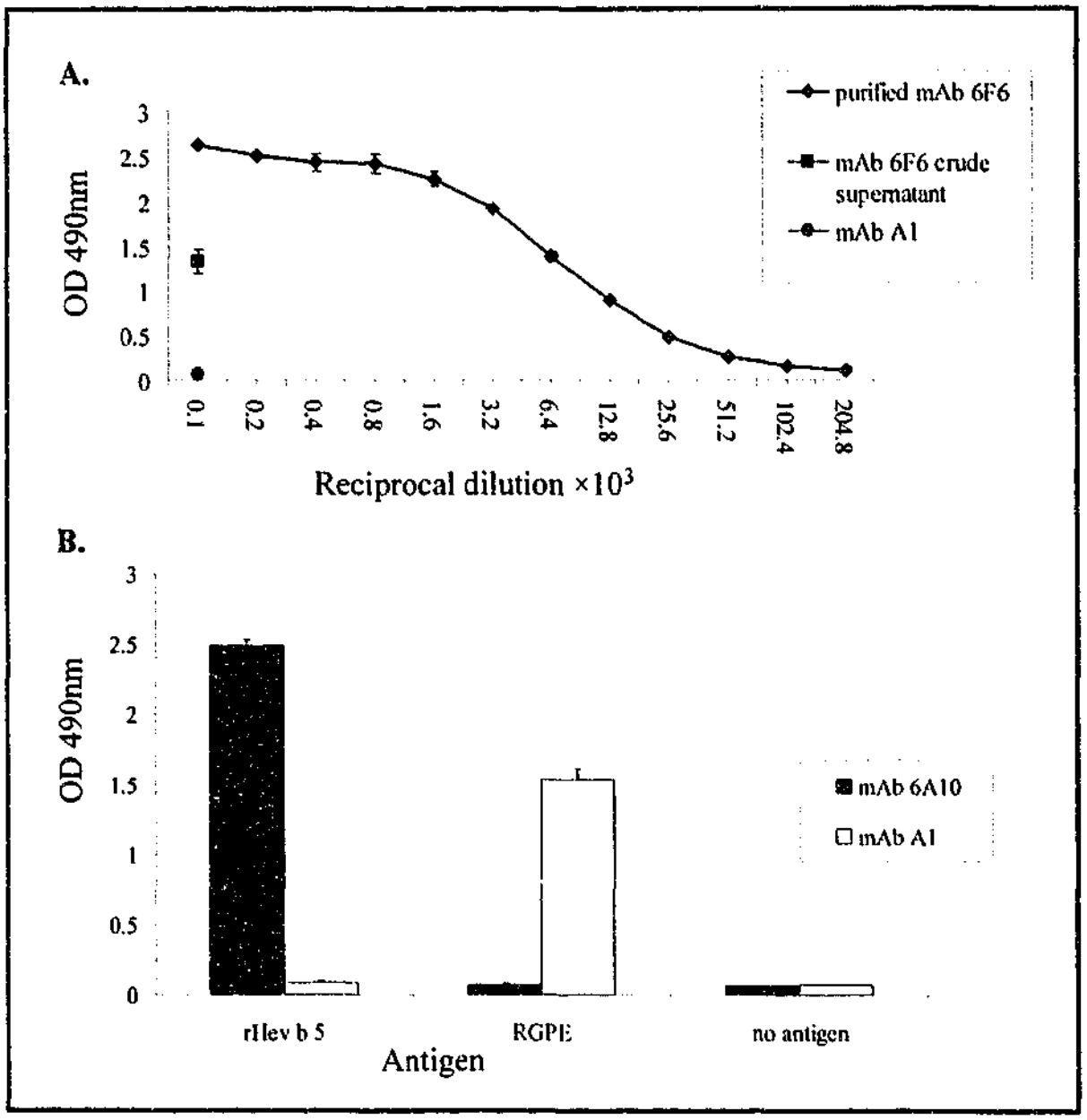


Figure 3.16 ELISA of rHev b 5 using purified Hev b 5-specific mAbs

Purified Hev b 5-specific mAb 6F6 (200 µg / ml) was added to ELISA plates coated with rHev b 5 at the dilutions shown (**A**). Crude mAb 6F6 hybridoma supernatant and rye grass-specific mAb A1 were used as positive and negative controls respectively. To demonstrate specificity, rHev b 5 specific mAb 6A10 (1:1000 dilution) was used to probe ELISA plates coated with rHev b 5, rye grass pollen extract or no antigen and compared with isotype control rye grass pollen (RGPE)-specific mAb A1 (**B**).

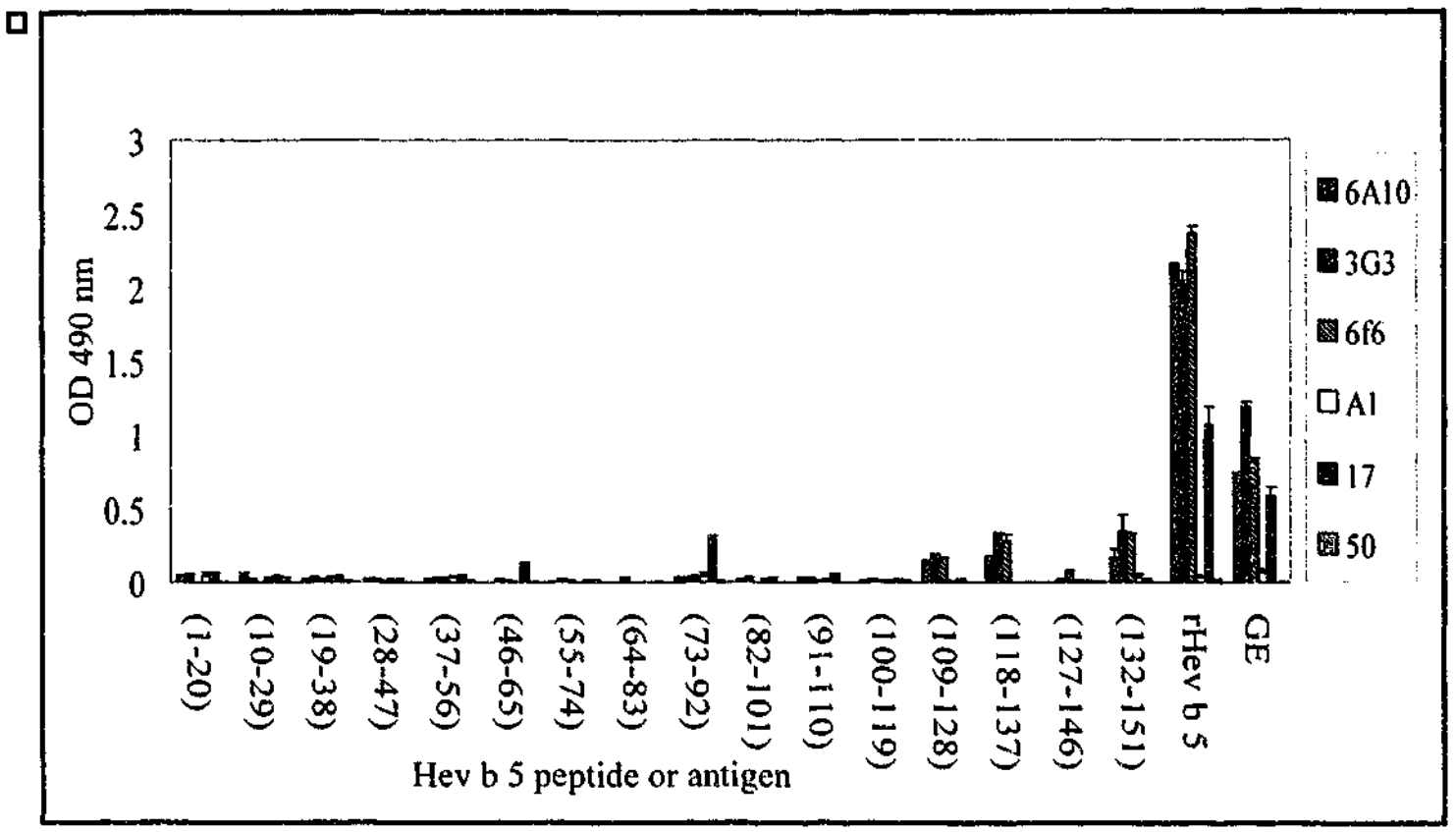


Figure 3.17 ELISA of Hev b 5 peptides using mAbs and human IgE

Direct ELISA of Hev b 5 peptides. Peptides and other antigens were coated overnight at 10 μg/ml. Detection was with the mAbs at 1:1000 dilution or control hybridoma supernatant (mAb A1) at 1:10 dilution. The human sera were added at a 1:10 dilution. Human subject 17 is latex allergic, subject 50 is latex non-allergic.

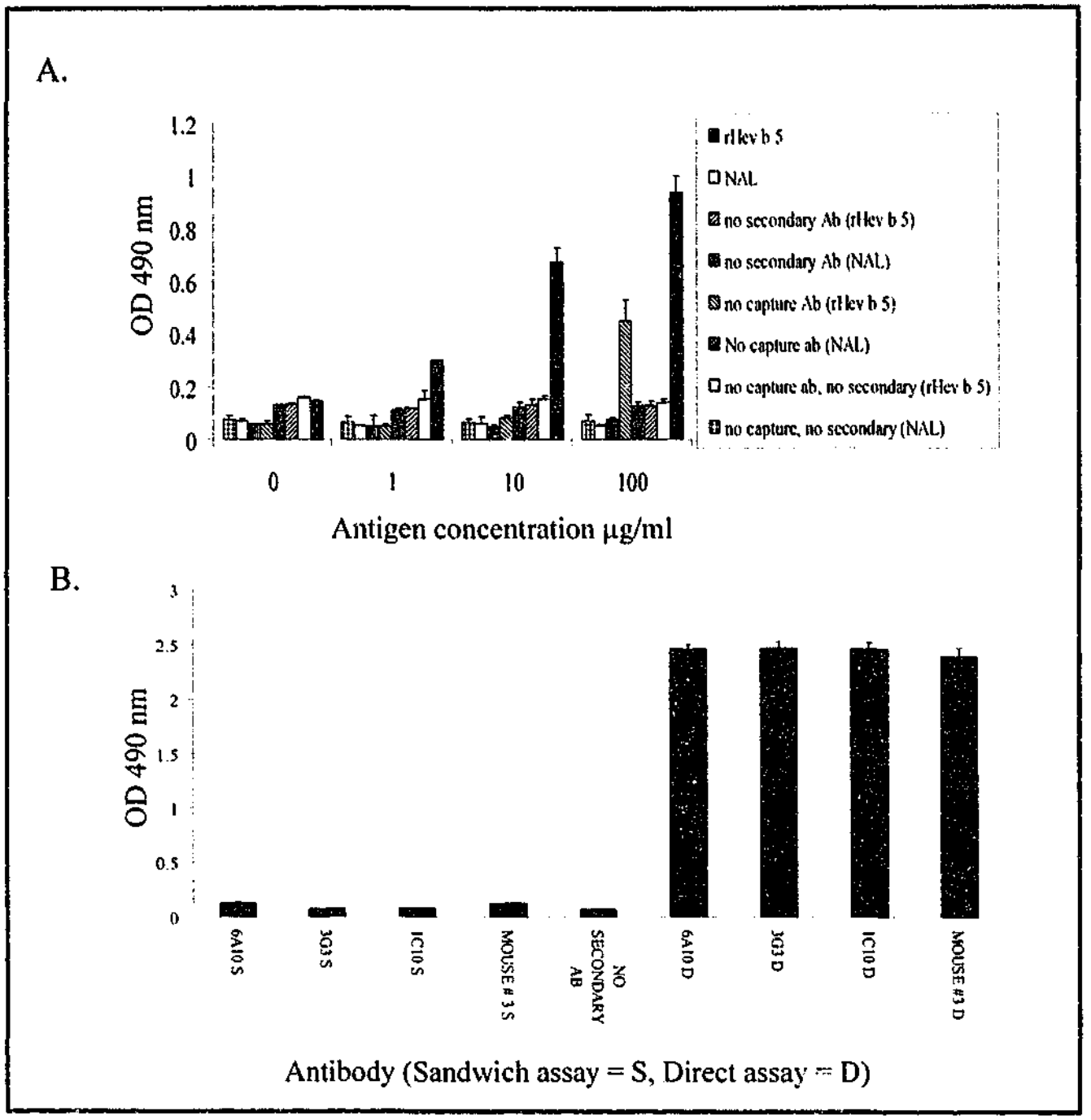


Figure 3.18 ELISA assay for detection of sandwich formation between Hev b 5-specific mAbs

Hev b 5-specific mAb 6F6 (isotype IgG2b), was used to coat ELISA plates at concentration of 10 µg/ml. The plates were then blocked and antigen (rHev b 5 or NAL at concentrations shown in {**A**}, rHev b 5 alone at 0.1 µg/ml in {**B**}) added. Following washing, detection occurred with Hev b 5 specific mAbs of IgG_1 class (mAb 6A10 in {**A**}, mAbs 6A10, 3G3, 1C10 or mouse polyclonal immune serum in {**B**}) followed by an HRP labelled IgG_1 isotype-specific mAb and OPD colour development observed. As a positive control for the isotype specific mAb, direct binding to rHev b 5 coated ELISA plates was also performed and is indicated with the D after each of the antibodies {**B**}.

however was weak compared with that observed with direct binding to the plate-bound rHev b 5, and there was significant reactivity even with no coating antibody. Additionally, at lower concentrations of 1 µg/ml, minimal sandwich formation was observed using mAb 6F6 for capture and mAb 6A10 or mAb 3G3 for detection (Figure 3.18 B).

3.3.17. Sandwich ELISA: rHev b 5 specific mAbs and human IgE

Using rHev b 5-specific mAbs as capture mAbs to coat ELISA plates (see section 2.2.4.6.5), some sandwich formation was observed using allergic subject 17 IgE as the detection antibody and rHev b 5 and GE as antigen (Figure 3.19). Not unexpectedly, because GE is not a pure Hev b 5 containing preparation (i.e. it contains additional latex allergens other than Hev b 5), the assay had a greater sensitivity for rHev b 5 (100 pg/ml) than GE (100 ng/ml).

3.4. Discussion

Specific mAbs to Hev b 5 were previously lacking. This chapter has described the generation, purification and characterisation of specific mAbs to Hev b 5 (Sutherland *et al.*, 2002), in addition to the expression of rHev b 5 in the pProX-HTa expression vector and its analysis using the mAbs. Following our report of Hev b 5-specific mAbs, a Finnish group has also reported Hev b 5-specific mAbs and used them in assays of quantification of latex extracts (Palosuo *et al.*, 2002). In their report, no characterisation of the mAbs or the epitopes recognised was performed, making direct comparison with the work described here difficult (Palosuo *et al.*, 2002). The findings of the Finnish group in their analysis of latex extracts are discussed and compared with the current study in Chapter 6 (Palosuo *et al.*, 2002; Sutherland *et al.*, 2002).

The murine polyclonal immune response to immunisation with rHev b 5/MBP, described at the beginning of this chapter illustrated the immunogenicity of the Hev b 5 allergen. This analysis showed that the rHev b 5 is more immunogenic than the MBP portion of the rHev b

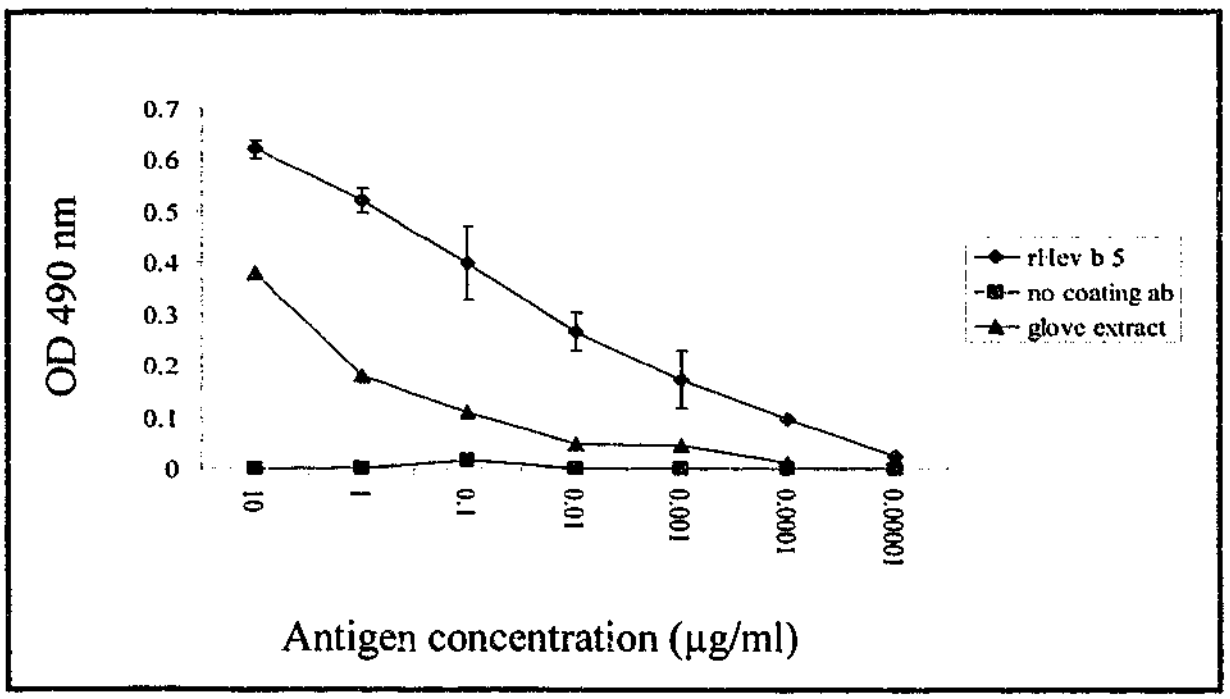


Figure 3.19 Sandwich ELISA for rHev b 5 using mAb capture and human IgE detection

The ELISA plate was coated with mAb 6A10 at a concentration of 10 µg/ml. Following blocking, varying concentrations of rHev b 5 (◆) or GE (♦)were added. Detection was then with serum (1:10 dilution) from latex allergic subject 17. The no coating antibody control(■) indicated there was no non-specific binding when rHev b 5 was added without capture antibody.

5/MBP fusion protein, which though larger by molecular mass, resulted in a lesser antigen-specific immune response.

It had originally been hoped that generation of the mAbs could have utilised purified rHev b 5 following cleavage from the MBP fusion partner. Optimisation of cleavage conditions to improve yield proved unsuccessful, with no increase in cleavage efficiency achieved beyond that published by Slater using 0.05% SDS (Slater *et al.*, 1996) and indeed no improvement on using no detergent at all. Because of this problem, three strategies were described to overcome the problems of MPB: firstly immunising with rHev b 5/MBP, boosting and screening with NAL; secondly, a double screening technique for hybridomas with negative selection for MBP and positive selection for Hev b 5/MBP; and finally, expression of Hev b 5 within the pProX-HTa expression vector to yield a recombinant protein free from a large molecular mass fusion partner.

Generation of the mAbs required optimisation of screening and hybridoma culture techniques prior to the final successful mAb generation. Screening directly with NAL following boosting with this antigen (as was done in the first fusion) was unsuccessful and resulted in antibodies specific for NAL but not the recombinant antigen (i.e. proteins other that Hev b 5). The final fusion was successful both because of the double screening method used, and also because of the method culturing the fusion in fewer ELISA plates (six in total); there was therefore a higher density of splenocytes as "feeders", resulting in a higher number of hybridomas for screening.

Isotype analysis of the mAbs revealed that three of the four tested were IgG_1 and the other, IgG_{2b}. The lack of IgM antibodies suggests a mature immune response. It is interesting to speculate as to whether the predominance of IgG_1 antibodies indicates a TH2 predominant response to the rHev b 5/MBP. Certainly it is consistent with this, however numbers are too small to make firm conclusions. In his analysis of murine T cell responses to latex antigens, Slater found strong IL-4

secretion from splenocytes from mice immunised with rHev b 5/MBP, consistent with it being a strong TH2 inducer (Slater *et al.*, 1999).

Characterisation of the mAbs indicated that they were specific for Hev b 5, with no reactivity demonstrated to MBP by ELISA or Western blot, an advance on previous mAbs reported against Hev b 5 (Slater *et al.*, 1996). They also recognised Hev b 5 in its native form, with reactivity demonstrable on Western blotting against natural latex extracts and by inhibition with natural latex extracts. The apparent molecular mass of the natural Hev b 5 (nHev b 5) observed of 26 kDa is at odds with the sequence data suggesting a molecular mass of 16 kDa. It is however, a similar mass to that described by Akasawa after the purification of nHev b 5, which was 24 kDa (Akasawa *et al.*, 1996). As can be seen from the expression of rHev b 5 without MBP, rHev b 5 migrates aberrantly on SDS PAGE depending on gel density. This is most likely due to its high content of proline residues that results in a strongly negative overall charge and makes SDS-PAGE analysis and Western blotting problematic. The mAbs raised against rHev b 5/MBP all recognised the rHev b 5 when expressed without MBP both by Western blotting and more strongly, by ELISA, again supporting their specificity for Hev b 5. The strength of binding to ELISA bound rHev b 5 may be in part due to the rHev b 5's superior binding to solid phase under ELISA conditions when compared with Western blotting, where, because of its charge, some rHev b 5 may be lost through its ability to pass through NC membranes (Akasawa *et al.*, 1996). Alternatively, the rHev b 5 antigen may be denatured more following Western blotting, whereas both denatured and native antigen are usually present for binding in ELISA format. Analysis of the rHev b 5 indicated that the protein forms aggregates and breakdown products. The mAbs however recognised the majority of these products suggesting retention of immunological activity.

The further analysis of the Hev b 5-specific mAbs showed that they appeared to have greater affinity for antigen bound to the ELISA plate

as compared with antigen in solution, as demonstrated by the very small amounts of bound antigen that still resulted in detectable reactivity with mAbs 6F6 and 6A10. Preliminary linear epitope analysis experiments indicated that the 3 mAbs were directed against the N terminal region of the molecule. Both the fact that the linear epitopes recognised were discontinuous and that the whole molecule gave a much larger signal indicate that the epitopes recognised are likely conformational in nature, as has been previously described with antibody antigen interactions (Laver *et al.*, 1990). Analysis of these conformational epitopes would require further inhibition ELISA experiments. Interestingly, all mAbs tested recognised the same peptides, indicating that they likely recognise a similar region within the N terminus. No significant sandwich ELISA formation at low antigen concentration could be demonstrated between the Hev b 5-specific mAbs either, also supporting the fact that they recognise a similar epitope. It is possible that the method described of screening for rHev b 5-specific hybridoma clones using the characteristic of being rHev b 5/MBP positive but MBP negative may have biased selection toward those antibodies that recognise the N-terminal region of the molecule. Additionally, through the effort to avoid raising antibodies to contaminating *E.coli* proteins, the low antigen coating concentrations used in hybridoma screening assays may have also biased selection of clones toward those of highest affinity for the plate-bound antigen.

The ability of human IgE to form a sandwich assay with the rHev b 5-specific mAbs and the fact that the Hev b 5-reactive serum chosen recognised different linear peptide epitopes in ELISA assays is suggestive that human IgE recognises different epitopes to the Hev b 5-specific mAbs. This is not unexpected when comparing a mAb with a polyclonal serum. It is however, also consistent with previously published linear peptide studies of Hev b 5. Using octamers overlapping by three amino acids and bound to NC membranes, Slater et al analysed the B cell epitopes of Hev b 5 in mice immunised with

rHev b 5/MBP fusion protein (Slàter *et al.*, 1999). They found IgE reactive regions within Hev b 5(1-38), Hev b 5(55-74), Hev b 5(109-128) and Hev b 5(132-151) (Slater *et al.*, 1999). As can be seen, the last two binding regions described are identical with the rHev b 5-specific mAbs linear peptide binding regions described in the present study. With regard to human epitopes, Beezhold also used octamer peptides of Hev b 5 with 3 amino acid overlap synthesised on a derivatised NC membrane (Beezhold *et al.*, 1999). Linear IgE binding epitopes were found using Hev b 5(15-22), Hev b 5(28-32), Hev b 5(50-56), Hev b 5(76-81), Hev b 5(90-95), Hev b 5(132-139), though inhibition assays using the peptides to inhibit IgE binding to the whole Hev b 5 molecule showed that inhibition was incomplete (Beezhold *et al.*, 1999). The human serum IgE binding peptides demonstrated within the current study Hev b 5(46-65) and Hev b 5(82-101) are entirely consistent with this previously described work and provide further corroborative evidence that the Hev b 5 specific mAb and human IgE epitopes are substantially different.

The strong reactivity of the mAbs described in assays directed against rHev b 5 expressed in the pProX-HTa system, particularly by ELISA, in addition to the recognition of Hev b 5 peptides are all powerful supporting evidence for their specificity for Hev b 5. Further applications of the mAbs in the analysis of latex extracts and aeroallergen samples, and their correlation with human IgE assays are presented in Chapter 5.

4. The human humoral response to latex allergen Hev b 5

4.1. Introduction

The humoral response to Hev b 5 is of considerable interest because of its important role in the diagnosis of latex allergy. Because current *in vitro* assay preparations, such as that of the Pharmacia UniCAP® system, have low levels of immunoreactive Hev b 5 (Chen *et al.*, 2000), it may be for this reason that they lack sensitivity (Hamilton *et al.*, 1999). Previous studies examining Hev b 5 reactivity have used recombinant Hev b 5 expressed in the MBP fusion protein system (Slater *et al.*, 1996; Yip *et al.*, 2000). These are the only studies of the percent prevalence of rHev b 5 IgE reactivity among adults, the first finding a prevalence of 92% in 13 latex-allergic HCW (Slater *et al.*, 1996), while the second a prevalence 62% rHev b 5 reactivity in a skin prick study of 31 latex-allergic adults, predominantly HCW (Yip *et al.*, 2000). This chapter presents data on the clinical characteristics of the study population, their IgE reactivity to diagnostic latex reagents, and finally their IgE and IgG_4 reactivity to rHev b 5.

4.2. Materials and methods

Human subjects were recruited from the Asthma and Allergy Clinic at the Alfred Hospital and gave informed consent as described (see section 2.1.7). Clinical information was collected using a questionnaire (Figure 2.1) and standardised latex-specific IgE assays and SPT performed to define the clinical phenotype (see section 2.2.2). Latex allergy was defined as a positive clinical history of immediate symptoms on exposure to latex and a positive SPT to latex, or where SPT was unavailable, or not performed for safety reasons, a positive latex-specific IgE assay. The sera were also analysed for rHev b 5-specific IgE by Western blotting and ELISA and for IgG_4 by ELISA as described previously (see sections 2.2.5.3.3, 2.2.6.1 and 2.2.6.2).

4.3. Results

4.3.1. General clinical characteristics and risk factors for latex allergy

Thirty-two subjects with latex allergy and nineteen control subjects were recruited from the Asthma and Allergy Clinics at the Alfred Hospital (Table 4.1; Appendix I). Both groups were well matched for age. There was a striking preponderance of females among the latex allergic group; however, this did not differ significantly from the control population. The latex allergic group also did not differ in the percentage of HCW, years in current occupation or operations with general anaesthetic (GA). None of the subjects had spina bifida. As far as pre-existing atopic disease is concerned, there was no significant difference in the prevalence of self reported rhinitis or asthma among the two groups, but the latex allergic group reported higher prevalence of a history of self reported eczema and food allergy when compared with the control group (Table 4.1; Appendix IV).

4.3.2. Prevalence of self-reported symptoms on latex exposure

Latex-allergic subjects reported significantly more symptoms on latex exposure than the control individuals (Pearson Chi-Square Test; Table 4.2; Appendix II). There was a high level of self reported angioedema, anaphylaxis and asthma among the latex-allergic subjects consistent with this subject group being a severely affected one. As expected this was significantly different from the control individuals. Interestingly, local skin itching and erythema was significantly different between the two groups, with only 20% of latex non-allergic individuals reporting these symptoms, compared with 91% of the latex-allergic group (Table 4.2). The latex allergic individuals reported symptoms on exposure to latex on more than one occasion in 100% of cases and on more than five occasions in 84% of cases.

Table 4.1 Clinical characteristics of human subjects

Parameter	Latex allergic	Latex non-allergic	*p*
n	32	19	
Age; mean (SD)	39.2 (10.7)	38.3 (11.9)	0.80
Percentage female	97	84	0.11
Percentage HCW	78	68	0..58
Years in current occupation; mean (SD)	10.8 (7.1)	8.9 (8.5)	0.42
Operations with GA; mean (SD)	3.7 (4.7)	2.2 (1.8)	0.21
Spina bifida	0	0	NA
Past history of food allergy	20 (63%)	5 (26%)	**0.01**
Past history of rhinitis	22 (69%)	11 (58%)	0.44
Past history of asthma	17 (53%)	5 (26%)	**0.04**
Past history of eczema	21 (66%)	3 (16%)	**<0.01**
Percentage atopic	27 (84%)	13 (68%)	0..32

Table 4.2 Self-reported symptoms on latex exposure

Symptom	Latex allergic (n = 32)	Latex non-allergic (n = 19)	*p*
Local skin itching and erythema	29 (91%)	4 (20%)	**0.001**
Local skin hives	28 (88%)	0	**0.001**
Generalised erythema	14 (44%)	1 (5%)	**0.001**
Sneezing	20 (63%)	0	**0.001**
Facial swelling	22 (69%)	0	**0.001**
Throat swelling	14 (44%)	0	**0.001**
Asthma, shortness of breath or wheezing	10 (72%)	1 (5%)	**0.001**
Anaphylaxis	13 (41%)	0	**0.001**

4.3.3. Latex-specific serum IgE assays, latex SPT and total IgE

Latex-allergic individuals had significantly higher levels of serum latex-specific IgE as assayed by the latex EAST (Sanofi-Pasteur Diagnostics, USA) and the Pharmacia UniCAP® (Pharmacia, Sweden; Table 4.3; Appendix III). Both assays were specific with the UniCAP® showing 100% specificity (no false positives) in this population as defined by no symptoms and negative skin test. The latex EAST however had one false positive value for a spe.ificity of 92.3% from the sample of 13 non-allergics tested using this assay. The subject concerned (Subject 6) had symptoms of hand itching on latex exposure but had a negative skin test and went on to have a negative blinded latex glove challenge.

The latex-allergic group had a significant increase in the size of the latex SPT reaction upon testing with the Stallergenes latex reagent (Stallergenes, France; Table 4.3). The result appears lower than may have been expected among the allergic group, however subjects with a history of anaphylaxis and a strongly positive EAST, who would have likely had a larger skin test reaction (Kim and Safadi, 1999), were generally excluded from skin testing for safety reasons because of the documented risk of anaphylaxis with latex skin testing (Valyasevi *et al.*, 1999). Both groups had a wide variation in total IgE levels, though the latex allergic group had significantly higher IgE levels, likely reflecting their greater level of atopy.

4.3.4. Hev b 5-specific IgE assays

4.3.4.1. rHev b 5-specific IgE levels (ELISA)

An ELISA was established to assay Hev b 5 specific IgE as described (see section 2.2.6.1). The ELISA for rHev b 5-specific IgE showed only subjects with true latex allergy (relevant symptoms on latex exposure and demonstrable latex-specific IgE) had elevated rHev b 5-specific IgE levels when tested by ELISA (Figure 4.1). This ELISA was a

Table 4.3 Latex-specific serum IgE assays, latex SPT and total IgE

Assay	Latex allergic	Latex non-allergic	*p*
Latex EAST in AEU/ml; mean(SD); (n=40)	8.6 (6.0) (n=27)	0.09 (0.0) (n=13)	**<0.001**
Latex UniCAP in IU/ml; mean(SD); (n=50)	4.4 (4.4) (n=32)	0.18 (0.0) (n=18)	**<0.001**
Latex SPT diameter in mm; mean (SD); (n=22)	5.1 (2.1) (n=8)	0.1 (0.3) (n=14)	**<0.001**
Total IgE in IU/ml; mean (SD); (n=50)	1180 (2475) (n=32)	117 (198) (n=18)	**0.001**

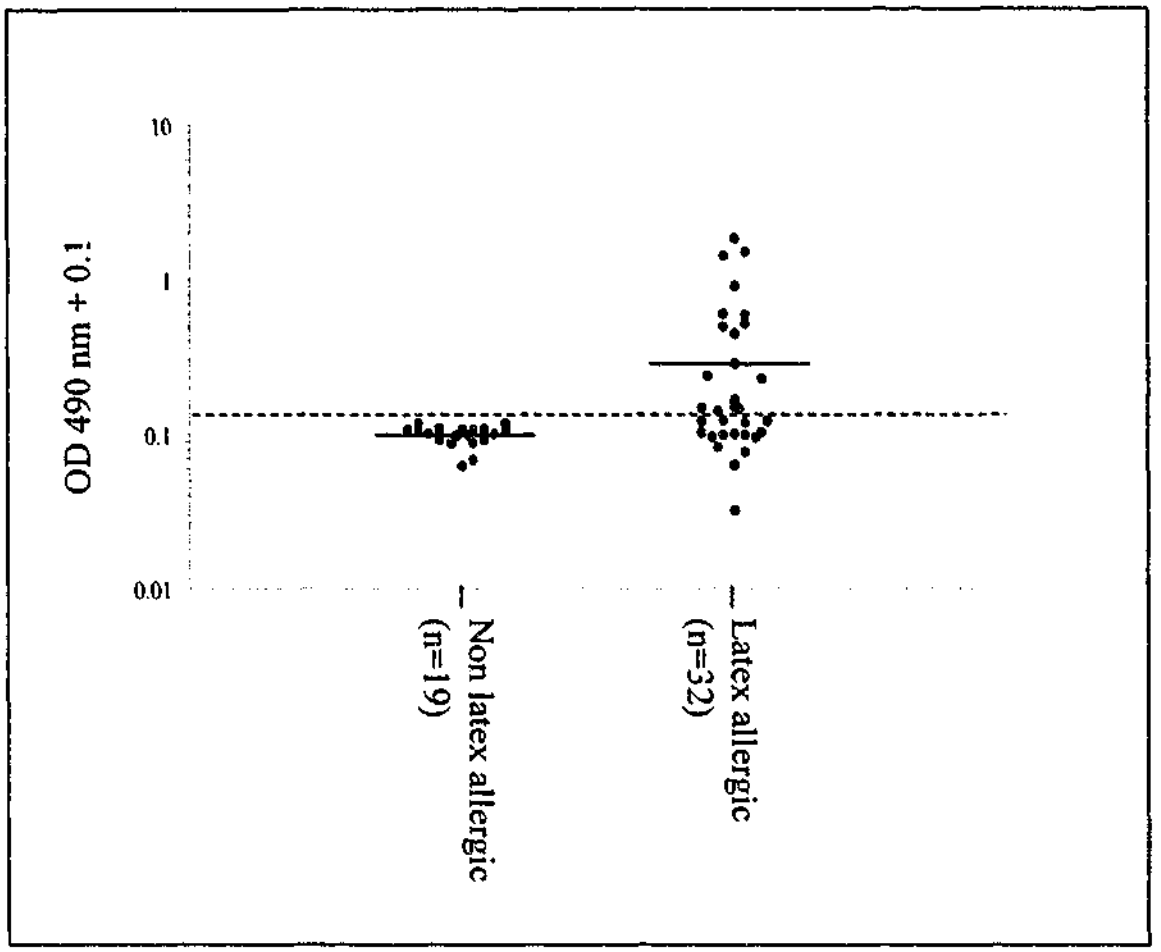


Figure 4.1 Direct ELISA of rHev b 5-specific IgE among study population

Human sera diluted 1:10 were added to rHev b 5-coated ELISA plates and rHev b 5 specific IgE quantified by colourimetric readout. Points shown are means of triplicates minus triplicate no antigen control values + 0.1. Horizontal bars indicate mean values (0.1, non-allergic; 0.39, allergic). Dashed line indicates cutoff value for positivity (2SD of non latex allergic group: 0.14). None of the non latex-allergic group had detectable rHev b 5-specific IgE, compared with 17/32 (53%) of the latex allergic group ($p < 0.001$, Mann Whitney U).

reliable assay when tested for inter-assay variation using IgE from highly reactive subject 17, with a mean OD of 1.80, standard deviation of 0.12 and a coefficient of variation of 6.6% when 6 individual rHev b 5 IgE ELISA were performed on separate ELISA plates.

4.3.4.2. Inhibition ELISA for rHev b 5

To ensure the specificity of the observed reactivity among latex-allergic HCW, inhibition ELISA were performed (Figure 4.2). After coating ELISA plates with rHev b 5 and pre-incubating sera with latex proteins or controls, a dose dependent inhibition was observed with rHev b 5, NAL and GE but not ryegrass pollen extract, confirming the specificity of the IgE reactivity observed, and also indicating the presence of Hev b 5 reactive epitopes within the natural latex extracts (Figure 4.2). As expected, much larger concentrations of natural extracts were required as inhibitors than the pure rHev b 5, indicating that the crude latex extracts contain other proteins in addition to Hev b 5.

4.3.5. Clinical characteristics of rHev b 5 positive, latex allergic subjects

As defined by the rHev b 5-specific IgE ELISA reactivity, the latex allergic subjects can be divided into Hev b 5-specific IgE positive or negative. The rHev b 5-specific IgE positive group were significantly older and had had more operations with general anaesthetic than the Hev b 5 negative latex allergic individuals when compared at a univariate level (Student's t test; Table 4.4).

There was no significant difference in total IgE levels between the rHev b 5-specific IgE positive and negative groups. With regard to the latex-specific IgE by Pharmacia UniCAP®, there was lower latex-specific UniCAP® class in the rHev b 5-reactive group and additionally, a significantly higher number of latex specific UniCAP® negative subjects within the rHev b 5-reactive group. There was also a significant association of rHev b 5 reactivity being associated with the serious allergic syndromes of angioedema and anaphylaxis (Table 4.4).

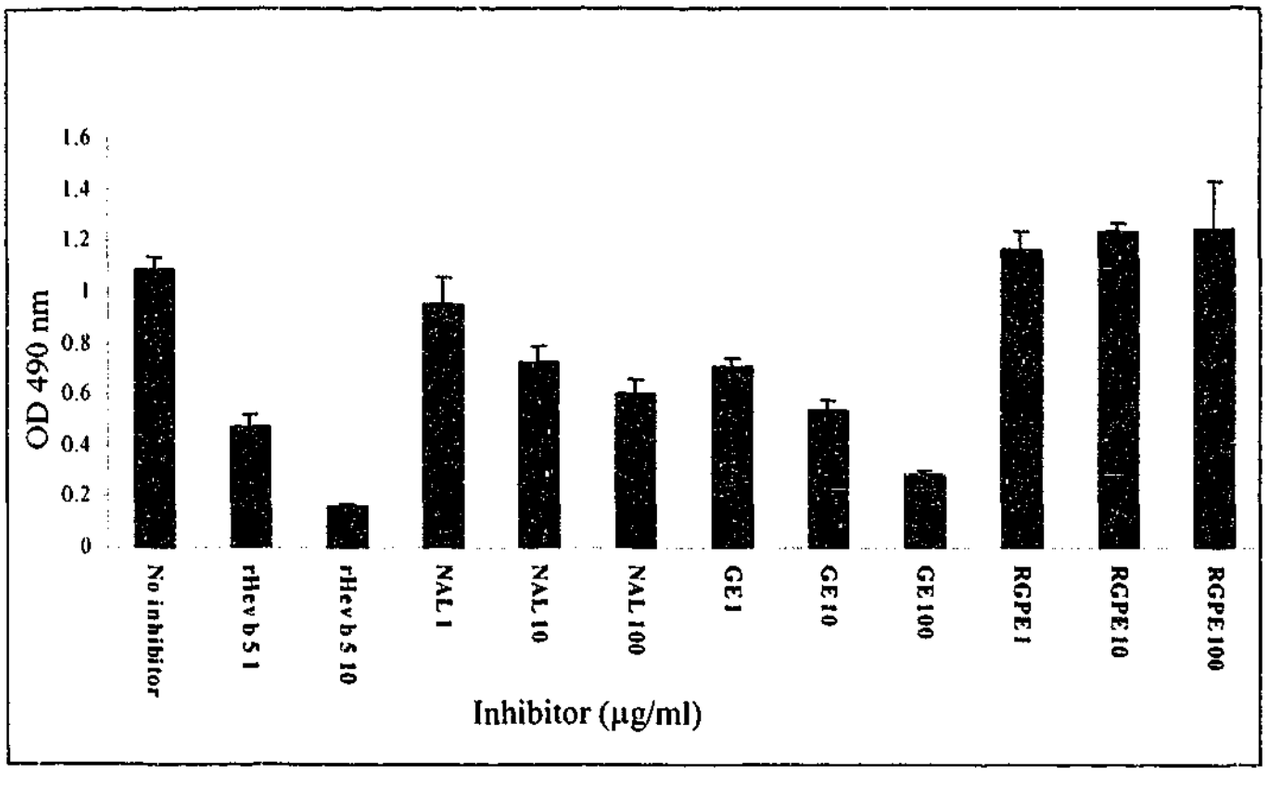


Figure 4.2 Inhibition ELISA for rHev b 5-specific human IgE

ELISA plates were coated with rHev b 5 before the addition of serum from latex allergic subject 17 diluted 1:20 and pre-incubated with inhibitors. Colour development was observed and quantified on a plate reader at OD 490nm; results shown are means (+SD) of triplicate values.

Table 4.4 Characteristics of latex allergic subjects according to Hev b 5 status

	Hev b 5-specific IgE positive (n=17)	Hev b 5-specific IgE negative (n=15)	*p* value
Age; mean (SD)	44.5(4.1)	33.5(7.9)	**0.003**
Operations with GA; mean (SD)	5.2 (5.7)	1.9 (2.1)	**0.04**
Current food allergy	10 (59%)	11 (73%)	0.46
Total IgE (kU/l)	551	1893	NA
Latex specific IgE EAST; mean (SD)	9.4 (6.4)	7.6 (5.6)	NA
Latex specific IgE EAST class; mean (SD)	2.5 (1.3)	2.3 (1.2)	0.65
Subjects with a negative latex-specific EAST	3 (18%)	2 (13%)	
Latex specific IgE UniCAP; mean (SD)	2.3 (4.2)	7.2 (3.3)	0.07
Latex specific UniCAP class; mean (SD)	1.5 (1.6)	2.5 (1.0)	**0.046**
Subjects with negative latex-specific UniCAP	8 (47%)	0	**0.001**
Skin hives	15 (88%)	13 (87%)	0.87
Facial swelling	14 (82%)	8 (53%)	**0.03**
Throat swelling	9 (53%)	3 (33%)	**0.03**
Asthma	12 (71%)	11 (73%)	0.98
Anaphylaxis	9 (53%)	4 (27%)	**0.02**

4.3.6. rHev b 5 immunoblots

Immunoblotting of rHev b 5 with a selection of the population to confirm the ELISA results according to the previously described method (see section 2.2.5.3.3) was performed. This data validated the rHev b 5-specific ELISA: only the subjects with rHev b 5-specific specific IgE by ELISA showed any reactivity on Western blotting (Figure 4.3). In addition, this reactivity could be inhibited by pre-incubating the sera with rHev b 5, confirming specificity. When assays were performed in parallel with the rHev b 5-specific mAbs, there was a similar pattern of specific reactivity observed when probing with pooled serum from latex allergic, Hev b 5 responsive subjects, and this was specifically inhibited with the pre-incubation of the pooled serum with rHev b 5 (Figure 4.4).

4.3.7. rHev b 5-specific IgG_4 in human subjects

Analysis of rHev b 5 specific IgG_4 levels was carried out by ELISA (see section 2.2.6.3; Figure 4.5). Generally, this assay had poorer discrimination for the diagnosis of latex allergy than the rHev b 5-specific IgE ELISA. The IgG_4 assay had a mean (SD) OD 490nm of 0.14 (0.10) for the non-latex allergic group and 0.25 (0.21) for the latex allergic group (p=0.36, 2 tailed t test). Using a cut-off of the mean + 2 SD of the non-allergic group (OD 490nm 0.43), however, only three latex allergic subjects and one latex non-allergic subject had positive rHev b 5-specific IgG_4 levels (Figure 4.5 A). Interestingly, the highest level of rHev b 5-specific IgG_4 was in a latex allergic HCW (Subject 30) who had undetectable Hev b 5-specific IgE. The other two rHev b 5-specific IgG_4 positive, latex-allergic subjects had high levels of rHev b 5-specific IgE (subjects 15 and 17; Figure 4.5). One of the non-allergic HCW (subject 44) had a high level of rHev b 5-specific-IgG_4 and undetectable rHev b 5-specific IgE. There was a weak correlation between rHev b 5-specific IgG_4 and IgE levels (R^2=0.27, Pearson moment correlation; Figure 4.5 B).

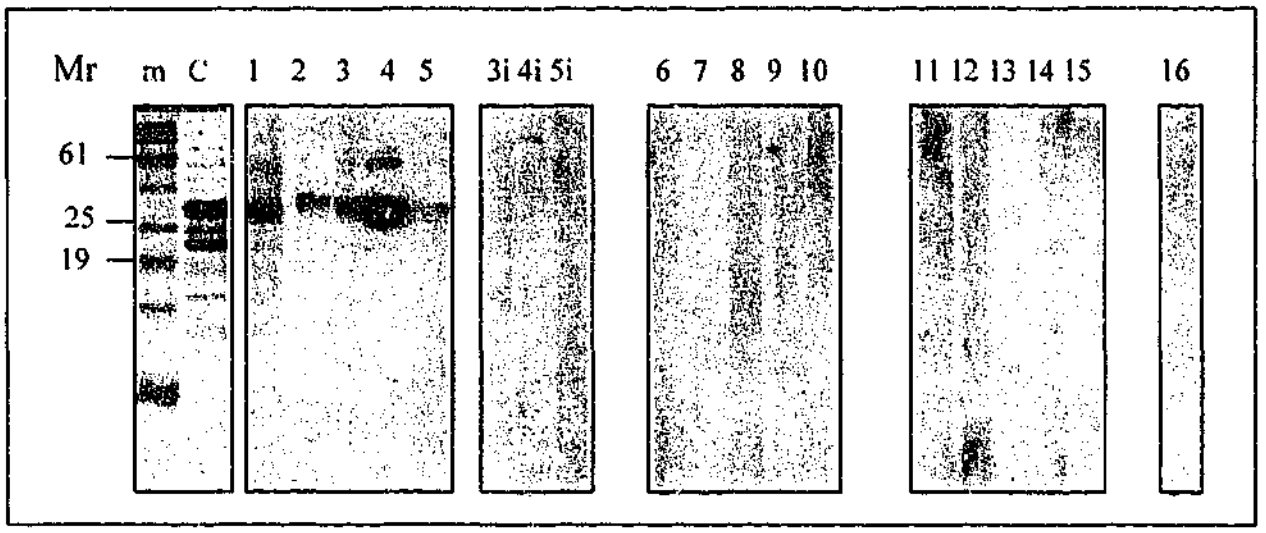


Figure 4.3 Western blotting of rHev b 5 with human IgE

Recombinant Hev b 5 was resolved on 16% SDS-PAGE and stained with CBB (C) for visualisation of total protein with relative mobility determined with See Blue® prestained molecular mass markers (m). Protein was then electro-blotted to NC membranes, blocked and diluted human sera (1:10) added for blotting as follows: sera from subjects with rHev b 5-specific IgE (lanes 1-5); sera from subjects with rHev b 5-specific IgE pre-incubated with rHev b 5 (lanes 3i, 4i, 5i); sera from latex allergic but rHev b 5 negative subjects (lanes 6-10); sera from non latex allergic subjects (lanes 11-15); no serum, detection antibody only (lane 16).

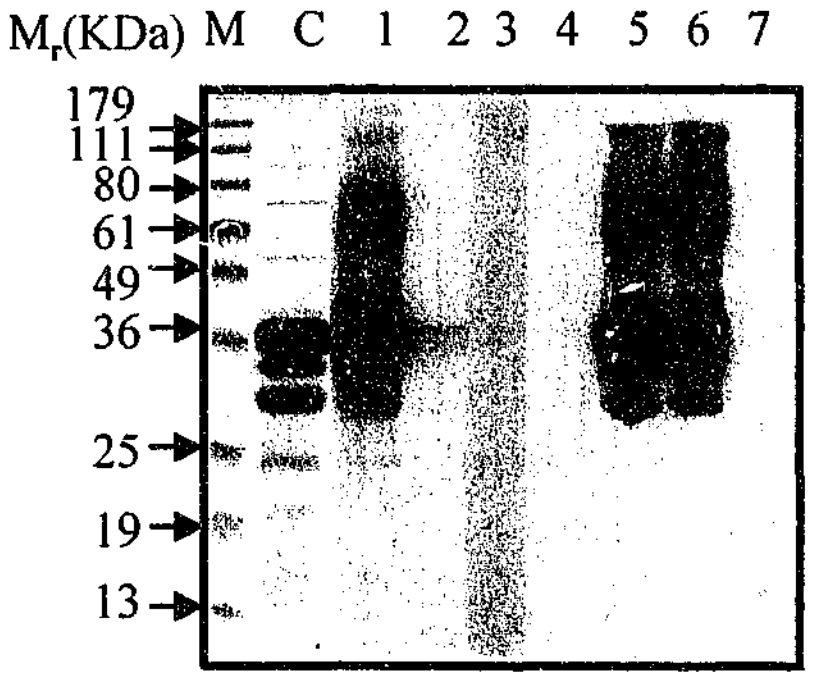


Figure 4.4 Inhibition immunoblot of rHev b 5 probed in parallel with rHev b 5-reactive human IgE and mAbs

Recombinant Hev b 5 was stained with CBB (lane C) or electroblotted to NC and probed with a pooled serum (1:5 dilution) of Hev b 5-reactive latex allergic subjects (lane 1); pooled serum pre-incubated with rHev b 5 (lane 2); a pooled serum of non-latex allergic subjects (lane 3); IgE detection antibody only (lane 4); rHev b 5-specific mAb 6F6 (lane 5); rHev b 5-specific mAb 6A10 (lane 6); ryegrass-specific mAb A1 IgG1 isotype control (lane 7). Molecular mass markers are indicated by lane M (Sutherland *et al.*, 2002).

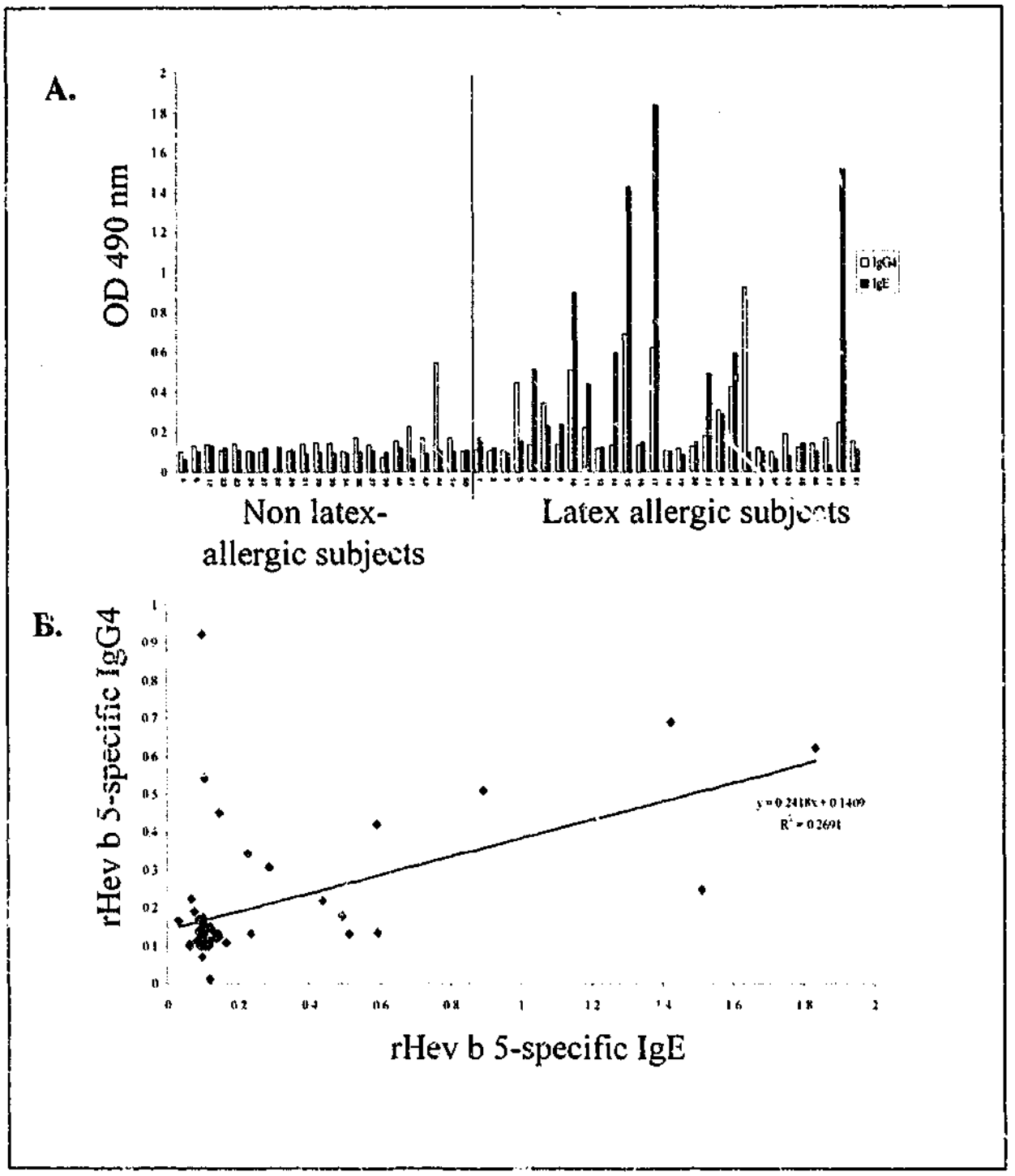


Figure 4.5 Comparison of rHev b 5-specific IgE and IgG_4

Sera from subjects were diluted 1:10 and added to ELISA plates coated with rHev b 5. Following detection with anti-human IgG_4 or IgE, colour development with OPD was measured at OD 490 nm (**A.**). Results are expressed as means of triplicates with background subtracted + 0.1 to prevent negative values. Figure **B.** shows the correlation with between the two assays (Pearson).

4.4. Discussion

This study examining the humoral immune response to Hev b 5 has confirmed Hev b 5 as a major latex allergen in this Australian population of predominantly HCW. The reactivity reported here of 53% by rHev b 5-specific IgE ELISA, is lower than the 92% reported by Slater in his original study (Slater *et al.*, 1996) among HCW, but is comparable to the reactivity reported by Yip of 66% (Yip *et al.*, 2000) and still qualifies Hev b 5 as a major antigen by WHO criteria (King *et al.*, 1994). On closer examination of Slater and co-workers' report of rHev b 5-specific IgE frequency, they divided their groups into latex allergic with a negative latex RAST (n=10) of which 2 had positive RAST to rHev b 5/MBP, and latex allergic with a positive latex RAST (n=13), of which 12 were positive by RAST to rHev b 5/MBP (Slater *et al.*, 1996). When these figures are combined as a total of latex allergic subjects (similar to the study presented here) the number of rHev b 5/MBP positive, latex allergic subjects described by Slater was 14/23 or 61% (Slater *et al.*, 1996), which is very similar to the results in the current study. What the results of the current study also show, however, is that as well as being a major allergen by population prevalence criteria; Hev b 5 is also a major latex allergen by way of its clinical importance. This importance manifests in two ways. Firstly, those individuals who have Hev b 5-specific IgE have a clinically more severe phenotype despite lower overall total IgE as shown by the higher incidence of clinically important allergic syndromes of angioedema and anaphylaxis. Secondly the results from this chapter indicate that many subjects with clinically important latex allergy may be negative by the most widely utilised latex diagnostic test in current use within the world today, the Pharmacia UniCAP®, and in all cases, these subjects were positive for rHev b 5 by the ELISA system described. This information is important, indicating that a negative UniCAP® alone is insufficient to exclude latex allergy. Taken together, these results indicate that Hev b 5 is a highly immunogenic component of natural rubber latex and is responsible for much of the

latex-specific IgE generation among latex allergic individuals. Secondly, it indicates that Hev b 5 may be more important as an allergen in latex gloves than in NAL. It has been reported in abstract form that Hev b 5 may be absent from the Pharmacia UniCAP®(Chen *et al.*, 2000). It has also been shown that the k82 NAL reagent used in the latex UniCAP® has greater diagnostic sensitivity when spiked with rHev b 5/MBP (Lundberg *et al.*, 2001) and this reagent has recently become commercially available following completion of the current study. It would be interesting to utilise this reagent to test the rHev b 5-specific IgE positive but UniCAP® negative group identified here. An alternative hypothesis raised by the current results is that rather than absence, (which has been disproved by the mAb results and the IgE inhibition ELISA presented in this thesis), Hev b 5 is altered during the glove manufacturing process. Further evidence to support this is presented in Chapter 6. Additional support for this argument is provided by the observation noted here that the Allercoat EAST had a superior sensitivity in the diagnosis of latex allergy compared with the UniCAP in this population and more particularly with reference to Hev b 5-positive individuals. The latex antigen used in the Allercoat EAST consists of a commercially available latex glove ground up and bound to a paper disc (Lex Lancaster, regional manager of Sanofi-Pasteur Diagnostics, USA; personal communication).

The results presented in this chapter also point towards characteristics of Hev b 5 that make it a highly potent allergen, reinforcing its clinical relevance. The high prevalence of anaphylaxis amongst Hev b 5 positive individuals suggest that Hev b 5 has a heightened capacity to cross-link effector-cell bound IgE and induce mast cell and basophil degranulation. This may be in part through its high water solubility and high negative charge (Slater *et al.*, 1996) which allows it to leach rapidly from gloves during wear and also bind to cornstarch donning powder and thus have high bioavailability. In addition, it has been suggested that because of its frequently repeating XEEX motifs it exhibits multivalencey and can thus effectively cross-link more than

one IgE molecule (Beezhold *et al.*, 1999). While there is no *in vivo* evidence for this presented here, it has been shown here Hev b 5 does have a propensity to form aggregates under SDS-PAGE conditions, and these are immunoreactive in Western blotting assays (see Figure 4.4).

The IgG_4 assays indicated only a weak correlation between IgE and IgG_4 levels ($R^2 = 0.27$; Figure 4.5B). As expected, the IgG_4 assay was poor at discriminating latex allergic from non-allergic individuals when a cut-off of 2 SD of the non-allergic group was used. It is difficult to make any firm conclusions from this rHev b 5-specific IgG_4 data. It has been suggested that IgG_4 is a marker for successful immunotherapy by acting as blocking antibodies (van Neerven *et al.*, 1999). There is perhaps some support for this here, in that some patients (such as subject 44) mounted a strong rHev b 5-specific IgG_4 response in the absence of symptoms on latex exposure or of specific IgE response. In addition, rHev b 5-specific IgG_4 levels were higher as a group among non-allergics than rHev b 5-specific IgE levels. Alternatively, rather than blocking antibodies, this may simply be an epi-phenomenon, where non-allergic cellular immune responses direct B cell antibody formation away from IgE towards IgG_4. It has also been suggested that IgG_4 in humans may have a role as an "anaphylactic antibody", mainly through complement activation (Bergamaschini *et al.*, 1996). The results presented here do not support this, as many of the non-allergics showed an IgG_4 response.

This study has shown that Hev b 5 is a major latex allergen in a group of Australian HCW. It has also shown the clinical importance of Hev b 5 as a latex allergen and indicated the limitations of currently available IgE assays as diagnostics. Further evidence of the importance of nHev b 5 within latex GE will be presented in Chapter 6, providing a rational explanation for the importance of Hev b 5 as an allergen among HCW who are exposed to natural rubber latex gloves as their major allergen source for sensitisation and symptoms.

5. Human cellular immune response to Hev b 5

5.1. Introduction

Latex allergy is a potentially severe condition (Pumphrey *et al.*, 2001) for which no safe and effective SIT is available. The mainstay of current management consists of allergen avoidance, but for severely affected individuals and especially those with concomitant food allergy, this may be insufficient (Sutherland *et al.*, 2002). Thus, safe and effective SIT is desirable, though recent experience with crude latex extracts for use in SIT in latex allergy has shown an unacceptable rate of systemic side effects (Leynadier *et al.*, 2000). The main drawbacks of SIT in general, centre on IgE-mediated side effects (Adkinson, 2001). As a means to reduce these side effects, novel approaches to SIT are in development which aim to decrease the cross-linking of effector cell-bound IgE. These methods include allergen peptides (Oldfield *et al.*, 2001), DNA vaccination (Slater and Colberg-Poley, 1997) and conjugation to immunostimmulatory sequences of DNA (ISS) or "CpG" motifs (Tighe *et al.*, 2000). As a prelude to the potential application of these technologies to the latex allergy problem, delineation of the human cellular immune response to Hev b 5 is mandatory. Hev b 5 is a rational target for novel immunotherapeutic approaches because of its status as a major latex allergen (Slater *et al.*, 1996) and the significant incidence of monosensitisation to Hev b 5 that exists among HCW (Yip *et al.*, 2000).

There were little previous data on the human cellular immune response to latex allergens in general (see section 1.3.5.1.2) and, prior to studies within our laboratory (de Silva *et al.*, 2000), none on the response to Hev b 5. In the study of 6 latex allergic HCW, de Silva found that Hev b 5(46-65) showed strong reactivity in 5/6 latex allergic HCW and in all cases where the subjects had rHev b 5-specific IgE. This suggested that this peptide molecule contained a dominant T cell epitope. The numbers in this study were small, however, making firm conclusions difficult. This chapter seeks to validate de Silva's initial work and

expands the analysis of the cellular responses to a larger number of subjects including latex non-allergic control individuals. In this chapter, the polyclonal cellular response to latex allergens is first examined, followed by oligoclonal T cell responses to Hev b 5.

5.2. Results

5.2.1. Toxicity assays of latex antigens

These studies showed that GE was not toxic to T cells at concentrations of up to 30 μg/ml, but was toxic at 100 μg/ml (Figure 5.1). Not all Hev b 5 peptides were tested, as these had been found previously to be non-toxic (de Silva *et al.*, 2000). Recombinant Hev b 5 and Hev b 5(46-65) were found to be non-toxic over the concentration range for testing in this study (Figure 5.1).

5.2.2. Mitogenicity assays of latex antigens

All latex allergens tested {GE, rHev b 5 and Hev b 5 (46-65)} were non-mitogenic at the concentrations used in assays (Figure 5.2). All Hev b 5 peptides had been found to be non-mitogenic in previous studies (de Silva *et al.*, 2000).

5.2.3. Polyclonal assays of latex allergens

5.2.3.1. PBMC proliferation to latex antigens

Analysis of PBMC proliferation assays to rHev b 5 and GE indicated that, as a whole, there was significantly greater proliferation to rHev b 5 and GE among latex allergic individuals than in non-allergic subjects (p=0.03 for Hev b 5 at 1 μg/ml, p=0.002 at 10 μg/ml, Mann Whitney U test; Figure 5.3). There were however, individual latex non-allergic subjects, who showed strong PBMC proliferation to rHev b 5 (Figure 5.3).

5.2.3.2. Cytokine responses to latex antigens

Analysis of cytokine responses showed that with PBMC assays, generally low levels of IL-5 were secreted in response to stimulation with rHev b 5 following the subtraction of the levels seen with the no-

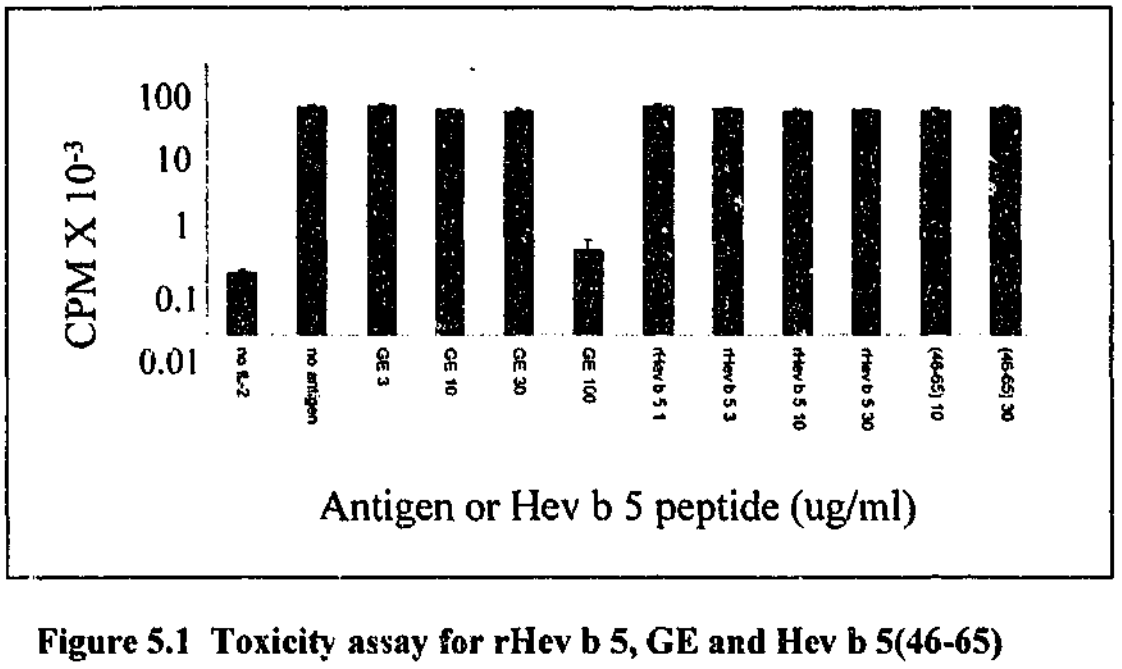


Figure 5.1 Toxicity assay for rHev b 5, GE and Hev b 5(46-65)

T cells from a two week couch grass pollen-specific T cell line kindly provided by Ms N. Eusebius were stimulated with 50 IU/ml IL-2 and in the presence of the various antigens shown. Proliferation was measured by ^{3}H-Thymidine incorporation.

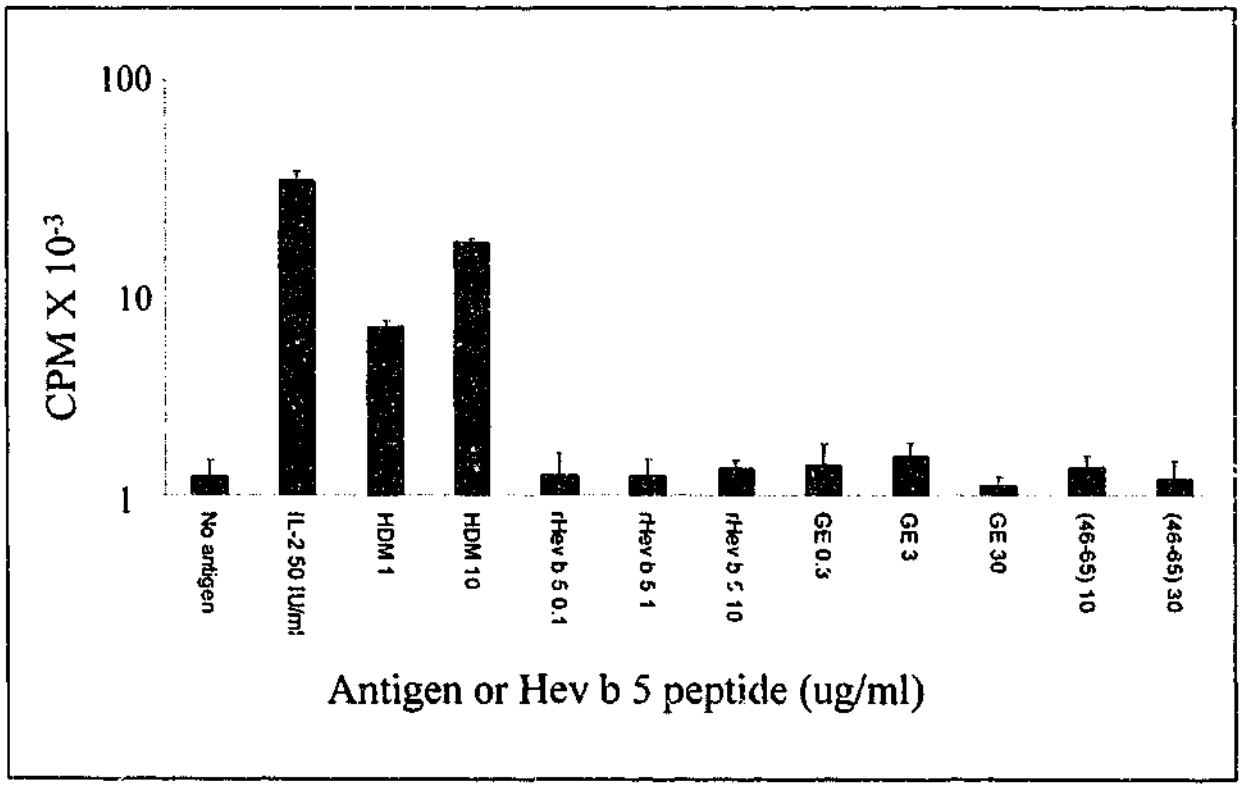


Figure 5.2 Mitogenicity assay for rHev b 5, GE and Hev b 5(46-65)

T cells from a 3 week house dust mite-specific T cell line kindly provided by Dr H. de Silva were stimulated with IL-2, house dust mite (HDM; positive control) or rHev b 5, GE or Hev b 5 (46-65) in the presence of irradiated, autologous PBMC and proliferation was assessed by measurement of ^{3}H-Thymidine uptake.

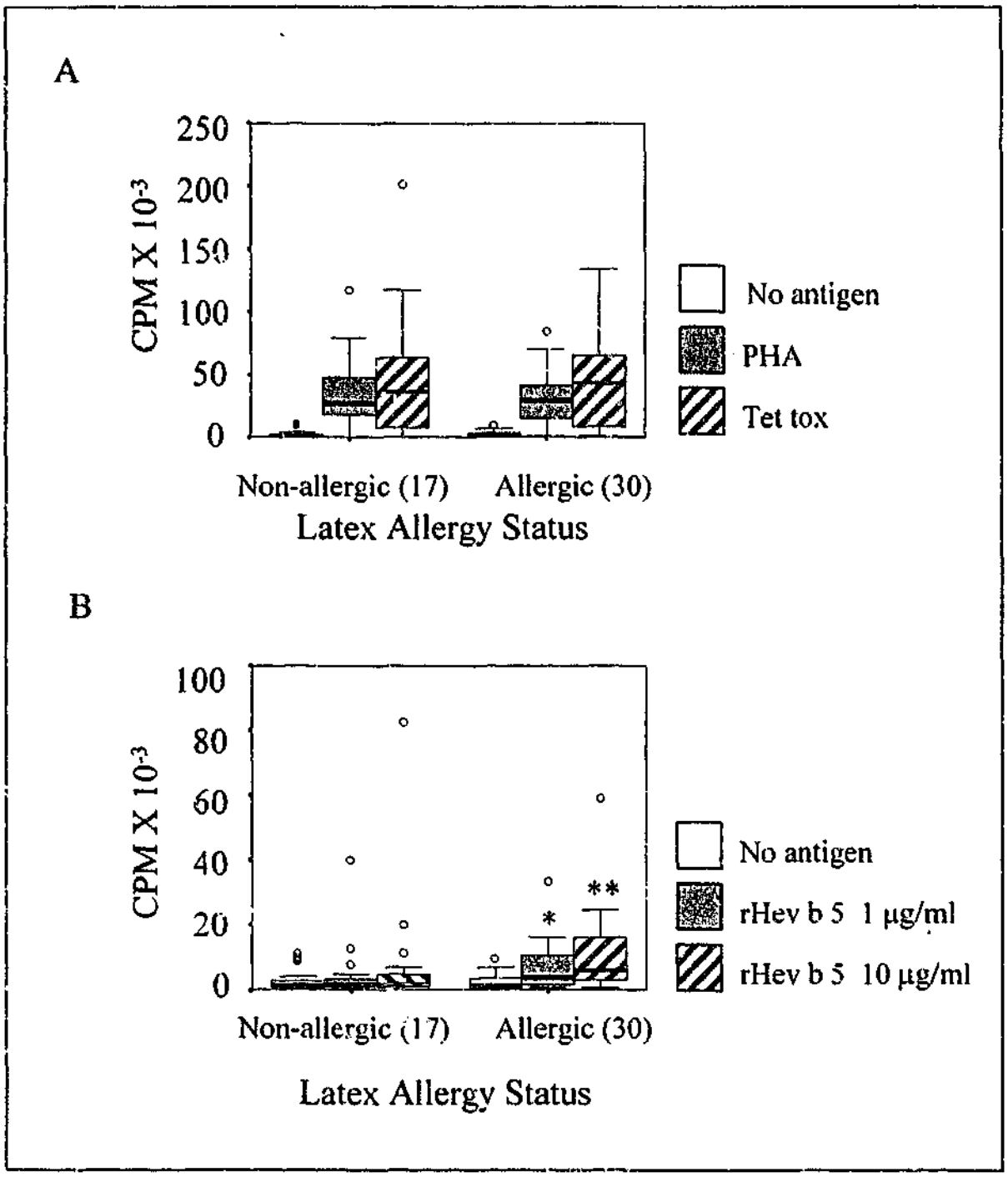


Figure 5.3 PBMC proliferation to rHev b 5

Boxplots showing summary of PBMC proliferation against control antigens (A) and rHev b 5 (B) in human subjects. Boxes represent 25th to 75th centiles. Medians are shown as horizontal lines within boxes. Whiskers represent expected distribution and open circles outlying & extreme observations (still included in analysis). There was no statistical difference in the proliferation to control antigens between latex-allergic and latex non-allergic subjects (Mann-Whitney U test). PBMC from latex allergic subjects proliferated significantly more against rHev b 5 at 1 µg/ml (p=0.032) and 10 µg/ml (p=0.002) than those from latex non-allergic subjects as indicated by asterisks (Mann-Whitney U test).

antigen control (ΔIL-5=IL-5 with antigen stimulation – IL-5 with no antigen stimulation). There was however significantly greater ΔIL-5 secretion at 72 hours upon stimulation with rHev b 5 10 μg/ml among latex allergic subjects (p = 0.034, Mann Whitney U; Figure 5.4A). Higher levels of ΔIL-5 secretion were seen with PHA stimulation compared with antigen specific stimulation, but there was no significant difference in levels between the latex allergic (median ΔIL-5 788 pg/ml) and non-allergic groups (median ΔIL-5 = 618 pg/ml; p=0.719, Mann Whitney U) as a whole for this positive control mitogen.

There was no significant difference in PBMC Day 6 ΔIFN-γ secretion in response to rHev b 5 or PHA between latex allergic and latex non-allergic subjects (p = 0.264 and 1.0 respectively, Mann Whitney U test; Figure 5.4B).

5.2.4. T cell assays

5.2.4.1. Optimisation of T cell line culture conditions

In the epitope mapping study using rHev b 5/MBP as an antigen, de Silva et al used LAL to drive PBMC for 2 weeks then further enriched for a rHev b 5 -specific response by driving the TCL with rHev b 5/MBP for one further week before peptide assay (de Silva *et al.*, 2000). The original batch of LAL used in de Silva et al's study was not available for the current study, and preliminary results indicated that the replacement LAL was less efficient at driving Hev b 5 latex specific antigen proliferation. Indeed, in inhibition ELISA experiments LAL had low levels of immunoreactive nHev b 5 (see section 6.3.2). Therefore, a high protein, Hev b 5-containing non-sterile powdered latex examination GE was used (Uniglove, Malaysia). Using this, a satisfactory Hev b 5-specific peptide response was achieved after 2 weeks of stimulation with GE for the previously identified rHev b 5-responsive, latex-allergic subject 17 (Figure 5.5). In addition, there were similar levels of stimulation achieved with 10 μg/ml and 30 μg/ml peptide concentrations; therefore for ease of performance in the

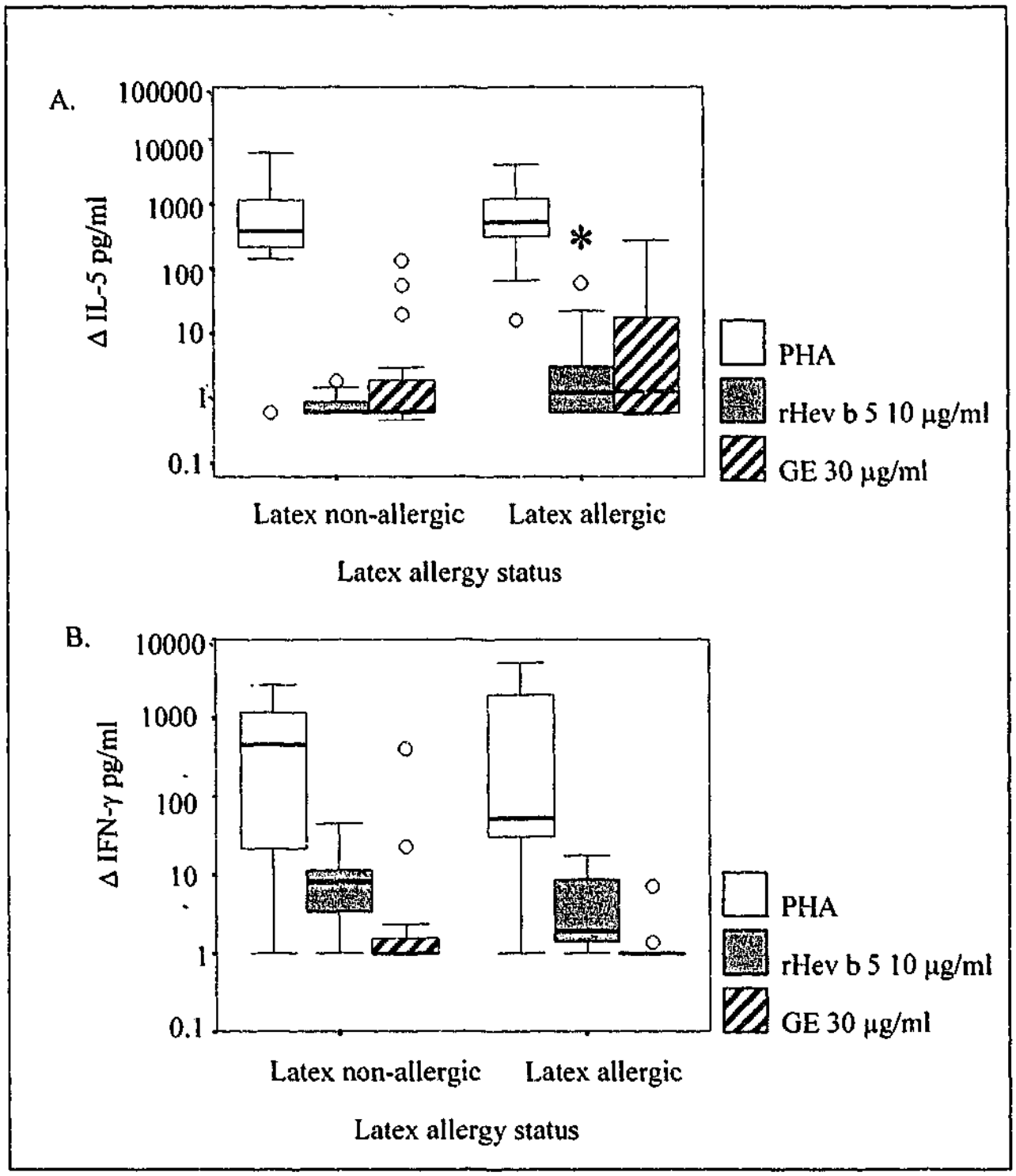


Figure 5.4 PBMC cytokine secretion on stimulation with rHev b 5

PBMC were stimulated with PHA or antigen at the concentrations indicated and supernatants collected at 3 days for IL-5 (A) and 6 days for IFN-γ (B) and asssayed for these cytokines using sandwich ELISA. Boxplots show medians and interquartile ranges; for each antigen and cytokine, latex allergic groups and non-allergic groups were compared using a Mann Whitney U test, with $p<0.05$ indicated with asterisk.

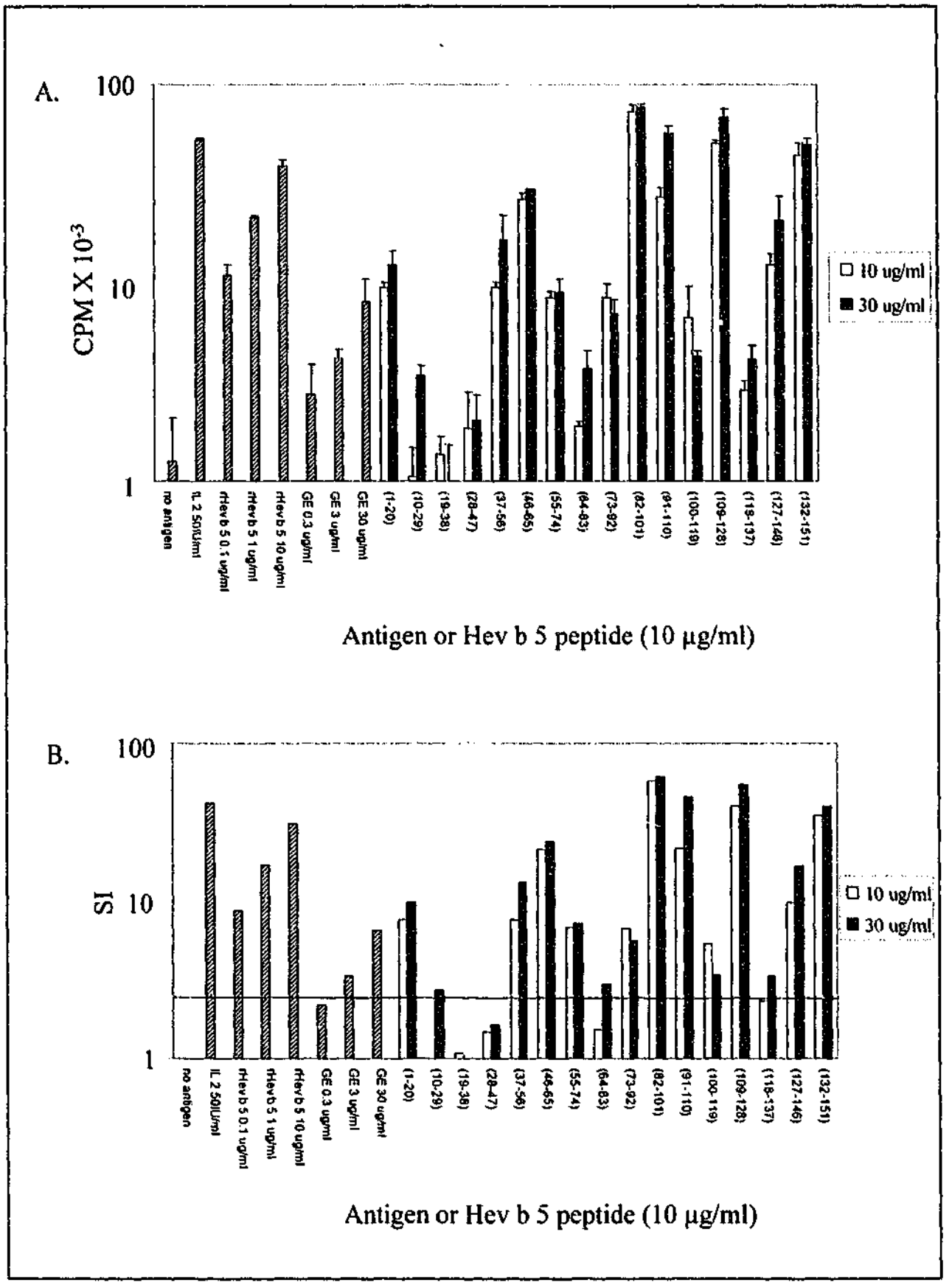


Figure 5.5 Hev b 5 peptide stimulation of short term TCL, subject 17

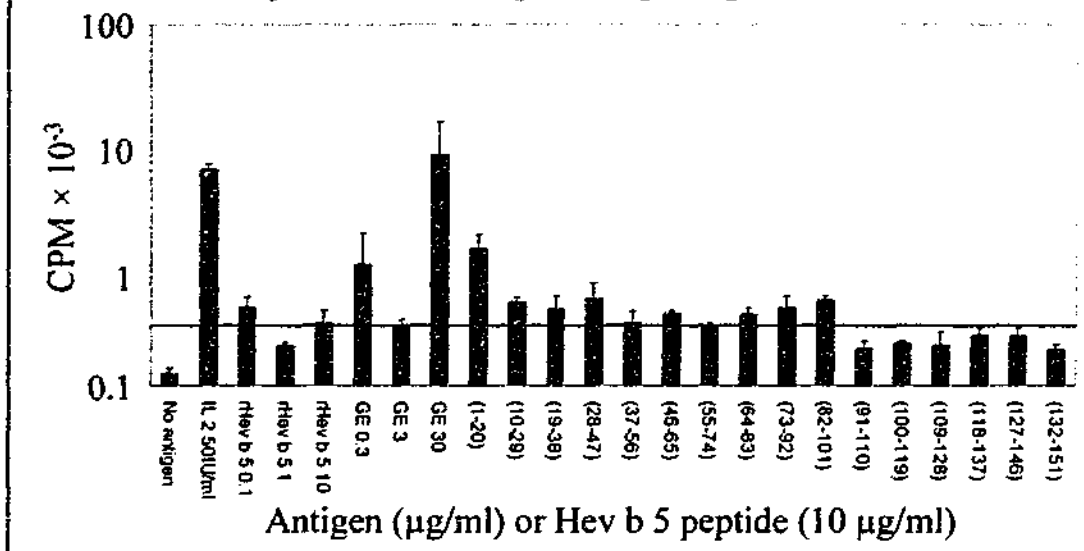
A short term TCL specific for GE, from latex allergic subject 17, was stimulated with control antigens or Hev b 5 peptides at the concentrations shown. Proliferation was measured by ^{3}H-Thymidine incorporation and is shown as raw counts (mean of triplicates + SEM; A) or SI (B). Significant SI of 2.5 is indicated by the horizontal line (B).

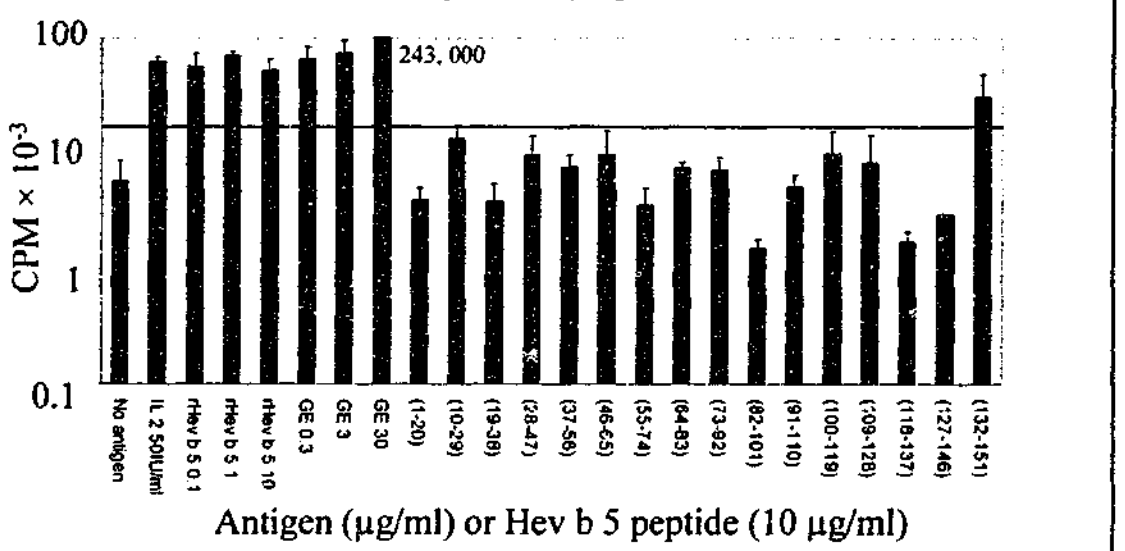
expanded number of subject assays, 10 μg/ml alone was used for subsequent experiments.

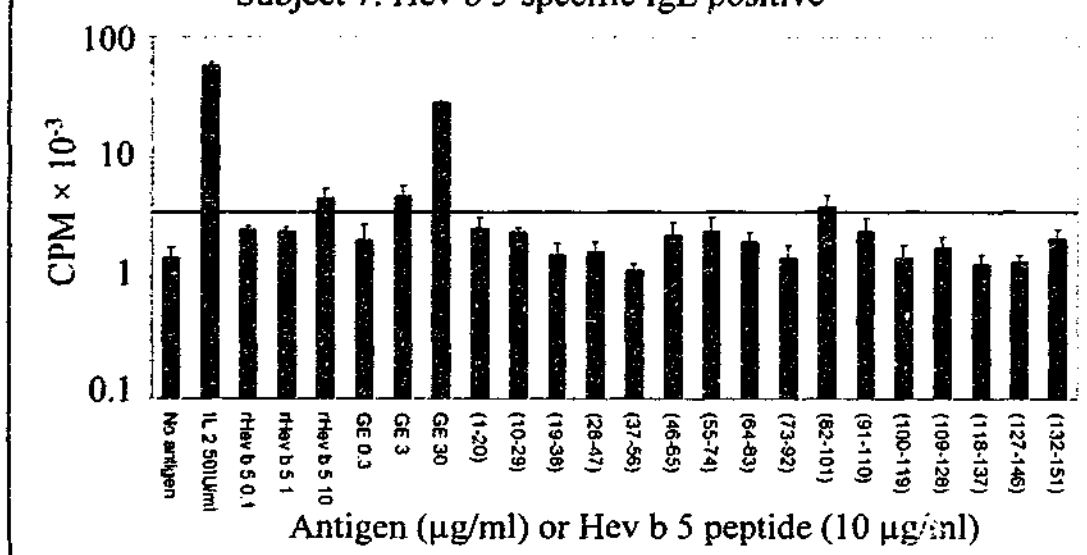
5.2.4.2. Hev b 5 peptide-induced T cell proliferative responses

Sufficient cells were available after 2 rounds of stimulation with GE for Hev b 5 peptide assays for 27 latex allergic subjects and 16 non-latex allergic subjects (Figures 5.6A, 5.6B and 5.7). All assays were performed in triplicate with positive and negative controls and Hev b 5 peptides at 10 μg/ml. A positive peptide response was defined as proliferation of 1 or more peptides at an SI of greater than or equal to 2.5. Using this criterion, there were 14 latex allergic subjects and 7 latex non-allergic subjects who mounted a significant peptide response to one or more Hev b 5 peptides. Analysis of patterns of peptide response indicated that 2 peptides were dominant (greater than 50% reactivity) among latex-allergic subjects: Hev b 5 (1-20; 71.4%) and Hev b 5 (46-65; 76.5%; Figure 5.8A). Moreover, 86% of latex-allergic individuals reacted to one or both of these two dominant peptides. In addition, four other peptides were each recognised by 50% of subjects in the allergic group: Hev b 5 (37-56), Hev b 5 (57-74), Hev b 5 (82-101) and Hev b 5 (109-128; Figure 5.8A). In contrast, there were no peptides identified as dominant on frequency criteria among the latex non-allergic group (Figure 5.8B). The peptide achieving the highest frequency of a significant proliferative response among the latex non-allergic group was Hev b 5 (82-101) at 42.9% (Figure 5.8B). In addition to peptide Hev b 5 (46-65) having the highest frequency of a significant proliferative response, it also induced the highest frequency of proliferative responses greater than SI 5 (50% of latex allergic subjects) and 10 (35.7% of latex allergic subjects; Figure 5.8A). When responses were considered as means of SI across the whole population, again Hev b 5 (1-20) and Hev b 5 (46-65) were the only peptides which showed significantly different responses between latex allergic and non-allergic subjects (p = 0.02 and 0.04 respectively, 2 tailed t test; Figure 5.9). This comparison also showed that mean proliferation at the

Figure 5.6A Proliferation assays to Hev b 5 peptides in latex allergic subjects mounting one or more positive peptide responses. Short term GE-specific TCL were stimulated for 48 hours with Hev b 5 peptides and control antigens as indicated. Proliferation was measured by ^{3}H-Thymidine incorporation. Significant proliferation (SI≥2.5) is indicated by the horizontal line and Hev b 5-specific IgE status is also shown.







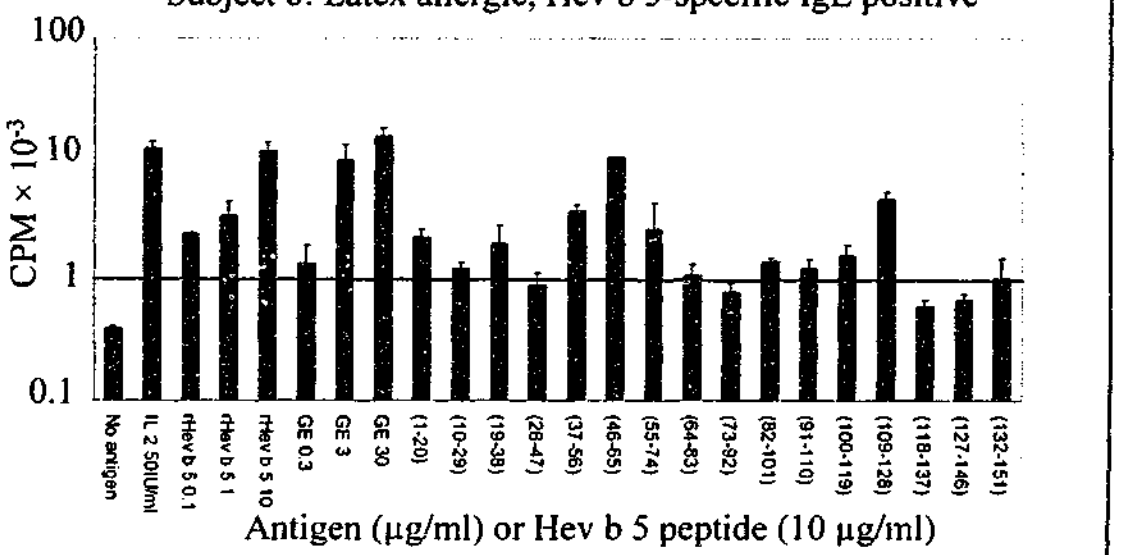
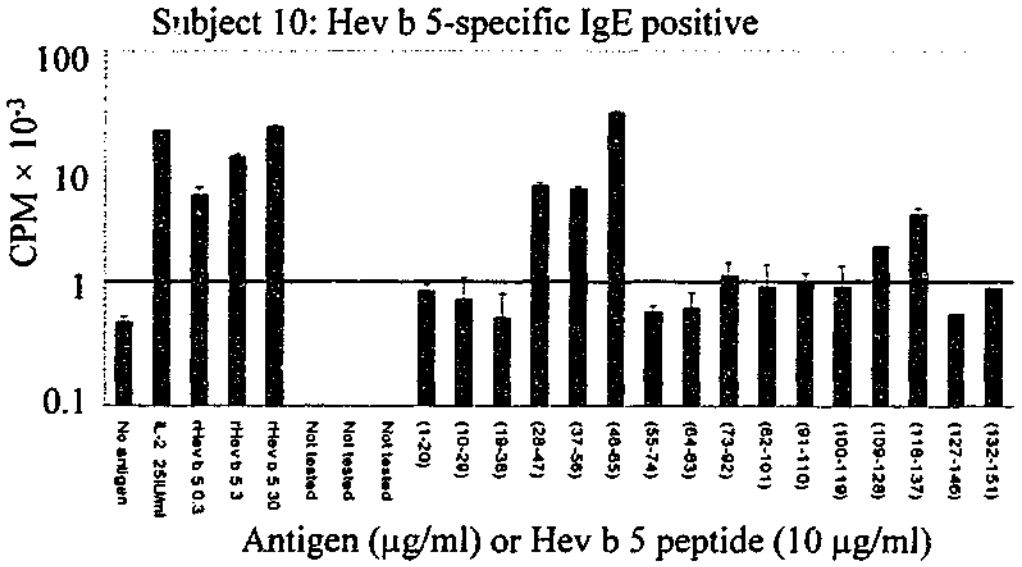
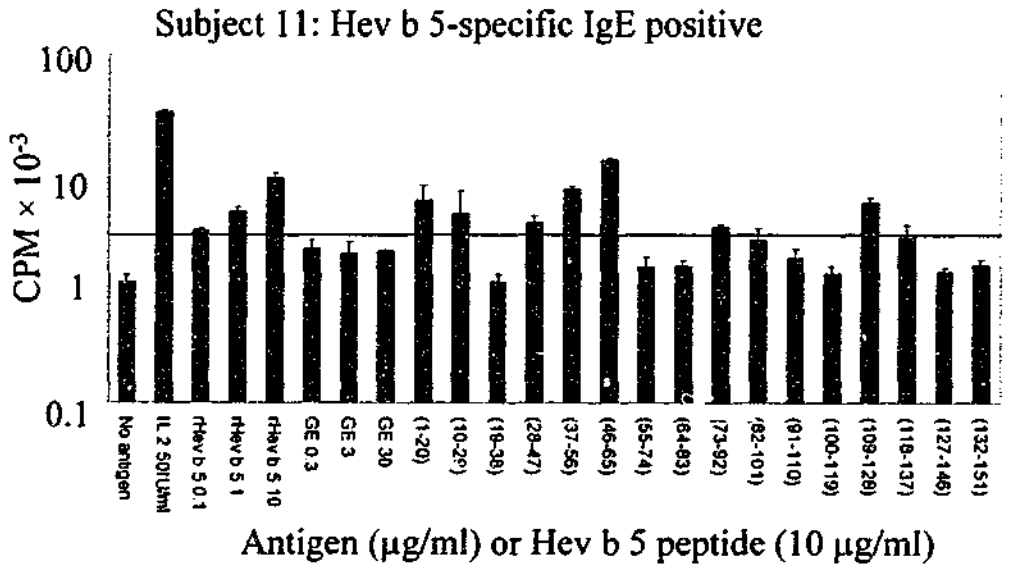
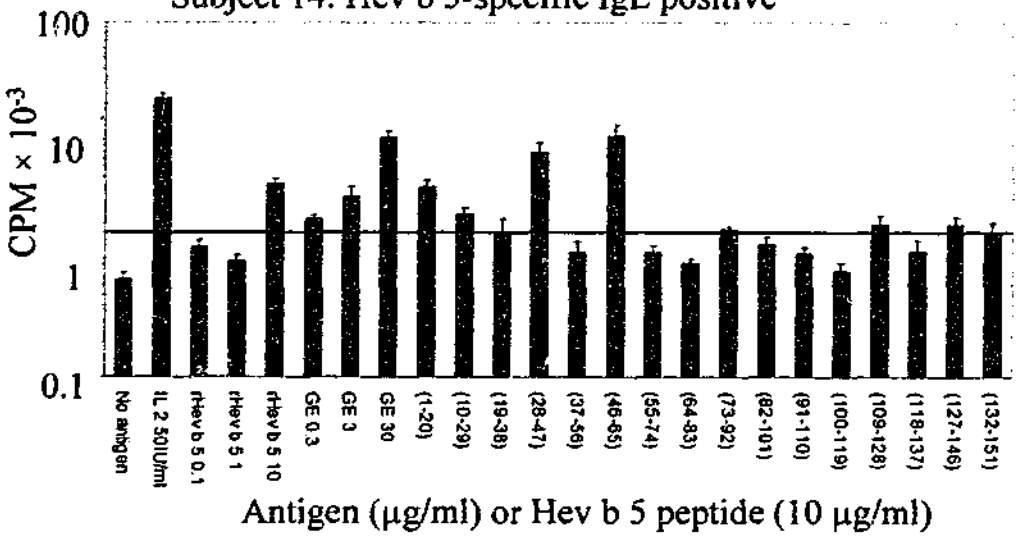


Figure 5.6A Proliferation assays to Hev b 5 peptides in latex allergic subjects mounting one or more positive peptide responses. Short term GE-specific TCL were stimulated for 48 hours with Hev b 5 peptides and control antigens as indicated. Proliferation was measured by ^{3}H-Thymidine incorporation. Significant proliferation (SI≥2.5) is indicated by the horizontal line and Hev b 5-specific IgE status is also shown.







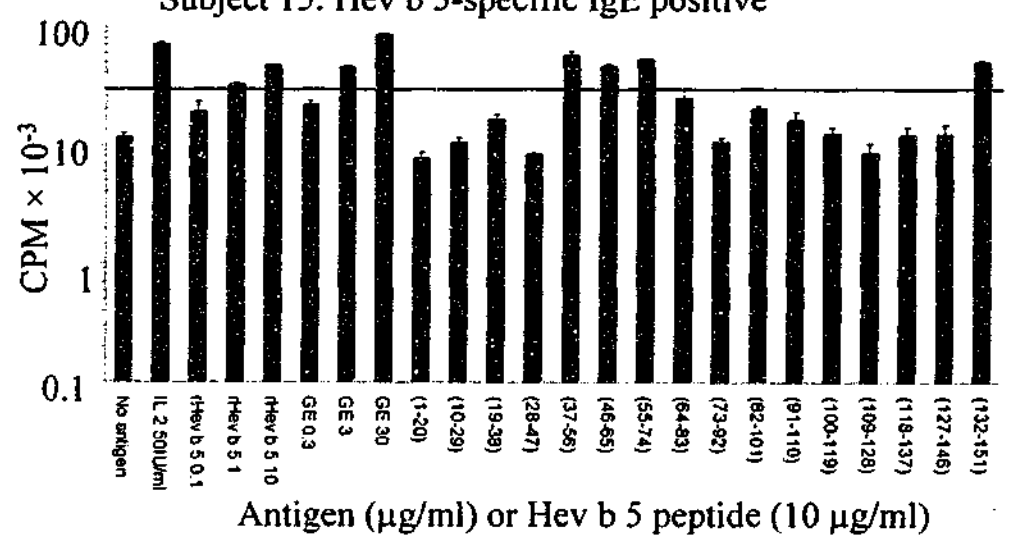
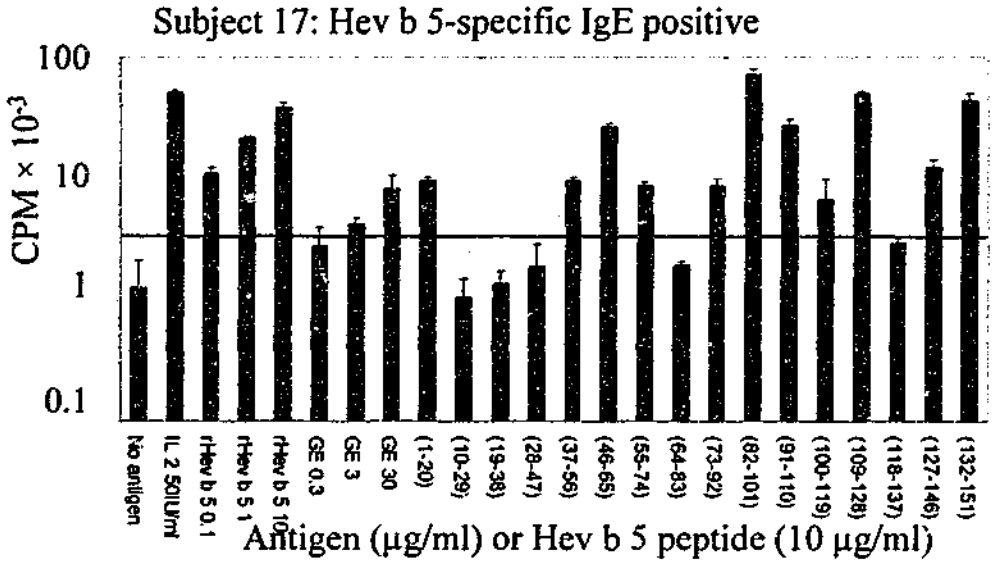
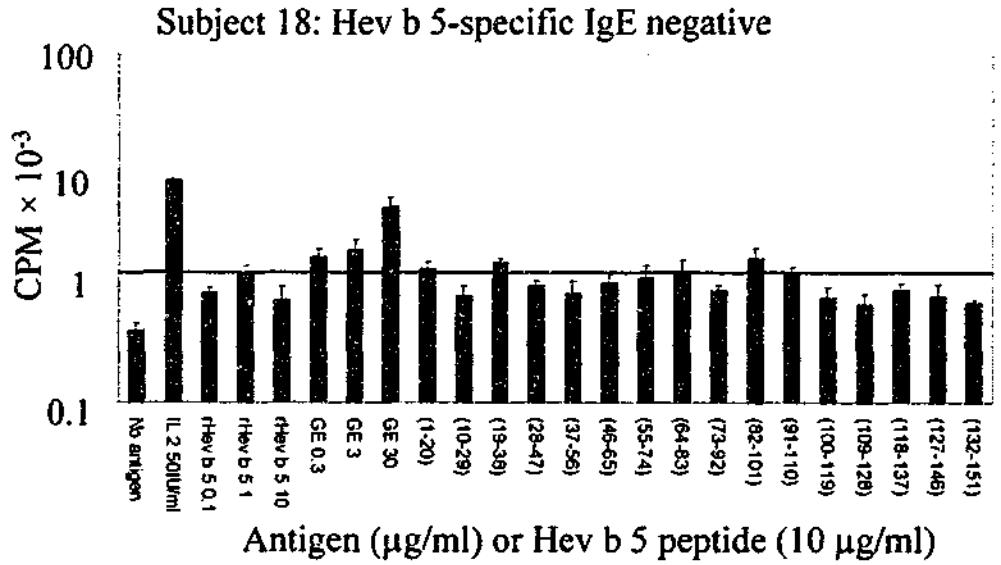
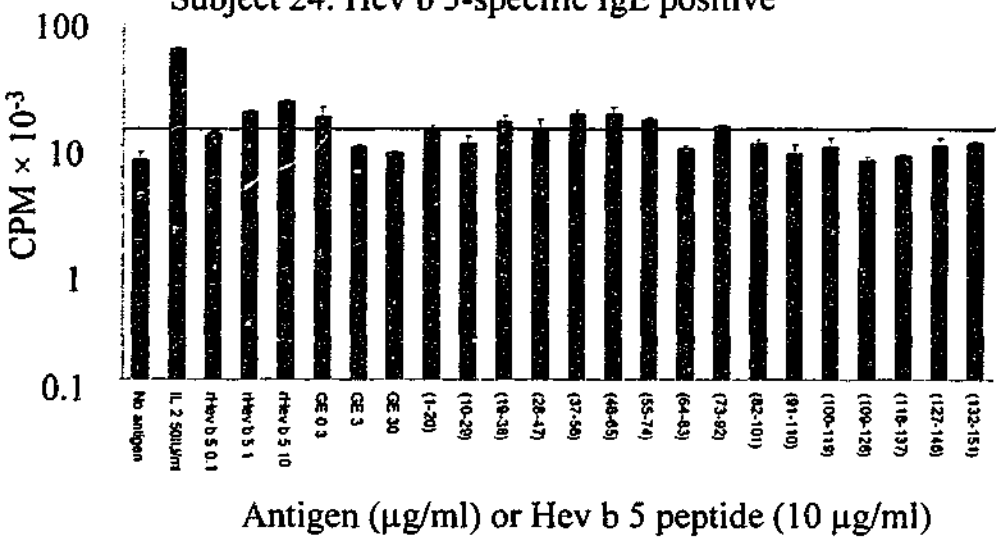


Figure 5.6A Proliferation assays to Hev b 5 peptides in latex allergic subjects mounting one or more positive peptide responses. Short term GE-specific TCL were stimulated for 48 hours with Hev b 5 peptides and control antigens as indicated. Proliferation was measured by ^{3}H-Thymidine incorporation. Significant proliferation (SI≥2.5) is indicated by the horizontal line and Hev b 5-specific IgE status is also shown.







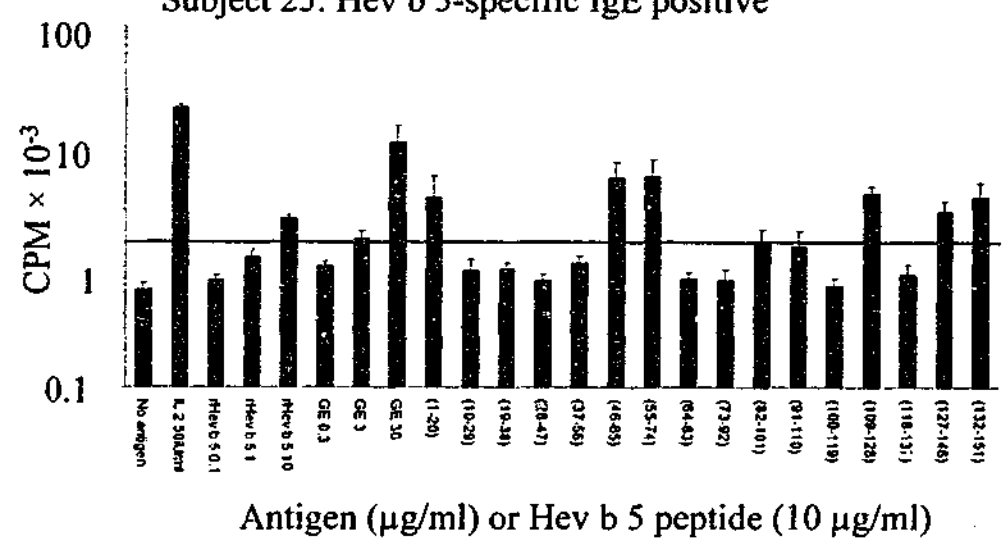
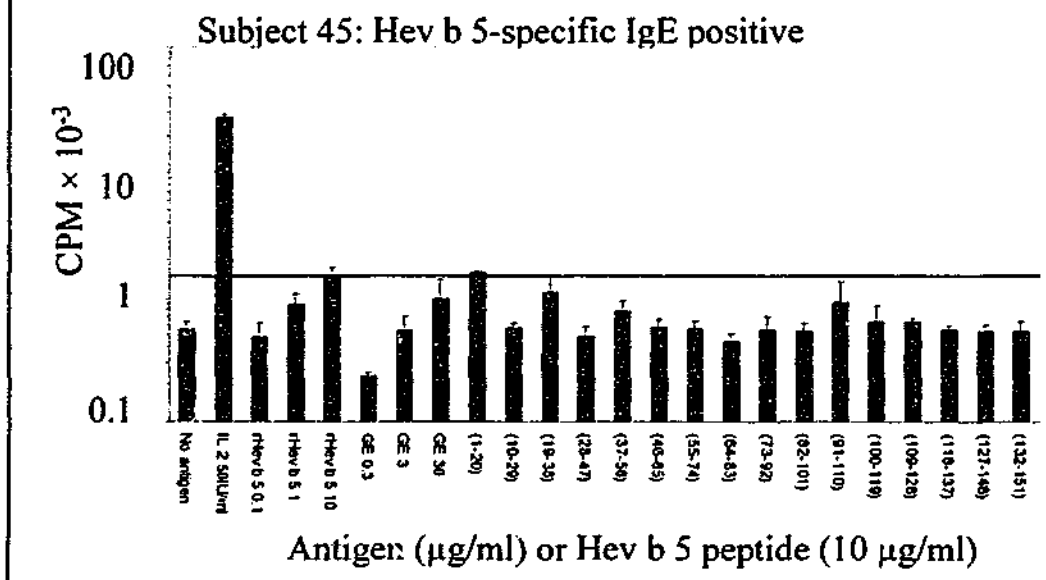


Figure 5.6A Proliferation assays to Hev b 5 peptides in latex allergic subjects mounting one or more positive peptide responses. Short term GE-specific TCL were stimulated for 48 hours with Hev b 5 peptides and control antigens as indicated. Proliferation was measured by ^{3}H-Thymidine incorporation. Significant proliferation (SI≥2.5) is indicated by the horizontal line and Hev b 5-specific IgE status is also shown.



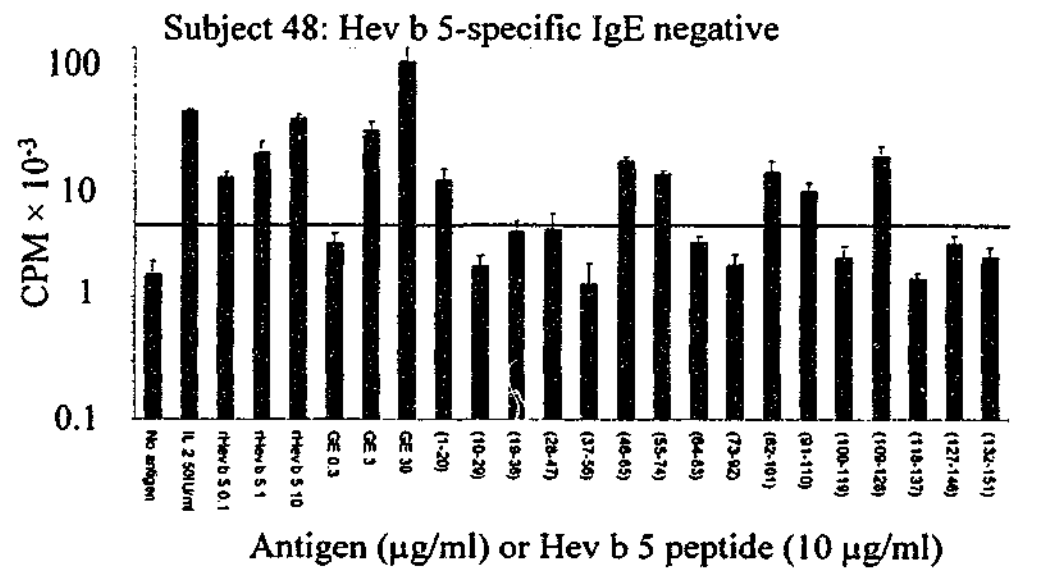
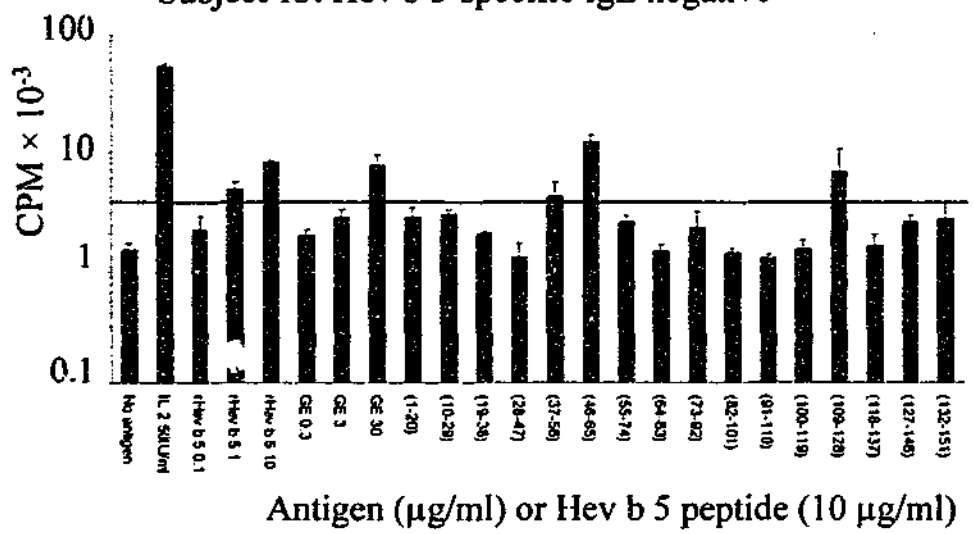
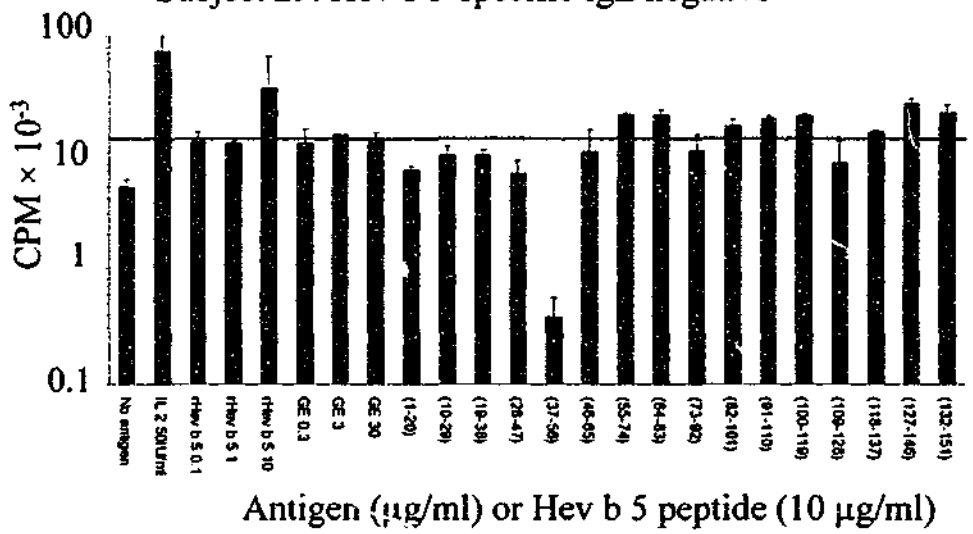
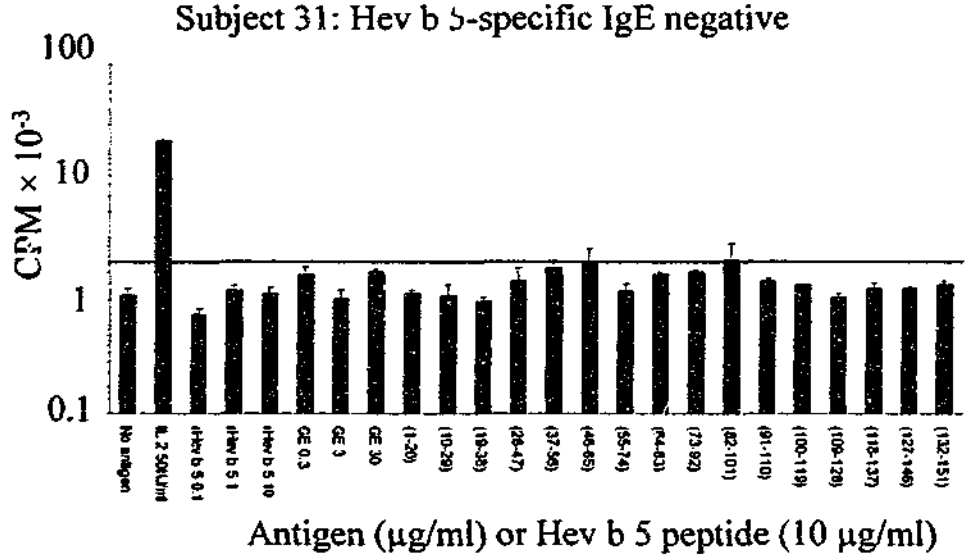


Figure 5.6B Proliferation assays to Hev b 5 peptides in latex non-allergic subjects mounting one or more positive peptide responses. Short term GE-specific TCL were stimulated for 48 hours with Hev b 5 peptides and control antigens as indicated. Proliferation was measured by ^{3}H-Thymidine incorporation. Significant proliferation (SI≥2.5) is indicated by the horizontal line and Hev b 5-specific IgE status is also shown.







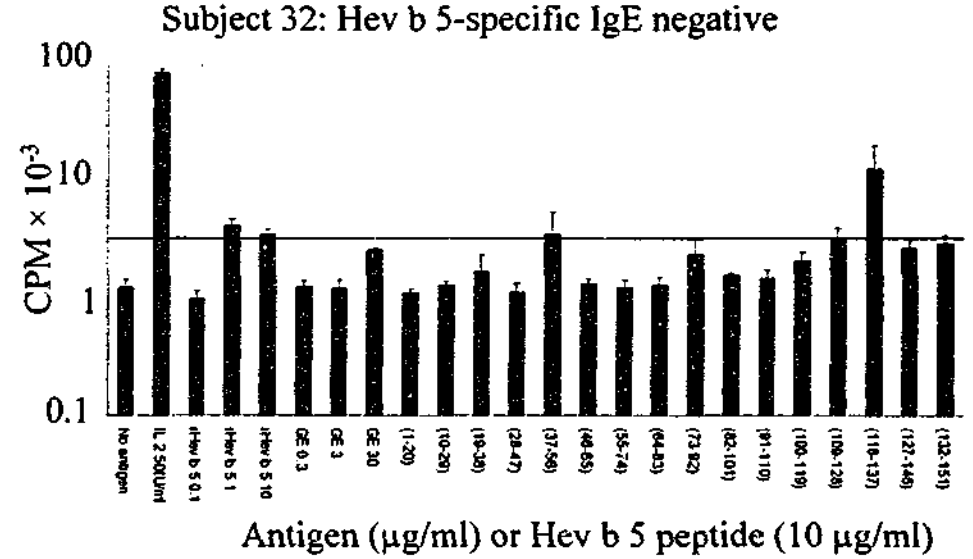
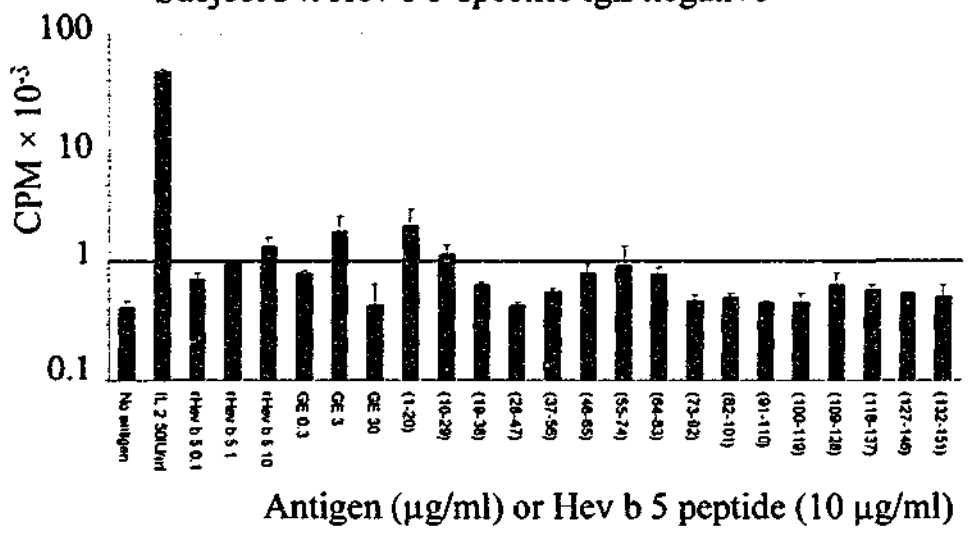
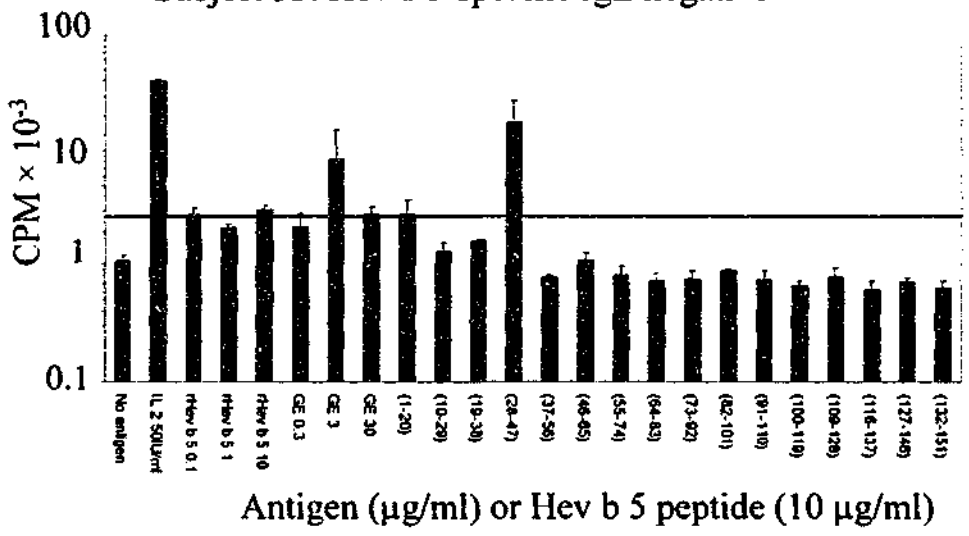


Figure 5.6B Proliferation assays to Hev b 5 peptides in latex non-allergic subjects mounting one or more positive peptide responses. Short term GE-specific TCL were stimulated for 48 hours with Hev b 5 peptides and control antigens as indicated. Proliferation was measured by ^{3}H-Thymidine incorporation. Significant proliferation (SI≥2.5) is indicated by the horizontal line and Hev b 5-specific IgE status is also shown.





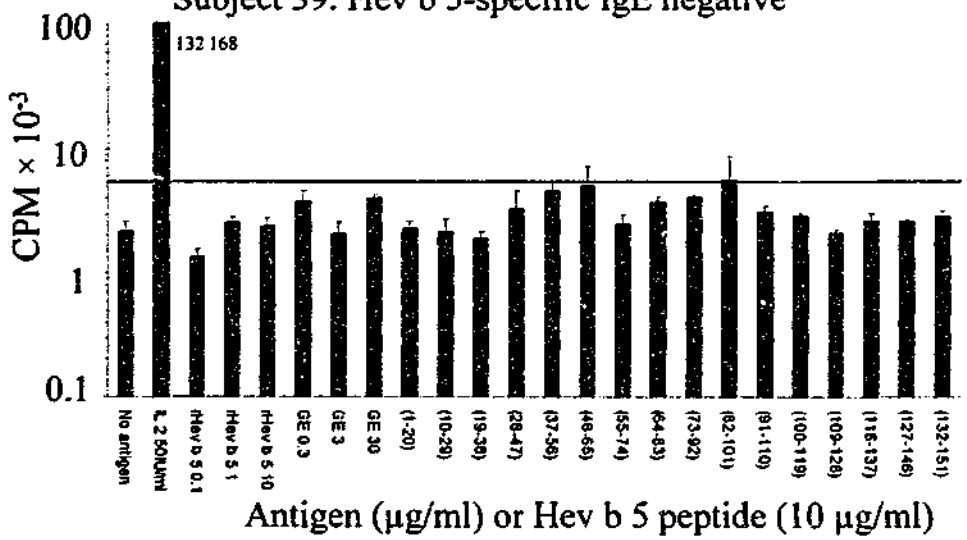


Figure 5.7 Summary of proliferation assays of short term TCL against Hev b 5 peptides

Raw data of proliferation assay for whole population presented in SI format. Significant proliferation (SI≥2.5) is indicated by blue shaded cells with bolded text. Latex allergic subjects are indicated by the cells shaded red in the subject ID number column.

ID no	(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)	(132-151)
	0.7	0.8	0.9	1.0	1.0	1.1	0.8	1.1	1.0	1.4	1.0	0.9	0.9	0.9	0.1	0.0
	NT															
							2.4				1.6	1.8	1.7	2.1	2.1	1.6
4	NT															
	0.7	2.3	0.7	1.7	1.3	1.7	0.6	1.3	1.2	0.3	0.9	1.7	1.4	0.3	0.5	
6	0.7	0.6	0.7	0.7	0.8	0.7	0.7	0.9	0.7	0.9	0.7	0.6	0.8	1.0	1.0	0.9
	1.8	1.7	1.1	1.1	0.8	1.6	1.7	1.4	1.0		1.7	1.0	1.2	0.9	0.9	1.4
				2.3					2.0					1.5	1.7	
	0.3	0.8	0.6	0.8	0.5	1.2	0.7	0.7	0.4	0.6	0.7	1.0	0.6	0.8	0.9	1.2
			1.0				1.3	1.4		2.3	1.6	1.2		2.4	1.2	1.4
	1.8	1.6	1.1				1.2	1.3	2.5	2.0	2.2	2.0			1.2	2.0
	1.1	1.1	1.0	1.1	1.0	1.1	1.1	1.5	1.1	1.4	1.2	1.3	1.3	1.2	1.4	1.6
13	1.9	2.0	1.4	0.8			1.7	1.0	1.5	0.9	0.8	1.0		1.1	1.7	1.8
			2.5		1.7		1.7	1.4		2.0	1.7	1.2		1.7		
	0.7	0.9	1.4	0.7				2.1	0.9	1.7	1.4	1.1	0.7	1.0	1.1	
	1.7	0.9	1.4	1.0	1.2	1.6	0.9	0.9	1.2	1.3	1.2	1.2	1.1	1.4	1.2	2.4
		0.8	1.1	1.5				1.5						2.3		
		2.0		2.4	2.1				2.2			1.9	1.7	2.2	2.0	1.7
	NT															
	1.0	0.8	0.8	0.9	0.7	1.1	0.9	1.0	0.9	0.9	0.9	1.1	1.0	0.9	1.0	1.2
	NT															
22	1.1	1.2	1.1	1.5	1.7	1.7	1.8	1.5	1.7	1.6	2.2	1.7	1.6	1.5	1.7	1.8
23	1.0	0.8	0.9	1.0	1.1	0.9	1.0	0.8	0.9	1.0	1.1	1.3	1.2	1.0	0.9	0.8
		1.7						1.4		1.7	1.2	1.5	1.0	1.2	1.6	1.7
		1.4	1.5	1.2	1.7			1.2	1.2		2.3	1.1		1.3		
	2.2	2.2	0.9	0.9	0.7	0.9	1.0	0.7	1.1	1.1	0.9	1.0	0.0	0.0	0.0	0.0
27	0.9	1.2	1.1	0.7	1.0	1.0	0.8	1.1	1.1	0.7	1.1	1.2	0.8	1.1	1.4	1.5
	0.8	1.2	1.4	1.6	1.1	2.3	1.3	1.2	1.0	1.1	1.4	1.3	1.2	1.1	1.4	1.4
29	1.4	1.9	1.9	1.3	0.1	2.1			2.1				1.6			
	2.4	1.4	1.7	2.2	0.6	2.3	1.6	0.9	0.6	0.6	1.0	0.8	0.5	0.6	0.6	0.9
31	1.0	1.0	0.9	1.5	2.1	2.4	1.1	1.7	1.8		1.4	1.3	1.0	1.2	1.2	1.3
32	0.9	1.0	1.3	0.9		1.1	1.0	1.1	1.9	1.3	1.2	1.7			2.1	2.3
33	0.8	0.9	0.6	0.8	1.0	0.6	1.1	0.9	0.7	0.8	0.7	0.6	1.0	1.3	1.0	0.6
34			1.6	1.0	1.4	2.0	2.3	1.9	1.2	1.2	1.1	1.1	1.6	1.5	1.3	1.3
35		1.2	1.5		0.7	1.0	0.8	0.7	0.7	0.8	0.7	0.6	0.7	0.6	0.7	0.6
	1.8	0.9	1.7	0.8	1.1	0.9	0.9	0.7	1.0	0.9	1.4	1.0	1.0	0.9	0.8	0.9
37	1.3	0.8	0.7	0.7	0.7	0.6	0.3	0.7	0.7	0.5	0.6	0.7	0.7	0.8	0.5	0.8
	0.8	0.8	0.5	0.0	1.3	1.2	0.8	0.7	0.7	0.6	0.7	0.7	0.8	0.6	0.7	0.5
39	1.1	1.0	0.9	1.5	2.1	2.3	1.1	1.7	1.9		1.5	1.4	1.0	1.2	1.2	1.3
	NT															
41	NT															
42	0.9	1.0	1.3	1.0	0.9	1.0	1.5	1.3	0.9	1.6	1.4	1.0	0.8	1.0	1.0	0.8
	NT															
44	1.6	2.1	1.4	1.1	0.8	1.2	1.5	0.5	1.1	0.7	0.7	0.8	0.6	0.8	0.8	0.4
		1.0	2.0	0.9	1.4	1.1	1.0	0.8	1.0	1.0	1.7	1.2	1.2	1.0	1.0	1.0
	1.1	1.1	1.0	1.3	0.6	1.1	0.8	0.7	0.7	0.7	0.5	0.6	0.5	0.5	0.6	0.6
	0.6	0.7	0.6	0.6	0.4	0.5	0.5	0.6	0.6	0.5	0.6	0.7	0.6	0.5	0.6	0.7
		1.2	2.2	2.3	0.8			1.8	1.2			1.4		0.9	1.7	1.4
49	0.5	0.7	0.9	0.7	0.7	0.8	1.0	0.8	0.8	1.0	0.9	1.1	0.9	1.0	0.8	0.8
50	NT															
	0.7	0.8	0.5	0.8	0.4	0.6	0.5	0.6	0.7	0.6	0.2	0.5	0.4	0.5	0.5	0.5

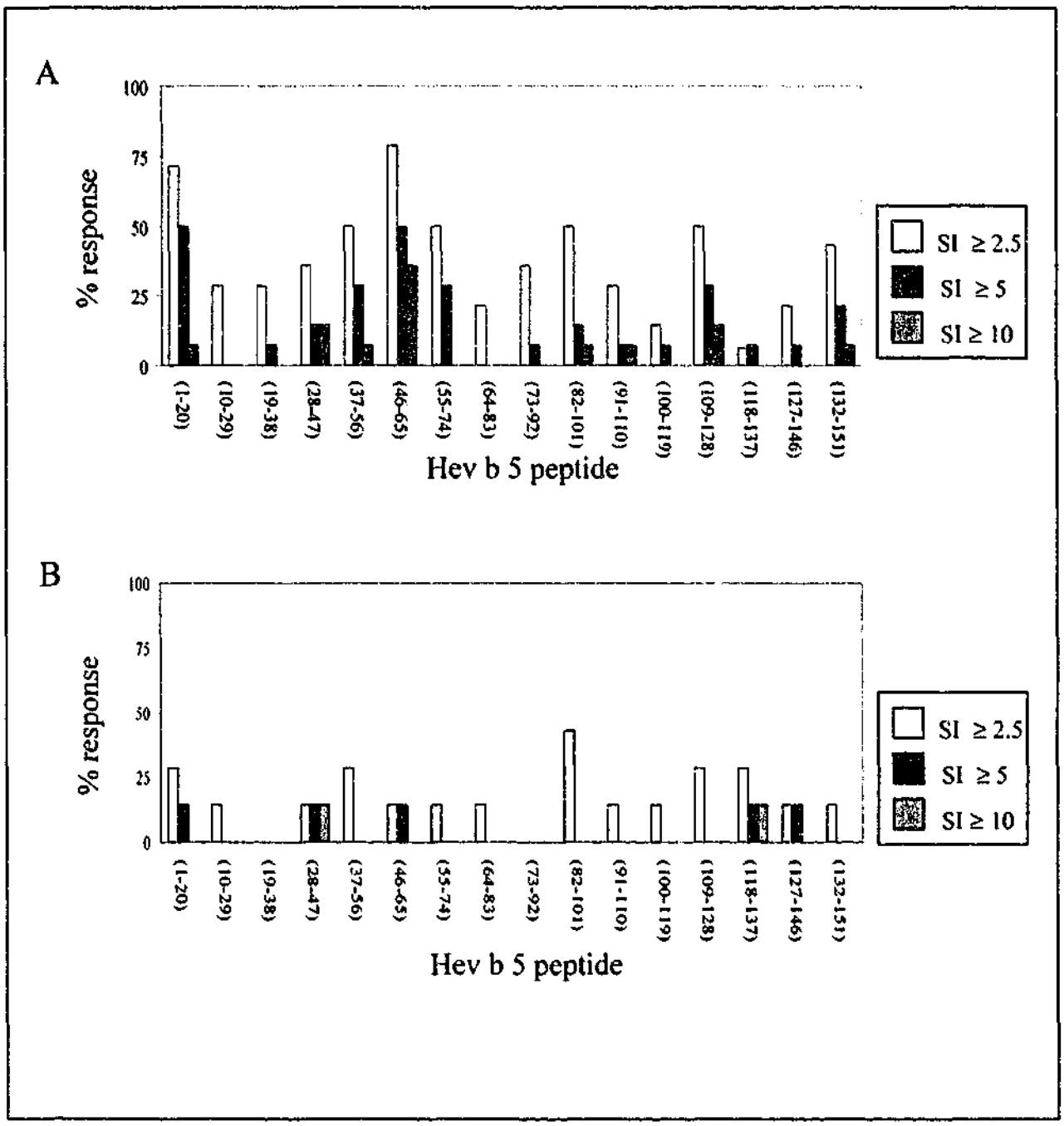


Figure 5.8 Frequency of individual Hev b 5 peptide responses

Frequency histogram of individual peptide responses among latex allergic peptide responders (n=14; A) and latex non-allergic peptide responders (n=7; B). A positive peptide response was defined as a stimulation index (SI) of greater than 2.5 (ratio of proliferation of cells with peptide antigen against proliferation of cells with no peptide antigen).

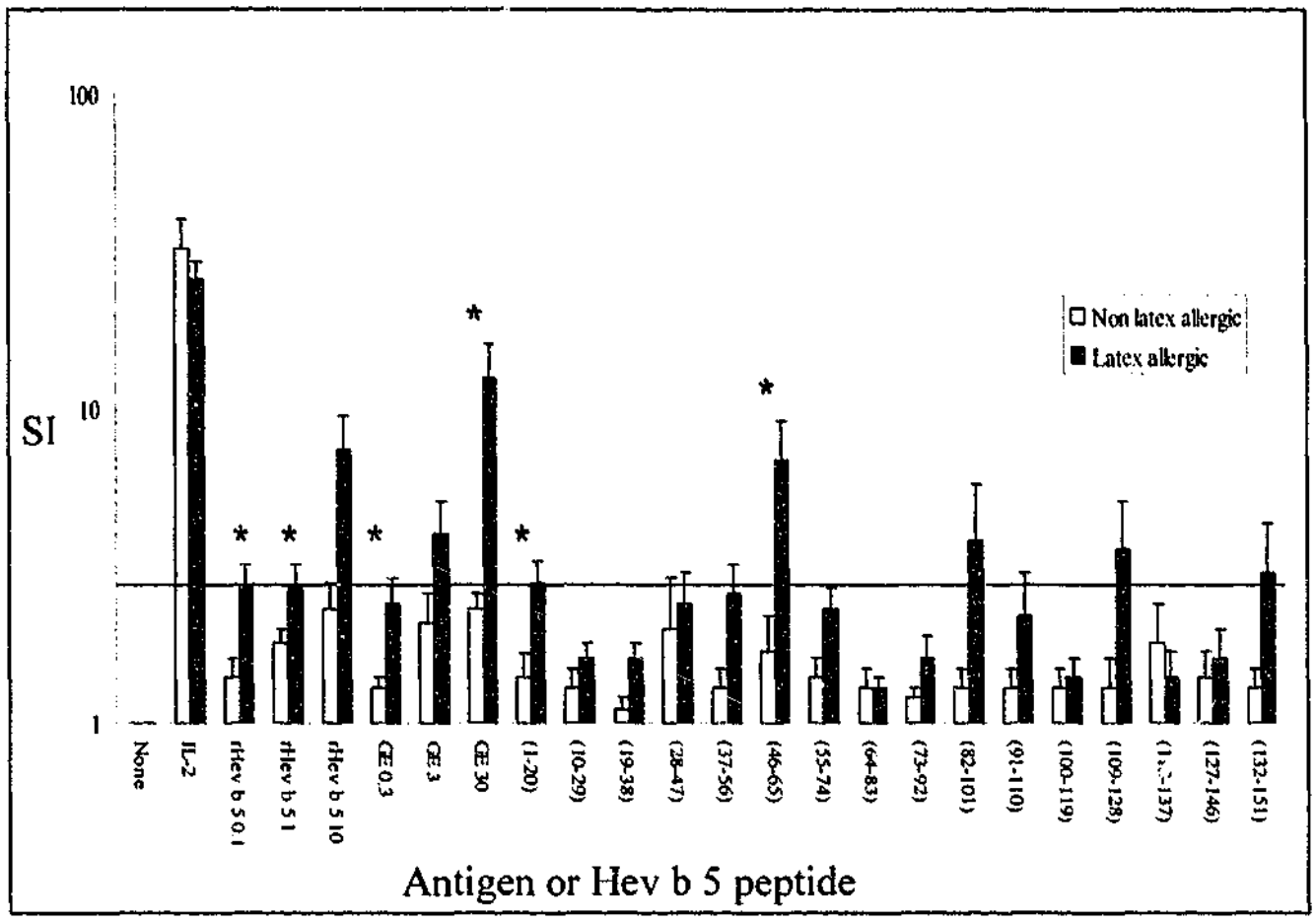


Figure 5.9 Comparison of mean T cell proliferative responses to Hev b 5 peptides in latex allergic and non latex allergic groups.

Summary graph of T cell stimulation index (SI) data of latex allergic (n=27) and latex non-allergic subjects (n=15) in response to stimulation with Hev b 5 peptides and control antigens. Hev b 5 peptides are at a concentration of 10 μg/ml and IL-2 at 50 IU/ml. Bars represent means +1 SEM. The horizontal line represents SI = 2.5. Statistically significant ($p<0.05$, two tailed t test) differences in SI between latex allergic and non latex allergic groups are notated with an asterisk.

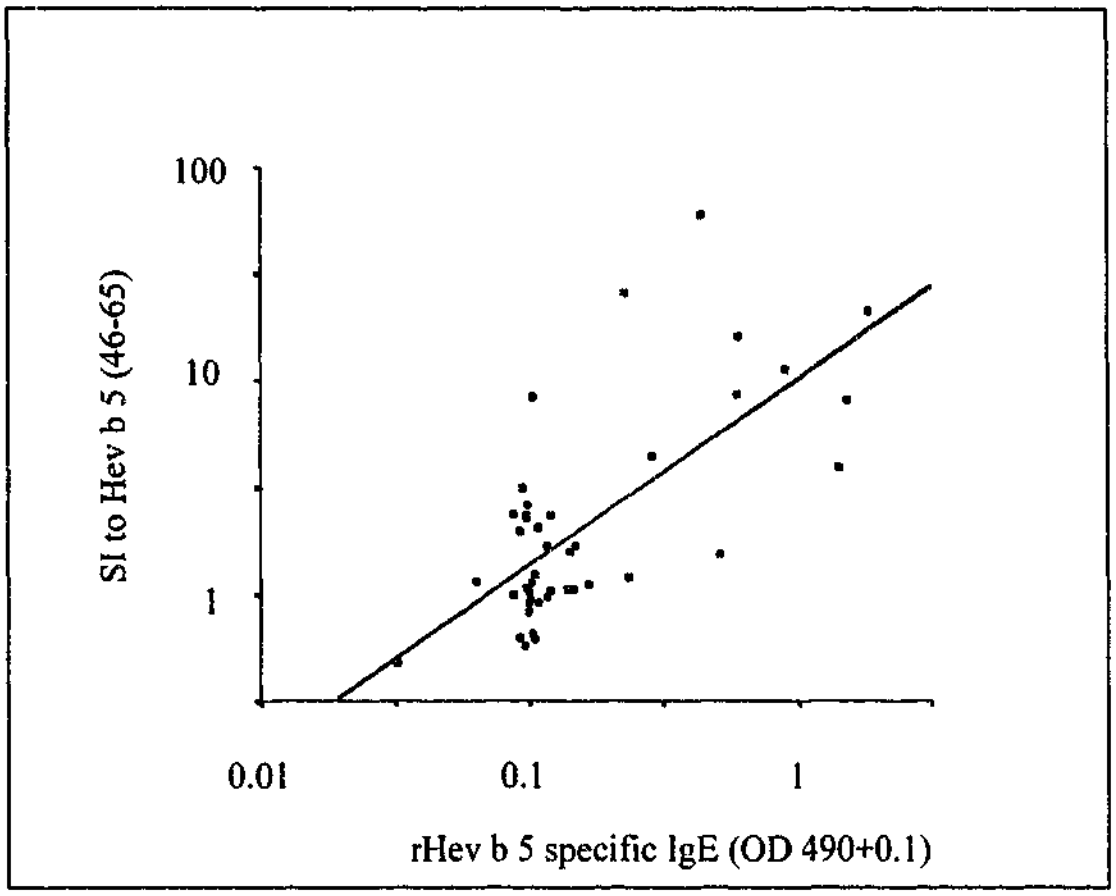


Figure 5.10 Correlation between proliferation to Hev b 5 (46-65) and rHev b 5-specific IgE

There was a moderately strong and statistically significant correlation between log {SI to Hev b 5(46-65)} and log {(rHev b 5-specific IgE (OD 490+0.1)}; R^2=0.484, p<0.001.

lowest antigen concentrations of both rHev b 5 and GE was a significant discriminator between the latex allergic and latex non-allergic groups (p=0.02 and 0.049 respectively, 2 tailed t test; Figure 5.9)

In general, subjects with the highest proliferative responses also had high levels of rHev b 5-specific IgE. There was a moderately strong correlation between log (rHev b 5 specific IgE) and log {SI to peptide Hev b 5 (46-65)}: R^2 = 0.48, p<0.001 (Figure 5.10). Interestingly, there was no such correlation for the other dominant peptide, Hev b 5 (1-20): R^2 = 0.002.

5.2.4.3. Hev b 5 peptide-induced T cell cytokine responses

Cytokine assays were performed for IL-5 and IFN-γ at 48 hr by capture ELISA (Figures 5.11 and 5.12). For subject 17, all peptides were assayed for IL-5 and IFN-γ, indicating an IL-5 - dominant pattern of cytokine secretion in this highly latex-allergic subject (Tables 5.11 and 5.12). To reduce the total number cytokine assays, only the highest 3 proliferating peptides and of 1 non-proliferating peptide, in addition to the positive (IL-2, rHev b 5 10 μg/ml, GE 30 μg/ml) and negative (no antigen) controls, were assayed for IL-5 and IFN-γ, for each of the subjects who mounted a positive proliferative response to one or more Hev b 5 peptides. These results indicated that there was a significantly increased IL-5 secretion for Hev b 5(46-65) and Hev b 5(109-128) only (p = 0.02 and 0.024 respectively, Mann Whitney U) comparing latex allergic and latex non-allergic subjects. For all other peptides and the positive and negative controls, there was no significant difference in IL-5 secretion. In addition, Hev b 5(46-65) showed the highest level of IL-5 secretion of any peptide (Figure 5.13), and IL-5 secretion was correlated significantly with proliferation (R^2=0.49, p = 0.01, Pearson; Figure 5.14A). There was a lesser, and non-significant correlation between T cell IL-5 secretion in response to Hev b 5(46-65) and rHev b 5-specific IgE (R^2= 0.30, p = 0.065, Pearson; Figure 5.11B). The amount of IL-5 secreted by non-allergic subjects on stimulation with

Figure 5.11 IL-5 secretion by Short term TCl in response to Hev b 5 peptides

Short term GE-specific TCL were stimulated with Hev b 5 peptides and supernatants collected at 48 hours. IL-5 levels were measured by ELISA for the 3 Hev b 5 peptides causing the greatest T cell proliferation for each subject that mounted a positive peptide response, and one control peptide that induced no proliferation (cell outline in bold). For subject 17, all peptide cytokine responses were asssayed. Results are expressed in pg/ml and represent means of duplicate values with background subtracted. Blank cells indicate the cytokine secretion was not assayed. Cytokine values between 0 and 1 inclusive have been assigned an arbitrary value of 1. Cells shaded yellow indicates peptides inducing an overall TH0 cytokine profile, those blue TH1, while pink indicates a TH2 cytokine profile.

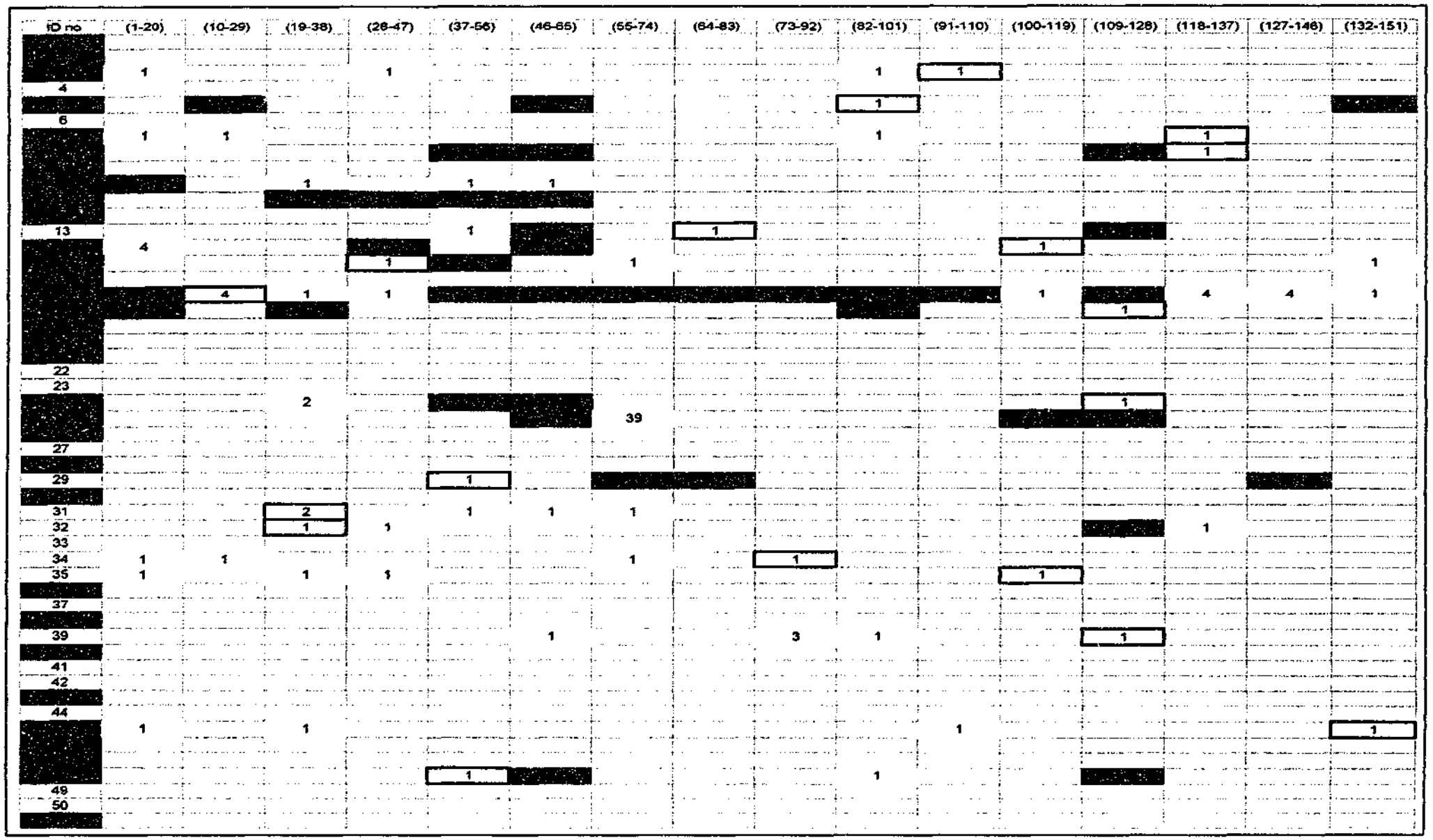
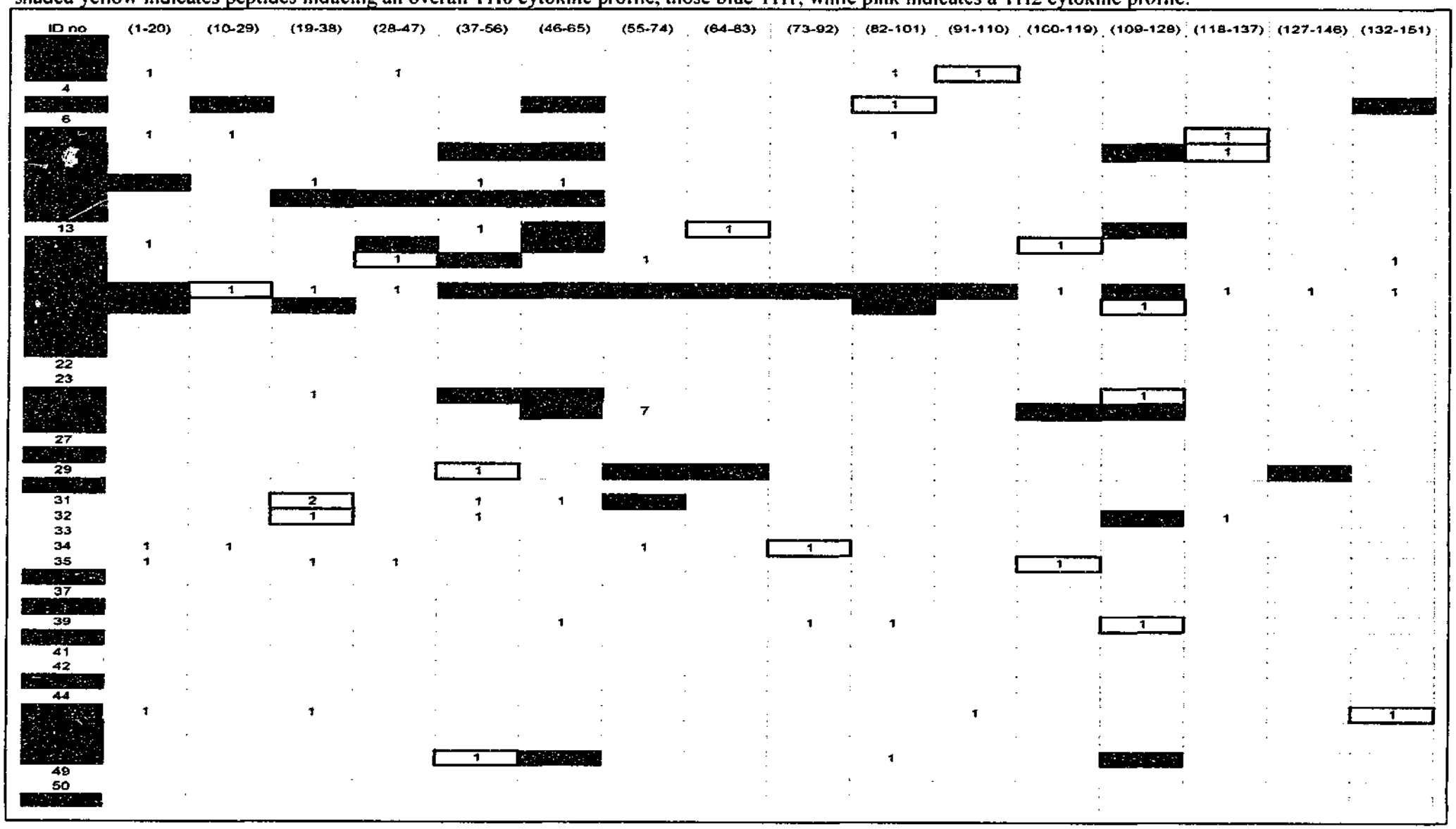


Figure 5.12 IFN-γ secretion by short term TCL in response to Hev b 5 peptides

Short term GE-specific TCL were stimulated with Hev b 5 peptides and supernatants collected at 48 hours. IFN-γ levels were measured by ELISA for the 3 Hev b 5 peptides causing the greatest T cell proliferation for each subject that mounted a positive peptide response, and one control peptide that induced no proliferation (cell outline in bold). For subject 17, all peptide cytokine responses were asssayed. Results are expressed in pg/ml and represent means of duplicate values with background subtracted. Blank cells indicate the cytokine secretion was not assayed. Cytokine values between 0 and 1 inclusive have been assigned an arbitrary value of 1. Cells shaded yellow indicates peptides inducing an overall TH0 cytokine profile, those blue TH1, while pink indicates a TH2 cytokine profile.



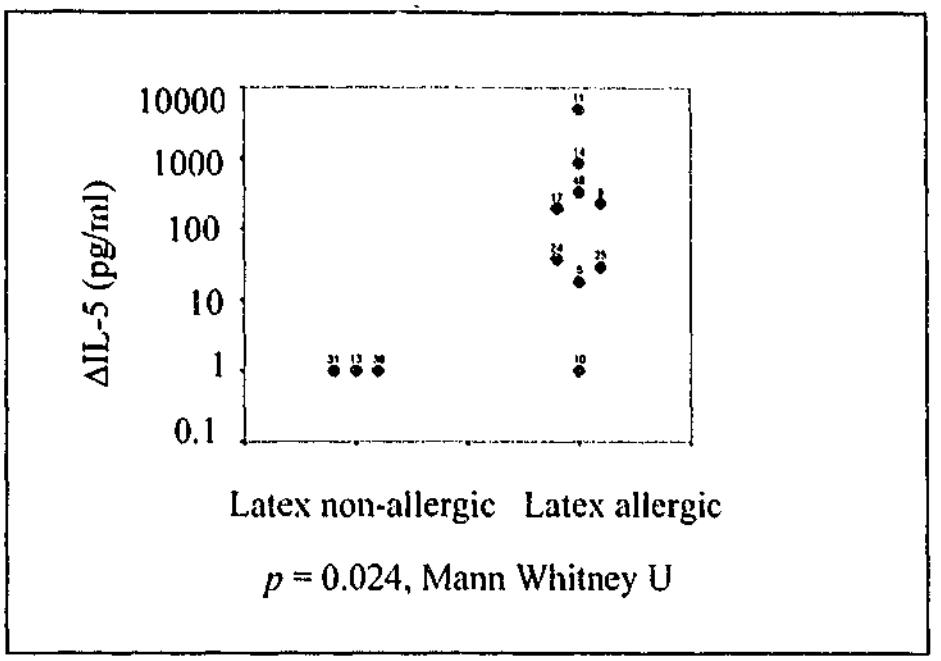


Figure 5.13 Short term TCL IL-5 response on stimulation with Hev b 5 (46-65)

Short term TCL were stimulated for 48 hours with rHev b 5(46-65) at 10 µg/ml. Supernatants were harvested and IL-5 levels assayed by capture ELISA. Data points are means of duplicates with no-antigen (background) IL-5 levels subtracted and are marked with subject number.

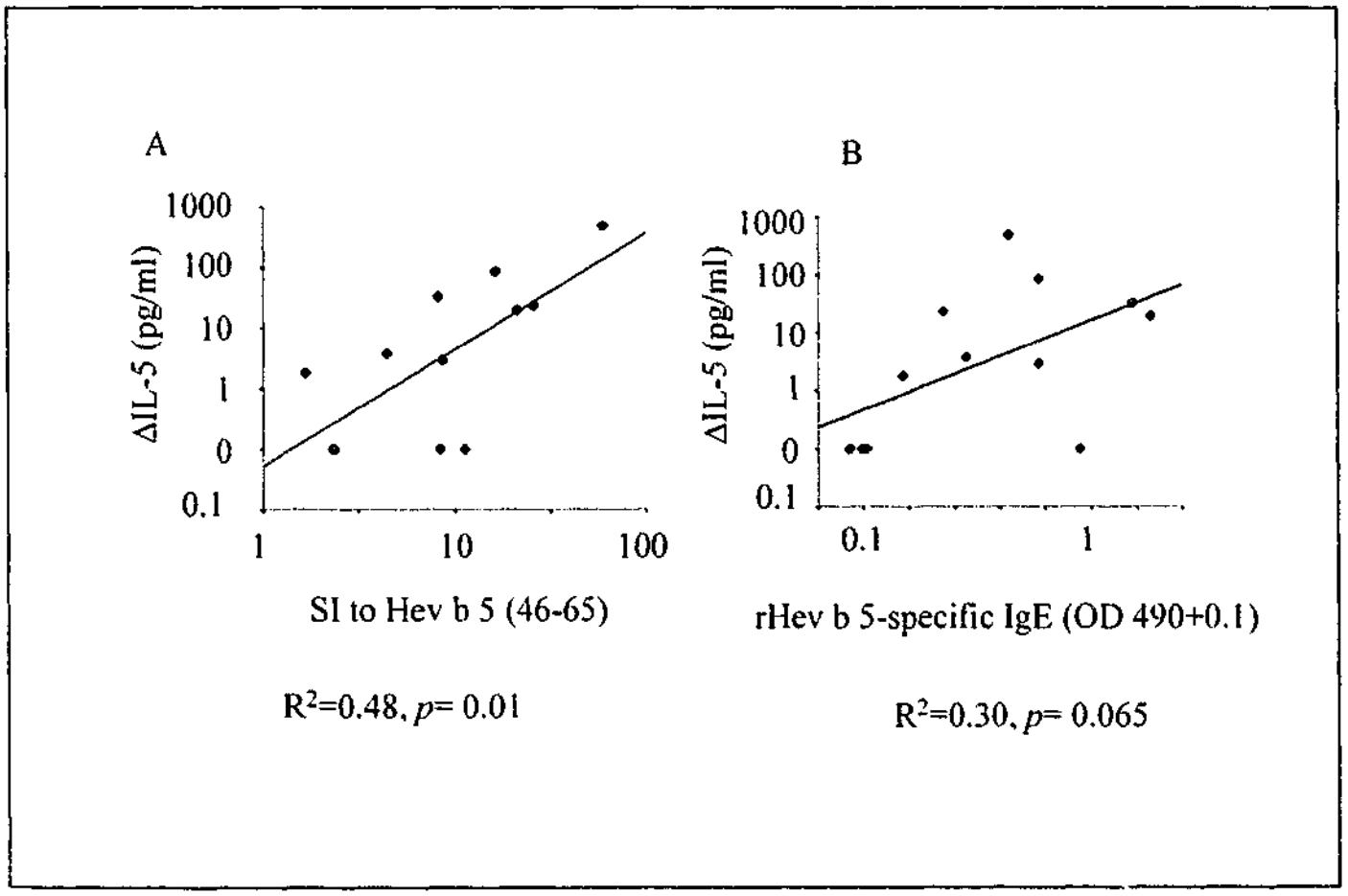


Figure 5.14 Correlations between T cell ΔIL-5 secretion on stimulation with Hev b 5 (46-65) and T cell proliferation in response to Hev b 5 (46-65; A) or rHev b 5-specific IgE (B).

Hev b 5 peptides was generally low. With regard to IFN-γ, there was no significant difference in the levels of IFN-γ secreted on stimulation with Hev b 5 peptides or any of the control antigens when comparing latex allergic and latex non-allergic subjects and levels were generally low (Figure 5.12). T cell phenotype was arbitrarily defined according to the ratio of IL-5 to IFN-γ based on the ratios used by Bohle and colleagues for ratios of IL-4 to IFN-γ (Bohle *et al.*, 2000). The ratio of IL-5 to IFN-γ indicated that Hev b 5(46-65) was the strongest TH2 (IL-5: IFN-γ ratio ≥ 5)-inducing peptide, while in comparison Hev b 5(1-20) elicited generally low cytokine responses that were a mixture of TH1 (IL-5: IFN-γ ratio < 1), TH0 (IL-5: IFN-γ ratio ≥ 1 and < 5) and TH2, despite the relatively strong proliferative responses (Figures 5.11, 5.12).

5.3. Discussion

This chapter has shown that generating oligoclonal TCL by driving PBMC with a GE with high levels of nHev b 5 (see Chapter 6) elicits a strong Hev b 5-specific TH2 response among latex allergic subjects and also strong TH2 antigen-specific Hev b 5 peptide responses. Moreover, dominant T cell-reactive regions of the Hev b 5 molecule have been identified which may facilitate the development of novel SIT for latex allergy.

There were no previous data on PBMC proliferation to rHev b 5. Studies of PBMC proliferation to the other latex allergens Hev b 1,2,3,6 and 7 (Raulf-Heimsoth *et al.*, 1998; Johnson *et al.*, 1999) had shown that latex non-allergic individuals did not mount a significant proliferative response to the latex allergens (Johnson *et al.*, 1999), though for Hev b 1, 25% of latex non-allergic individuals did show proliferation to this latex allergen (Raulf-Heimsoth *et al.*, 1998). The current investigation of PBMC responses to Hev b 5 has shown that proliferation is significantly less among latex non-allergics, however some individual latex non-allergic subjects do mount a significant proliferative response to rHev b 5. Some investigators using other

allergen systems such as HDM have suggested that most individuals, regardless of sensitisation state will mount PBMC responses to allergens on stimulation (Upham, 1997). What the current study indicates is that PBMC proliferation to rHev b 5 has poor specificity and therefore little clinical utility for the diagnosis of latex allergy, in keeping with previous work with crude latex antigens (Turjanmaa *et al.*, 1989).

Analysis of PBMC cytokine secretion indicated that there was significantly greater IL-5 secretion on stimulation with rHev b 5 among latex allergic compared with latex non-allergic individuals. In contrast, levels of IFN-γ did not differ significantly between allergic and non-allergic individuals, however there was a non-significant trend toward increased IFN-γ production with rHev b 5 stimulation in the latex non-allergic group (p=0.056). Other investigators have noted increased allergen-specific IFN-γ secretion by PBMC among individuals sensitised to allergens (O'Brien *et al.*, 2000). The results presented here would suggest that this phenomenon is less marked than an increase in allergen-specific IL-5 production as noted by other investigators (Till *et al.*, 1997; Lagging *et al.*, 1998).

The T cell epitope mapping investigations showed that it was possible to elicit a Hev b 5 peptide-specific response in around 50 percent of subjects, whether latex allergic or non-allergic. This is in line with previous studies using other latex allergens (Bohle *et al.*, 2000). The dominant peptides identified were Hev b 5(1-20) and Hev b 5(46-65) on frequency criteria among the latex allergic subjects. The dominant peptide Hev b 5(46-65) was also identified in De Silva's study (de Silva *et al.*, 2000). Interestingly, the peptide Hev b 5(1-20) was not, only causing significant proliferation in 1 out of 6 of the latex allergic subjects. This difference may reflect the different stimulus to the cell lines (GE rather than LAL and rHev b 5/MBP). It is interesting to speculate as to whether the MBP which is connected at the C terminus of the molecule, i.e. adjacent to Hev b 5 (1-20), caused interference in

antigen presentation or processing during the 3rd week during which cells were driven with Hev b 5/MBP in de Silva's study (de Silva *et al.*, 2000). Apart from this discrepancy, the current study has validated de Silva's study, showing Hev b 5(46-65) to be dominant in a larger group of latex allergic subjects. Interestingly, Hev b 5(1-20) was also noted to be a dominant T cell epitope in the BALB/c mouse in a study which utilised the same peptides as the current study (Slater *et al.*, 1999). Given the high percentage frequency of response to Hev b 5(1-20) and Hev b 5(46-65), these peptides would be critical to retain in any peptide vaccine approach. There was no dominant peptide among the latex non-allergic subjects and any significant proliferation was generally weaker than in the latex allergic group. This is likely to reflect the greater precursor frequency of primed memory T cells in the PBMC of the latex allergic individuals (Nakamura *et al.*, 2001), and therefore a more vigourous *in vitro* response than the non-allergic group. Additional mechanisms which may play an important role in vitro include IgE-mediated antigen presentation (Maurer *et al.*, 1995), which may partially explain the greater proliferative responses seen among subjects with a high rHev b 5-specific IgE. When compared with Hev b 5(1-20), Hev b 5(46-65) generated stronger proliferative responses among latex allergic individuals, induced greater IL-5 secretion which was significantly correlated with rHev b 5-specific IgE secretion. Taken together, these results indicate that Hev b 5(46-65) is the dominant peptide within Hev b 5 in generating TH2 specific T cell responses. The exact sequence of the T cell epitope contained within this peptide will require further studies of fine epitope mapping that are beyond the scope of this thesis, such as has been undertaken for Lol p 5 (Burton *et al.*, 1999). In addition, it would be useful to examine MHC Class II haplotypes for the whole population, which was not examined in this study. It is likely, as for other allergens such as ryegrass pollen, that in latex allergic subjects there are different MHC molecules that can present Hev b 5 peptides, so called promiscuity of peptide presentation (de Lalla *et al.*, 1999). The dominant peptide Hev b 5(46-65), is also of interest as it shares 60% sequence identity with a

protein from the parasite *Strongyloides stercoralis* (Ramachandram *et al.*, 1998), suggesting a common motif which induces strong IL-5 secretion and TH2 responses. Also of importance is the knowledge that a dominant peptide such as Hev b 5(46-65) through increased immunogenicity may be a superior tolerogen and therefore potentially useful for SIT in Hev b 5-reactive, latex allergic subjects. There is evidence from mouse models that a dominant peptide can induce tolerance to a whole allergen molecule, so-called intramolecular suppression (Hoyne *et al.*, 1997). However, a broader approach for peptide SIT may be required, such as has been used for the major cat allergen Fel d 1, where an equimolar mixture of Fel d 1 peptides has been used (Oldfield *et al* , 2001).

6. Analysis of latex extracts using monoclonal antibodies and human IgE

6.1. Background

Latex allergy is characterised by sensitivity to the protein fraction of natural rubber latex (NRL) used in the production of gloves and other medical products (Poley and Slater, 2000). The predominant cause of sensitisation and symptoms amongst HCW is the use of latex gloves (Sussman *et al.*, 1998), but much previous research has focussed on crude latex sap extracts such as non-ammoniated latex (NAL) and low-ammoniated latex (LAL). The effects of the glove manufacturing process on the immunogenicity of NRL and nHev b 5 are poorly understood. Previous studies have measured allergenicity of latex gloves using inhibition ELISA using serum from latex allergic subjects (Yunginger *et al.*, 1994; Palosuo *et al.*, 1998). Although a sensitive technique, inhibition ELISA lacks standardisation because it depends on a pooled serum, the constituents of which may vary in time and between sites. Monoclonal antibody (mAb) based assays have the advantage of standardisation, and are available for a number of allergens such as house dust mite (Luczynska *et al.*, 1989) and cat allergens (Chapman *et al.*, 1988). A sandwich ELISA was described for the quantification of Hev b 1 (Raulf-Heimsoth *et al.*, 2000). Prior to the current study (Sutherland *et al.*, 2002) and a more recent study by a Finnish group (Palosuo *et al.*, 2002), there was no information regarding allergen quantification of Hev b 5 in NRL preparations. The results presented in this chapter describe the use of rHev b 5-specific mAbs to characterise and determine the relative amount of immunoreactive Hev b 5 in different NRL preparations and correlate this with the rHev b 5-specific serum IgE reactivity of latex allergic patients. In addition, the use of Hev b 5-specific mAbs is described in the detection of Hev b 5-bearing particles from latex gloves in

Halogen© immunoassays (Mitakakis *et al.*, 2002). Taken together, these data provide important information to guide allergen avoidance measures in the prevention and management of latex allergy.

6.2. Materials and Methods

6.2.1. Glove Extracts (GE)

Glove extracts were prepared as described previously (see section 2.2.1.3). For the Hev b 5 quantification experiments, 5 different commercially available gloves were used. For convenience these were designated G1 to G5. Gloves G1 and G2 were powdered, non-sterile latex gloves: G1 a laboratory utility glove (Uniglove, Malaysia), G2 a non-medical grade household disposable glove available from supermarkets in Australia (Handigloves, Ansell, Australia). Gloves G3, G4 and G5 were all sterile surgical gloves currently in use in our hospital: G4 was non-powdered (Nutex, Ansell, Australia) and G3 (Gammex, Ansell, Australia) and G5 (Profeel, Unimex, Malaysia) were powdered.

6.2.2. Human subjects

Five subjects who had been previously shown to recognise rHev b 5/MBP by immunoblotting and T cell proliferation assays (de Silva *et al.*, 2000) were utilised for the IgE assays. The strongest responder (subject 17) was used for the IgE inhibition ELISA experiments, while the sera of these rHev b 5/MBP responsive individuals were pooled for the IgE immunoblots (subjects 5, 8, 11, 14 and 17). A serum pool of latex-exposed, latex non-allergic subjects was used as a negative control serum in the IgE immunoblots (subjects 32, 35, 37, 42, 49).

6.2.3. Inhibition ELISA for mAbs and human IgE

Dilution curves with purified Hev b 5 specific mAbs and latex allergic HCW IgE were performed to determine the optimal concentrations; 0.017 µg/ml for mAb 6F6, and 1:20 for HCW IgE. Inhibitors were added and incubated with antibody/sera at 37 °C for 1 hr prior to addition to the assay plate. The GE inhibitors were used neat or diluted

1:10 in PBS before addition to an equal volume of the diluted human sera or mAb, giving a final dilution of 1:2 or 1:20 of the extract. Final effective protein concentrations therefore were for G1 274 µg/ml and 27.4 µg/ml, G2 370 µg/ml and 37 µg/ml or, where the protein concentration was beneath the levels of detection, GE inhibitors were expressed as dilutions of 1:2 and 1:20 (Fig. 6.2B, Table 6.1). The NAL and LAL extracts were similarly diluted in PBS before addition to the sera or mAb, giving final effective concentrations for both of 100 µg/ml and 10 µg/ml. Ryegrass pollen extract was used as a negative control protein at a final effective concentration of 100 µg/ml.

In addition, to ensure that observed inhibitions were specific, a control ELISA was performed using ryegrass pollen extract to coat the plates (10 µg/ml) and mAb A1, a Lol p 1-specific mAb of isotype IgG1, for detection (Smart *et al.*, 1983).

6.2.4. Analysis of bronchoscopy suite aero-allergen samples using Halogen® assays

These were performed according to previously published protocols using Hev b 5-specific mAb 6F6 generated by the author (Sutherland *et al.*, 2002) using previously published protocols for the Halogen® assay (Graham *et al.*, 2000). This work was performed principally by Dr Theresa Mitakakis from the Institute of Respiratory Medicine, Sydney, Australia. The method protocol is fully described in the published manuscript (Mitakakis *et al.*, 2002), filed following the references, and the author gratefully acknowledges the kind collaboration of Dr Mitakakis and Dr Euan Tovey, also from the Institute of Respiratory Medicine, Sydney, Australia.

6.3. Results

6.3.1. Analysis of latex GE using Western blotting

The immunoblot reactivity to GE was strong when probed with a serum from latex allergic subjects, with a principal band at approximately 36 kDa and a smear of reactivity extending from 36 to 80 kDa. This IgE

reactivity was Hev b 5-specific (inhibitable) and a similar pattern of smeared reactivity was shown by the Hev b 5-specific mAbs (Figure 6.1) although the smear extended to a higher molecular mass of around 110 kDa. Strong reactivity to the glove extract was observed despite a low protein amount (5 µg per lane) of the glove extract being loaded on the gel and almost no protein being visualised on CBB stain, though some protein was observed with silver staining (Figure 6.1).

6.3.2. Analysis of latex extracts using inhibition ELISA

The inhibition ELISA showed that rHev b 5 gave a strong dose dependent inhibition of antibody reactivity (Fig. 6.2A) with a strong correlation between the 2 standard curves (IgE and mAb6F6) R^2=0.94 (p<0.001; Figure 6.3). The IgE ELISA was more sensitive with a limit of sensitivity (statistically significant difference from 0 inhibitor) of 0.2 µg/ml rHev b 5; the mAb inhibition ELISA sensitivity was 0.8 µg/ml rHev b 5. The high protein powdered glove extracts gave relatively more inhibition even after adjusting for protein content than NAL or LAL (Fig. 6.2B and Table 6.1). Ryegrass pollen extract used as a negative control gave no inhibition. Similarly, the high quality surgical gloves with undetectable total protein, both powdered and non-powdered also gave little or no inhibition, though there was a trend towards G5 having a greater nHev b 5 content than G3 or G4. Natural and recombinant latex proteins gave minimal or no inhibition of a ryegrass pollen specific mAb in a control ryegrass pollen-specific ELISA (Figure 6.4).

6.3.3. Detection of Hev b 5-bearing particles from latex glove powder using Hev b 5-specific Halogen immunoassays

Examples of Halogen® staining of latex glove powder are shown in Figure 6.5. As can be seen, the majority of bioabsorbable cornstarch particles from the Livingstone glove, bear Hev b 5 allergen (Figure 6.5A). The Uniglove powdered latex glove is also shown, indicating that this glove is powdered with talcum (Figure 6.5B). There is

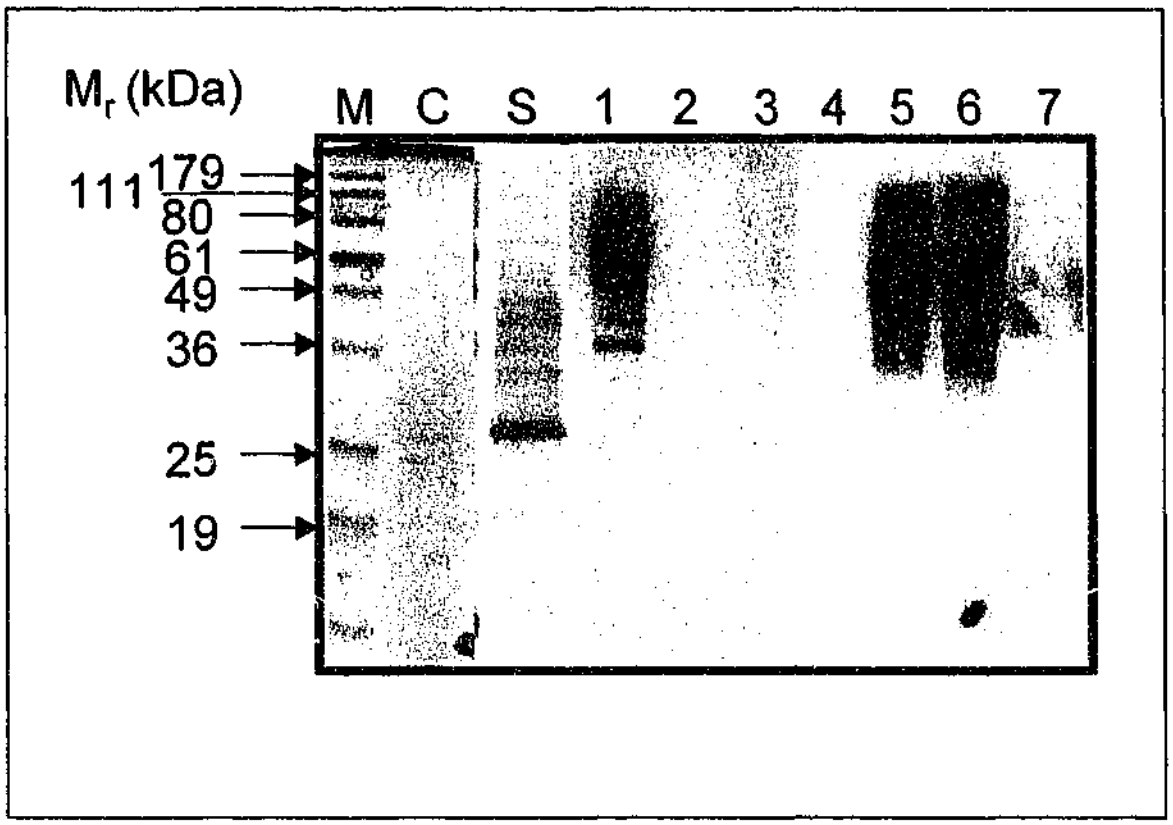


Figure 6.1 Inhibition immunoblot of latex GE with human IgE and mAbs

A high protein latex GE (Uniglove, Malaysia) was resolved by 12% SDS-PAGE and transferred onto NC for Western analysis. NC blots were probed for antibody binding with pooled sera from latex allergic patients (lane 1), pooled sera from latex allergic patients pre-incubated with 20 μg rHev b 5 per ml of diluted (1:5) serum pool (lane 2), pooled sera from non-allergic patients (lane 3), no serum control (lane 4), mAb 6A10 (lane 5), mAb 6F6 (lane 6) and mAb A1 isotype control (lane 7). Molecular mass markers, Coomassie brilliant blue-stained gel slices and silver-stained gel slices are denoted by M, C and S, respectively.

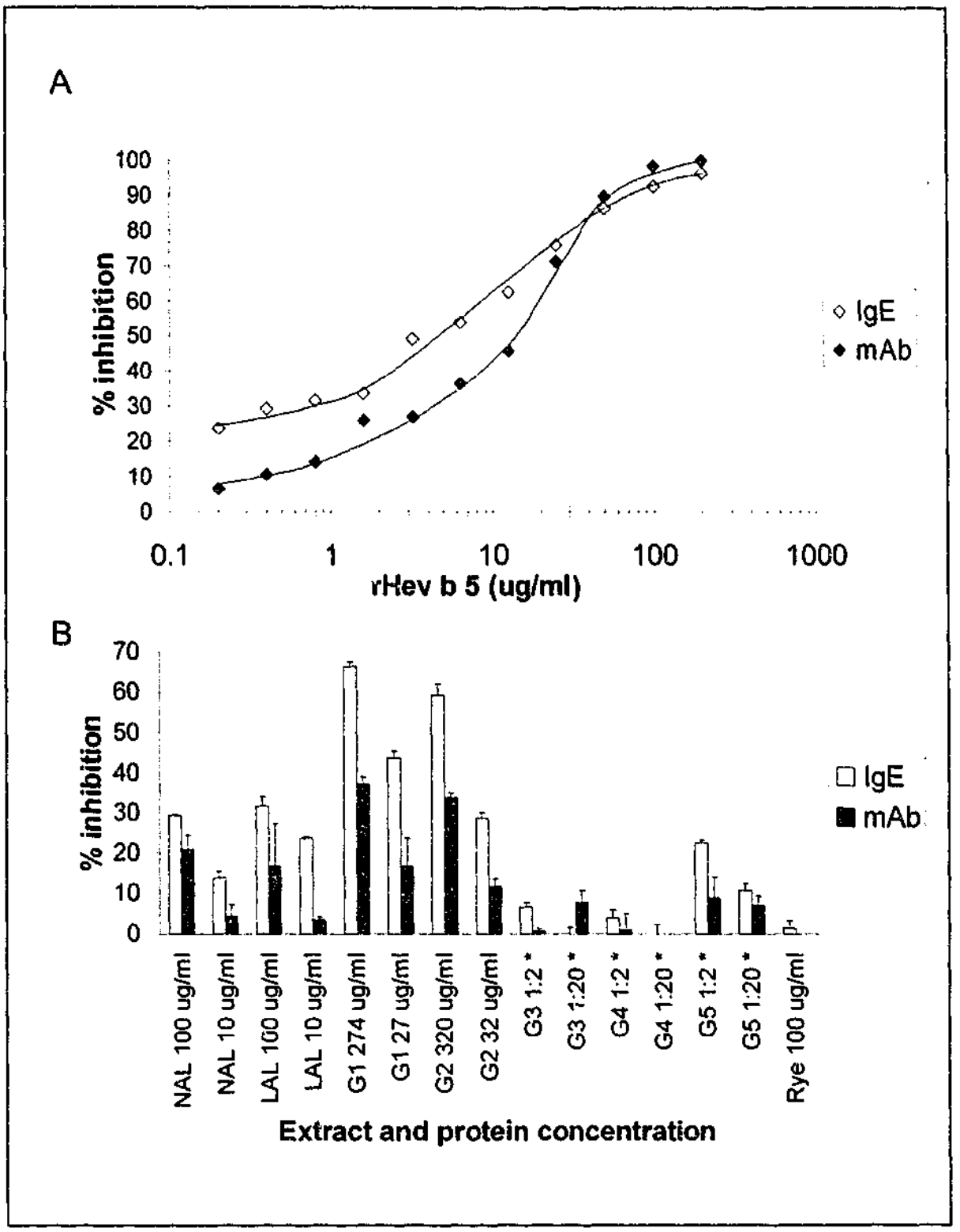


Figure 6.2 Inhibition ELISA analysis of rHev b 5 with serum IgE of latex allergic subject and mAb 6F6

Recombinant Hev b 5 was immobilised on an ELISA plate and human IgE/mAb 6F6 binding was inhibited with different amounts of rHev b 5 to generate standard curves (A) for the determination of rHev b 5 equivalents in the different latex extracts (B). Latex extracts used (as potential inhibitors of antibody binding to rHev b 5) included NAL, LAL, different glove extracts (GE; G1 and G2 are powdered non-sterile utility gloves, G3 and G5 are powdered sterile surgical gloves and G4 is a non-powdered sterile surgical glove) and ryegrass pollen extract (irrelevant allergen negative control). Measurements are means (+/-SEM) of triplicates. The sensitivity of the human IgE assay was 0.2 µg/ml and of the mAb assay was 0.8 µg/ml. For glove extracts (GE) where protein was undetectable, concentration is expressed as an extract dilution (*).

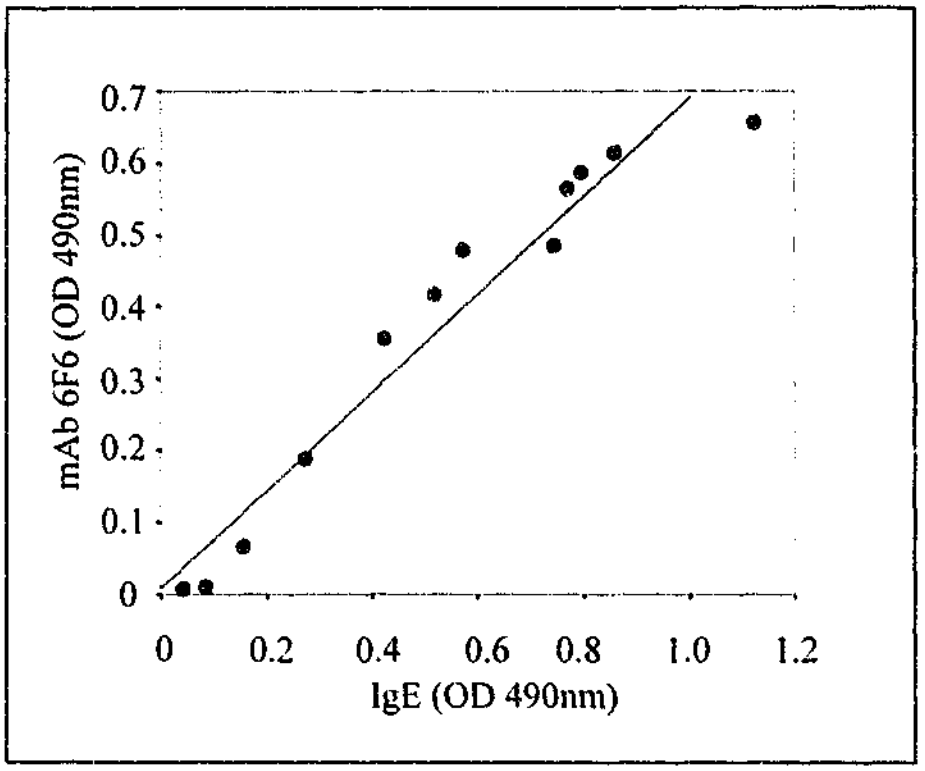


Figure 6.3 Correlation between mAb 6F6 and subject 17 IgE rHev b 5-specific inhibition assay standard curves

Standard curves inhibiting with rHev b 5 were compared with a Pearson test, yielding a strong correlation ($R^2 = 0.94$, $p < 0.001$).

Table 6.1 Relative Hev b 5 content of latex extracts

Latex preparation	Total protein concentration (ug/ml)	rHev b 5 equivalents by mAb ELISA (ug/ml)	rHev b 5 equivalents by IgE ELISA (ug/ml)	Mean % rHev b 5 equivalents by mAb ELISA #	Mean % rHev b 5 equivalents by IgE ELISA #
G1	274	6.5	12.5	3.0	7.5
	27.4	1.0	2.8		
G2	320	5.0	8.0	1.6	2.2
	32	†	0.6		
G3	*	†	†	†	†
G4	*	†	†	†	†
G5	*	†	†	†	†
NAL	100	1.8	0.6	1.8	0.6
LAL	100	1.0	1.0	1.0	1.0

G1, G2 – powdered non-sterile utility gloves; G3, G4, G5 – sterile surgical gloves. Levels of total protein (*) or rHev b 5 equivalents (†) below limits of detection. Mean percentage rHev b 5 equivalents (#) were obtained by calculating the mean of [(rHev b 5 equivalent/total protein concentration) × 100] for 2 concentrations of extract where rHev b 5 equivalent values were detectable, or one value only where lower dilution had undetectable rHev b 5 levels (eg NAL or LAL).

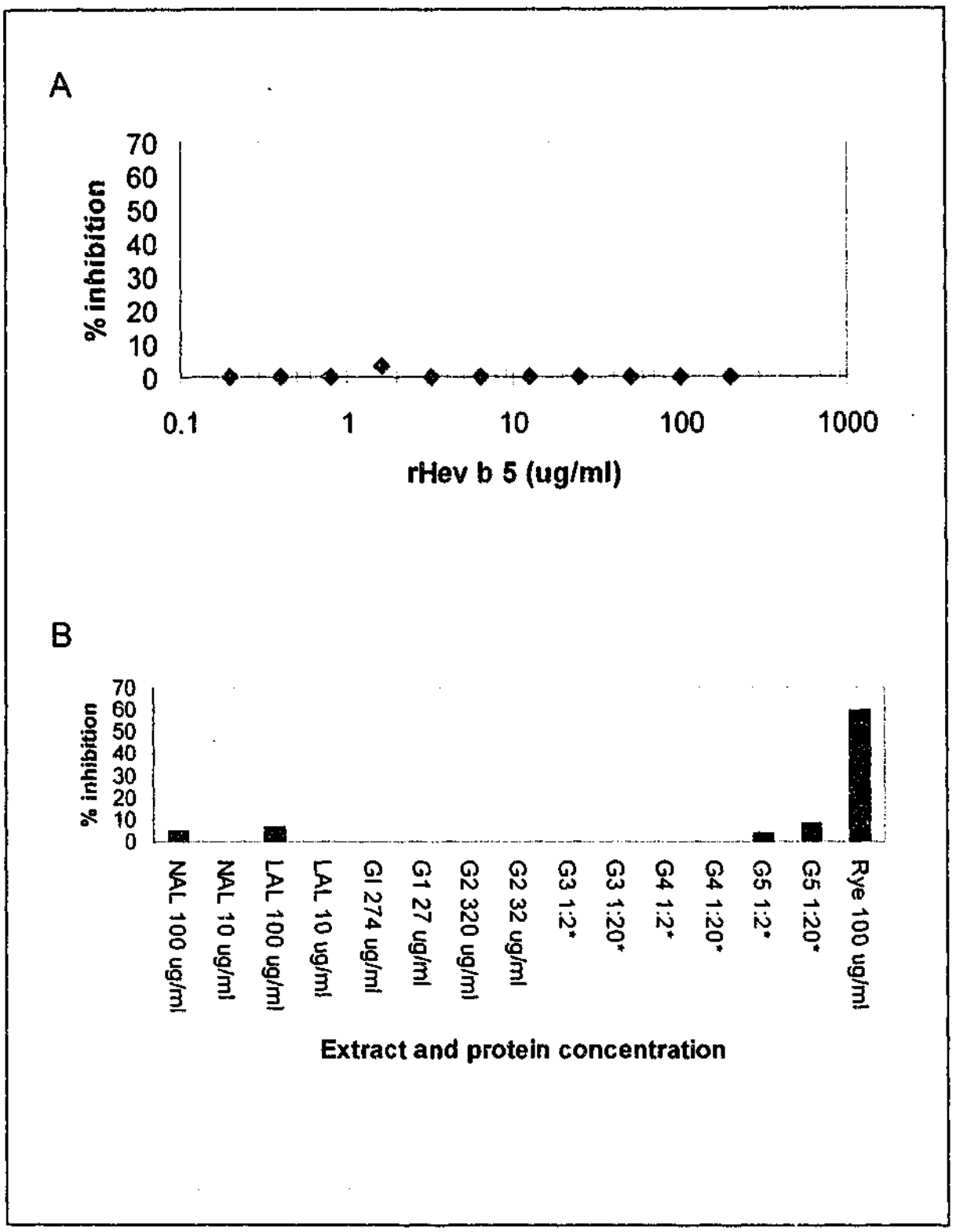


Figure 6.4 Control inhibition ELISA using ryegrass-specific mAb A1

Ryegrass pollen extract was immobilised on an ELISA plate and human ryegrass-specific mAb A1 binding was inhibited with different amounts of rHev b 5 to generate the standard curve (A) or latex extracts and ryegrass pollen extract (B). Latex extracts used (as potential inhibitors of antibody binding to rHev b 5) included NAL, LAL, different glove extracts (GE; G1 and G2 are powdered non-sterile utility gloves, G3 and G5 are powdered sterile surgical gloves and G4 is a non-powdered sterile surgical glove) and ryegrass pollen extract. For glove extracts (GE) where protein was undetectable, concentration is expressed as an extract dilution (*).

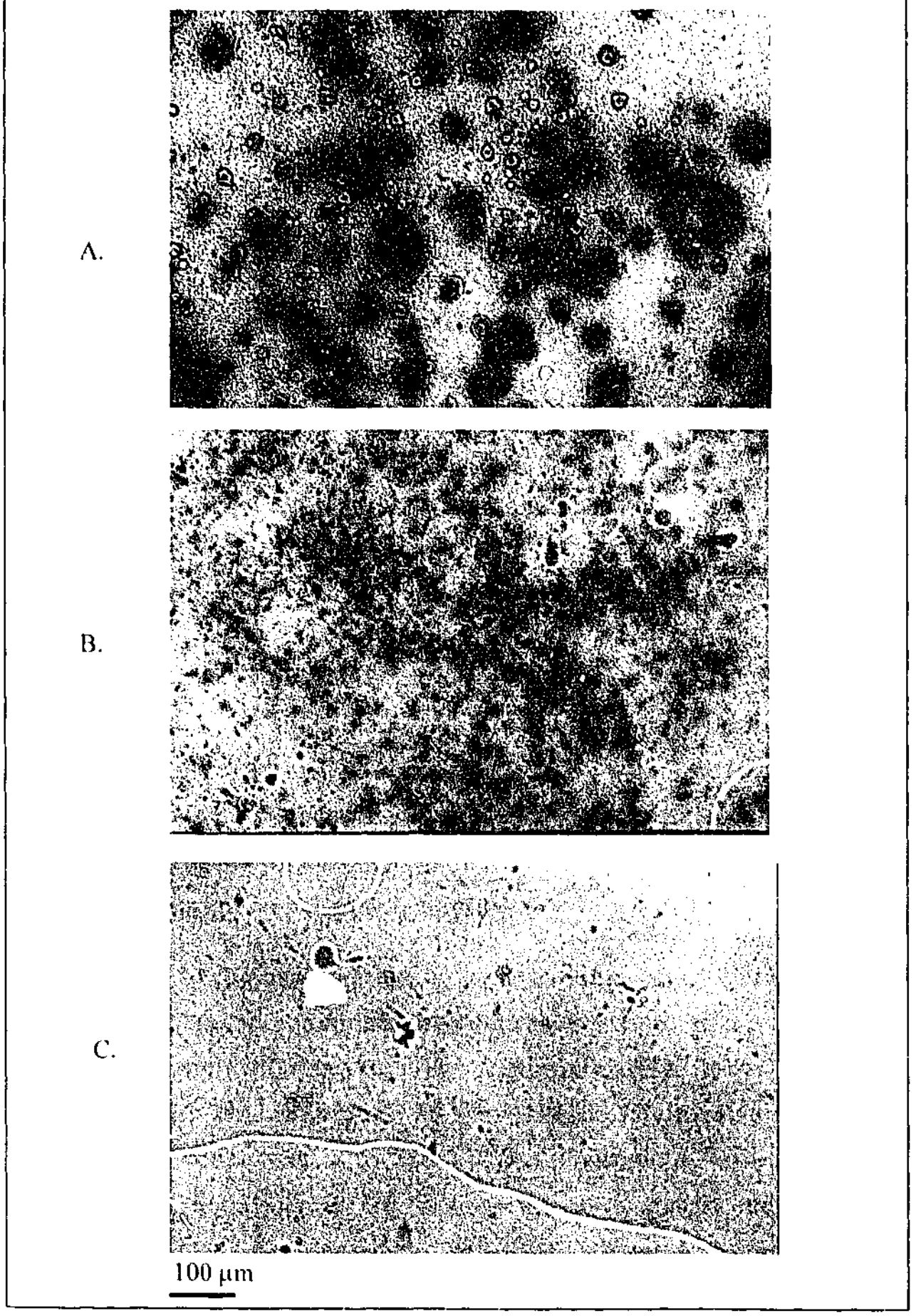


Figure 6.5 Halogen® assays of glove particles using Hev b 5-specific mAb.

A. Livingstone powdered latex examination glove, B: Uniglove powdered latex utility glove C. Vinyl examination glove. Gloves were placed against adhesive surface and then transferred to nitrocellulose membrane, probed with Hev b 5-specific mAb 6F6 at a dilution of 1:1000 and then washed and probed with peroxidase labelled anti-mouse antibody for development according to Halogen® assay protocol (Mitikakis *et al.*, 2002).

extensive staining of Hev b 5 eluted from these particles, some of which are of respirable (< 7 μm diameter) size, while a vinyl glove shows no halo staining for Hev b 5 (Figure 6.5C).

The Halogen® assays staining with the Hev b 5 specific mAb 6F6 were then used for personal nasal sampling assays in the bronchoscopy suite (Mitakakis *et al.*, 2002). This study indicated that, as expected, wearing non-latex gloves was the most effective means of reducing inhaled Hev b 5-bearing cornstarch particles (Figure 6.6). Secondly, the study found that where powdered gloves were used, particulate filter masks were effective in reducing the number of inhaled, Hev b 5 bearing particles (Figure 6.6, 6.7). Interestingly, there was a low, but measureable, background level of inhaled Hev b 5 particles when no gloves were worn (Mitakakis *et al.*, 2002).

6.4. Discussion

This chapter has described the use of Hev b 5-specific mAbs and human IgE to show that immunoreactive rHev b 5 equivalents are more abundant in some latex GEs than in crude latex sap extracts. It has also shown that Hev b 5-specific mAbs can be used successfully as probes of the latex glove powder aeroallergen samples using Halogen® assay technology and personal nasal samplers.

With regard to the examination of latex extracts using Western blotting experiments, the predominant immunoreactivity observed in GE is in the higher molecular mass range (approximately 36 to 110 kDa), compared with NAL at around 26 kDa. These proteins also stain poorly with CBB, consistent with Hev b 5 (Akasawa *et al.*, 1996). The predominant reactivity in GE is observed as a smear, indicating that Hev b 5 may be degraded following glove manufacture, form aggregates and/or interact with other latex proteins. The rHev b 5 also appeared to form higher molecular mass aggregates seen on Western blotting (see Chapter 3). The smeared pattern of rHev b 5-specific IgE reactivity observed in this study is also seen in IgE immunoblots of latex glove extracts reported by other investigators (Swanson *et al.*,

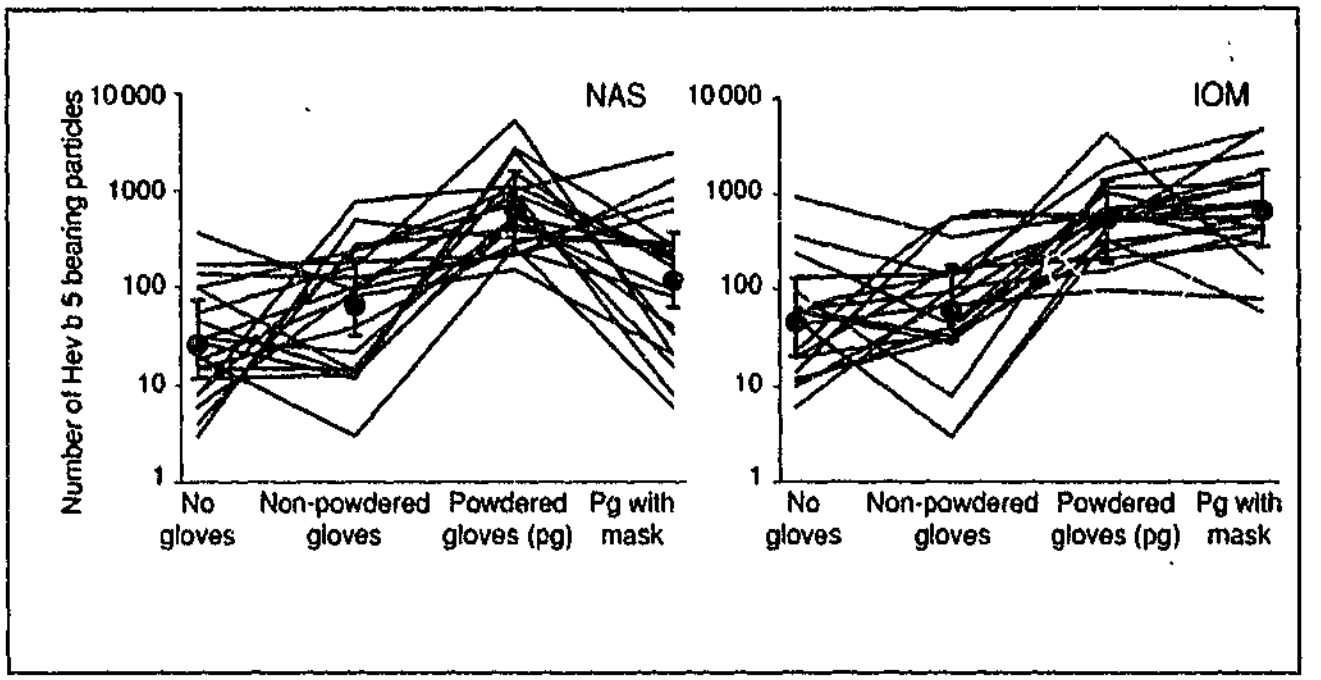


Figure 6.6 Influence on latex glove type on number of inhaled Hev b 5 bearing particles in bronchoscopy suite

Particles were collected during normal activity in bronchoscopy suite while wearing nasal sampling (NAS) personal allergen monitors or Institute of Occupational Medicine (IOM) lapel-worn sampler and Hev b 5-bearing particles assayed by Halogen assay using Hev b 5-specific mAb 6F6 (Mitakakis, *et al.*, 2002).

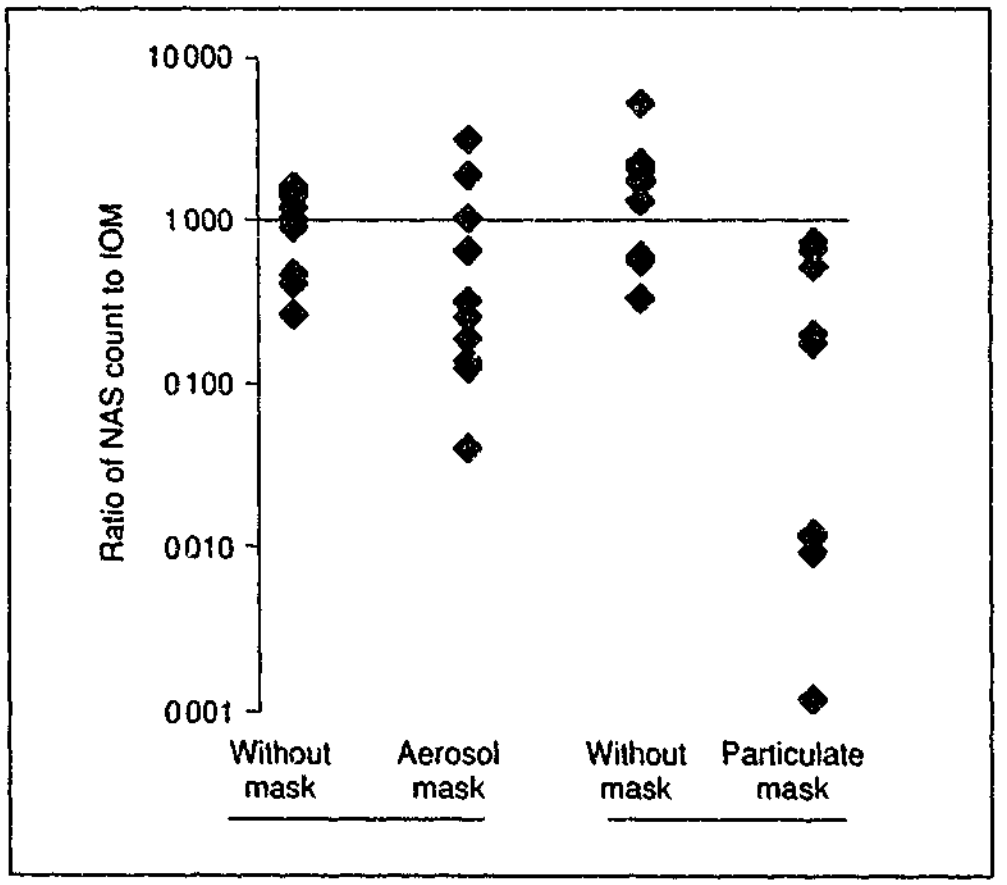


Figure 6.7 Influence of mask type on inhaled inhaled Hev b 5 particles in bronchoscopy suite (Mitakakis, *et al.*, 2002)

1994; Grote *et al.*, 2000; Mahler *et al.*, 2000) but has not previously been attributed to nHev b 5. Interestingly, this smear is also similar to that noted by Swanson *et al.* in their IgE immunoblots of latex aeroallergen samples (Swanson *et al.*, 1994). This suggests an important role for Hev b 5 as a latex aeroallergen bound to glove donning powder and may explain its noted higher frequency of sensitisation amongst HCW compared with spina bifida children (Slater *et al.*, 1996).

Both the Hev b 5-specific mAb and human IgE ELISAs successfully determined rHev b 5 equivalents in the different latex extracts. However, the human IgE ELISA gave higher values. This may be due to the higher sensitivity of the human IgE ELISA (i.e. 0.2 µg/ml) than the mAb ELISA (i.e. 0.8 µg/ml). In addition, unlike mAbs which may recognise only one rHev b 5 epitope, due to its polyclonal nature, human IgE may recognise more rHev b 5 epitopes, therefore contributing to the observed higher detection levels. Nevertheless, there was a strong correlation between the rHev b 5-specific mAb and IgE ELISAs. The advantage of standardisation available with the mAb make it a potentially useful tool in assays of Hev b 5 content of latex extracts and aeroallergen samples.

Our method of glove protein elution (short incubation time, PBS alone on interior of glove) may have favoured the elution of Hev b 5 over other latex allergens, however it may represent a more "physiological" method compared with cutting gloves up or using detergents such as SDS to elute particle bound proteins. Recent publications have shown a higher level of protein on the interior of gloves and in powdered latex gloves (Grote *et al.*, 2000). It may be speculated that Hev b 5 proteins are altered during the glove manufacturing process (high temperatures, addition of cornstarch donning powder) resulting in greater immunoreactivity through exposure of IgE binding cryptopes (Laver *et al.*, 1990) or alternatively the production of advanced glycation end products (Chung and Champagne, 2001). Further studies would be

required to conclusively prove these speculations. Other investigators have found some allergens, such as peanut, more IgE reactive after roasting (Maleki *et al.*, 2000). Unfortunately, glove-manufacturing processes are non-standardised and often considered proprietary, making systematic evaluation difficult. Unlike the latex allergen Hev b 1, which is more abundant in crude latex extracts (7% of NAL) compared with 2-4% of NRL glove extracts (Raulf-Heimsoth *et al.*, 2000), the current results indicate that Hev b 5 may be enriched for by the glove manufacturing process in some high protein powdered gloves.

The high quality surgical gloves assayed showed levels of both protein and rHev b 5 equivalents that were beneath the levels of detection. It is likely that the modern processes of extensive high temperature washing and leaching used in the manufacture of such gloves and other medical rubber products (Lundberg *et al.*, 2001); (Baur *et al.*, 1998) have been highly effective with regard to reduction in immunoreactive nHev b 5 levels. Finnish investigators have recently described a 2 site binding ELISA for the quantification of Hev b 1, 3, 5 and 6 and this is available as a commercially available assay known as the FITkit® (Palosuo *et al.*, 2002). Their investigation of latex gloves in Finland gave similar results to the current study (Sutherland *et al.*, 2002), indicating that Hev b 5 was relatively abundant in high protein powdered latex gloves (Palosuo *et al.*, 2002). Interestingly, their study found that Hev b 5 levels were around one tenth of Hev b 6 levels and only in one glove out of the 22 gloves measured was there detectable Hev b 5 levels when Hev b 6 was undetectable. This may indicate that Hev b 5 is relatively more immunogenic for a given mass than Hev b 6. Additionally, there was a strong correlation between Hev b 5 levels and Hev b 6 levels ($R^2 = 0.68$, $p < 0.001$). Palosuo and co-workers also showed that glove allergen levels had reduced in medical gloves between 1996 and 1999 and that SPT reactions were generally achievable when the sum of allergens detected was greater than or equal to 1 µg/g of latex glove (Palosuo *et al.*, 2002). The results presented in the current chapter are in general agreement with this

study and others indicating that the high quality surgical latex gloves generally available now have low extractable allergen content (Sussman *et al.*, 1998). It cautions however, that lower quality, non-medical grade powdered latex gloves are a rich source of immunoreactive nHev b 5 and represent a potential hazard if inappropriately used (e.g. repeated high level use by atopic individuals or food handlers) in the community. This information is an important reminder that as medical glove technology and awareness in the health care setting improves, we must be vigilant to ensure that inappropriate high allergen glove use by non-HCWs does not expand the latex allergy epidemic unnecessarily into the wider community. The availability of specific mAbs offers the potential to monitor the Hev b 5 content of different latex extracts and, together with Halogen® assays and personal nasal samplers (Mitakakis *et al.*, 2002), offers the prospect of improved allergen avoidance in latex allergy.

7. General discussion

7.1. Introduction

Latex allergy has been a newly described entity of the late 20th Century (Nutter, 1979). As such, it offers a fascinating insight into allergic disease. Allergy occurs to the protein fraction of *Hevea brasiliensis* sap; proteins which undergo harsh physical conditions during the manufacturing process of natural rubber latex products. Such conditions could be expected to alter the tertiary structure of these proteins and thus their recognition by the immune system. Recent advances in molecular biology have resulted in the cloning of 11 latex allergens, but the clinical importance of each relative to one another remains unclear. The spectrum of disease severity in latex allergy is also striking, with some latex allergic patients suffering anaphylaxis from minute latex exposures, while other latex allergic patients may tolerate wearing latex gloves if low in protein and powder free. For a minority of severely affected latex allergic patients, allergen avoidance may be insufficient, necessitating the patient's removal from the workplace, often a tragedy for highly trained health care workers (HCW). Allergen specific immunotherapy (SIT) would be desirable for such patients, but current experience with crude latex extracts suggests IgE-mediated side effects preclude their widespread application. Thus, new approaches, such as peptide-based vaccines, allergen conjugation to CpG-motifs, DNA vaccination or allergen mutants, are needed to diminish IgE mediated side effects. This chapter summarises the contribution made by this thesis to understanding the critical role of Hev b 5 in latex allergy through the generation of specific mAbs and use of human IgE, together with the elucidation of the cellular responses to Hev b 5 peptides; the latter information important for the design of novel immunotherapeutic approaches to this as yet incurable allergy. Moreover, it encompasses discussion of future directions that research may take in the investigation of this pivotal latex allergen.

7.2. The rise of the latex allergy problem and the powdered examination glove

The rise to prominence of latex allergy as a disorder has been driven by a number of factors. These include, firstly, a transient drop in glove quality, as the advent of the AIDS epidemic saw an insatiable rise in demand for latex gloves to utilise in universal precautions (Granady and Slater, 1995). Furthermore, there was a change in glove manufacturing processes in powdered gloves to the use of bioabsorbable cornstarch rather than talc, resulting in more respirable particles that were able to transmit sensitising and symptom-inducing latex allergens to the respiratory tracts of HCW (Subramaniam, 1995). Thirdly, there has been an increase in awareness, fuelled in part by litigation, of the latex allergy problem (Poley and Slater, 2000). Finally, there has been a documented rise in the prevalence of atopic diseases generally within Western societies (ISAAC, 1998).

This thesis has shown that high-protein, powdered latex gloves are a rich source of immunoreactive nHev b 5 and as such, lend weight to other commentators who have suggested that the widespread use of powdered latex examination gloves, in response to the need for universal precautions, is likely the most important contributer to the latex epidemic (Kelly *et al.*, 1996). The generation of Hev b 5-specific mAbs, as described in this thesis, together with the use of serum IgE from Hev b 5-responsive, latex-allergic HCW, showed that powdered latex utility gloves were relatively more abundant in nHev b 5 than the raw latex extracts NAL or LAL (Sutherland *et al.*, 2002). As was shown, unfortunately it was not possible to develop a two-site binding ELISA for the detection of Hev b 5 using the mAbs described for Hev b 5, likely because they recognise similar epitopes on Hev b 5. Other investigators have since described a two-site binding ELISA for Hev b 5 detection (Palosuo *et al.*, 2002), and their results are in general agreement with those described here, indicating that powdered latex gloves high in overall protein are usually high in Hev b 5 levels; that is, Hev b 5 is a major allergenic component of latex gloves.

There is some evidence that the removal of high protein powdered latex examination gloves and their replacement by medical grade powder free latex gloves that undergo extractable protein reduction processing has resulted in a decrease in new sensitisation rates (Tarlo *et al.*, 2001). Further studies are needed to confirm whether this strategy will halt the progression of the latex allergy epidemic. It also warns that these powdered latex gloves should not be used in any community situation where repeated glove changes and thus recurring exposure may occur, such as childcare facilities, food preparation, cleaning, hairdressing or even housework. Food preparation is of particular relevance as many of the latex proteins, including Hev b 5 are highly water soluble and have been shown to easily transfer to salad vegetables during handling with high protein powdered latex gloves (Beezhold *et al.*, 2000). Such small quantities of protein, while unlikely to cause sensitisation or symptoms when ingested by normal individuals may result in anaphylaxis in highly latex allergic subjects (Nixon and Lee, 2001). Avoidance of spreading the epidemic to the wider community will require both education and likely legislation regarding safe exposure levels and extractable protein and latex allergen levels. Interestingly, in the latex-allergic subject group described in this thesis, 3/32 (9%) described their occupation as home duties, and had no past history of being HCW, though one of these subjects reported having had 15 operations with a general anaesthetic. All these three subjects had histories of eczema and food allergy and this suggests that such patients may be sensitised in the community without traditional risk factors, likely through the use of latex gloves for washing and cleaning.

7.3. Hev b 5 is a major latex allergen

This thesis has confirmed that Hev b 5 is a major latex allergen, with more than 50% of latex-allergic subjects, the majority (78%) of whom were HCW, showed reactivity with Hev b 5 by IgE ELISA and none of the negative control population of latex non-allergic subjects. It is interesting to speculate why not all subjects with latex allergy are

sensitised to this highly potent allergen. The current study did not examine tissue typing in all the subject groups, but it is likely that differing HLA specificities among individuals account for, at least in part, the different patterns of sensitisation observed. Additionally, different glove extracts that subjects were sensitised with in the community may have contained differing amounts of Hev b 5 when compared with other allergens. Recent studies have suggested that gloves can vary widely in their allergen content (Palosuo *et al.*, 2002; Sutherland *et al.*, 2002) and indeed the current thesis has shown that some latex gloves, in particular the sterile surgical gloves assayed here, may have undetectable levels of immunoreactive nHev b 5 (see Chapter 6).

Given Hev b 5's major allergen status, immunotherapeutic approaches to the treatment of latex allergy among HCW will need to incorporate Hev b 5 T cell determinants to achieve efficacy.

7.4. Hev b 5 may be an important protein in explaining the observed potency of latex allergens

The immunotherapy experience with crude latex extracts (Pereira *et al.*, 1999; Leynadier *et al.*, 2000) combined with the instances of latex balloon-induced barium enema anaphylaxis (Ownby *et al.*, 1991) and intra-operative anaphylaxis (Slater, 1989) indicate that latex proteins are extremely potent allergens in their ability to induce severe IgE-mediated reactions. Other experimental evidence further supports this claim. For example, in a skin testing study using recombinant and natural latex extracts, Yip *et al.* found that the most latex sensitive patient reacted to a Bencard LAL extract on simple skin testing down to a concentration of 70 pg/ml (Yip *et al.*, 2000). In addition, it has been recently shown that latex proteins eluted from rubber medication vial closures are enough to elicit wheal and flare reactions when used in skin testing of latex allergic individuals (Primeau *et al.*, 2001). Latex is also the most common allergen to induce systemic reactions on

skin testing, when compared with all others including drugs, foods and pollens (Valyasevi *et al.*, 1999).

Evidence from the current thesis and other recent work point to the importance of Hev b 5 as one possible reason for this observed potency. This thesis found that latex allergic individuals sensitised to Hev b 5 had a more severe clinical phenotype, with a greater incidence of self-reported angioedema and anaphylaxis, suggesting that Hev b 5 is highly potent in its action *in vivo* in humans (see Chapter 4).

Also supporting this was the observation made, during the preparation of the rHev b 5-specific mAbs, that the immunisation schedule and test bleeding showed Hev b 5 as highly immunogenic; more so than its larger MBP fusion partner.

Other investigators have recently shown that Hev b 5 is less abundant in most latex gloves compared with Hev b 6 by a factor of around 10 (Palosuo *et al.*, 2002). Notwithstanding this, Hev b 5 is often a monosensitiser (Yip *et al.*, 2000) and the current study indicates that it can stimulate strong TH2 and IgE responses in sensitive individuals, despite its lesser abundance than Hev b 6 (See chapters 5 and 6).

Hev b 5, being proline rich and highly charged (Akasawa *et al.*, 1996; Slater *et al.*, 1996), is highly water soluble, and this may enhance its ability to leach from latex gloves on donning and also from glove powder particles when these contact mucous membranes. The bioavailability of allergens and how they interact with carrier particles and the body's mucous membranes and tissues is of great interest and would be a valuable area of future research with regard to Hev b 5.

It has been suggested that Hev b 5 may have enhanced ability through multivalency to cross-link multiple effector cell-bound IgE molecules (Beezhold *et al.*, 2001). This claim is hard to substantiate in the absence of the crystal structure, and suggests a possible line of future research to elucidate more fully, molecular mechanisms for the potency of Hev b 5. The crystal structure of the other major latex allergen

among HCW, Hev b 6, has been solved, indicating a rigid tertiary structure, particularly in the hevein domain, stabilised by 4 disulphide bonds (Hernandez-Arana *et al.*, 1995), whereas computer modelling of Hev b 5 predicts a random tertiary structure (Slater *et al.*, 1996). Perhaps more important than the structure of the native molecule will be research into the nature of the aggregates/ advanced glycation end products (AGEPs) observed in the GE on probing with Hev b 5-specific mAbs (See chapter 6). This could be further investigated with specific probes such as anti-sera against AGEPs (Chung and Champagne, 2001). It may be that Hev b 5, by forming these aggregates, enhances its allergenicity, despite its lower abundance, through improved bioavailablity, antigen uptake and/or presentation, or adjuvant effects.

7.5. The role of Hev b 5 immunoreactivity in the diagnosis of latex allergy

Latex glove proteins are, in many cases, altered by the glove manufacturing process. This difference has given rise, at least in part, to some of the difficulties that exist with the *in vitro* diagnosis of latex allergy, which hitherto have, in the main, utilised raw latex extracts such as NAL.

The implication of the current thesis for the diagnosis of latex allergy, is to confirm that current in vitro assays are imperfect for the diagnosis of this disorder (Chen *et al.*, 2000). In Chapter 4, data were presented showing that eight subjects with histories consistent with latex allergy and positive latex-specific EASTs and/or skin prick tests, were negative by IgE assay using the latex-specific Pharmacia UniCAP®, a reagent using NAL. All these subjects were positive by the rHev b 5-specific IgE ELISA. This suggests that diagnostic methods may need to be adjusted to enrich Hev b 5 content (Lundberg *et al.*, 2001) and confirms that the presence of a credible history of immediate symptoms on exposure to natural rubber latex in the absence of latex-specific IgE should always prompt further investigation with latex SPT. The general

unavailability of the latex SPT in Australia, however, emphasises the need for enhanced *in vitro* assays such as those including Hev b 5.

Future improvements in the diagnosis of latex allergy may involve the use of rHev b 5 in basophil activation assays (Sainte-Laudy *et al.*, 1996), which have shown promise in the diagnosis of latex allergy but have not shown widespread application, mainly due to problems of specificity (Sainte-Laudy *et al.*, 1996). In addition, purified rHev b 5 may be a useful skin test reagent, as has been shown with rHev b 5/MBP (Yip *et al.*, 2000).

7.6. Specific monoclonal antibodies to Hev b 5 have shown its relative abundance and have suggested its structural alteration in glove extracts

The generation of mAbs specific for rHev b 5 as presented in this thesis have been an advance upon previous antibodies, which were not reported to be mono-specific (Slater *et al.*, 1996). Since the current work was performed (Sutherland *et al.*, 2002), others have published reports of Hev b 5-specific mAbs (Palosuo *et al.*, 2002). The reactivity of the mAbs presented in this thesis, suggested that the process of glove manufacture both enriches for and alters the Hev b 5 content within latex gloves. It was shown in Chapter 6 that the nHev b 5 content was increased in high protein powdered latex GE. This is most likely because of the protein's previously demonstrated ability to survive autoclaving conditions (Akasawa *et al.*, 1996). As well as an increased content, the mAbs indicated that nHev b 5 was altered or formed aggregates on SDS-PAGE that were still immunoreactive on Western blotting and also correlated with Hev b 5-specific IgE activity. Further clinical correlation was offered by the observation that among Hev b 5-responsive individuals, driving PBMC with a high protein powdered latex GE resulted in a strong rHev b 5-specific and Hev b 5 peptide-specific immune response. Additionally, it has been demonstrated in Chapter 6 that high quality surgical gloves, which undergo extensive leaching during production, have low levels of immunoreactive Hev b 5. Both the Hev b 5-specific human IgE and

mAbs recognised the immunoreactive Hev b 5 as a smear within the higher molecular weight ranges in the GE on Western blotting. This suggests that Hev b 5 is altered during glove manufacturing by processes such as heating, which may result in, as has been speculated, the formation of AGEPs. It also indicates that the Hev b 5 as it is present in GE has the conformation that results in a strong humoral immune response from patients. The prospect of mAb-based assays to monitor Hev b 5 content of latex gloves gives hope for improved allergen avoidance in latex allergy.

7.7. Hev b 5 can be delivered as an aeroallergen

It has been well known that latex can act as an aero-allergen (Baur *et al.*, 1998). Swanson and colleagues noted in their study of aeroallergen levels within a teaching hospital facility that when analysed by Western blotting using IgE from human latex allergic subjects that the resultant bands ran as a high molecular weight smear (Swanson *et al.*, 1994), similar to that observed on blotting with the rHev b 5-specific mAbs (Sutherland *et al.*, 2002). They were unable to identify this allergen as Hev b 5, however. Using the rHev b 5-specific mAbs described in this thesis, collaboration with scientists from the Institute of Respiratory Medicine in Halogen® assays, as presented in this thesis, has shown unequivocally that Hev b 5 is present on latex glove donning powder from powdered latex gloves (Mitakakis *et al.*, 2002). This important information indicates that Hev b 5 may act as a respiratory sensitiser and symptom-inducer. Furthermore, this study showed that wearing latex powder-free gloves was as effective as wearing no gloves in reducing respirable Hev b 5-bearing particles, again supporting current recommendations on the use of powder-free latex gloves (Kelly *et al.*, 1996). Moreover, a particulate filtering mask was shown to be more effective that a normal surgical mask in reducing personal inhaled allergen. Given the sensitivity of some individuals however, this finding is likely of limited significance, and powder free glove use is likely to provide a safer working environment with regard to inhaled aeroallergen levels (Swanson *et al.*, 1994). In the future it

would be interesting to determine how much Hev b 5 is contained within aeroallergen samples relative to other latex allergens. In addition, the background levels of nHev b 5 aeroallergen detected in the Mitakakis study would be interesting to further evaluate. Comparing these levels detected within the bronchoscopy suite compared with ambient environmental levels outside the hospital would be a first step. If real measurable levels were detectable it would be interesting to do further studies of environmental samples around Melbourne. Studies within the US have detected latex proteins within tyre dust samples collected from Los Angeles air sampling (Miguel *et al.*, 1996), however the clinical importance of this is unclear. Certainly, the epidemiology of latex allergy does not support a prominent role of ambient latex particles within the environmental atmosphere of cities (Liss and Sussman, 1999), however anecdotal clinical experience indicates that some patients complain of latex induced symptoms on high pollution days. Other factors (or allergens), however, may be inducing these symptoms. Well-conducted epidemiological studies of latex allergy prevalence within the Australian population as a whole are currently lacking. Further aeroallergen sampling studies could also be performed with regard to the background levels of exposure within the hospital, to monitor the return to work of latex sensitive individuals within a latex powder free environment. Current, largely anecdotal evidence suggests that the majority of individuals can return to work under these circumstances (Bubak, 2000). What is not known however, is whether the low levels of Hev b 5 particles detected as a background represents any ongoing risk, and studies correlating subject symptoms and particle levels would provide valuable insights.

7.8. The human T cell response to Hev b 5 is strong in Hev b 5 sensitive individuals and dominant T cell determinants are present

The T cell and PBMC data presented have indicated that Hev b 5 is highly potent as a T cell immunogen. In sensitised individuals, it

elicits both proliferation and an enhanced IL-5 cytokine secretion profile consistent with a TH2 response. It has also been demonstrated within this thesis that there are dominant regions within the molecule that are recognised by the majority of subjects with a peptide response. These include Hev b 5(46-65) and Hev b 5(1-20). Sequences contained within these peptides would thus be important to maintain in any vaccine for the treatment of latex allergy through immunotherapy with peptides or modified allergen mutants.

This thesis also suggests further lines of future enquiry. Firstly, although dominant T cell determinants have been found, further fine epitope-mapping studies are required to definitively delineate the Hev b 5 epitopes and in particular, Hev b 5(46-65). Moreover, before the application of Hev b 5 peptides in SIT, it will be necessary to perform basophil histamine release experiments and skin testing assays to determine if these peptides release effector cell-bound IgE.

7.9. Future approaches to immunotherapy with Hev b 5

In an effort to combat the side effects (which are IgE-mediated and limit the clinical utility of SIT), a number of novel methods of SIT are under development in other allergen systems. These novel methods illustrate mechanisms of SIT and provide proof of concept for similar approaches that have potential in the treatment of latex allergy.

T-cell epitope peptides are intrinsically attractive for use in immunotherapy because of their ability to stimulate T cells and thus modulate the cellular immune response, without significant cross-linking effector cell-bound IgE. Peptide immunotherapy has been used in honeybee venom allergy, where a mixture of the three dominant T cell epitopes of phospholipase A_2 (PLA_2) was administered to 5 honeybee venom-allergic subjects (Muller *et al.*, 1998). There were no systemic side effects from the subcutaneous injections of the peptides, and the patients were protected from challenge with PLA_2 in all cases and in 3/5 against sting challenge. The authors demonstrated decreased

in vitro T cell proliferation and cytokine secretion consistent with anergy to the whole PLA_2 molecule (Muller *et al.*, 1998).

Tempering this enthusiasm has been the knowledge that peptide injections have been reported to induce adverse events in cat allergic patients (Norman *et al.*, 1996). More recently, Haselden *et al.* showed that a mixture of Fel d 1 T-cell epitope peptides in equi molar amounts was able to induce MHC-restricted T-cell dependent late asthmatic responses independent of IgE (Haselden *et al.*, 1999). While these responses are of concern they indicate that Fel d 1 specific T cells are being activated without effector-cell IgE cross-linking. More encouragingly, the same group has reported both safety and efficacy of immunotherapy with Fel d 1 T-cell epitope peptides in a double blind placebo controlled trial (Oldfield *et al.*, 2001), and in addition, developed a dosage schedule that avoids the induction of late asthmatic responses (Alexander *et al.*, 2001). The use of a dominant peptide or small number of peptides may be advantageous in avoiding some of the potential problems of aggregation of a complex mixture of 20 or more peptides. The dominant peptides, being better immunogens may be better tolerogens and there is evidence from animal models that a dominant peptide can induce tolerance against the whole molecule through linked suppression (Hoyne *et al.*, 1997). This was also seen in the honeybee venom peptide SIT trial (Muller *et al.*, 1998). With regard to Hev b 5 and the potential peptide immunotherapy of latex allergy, T cell determinants contained within Hev b 5(1-20) and Hev b 5(46-65) will likely need to be retained to maintain efficacy. Whether these two peptides would be sufficient will require further study, as will ensuring that they do not cross-link effector cell bound IgE and release histamine. Alternatively, since the current study has shown that among latex-allergic subjects a total of 6 peptides had 50% prevalence of significant reactivity, it may be that an equimolar mixture of all peptides such as used in the Fel d 1 work (Haselden *et al.*, 1999), is more effective.

Another approach to decrease the effector cell-bound IgE cross-linking with the aim of reducing potential side effects is to modify the primary, secondary or tertiary structure via engineering of recombinant allergens. To maintain efficacy, T cell reactivity must be preserved. This has been reported with house dust mite (Smith and Chapman, 1996), birch pollen (Ferreira *et al.*, 1998) and timothy grass pollen (Schramm *et al.*, 1999) allergens. To date, there is no evidence of the efficacy of this approach in modifying an established TH2 response, which must be the aim of any vaccine. As discussed earlier, characterisation of the crystal structure of nHev b 5 may help in this endeavour by helping to rationally target modifications, which will effect structure. Computer modelling suggests that Hev b 5 has a predicted random tertiary structure (Slater *et al.*, 1996) which may hamper this approach. Hev b 5 has been modified by Beezhold *et al.* to reduce IgE binding (Beezhold *et al.*, 2001). They found that a total of 7 alanine substitutions were required within 3 of the linear IgE binding epitopes of Hev b 5 to obtain a 100-fold reduction in IgE binding to the whole molecule by inhibition ELISA. With a total of 14 alanine substitutions in 8 epitopes, IgE binding could be decreased by 4,500 fold. Consequently for the generation of successful candidate allergen mutant vaccines, future studies will need to address whether such mutants retain T cell reactivity in addition to the demonstration of decreased IgE binding and then move forward to testing likely in animal models to see if established Hev b 5-specific TH2 responses can be abrogated.

On the other hand, Hev b 6 which has a well defined and rigid tertiary structure (Hernandez-Arana *et al.*, 1995), is a more attractive target for allergen mutant generation.

Finally, in addition to mutant recombinant allergen vaccines, DNA vaccination or conjugation of allergens to immunostimulatory sequences (ISS) of DNA hold promise as alternative methods of

delivering SIT. Animal studies (Magone *et al.*, 2000; Tighe *et al.*, 2000) and *in vitro* human studies (Marshall *et al.*, 2001) using ISS in ragweed pollen allergy and DNA vaccination in peanut allergy (Roy *et al.*, 1999) have demonstrated decreased allergenicity with increased immunogenicity and the ability to modulate established allergic states, by the up-regulation of allergen-specific TH1 responses and concomitant reduction in TH2 responses. With regard to latex allergy, Slater has utilised DNA vaccination in a murine model (Slater and Colberg-Poley, 1997). This study showed successful distribution of the Hev b 5 transcript in the lungs, spleen, lymph nodes, blood and tongue of the mouse and the mounting of both an allergen-specific cellular and humoral immune response of TH1 type. Toxicity was not seen, but severe local reactions had been observed by the same author during optimisation experiments (Slater *et al.*, 1998). The fear of powerful TH1 type reactions including auto-immunity is one of the major concerns that is hampering the application of this approach in humans. It is likely that the critical questions of safety and efficacy of DNA vaccination or ISS conjugate vaccines in humans will be established in other allergen systems such as ragweed pollen allergy, where cautious and well-conducted trials of an ISS conjugated Amb a 1 have begun (Creticos *et al.*, 2001), prior to the possible application of this technology in the immunotherapy of latex allergy.

7.10. Conclusion

This thesis has confirmed Hev b 5 as a major latex allergen for which dominant T cell determinants exist. Moreover, it has suggested that Hev b 5 is a highly potent allergen, relatively abundant in high protein powdered latex glove extracts, but which may be inadequately represented in some current diagnostic assays that utilise raw latex extracts. Overall, it has suggested cause for optimism in the future management of latex allergy. This will manifest firstly, through improved diagnosis of latex allergy utilising rHev b 5. Secondly, the

reduction in latex glove extractable Hev b 5 levels and airborne Hev b 5, with the adoption of improvements in glove technology and powder free gloves, should lead to improved allergen avoidance and workplace safety. Finally, there is the prospect that the dominant T cell determinants identified within Hev b 5 may one day herald safe and effective immunotherapy for the management of latex-allergic individuals.

8. Appendices

Appendix I Human subjects – demographic features and self reported atopic disease. For categorical variables (yes/no), 1= yes, 0 = no. Where a the subject failed to respond to a question, the cell is left blank.

ID number	latex allergic	age	female	HCW	occupation	years in current occupation	operations with general anaesthetic	hay fever	asthma	eczema	food allergies
1	1	51	0	0	rubber industry worker		0	1	0	0	0
2	1	38	1	1	general nurse	10	0	0	0	1	1
3	1	32	1	1	cleaner in hospital	8	0	0	0	1	1
4	0	29	1	0	receptionist	5	0	0	0	0	0
5	1	45	1	1	intellectual disability nurse	19	20	1	1	0	1
6	0	23	1	1	general nurse	4	0	1	1	0	1
7	1	42	1	1	general nurse	23	6	0	0	1	0
8	1	58	1	1	theatre nurse	18	0	1	1	1	0
9	1	34	1	1	general nurse	4	5	1	0	0	0
10	1	38	1	1	midwife	20	1	1	1	0	1
11	1	41	1	1	icu nurse	15	0	1	1	1	1
12	1	32	1	0	home duties	15	2	1	1	1	1
13	0	41	1	1	theatre nurse	20	2	1	1	0	0
14	1	31	1	1	medical scientist	10	0	1	0	1	1
15	1	31	1	1	emergency nurse	10	7	1	0	0	0
16	1	55	1	1	retired nurse	20	10	0	0	0	0
17	1	68	1	0	home duties	7	15	0	1	0	1
18	1	28	1	1	former surgeon	2	2	1	1	1	1
19	1	41	1	1	anaesthetist		8	1	1	1	1
20	1	30	1	1	theatre nurse	8	0	1	1	1	1
21	1	53	1	1	nurse in nursing home	7	6	1	0	0	0
22	0	28	1	1	endoscopy nurse	2	2	0	0	0	0
23	0	67	1	0	home duties	U	5	1	0	0	0
24	1	43	1	1	anaesthetist	18	3	0	0	1	1
25	1	52	1	1	general nurse	15	4	1	1	1	1
26	1	26	1	1	surgical nurse	5	0	1	1	1	0
27	0	37	1	1	general nurse	15	2	1	1	0	0
28	1	50	1	1	theatre nurse	29	1	1	1	1	1
29	0	37	1	0	cleaner in hotel	7	0	1	0	0	0
30	1	27	1	1	general nurse	4	3	0	0	0	0
31	0	26	1	0	animal house worker	1	1	0	0	0	0
32	0	23	1	1	medical student	5	2	0	0	0	0
33	0	44	0	0	postman		1	0	0	0	0
34	0	26	1	1	anaesthetic nurse	5	2	1	0	1	1
35	0	34	0	1	icu nurse	2	3	1	1	1	1
36	1	31	1	1	general nurse	5	2	1	0	0	0
37	0	51	1	1	general nurse	32	3	1	1	1	1
38	1	28	1	0	hairdresser	2	2	1	1	1	0
39	0	35	0	1	physician	10	2	0	0	0	0
40	1	36	1	1	general nurse	10	0	0	0	0	1
41	0	48	1	0	secretary	2	2	0	0	0	0
42	0	57	1	1	endoscopy nurse	2	8	1	0	0	1
43	1	25	1	0	childcare worker	1	2	0	1	1	1
44	0	45	1	1	physician	15	2	0	0	0	0
45	1	34	1	1	endoscopy nurse	6	2	1	0	1	0
46	1	49	1	0	shop assistant (pharmacy)	15	0	1	1	1	1
47	1	27	1	0	home duties	4	2	1	1	1	1
48	1	46	1	1	general nurse	10	10	0	1	1	1
49	0	39	1	1	anaesthetic technician		2	1	0	0	0
50	0	40	1	1	general nurse	15	3	1	0	0	0
51	1	33	1	1	general nurse	4	4	1	0	1	1

Appendix II Human subjects – self reported symptoms on latex exposure and reactions to latex products.PLG(powdered latex gloves), NPLG (non-powdered latex gloves). For categorical variables (yes/no),1= yes, 0 = no.

ID number	latex allergic	local skin itch	skin hives	general erythema	sneeze	facial swelling	throat swelling	shortness of breath/asthma	anaphylaxis	PLG	NPLG	balloons	condoms
1	1	1	1	1	1	1	0	1	1	0	0	0	0
2	1	1	1	1	1	1	0	1	0	1	1	1	1
3	1	1	1	0	1	1	0	1	0	1	1	1	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0
5	1	0	0	1	0	1	1	0	1	0	0	1	0
6	0	1	0	1	0	0	0	1	0	1	1	0	0
7	1	1	1	1	1	1	1	1	1	1	1	1	1
8	1	1	1	0	1	0	0	0	0	0	0	1	0
9	1	1	1	0	1	1	0	0	0	1	1	1	1
10	1	1	1	1	1	1	1	1	0	1	0	1	0
11	1	1	1	1	1	1	1	1	1	1	1	1	1
12	1	1	1	0	0	0	0	0	0	1	1	1	1
13	0	0	0	0	0	0	0	0	0	0	0	0	0
14	1	1	1	1	1	1	0	1	1	1	1	1	0
15	1	1	1	1	1	1	1	1	1	1	1	1	1
16	1	1	0	1	1	1	1	1	1	1	1	1	0
17	1	1	1	0	0	0	0	1	0	1	1	1	0
18	1	1	1	1	1	1	1	1	1	1	1	0	1
19	1	1	1	0	0	1	1	1	1	1	1	1	0
20	1	1	1	0	1	0	0	0	0	1	0	0	0
21	1	1	1	1	0	1	0	1	0	1	1	1	0
22	0	1	0	0	0	0	0	0	0	1	1	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0	0
24	1	1	1	0	1	1	1	0	0	1	1	1	0
25	1	1	1	0	0	1	1	1	1	1	0	1	0
26	1	1	0	0	1	1	0	1	0	1	1	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0	0
28	1	1	1	0	1	0	0	1	0	1	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0	0
30	1	1	1	0	1	0	0	0	0	1	0	1	0
31	0	1	0	0	0	0	0	0	0	1	0	0	0
32	0	0	0	0	0	0	0	0	0	0	1	0	0
33	0	1	0	0	0	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0	0	0	0	0	0
35	0	1	0	0	0	0	0	0	0	0	0	0	0
36	1	1	0	0	0	0	0	0	0	1	0	0	0
37	0	0	0	0	0	0	0	0	0	0	0	0	0
38	1	1	1	0	0	0	0	1	0	1	0	1	1
39	0	0	0	0	0	0	0	0	0	0	0	0	0
40	1	1	1	1	1	1	1	1	1	1	1	1	0
41	0	0	0	0	0	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0	0	0	0	0	0
43	1	0	1	0	0	0	0	0	0	1	0	1	1
44	0	0	0	0	0	0	0	0	0	0	0	0	0
45	1	1	1	1	0	1	1	1	1	1	1	1	0
46	1	1	1	0	0	0	0	1	0	1	1	0	0
47	1	1	1	1	1	1	1	1	1	1	1	1	0
48	1	0	1	0	1	1	0	1	0	1	1	1	0
49	0	0	0	0	0	0	0	0	0	0	1	0	0
50	0	0	0	0	0	0	0	0	0	1	0	0	0
51	1	1	1	0	0	1	1	1	0	1	0	1	0

Appendix III Human subjects – latex diagnostic serological assays, skin testing in mm (columns 2nd to 8th from the right hand side of the table) and atopic status. For categorical variables (yes/no),1= yes, 0 = no. Where a the subject failed to respond to a question, or did not undergo the assay or test, the cell is left blank.

ID number	latex allergic	latex EAST(AEU/ml)	latex EAST class	Pharmacia UniCAP latex	UniCAP class	total IgE kU/L	histamine	saline	latex	mixed pollen	hdm	aspergillus	cat	atopic
1	1	0.09	0	0.18	1	311	4	0	5	0	4	0	0	1
2	1	9.53	3	9.64	3	116	5	0		0	0	0	2	0
3	1	13.2	3	3.50	2	470	4	0	4	0	0	0	0	0
4	0			0.18	0	26	0	0	0	0	0	0	0	0
5	1	5.61	3	0.18	0	14	5	0						1
6	0	0.19	0	0.18	0	135	4	0	0	8	9	0	7	1
7	1	0.09	0	8.69	3	273	4	0		10	13	0	10	1
8	1	11.86	3	0.18	0	11	5	0	6	8	8	3	3	1
9	1	7.06	3	0.18	0	25				13	6	2	5	1
10	1	16.24	3	0.18	0	112	5	0		8	10	4	10	1
11	1	9.52	3	9.03	3	291								1
12	1	10	3	1.38	2	1827	7	0		20	12	3	9	1
13	0	0.09	0	0.18	0	141								1
14	1	16.31	3	15.5	3	244	4	0	10	0	0	0	4	1
15	1	17.5	4	7.35	3	241	4	1		1	1	1	1	0
16	1	1.9	2	0.18	0	11	5	0	4	0	0	0	0	0
17	1	17.5	4	1.94	2	645	10	0		0	9	6	5	1
18	1	16.64	3	10.9	3	1706	3	0		4	9	2	5	1
19	1	9.5	3	17.9	4	7114	7	0		12	10	0	0	1
20	1	11.36	3	8.82	3	5760	0	0		9	3	0	2	1
21	1	11.25	3	2.63	2	148								1
22	0	0.09	0	0.18	0	129	5	0	0	0	0	0	0	0
23	0	0.09	0	0.18	0	808								1
24	1	11.84	3	0.18	0	96	7	0		0	8	0	7	1
25	1	4.72	3	0.59	1	78	7	0		10	7	3	0	1
26	1	5.83	3	2.43	2	1327	5	0		10	11	1	8	1
27	0						6	0	0	10	10	0	0	1
28	1	0.36	1	0.4	1	16	6	0		0	5	5	10	1
29	0	0.09	0	0.18	0	49	7	2	0	8	7	2	6	1
30	1	0.81	2	2.56	2	185	5	0	5	0	7	0	0	1
31	0	0.09	0	0.18	1	153	6	0	0	10	0	0	0	1
32	0			0.18	1	13	5	0	0	0	0	0	0	0
33	0	0.09	0	0.18	1	70	6	0	0	0	0	0	0	0
34	0	0.09	0	0.18	1	65	6	0	0	7	5	0	0	1
35	0	0.09	0	0.18	1	115	7	0	0	0	16	0	0	1
36	1	0.09	0	0.18	1	157	6	0	4	3	4	0	10	1
37	0			0.18	1	42				7	0	0	0	1
38	1	17.5	4	29	4	2188				10	10	0	0	1
39	0			0.18	0	63	6	0	0	5	0	0	0	1
40	1	4.5	3	1.38	2	19	5			0	0	0	0	0
41	0			0.18	0	4	7	0	0	0	0	0	0	0
42	0			0.18	0	10	7	0	1	8	0	0	0	1
43	1	7.67	3	2.07	2	367				10	7	2	5	1
44	0	0.09	0	0.18	0	1	6	0	0	0	0	0	0	0
45	1	0.09	0	0.18	0	110	3	0	3	0	10	0	0	1
46	1	11.03	0	9.4	3	578	4	0		12	7	0	8	1
47	1	3.39	2	31.5	4	11610	5	0		9	11	3	7	1
48	1	17.5	3	99	5	1000	4	0		12	7	0	0	1
49	0	0.09	0	0.18	0	65				10	15	5	7	1
50	0	0.09	0	0.18	0	52				0	3	0	12	1
51	1	3.00	2	8.47	3	717				10	6	0	5	1

Appendix IV Serum assays for fruit allergens, total IgE and self reported food allergy (4 columns on far right of table). For categorical variables (yes/no), 1= yes, 0 = no. Where an assay was not performed or a question not answered the cell has been left blank.

ID number	LATEX ALLERGIC	banana EAST(AEU/ml)	banana EAST class	avocado EAST(AEU/ml)	avocado EAST class	kiwi EAST (AEU/ml)	kiwi EAST class	total IgE kU/L	foods reported to cause allergy	banana	avocado	kiwi
1	1							311		0	0	0
2	1			4.42	3			116	tomato, pepper, avocado, banana	1	1	1
3	1	0.51	1			0.35	1	470		1	1	1
4	0							26		0	0	0
5	1	0.09	0	0.09				14	nuts, shellfish, eggs, tomatoes+ others	1	1	1
6	0				0			136	milk products	0	0	0
7	1	1.22	2					273	bananas, raw potato, MSG	1	0	0
8	1							11		0	0	0
9	1							25		0	0	0
10	1							112	wine, champagne, mustard, stonefruits	0	0	0
11	1	2.43	2	0.66	2	3.43	2	291		1	1	1
12	1			17.5	4			1827	avocado	0	1	0
13	0	0.26	0	0.66	1			141	bananas, avocados	1	1	0
14	1	11.79	3	5.96	3			244	banana,avocado,tomato	1	1	0
15	1							241	No	0	0	0
16	1							11		0	0	0
17	1			0.3	1	0.09	0	645	fish, crustaceans, peanuts	1	1	1
18	1	0.66	1	1.1	2			1708	No	0	0	0
19	1	1	1	2	2			7114	apple, avocado	0	1	0
20	1							5760	chocolate, eggs,yeast	0	0	0
21	1							148	No	0	0	0
22	0	0.09	0	0.09	0	0.09	0	129		0	0	0
23	0	0.09	0	0.09	0	0.09	0	808	No	0	0	0
24	1	0.09	0	0.25	1	0.1	0	96	avocado	0	1	0
25	1	0.29	1	0.19	1	1.62	2	78	nuts, chocolate, wheat, preservatives	0	0	0
26	1							1327		0	0	0
27	0									0	0	0
28	1							16		0	1	0
29	0	0.09	0	0.09	0	0.09	0	49		0	0	0
30	1							185		0	0	0
31	0							153		0	0	0
32	0							13		0	0	0
33	0	0.09	0	0.09	0	0.09	0	70		0	0	0
34	0	0.09	0	0.09	0	0.22	0	65	onions and garlic	0	0	1
35	0	0.09	0	0.09	0	0.09	0	115	pickled onions	0	0	0
36	1	0.79	2	0.09	0	0.09	0	157	bananas	1	0	0
37	0							42	prawns	0	0	0
38	1	0.09	0	2.48	2	0.62	1	2168		0	1	1
39	0				0			63		0	0	0
40	1	0.44	0	0.53	1			19	avocado	0	1	0
41	0							4		0	0	0
42	0	0.09	0				0	10	milk products	0	0	0
43	1	0.38	1	0.8	2	0.22	0	367	nuts, poppy seeds	0	0	0
44	0	0.09	0	0.09	0	0.09	0	1		0	0	0
45	1	0.09	0	0.09	0	0.09	0	110		0	0	0
46	1	0.22	1	1.33	2	0.09	1	579	wine	0	1	0
47	1	0.84	2	0.22	1	0.09	0	11810	chocolate, tomatoes,beef, dairy	1	1	1
48	1	1.75	2	2.18	2	0.51	1	1000	avocado	0	1	0
49	0							65		0	0	0
50	0							52		0	0	0
51	1	0.09	0	0.09	0	0.09	0	717		1	1	1

9. References

Abeck, D., M. Borries, C. Kuwert, V. Steinkraus, D. Vieluf and J. Ring (1994). "Food-induced anaphylaxis in latex allergy." Hautarzt **45**(6): 364-7.

Abramson, M. J., R. M. Puy and J. M. Weiner (2000). "Allergen immunotherapy for asthma." Cochrane Database Syst Rev **2**.

Adkinson, N. F., Jr. (2001). "Con: Immunotherapy is not clinically indicated in the management of allergic asthma." Am J Respir Crit Care Med **164**(12): 2140-1; discussion 2141-2.

Aichane, A., Z. Bouayad, A. el Outmani, H. Afif, N. Trombati and A. Bahlaoui (1997). "Latex allergy in a hospital setting. Results of a study in Casablanca." Rev Mal Respir **14**(6): 451-5.

Akasawa, A., L. S. Hsieh, B. M. Martin, T. Liu and Y. Lin (1996). "A novel acidic allergen, Hev b 5, in latex. Purification, cloning and characterization." J Biol Chem **271**(41): 25389-93.

Akdis, C. A. and K. Blaser (2001). "Mechanisms of interleukin-10-mediated immune suppression." Immunology **103**(2): 131-6.

Alenius, H., N. Kalkkinen, T. Reunala, K. Turjanmaa and T. Palosuo (1996). "The main IgE-binding epitope of a major latex allergen, prohevein, is present in its N-terminal 43-amino acid fragment, hevein." J Immunol **156**(4): 1618-25.

Alenius, H., S. Makinen-Kiljunen, M. Ahlroth, K. Turjanmaa, T. Reunala and T. Palosuo (1996). "Crossreactivity between allergens in natural rubber latex and banana studied by immunoblot inhibition." Clin Exp Allergy **26**(3): 341-8.

Alexander, C., W. L. G. Oldfield, K. Shirley, M. Larche and A. B. Kay (2001). "A Dosing Protocol of Allergen-derived T Cell Peptide

Epitopes for the Treatment of Allergic Disease." J Allergy Clin Immunol **102**(7): S217.

Allmers, H., R. Brehler, Z. Chen, M. Raulf-Heimsoth, H. Fels and X. Baur (1998). "Reduction of latex aeroallergens and latex-specific IgE antibodies in sensitized workers after removal of powdered natural rubber latex gloves in a hospital." J Allergy Clin Immunol **102**(5): 841-6.

Allmers, H., B. Kirchner, H. Huber, Z. Chen, J. W. Walther and X. Baur (1996). "The latency period between exposure and the symptoms in allergy to natural latex. Suggestions for prevention." Dtsch Med Wochenschr **121**(25-26): 823-8.

Allmers, H., J. Schmengler and C. Skudlik (2002). "Primary prevention of natural rubber latex allergy in the German health care system through education and intervention." J Allergy Clin Immunol **110**(2): 318-23.

Anliker, M. D., J. Reindl, S. Vieths and B. Wuthrich (2001). "Allergy caused by ingestion of persimmon (Diospyros kaki): detection of specific IgE and cross-reactivity to profilin and carbohydrate determinants." J Allergy Clin Immunol **107**(4): 718-23.

Antico, A. (1996). "Oral allergy syndrome induced by chestnut (Castanea sativa)." Ann Allergy Asthma Immunol **76**(1): 37-40.

Archambault, S., J. L. Malo, C. Infante-Rivard, H. Ghezzo and D. Gautrin (2001). "Incidence of sensitization, symptoms, and probable occupational rhinoconjunctivitis and asthma in apprentices starting exposure to latex." J Allergy Clin Immunol **107**(5): 921-3.

Arshad, S. H., K. S. Babu and S. T. Holgate (2001). Anti-IgE therapy in allergy and asthma. London, Martin Dunitz.

Axelsson, J. G., S. G. Johansson and K. Wrangsjo (1987). "IgE-mediated anaphylactoid reactions to rubber." Allergy **42**(1): 46-50.

Baldini, M., I. C. Lohman, M. Halonen, R. P. Erickson, P. G. Holt and F. D. Martinez (1999). "A Polymorphism in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E." Am J Respir Cell Mol Biol **20**(5): 976-83.

Baselmans, P. J., E. Pollabauer, F. C. van Reijsen, H. C. Heystek, A. Hren, P. Stumptner, M. G. Tilanus, W. C. Vooijs and G. C. Mudde (2000). "IgE production after antigen-specific and cognate activation of HLA- DPw4-restricted T-cell clones, by 78% of randomly selected B-cell donors." Hum Immunol **61**(8): 789-98.

Baur, X., Z. Chen and H. Allmers (1998). "Can a threshold limit value for natural rubber latex airborne allergens be defined?" J Allergy Clin Immunol **101**(1 Pt 1): 24-7.

Beasley, R., J. Crane, C. K. Lai and N. Pearce (2000). "Prevalence and etiology of asthma." J Allergy Clin Immunol **105**(2 Pt 2): S466-72.

Beezhold, D. H., V. L. Hickey, J. E. Slater and G. L. Sussman (1999). "Human IgE-binding epitopes of the latex allergen Hev b 5." J Allergy Clin Immunol **103**(6): 1166-72.

Beezhold, D. H., V. L. Hickey and G. L. Sussman (2001). "Mutational analysis of the IgE epitopes in the latex allergen Hev b 5." J Allergy Clin Immunol **107**(6): 1069-76.

Beezhold, D. H., J. E. Reschke, J. H. Allen, D. A. Kostyal and G. L. Sussman (2000). "Latex protein: a hidden "food" allergen?" Allergy Asthma Proc **21**(5): 301-6.

Belsito, D. V. (1990). "Contact urticaria caused by rubber. Analysis of seven cases." Dermatol Clin **8**(1): 61-6.

Bergamaschini, L., T. Santangelo, A. Faricciotti, N. Ciavarella, P. M. Mannucci and A. Agostoni (1996). "Study of complement-mediated anaphylaxis in humans. The role of IgG subclasses (IgG1 and/or IgG4)

in the complement-activating capacity of immune complexes." J Immunol **156**(3): 1256-61.

Birmingham, P. K., R. M. Dsida, J. J. Grayhack, J. Han, M. Wheeler, J. A. Pongracic, C. J. Cote and S. C. Hall (1996). "Do latex precautions in children with myelodysplasia reduce intraoperative allergic reactions?" J Pediatr Orthop **16**(6): 799-802.

Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger and D. C. Wiley (1987). "Structure of the human class I histocompatibility antigen, HLA-A2." Nature **329**(6139): 506-12.

Blanco, C., T. Carrillo, R. Castillo, J. Quiralte and M. Cuevas (1994). "Avocado hypersensitivity." Allergy **49**(6): 454-9.

Blanco, C., T. Carrillo, R. Castillo, J. Quiralte and M. Cuevas (1994). "Latex allergy: clinical features and cross-reactivity with fruits." Ann Allergy **73**(4): 309-14.

Blanco, C., A. Diaz-Perales, C. Collada, R. Sanchez-Monge, C. Aragoncillo, R. Castillo, N. Ortega, M. Alvarez, T. Carrillo and G. Salcedo (1999). "Class I chitinases as potential panallergens involved in the latex- fruit syndrome." J Allergy Clin Immunol **103**(3 Pt 1): 507-13.

Bode, C. P., U. Fullers, S. Roseler, A. Wawer, C. Bachert and V. Wahn (1996). "Risk factors for latex hypersensitivity in childhood." Pediatr Allergy Immunol **7**(4): 157-63.

Boehncke, W. H., C. Loeliger, P. Kuehnl, H. Kalbacher, B. O. Bohm and H. Gall (1998). "Identification of HLA-DR and -DQ alleles conferring susceptibility to pollen allergy and pollen associated food allergy." Clin Exp Allergy **28**(4): 434-41.

Bohle, B., B. Wagner, U. Vollmann, D. Buck, B. Niggemann, Z. Szepfalusi, G. Fischer, O. Scheiner, H. Breiteneder and C. Ebner (2000). "Characterization of T cell responses to Hev b 3, an allergen

associated with latex allergy in spina bifida patients." J Immunol **164**(8): 4393-8.

Borish, L. C., H. S. Nelson, M. J. Lanz, L. Claussen, J. B. Whitmore, J. M. Agosti and L. Garrison (1999). "Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial." Am J Respir Crit Care Med **160**(6): 1816-23.

Bousquet, J. (2001). "Allergic Rhinitis and Its Impact on Asthma." J Allergy Clin Immunol **108**: S147-336.

Boxer, M. (1996). "Hand dermatitis: a risk factor for latex hypersensitivity." J Allergy Clin Immunol **98**(4): 855-6.

Brown, J. H., T. S. Jardetzky, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger and D. C. Wiley (1993). "Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1." Nature **364**(6432): 33-9.

Brown, R. H., J. F. Schauble and R. G. Hamilton (1998). "Prevalence of latex allergy among anesthesiologists: identification of sensitized but asymptomatic individuals." Anesthesiology **89**(2): 292-9.

Bryan, S. A., B. J. O'Connor, S. Matti, M. J. Leckie, V. Kanabar, J. Khan, S. J. Warrington, L. Renzetti, A. Rames, J. A. Bock, M. J. Boyce, T. T. Hansel, S. T. Holgate and P. J. Barnes (2000). "Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response." Lancet **356**(9248): 2149-53.

Bubak, M. E. (2000). "Allergy to natural rubber latex." Ann Allergy Asthma Immunol **84**(2): 175.

Bungy, G. A., S. Rodda, I. Roitt and J. Brostoff (1994). "Mapping of T cell epitopes of the major fraction of rye grass using peripheral blood mononuclear cells from atopics and non-atopics. II. Isoallergen clone

5A of Lolium perenne group I (Lol p 1)." Eur J Immunol **24**(9): 2098-103.

Burton, M. D., B. Blaher, C. Suphioglu, R. E. O'Hehir, F. R. Carbone and J. M. Rolland (1999). "T-cell receptor contact and MHC binding residues of a major rye grass pollen allergen T-cell epitope." J Allergy Clin Immunol **103**(2 Pt 1): 255-61.

Burton, M. D., L. Papalia, N. P. Eusebius, E. O'Hehir R and J. Rolland (2002). "Characterisation of the human T cell response to rye grass pollen allergens Lol p 1 and Lol p 5." Allergy **In press**.

Busse, W. W. and R. F. Lemanske, Jr. (2001). "Asthma." N Engl J Med **344**(5): 350-62.

Car, B. D., V. M. Eng, B. Schnyder, L. Ozmen, S. Huang, P. Gailay, D. Heumann, M. Aguet and B. Ryffel (1994). "Interferon gamma receptor deficient mice are resistant to endotoxic shock." J Exp Med **179**(5): 1437-44.

Carballido, J. M., N. Carballido-Perrig, M. K. Kagi, R. H. Meloen, B. Wuthrich, C. H. Heusser and K. Blaser (1993). "T cell epitope specificity in human allergic and nonallergic subjects to bee venom phospholipase A2." J Immunol **150**(8 Pt 1): 3582-91.

Carswell, F., J. Oliver and J. Weeks (1999). "Do mite avoidance measures affect mite and cat airborne allergens?" Clin Exp Allergy **29**(2): 193-200.

Chapman, M. D., R. C. Aalberse, M. J. Brown and T. A. Platts-Mills (1988). "Monoclonal antibodies to the major feline allergen Fel d I. II. Single step affinity purification of Fel d I, N-terminal sequence analysis, and development of a sensitive two-site immunoassay to assess Fel d I exposure." J Immunol **140**(3): 812-8.

Chen, Z., R. Cremer, A. Posch, M. Raulf-Heimsoth, H. P. Rihs and X. Baur (1997). "On the allergenicity of Hev b 1 among health care

workers and patients with spina bifida allergic to natural rubber latex." J Allergy Clin Immunol **100**(5): 684-93.

Chen, Z., H. P. Rihs, J. E. Slater, E. J. Paupore, E. M. Schneider and X. Baur (2000). "The absence of Hev b 5 in capture antigen may cause false negative results in serologic assays for latex-specific IgE antibodies (abstract)." J Allergy Clin Immunol **105**: S83.

Chtanova, T., R. A. Kemp, A. P. Sutherland, F. Ronchese and C. R. Mackay (2001). "Gene microarrays reveal extensive differential gene expression in both CD4(+) and CD8(+) type 1 and type 2 T cells." J Immunol **167**(6): 3057-63.

Chung, K. F. and P. J. Barnes (1999). "Cytokines in asthma." Thorax **54**(9): 825-57.

Chung, S. Y. and E. T. Champagne (2001). "Association of end-product adducts with increased IgE binding of roasted peanuts." J Agric Food Chem **49**(8): 3911-6.

Cogswell, J. J. (2000). "Influence of maternal atopy on atopy in the offspring." Clin Exp Allergy **30**(1): 1-3.

Conde-Salazar, L., E. del-Rio and D. Guimareens (1993). "Type IV allergy to rubber additives: A 10 year study of 686 Cases." J Am Acad Dermatol **29**(176).

Cookson, W. (1999). "The alliance of genes and environment in asthma and allergy." Nature **402**(6760 Suppl): B5-11.

Cookson, W. O. C. and M. F. Moffat (2000). "Genetics of asthma and allergic disease." Human Mol Genetics **9**(16): 2354-64.

Cottrez, F., S. D. Hurst, R. L. Coffman and H. Groux (2000). "T regulatory cells 1 inhibit a Th2-specific response in vivo." J Immunol **165**(9): 4848-53.

Counsell, C. M., J. F. Bond, J. L. Ohman, Jr., J. L. Greenstein and R. D. Garman (1996). "Definition of the human T-cell epitopes of Fel d 1, the major allergen of the domestic cat." J Allergy Clin Immunol **98**(5 Pt 1): 884-94.

Creticos, P. S., S. L. Balcer, J. T. Schroeder, R. G. Hamilton, B. Chung, P. S. Norman, L. M. Lichtenstein and J. J. Eiden (2001). "Initial Immunotherapy Trial to Explore the Safety, Tolerability and Immunogenicity of Subcutaneous Injections of an Amb a 1 Immunostimulatory Oligonucleotide Conjugate (AIC) in Ragweed Allergic Adults." J Allergy Clin Immunol **107**(2): S216.

D'Andrea, A., X. Ma, M. Aste-Amezaga, C. Paganin and G. Trinchieri (1995). "Stimulatory and inhibitory effects of interleukin (IL)-4 and IL-13 on the production of cytokines by human peripheral blood mononuclear cells: priming for IL-12 and tumor necrosis factor alpha production." J Exp Med **181**(2): 537-46.

De Greef, J. M., F. Lieutier-Colas, J. C. Bessot, A. Verot, A. M. Gallerand, G. Pauli and F. de Blay (2001). "Urticaria and Rhinitis to Shrubs of Ficus benjamina and Breadfruit in a Banana-Allergic Road Worker: Evidence for a Cross-Sensitization between Moracea, Banana and Latex." Int Arch Allergy Immunol **125**(2): 182-4.

de Lalla, C., T. Sturniolo, L. Abbruzzese, J. Hammer, A. Sidoli, F. Sinigaglia and P. Panina-Bordignon (1999). "Cutting edge: identification of novel T cell epitopes in Lol p5a by computational prediction." J Immunol **163**(4): 1725-9.

de Paulis, A., F. Annunziato, L. Di Gioia, S. Romagnani, M. Carfora, C. Beltrame, G. Marone and P. Romagnani (2001). "Expression of the chemokine receptor CCR3 on human mast cells." Int Arch Allergy Immunol **124**(1-3): 146-50.

de Silva, H. D., M. F. Sutherland, C. Suphioglu, S. C. McLellan, J. E. Slater, J. M. Rolland and E. O'Hehir R (2000). "Human T-cell epitopes

of the latex allergen Hev b 5 in health care workers." J Allergy Clin Immunol **105**(5): 1017-24.

Del Prete, G., M. De Carl., F. Almerigogna, M. G. Giudizi, R. Biagiotti and S. Romagnani (1993). "Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production." J Immunol **150**(2): 353-60.

Dhillon, M., C. Roberts, T. Nunn and M. Kuo (1992). "Mapping human T cell epitopes on phospholipase A2: the major bee-venom allergen." J Allergy Clin Immunol **90**(1): 42-51.

Douglas, R., J. Morton, D. Czarny and R. E. O'Hehir (1997). "Prevalence of IgE-mediated allergy to latex in hospital nursing staff." Aust N Z J Med **27**(2): 165-9.

Dow, S. W., J. Schwarze, T. D. Heath, T. A. Potter and E. W. Gelfand (1999). "Systemic and local interferon gamma gene delivery to the lungs for treatment of allergen-induced airway hyperresponsiveness in mice." Hum Gene Ther **10**(12): 1905-14.

Duffy, D. L., N. G. Martin, D. Battistutta, J. L. Hopper and J. D. Mathews (1990). "Genetics of asthma and hay fever in Australian twins." Am Rev Respir Dis **142**(6 Pt 1): 1351-8.

Duque, S., L. Fernandez-Pellon and F. Rodriguez (1999). "Mango allergy in a latex-sensitized patient." Allergy **54**(9): 1004-5.

Durham, S. R., S. M. Walker, E. M. Varga, M. R. Jacobson, F. O'Brien, W. Noble, S. J. Till, Q. A. Hamid and K. T. Nouri-Aria (1999). "Long-term clinical efficacy of grass-pollen immunotherapy." N Engl J Med **341**(7): 468-75.

Ebo, D. G., W. J. Stevens, C. H. Bridts and L. S. De Clerck (1997). "Latex-specific IgE, skin testing, and lymphocyte transformation to latex in latex allergy." J Allergy Clin Immunol **100**(5): 618-23.

ECRHS (1996). "Variations in the prevalence of respiratory symptoms, self-reported asthma attacks, and use of asthma medication in the European Community Respiratory Health Survey (ECRHS)." Eur Respir J **9**(4): 687-95.

Ellsworth, P. I., P. A. Merguerian, R. B. Klein and A. A. Rozycki (1993). "Evaluation and risk factors of latex allergy in spina bifida patients: is it preventable?" J Urol **150**(2 Pt 2): 691-3.

Faith, A., J. A. Higgins, R. E. O'Hehir and J. R. Lamb (1995). "Differential dependence of TH-0, TH-1 and TH-2 CD4+ T cells on co-stimulatory activity provided by the accessory molecule LFA-1." Clin Exp Allergy **25**(12): 1163-70.

Ferreira, F., C. Ebner, B. Kramer, G. Casari, P. Briza, A. J. Kungl, R. Grimm, B. Jahn-Schmid, H. Breiteneder, D. Kraft, M. Breitenbach, H. J. Rheinberger and O. Scheiner (1998). "Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy." Faseb J **12**(2): 231-42.

Fiocchi, A., P. Restani, C. Ballabio, G. R. Bouygue, A. Serra, M. Travaini and L. Terracciano (2001). "Severe anaphylaxis induced by latex as a contaminant of plastic balls in play pits." J Allergy Clin Immunol **108**(2): 298-300.

Forstrom, L. (1980). "Contact urticaria from latex surgical gloves." Contact Dermatitis **6**(1): 33-4.

Foster, P. S., S. P. Hogan, A. J. Ramsay, K. I. Matthaei and I. G. Young (1996). "Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model." J Exp Med **183**(1): 195-201.

Fowler, M. R., J. Gartland, W. Norton, A. Slater, M. C. Elliott and N. W. Scott (2000). "RS2: a sugar beet gene related to the latex allergen Hev b 5 family." J Exp Bot **51**(353): 2125-6.

Fuchs, T., S. Spitzauer, C. Vente, J. Hevler, S. Kapiotis, H. Rumpold, D. Kraft and R. Valenta (1997). "Natural latex, grass pollen, and weed pollen share IgE epitopes." J Allergy Clin Immunol **100**(3): 356-64.

Galinsky, T. and D. Kleinhans (1982). "[Contact urticaria due to rubber gloves]." Derm Beruf Umwelt **30**(4): 118-20.

Garcia Ortiz, J. C., J. C. Moyano, M. Alvarez and J. Bellido (1998). "Latex allergy in fruit-allergic patients." Allergy **53**(5): 532-6.

Ghaemmaghami, A. M., A. Robins, L. Gough, H. F. Sewell and F. Shakib (2001). "Human T cell subset commitment determined by the intrinsic property of antigen: the proteolytic activity of the major mite allergen Der p 1 conditions T cells to produce more IL-4 and less IFN-gamma." Eur J Immunol **31**(4): 1211-6.

Gimmi, C. D., G. J. Freeman, J. G. Gribben, G. Gray and L. M. Nadler (1993). "Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation." Proc Natl Acad Sci U S A **90**(14): 6586-90.

Goding, J. (1996). Monoclonal Antibodies: principles and practice. London, Academic Press.

Gosset, P., C. Lamblin-Degros, I. Tillie-Leblond, A. S. Charbonnier, M. Joseph, B. Wallaert, J. P. Kochan and A. B. Tonnel (2001). "Modulation of high-affinity IgE receptor expression in blood monocytes: opposite effect of IL-4 and glucocorticoids." J Allergy Clin Immunol **107**(1): 114-22.

Graham, J. A., P. K. Pavlicek, J. K. Sercombe, M. L. Xavier and E. R. Tovey (2000). "The nasal air sampler: a device for sampling inhaled aeroallergens." Ann Allergy Asthma Immunol **84**(6): 599-604.

Granady, L. C. and J. E. Slater (1995). "The history and diagnosis of latex allergy." Immunol Allergy Clin North America **15**(1): 21-30.

Graves, P. E., M. Kabesch, M. Halonen, C. J. Holberg, M. Baldini, C. Fritzsch, S. K. Weiland, R. P. Erickson, E. von Mutius and F. D. Martinez (2000). "A cluster of seven tightly linked polymorphisms in the IL-13 gene is associated with total serum IgE levels in three populations of white children." J Allergy Clin Immunol **105**(3): 506-13.

Grote, M., V. Mahler, S. Spitzauer, T. Fuchs, R. Valenta and R. Reichelt (2000). "In situ localization of latex allergens in 3 different brands of latex gloves by means of immunogold field emission scanning and transmission electron microscopy." J Allergy Clin Immunol **105**(3): 561-9.

Grunewald, S. M., A. Werthmann, B. Schnarr, C. E. Klein, E. B. Brocker, M. Mohrs, F. Brombacher, W. Sebald and A. Duschl (1998). "An antagonistic IL-4 mutant prevents type I allergy in the mouse: inhibition of the IL-4/IL-13 receptor system completely abrogates humoral immune response to allergen and development of allergic symptoms in vivo." J Immunol **160**(8): 4004-9.

Grzybowski, M., D. R. Ownby, P. A. Peyser, C. C. Johnson and M. A. Schork (1996). "The prevalence of anti-latex IgE antibodies among registered nurses." J Allergy Clin Immunol **98**(3): 535-44.

Hack, M. E. (2001). "The prevalence of latex allergy in operating theatre staff." Anaesth Intensive Care **29**(1): 43-7.

Hamilton, R. G. and N. F. Adkinson, Jr. (1998). "Diagnosis of natural rubber latex allergy: multicenter latex skin testing efficacy study. Multicenter Latex Skin Testing Study Task Force." J Allergy Clin Immunol **102**(3): 482-90.

Hamilton, R. G., R. E. Biagini and E. F. Krieg (1999). "Diagnostic performance of Food and Drug Administration-cleared serologic assays for natural rubber latex-specific IgE antibody. The Multi-Center Latex Skin Testing Study Task Force." J Allergy Clin Immunol **103**(5 Pt 1): 925-30.

Hamilton, R. G. and R. H. Brown (2000). "Impact of personal avoidance practices on health care workers sensitized to natural rubber latex." J Allergy Clin Immunol **105**(4): 839-41.

Hanifin, J. M., L. C. Schneider, D. Y. Leung, C. N. Ellis, H. S. Jaffe, A. E. Izu, L. R. Bucalo, S. E. Hirabayashi, S. J. Tofte, G. Cantu-Gonzales and et al. (1993). "Recombinant interferon gamma therapy for atopic dermatitis." J Am Acad Dermatol **28**(2 Pt 1): 189-97.

Hansen, G., J. J. McIntire, V. P. Yeung, G. Berry, G. J. Thorbecke, L. Chen, R. H. DeKruyff and D. T. Umetsu (2000). "CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation." J Clin Invest **105**(1): 61-70.

Harlow, E. and D. Lane (1988). Antibodies. New York, Cold Spring Harbor Laboratory.

Harris, N., R. Peach, J. Naemura, P. S. Linsley, G. Le Gros and F. Ronchese (1997). "CD80 costimulation is essential for the induction of airway eosinophilia." J Exp Med **185**(1): 177-82.

Harvey, P., S. Sperber, F. Kette, R. J. Heddle and P. J. Roberts-Thomson (1984). "Bee-sting mortality in Australia." Med J Aust **140**(4): 209-11.

Haselden, B. M., A. B. Kay and M. Larche (1999). "Immunoglobulin E-independent major histocompatibility complex- restricted T cell peptide epitope-induced late asthmatic reactions." J Exp Med **189**(12): 1885-94.

Haselden, B. M., E. Syrigou, M. Jones, D. Huston, K. Ichikawa, M. D. Chapman, A. B. Kay and M. Larche (2001). "Proliferation and release of IL-5 and IFN-gamma by peripheral blood mononuclear cells from cat-allergic asthmatics and rhinitics, non-cat- allergic asthmatics, and normal controls to peptides derived from Fel d 1 chain 1." J Allergy Clin Immunol **108**(3): 349-56.

Hayden, C., E. Pereira, P. Rye, L. Palmer, N. Gibson, M. Palenque, I. Hagel, N. Lynch, J. Goldblatt and P. Lesouef (1997). "Mutation screening of interferon-gamma (IFNgamma) as a candidate gene for asthma." Clin Exp Allergy **27**(12): 1412-6.

Heilman, D. K., R. T. Jones, M. C. Swanson and J. W. Yunginger (1996). "A prospective, controlled study showing that rubber gloves are the major contributor to latex aeroallergen levels in the operating room." J Allergy Clin Immunol **98**(2): 325-30.

Hernandez-Arana, A., A. Rojo-Dominguez, M. Soriana-Garcia and A. Rodriguez-Romero (1995). "The thermal unfolding of hevein, a small disulfide-rich protein." Eur J Biochem **228**: 649-652.

Higgins, J. A., C. J. Thorpe, J. D. Hayball, R. E. O'Hehir and J. R. Lamb (1994). "Overlapping T-cell epitopes in the group I allergen of Dermatophagoides species restricted by HLA-DP and HLA-DR class II molecules." J Allergy Clin Immunol **93**(5): 891-9.

Hnizdo, E., T. M. Esterhuizen, D. Rees and U. G. Lalloo (2001). "Occupational asthma as identified by the Surveillance of Work-related and Occupational Respiratory Diseases programme in South Africa." Clin Exp Allergy **31**(1): 32-9.

Hogan, S. P., K. I. Matthaei, J. M. Young, A. Koskinen, I. G. Young and P. S. Foster (1998). "A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5." J Immunol **161**(3): 1501-9.

Holt, P. G. and C. Macaubas (1997). "Development of long-term tolerance versus sensitisation to environmental allergens during the perinatal period." Curr Opin Immunol **9**(6): 782-7.

Hoyne, G., A. Jarnicki, W. Thomas and J. Lamb (1997). "Characterization of the specificity and duration of T cell tolerance to

intranasally administered peptides in mice: a role for intramolecular epitope suppression." Int Immunol 9: 1165-73.

Ikagawa, S., S. Matsushita, Y. Z. Chen, T. Ishikawa and Y. Nishimura (1996). "Single amino acid substitutions on a Japanese cedar pollen allergen (Cry j 1)-derived peptide induced alterations in human T cell responses and T cell receptor antagonism." J Allergy Clin Immunol **97**(1 Pt 1): 53-64.

ISAAC (1998). "Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee." Lancet **351**(9111): 1225-32.

Janeway, C. A., P. Travers, M. Walport and M. Shlomchik (2001). Immunobiology. New York, Garland Publishing.

Johnson, B. D., V. P. Kurup, G. L. Sussman, S. A. Arif, K. J. Kelly, D. H. Beezhold and J. N. Fink (1999). "Purified and recombinant latex proteins stimulate peripheral blood lymphocytes of latex allergic patients." Int Arch Allergy Immunol **120**(4): 270-9.

Joss, A., M. Akdis, A. Faith, K. Blaser and C. A. Akdis (2000). "IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway." Eur J Immunol **30**(6): 1683-90.

Kalliomaki, M., S. Salminen, H. Arvilommi, P. Kero, P. Koskinen and E. Isolauri (2001). "Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial." Lancet **357**(9262): 1076-9.

Kammerer, R., A. Kettner, Y. Chvatchko, N. Dufour, J. M. Tiercy, G. Corradin and F. Spertini (1997). "Delineation of PLA2 epitopes using short or long overlapping synthetic peptides: interest for specific immunotherapy." Clin Exp Allergy **27**(9): 1016-26.

Katelaris, C. H., R. P. Widmer and R. M. Lazarus (1996). "Prevalence of latex allergy in a dental school." Med J Aust **164**(12): 711-4.

Kay, A. B. (2001). "Allergy and allergic diseases. First of two parts." N Engl J Med **344**(1): 30-7.

Kay, A. B. (2001). "Allergy and allergic diseases. Second of two parts." N Engl J Med **344**(2): 109-13.

Keane-Myers, A., W. C. Gause, P. S. Linsley, S. J. Chen and M. Wills-Karp (1997). "B7-CD28/CTLA-4 costimulatory pathways are required for the development of T helper cell 2-mediated allergic airway responses to inhaled antigens." J Immunol **158**(5): 2042-9.

Kelly, K. J., V. Kurup, M. Zacharisen, A. Resnick and J. N. Fink (1993). "Skin and serologic testing in the diagnosis of latex allergy." J Allergy Clin Immunol **91**(6): 1140-5.

Kelly, K. J., M. L. Pearson, V. P. Kurup, P. L. Havens, R. S. Byrd, M. A. Setlock, J. C. Butler, J. E. Slater, L. C. Grammer, A. Resnick and et al. (1994). "A cluster of anaphylactic reactions in children with spina bifida during general anesthesia: epidemiologic features, risk factors, and latex hypersensitivity." J Allergy Clin Immunol **94**(1): 53-61.

Kelly, K. J., G. Sussman and J. N. Fink (1996). "Rostrum. Stop the sensitization." J Allergy Clin Immunol **98**(5 Pt 1): 857-8.

Kim, K. T. and H. Hussain (1999). "Prevalence of food allergy in 137 latex-allergic patients." Allergy Asthma Proc **20**(2): 95-7.

Kim, K. T. and G. S. Safadi (1999). "Relation of latex-specific IgE titer and symptoms in patients allergic to latex." J Allergy Clin Immunol **103**(4): 671-7.

King, T. P., D. Hoffman, H. Lowenstein, D. G. Marsh, T. A. Platts-Mills and W. Thomas (1994). "Allergen nomenclature. WHO/IUIS Allergen Nomenclature Subcommittee." Int Arch Allergy Immunol **105**(3): 224-33.

King, T. P. and M. D. Spangfort (2000). "Structure and biology of stinging insect venom allergens." Int Arch Allergy Immunol **123**(2): 99-106.

Kips, J. C., G. J. Brusselle, G. F. Joos, R. A. Peleman, J. H. Tavernier, R. R. Devos and R. A. Pauwels (1996). "Interleukin-12 inhibits antigen-induced airway hyperresponsiveness in mice." Am J Respir Crit Care Med **153**(2): 535-9.

Kita, H., Z. X. Lian, J. Van de Water, X. S. He, S. Matsumura, M. Kaplan, V. Luketic, R. L. Coppel, A. A. Ansari and M. E. Gershwin (2002). "Identification of HLA-A2-restricted CD8(+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells." J Exp Med **195**(1): 113-23.

Kohler, G. and C. Milstein (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity." Nature **256**(5517): 495-7.

Kondo, N., E. Matsui, H. Kaneko, T. Fukao, T. Teramoto, R. Inoue, M. Watanabe, K. Kasahara and N. Morimoto (2001). "Reduced interferon-gamma production and mutations of the interleukin-12 receptor beta(2) chain gene in atopic subje ts." Int Arch Allergy Immunol **124**(1-3): 117-20.

Kopf, M., G. Le Gros, M. Bachmann, M. C. Lamers, H. Bluethmann and G. Kohler (1993). "Disruption of the murine IL-4 gene blocks Th2 cytokine responses." Nature **362**(6417): 245-8.

Koppelman, G. H., N. E. Reijmerink, O. Colin Stine, T. D. Howard, P. A. Whittaker, D. A. Meyers, D. S. Postma and E. R. Bleecker (2001). "Association of a promoter polymorphism of the CD14 gene and atopy." Am J Respir Crit Care Med **163**(4): 965-9.

Kulkarni, A. B., C. G. Huh, D. Becker, A. Geiser, M. Lyght, K. C. Flanders, A. B. Roberts, M. B. Sporn, J. M. Ward and S. Karlsson

(1993). "Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death." Proc Natl Acad Sci U S A **90**(2): 770-4.

Kurtz, K. M., R. G. Hamilton, J. A. Schaefer and N. F. Adkinson, Jr. (2001). "A hooded exposure chamber method for semiquantitative latex aeroallergen challenge." J Allergy Clin Immunol **107**(1): 178-84.

Kurup, V. P., H. Y. Yeang, G. L. Sussman, N. K. Bansal, D. H. Beezhold, K. J. Kelly, D. R. Hoffman, B. Williams and J. N. Fink (2000). "Detection of immunoglobulin antibodies in the sera of patients using purified latex allergens." Clin Exp Allergy **30**(3): 359-69.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(259): 680-5.

Lagging, E., M. van Hage-Hamsten, R. Gronneberg, L. Elfman and B. Harfast (1998). "Cytokine production in PBMC from allergics and non-allergics following in vitro allergen stimulation." Immunol Lett **60**(1): 45-9.

Lagier, F., M. Badier, J. Martigny, D. Charpin and D. Vervloet (1990). "Latex as aeroallergen." Lancet **336**(8713): 516-7.

Laver, G., G. M. Air, R. G. Webster and S. J. Smith-Gill (1990). "Epitopes on protein antigens:misconceptions and realities." Cell **61**: 553-6.

Leckie, M. J., A. ten Brinke, J. Khan, Z. Diamant, B. J. O'Connor, C. M. Walls, A. K. Mathur, H. C. Cowley, K. F. Chung, R. Djukanovic, T. T. Hansel, S. T. Holgate, P. J. Sterk and P. J. Barnes (2000). "Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response." Lancet **356**(9248): 2144-8.

Ledger, S. E. and R. C. Gardner (1994). "Cloning and characterization of five cDNAs for genes differentially expressed during fruit

development of kiwifruit (Actinidia deliciosa var. deliciosa)." Plant Mol Biol **25**(5): 877-86.

Leonard, J. P., M. L. Sherman, G. L. Fisher, L. J. Buchanan, G. Larsen, M. B. Atkins, J. A. Sosman, J. P. Dutcher, N. J. Vogelzang and J. L. Ryan (1997). "Effects of single-dose interleukin-12 exposure on interleukin-12- associated toxicity and interferon-gamma production." Blood **90**(7): 2541-8.

Leung, R., A. Ho, J. Chan, D. Choy and C. K. Lai (1997). "Prevalence of latex allergy in hospital staff in Hong Kong." Clin Exp Allergy **27**(2): 167-74.

Levings, M. K. and M. G. Roncarolo (2000). "T-regulatory 1 cells: a novel subset of CD4 T cells with immunoregulatory properties." J Allergy Clin Immunol **106**(1 Pt 2): S109-12.

Levy, D. A., N. Mounedji, C. Noirot and F. Leynadier (2000). "Allergic sensitization and clinical reactions to latex, food and pollen in adult patients." Clin Exp Allergy **30**(2): 270-5.

Leynadier, F., D. Herman, D. Vervloet and C. Andre (2000). "Specific immunotherapy with a standardized latex extract versus placebo in allergic healthcare workers." J Allergy Clin Immunol **106**(3): 585-90.

Liew, F. Y. (2002). "Th1 and Th2 cells: a historical perspective." Nat Rev Immunol **2**: 55-60.

Liss, G. M. and G. L. Sussman (1999). "Latex sensitization: occupational versus general population prevalence rates." Am J Ind Med **35**(2): 196-200.

Liss, G. M., G. L. Sussman, K. Deal, S. Brown, M. Cividino, S. Siu, D. H. Beezhold, G. Smith, M. C. Swanson, J. Yunginger, A. Douglas, D. L. Holness, P. Lebert, P. Keith, S. Wasserman and K. Turjanmaa (1997). "Latex allergy: epidemiological study of 1351 hospital workers." Occup Environ Med **54**(5): 335-42.

Luczynska, C. M., L. K. Arruda, T. A. Platts-Mills, J. D. Miller, M. Lopez and M. D. Chapman (1989). "A two-site monoclonal antibody ELISA for the quantification of the major Dermatophagoides spp. allergens, Der p I and Der f I." J Immunol Methods **118**(2): 227-35.

Lundberg, M., Z. Chen, H. Rihs and K. Wrangsjo (2001). "Recombinant spiked allergen extract." Allergy **56**: 794-5.

Macaubas, C., S. L. Prescott, T. J. Venaille, B. J. Holt, T. B. Smallacombe, P. D. Sly and P. G. Holt (2000). "Primary sensitization to inhalant allergens." Pediatr Allergy Immunol **11**(Suppl 13): 9-11.

Madsen, K. L. (2001). "Inflammatory bowel disease: lessons from the IL-10 gene-deficient mouse." Clin Invest Med **24**(5): 250-7.

Magone, M. T., C. C. Chan, L. Beck, S. M. Whitcup and E. Raz (2000). "Systemic or mucosal administration of immunostimulatory DNA inhibits early and late phases of murine allergic conjunctivitis." Eur J Immunol **30**(7): 1841-50.

Magram, J., S. E. Connaughton, R. R. Warrier, D. M. Carvajal, C. Y. Wu, J. Ferrante, C. Stewart, U. Sarmiento, D. A. Faherty and M. K. Gately (1996). "IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses." Immunity **4**(5): 471-81.

Mahler, V., S. Fischer, T. Fuchs, M. Ghannadan, P. Valent, M. Fartasch, D. Kraft, G. Schuler and R. Valenta (2000). "Prevention of latex allergy by selection of low-allergen gloves." Clin Exp Allergy **30**(4): 509-20.

Makela, M. J., A. Kanehiro, L. Borish, A. Dakhama, J. Loader, A. Joetham, Z. Xing, M. Jordana, G. L. Larsen and E. W. Gelfand (2000). "IL-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization." Proc Natl Acad Sci U S A **97**(11): 6007-12.

Maleki, S. J., S. Y. Chung, E. T. Champagne and J. P. Raufmann (2000). "The effects of roasting on the allergenic properties of peanut proteins." J Allergy Clin Immunol **106**: 763-8.

Maleki, S. J., R. A. Kopper, D. S. Shin, C. W. Park, C. M. Compadre, H. Sampson, A. W. Burks and G. A. Bannon (2000). "Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation." J Immunol **164**(11): 5844-9.

Marshall, J. D., S. Abtahi, J. J. Eiden, S. Tuck, R. Milley, F. Haycock, M. J. Reid, A. Kagey-Sobotka, P. S. Creticos, L. M. Lichtenstein and G. Van Nest (2001). "Immunostimulatory sequence DNA linked to the Amb a 1 allergen promotes TH1 cytokine expression while downregulating TH2 cytokine expression in PBMCs from human patients with ragweed allergy." J Allergy Clin Immunol **108**(2 Pt 1): 191-7.

Mattes, J., M. Yang, A. Siqueira, K. Clark, J. MacKenzie, A. N. McKenzie, D. C. Webb, K. I. Matthaei and P. S. Foster (2001). "IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung." J Immunol **167**(3): 1683-92.

Matzinger, P. (1994). "Tolerance, danger, and the extended family." Annu Rev Immunol **12**: 991-1045.

Maurer, D., C. Ebner, B. Reininger, E. Fiebiger, D. Kraft, J. P. Kinet and G. Stingl (1995). "The high affinity IgE receptor (Fc epsilon RI) mediates IgE-dependent allergen presentation." J Immunol **154**(12): 6285-90.

Mazon, A., A. Nieto, J. J. Linana, J. Montoro, F. Estornell and F. Garcia-Ibarra (2000). "Latex sensitization in children with spina bifida: follow-up comparative study after two years." An. Allergy Asthma Immunol **84**(2): 207-10.

McDonald, J. C., H. L. Keynes and S. K. Meredith (2000). "Reported incidence of occupational asthma in the united kingdom, 1989- 97." Occup Environ Med **57**(12): 823-9.

McKenzie, G. J., P. G. Fallon, C. L. Emson, R. K. Grencis and A. N. McKenzie (1999). "Simultaneous disruption of interleukin (IL)-4 and IL-13 defines individual roles in T helper cell type 2-mediated responses." J Exp Med **189**(10): 1565-72.

Meding, B. and S. Fregert (1984). "Contact urticaria from natural latex gloves." Contact Dermatitis **10**(1): 52-3.

Miguel, A. G., G. R. Cass, J. Weiss and M. M. Glovsky (1996). "Latex allergens in tire dust and airborne particles." Environ Health Perspect **104**(11): 1180-6.

Mikkola, J. H., H. Alenius, N. Kalkkinen, K. Turjanmaa, T. Palosuo and T. Reunala (1998). "Hevein-like protein domains as a possible cause for allergen cross- reactivity between latex and banana." J Allergy Clin Immunol **102**(6 Pt 1): 1005-12.

Milgrom, H., R. B. Fick, Jr., J. Q. Su, J. D. Reimann, R. K. Bush, M. L. Watrous and W. J. Metzger (1999). "Treatment of allergic asthma with monoclonal anti-IgE antibody. rhuMAb- E25 Study Group." N Engl J Med **341**(26): 1966-73.

Minty, A., P. Chalon, J. M. Derocq, X. Dumont, J. C. Guillemot, M. Kaghad, C. Labit, P. Leplatois, P. Liauzun, B. Miloux and et al. (1993). "Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses." Nature **362**(6417): 248-50.

Mitakakis, T. Z., E. R. Tovey, D. H. Yates, B. G. Toelle, A. Johnson, M. F. Sutherland, E. O'Hehir R and G. B. Marks (2002). "Particulate masks and non-powdered gloves reduce latex allergen inhaled by healthcare workers." Clin Exp Allergy **32**: 1166-69.

Mochizuki, M., J. Bartels, A. I. Mallet, E. Christophers and J. M. Schroder (1998). "IL-4 induces eotaxin: a possible mechanism of selective eosinophil recruitment in helminth infection and atopy." J Immunol **160**(1): 60-8.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin and R. L. Coffman (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." J Immunol **136**(7): 2348-57.

Muller, U., C. A. Akdis, M. Fricker, M. Akdis, T. Blesken, F. Bettens and K. Blaser (1998). "Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom." J Allergy Clin Immunol **101**(6 Pt 1): 747-54.

Muller, W. D., T. Karamfilov, A. Bufe, B. Fahlbush, I. Wolf and L. Jager (1996). "Group 5 allergens of timothy grass (Phl p 5) bear cross-reacting T cell epitopes with group 1 allergens of rye grass (Lol p 1)." Int Arch Allergy Immunol **109**(4): 352-5.

Nakamura, Y., S. Takagi, M. Suzuki, H. Ito, S. Murakami and N. Ohta (2001). "Survival of memory T cells specific for Japanese cypress pollen allergen is maintained by cross-stimulation of putative pectate lyases from other plants." Allergy **56**(5): 385-92.

Naya, A., K. Kobayashi, M. Ishikawa, K. Ohwaki, T. Saeki, K. Noguchi and N. Ohtake (2001). "Discovery of a novel CCR3 selective antagonist." Bioorg Med Chem Lett **11**(9): 1219-23.

Nelde, A., M. Teufel, C. Hahn, A. Duschl, W. Sebald, E. B. Brocker and S. M. Grunewald (2001). "The impact of the route and frequency of antigen exposure on the IgE response in allergy." Int Arch Allergy Immunol **124**(4): 461-9.

Nettis, E., P. Dambra, P. L. Traetta, M. P. Loria, A. Ferrannini and A. Tursi (2001). "Systemic reactions on SPT to latex." Allergy **56**(4): 355-6.

Niggemann, B., D. Buck, T. Michael, H. Haberl and U. Wahn (2000). "Latex allergy in spina bifida: at the turning point?" J Allergy Clin Immunol **106**(6): 1201.

Niggemann, B., M. Kulig, R. Bergmann and U. Wahn (1998). "Development of latex allergy in children up to 5 years of age: a retrospective analysis of risk factors." Pediatr Allergy Immunol **9**(1): 36-9.

Nixon, R. L. and A. Y. Lee (2001). "Latex allergy and food handlers." Med J Aust **174**(9): 482.

Noon, L. (1911). "Prophylactic inoculation against hay fever." Lancet: 1572-3.

Norman, P S., J. L. Ohman, Jr., A. A. Long, P. S. Creticos, M. A. Gefter, Z. Shaked, R. A. Wood, P. A. Eggleston, K. B. Hafner, P. Rao, L. M. Lichtenstein, N. H. Jones and C. F. Nicodemus (1996). "Treatment of cat allergy with T-cell reactive peptides." Am J Respir Crit Care Med **154**(6 Pt 1): 1623-8.

Nucera, E., D. Schiavino, A. Buonomo, C. Roncallo, M. Del Ninno, A. Milani, E. Pollastrini and G. Patriarca (2001). "Latex rush desensitization." Allergy **56**(1): 86-7.

Nutter, A. F. (1979). "Contact urticaria to rubber." Br J Dermatol **101**(5): 597-8.

O'Brien, R. M., H. Xu, J. M. Rolland, K. A. Byron and W. R. Thomas (2000). "Allergen-specific production of interferon-gamma by peripheral blood mononuclear cells and CD8 T cells in allergic disease and following immunotherapy." Clin Exp Allergy **30**(3): 333-40.

Ochi, H., N. H. De Jesus, F. H. Hsieh, K. F. Austen and J. A. Boyce (2000). "IL-4 and -5 prime human mast cells for different profiles of IgE- dependent cytokine production." Proc Natl Acad Sci U S A **97**(19): 10509-13.

O'Hehir, R. E., A. Verhoef, E. Panagiotopoulou, S. Keswani, J. D. Hayball, W. R. Thomas and J. R. Lamb (1993). "Analysis of human T cell responses to the group II allergen of Dermatophagoides species: localization of major antigenic sites." J Allergy Clin Immunol **92**(1 Pt 1): 105-13.

Oldfield, W. L. G., K. Shirley, M. Larche and A. B. Kay (2001). "A Double-blind, Placebo-controlled Study of Short Peptides Derived from Fel d 1 in Cat Allergic Subjects." J Allergy Clin Immunol **107**(2): S216.

O'Mahony, L., M. Feeney, S. O'Halloran, L. Murphy, B. Kiely, J. Fitzgibbon, G. Lee, G. O'Sullivan, F. Shanahan and J. K. Collins (2001). "Probiotic impact on microbial flora, inflammation and tumour development in IL-10 knockout mice." Aliment Pharmacol Ther **15**(8): 1219-25.

O'Riordain, G., C. Radauer, S. Wagner, C. Blanco, K. Hoffman-Sommergruber, O. Scheiner, C. Ebner and H. Breiteneder (2001). "Hev B 11, a Class I Chitinase From Hevea Brasiliensis, and Its Cross Reactivity With Hev B 6.02, Hevein." J Allergy Clin Immunol **107**(2): S57.

Ownby, D. R., B. Magera and P. B. Williams (2000). "A blinded, multi-center evaluation of two commercial in vitro tests for latex-specific IgE antibodies." Ann Allergy Asthma Immunol **84**(2): 193-6.

Ownby, D. R., M. Tomlanovich, N. Sammons and J. McCullough (1991). "Anaphylaxis associated with latex allergy during barium enema examinations." AJR Am J Roentgenol **156**(5): 903-8.

Palosuo, T., H. Alenius and K. Turjanmaa (2002). "Quantitation of latex allergens." Methods **27**(1): 52-8.

Palosuo, T., S. Makinen-Kiljunen, H. Alenius, T. Reunala, E. Yip and K. Turjanmaa (1998). "Measurement of natural rubber latex allergen levels in medical gloves by allergen-specific IgE-ELISA inhibition, RAST inhibition, and skin prick test." Allergy **53**(1): 59-67.

Parronchi, P., D. Macchia, M. P. Piccinni, P. Biswas, C. Simonelli, E. Maggi, M. Ricci, A. A. Ansari and S. Romagnani (1991). "Allergen- and bacterial antigen-specific T-cell clones established from atopic donors show a different profile of cytokine production." Proc Natl Acad Sci U S A **88**(10): 4538-42.

Pereira, C., P. Rico, M. Lourenco, M. Lombardero, J. Pinto-Mendes and C. Chieira (1999). "Specific immunotherapy for occupational latex allergy." Allergy **54**(3): 291-3.

Pohl, D., C. Bockelmann, K. Forster, C. H. Rieger and U. Schauer (1997). "Neonates at risk of atopy show impaired production of interferon-gamma after stimulation with bacterial products (LPS and SEE)." Allergy **52**(7): 732-8.

Poley, G. E., Jr. and J. E. Slater (2000). "Latex allergy." Journal of Allergy & Clinical Immunology **105**(6 Pt 1): 1054-62.

Porri, F., M. Pradal, C. Lemiere, J. Birnbaum, J. L. Mege, A. Lanteaume, D. Charpin, D. Vervloet and J. Camboulives (1997). "Association between latex sensitization and repeated latex exposure in children." Anesthesiology **86**(3): 599-602.

Prescott, S. L., C. Macaubas, T. Smallacombe, B. J. Holt, P. D. Sly and P. G. Holt (1999). "Development of allergen-specific T-cell memory in atopic and normal children." Lancet **353**(9148): 196-200.

Prescott, S. L., C. Macaubas, T. Smallacombe, B. J. Holt, P. D. Sly, R. Loh and P. G. Holt (1998). "Reciprocal age-related patterns of

allergen-specific T-cell immunity in normal vs. atopic infants." Clin Exp Allergy **28 Suppl 5**: 39-44; discussion 50-1.

Primeau, M. N., N. F. Adkinson, Jr. and R. G. Hamilton (2001). "Natural rubber pharmaceutical vial closures release latex allergens that produce skin reactions." J Allergy Clin Immunol **107**(6): 958-62.

Pumphrey, R. S., M. Duddridge and J. Norton (2001). "Fatal latex allergy." J Allergy Clin Immunol **107**(3): 558.

Ramachandram, S., R. Thompson, A. Gam and F. Neva (1998). "Recombinant cDNA clones for the immunodiagnosis of strongyloidiasis." J Infect Dis **177**: 196-203.

Raulf-Heimsoth, M., Z. Chen, H. P. Rihs, H. Kalbacher, V. Liebers and X. Baur (1998). "Analysis of T-cell reactive regions and HLA-DR4 binding motifs on the latex allergen Hev b 1 (rubber elongation factor)." Clin Exp Allergy **28**(3): 339-48.

Raulf-Heimsoth, M., I. Sander, Z. Chen, G. Borowitzki, K. Diewald, V. van Kampen and X. Baur (2000). "Development of a monoclonal antibody-based sandwich ELISA for detection of the latex allergen Hev b 1." Int Arch Allergy Immunol **123**(3): 236-41.

Reindl, J., M. D. Anliker, F. Karamloo, S. Vieths and B. Wuthrich (2000). "Allergy caused by ingestion of zucchini (Cucurbita pepo): characterization of allergens and cross-reactivity to pollen and other foods." J Allergy Clin Immunol **106**(2): 379-85.

Reinherz, E. L., K. Tan, L. Tang, P. Kern, J. Liu, Y. Xiong, R. E. Hussey, A. Smolyar, B. Hare, R. Zhang, A. Joachimiak, H. C. Chang, G. Wagner and J. Wang (1999). "The crystal structure of a T cell receptor in complex with peptide and MHC class II." Science **286**(5446): 1913-21.

Rich, P., M. L. Belozer, P. Norris and F. J. Storrs (1991). "Allergic contact dermatitis to two antioxidants in latex gloves: 4,4'- thiobis(6-

tert-butyl-meta-cresol) (Lowinox 44S36) and butylhydroxyanisole. Allergen alternatives for glove-allergic patients." J Am Acad Dermatol **24**(1): 37-43.

Rihs, H. P., Z. Chen, R. Cremer and X. Baur (1997). "HLA class II antigens DR4 and DQ8 are associated with allergy to hevein, a major allergen of Hevea latex." Tissue Antigens **49**(1): 92-5.

Rihs, H. P., Z. Chen, P. Rozynek, X. Baur, M. Lundberg and R. Cremer (2000). "PCR-based cloning, isolation, and IgE-binding properties of recombinant latex profilin (rHev b 8) [In Process Citation]." Allergy **55**(8): 712-7.

Rihs, H. P., R. Cremer, Z. Chen and X. Baur (1998). "Molecular analysis of DRB and DQB1 alleles in German spina bifida patients with and without IgE responsiveness to the latex major allergen Hev b 1." Clin Exp Allergy **28**(2): 175-80.

Robertson, C. F., M. F. Dalton, J. K. Peat, M. M. Haby, A. Bauman, J. D. Kennedy and L. I. Landau (1998). "Asthma and other atopic diseases in Australian children. Australian arm of the International Study of Asthma and Allergy in Childhood." Med J Aust **168**(9): 434-8.

Rolland, J. and R. O'Hehir (1998). "Immunotherapy of allergy: anergy, deletion, and immune deviation." Curr Opin Immunol **10**(6): 640-5.

Ross, D. J., H. L. Keynes and J. C. McDonald (1998). "SWORD '97: surveillance of work-related and occupational respiratory disease in the UK. [Review] [9 refs]." Occupational Medicine (Oxford) **48**(8): 481-5.

Roy, K., H. Q. Mao, S. K. Huang and K. W. Leong (1999). "Oral gene delivery with chitosan - DNA nanoparticles generates immunologic protection in a murine model of peanut allergy." Nat Med **5**(4): 387-91.

Rueff, F., A. Kienitz, P. Schopf, W. Hugo Hartl, H. J. Andrebeta, D. Zaak, M. Menninger and B. Przybilla (2001). "Frequency of natural

rubber latex allergy in adults is increased after multiple operative procedures." Allergy **56**(9): 889-94.

Saary, M. J., A. Kanani, H. Alghadeer, D. L. Holness and S. M. Tarlo (2002). "Changes in rates of natural rubber latex sensitivity among dental school students and staff members after changes in latex gloves." J Allergy Clin Immunol **109**(1 Pt 1): 131-5.

Sainte-Laudy, J., C. Vallon and J. C. Guerin (1996). "Diagnosis of latex allergy: comparison of histamine release and flow cytometric analysis of basophil activation." Inflamm Res **45 Suppl 1**: S35-6.

Sakai, K., A. Yokoyama, N. Kohno and K. Hiwada (1999). "Effect of different sensitizing doses of antigen in a murine model of atopic asthma." Clin Exp Immunol **118**(1): 9-15.

Sanchez-Guerrero, I. M., A. I. Escudero, J. A. Tortosa and M. Lombardero (2000). "Anaphylaxis to cherimoya." Allergy **55**(10): 976-7.

Schappi, G. F., P. E. Taylor, M. C. Pain, P. A. Cameron, A. W. Dent, I. A. Staff and C. Suphioglu (1999). "Concentrations of major grass group 5 allergens in pollen grains and atmospheric particles: implications for hay fever and allergic asthma sufferers sensitized to grass pollen allergens." Clin Exp Allergy **29**(5): 633-41.

Schramm, G., H. Kahlert, R. Suck, B. Weber, H. T. Stuwe, W. D. Muller, A. Bufe, W. M. Becker, M. W. Schlaak, L. Jager, O. Cromwell and H. Fiebig (1999). ""Allergen engineering": variants of the timothy grass pollen allergen Phl p 5b with reduced IgE-binding capacity but conserved T cell reactivity." J Immunol **162**(4): 2406-14.

Seppala, U., T. Palosuo, N. Kalkkinen, L. Ylitalo, T. Reunala and K. Turjanmaa (2000). "IgE reactivity to patatin-like latex allergen, Hev b 7, and to patatin of potato tuber, Sol t 1, in adults and children allergic to natural rubber latex." Allergy **55**(3): 266-73.

Shakib, F., O. Schulz and H. Sewell (1998). "A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity." Immunol Today **19**(7): 313-6.

Shikano, H., Z. Kato, H. Kaneko, M. Watanabe, R. Inoue, K. Kasahara, M. Takemura and N. Kondo (2001). "IFN-gamma production in response to IL-18 or IL-12 stimulation by peripheral blood mononuclear cells of atopic patients." Clin Exp Allergy **31**(8): 1263-70.

Simons, E. (1994). Ancestors of Allergy. New York, Global Medical Communications.

Slater, J. E. (1989). "Rubber anaphylaxis." N Engl J Med **1126-1130**.

Slater, J. E. and A. Colberg-Poley (1997). "A DNA vaccine for allergen immunotherapy using the latex allergen Hev b 5." Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M **91**: 230-5.

Slater, J. E., E. Paupore, Y. T. Zhang and A. M. Colberg-Poley (1998). "The latex allergen Hev b 5 transcript is widely distributed after subcutaneous injection in BALB/c mice of its DNA vaccine." J Allergy Clin Immunol **102**(3): 469-75.

Slater, J. E., E. J. Paupore and R. E. O'Hehir (1999). "Murine B-cell and T-cell epitopes of the allergen Hev b 5 from natural rubber latex." Mol Immunol **36**(2): 135-43.

Slater, J. E., T. Vedvick, A. Arthur-Smith, D. E. Trybul and R. G. Kekwick (1996). "Identification, cloning, and sequence of a major allergen (Hev b 5) from natural rubber latex (Hevea brasiliensis)." J Biol Chem **271**(41): 25394-9.

Smart, I. J., R. J. Heddle, H. Zola and J. Bradley (1983). "Development of monoclonal mouse antibodies specific for allergenic components of Ryegrass (*Lolium perenne*) pollen." Int Arch Appl Immunol **72**: 243-248.

Smith, A. M. and M. D. Chapman (1996). "Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2." Mol Immunol **33**(4-5): 399-405.

Sone, T., K. Morikubo, M. Miyahara, N. Komiyama, K. Shimizu, H. Tsunoo and K. Kino (1998). "T cell epitopes in Japanese cedar (Cryptomeria japonica) pollen allergens: choice of major T cell epitopes in Cry j 1 and Cry j 2 toward design of the peptide-based immunotherapeutics for the management of Japanese cedar pollinosis." J Immunol **161**(1): 448-57.

Spiegelberg, H. L., L. Beck, D. D. Stevenson and G. Y. Ishioka (1994). "Recognition of T cell epitopes and lymphokine secretion by rye grass allergen Lolium perenne I-specific human T cell clones." J Immunol **152**(9): 4706-11.

Strachan, D. P. (1989). "Hay fever, hygiene, and household size." Bmj **299**(6710): 1259-60.

Subramaniam, A. (1995). "The chemistry of natural rubber latex." Immunol Allergy Clin North America **15**(1): 1-19.

Suphioglu, C., M. B. Singh, P. Taylor, R. Bellomo, P. Holmes, R. Puy and R. B. Knox (1992). "Mechanism of grass-pollen-induced asthma." Lancet **339**(8793): 569-72.

Sussman, G. L., G. M. Liss, K. Deal, S. Brown, M. Cividino, S. Siu, D. H. Beezhold, G. Smith, M. C. Swanson, J. Yunginger, A. Douglas, D. L. Holness, P. Lebert, P. Keith, S. Waserman and K. Turjanmaa (1998). "Incidence of latex sensitization among latex glove users." J Allergy Clin Immunol **101**(2 Pt 1): 171-8.

Sussman, G. L., G. M. Liss and S. Wasserman (1998). "Update on the Hamilton, Ontario latex sensitization study." J Allergy Clin Immunol **102**(2): 333.

Sutherland, M., D. Czarny and J. Douglass (1998). "Allergy in the airway." Australian Family Physician **27**(8): 681-5.

Sutherland, M., C. Suphioglu, J. Rolland and E. O'Hehir R (2002). "Latex allergy: towards immunotherapy for health care workers." Clin Exp Allergy **32**: 667-73.

Sutherland, M. F., A. Drew, J. M. Rolland, J. E. Slater, C. Suphioglu and R. E. O'Hehir (2002). "Specific monoclonal antibodies and human immunoglobulin E show that Hev b 5 is an abundant allergen in high protein powdered latex gloves." Clin Exp Allergy **32**(4): 583-9.

Suzuki, I., N. Hizawa, E. Yamaguchi and Y. Kawakami (2000). "Association between a C+33T polymorphism in the IL-4 promoter region and total serum IgE levels." Clin Exp Allergy **30**(12): 1746-9.

Swanson, M. C., M. E. Bubak, L. W. Hunt, J. W. Yunginger, M. A. Warner and C. E. Reed (1994). "Quantification of occupational latex aeroallergens in a medical center." J Allergy Clin Immunol **94**(3 Pt 1): 445-51.

Takabayashi, A., K. Ihara, Y. Sasaki, Y. Suzuki, S. Nishima, K. Izuhara, N. Hamasaki and T. Hara (2000). "Childhood atopic asthma: positive association with a polymorphism of IL- 4 receptor alpha gene but not with that of IL-4 promoter or Fc epsilon receptor I beta gene." Exp Clin Immunogenet **17**(2): 63-70.

Tan, B. B., D. Weald, I. Strickland and P. S. Friedmann (1996). "Double-blind controlled trial of effect of housedust-mite allergen avoidance on atopic dermatitis." Lancet **347**(8993): 15-8.

Tang, L., S. Benjaponpitak, R. H. DeKruyff and D. T. Umetsu (1998). "Reduced prevalence of allergic disease in patients with multiple sclerosis is associated with enhanced IL-12 production." J Allergy Clin Immunol **102**(3): 428-35.

Tang, M., A. Kemp and G. Varigos (1993). "IL-4 and interferon-gamma production in children with atopic disease." Clin Exp Immunol **92**(1): 120-4.

Tang, M. L., A. S. Kemp, J. Thorburn and D. J. Hill (1994). "Reduced interferon-gamma secretion in neonates and subsequent atopy." Lancet **344**(8928): 983-5.

Tarlo, S. M., A. Easty, K. Eubanks, C. R. Parsons, F. Min, S. Juvet and G. M. Liss (2001). "Outcomes of a natural rubber latex control program in an Ontario teaching hospital." J Allergy Clin Immunol **108**(4): 628-33.

Tarlo, S. M., G. Sussman, A. Contala and M. C. Swanson (1994). "Control of airborne latex by use of powder-free latex gloves." J Allergy Clin Immunol **93**(6): 985-9.

Tepper, R. I., D. A. Levinson, B. Z. Stanger, J. Campos-Torres, A. K. Abbas and P. Leder (1990). "IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice." Cell **62**(3): 457-67.

Tighe, H., K. Takabayashi, D. Schwartz, G. Van Nest, S. Tuck, J. J. Eiden, A. Kagey-Sobotka, P. S. Creticos, L. M. Lichtenstein, H. L. Spiegelberg and E. Raz (2000). "Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity." J Allergy Clin Immunol **106**(1 Pt 1): 124-34.

Till, S., R. Dickason, D. Huston, M. Humbert, D. Robinson, M. Larche, S. Durham, A. B. Kay and C. Corrigan (1997). "IL-5 secretion by allergen-stimulated CD4+ T cells in primary culture: relationship to expression of allergic disease." J Allergy Clin Immunol **99**(4): 563-9.

Tovey, E. R., M. D. Chapman and T. A. Platts-Mills (1981). "Mite faeces are a major source of house dust allergens." Nature **289**(5798): 592-3.

Tsuyuki, S., J. Tsuyuki, K. Einsle, M. Kopf and A. J. Coyle (1997). "Costimulation through B7-2 (CD86) is required for the induction of a lung mucosal T helper cell 2 (TH2) immune response and altered airway responsiveness." J Exp Med **185**(9): 1671-9.

Turjanmaa, K. (1995). "Natural rubber latex allergy:the European experience." Immunol Allergy Clin North America **15**(1): 71-88.

Turjanmaa, K., M. Kanto, H. Kautiainen, T. Reunala and T. Palosuo (2002). "Long-term outcome of 160 adult patients with natural rubber latex allergy." J Allergy Clin Immunol **110**(2 Suppl): S70-4.

Turjanmaa, K., T. Palosuo, H. Alenius, F. Leynadier, J. E. Autegarden, C. Andre, H. Sicard, M. Hrabina and T. X. Tran (1997). "Latex allergy diagnosis: in vivo and in vitro standardization of a natural rubber latex extract." Allergy **52**(1): 41-50.

Turjanmaa, K., L. Rasanen, M. Lehto, S. Makinen-Kiljunen and T. Reunala (1989). "Basophil histamine release and lymphocyte proliferation tests in latex contact urticaria. In vitro tests in latex contact urticaria." Allergy **44**(3): 181-6.

Upham, J. W. (1997). "T-cell proliferative responses to mite allergens are detectable in most individuals regardless of atopic status and age." Respirology **2**(2): 141-2.

Uveges, R. E., R. E. Grimwood, L. D. Slawsky and J. G. Marks, Jr. (1995). "Epidemiology of hand dermatitis in dental personnel." Mil Med **160**(7): 335-8.

Valentine, J. P., J. J. Kurinczuk, R. K. Loh and P. J. Chauvel (1999). "Latex allergy in an Australian population of children and adolescents with spinal dysfunction." Med J Aust **170**(1): 15-8.

Valsecchi, R., P. Leghissa, R. Cortinovis, L. Cologni and A. Pomesano (2000). "Contact urticaria from latex in healthcare workers." Dermatology **201**(2): 127-31.

Valyasevi, M. A., D. E. Maddox and J. T. Li (1999). "Systemic reactions to allergy skin tests." Ann Allergy Asthma Immunol **83**(2): 132-6.

van den Biggelaar, A. H., R. van Ree, L. C. Rodrigues, B. Lell, A. M. Deelder, P. G. Kremsner and M. Yazdanbakhsh (2000). "Decreased atopy in children infected with Schistosoma haematobium: a role for parasite-induced interleukin-10." Lancet **356**(9243): 1723-7.

van der Pouw Kraan, T. C., A. van Veen, L. C. Boeije, S. A. van Tuyl, E. R. de Groot, S. O. Stapel, A. Bakker, C. L. Verweij, L. A. Aarden and J. S. van der Zee (1999). "An IL-13 promoter polymorphism associated with increased risk of allergic asthma." Genes Immun **1**(1): 61-5.

van Herwerden, L., S. B. Harrap, Z. Y. Wong, M. J. Abramson, J. J. Kutin, A. B. Forbes, J. Raven, A. Lanigan and E. H. Walters (1995). "Linkage of high-affinity IgE receptor gene with bronchial hyperreactivity, even in absence of atopy." Lancet **346**(8985): 1262-5.

van Neerven, R., W. van t'Hof, J. H. Ringrose, H. M. Jansen, R. C. Aalberse, E. A. Wierenga and M. L. Kapsenberg (1993). "T cell epitopes of house dust mite major allergen Der p II." J Immunol **151**(4): 2326-35.

van Neerven, R., M. Wissenbach, H. Ipsen, A. Bufe, J. Arnved and P. A. Wurtzen (1999). "Differential recognition of recombinant Phl p 5 isoallergens by Phl p 5- specific T cells." Int Arch Allergy Immunol **118**(2-4): 125-8.

van Neerven, R. J., M. M. van de Pol, E. A. Wierenga, R. C. Aalberse, H. M. Jansen and M. L. Kapsenberg (1994). "Peptide specificity and

HLA restriction do not dictate lymphokine production by allergen-specific T-lymphocyte clones." Immunology **82**(3): 351-6.

van Neerven, R. J., T. Wikborg, G. Lund, B. Jacobsen, A. Brinch-Nielsen, J. Arnved and H. Ipsen (1999). "Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation." J Immunol **163**(5): 2944-52.

Vandenplas, O., J. P. Delwiche, G. Evrard, P. Aimont, X. van der Brempt, J. Jamart and L. Delaunois (1995). "Prevalence of occupational asthma due to latex among hospital personnel." Am J Respir Crit Care Med **151**(1): 54-60.

Vercelli, D., H. H. Jabara, K. Arai and R. S. Geha (1989). "Induction of human IgE synthesis requires interleukin 4 and T/B cell interactions involving the T cell receptor/CD3 complex and MHC class II antigens." J Exp Med **169**(4): 1295-307.

Verhoef, A., J. A. Higgins, C. J. Thorpe, S. G. Marsh, J. D. Hayball, J. R. Lamb and R. E. O'Hehir (1993). "Clonal analysis of the atopic immune response to the group 2 allergen of Dermatophagoides spp.: identification of HLA-DR and -DQ restricted T cell epitopes." Int Immunol **5**(12): 1589-97.

Vodovotz, Y., A. G. Geiser, L. Chesler, J. J. Letterio, A. Campbell, M. S. Lucia, M. B. Sporn and A. B. Roberts (1996). "Spontaneously increased production of nitric oxide and aberrant expression of the inducible nitric oxide synthase in vivo in the transforming growth factor beta 1 null mouse." J Exp Med **183**(5): 2337-42.

Von Ehrenstein, O. S., E. Von Mutius, S. Illi, L. Baumann, O. Bohm and R. von Kries (2000). "Reduced risk of hay fever and asthma among children of farmers." Clin Exp Allergy **30**(2): 187-93.

Wagner, B., D. Buck, C. Hafner, S. Sowka, B. Niggemann, O. Scheiner and H. Breiteneder (2001). "Hev b 7 is a Hevea brasiliensis protein associated with latex allergy in children with spina bifida." J Allergy Clin Immunol **108**(4): 621-7.

Walter, D. M., J. J. McIntire, G. Berry, A. N. McKenzie, D. D. Donaldson, R. H. DeKruyff and D. T. Umetsu (2001). "Critical role for IL-13 in the development of allergen-induced airway hyperreactivity." J Immunol **167**(8): 4668-75.

Wan, H., H. L. Winton, C. Soeller, E. R. Tovey, D. C. Gruenert, P. J. Thompson, G. A. Stewart, G. W. Taylor, D. R. Garrod, M. B. Cannell and C. Robinson (1999). "Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions." J Clin Invest **104**(1): 123-33.

Weiss, S. J. and J. F. Halsey (1996). "A nurse with anaphylaxis to stone fruits and latex sensitivity: potential diagnostic difficulties to consider." Ann Allergy Asthma Immunol 77(6): 504-8.

Westall, G. P., F. C. Thien, D. Czarny, R. E. O'Hehir and J. A. Douglas (2001). "Adverse events associated with rush hymenoptera venom immunotherapy." Med J Aust **174**(5): 227-30.

WHO-IUIS (2002). Latex Allergens. **2002**.

Wills-Karp, M., J. Santeliz and C. L. Karp (2001). "The germless theory of allergic disease: revisiting the hygeine hypothesis." Nat Rev Immunol **1**: 70-75.

Woodfolk, J. A., S. S. Sung, D. C. Benjamin, J. K. Lee and T. A. Platts-Mills (2000). "Distinct human T cell repertoires mediate immediate and delayed-type hypersensitivity to the Trichophyton antigen, Tri r 2." J Immunol **165**(8): 4379-87.

Wurtzen, P., M. Wissenbach, H. Ipsen, A. Bufe, J. Arnved and R. J. van Neerven (1999). "Highly heterogeneous Phl p 5-specific T cells

from patients with allergic rhinitis differentially recognize recombinant Phl p 5 isoallergens." J Allergy Clin Immunol **104**(1): 115-22.

Yabuhara, A., C. Macaubas, S. L. Prescott, T. J. Venaille, B. J. Holt, W. Habre, P. D. Sly and P. G. Holt (1997). "TH2-polarized immunological memory to inhalant allergens in atopics is established during infancy and early childhood." Clin Exp Allergy **27**(11): 1261-9.

Yeang, H. Y. (2000). "Prevalence of latex allergy may be vastly overestimated when determined by in vitro assays." Ann Allergy Asthma Immunol **84**(6): 628-32.

Yeang, H. Y., K. F. Cheong, E. Sunderasan, S. Hamzah, N. P. Chew, S. Hamid, R. G. Hamilton and M. J. Cardosa (1996). "The 14.6 kd rubber elongation factor (Hev b 1) and 24 kd (Hev b 3) rubber particle proteins are recognized by IgE from patients with spina bifida and latex allergy." J Allergy Clin Immunol **98**(3): 628-39.

Yip, L., V. Hickey, B. Wagner, G. Liss, J. Slater, H. Breiteneder, G. Sussman and D. Beezhold (2000). "Skin prick test reactivity to recombinant latex allergens." Int Arch Allergy Immunol **121**(4): 292-9.

Ylitalo, L., S. Makinen-Kiljunen, K. Turjanmaa, T. Palosuo and T. Reunala (1999). "Cow's milk casein, a hidden allergen in natural rubber latex gloves." J Allergy Clin Immunol **104**(1): 177-80.

Ylitalo, L., K. Turjanmaa, T. Palosuo and T. Reunala (1997). "Natural rubber latex allergy in children who had not undergone surgery and children who had undergone multiple operations." J Allergy Clin Immunol **100**(5): 606-12.

Yokota, A., H. Kikutani, T. Tanaka, R. Sato, E. L. Barsumian, M. Suemura and T. Kishimoto (1988). "Two species of human Fc epsilon receptor II (Fc epsilon RII/CD23): tissue-specific and IL-4-specific regulation of gene expression." Cell **55**(4): 611-8.

Yssel, H., K. E. Johnson, P. V. Schneider, J. Wideman, A. Terr, R. Kastelein and J. E. De Vries (1992). "T cell activation-inducing epitopes of the house dust mite allergen Der p I. Proliferation and lymphokine production patterns by Der p I- specific CD4+ T cell clones." J Immunol **148**(3): 738-45.

Yunginger, J. W., R. T. Jones, A. F. Fransway, J. M. Kelso, M. A. Warner and L. W. Hunt (1994). "Extractable latex allergens and proteins in disposable medical gloves and other rubber products." J Allergy Clin Immunol **93**(5): 836-42.

Clin Exp Allergy 2002; 32:583–589

Specific monoclonal antibodies and human immunoglobulin E show that Hev b 5 is an abundant allergen in high protein powdered latex gloves

M. F. Sutherland*‡, A. Drew*‡, J. M. Rolland†‡, J. E. Slater§, C. Suphioglu*‡ and R. E. O'Hehir*‡

*Departments of *Allergy, Asthma and Clinical Immunology, and †Pathology and Immunology, The Alfred Hospital and Monash University, Victoria, ‡Co-operative Research Centre for Asthma, Sydney, Australia; and the §Laboratory of Immunobiochemistry, Center for Biologics Evaluation and Research, US Food and Drug Administration, Rockville, MD, USA*

Summary

Background Hev b 5 is a major latex allergen recognized predominantly by latex-allergic health care workers (HCWs). Recombinant Hev b 5 (rHev b 5) was previously expressed as a fusion protein with maltose binding protein (MBP), itself an immunogenic molecule; therefore non-fusion rHev b 5 is desirable. Moreover, standardized immunological assays for the detection of Hev b 5 are currently lacking and may have important implications for both allergen avoidance and diagnosis in latex allergy.

Objectives To generate and use Hev b 5-specific mAbs to determine the relative abundance of Hev b 5 in different latex extracts, correlating this with the IgE reactivity of latex-allergic HCWs and to produce non-fusion rHev b 5.

Methods For the production of mAbs, mice were immunized with rHev b 5/MBP fusion protein and mAbs selected with rHev b 5/MBP but not MBP reactivity. The mAb reactivity was compared with polyclonal IgE from latex-allergic HCWs using direct and inhibition ELISA and immunoblot assays. Recombinant Hev b 5 was expressed and purified in the pPROEX-HTa bacterial expression system.

Results Four Hev b 5-specific mAbs were produced. Immunoblotting and ELISA using the mAbs indicate abundant Hev b 5 in high protein powdered latex glove extracts as compared with crude latex sap extracts. High quality surgical gloves with no detectable protein have no detectable Hev b 5. Inhibition ELISAs using serum IgE from latex-allergic HCWs and Hev b 5-specific mAbs gave strong correlation. Non-fusion recombinant Hev b 5 was successfully expressed and purified, showing reactivity with both the Hev b 5-specific mAbs and serum IgE of latex-allergic HCWs.

Conclusion Hev b 5-specific mAbs and human IgE from latex-allergic HCWs demonstrate the greater content of Hev b 5 in high protein powdered glove extracts. This may explain the observed higher frequency of sensitization to this allergen in HCWs.

Keywords Hev b 5, latex allergy, latex gloves, monoclonal antibodies, recombinant proteins.
Submitted 9 May 2001; revised 16 October 2001; accepted 23 October 2001

Introduction

Latex allergy is characterized by sensitivity to the protein fraction of natural rubber latex (NRL) [1] used in the production of gloves and other medical products. The major groups affected include health care workers (HCWs) [2], spina bifida children [3] and others undergoing multiple operations [4]. The predominant cause of sensitization and symptoms amongst HCWs is the use of latex gloves [5], but much previous research has focused on crude latex sap extracts such as non-ammoniated latex (NAL) and low-ammoniated latex (LAL). Hev b 5 is a major latex allergen, isolated and characterized by recombinant DNA technology [6,7] and initially expressed as a fusion protein with maltose binding protein (MBP). The majority of latex-allergic HCWs show reactivity to the recombinant Hev b 5/MBP fusion protein (rHev b 5/MBP) by serum IgE [6], T cell function [8] and skin testing assays [9]. The presence of immunoreactive natural Hev b 5 (nHev b 5) in NRL, however, has been difficult to clearly demonstrate [10], in part because of the physicochemical properties of the protein (highly negatively charged, proline rich) and the complexity of latex extracts, and in part because of the lack of specific probes. Hev b 5 may also be in low abundance in commercially available capture assays [11], reducing their diagnostic sensitivity. Moreover, the effects of the glove manufacturing process on the immunogenicity of NRL and nHev b 5 are poorly understood. Previous monoclonal antibodies (mAbs) to Hev b 5/MBP were not mono-specific for Hev b 5, with reactivity reported to MBP alone in addition to NAL and rHev b 5/MBP fusion protein [6]. In this study, we aimed to generate rHev b 5-specific mAbs to characterize and determine the relative amount of immunoreactive Hev b 5 in

Correspondence: Professor Robyn E. O'Hehir, Department of Allergy, Asthma and Clinical Immunology, Alfred Hospital, Commercial Road, Prahran, VIC 3181, Australia. E-mail: Robyn.OHehir@med.monash.edu.au

different latex preparations and compare this with the rHev b 5-specific serum IgE reactivity of latex-allergic patients. Since MBP is itself a large (42 kDa) and immunogenic molecule, we also aimed to express rHev b 5 without a MBP fusion partner and evaluate its IgE and mAb reactivity. Production of highly specific mAbs is mandatory for the determination of the relative abundance of Hev b 5 in different glove extracts. Such novel information is pivotal for allergen avoidance while the availability of IgE-reactive rHev b 5 is important for allergen standardization to permit accurate diagnosis of latex allergy.

Materials and methods

Antigens

rHev b 5/MBP The cDNA encoding Hev b 5 was expressed as a fusion protein with MBP in the pMAL/c-2 vector and purified on an amylose column (New England Biolabs, Beverly, MA, USA) as described previously [6]. An MBP-LacZα fusion protein produced by expressing the pMAL/c-2 vector alone was used as the negative control protein.

rHev b 5 The sequence encoding the Hev b 5 protein was amplified by the polymerase chain reaction (PCR) using plasmid pMAL/Hev b 5 DNA as the template [6]. In detail, 20 ng of plasmid DNA was amplified using the primers: HEVB5F (5′-GCGGAATTCATGGCCAGTGTTGAGGTTG-3′) and HEVB5R (5′-GCGGTCGACTTATTCCTCTGTTTTTTCC-ACC-3′). Thirty cycles of amplification were conducted with each cycle consisting of 1 min at 95 °C, 1 min at 50 °C and 1 min at 72 °C, followed by incubation at 72 °C for 10 min The pProX-HTa vector (Life Technologies, Carlsbad, CA, USA; possessing a hexahistidine tag to facilitate purification) and the Hev b 5 PCR product were cleaved with *Eco*RI and *Sal*I and purified by agarose gel electrophoresis followed by extraction of the DNAs from the gel (QIAGEN Gel purification kit; Germantown, MD, USA). Vector DNA and the PCR product were ligated using T4 DNA ligase and transformed into DH5α competent cells. *E. coli* DH5α cells were grown to logarithmic stage and expression of rHev b 5 induced by the addition of isopropyl-1-thio-β-D-galactoside to a final concentration of 0.6 mmol/L. Following induction of expression the cells were grown for 4 h at 37 °C. The bacteria were harvested by centrifugation for 15 min at 4000 *g* at 4 °C and the pellets frozen overnight at −20 °C. The pellets were resuspended in 20 mL of native lysis buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 10 mM imidazole) per litre of original culture. The cell suspension was sonicated 6 × 20 s on ice, using a Branson sonifier model 250 and then centrifuged for 30 min at 16000 *g*. at 4 °C. The supernatant was applied to a column containing Ni-NTA agarose (QIAGEN) with a bed volume of 5 mL. The column was washed with native lysis buffer and then eluted (50 mM $Na_2H_2PO_4$, 300 mM NaCl, 250 mM imidazole). Recombinant Hev b 5 was soluble with a yield of 10 mg per litre of broth culture. The concentration of rHev b 5 was determined by the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) with bovine gamma globulin (Bio-Rad, Hercules, CA, USA) as the standard. SDS-PAGE (under reducing and denaturing conditions) and Western analysis was used to assess purity and integrity of rHev b 5 (see Fig. 1a).

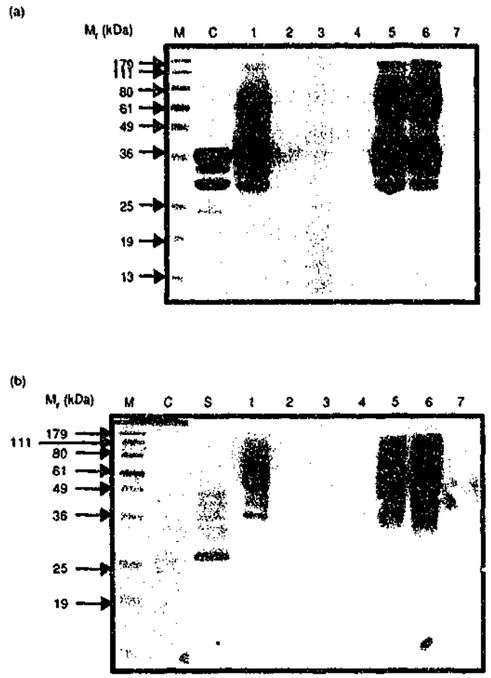


Fig. 1. Western blot and inhibition analysis of rHev b 5 and glove extract with human IgE and mAbs. Recombinant Hev b 5 (a) and glove extract (b) were resolved by 12% SDS-PAGE and transferred onto NC for Western analysis. NC blots were probed for antibody binding with pooled sera from latex-allergic patients (lane 1), pooled sera from latex-allergic patients pre-incubated with 20 μg rHev b 5 per mL of diluted (1:5) serum pool (lane 2), pooled sera from non-allergic patients (lane 3), no serum control (lane 4), mAb 6A10 (lane 5), mAb 6F6 (lane 6), and mAb A1 isotype control (lane 7). Molecular mass markers, coomassie brilliant blue-stained gel slices and silver-stained gel slices are denoted by M, C and S, respectively.

Non-ammoniated latex (NAL) This was obtained from the Rubber Research Institute of Malaysia. The sap was centrifuged for 1 h at 50000 *g* and the C-serum removed for protein estimation using the BCA assay described above. It was then aliquotted and stored at −20 °C. The concentration of the undiluted NAL extract was 7.3 mg/mL. The NAL extract was diluted in PBS, as appropriate, for the immunoassays described below.

Low-ammoniated latex (LAL) This was a gift of Ansell Australia and was processed as described above. The concentration of the undiluted LAL extract was 7.6 mg/mL.

Glove Extracts (GEs) These were obtained by adding phosphate buffered saline (PBS; 1 mL per gram of glove) to the interior of intact, commercially available latex gloves and incubating for 1 h at room temperature (20 °C) on an orbital shaker. The resultant glove eluate was then removed, centrifuged at 5000 *g* for 10 min to remove all glove donning powder, then filter-sterilized through a 0.2-μm filter. Protein concentration was estimated with the BCA assay as described above (assay sensitivity 20 μg/mL). Five different commercially available gloves were used. For convenience these were designated G1 to G5. Gloves G1 and G2 were powdered, non-sterile latex gloves: G1 a laboratory utility glove, G2 a non-medical grade household disposable glove available from supermarkets in Australia. Gloves G3, G4 and G5 were all sterile surgical gloves currently in use in our hospital: G4 was non-powdered and G3 and G5 were powdered.

Human subjects

Latex-allergic subjects were recruited from the Allergy and Asthma Clinic at the Alfred Hospital and all patients gave informed consent with the study being approved by the Alfred Hospital Ethics Committee. Five out of six subjects have been previously shown to recognize rHev b 5/MBP by immunoblotting and T cell proliferation assays [8]. The strongest responder was used for the IgE inhibition ELISA experiments, while the sera were pooled for the IgE immunoblots. A serum pool of latex-exposed, non-atopic HCWs was used as a negative control serum in the IgE immunoblots.

Monoclonal antibody production, isotyping and purification

Monoclonal antibody production was carried out according to established protocols [12,13] and all experiments were approved by the Monash University Animal Ethics Committee with animal care conducted according to National Health and Medical Research Guidelines. Briefly, six-week-old-female BALB/c mice received six injections intraperitoneally (IP) at 2–4 week intervals with 20 μg rHev b 5/MBP initially with, subsequently without, Complete Freund's adjuvant. Immune mouse splenocytes were fused with cells from the murine myeloma cell line X63-Ag8.653 and hybridomas screened by duplicate ELISA for each well against rHev b 5/MBP and MBP. ELISA plates (Costar, Acton, MA, USA) were coated overnight (O/N) at 4 °C with 50 μL per well of rHev b 5/MBP or MBP alone at 0.2 μg/mL in ELISA coating buffer (0.03 M $NaHCO_3$? 0.02 M Na_2CO_3, pH 9.5). The plates were then washed eight times in PBS with 0.05% Tween 20 (BDH, Poole, UK; PBST) and then blocked with 5% w/v skim milk powder (SMP) in PBST (SMP-PBST) O/N at 4 °C before being washed again eight times in PBST. Hybridoma supernatants were then incubated (50 μL/well) for 1 h at 37 °C. The plates then washed eight times in PBST then 50 μL per well of horseradish peroxidase (HRP) labelled sheep anti-mouse antibody (Silenius, Melbourne, Australia) diluted 1 : 5000 in 1% SMP-PBST was added and incubated for 1 h at 37 °C. The plates were then washed four times with PBST and then four times with PBS alone before the addition of 50 μL/well of a solution of 5 mg O-phenylenediamine dihydrochloride (OPD; Sigma, St Louis, MO, USA) in 12.5 mL of 0.05 M phosphate-citrate buffer with 0.03% sodium perborate (Sigma). This was incubated for 15 min at 37 °C and the reaction stopped with 4 M HCl. Colour development was then observed and the plate read at 490 nm. In all cases, one well of the ELISA plate had cell culture medium alone as a negative control and the terminal bleed of the fusion mouse at a 1 : 5000 dilution in 1% SMP-PBST as positive control. After expansion and cloning of hybridomas by limiting dilution, supernatants were assayed by ELISA for reactivity to NAL. The NAL ELISA was performed as above but the antigen coating concentration was 10 μg/mL.

Once monoclonality of hybridomas was achieved, supernatants were isotyped by ELISA (Pharmingen, San Diego, CA, USA) and mAbs purified on protein A (Biosepra©, Life Technologies) or protein G (Pharmacia, Uppsala, Sweden) columns as appropriate, following the manufacturer's instructions.

Immunoblotting

For SDS-PAGE immunoblots, extracts of NAL and LAL (50 μg/lane) or GE (G1; 5 μg/lane) or recombinant proteins (5 μg/lane) were resolved on 12% SDS-PAGE mini gels (Novex, Carlsbad, CA, USA) for 90 min at 125 V DC with Benchmark© prestained protein ladder (Life Technologies) as the molecular mass standards. Gels were then stained with Coomassie Brilliant Blue (CBB) or silver (Novex) for total protein visualization or, for Western blotting transferred to nitrocellulose (NC) membranes (Schleicher and Schuell, Dassel, Germany) for 60 min at 25 V DC. After transfer, NC membranes were dried then cut into strips. They were then blocked with 5% SMP-PBST for 1 h at room temperature, washed twice in PBST and then incubated with hybridoma supernatants (1 : 100), purified mAb (0.1–1 μg/mL) or pooled human serum (1 : 5) for 1–2 h at room temperature. After washing and incubation with horseradish peroxidase (HRP) labelled detection antibodies, blots were washed and developed with 4-chloro-1-naphthol as previously described [14] or enhanced chemiluminescence (ECL) reagent (NEN Life Sciences, Boston, MA, USA) with luminescence detected by autoradiography (Kodak, Melbourne, Australia). Inhibition immunoblot analysis was essentially the same as described above, except the serum pool was preincubated with 20 μg of rHev b 5 per ml of sera (diluted 1 : 5) for 1 h at 37 °C before incubation of the NC strips.

Inhibition ELISA for mAbs and human IgE

Inhibition ELISAs were performed as above but coating of ELISA plates with rHev b 5 was at 0.5 μg/mL and for the detection of human IgE, rabbit anti-human IgE (Dako, Carpinteria, CA, USA) was used at a dilution of 1 : 500 in 1% SMP-PBST. Dilution curves with purified Hev b 5 specific mAbs and latex-allergic HCW IgE were performed to determine the optimal concentrations (data not shown); 0.017 μg/mL for mAb 6F6, and 1 : 20 for HCW IgE. Inhibitors were added and incubated with antibody/sera at 37 °C for 1 h prior to addition to the assay plate. The GE inhibitors were used neat or diluted 1 : 10 in PBS before addition to an equal volume of the diluted human sera or mAb, giving a final dilution of 1 : 2 or 1 : 20 of the extract. Final effective protein concentrations therefore were for G1 274 μg/mL and 27.4 μg/mL, G2 370 μg/mL and 37 μg/mL or, where the protein concentration was beneath the levels of detection, GE inhibitors were expressed as dilutions of 1 : 2 and 1 : 20 (Fig. 3). The NAL and LAL extracts were similarly diluted in PBS before addition to the sera or mAb, giving final effective concentrations for both of 100 μg/mL and 10 μg/mL. Rye grass pollen extract was used as a negative control protein at a final effective concentration of 100 μg/mL.

In addition, to ensure that observed inhibitions were specific, a control ELISA was performed using rye grass pollen extract to coat the plates (10 μg/mL) and mAb A1, a Lol p 1-specific mAb of isotype IgG1, for detection [15].

Results

Four hybridomas were produced which showed strong reactivity to rHev b 5 fusion protein by ELISA and immunoblot, no

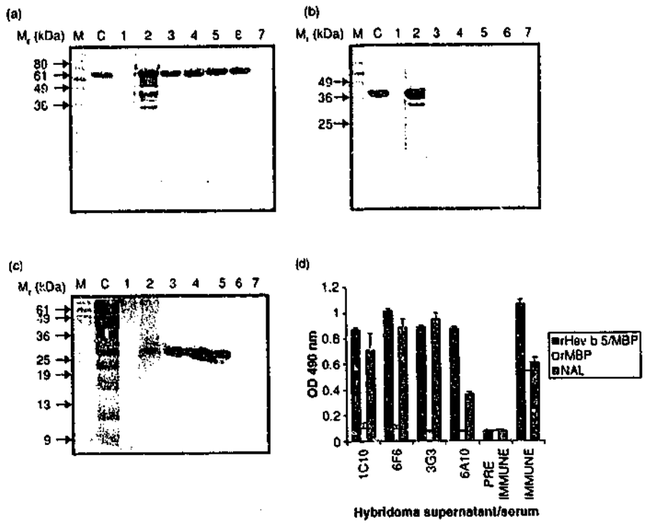


Fig. 2. Immunological analysis of recombinant and natural latex proteins. Recombinant Hev b 5/MBP fusion protein (a), rMBP (b) and NAL (c) were resolved by 12% SDS-PAGE and transferred onto nitrocellulose (NC) for Western analysis. NC blots were probed for antibody binding with pre-immune mouse serum (lane 1), polyclonal immune mouse serum (lane 2), mAb 6A10 (lane 3), mAb 6F6 (lane 4), mAb 3G3 (lane 5), mAb 1C10 (lane 6) and mAb A1 isotype control (lane 7). Molecular mass markers and Coomassie brilliant blue-stained gel slices are denoted by M and C, respectively. Monoclonal antibody binding to rHev b 5/MBP, rMBP and NAL was also assessed by ELISA (d).

reactivity to MBP alone and also reactivity to NAL (Fig 2). The immunoblot reactivity to NAL was maximal to a band at around 26 kDa on a 12% SDS-PAGE gel and was strongest for mAbs 6F6, 6A10, 3G3 and weak for 1C10. Isotyping showed that three of the mAbs were IgG1 subclass (6A10, 1C10 and 3G3), the other subclass IgG2b (6F6).

All the mAbs also recognized the rHev b 5 expressed without MBP. A representative Western blot with two of the antibodies is shown (Fig. 1a). The pattern of reactivity was almost identical to that of the pooled sera of latex-allergic individuals. The HCW IgE reactivity was also Hev b 5 specific with almost complete inhibition by preincubation with 20 µg/mL rHev b 5 (Fig. 1a). Although the majority of the protein was monomeric rHev b 5, breakdown products and aggregates were evident (lanes C, 1, 5 and 6 of Fig. 1a). However, the integrity of rHev b 5 (i.e. IgE and mAb 6F6 reactivity) was maintained (Fig. 1a). Western blotting of GE by HCW IgE and Hev b 5-specific mAbs showed strong reactivity to glove extract with a principal band at approximately 36 kDa and a smear of reactivity extending from 36 to 80 kDa. This IgE reactivity was Hev b 5-specific (inhibitable) and a similar pattern of smeared reactivity was shown by the Hev b 5-specific mAbs (Fig. 1b) although the smear extended to a higher molecular mass of around 110 kDa. Strong reactivity to the glove extract was observed despite a low protein amount (5 µg per lane) of the glove extract being loaded on the gel and almost no protein being visualized on CBB stain, though some protein was observed with silver staining (Fig. 1b).

The inhibition ELISAs showed that rHev b 5 gave a strong dose-dependent inhibition of antibody reactivity (Fig. 3a) with a strong correlation between the two standard curves (IgE and mAb6F6) $R^2 = 0.94$ ($P < 0.001$). The IgE ELISA was more sensitive with a limit of sensitivity (statistically significant difference from 0 inhibitor) of 0.2 µg/mL rHev b 5; the mAb inhibition ELISA sensitivity was 0.8 µg/mL rHev b 5. The high protein powdered glove extracts gave relatively more inhibition even after adjusting for protein content than NAL or LAL (Fig. 3b and Table 1). Rye grass pollen extract used as a negative control gave no inhibition. Similarly, the high quality surgical gloves with undetectable total protein, both powdered and non-powdered also gave little or no inhibition, though there was a trend towards G5 having a greater Hev b 5 content than G3 or G4. Natural and recombinant latex proteins gave no inhibition of a rye grass pollen specific mAb in a control rye grass pollen-specific ELISA (data not shown).

Discussion

Hev b 5-specific mAbs have important implications for allergen avoidance and diagnosis of latex allergy, but are currently lacking. In this study, we have produced and immunologically characterized Hev b 5-specific mAbs. With the use of these, we have shown that immunoreactive rHev b 5 equivalents are more abundant in some latex GEs than in crude latex sap extracts. The predominant immunoreactivity in GE is in the higher molecular mass range (approximately 36–110 kDa) as assessed by immunoblotting, compared with NAL at around 26 kDa. These proteins also stain poorly with CBB, consistent with Hev b 5 [7]. The predominant reactivity in GE is observed as a smear, indicating that Hev b 5 may be degraded following glove manufacture, form aggregates and/or interact with other latex proteins. The rHev b 5 also appeared to form higher molecular mass aggregates seen on our Western blotting. The smeared pattern of rHev b 5-specific IgE reactivity observed in this study is also seen in IgE immunoblots of latex glove extracts reported by other investigators [16–18] but has not previously been attributed to nHev b 5. Interestingly, this smear is also similar to that noted by Swanson et al. in their IgE immunoblots of latex aeroallergen samples [18]. This suggests an important role for Hev b 5 as a latex aeroallergen bound to glove donning powder and may explain its noted higher frequency of sensitization amongst HCWs compared with spina bifida children [6].

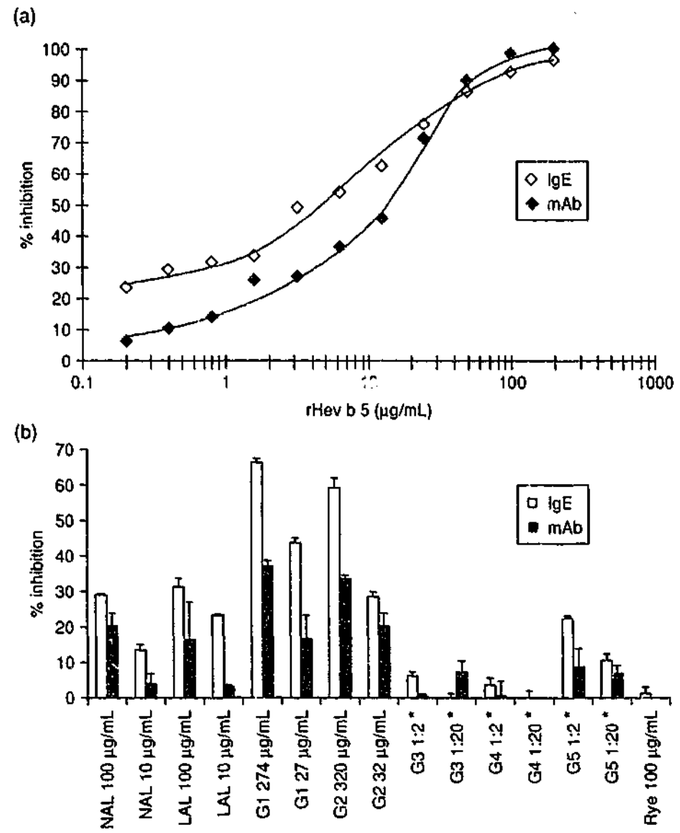


Fig. 3. Inhibition ELISA. analysis of rHev b 5 with serum IgE of latex-allergic HCWs and mAb 6F6. Recombinant Hev b 5 was immobilised on an ELISA plate and human IgE/mAb 6F6 binding was inhibited with different amounts of rHev b 5 to generate standard curves (a) for the determination of rHev b 5 equivalents in the different latex extracts (b). Latex extracts used (as potential inhibitors of antibody binding to rHev b 5) included NAL, LAL, different glove extracts (GE; G1 and G2 are powdered non-sterile utility gloves, G3 and G5 are powdered sterile surgical gloves and G4 is a non-powdered sterile surgical glove) and rye grass pollen extract (irrelevant allergen negative control). Measurements are means (+/-SEM) of triplicates. The sensitivity of the human IgE assay was, 0.2 µg/ml and of the mAb assay was, 0.8 µg/ml. For glove extracts (GE) where protein was undetectable, concentration is expressed as an extract dilution (*).

Table 1. Relative Hev b 5 content of latex extracts

	Total protein concentration (ug/mL)	rHev b 5 equivalents by mAb ELISA (ug/mL)	rHev b 5 equivalents by IgE ELISA (ug/mL)	Mean % rHev b 5 equivalents by mAb ELISA #	Mean % rHev b 5 equivalents by IgE ELISA#
G1	274	6.5	12.5	3.0	7.5
	27.4	1.0	2.8		
G2	320	5.0	8.0	1.6	2.2
	32	†	0.6		
G3	*	†	†	†	†
G4	*	†	†	†	†
G5	*	†	†	†	†
NAL	100	1.8	0.6	1.8	0.6
LAL	100	1.0	1.0	1.0	1.0

G1, G2: powdered non-sterile utility gloves; G3, G4, G5: sterile surgical gloves.Levels of total protein (*) or rHev b 5 equivalents (†) below limits of detection.Mean percentage rHev b 5 equivalents (#) were obtained by calculating the mean of [(rHev b 5 equivalent/total protein concentration) × 100] for two concentrations of extract where rHev b 5 equivalent values were detectable, or one value only where lower dilution had undetectable rHev b 5 levels (e.g. NAL or LAL).

Both our Hev b 5-specific mAb and human IgE ELISAs successfully determined rHev b 5 equivalents in the different latex extracts. However, the human IgE ELISA gave higher values. This may be due to the higher sensitivity of the human IgE ELISA (i.e. 0.2 µg/mL) than the mAb ELISA (i.e. 0.8 µg/mL). In addition, unlike mAbs which may recognize only one

rHev b 5 epitope, due to its polyclonal nature, human IgE may recognize more rHev b 5 epitopes, therefore contributing to the observed higher detection levels. Nevertheless, there was a strong correlation between the rHev b 5-specific mAb and IgE ELISAs. The advantage of standardization available with the mAb make it a potentially useful tool in assays of Hev b 5 content of latex extracts and aeroallergen samples. Considering that a number of different mAbs were produced, we attempted to establish a two-site ELISA for Hev b 5 detection using three of the mAbs: 6A10, 3G3 and 6F6 (1C10 not yet evaluated). However, no sandwich formation was observed using these antibodies suggesting that these mAbs may recognize the same region on the allergen molecule. Nevertheless, we did successfully develop a sensitive one-site mAb ELISA, as presented here, to determine relative Hev b 5 content of different latex extracts.

Our method of glove protein elution (short incubation time, PBS alone on interior of glove) may have favoured the elution of Hev b 5 over other latex allergens, however, it may represent a more 'physiological' method compared with cutting gloves up or using detergents such as SDS to elute particle bound proteins. Recent publications have shown a higher level of protein on the interior of gloves and in powdered latex gloves [16]. We speculate that Hev b 5 proteins are altered during the glove manufacturing process (high temperatures, addition of cornstarch donning powder) resulting in greater immunoreactivity through exposure of IgE binding cryptopes [19]. Other investigators have found some allergens more IgE reactive after roasting [20]. Unfortunately, glove-manufacturing processes are non-standardized and often considered proprietary, making systematic evaluation difficult. Unlike the latex allergen Hev b 1 which is more abundant in crude latex extracts (7% of NAL) compared with glove extracts (2–4%) [21], our results indicate that Hev b 5 may be enriched by the glove manufacturing process in some high protein powdered gloves.

The high quality surgical gloves assayed showed levels of both protein and rHev b 5 equivalents that were beneath the levels of detection. It is likely that the modern processes of extensive high temperature washing and leaching used in the manufacture of such gloves and other medical rubber products [22,23] have been highly effective with regard to reduction in immunoreactive nHev b 5 levels. This study is in agreement with other studies showing a reduction in surgical glove protein levels and allergenicity over the last decade [5]. It cautions however, that lower quality, non-medical grade powdered latex gloves are a rich source of immunoreactive nHev b 5 and represent a potential hazard if inappropriately used (e.g. repeated high level use by atopic individuals or food handlers) in the community. Such gloves may be also high in endotoxin [24] and this has been shown to exert a Th2-adjuvant effect in a mouse model of rHev b 5/MBP immunization [25] adding to their potential hazard. This information is an important reminder that as medical glove technology and awareness in the health care setting improves, we must be vigilant to ensure that inappropriate high allergen glove use by non-HCWs does not expand the latex allergy epidemic unnecessarily into the wider community. The availability of highly specific mAbs offers the potential to monitor the Hev b 5 content of different latex extracts to assist in allergen avoidance strategies and together with purified and IgE reactive rHev b 5 offers hope for improved diagnosis of latex allergy.

Acknowledgements

The authors would like to thank Professor Jim Goding for advice regarding the mAbs production procedure, Mr Michael Bailey for statistical advice, Sister Sue McLellan for her invaluable help in subject recruitment and data collection. Financial support from the National Health and Medical Research Council of Australia, Alfred Research Trusts and the Cooperative Research Center (CRC) for Asthma is gratefully acknowledged.

References

1 Poley GE, Slater JE. Latex allergy. J Allergy Clin Immunol 2000; 105:1054–62.
2 Liss GM, Sussman GL, Deal K et al. Latex allergy: epidemiological study of 1351 hospital workers. Occup Environ Med 1997; 54:335–42.
3 Bernardini R, Novembre E, Lombardi E et al. Prevalence of and risk factors for latex sensitisation in patients with spina bifida. J Urol 1998; 160:1775–8.
4 Ylitalo L, Turjanmaa K, Palosuo T, Reunala T. Natural rubber latex allergy in children who had not undergone surgery and children who had undergone multiple operations. J Allergy Clin Immunol 1997; 100:606–12.
5 Sussman GL, Liss GM, Deal K et al. Incidence of latex sensitisation among latex glove users. J Allergy Clin Immunol 1998; 101:171–8.
6 Slater JE, Vedvick T, Arthur-Smith A, Trybul DE, Kekwick RG. Identification, cloning, and sequence of a major allergen (Hev b 5) from natural rubber latex (*Hevea brasiliensis*) J Biol Chem 1996; 271:25394–9.
7 Akasawa A, Hsieh LS, Martin BM, Liu T, Lin Y. A novel acidic allergen, Hev b 5, in latex. Purification, cloning and characterisation. J Biol Chem 1996; 271:25389–93.
8 de Silva HD, Sutherland MF, Suphioglu C et al. Human T-cell epitopes of the latex allergen Hev b 5 in health care workers. J Allergy Clin Immunol 2000; 105:1017–24.
9 Yip L, Hickey V, Wagner B et al. Skin prick test reactivity to recombinant latex allergens. Int Arch Allergy Immunol 2000; 121:292–9.
10 Beezhold DH, Hickey VL, Slater JE, Sussman GL. Human IgE-binding epitopes of the latex allergen Hev b 5. J Allergy Clin Immunol 1999; 103:1166–72.
11 Chen Z, Rihs HP, Slater JE, Paupore EJ, Schneider EM, Baur X. The absence of Hev b 5 in capture antigen may cause false negative results in serologic assays for latex-specific IgE antibodies (Abstract). J Allergy Clin Immunol 2000; 105:S83.
12 Goding J. Monoclonal Antibodies: Principles and Practice, 3rd edn. London: Academic Press 1996.
13 Harlow E, Lane D. Antibodies, 1st edn. New York: Cold Spring Harbor Laboratory 1988.
14 Sutherland MF, O'Hehir RE, Czarny D, Suphioglu C. Macadamia nut anaphylaxis: demonstration of specific IgE reactivity and partial cross-reactivity with hazelnut. J Allergy Clin Immunol 1999; 104:88.
15 Smart IJ, Heddle RJ, Zola H, Bradley J. Development of monoclonal mouse antibodies specific for allergenic components of Ryegrass (Lolium perenne) Pollen Int Arch Appl Immunol 1983; 72:243–8.
16 Mahler V, Fischer S, Fuchs T et al. Prevention of latex allergy by selection of low-allergen gloves. Clin Exp Allergy 2000; 30:509–20.
17 Grote M, Mahler V, Spitzauer S, Fuchs T, Valenta R, Reichelt R. In situ localization of latex allergens in 3 different brands of latex gloves by means of immunogold field emission scanning and transmission electron microscopy. J Allergy Clin Immunol 2000; 105:561–9.

18 Swanson MC, Bubak ME, Hunt LW, Yunginger JW, Warner MA, Reed CE. Quantification of occupational latex aeroallergens in a medical center. J Allergy Clin Immunol 1994; 94:445–51.
19 Laver G, Air GM, Webster RG, Smith-Gill SJ. Epitopes on protein antigens: misconceptions and realities. Cell 1990; 61:553–6.
20 Maleki SJ, Chung SY, Champagne ET, Raufmann JP. The effects of roasting on the allergenic properties of peanut proteins. J Allergy Clin Immunol 2000; 106:763–8.
21 Raulf-Heimsoth M, Sander I, Chen Z et al. Development of a monoclonal antibody-based sandwich ELISA for detection of the latex allergen Hev b 1. Int Arch Allergy Immunol 2000; 123:236–41.
22 Lundberg M, Wrangsjo K, Eriksson-Widblom K, Johansson SG. Reduction of latex-allergen content in Swedish medical catheter balloons - a survey of 3 years' production. Allergy 1997; 52:1057–62.
23 Baur X, Rennert J, Chen Z. Latex allergen elimination in natural latex sap and latex gloves by treatment with alkaline potassium hydroxide solution. Allergy 1997; 52:306–11.
24 Williams PB, Halsey JF. Endotoxin as a factor in adverse reactions to latex gloves. Ann Allergy Asthma Immunol 1997; 79:303–10.
25 Slater JE, Paupore EJ, Elwell MR, Truscott W. Lipopolysaccharide augments IgG and IgE responses of mice to the latex allergen Hev b 5. J Allergy Clin Immunol 1998; 102:977–83.

Clin Exp Allergy 2002; 32:667–673

REVIEW

Latex allergy: towards immunotherapy for health care workers

M. F. Sutherland*†‡, C. Suphioglu*†‡, J. M. Rolland†‡ and R. E. O'Hehir*†‡

*Departments of *Allergy, Asthma and Clinical Immunology, and †Pathology and Immunology, The Alfred Hospital and Monash University, Victoria, and the ‡Co-operative Research Centre for Asthma, Sydney, Australia*

Summary

Latex allergy is an important allergic disease for which safe and readily available immunotherapy is currently lacking. Despite advances in latex glove technology and reduction in allergen content, there remains a core of severely allergic health care workers (HCW), particularly with concominant food allergy, for whom allergen avoidance is insufficient. Current experience with immunotherapy using crude latex extracts has shown an unacceptable level of local and systemic side-effects. Latex allergens are extremely potent with a heightened capacity to cross-link effector cell-bound IgE and induce anaphylaxis. The predominant pattern of allergen reactivity among HCW is different from that among children with spina bifida, perhaps due to exposure to latex glove proteins, particularly via inhalation, rather than particle bound latex proteins present in urinary catheters. Recent studies using purified skin testing reagents have indicated that the most clinically important latex allergens amongst HCW are Hev b 5, 6 and 7. Elucidation of the molecular and cellular mechanisms of the immune response to these allergens is pivotal to facilitate the search for safer immunotherapy of latex allergy among HCW.

Background

Latex allergy is principally a late 20th century phenomenon, rising to prominence through the convergence of a number of factors, including the rise of atopy in general, increased awareness by both patients and clinicians, and most importantly, the Human Immunodeficiency Virus (HIV) epidemic which resulted in an exponential increase in the use of latex gloves as a barrier protection among HCW. The disorder has attracted attention for its potentially catastrophic consequences when mucosal surfaces are breached by latex proteins in allergic individuals. This happened with the sentinel barium enema deaths [1], and is seen in intraoperative anaphylaxis to latex [2]. Moreover, latex allergy causes considerable morbidity in affected individuals and may be associated with asthma [3], rhinoconjunctivitis [4], urticaria [5] and food allergy [6].

This article will focus on the current and possible future approaches towards safer immunotherapy of latex allergy, concentrating on HCW. It will discuss the current status of immunotherapy in latex allergy and review the latex allergens of clinical importance among HCW, which are thus potential immunotherapy candidates. Finally, it will look at the possible future approaches to immunotherapy including T cell epitope peptides, hypoallergenic mutants, allergens conjugated to immunostimulatory sequences (ISS) of DNA and DNA vaccination.

Correspondence: Professor R. E. O'Hehir, Department of Allergy, Asthma and Clinical Immunology, Alfred Hospital, Commercial Road, Prahran, VIC 3181, Australia. E-mail: Robyn.OHehir@med.monash.edu.au

Traditional immunotherapy for latex allergy

Three studies have reported immunotherapy in latex allergy, and all have used crude ammoniated latex preparations. The most important of these studies was a randomized, placebo-controlled trial using the Stallergenes low ammoniated latex (LAL) preparation [7]. This is a standardized extract used widely in Europe and on a restricted basis in Australia for skin testing in the diagnosis of latex allergy. It is manufactured from Malaysian rubber (RRIM clone 600), has a 100% specificity and 93% sensitivity when used at 100 index of reactivity (IR) units (22 μg/mL concentration) in skin testing for latex allergy diagnosis and contains all the known IgE binding latex components [8]. This was a multicentre study in which 17 HCW with latex allergy were randomized to immunotherapy or placebo. The immunotherapy regimen involved a two day rush protocol where patients were up-dosed to their maximum tolerated dose with the 10 IR or 100 IR vial depending on sensitivity. This was followed by a 12-month maintenance phase of injections at the maximum tolerated dose, initially fortnightly and then monthly. The active treatment group reported a statistically significant decrease in symptoms of rhinoconjunctivitis and urticaria but not of asthma, though it was noteworthy that the placebo group had an increased incidence of asthma at the commencement of the study compared with the active group. Although efficacy for the treatment was shown, side-effects were noted. These included a local reaction rate of over 40% in the active treatment group compared with 15% in the placebo group. In addition, 15% of injections in the active group induced episodes of rhinitis, 2.7% induced asthma, 0.6% angioedema and 0.3% systemic anaphylaxis. Most importantly, these reactions occurred just as frequently in the maintenance phase of injections as compared with the up-dosing phase of treatment.

The two other reports of immunotherapy in latex allergy are case reports. In the first, Pereira et al. [9] described the desensitization of a 31-year-old-radiology technician using a low

ammoniated latex extract (ALK-Abello). During the up-dosing phase of immunotherapy she had a systemic reaction with hypotension and voice hoarseness requiring adrenaline. Again, once in the maintenance phase, symptoms upon latex exposure were reported by the patient to be much reduced and this was validated by a reduction in reactivity on latex provocation testing and skin testing. Interestingly, there were also decreases in *in vitro* reactivity to banana, kiwi and chestnut. The second of the case reports described the sublingual desensitization of a latex-allergic medical student using the ALK-Abello LAL extract [10]. There were no systemic side-effects reported up to a cumulative dose of 500 μg of the extract, while there was a reduction in symptoms and reactivity to latex-specific provocation of the subject.

These studies indicate some of the inherent difficulties with immunotherapy of latex allergy. Firstly, paradoxically, those patients who are the most latex-sensitive, and thus have the greatest potential benefit from desensitization, are most at risk from therapy. Patients with mild or moderate latex allergy may be managed successfully with allergen avoidance measures alone [11]. Immunotherapy, to be of real benefit, must safely help subjects with severe allergy for whom allergen avoidance is more difficult because of their propensity to get symptoms on exposure to even minute quantities of latex protein. Latex is a ubiquitous material within our modern day environment and these severely affected individuals may get symptoms where mildly affected individuals have no difficulty. In fact, they may be put at risk of anaphylaxis through everyday activities in the community [12, 13]. The studies to date have indicated that there is a high risk of anaphylaxis, or at least severe local side-effects with immunotherapy using crude latex extracts. Thus, highly latex-sensitive individuals may be exposed to considerable risk by immunotherapy using crude extracts. At present, immunotherapy with crude extracts can only be considered experimental and must be performed by an experienced allergist in a hospital setting where intensive care backup is readily available. Further trials of immunotherapy with crude extracts are awaited with interest and are underway in Europe with the Stallergenes extract and in the USA with the Greer non-ammoniated latex (NAL) extract.

Latex allergens are extremely potent and have a high capacity to cross-link effector cell-bound IgE

The immunotherapy experience with crude extracts combined with the instances of barium enema and intraoperative anaphylaxis indicate that latex proteins are extremely potent allergens in their ability to induce severe IgE-mediated reactions. Other experimental evidence further supports this claim. For example, in a skin testing study using recombinant and natural latex extracts, Yip et al. found that the most latex-sensitive patient reacted to a Bencard LAL extract on simple skin testing down to a concentration of 70 pg/mL [14]. In addition, it has been recently shown that latex proteins eluted from rubber medication vial closures are enough to elicit weal and flare reactions when used in skin testing of latex-allergic individuals [15]. Latex is also the most common allergen to induce systemic reactions on skin testing, when compared with all others including drugs, foods and pollens [16]. The exact molecular mechanisms for this potency are yet to be fully elucidated. It has been suggested that the major latex allergen Hev b 5, may have enhanced ability through multivalency to cross-link multiple effector cell-bound IgE molecules [17]. Other possible reasons for potency of latex allergens may relate to their bio-availability or enhanced ability to traverse mucous membranes, but little firm data is available.

The latex–fruit syndrome makes allergen avoidance difficult for some latex-allergic individuals

Up to 52% of latex allergy sufferers have sensitivity to various fruits and vegetables [6]. Also, latex-allergic individuals have four times the risk of the general population of food allergy. Food allergy amongst latex-sensitive subjects frequently manifests as anaphylaxis (36% in the Blanco study) [6]. Therefore, the latex–fruit syndrome is of considerable clinical importance. A Medline search identified more than 20 foods, fruits or plants that have been reported to be immunologically cross-reactive with latex [6, 18–32]. The most commonly described are banana, avocado, kiwifruit and chestnut. Figure 1 however, shows the fruits and vegetables reported cover a wide proportion of the plant kingdom. This is indicative of two important issues: firstly, that a pan-allergen (or allergens) is likely important, and secondly, the breadth of dietary components that may put severely latex-allergic individuals at risk of food allergy symptoms. It is not currently advised for latex-allergic patients to avoid all such foods unless they have specific symptoms. However, it is incumbent upon clinicians to warn patients of the potential for these reactions. The major latex allergen Hev b 6 has been identified as being the principal latex allergen responsible for this cross-reactivity [33]. The hevein molecule (Hev b 6.02) has striking homology with Class 1 chitinases [34] which have a hevein-like domain and occur widely in plants, thus fitting the description of a 'pan-allergen'. RAST inhibition and skin testing studies have provided further correlation of this molecular observation. Although primary sensitization via fruit exposure has been reported and is a risk factor for latex allergy [26], current evidence indicates that primary sensitization to latex accounts for the vast bulk of the latex-fruit syndrome. Whether other latex allergens are responsible for food cross-reactivity is not so clear. Hev b 5 has sequence homology to an acidic protein in kiwi fruit [35] and also in sugar beet [36] but no evidence for cross-reactivity has been found. Hev b 7 has sequence homology to patatin, which can cause positive skin test results and inhibition of IgE binding to Hev b 7 among Hev b 7-sensitized individuals [29]. Hev b 8 is a profilin; homologues of which are identified as allergens in many fruits and vegetables. Clear evidence of primary sensitization to Hev b 8 via latex exposure with subsequent food allergy has not been demonstrated and therefore for Hev b 8-sensitive individuals, primary sensitization to grass pollen profilins are the likely cause [37].

The most clinically important allergens in HCW are Hev b 5, 6 and 7

As described in the previous section, the qualification of Hev b 6 as a pan-allergen cements its clinical importance. IgE-binding, and skin test data also add support. The 4.7 kDa Hev b 6.02 (hevein) is cleaved from the *N*-terminus of Hev b 6.01

Kingdom	Sub-kingdom	Super-division	Division	Class	Sub-class	Order	Family	Genus, species (Common name)
Plantae (Plants)	Tracheobionta (Vascular plants)	Spermatophyta (Seed plants)	Magnoliophyta (Flowering plants)	Magnoliopsida (Dicotyledons)	Rosidae	Apiales	Apiaceae (Carrot family)	*Apium* L. **(Celery)** [27]
						Euphorbiales	Euphorbiaceae (Spurge family)	*Hevea brasiliensis* (Willd. Ex Adr. Juss) Muell. Arg. **(Rubber tree)**
						Rosales	Rosaceae (Rose family)	*Malnus*. P. Mill. **(Apple)** [27] *Prunus* (L.) **(Plum)** [30] *Prunus avium* (L.) L. **(Sweet cherry)** [23] *Prunus persica* (L.) Batsch **(Peach)** [26] *Prunus persica* (L.) Batsch var. nucipersica (Suckow) C. Sehneider **(Nectarine)** [30]
						Sapindales	Anacardiaceae (Sumac family)	*Managifera Indica* L. **(Mango)** [25]
					Magnoliidae	Lauraceae	Lauraceae (Laurel family)	*Persea americana* P. Mill. **(Avocado)** [19]
						Magnoliales	Annonaceae (Custard-apple family)	*Annona cheirimola* P Mill. **(Cherimoya)** [28] *Asimina triloba* L. **(Pawpaw)** [6]
					Hamamelidae	Fagales	Fagaceae (Beech family)	*Castanea sativa* P.Mill. **(European chestnut)** [6]
						Urticales	Moraceae (Mulberry family)	*Artocarpus altilis* (**Parkinson**) Fosberg **(Breadfruit)** [24] *Ficus benjamina* L. **(Weeping fig)** [24]
					Dilleniidae	Ebenales	Ebenaceae (Ebony family)	*Diospyros virginiana* L. **(Persimmon)** [21]
						Theales	Actinidiaceae (Chinese gooseberry family)	*Actinidia chinensis* Planchon. **(Kiwi fruit)** [6]
						Violales	Cucurbitaceae (Cucumber family)	*Cucumis* L. **(Melon)** [26] *Cucurbita pepo* L. **(Zucchini)** [31]
					Caryophyllidae	Polygonales	Polygonaceae (Buckwheat family)	*Eriogonum Michx.* **(Buckwheat)** [20]
						Solanales	Solanaceae (Potato family)	*Solanum lycopersicum* L. **(Tomato)** [20] *Solanum tuberosum* L. **(Potato)** [29]
				Liliopsida (Monocotyledon)	Zingiberidae	Bromeliales	Bromeliaceae (Bromeliad family)	*Ananas comosus* (L.) Merr. **(Pineapple)** [27]
						Zingiberales	Musaceae (Banana family)	*Musa acuminata* Colla **(Banana)** [18]
					Commelinidae	Cyperales	Poaceae (Grass family)	*Phleum pratense* L. **(Timothy grass)** [32]

Fig. 1. Taxonomic classification of fruits, vegetables and other plant tissues reported to cross-react with latex. References are cited by numbers in square brackets following common names.

(prohevein). Being hydrophilic, hevein is easily leached from gloves during wear and forms a large percentage of extractable protein of gloves [38]. Purified hevein has also been found on skin testing to induce a positive reaction in 81% of patients with latex allergy [39].

Hev b 5 is also an important allergen amongst HCW. Our group has shown that Hev b 5 is relatively more abundant in high protein powdered glove extracts than in raw latex extracts [40]. This is likely due to its ability to survive the harsh conditions of glove manufacture and, as for hevein, its hydrophilicity. Latex allergy among HCW may result from both sensitization through the respiratory tract by inhalation of latex proteins adsorbed to carrier particles such as cornstarch donning powder, and sensitization through direct glove contact via the transcutaneous route. Therefore, this results in the predominant reactivity to Hev b 5, 6 and 7 rather than the so-called rubber particle bound proteins Hev b 1 and 3, that are important allergens among children with spina bifida. Slater et al. found that 92% of latex-allergic HCW reacted with the fusion protein of rHev b 5 with maltose binding protein (MBP) by RAST [41].

The skin testing study performed by Yip et al. used recombinant and purified latex allergens in skin testing of latex allergic patients. This was a valuable study since it employed an *in vivo* method and the allergens were systematically evaluated in serial dilutions. The allergens tested were Hev b 2, 5, 6, 8 (recombinant) and Hev b 3 and 7 (purified natural protein). All allergens were skin tested at 10-fold serial dilutions in 31 latex-allergic individuals (predominantly HCWs), two of whom were excluded from the analysis (one due to dermographism, the other due to non-reactivity to the LAL-positive control), and 10 non-latex allergic controls. The important findings of the study were that 93% of subjects with a positive skin test to the raw latex extract (Bencard, LAL) reacted to one or more of the recombinant or purified latex allergens. Furthermore, the most frequent reactivity was to Hev b 6 (66%), Hev b 5 (62%) or Hev b 7 (41%) and all subjects who reacted to one or more recombinant or purified latex allergens reacted to one or more of Hev b 5, 6 and 7, giving a 93% diagnostic sensitivity by using these three recombinant allergens alone. None of the 10 control individuals reacted to the recombinant or purified allergens, giving 100% specificity. More importantly, no adverse reactions were reported on skin testing with the recombinant allergens, though detailed analysis of adverse events was not given. Another interesting finding of the study was the presence of monosensitization, particularly to Hev b 5 (17%), 6 (10%) and 7 (10%); again making immunotherapy with a combination of these allergens more attractive. One weakness of the study was that not all the cloned and sequenced latex allergens were used; particularly Hev b 1 and Hev b 4. Hev b 1 appears to be more important among the *spina bifida* population and shows cross-reactivity with Hev b 3 (which was used) while Hev b 4 may be an important allergen among HCW but further skin testing studies are currently underway. Also, only a small number of controls were used, and larger studies will be needed to establish the sensitivity and safety of these recombinant and natural latex allergens.

These studies, combined with the fact that Hev b 5 and 6 are the relatively abundant latex glove proteins, qualify Hev b 5, 6 and 7 as the most clinically important allergens among HCW. Therefore, the successful development of immunotherapeutic reagents representing these allergens would be, at the very least, a considerable advance, and may be sufficient to ensure effective treatment for the majority of HCW.

Reducing the potential for anaphylaxis: (I) peptide immunotherapy for latex allergy

There has been a renewal of interest in peptide immunotherapy of allergy following two encouraging reports on cat [42] and honey bee venom allergy [43].

T cell epitope peptides are intrinsically attractive for use in immunotherapy because of their ability to stimulate T cells and thus modulate the cellular immune response, without cross-linking effector cell-bound IgE. It is already known that immunotherapy for latex and other diseases is effective, however, the risks of IgE mediated morbidity and possibly mortality makes immunotherapy for latex allergy in its current form unacceptable [7]. Honey bee venom allergy has some parallels to latex allergy in that traditional immunotherapy with natural antigens has, at times, a high risk of systemic side-effects [44]. In honey bee venom allergy, however, this risk is warranted because of the difficulty of absolute avoidance and the measurable and real risk of fatality amongst unprotected honey bee allergic individuals. In an effort to reduce systemic side-effects, Muller et al. administered a mixture of the three dominant T cell epitopes of phospholipase A_2 (PLA_2) to five honey bee venom-allergic subjects. There were no systemic side-effects from the subcutaneous injections of the peptides, and the patients were protected from challenge with PLA_2 in all cases and in 3/5 against sting challenge. The authors demonstrated decreased *in vitro* T cell proliferation and cytokine secretion consistent with anergy to the whole PLA_2 molecule.

Tempering this enthusiasm has been the knowledge that peptide injections have been reported to induce adverse events in cat allergic patients [45]. More recently, Haselden et al. showed that a mixture of Fel d 1 T cell epitope peptides in equimolar amounts was able to induce MHC-restricted T cell-dependent late asthmatic responses independent of IgE [42]. While these responses are of concern they indicate that Fel d 1 specific T cells are being activated without effector-cell IgE cross-linking. More encouragingly, the same group has reported both safety and efficacy of immunotherapy with Fel d 1 T cell epitope peptides in a double-blind, placebo-controlled trial [46], and in addition, developed a dosage schedule that avoids the induction of late asthmatic responses [47]. The use of a dominant peptide or small number of peptides may be advantageous in avoiding some of the potential problems of aggregation of a complex mixture of 20 or more peptides. The dominant peptides, being better immunogens may be better tolerogens and there is evidence from animal models that a dominant peptide can induce tolerance against the whole molecule through linked suppression [48]. This was also seen in the honey bee venom peptide immunotherapy trial [43].

What of peptide approaches in latex allergy? Our work with short-term LAL- and rHev b 5-specific T cell lines has shown that Hev b 5 has a dominant T cell epitope [49]. Hev b 5 peptide 46–65 caused highly significant T cell proliferation and increased IL-5 cytokine secretion in 5/6 highly latex-allergic HCW studied. A T cell proliferation assay to Hev b 5 peptides from a HCW with high IgE-specific ELISA response to rHev b 5

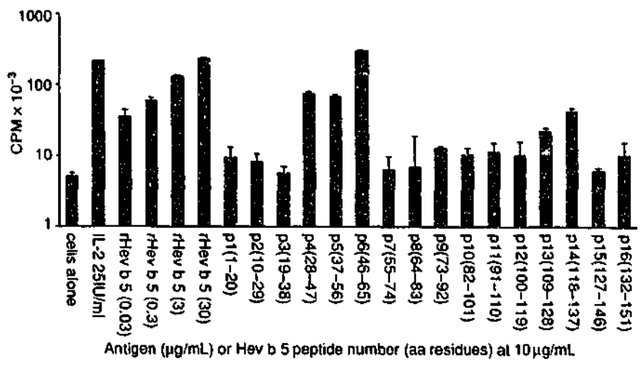


Fig. 2. T cell proliferation assay from short-term T cell line of latex-allergic HCW. PBMCs were stimulated for 1 week with a Hev b 5-rich powdered examination glove extract (GE) at 20 μg/mL. Cells were then recovered, autologous irradiated PBMC added in 1 : 1 ratio and restimulated for a further week with GE 20 μg/mL. Suboptimal IL-2 (25 IU/mL) was added on day 1 and 3 following restimulation. T cells were then recovered once more, autologous irradiated PBMC added in 1 : 1 ratio and the cells stimulated with different concentrations of rHev b 5 or Hev b 5 peptides at 10 μg/mL for 72 h. Proliferation was measured by tritiated thymidine incorporation and expressed as counts per minute (CPM). Mean CPM (+ SEM) for triplicate cultures are shown.

is shown (Fig. 2). Whether a single, highly dominant peptide will be sufficient to induce T cell anergy against the whole Hev b 5 molecule is at present unknown. Hev b 5 is an attractive initial target for immunotherapy strategies due to the appreciable incidence (17%) of monosensitization seen amongst latex allergic HCW [14]. However it is likely, as already stated, that effective immunotherapy for latex allergy in HCW would need to target at least Hev b 6 and 7 in addition to Hev b 5. Apart from Hev b 5, T cell epitope mapping has only been reported for Hev b 1 [50] and 3 [51] in children with spina bifida, and as discussed, these allergens appear much less important in the HCW population.

Reducing the potential for anaphylaxis: (II) hypoallergenic mutants

Another approach to decrease the effector cell-bound IgE cross-linking with the aim of reducing potential side-effects, is to modify the primary, secondary or tertiary structure via engineering of recombinant allergens. To maintain efficacy, T cell reactivity must be preserved. This has been reported in other allergen systems, notably house dust mite [52], birch pollen [53] and timothy grass pollen [54].

With regard to the clinically relevant latex allergens in HCW, Hev b 5 has been modified by Beezhold et al. to reduce IgE binding [17]. They found that a total of seven alanine substitutions were required within three of the linear IgE binding epitopes of Hev b 5 to obtain a 100-fold reduction in IgE binding to the whole molecule by inhibition ELISA. With a total of 14 alanine substitutions in 8 epitopes, IgE binding could be decreased by 4500 fold. Figure 3 shows schematically the combination of the major T cell [49] and linear B cell [17, 55] epitopes of Hev b 5 that have been reported. As can be seen there is some overlap. Consequently for the generation of successful candidate allergen mutant vaccines, future studies will need to address whether such mutants retain T cell reactivity in addition to the demonstration of decreased IgE binding.

Hev b 6 is an attractive target for modification of tertiary structure via mutagenesis, particularly in the highly reactive hevein domain which is cysteine rich and contains four disulphide bridges [56]. At this stage there are no published studies describing mutation of Hev b 6 and its effect on IgE binding. Similarly, mutational studies of Hev b 7 have not been reported.

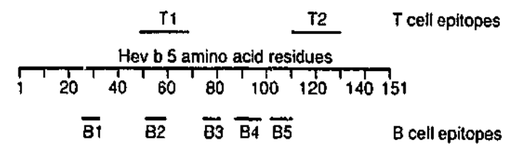


Fig. 3. Schematic diagram of major T cell and B cell epitopes for Hev b 5. Epitope sequences were classified as major if they reacted with greater than 50% of Hev b 5 reactive patients [17, 49]. Amino acid residues: T1 = 45–65, T2 = 109–128; B1 = 27–34, B2 = 50–59, B3 = 75–82, B4 = 86–98, B5 = 103–111.

Reducing the potential for anaphylaxis: (III) DNA vaccination and conjugation of allergens to immunostimulatory DNA

Finally, in addition to mutant recombinant allergen vaccines, DNA vaccination or conjugation of allergens to immunostimulatory sequences (ISS) of DNA hold promise as alternative methods of delivering allergen immunotherapy. Animal studies [57, 58] and *in vitro* human studies [59] using ISS in ragweed pollen allergy and DNA vaccination in peanut allergy [60] have demonstrated decreased allergenicity with increased immunogenicity and the ability to modulate established allergic states, by the up-regulation of allergen-specific Th1 responses and concomitant reduction in Th2 responses. In latex allergy, studies have thus far been limited to a murine model utilizing DNA vaccination for Hev b 5 [61]. This study showed successful distribution of the Hev b 5 transcript in the lungs, spleen, lymph nodes, blood and tongue and the mounting of both an allergen-specific cellular and humoral immune response of Th1 type. Toxicity was not seen, but severe local reactions had been observed by the same author during optimization experiments [61]. It is likely that safety and efficacy of DNA vaccination or ISS conjugate vaccines in humans will be established in other allergen systems such as ragweed pollen allergy, where trials of an ISS conjugated Amb a 1 have begun [62], prior to its possible application in the immunotherapy of latex allergy.

Summary and conclusions

Latex allergy is a significant health hazard for HCW. As yet, specific therapy that is both safe and effective is lacking.

Immunotherapy with crude extracts has been shown to be effective in symptom reduction, but has an unacceptable risk of local and systemic side-effects. Latex allergy among HCW is characterized by a different pattern of reactivity to latex allergens from that seen in spina bifida children. The pattern of reactivity among HCW is predominantly to Hev b 5, 6 and 7, and Hev b 5 and 6, appear to be the important allergens in latex gloves, the main route of sensitization and symptoms in HCW. While allergen avoidance may be sufficient for mildly latex-allergic individuals, for more severely affected individuals, particularly with associated food allergy, adequate avoidance may be difficult or impossible and safe immunotherapy is desirable. Hev b 5, 6 and 7 are attractive candidates for immunotherapy approaches both for their frequency of reactivity and also because of the significant incidence of mono-sensitization. Novel approaches to therapy include peptide immunotherapy or modifications of whole allergens such that T cell reactivity is maintained but IgE binding is abrogated. Further studies of *in vitro* T cell reactivity, basophil histamine release and *in vivo* skin testing of candidate peptides and whole allergen mutants are awaited with great interest.

Acknowledgements

The authors would like to thank Co-operative Research Centre (CRC) for Asthma, National Health and Medical Research Council (NH & MRC) of Australia, Alfred Hospital Research Trusts.

References

1 Ownby DR, Tomlanovich M, Sammons N, McCullough J. Anaphylaxis associated with latex allergy during barium enema examinations. AJR Am J Roentgenol 1991; 156:903–8.
2 Swartz J, Braude BM, Gilmour RF, Shandling B, Gold M. Intraoperative anaphylaxis to latex. Can J Anaesth 1990; 37:589–92.
3 Vandenplas O. Occupational asthma caused by natural rubber latex. Eur Respir J 1995; 8:1957–65.
4 Archambault S, Malo JL, Infante-Rivard C, Ghezzo H, Gautrin D. Incidence of sensitization, symptoms, and probable occupational rhinoconjunctivitis and asthma in apprentices starting exposure to latex. J Allergy Clin Immunol 2001; 107:921–3.
5 Nutter AF. Contact urticaria to rubber. Br J Dermatol 1979; 101:597–8.
6 Blanco C, Carrillo T, Castillo R, Quiralte J, Cuevas M. Latex allergy. clinical features and cross-reactiv ity with fruits. Ann Allergy 1994; 73:309–14.
7 Leynadier F, Herman D, Vervloet D, Andre C. Specific immunotherapy with a standardized latex extract versus placebo in aliergic healthcare workers. J Allergy Clin Immunol 2000; 106:585–90.
8 Turjanmaa K, Palosuo T, Alenius H et al. Latex allergy diagnosis: in vivo and in vitro standardization of a natural rubber latex extract. Allergy 1997; 52:41–50.
9 Pereira C, Rico P, Lourenco M et al. Specific immunotherapy for occupational latex allergy. Allergy 1999; 54:291–3.
10 Nucera E, Schiavino D, Buonomo A et al. Latex rush desensitization. Allergy 2001; 56:86–7.
11 Bubak ME. Allergy to natural rubber latex. Ann Allergy Asthma Immunol 2000; 84:175.
12 Pumphrey RS, Duddridge M, Norton J. Fatal latex allergy. J Allergy Clin Immunol 2001; 107:558.
13 Fiocchi A, Restani P, Ballabio C et al. Severe anaphylaxis induced by latex as a contaminant of plastic balls in play pits. J Allergy Clin Immunol 2001; 108:298–300.
14 Yip L, Hickey V, Wagner B, Liss G et al. Skin prick test reactivity to recombinant latex allergens. Int Arch Allergy Immunol 2000; 121:292–9.
15 Primeau MN, Adkinson NF, Hamilton RG. Natural rubber pharmaceutical vial closures release latex allergens that produce skin reactions. J Allergy Clin Immunol 2001; 107:958–62.
16 Valyasevi MA, Maddox DE, Li JT. Systemic reactions to allergy skin tests. Ann Allergy Asthma Immunol 1999; 83:132–6.
17 Beezhold DH, Hickey VL, Sussman GL. Mutational analysis of the IgE epitopes in the latex allergen Hev b 5. J Allergy Clin Immunol 2001; 107:1069–76.
18 Alenius H, Makinen-Kiljunen S, Ahlroth M, Turjanmaa K, Reunala T, Palosuo T. Crossreactivity between allergens in natural rubber latex and banana studied by immunoblot inhibition. Clin Exp Allergy 1996; 26:341–8.
19 Blanco C, Carrillo T, Castillo R, Quiralte J, Cuevas M. Avocado hypersensitivity. Allergy 1994; 49:454–9.
20 Abeck D, Borries M, Kuwert C, Steinkraus V, Vieluf D, Ring J. [Food-induced anaphylaxis in latex allergy]. Hautarzt 1994; 45:364–7.
21 Anliker MD, Reindl J, Vieths S, Wuthrich B. Allergy caused by ingestion of persimmon (*Diospyros kaki*): detection of specific IgE and cross-reactivity to profilin and carbohydrate determinants. J Allergy Clin Immunol 2001; 107:718–23.
22 Antico A. Oral allergy syndrome induced by chestnut (Castanea sativa) Ann Allergy Asthma Immunol 1996; 76:37–40.
23 Kim KT, Hussain H. Prevalence of food allergy in 137 latex-allergic patients. Allergy Asthma Proc 1999; 20:95–7.
24 De Greef JM, Lieutier-Colas F, Bessot JC et al. Urticaria and Rhinitis to Shrubs of Ficus benjamina and Breadfruit in a Banana-Allergic Road Worker: Evidence for a Cross-Sensitization between Moracea, Banana and Latex. Int Arch Allergy Immunol 2001; 125:182–4.
25 Duque S, Fernandez-Pellon L, Rodriguez F. Mango allergy in a latex-sensitized patient. Allergy 1999; 54:1004–5.
26 Garcia Ortiz JC, Moyano JC, Alvarez M, Bellido J. Latex allergy in fruit-allergic patients. Allergy 1998; 53:532–6.
27 Levy DA, Mounedji N, Noirot C, Leynadier F. Allergic sensitization and clinical reactions to latex, food and pollen in adult patients. Clin Exp Allergy 2000; 30:270–5.
28 Sanchez-Guerrero IM, Escudero AI, Tortosa JA, Lombardero M. Anaphylaxis to cherimoya. Allergy 2000; 55:976–7.
29 Seppala U, Palosuo T, Kalkkinen N, Ylitalo L, Reunala T, Turjanmaa K. IgE reactivity to patatin-like latex allergen, Hev b 7, and to patatin of potato tuber, Sol t 1, in adults and children allergic to natural rubber latex. Allergy 2000; 55:266–73.
30 Weiss SJ, Halsey JF. A nurse with anaphylaxis to stone fruits and latex sensitivity: potential diagnostic difficulties to consider. Ann Allergy Asthma Immunol 1996; 77:504–8.
31 Reindl J, Anliker MD, Karamloo F, Vieths S, Wuthrich B. Allergy caused by ingestion of zucchini (*Cucurbita pepo*): characterization of allergens and cross-reactivity to pollen and other foods. J Allergy Clin Immunol 2000; 106:379–85.
32 Fuchs T, Spitzauer S, Vente C et al. Natural latex, grass pollen, and weed pollen share IgE epitopes. J Allergy Clin Immunol 1997; 100:356–64.
33 Mikkola JH, Alenius H, Kalkkinen N, Turjanmaa K, Palosuo T, Reunala T. Hevein-like protein domains as a possible cause for allergen cross-reactivity between latex and banana. J Allergy Clin Immunol 1998; 102:1005–12.

34 Blanco C, Diaz-Perales A, Collada C et al. Class I chitinases as potential panallergens involved in the latex– fruit syndrome. J Allergy Clin Immunol 1999; 103:507–13.
35 Ledger SE, Gardner RC. Cloning and characterization of five cDNAs for genes differentially expressed during fruit development of kiwifruit (*Actinidia deliciosa var. deliciosa*). Plant Mol Biol 1994; 25:877–86.
36 Fowler MR, Gartland J, Norton W, Slater A, Elliott MC, Scott NW. RS2: a sugar beet gene related to the latex allergen Hev b 5 family. J Exp Bot 2000; 51:2125–6.
37 Rihs HP, Chen Z, Rozynek P, Baur X, Lundberg M, Cremer R. PCR-based cloning, isolation, and IgE-binding properties of recombinant latex profilin (rHev b 8). Allergy 2000; 55:712–7.
38 Alenius H, Kalkkinen N, Reunala T, Turjanmaa K, Palosuo T. The main IgE-binding epitope of a major latex allergen, prohevein, is present in its N-terminal 43-amino acid fragment, hevein. J Immunol 1996; 156:1618–25.
39 Chen Z, Posch A, Lohaus C, Raulf-Heimsoth M, Meyer HE, Baur X. Isolation and identification of hevein as a major IgE-binding polypeptide in Hevea latex. J Allergy Clin Immunol 1997; 99:402–9.
40 Sutherland MF, Drew A, Suphioglu C, Slater J, Rolland JM, O'Hehir RE. Specific monoclonal antibodies and human IgE show Hev b 5 is an abundant allergen in high protein powdered latex gloves. Clin Exp Allergy 2002; 32:583–8.
41 Slater JE, Vedvick T, Arthur-Smith A, Trybul DE, Kekwick RG. Identification, cloning, and sequence of a major allergen (Hev b 5) from natural rubber latex (Hevea brasiliensis) J Biol Chem 1996; 271:25394–9.
42 Haselden BM, Kay AB, Larche M. Immunoglobulin E-independent major histocompatibility complex-restricted T cell peptide epitope-induced late asthmatic reactions. J Exp Med 1999; 189:1885–94.
43 Muller U, Akdis CA, Fricker M et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. J Allergy Clin Immunol 1998; 101:747–54.
44 Westall GP, Thien FC, Czarny D, O'Hehir RE, Douglas JA. Adverse events associated with rush hymenoptera venom immunotherapy. Med J Aust 2001; 174:227–30.
45 Norman PS, Ohman JL, Long AA et al. Treatment of cat allergy with T-cell reactive peptides. Am J Respir Crit Care Med 1996; 154:1623–8.
46 Oldfield WLG, Shirley K, Larche M, Kay AB. A Double-blind, Placebo-controlled Study of Short Peptides Derived from Fel d 1 in Cat Allergic Subjects. J Allergy Clin Immunol 2001; 107:S216.
47 Alexander C, Oldfield WLG, Shirley K, Larche M, Kay AB. A Dosing Protocol of Allergen-derived T Cell Peptide Epitopes for the Treatment of Allergic Disease. J Allergy Clin Immunol 2001; 102:S217.
48 Hoyne G, Jarnicki A, Thomas W, Lamb J. Characterization of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression. Int Immunol 1997; 9:1165–73.
49 de Silva HD, Sutherland MF, Suphioglu C et al. Human T-cell epitopes of the latex allergen Hev b 5 in health care workers. J Allergy Clin Immunol 2000; 105:1017–24.
50 Raulf-Heimsoth M, Chen Z, Rihs HP, Kalbacher H, Liebers V, Baur X. Analysis of T-cell reactive regions and HLA-DR4 binding motifs on the latex allergen Hev b 1 (rubber elongation factor). Clin Exp Allergy 1998; 28:339–48.
51 Bohle B, Wagner B, Vollmann U et al. Characterization of T cell responses to Hev b 3, an allergen associated with latex allergy in spina bifida patients. J Immunol 2000; 164:4393–8.
52 Smith AM, Chapman MD. Reduction in IgE binding to allergen variants generated by site-directed mutagenesis. contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. Mol Immunol 1996; 33:399–405.
53 Ferreira F, Ebner C, Kramer B et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. Faseb J 1998; 12:231–42.
54 Schramm G, Kahlert H, Suck R et al. 'Allergen engineering': variants of the timothy grass pollen allergen Phl p 5b with reduced IgE-binding capacity but conserved T cell reactivity. J Immunol 1999; 162:2406–14.
55 Beezhold D, Hickey V, Slater J, Sussman G. Human IgE binding epitopes of the latex allergen Hev b 5. J Allergy Clin Immunol 1999; 103:1166–72.
56 Hernandez-Arana A, Rojo-Dominguez A, Soriana-Garcia M, Rodriguez-Romero A. The thermal unfolding of hevein, a small disulfide-rich protein. Eur J Biochem 1995; 228:649–52.
57 Magone MT, Chan CC, Beck L, Whitcup SM, Raz E. Systemic or mucosal administration of immunostimulatory DNA inhibits early and late phases of murine allergic conjunctivitis. Eur J Immunol 2000; 30:1841–50.
58 Tighe H, Takabayashi K, Schwartz D et al. Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. J Allergy Clin Immunol 2000; 106:124–34.
59 Marshall JD, Abtahi S, Eiden JJ et al. Immunostimulatory sequence DNA linked to the Amb a 1 allergen promotes TH1 cytokine expression while downregulating TH2 cytokine expression in PBMCs from human patients with ragweed allergy. J Allergy Clin Immunol 2001; 108:191–7.
60 Roy K, Mao HQ, Huang SK, Leong KW. Oral gene delivery with chitosan – DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. Nat Med 1999; 5:387–91.
61 Slater JE, Paupore E, Zhang YT, Colberg-Poley AM. The latex allergen Hev b 5 transcript is widely distributed after subcutaneous injection in BALB/c mice of its DNA vaccine. J Allergy Clin Immunol 1998; 102:469–75.
62 Creticos PS, Balcer SL, Schroeder JT et al. Initial Immunotherapy Trial to Explore the Safety, Tolerability and Immunogenicity of Subcutaneous Injections of an Amb a 1 Immunostimulatory Oligonucleotide Conjugate (AIC) in Ragweed Allergic Adults. J Allergy Clin Immunol 2001; 107:S216.

Clin Exp Allergy 2002; 32:1166–1169

Particulate masks and non-powdered gloves reduce latex allergen inhaled by healthcare workers

T. Z. Mitakakis, E. R. Tovey, D. H. Yates, B. G. Toelle, A. Johnson, M. F. Sutherland, R. E. O'Hehir and G. B. Marks
Cooperative Research Centre for Asthma, Sydney, Australia

Summary

Background Although allergy to latex is a well-characterized phenomenon, some hospitals continue to provide staff with powdered latex gloves as an option to low- or non-powdered gloves.

Objective We aimed to measure the extent to which inhalation of latex particles could be reduced by the use of protective masks or by replacing powdered latex gloves with non-powdered latex gloves.

Methods Twenty healthcare workers in a hospital setting wore nasal air samplers (NAS) and Institute of Occupational Medicine (IOM) samplers for four 20-min periods. Subjects wore powdered gloves, non-powdered gloves and no gloves during three sampling periods, and in the fourth, subjects applied an aerosol barrier face-mask or a particulate face-mask (N95) while wearing powdered gloves. All samples were stained for particles bearing Hev b 5 allergen by the Halogen assay.

Results All subjects inhaled Hev b 5 bearing particles in all sampling periods. IOM samplers collected particles at 70% of the rate of NAS. The number of particles inhaled while wearing powdered gloves was 23.8-fold higher than when not wearing gloves and 9.7-fold higher than when wearing non-powdered latex gloves ($P<0.0001$). Wearing an aerosol barrier mask did not significantly reduce the number of particles inhaled ($P=0.108$), while use of particulate masks significantly reduced the number of particles inhaled by 17.4-fold ($P=0.003$).

Conclusions Use of non-powdered gloves is the most effective method of reducing occupational aeroallergen exposure to latex arising from gloves. However, secondary protection using particulate masks is a valid alternative, and may be helpful for preventing respiratory sensitization.

Keywords allergen, exposure, Hev b 5, intervention, IOM, latex, mask, nasal air sampler
Submitted 14 November 2001; revised 22 February 2002; accepted 10 March 2002

Introduction

IgE-mediated hypersensitivity to natural rubber latex remains a serious occupational health problem in healthcare workers, especially since the adoption of universal exposure precautions in the mid-1980s [1]. Exposure to latex allergens adsorbed to powder from latex gloves can cause urticaria, rhinoconjunctivitis, airborne contact dermatitis, asthma or even life-threatening anaphylaxis in sensitized people. Hev b 5, a major latex allergen, has been identified as an important sensitizing antigen among healthcare workers [2–5].

Currently the only form of treatment available is allergen avoidance and symptomatic relief with no safe, effective specific immunotherapy for this condition. Despite recommendations to avoid powdered latex gloves [6], both for personal and patient health, some healthcare workers are still provided with powdered latex gloves by their employer. While it is recognized that use of non-powdered gloves can substantially reduce concentrations of latex allergen in the air [7] and latex-specific IgE antibodies in workers [8], it is unknown how this intervention influences the amount of inhaled allergenic particles during typical hospital duties and medical procedures.

Personal protective equipment has been used to reduce inhalation of allergenic particles. HEPA-laminar flow helmets have been shown to prevent nearly all allergen from approaching the face [9]. However, such a measure is too cumbersome to be applied during medical procedures. The effect of simple face masks on reducing inhalation of particles bearing latex allergen is not known. Two types of face masks are used in hospital settings: aerosol barrier masks and particulate masks. The former are widely available and are principally used to protect surgical patients from aerosols generated by healthcare workers. Particulate masks, on the other hand, are designed to protect healthcare workers from inhaling infectious material (such as mycobacteria) emanating from patients or biological specimens. These are available in specialized settings within healthcare environments. We hypothesized that these particulate masks may be more effective than the aerosol masks in reducing inhalation of particles bearing latex allergen.

The aim of this study was to measure the extent to which inhalation of particles carrying Hev b 5 was reduced by the use of protective masks or by replacing powdered gloves with

Correspondence: Euan Tovey, Institute of Respiratory Medicine, Blackburn Building D06, University of Sydney, NSW, Australia 2006

non-powdered latex gloves. The study was conducted among healthcare workers performing various duties in a bronchoscopy suite.

Materials and methods

Latex exposure of staff at a bronchoscopy suite in a hospital was examined using nasal air samplers [10] and Institute of Occupational Medicine (IOM) filter samplers [11]. The nasal air samplers are small devices that fit snugly into each nostril and collect inhaled particles by impacting them onto an internal collection plate covered with an adhesive strip. IOM filter samplers consist of a small battery-operated pump (model PCXR4, SKC Limited, Blandford Forum, Dorset, UK) drawing a constant flow of air at 2.0 L/min through a 0.8-μm membrane filter (Millipore, Lane Cove, NSW, Australia) held within an IOM sampling head (SKC Limited). The sampling head was worn on the lapel within the breathing zone, while the pump was worn on the belt. The samplers were used in accordance with the occupational standards AS2985-1987 and AS3640-1989. Thus, the nasal air samplers permit the detection of inhaled particles, while the IOM collects particles at the lapel. By using both techniques we were able to examine the effect of wearing masks, while controlling for variation in local ambient exposure to allergenic particles.

Twenty healthcare workers wore a pair of nasal air samplers and an IOM sampler (rate of 2 L air/min) simultaneously for 4 × 20 min sampling periods. These periods consisted of:

- Resting quietly in the tea room or office, without wearing gloves.
- Performing duties such as bronchoscopies, cleaning of equipment or tending to patients while wearing powdered latex gloves ('Ambi-Tex', Ansell, Victoria, Australia).
- Performing similar duties while wearing non-powdered latex gloves ('Flexi', Mediflex, Islandia, NY, USA).
- Performing similar duties while wearing the powdered latex gloves and either a particulate-filtering mask (N95 Particulate Respirator, Kimberly Clark Corp, North Ryde, New South Wales, Australia) or aerosol barrier mask (Tecnol® PCM 2000 0.1 μm fluid shield, Kimberly Clark Corp). Ten subjects wore the particulate mask and 10 subjects wore the aerosol barrier mask.

All other equipment used by the healthcare workers during the experiments was labelled latex-free and ventilation within the suite was constant throughout the rooms.

Nasal air samples, which consisted of particles collected onto adhesive (Inhalix, Sydney, Australia), were laminated onto 0.45 μm Polyvinylidene difluoride (PVDF) membrane. IOM sampler filters (1.0 μm PVDF) were laminated with the same adhesive. All samples were then processed using the Halogen immunoassay (Inhalix, Sydney, Australia).

The protocol for the Halogen assay, detailed elsewhere [12], was optimized for the detection of particles bearing Hev b 5 and reduced to a 1-day protocol. Briefly, laminated samples were wetted, then incubated for 30 min in borate buffer pH 8.2 to extract the allergen from the particles onto the membrane, followed by blocking in 5% skim milk in phosphate buffered saline/0.05% Tween 20 for 45 min. Samples were incubated with a monoclonal ('6F6', 1 μg/mL) directed against Hev b 5 [13] (CRC for Asthma, Sydney, Australia) diluted 1 in 2000 for 2 h, followed by 1.5 h in biotinylated anti-mouse IgG (1 in 500, Sigma, St Louis, MO, USA) then 1.5 h in Streptavidin alkaline phosphatase conjugate (1 in 1000, Sigma). Samples were then washed three times in PBST between each incubation step. Samples were then developed with BCIP/NBT (Sigma) for 15 min. A control isotype antibody confirmed specificity of the primary antibody. Positive detection of allergen was determined by a 'halo' of stain around a particle. Entire samples were counted.

The study was approved by the Central Sydney Area Health Service Ethics Review Committee (RPA Zone) and written consent was obtained from all participants.

Statistical analysis

Counts were log$_{10}$ transformed to normalize the data. Comparison between all four sampling conditions for nasal air samplers and IOM filters was by repeated measures analysis of variance using Analyse-It for Microsoft Excel software (Analyse-It Software Ltd, Leeds, UK). Pair-wise comparisons between the conditions were tested by paired *t*-tests (Analyse-It). The correlation between nasal air samplers and IOM filter samples was examined using intraclass correlation coefficients [14], for the three conditions, excluding when masks were worn.

Results

All subjects inhaled Hev b 5 bearing particles in every condition. Particle counts ranged from 3 to 5278 on the nasal air samplers and 3 to 5018 on the IOM filters.

Overall, significant differences were detected between the four sampling conditions for both NAS and IOM ($P < 0.0001$). Both NAS and IOM samples demonstrated substantially higher latex particle counts during periods in which powdered gloves were used, compared with periods when no gloves or non-powdered gloves were used (Fig. 1 and Table 1). There was no significant difference in particle counts between the no gloves and the non-powdered gloves conditions although there was a trend towards higher counts when wearing the gloves (Table 1).

As expected, IOM sampling detected similar particle numbers when powdered gloves were worn with or without a mask ($P = 0.49$, Fig. 1). The effectiveness of the mask was assessed by examining the ratio of NAS to IOM filter counts with and without wearing a mask. The use of this ratio adjusted for variation in the ambient exposure to latex. The analyses showed that wearing an aerosol barrier mask did not significantly reduce the number of particles inhaled ($P = 0.108$); however, wearing particulate masks significantly reduced the number of particles inhaled by 17.4-fold (95% CI 3.4 to 88.5, $P = 0.003$, Fig. 2). Of the 10 subjects who wore particulate masks, there was no significant difference in the amount inhaled while wearing powdered gloves with a particulate mask compared with not wearing gloves or wearing non-powdered gloves (all $P > 0.05$).

By combining all sampling conditions except when masks were worn, the intraclass correlation coefficient between NAS and IOM sampler was 0.82 (Fig. 3). The average ratio of NAS to IOM sampler counts across these samples was 1.43.

Table 1. Fold differences of Hev b 5 carrying particles collected by healthcare workers by nasal air sampling and IOM filter sampling in a hospital bronchoscopy suite. Twenty subjects each wore powdered gloves, non-powdered gloves or no gloves. Significance was detected by paired sample *t*-tests

Fold difference (95% CI)	No gloves	Non-powdered gloves	Powdered gloves
No gloves	–	2.47*	2.38*
	–	(0.92–6.65)*	(11.5–49.6)*
Non-powdered gloves	1.31†	–	9.66*
	(0.53–3.23)†	–	(4.11–22.7)*
Powdered gloves	12.3†	9.41†	–
	(6.71–22.5)†	(4.29–20.7)†	–

*NAS samples. †IOM samples.

Discussion

We have demonstrated that, in a hospital setting, substantial reductions in the number of latex allergenic particles that are inhaled can be achieved through use of either non-powdered gloves or by wearing breathing masks.

Such observations regarding the reduced levels of airborne latex allergen, following the replacement of powdered gloves with non-powdered gloves, has been shown previously by static air (area) sampling and by personal air sampling [6,15], identifying the allergen using pooled IgE reactive to crude latex or by counting numbers of corn starch particles [16]. We have shown that exposure to the major latex allergen, Hev b 5, is decreased substantially by the use of non-powdered gloves. We have also shown that the inhaled dose follows the pattern of exposure determined by sampling at the lapel.

It is noteworthy that allergenic particles were sampled and inhaled while subjects were not wearing gloves. This indicates that all staff in healthcare settings, such as theatres and endoscopy suites, are probably exposed to low, background levels of latex particles, independent of their choice of gloves worn. Low and persistent levels of latex allergen are sufficient to induce sensitization and latex-specific allergic symptoms, including asthma [17]. It is also possible that the allergenic particles are carried on clothing and gowns and may be transferred between people, as has been shown for other allergenic particulates [18]. In addition, particles are likely to accumulate in settled dust and be redispersed into the air with disturbance. Thus a personal choice to reduce airborne latex allergen by use of non-powdered

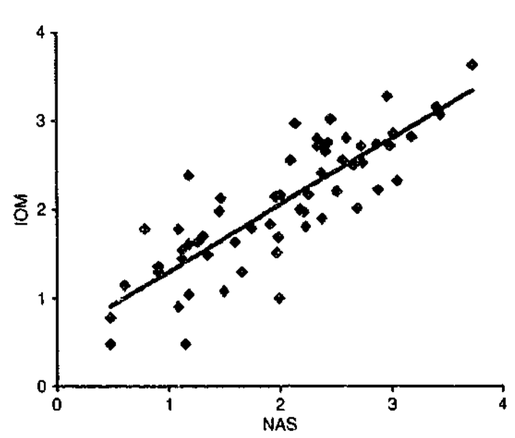


Fig. 1. Numbers of particles bearing Hev b 5 collected by (a) nasal air sampling and (b) IOM filter sampling for each subject ($n = 20$) over 20-min sampling periods, for the three glove conditions. Geometric means and 95% confidence intervals of the mean are shown for each condition.

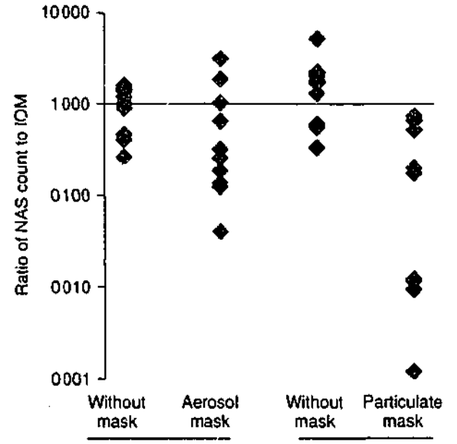


Fig. 3. Nasal air sampling counts compared with IOM filter sampling counts of Hev b 5 bearing particles collected ($n = 20$) under three conditions: (a) while wearing powdered latex gloves; (b) while wearing non-powdered latex gloves; and (c) not wearing gloves.

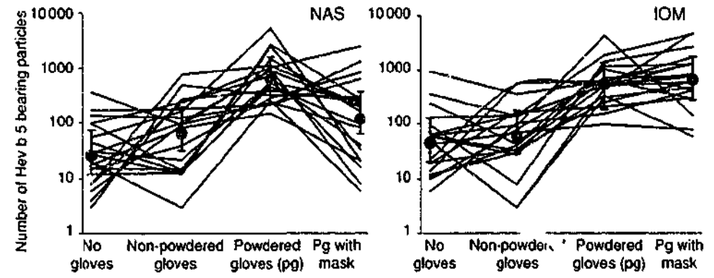


Fig. 2. Comparison of effect of aerosol barrier masks and particulate masks on the number of inhaled particles bearing Hev b 5. (a) Ratio of nasal air sampling (NAS) count to IOM count showing paired groups without mask ($n = 10$ each) and either aerosol barrier mask ($n = 10$) or particulate mask ($n = 10$).

gloves will not remove the entire source of exposure if co-workers choose to wear powdered gloves.

Our hypothesis that the particulate masks would offer more effective protection than the aerosol barrier masks was supported. These results provide evidence that healthcare workers who are allergic to latex allergen and need to wear gloves can reduce their exposure by wearing non-powdered gloves or alternatively by wearing a particulate mask when wearing powdered gloves. This may be a simple and useful alternative to workers who prefer the ease of donning powdered gloves but are sensitized to the allergen adsorbed to the powder, and may be particularly relevant for the prevention of occupational asthma.

In some cases, more particles were inhaled while wearing a mask than when not, suggesting that the seal of the mask must determine the effectiveness. While staff were aware of the correct application and fit of the masks and applied them before donning gloves, clearly particles were still inhaled. Alternatively, the results may be indicative that people tend to inhale faster or deeper when wearing the perceived breathing obstruction. In either case, it is likely that this observation holds for other types of particulates and possibly aerosols [19], and therefore in avoidance of allergen or potentially infectious aerosols, the emphasis on the mask fit is important.

Compared with nasal air sampling, IOM samplers undersampled by approximately 30%. This percentage difference between nasal air samplers and IOM samplers was found with the collection of *Alternaria* spores [20]. The design of the IOM sampling head was to collect 'the same measured dust concentration and aerodynamic size distribution as that inspired by the wearer, regardless of dust source, location and wind condition' [11]. Given a ratio that nears 1, and given the collection efficiencies and nature of the two samplers, the amounts detected by sampling are likely to be close to actual amounts of particles inhaled under these circumstances.

In this study, we tested the samples examining only one of 11 known latex allergens. As a major latex allergen, Hev b 5 is present in many of the powdered latex gloves that have been examined [2,21] and few, if any, of the observed particles that resembled corn starch did not have an associated allergen-staining halo. This suggests that choice of counting particles bearing Hev b 5 was a valid measure of latex-bearing particles.

In conclusion, the use of non-powdered gloves is the most effective method of reducing occupational exposure to airborne latex allergen arising from gloves. However, secondary protection using particulate masks is also effective, and may be helpful for preventing latex-induced symptoms and respiratory sensitization.

Acknowledgements

We thank the volunteers for participating and the Cooperative Research Centre for Asthma, Australia, for their funding support.

References

1 Meyer JD, Holt DL, Chen Y, Cherry NM, Mcdonald JC. SWORD '99: surveillance of work-related and occupational respiratory disease in the UK. Occup Med 2001; 51:204–8.

2 Akasawa A, Hsieh LS, Martin BM, Liu T, Lin Y. A novel acidic allergen, Hev b 5, in latex. Purification, cloning and characterization. J Biol Chem 1996; 271:25389–93.

3 Slater JE, Vedvick T, Arthur-Smith A, Trybul DE, Kekwick RG. Identification, cloning, and sequence of a major allergen (Hev b 5) from natural rubber latex (Hevea brasiliensis) J Biol Chem 1996; 271:25394–9.

4 De Silva HD, Sutherland MF, Suphioglu C et al. Human T-cell epitopes of the latex allergen Hev b 5 in health care workers. J Allergy Clin Immunol 2000; 105:1017–24.

5 Nettis E, Colanardi MC, Ferrannini A, Tursi A. Airborne allergic contact dermatitis. Allergy 2001; 56:583–4.

6 New South Wales Department of Health. Policy Framework and Guidelines for the Prevention and Management of Latex Allergy. Retrieved 15 October 2001 from www.Health.Nsw.Gov.Au/Fesd/Rmc/Cib/Circulars/ 2000/cir2000 –99.pdf.

7 Tarlo SM, Sussman G, Contala A, Swanson MC. Control of airborne latex by use of powder-free latex gloves. J Allergy Clin Immunol 1994; 93:985–9.

8 Allmers H, Brehler R, Chen Z, Raulf-Heimsoth M, Fels H, Baur X. Reduction of latex aeroallergens and latex-specific IgE antibodies in sensitised workers after removal of powdered natural rubber latex gloves in a hospital. J Allergy Clin Immunol 1998; 102:841–6.

9 Laoprasert N, Swanson MC, Jones RT, Schroeder DR, Yunginger JW. Inhalation challenge testing of latex-sensitive health care workers and the effectiveness of laminar flow HEPA-filtered helmets in reducing rhinoconjunctival and asthmatic reactions. J Allergy Clin Immunol 1998: 102:998–1004.

10 Graham JA, Pavlicek PK, Sercombe JK, Xavier ML, Tovey ER. The nasal air sampler: a device for sampling inhaled aeroallergens. Ann Allergy Asthma Immunol 2000; 84:599–604.

11 Mark D, Vincent JH. A new personal sampler for airborne total dust in workplaces. Ann Occup Hygiene 1986; 30:89–102.

12 O'Meara TJ, Tovey ER. Monitoring personal allergen exposure. Clin Rev Allergy Immunol 2000; 18:341–95.

13 Sutherland MF, Drew A, Rolland JM, Slater JE, Suphioglu C, O'Hehir RE. Specific monoclonal antibodies and human immunoglobulin E show that Hev b 5 is an abundant allergen in high protein powdered latex gloves. Clin Exp Allergy 2002; 32:583–9.

14 Deyo RA, Diehr P, Patrick DL. Reproducibility and responsiveness of health status measures. Statistics and strategies for evaluation. Control Clin Trials 1991; 12:142S–158S.

15 Sussman GL, Liss GM, Deal K et al. Incidence of latex sensitization among latex glove users. J Allergy Clin Immunol 1998; 101:171–8.

16 Hermesch CB, Spackman GK, Dodge WW, Salazar A. Effect of powder-free latex examination glove use on airborne powder levels in a dental school clinic. J Dental Educ 1999; 63:814–20.

17 Vandenplas O, Delwiche JP, Sibille Y. Occupational asthma due to latex in a hospital administrative employee. Thorax 1996; 51:452–3.

18 De Lucca SD, O'Meara TJ, Tovey ER. Exposure to mite and cat allergens on a range of clothing items at home and the transfer of cat allergen in the workplace. J Allergy Clin Immunol 2000; 106:874–9.

19 Renstrom A, Karlsson A, Tovey ER. Measuring inhaled allergen: comparison between nasal sampling and conventional air sampling on filters using pumps to evaluate occupational allergen exposure. Eur Respir J 1999; 14:538S.

20 Mitakakis TZ, Tovey ER, Xuan W, Marks GB. Personal exposure to allergenic pollen and mould spores in inland New South Wales, Australia. Clin Exp Allergy 2000; 30:1733–9.

21 Alenius H, Makinen-Kiljunen S, Turjanmaa K, Palosuo T, Reunala T. Allergen and protein content of latex gloves. Ann Allergy 1994; 73:315–20.

Human T-cell epitopes of the latex allergen Hev b 5 in health care workers

Nalini D. de Silva, PhD,[a] Michael F. Sutherland, FRACP,[a] Cenk Suphioglu, PhD,[a] Susan C. McLellan, SRN,[a] Jay E. Slater, MD,[c] Jennifer M. Rolland, PhD,[b] and Robyn E. O'Hehir, FRACP, PhD[a] *Victoria, Australia, and Rockville, Md*

Background: Latex allergy affects health care workers as a high-risk cohort. Hev b 5 is a major latex allergen reacting with serum IgE from 92% of latex-allergic health care workers. Because CD4⁺ T-cell recognition is central to the specific immune response to allergens, identification of dominant T-cell epitopes is important for the development of specific immunotherapy for latex allergy.

Objective: Our purpose was to map T-cell epitopes of Hev b 5 in health care workers.

Methods: Six latex-allergic health care workers (grade 3 to 4 enzyme allergosorbent test score) were studied. Peripheral blood latex specific 3-week T-cell lines were generated and screened for proliferative response to overlapping 20-mer peptides of Hev b 5. Supernatants collected at 48 hours were analyzed by ELISA for IL-5 and IFN-γ.

Results: Dot immunoblotting with use of recombinant Hev b 5/maltose-binding protein indicated serum-specific IgE in 5 of 6 patients. T-cell reactivity to one or more Hev b 5 peptides was identified in these 5 donors, but not in the sixth. Hev b 5 (46-65) induced T-cell proliferation in all 5 donors. Hev b 5 (109-128) stimulated T cells from 3 of these patients. Proliferative responses were accompanied by substantial IL-5 secretion and minimal IFN-γ, indicating a T_H2-predominant cytokine profile.

Conclusions: Five of 6 latex-allergic patients demonstrated T-cell responsiveness to Hev b 5 consistent with a major T-cell reactive latex allergen. Two T-cell immunodominant regions of Hev b 5 were identified, and reactivity to these sites was associated with strong IL-5 but minimal IFN-γ production. (J Allergy Clin Immunol 2000;105:1017-24.)

Key words: T cells, latex, Hev b 5, peptides, T-cell epitopes, allergen, allergy

Abbreviations used

AEU:	Allercoat enzyme allergosorbent units
APC:	Antigen-presenting cells
cpm:	Counts per minute
EAST:	Enzyme allergosorbent test
HCW:	Health care worker
^{3}HTdR:	Tritiated thymidine
LAL:	Low ammoniated latex
MBP:	Maltose-binding protein
rHev b 5/MBP:	Fusion protein of recombinant Hev b 5 and maltose-binding protein
T_H2:	T helper cell type 2

IgE-mediated hypersensitivity to latex has emerged as a serious occupational health problem since the introduction of universal precautions in the mid-1980s.[1,2] The use of latex gloves for barrier protection may lead to sensitization of health care workers (HCW) to protein allergens present in the natural rubber latex. The prevalence of latex sensitization among HCWs has been reported from 8.2% to 22%[3-7] with a prevalence of up to 49% for children with spina bifida.[8] Allergic reactions to latex range from urticaria, rhinoconjunctivitis, asthma,[9] and angioedema to severe generalized anaphylaxis in some cases.[10] Because the only form of treatment currently available is allergen avoidance and symptomatic relief, there is an urgent need for the development of specific immunotherapy for this condition. Many hospitals are developing a latex powder-free glove policy and replacing latex products with latex-free alternatives wherever possible to develop a "latex-safe" environment.[11] However, for some highly sensitized patients these measures remain insufficient to allow continued occupational exposure to latex products, even in the presence of a powder-free glove policy. The immunologic characterization of latex allergens is an important initial step in the development of specific immunotherapy regimens for latex allergy that are both safe and efficacious. Several allergens from the rubber plant *Hevea brasiliensis* have been identified.[12-20] On the basis of IgE binding studies, certain latex allergens seem to be preferentially recognized by particular risk groups (Hev b 1 and 3 by children with spina bifida and Hev b 5 and 6.02 by HCWs[21]). Hev b 5, a highly acidic and proline-rich protein with a predicted predominantly random secondary structure, has been shown to react with IgE from 92% of HCWs and 56% of spina bifida patients with latex allergy.[18] Thus Hev b 5 has been identified as a major allergen in natural rubber latex and is a rational first target for specific immunotherapy.

From the Departments of [a]Allergy, Asthma, and Clinical Immunology and [b]Pathology and Immunology, Alfred Hospital and Monash University, and the [c]Laboratory of Immunobiochemistry, Center for Biologics Evaluation and Research, US Food and Drug Administration, Rockville, Md.

Supported by the National Health and Medical Research Council of Australia, the Alfred Hospital Research Trusts, and the Australian Allergy Foundation. The Australian authors are members of the Cooperative Research Centre for Asthma.

Received for publication Sept 14, 1999; revised Jan 13, 2000; accepted for publication Jan 18, 2000.

Reprint requests: R. E. O'Hehir, FRACP, PhD, Department of Allergy, Asthma, and Clinical Immunology, Alfred Hospital, Commercial Road, Prahran, 3181, Victoria, Australia.

1/1/105806

doi:10.1067/mai.2000.105806

TABLE I. Clinical characteristics of latex-allergic patients

Patient No.	Age(y)	Sex	Clinical symptoms on contact with latex	Clinical food allergy	Clinical kiwi fruit allergy	Latex EAST score out of 4 (AEU/mL)	Kiwi EAST score out of 4 (AEU/mL)	Total IgE (IU/mL)
1	37	F	Urticaria, asthma, angioedema, anaphylaxis	Yes	No	4 (16.4)	0 (<0.18)	98
2	55	F	Urticaria, angioedema	Yes	Yes	3 (7.1)	0 (<0.18)	22
3	41	F	Urticaria, rhinitis, asthma, angioedema, anaphylaxis	Yes	Yes	3 (9.5)	2 (1.65)	291
4	65	F	Urticaria, rhinitis, asthma, angioedema, anaphylaxis	Yes	Yes	4 (>17.5)	0 (<0.18)	645
5	30	F	Urticaria, angioedema	Yes	Unknown	4 (>17.5)	0 (<0.18)	244
6	41	F	Urticaria, rhinitis, asthma, angioedema, anaphylaxis	Yes	Unknown	3 (9.5)	0/1 (0.23)	>1000

F, Female.

Interestingly, the amino acid sequence of Hev b 5 shows 46% identity to another acidic protein identified in kiwi fruit (*Actinidia deliciosa*)[18] and this may provide a molecular explanation for the high frequency of fruit hypersensitivity seen in latex-allergic patients.[22]

The production of allergen-specific IgE by B cells and release of inflammatory mediators by mast cells and eosinophils results in the effector response of allergic disease. However, it is well established that these events are orchestrated by allergen-specific CD4+ T cells with a T helper cell type 2 (T_H2) cytokine profile. T-cell reactive determinants have been reported for another major latex allergen Hev b 1[23] but not for Hev b 5. Thus characterization of the T-cell response to Hev b 5 is important as an initial step in the development of specific immunotherapy.

In this study we examined T-cell responses to overlapping peptides that span the entire length of the Hev b 5 molecule with use of latex-specific T-cell lines generated from the peripheral blood of latex-allergic HCWs.

METHODS

Subjects

Latex-allergic HCWs were recruited from the Alfred Hospital Allergy Clinic (with informed consent after approval by the Alfred Hospital Ethics Committee). All subjects had severe clinical symptoms of IgE-mediated latex hypersensitivity with confirmation of sensitization by a grade 3-4/4 score of latex-specific serum IgE (Table I) measured with the Kallestad Allercoat enzyme allergosorbent test (EAST) system (Sanofi-Pasteur Diagnostics). The latex allergen source used in the manufacture of the solid phase of the allergen is a pulverized nonpowdered commercial glove extract. The latex EAST score in nonallergic individuals is 0/4 and <0.18 Allercoat EAST units (AEU) per milliliter.

Antigens

Low-ammoniated latex. Low ammoniated latex (LAL) (Ansell) was centrifuged at 20,000 revolutions/min for 20 minutes at room temperature. The middle clear layer was collected, dialyzed against PBS overnight at 4°C, and filter sterilized, and the protein concentration was determined with use of the BCA protein assay kit with BSA as the standard (Pierce).

Hev b 5. A construct composed of the Hev b 5–encoding complementary DNA in the pMAL/c-2 vector was generated as part of a maltose binding protein (MBP) fusion protein (rHev b 5/MBP) and purified as described previously.[18] As a control protein, MBP fused to the LacZα protein was produced by expressing the pMAL/c-2 vector alone as described previously.[18]

Tetanus toxoid and PHA. These control antigens were purchased from Sigma.

Peptides. Synthetic peptides (20-mers overlapping by 11 or, for the 2 N-terminal peptides, 15 residues) spanning the entire length of the Hev b 5 molecule[18] were purchased from Chiron Technologies (Clayton, Australia).

Immunoblotting

Patient serum IgE reactivity to latex allergens LAL (25 μg), rHev b 5/MBP (7 μg), and MBP (4.5 μg) was analyzed by dot immunoblots according to our established protocols.[24] The amount of MBP was estimated to be equivalent to that in the rHev b 5/MBP aliquot.

Generation of latex-specific T-cell lines

Latex-specific T-cell lines were isolated with use of our well-established methods for the generation of allergen-specific oligoclonal T-cell populations.[25,26] Briefly, PBMCs (2.5×10^6/well) were separated from heparinized venous blood by density centrifugation and initially stimulated for 1 week with LAL at 20 μg/mL in 24-well tissue culture plates (Costar) in complete medium (RPMI 1640, Gibco Life Technologies, supplemented with 2 mmol/L L-glutamine, 100 IU/mL penicillin-streptomycin, and 5% screened heat-inactivated human AB serum [Sigma]). After 7 days lymphoblasts (1×10^6/well) were restimulated for 1 week with LAL at a concentration of 20 μg/mL in the presence of an equal number of irradiated (3000 rad) autologous PBMCs as antigen-presenting cells (APC). On days 2 and 4 cultures were supplemented with Lymphocult-T (5% vol/vol, Biotest Folex) and fresh medium. At the end of the second week lymphoblasts were restimulated for 1 week with rHev b 5/MBP at a concentration of 20 μg/mL as described above. Oligoclonal T cells were recovered, washed, and tested in proliferation and cytokine assays (as described below). We have previously shown that $CD4^+$ T cells are preferentially expanded in these cultures.

Oligoclonal T-cell proliferation assays

Oligoclonal T-cell blasts (5×10^4/well) from the 3-week cultures were incubated in 96-well round-bottom plates (Linbro, ICN Biomedicals) in triplicate with equal numbers of irradiated APCs in the presence of LAL at concentrations ranging from 0.03 to 100 μg/mL, rHev b 5/MBP (10 and 20 μg/mL), MBP (10 and 20 μg/mL), overlapping peptides of the Hev b 5 sequence (10 and 30 μg/mL), Lymphocult-T (10 IU/ml), or PHA (3 μg/ml). Cultures of T cells and APCs in the absence of antigen, T cells alone, and APCs alone in the absence of antigen were included as controls. After 72 hours cultures were pulsed with 1 μCi of tritiated thymidine (^{3}HTdR) (DuPont, NEN) and harvested 12 to 16 hours later. Proliferation as correlated with ^{3}HTdR incorporation was measured by liquid scintillation spectroscopy. Results are expressed as mean counts per minute (cpm) for triplicate cultures with SD (≤20% for all experiments).

Production of IL-5 and IFN-γ by latex-specific oligoclonal T cells

The secretion of IL-5 and IFN-γ by oligoclonal latex-specific T cells was determined by ELISA on culture supernatants. Representative stimulatory and nonstimulatory peptides were selected and T cells were cultured in triplicate with these peptides. At 48 hours 50 μL of supernatant was harvested from the cultures, and IL-5 and IFN-γ were assayed by ELISA with use of paired cytokine antibodies (from Pharmingen, Becton Dickinson and Endogen, respectively). For this, opaque flat-bottom ELISA plates (Nalgene, Nunc International) were coated with 30 μL of capture antibody at 2 μg/mL in 0.1 mol/L sodium bicarbonate, pH 8.2, coating buffer overnight at 4°C. After being washed 3 times with PBS/0.5% Tween the wells were blocked with 3% BSA/PBS at room temperature for 2 hours. The plates were washed and 30 μL of pooled triplicate culture supernatants was added in duplicate. After incubation overnight at 4°C and washing, bound cytokine was revealed by incubation with 30 μL of biotinylated detecting antibody (1 μg/mL for IL-5, 0.5 μg/mL for IFN-γ) for 45 minutes at room temperature followed by streptavidin-biotinylated horseradish peroxidase (Amersham Pharmacia Biotech) at a 1:2000 dilution for 30 minutes and enhanced chemiluminescence substrate (NEN Life Science). Light emission was read immediately on a Lumicount microplate Glow Luminometer (Packard Instrument Company). A standard curve was run each time with known concentrations of standard cytokine samples, and the concentration of IL-5 and IFN-γ in the culture supernatants was calculated accordingly. The lower limit of detection of the IL-5 assay was 20 pg/mL and the lower limit of detection of the IFN-γ assay was 2 pg/mL.

RESULTS

Serum IgE response to Hev b 5

Sera from the latex-allergic HCWs were tested for IgE reactivity to latex allergens by dot immunoblotting with use of LAL and rHev b 5/MBP as antigens. MBP was included as a control to exclude the possibility that binding of patient sera to the fusion protein was the result of anti-MBP antibodies. All patients except patient 2 demonstrated IgE reactivity to LAL (Fig 1). Sera from patients 1, 2, 3, 4, and 5 showed IgE reactivity to rHev b 5/MBP, which was markedly stronger than that to MBP alone, indicating the presence of a B-cell response to Hev b 5 in these patients. Patient 6 showed IgE reactivity to LAL but not to the rHev b 5/MBP fusion protein, indicating that Hev b 5 was unlikely to be a significant allergen in this case.

T cell responses to latex allergens

In preliminary primary cultures of PBMCs, the optimal response to LAL was observed at 10 to 30 μg/mL in all cases (data not shown). The polyclonal responses to rHev b 5/MBP were considerably less than those to LAL. A similar response was observed to MBP alone, indicating the presence of MBP-specific T cells in the peripheral blood. Therefore, to enrich for latex-specific T cells while preventing the selective expansion of MBP-specific T cells, LAL at 20 μg/mL was used for primary and secondary stimulations. Finally, Hev b 5–specific T cells were enriched by incubating the LAL-specific 2-week T-cell line with rHev b 5/MBP at 20 μg/mL in the third antigen stimulation. The 3-week latex-specific T-cell lines from all 6 latex-allergic donors responded to LAL (Table II). The T-cell lines from patients 1 and 2 were also tested for their proliferative responses to rHev b 5/MBP and MBP alone. In contrast to the polyclonal responses, the oligoclonal responses to rHev b 5/MBP were significantly higher than those to MBP alone, indicating a selective expansion of Hev b 5–specific T cells. In fact, MBP responses were only at background levels. Mitogenicity and toxicity of latex antigens were excluded (data not shown).

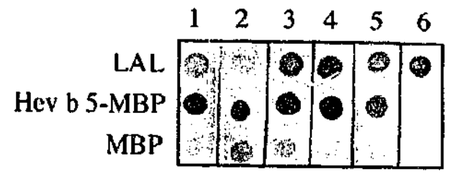


FIG 1. Dot immunoblot analysis of serum IgE reactivity with latex allergens for the latex-allergic patients 1 through 6.

Hev b 5 T-cell epitope mapping

The latex-specific 3-week T-cell lines were tested for proliferative responses to the Hev b 5 peptides. T-cell reactivity to one or more Hev b 5 peptides was identified in all 5 donors who demonstrated IgE binding to rHev b 5/MBP (Table II). Patient 6 failed to respond to any peptide (Table II). Hev b 5(46-65) induced T-cell proliferation in all 5 donors with IgE reactivity to rHev b 5/MBP (Table II). The stimulation index values observed for these responses were 5.9, 10.8, 5.0, 5.9, and 5.6. Additionally, peptide Hev b 5 (109-128) stimulated T cells from 3 of the 5 donors.

Production of IL-5 and IFN-γ by oligoclonal latex-specific T cells

IL-5 was the predominant cytokine produced by the latex-specific T-cell lines in response to stimulation with selected Hev b 5 peptides that caused T-cell proliferation (Table III). Peptides that failed to stimulate T-cell proliferation induced minimal or no cytokine production. Illustrative data from patient 4 is shown (Fig 2). Peptides Hev b 5 (37-56), (46-65), (55-74), (64-83), and (109-128) induced T-cell proliferation (stimulation index values greater than or equal to 2.5) and also secretion of IL-5, whereas peptides Hev b 5 (100-119) and (118-137) failed to stimulate either T-cell proliferation or cytokine secre-

TABLE II. Oligoclonal proliferative responses to Hev b 5 peptides of latex-allergic donors

Antigen	P1	P2	P3	P4	P5	P6
T + APC alone	3.6	2.1	5.2	0.9	1.0	1.0
LAL						
100	**9.5**	**12.6**	**55.9**	2.2	**4.1**	**29.9**
30	7.8	**9.8**	**65.9**	**2.9**	**4.6**	**27.9**
10	8.8	**9.4**	**63.4**	**3.1**	**3.3**	**14.6**
3	7.1	**7.7**	**37.1**	2.0	1.7	**9.9**
Hev b 5/MBP	**9.0**	**5.7**	NT	NT	NT	NT
MBP	2.5	1.5	NT	NT	NT	NT
(1-20)						
30	2.5	2.1	8.2	2.0	**2.6**	2.2
10	3.0	1.6	7.6	1.6	0.9	1.0
(10-29)						
30	3.3	1.7	5.2	1.2	0.8	1.7
10	3.2	1.4	7.7	1.6	1.5	1.1
(19-38)						
30	2.4	2.4	3.0	1.6	**4.0**	2.0
10	2.4	1.1	6.4	1.2	0.6	0.9
(28-47)						
30	5.0	**41.7**	6.9	1.4	1.6	1.1
10	7.2	**25.8**	6.4	1.2	0.8	0.9
(37-56)						
30	8.5	**38.8**	2.7	**3.2**	1.1	0.9
10	7.1	**18.3**	8.8	**3.0**	1.4	1.4
(46-65)						
30	**19.6**	**22.6**	**19.2**	**5.3**	**5.6**	1.2
10	**21.3**	**13.4**	**25.8**	**4.4**	**4.9**	1.5
(55-74)						
30	7.7	**5.6**	8.2	**2.8**	0.7	0.9
10	5.0	2.8	9.1	2.0	1.1	0.7
(64-83)						
30	5.6	2.1	4.3	**2.6**	2.4	1.0
10	3.5	1.8	7.0	1.9	0.5	0.8
(73-92)						
30	2.9	**14.6**	3.1	1.2	0.5	1.3
10	2.5	**9.8**	5.3	1.2	0.8	1.0
(82-101)						
30	5.2	2.1	6.5	**2.6**	1.6	1.2
10	4.1	3.2	7.3	**2.4**	1.3	0.8
(91-110)						
30	2.6	3.2	8.1	2.2	**3.2**	1.2
10	2.4	2.4	7.4	1.6	1.1	0.7
(100-119)						
30	2.0	2.2	8.0	1.1	0.8	1.0
10	2.4	1.3	6.8	1.2	1.0	0.7
(109-128)						
30	5.6	**42.7**	**17.3**	**3.0**	0.9	0.6
10	8.2	**36.9**	**13.7**	**2.3**	1.3	0.9
(118-137)						
30	2.5	**20.8**	8.5	1.2	1.3	0.8
10	3.9	**19.0**	6.8	0.9	0.5	0.9
(127-146)						
30	4.4	**6.6**	8.6	1.6	**2.5**	0.8
10	3.3	3.8	7.1	2.2	**2.7**	1.0
(132-151)						
30	6.5	3.3	7.0	1.7	1.0	0.8
10	4.1	2.7	6.7	1.1	1.4	1.2

Latex-specific 3-week T-cell lines from latex-allergic patients (P1-P6) were stimulated with the Hev b 5 peptides at 10 and 30 μg/mL, LAL at 3, 10, 30 and 100 μg/mL, and rHev b 5/MBP at 10 μg/mL in 4-day cultures. The responses for each peptide and protein antigen are given as cpm × 10^{-3} and compared with the control response where T cells and APCs were incubated alone in the absence of antigen. Stimulation index values of ≥2.5 are shown in bold. *NT,* Not tested.

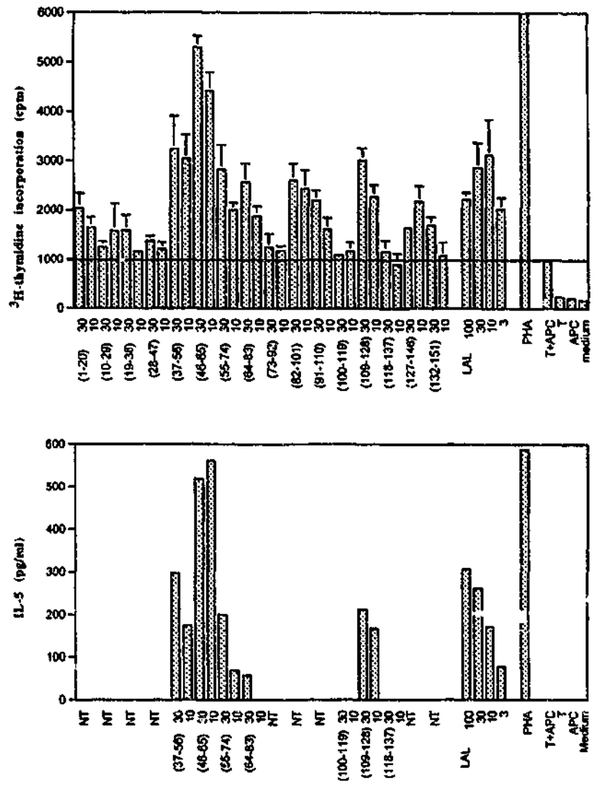


FIG 2. A, Proliferation and **(B)** IL-5 production by latex-specific T-cell line of latex-allergic patient 4. **A,** Proliferation of T cells in response to Hev b 5 peptides (30, 10 μg/mL), LAL (100, 30, 10, 3 μg/mL), and PHA (3 μg/mL) assessed ^{3}HTdR incorporation (values shown are averages of triplicate samples with SD indicated). Background level of cell proliferation (T + APC) is indicated by the horizontal line at 1000 cpm. **B,** Production of IL-5 in the culture supernatants determined by ELISA. Only the indicated peptides were tested for their ability to stimulate T-cell production of IL-5. *NT,* Peptides that were not tested in the IL-5 assay. Values indicate averages of duplicate samples.

tion. In contrast, secretion of IFN-γ by T cells that proliferated in response to Hev b 5 peptides was decreased in comparison with IL-5 secretion in all cases.

DISCUSSION

Latex allergy affects certain high-risk groups including HCWs and patients with spina bifida or a history of multiple surgical procedures. Currently latex avoidance and symptomatic treatment are the only available forms of treatment. In severely affected patients this may be insufficient to allow continued employment in a latex-containing environment. Effective latex-specific immunotherapy is a highly desirable objective.

Hev b 5 is a major latex allergen reacting with serum IgE from 92% of latex-allergic HCWs and 56% of patients with spina bifida. Although the predicted molecular weight of Hev b 5 is 17 kd, this protein migrates at around 24 kd on SDS-PAGE.[17,18] This aberrant migration has been observed for other proteins that are highly acidic and proline rich.[18] Although mAbs to rHev b 5/MBP have been generated,[18] it has been difficult to specifically identify natural Hev b 5 on Western blots of latex extracts.[17,18,27] Therefore we carried out dot-immunoblots with use of LAL and rHev b 5/MBP as antigen. Dot immunoblot analysis showed LAL-specific and rHev b 5/MBP-specific IgE in sera from patients 1, 3, 4, and 5, indicating the presence of a B-cell response to Hev b 5 in these patients. On the other hand, patient 6 showed IgE reactivity to LAL but not to the rHev b 5/MBP fusion protein, indicating that Hev b 5 was not a significant allergen in this case. Patient 2 had IgE reactivity to rHev b

TABLE III. IL-5 and IFN-γ levels produced by latex-specific T-cell lines when stimulated with Hev b 5 peptides

Patient	Hev b 5 peptides tested (30, 10 μg/ml)	IL-5 (pg/mL) 30	IL-5 (pg/mL) 10	IFN-γ (pg/mL) 30	IFN-γ (pg/mL) 10	IL-5/IFN-γ ratio 30	IL-5/IFN-γ ratio 10
P1	28-47 (–)	68	0	0	0	–	–
	37-56 (–)	0	0	0	0	–	–
	46-65 (+)	279	344	2	2	139.5	172
	55-74 (–)	0	0	0	0	–	–
	64-83 (–)	0	14	0	0	–	–
	73-92 (–)	0	0	0	0	–	–
	100-109 (–)	0	0	0	0	–	–
	109-128 (–)	69	0	0	0	–	–
P2	28-47 (+)	678	706	112	122	6.1	5.8
	37-56 (+)	767	744	77	74	10	10.1
	46-65 (+)	408	58	54	18	7.6	3.2
	55-74 (–)	0	0	4	2	–	–
	64-83 (–)	0	0	4	2	–	–
	73-92 (+)	287	141	45	18	6.4	7.8
	100-119 (–)	17	0	4	2	4.2	–
	109-128 (+)	595	293	97	73	6.1	4
P3	46-65 (+)	3186	3799	227	388	14	9.8
	64-83 (–)	205	109	129	132	1.6	0.8
	73-92 (–)	24	27	83	112	0.3	0.2
	109-128 (+)	1559	1403	292	257	5.3	5.5
P4	37-56 (+)	296	173	6	0	49.3	173
	46-65 (+)	519	561	12	25	43.2	22.4
	55-74 (+)	199	70	0	0	199	70
	64-83 (+)	56	0	4	0	14	0
	82-101 (+)	280	285	0	0	280	285
	110-119 (–)	0	0	0	0	–	–
	109-128 (+)	212	166	0	0	212	166
	118-137 (–)	0	0	0	0	–	–
P5				Not tested			

Supernatants were removed at 48 hours from T-cell cultures and tested in an ELISA for the presence of IL-5 and IFN-γ. Proliferation inducing and noninducing peptides are indicated by (+) and (–), respectively. The levels of IL-5 and IFN-γ produced in picograms per milliliter are shown at peptide concentrations of 30 and 10 μg/mL. Where both cytokines were detectable, the IL-5/IFN-γ ratio is shown at peptide concentrations of 30 and 10 μg/mL.

5/MBP but not to LAL. A possible explanation for this observation is that the natural Hev b 5 in LAL does not present IgE-binding epitopes for this patient's serum. This may be a result of degradation of the natural Hev b 5 protein caused by the process of ammoniation. Nevertheless, the T-cell repertoire of patient 2 included recognition of Hev b 5 (Table II). This is consistent with the fact that B-cell recognition of antigen is conformationally dependent, whereas T cells recognize processed linear peptides with no recognition of conformational determinants.

We generated latex-specific T-cell lines from 6 latex-allergic HCWs and analyzed the T-cell response to Hev b 5 with use of overlapping peptides that spanned the entire length of this molecule. The 5 patients with IgE reactivity to the rHev b 5/MBP by dot blot analysis demonstrated T-cell responses to the Hev b 5 peptides. As expected, the latex-specific T-cell line from patient 6 who lacked IgE reactivity to rHev b 5/MBP failed to proliferate to the Hev b 5 peptides. Because T-cell lines from 5 of the 6 latex-allergic patients responded to Hev b 5 peptides, we have established that Hev b 5 is a major T-cell allergen in the highly allergic HCW group. It will also be of interest to determine whether other patient groups (for example, atopic but not latex-allergic controls and spina bifida patients) have a similar T-cell repertoire, and longitudinal studies of this nature are the subject of continuing research in our department.

Several peptides of the Hev b 5 molecule stimulated T-cell proliferation in our patient group. Of these peptides, Hev b 5 (46-65) induced proliferation in the T-cell lines of all rHev b/MBP IgE-reactive donors, whereas Hev b 5 (109-128) stimulated T cells from 3 of these 5 donors. Both the magnitudes and the frequencies of responses to these peptides suggest they contain immunodominant T-cell epitopes of Hev b 5. The high frequency of reactivity is consistent with degenerate binding of this peptide to different MHC class II molecules in an outbred human population.[28] Such degeneracy is highly desirable for the development of immunotherapeutic approaches in human allergic diseases.[29]

Several other peptides, Hev b 5 (37-56), Hev b 5 (55-74), and Hev b 5 (118-137), that were overlapping with the 2 immunodominant regions above stimulated responses in 1 or more subjects (Table II). These may represent shared epitopes or 2 separate epitopes, and further fine mapping would be required for clarification.

A database search showed Hev b 5 (46-65) to have 60% sequence identity with a peptide sequence of a parasite protein from *Strongyloides stercoralis*, which binds IgG and IgE from patients with strongyloidiasis.[30] This is of interest because both allergenic and parasitic antigens induce a T_H2 polarized cytokine response and induce IgE synthesis. None of our patients had visited a region endemic for *Strongyloides* in the previous 2 years and none had symptoms or peripheral blood eosinophilia to suggest subclinical infection.

A search for Hev b 5 (109-128) identified 62.5% sequence identity with a region of the kiwi fruit protein pKIWI501. The sequence similarity observed between the whole Hev b 5 and pKIWI501 molecules is most striking in the N- and C-terminal regions. Therefore the C-terminal region Hev b 5 (109-128) may contain a T-cell epitope that explains the dual reactivity seen clinically with latex and kiwi fruit. All 5 patients in our study with Hev b 5 IgE reactivity were also known to have food allergy. Interestingly, 3 of these patients, in addition to latex allergy, described angioedema or anaphylaxis on contact with kiwi fruit. However, kiwi fruit allergy did not precede the development of clinical latex allergy in any of these patients. Of the other 2 patients, one denied kiwi fruit allergy and the other patient had avoided kiwi fruit because of fear of possible cross-reactivity. Recognition of antigen components of related allergens (including avocado, banana, kiwi fruit, and chestnut) by cross-reactive IgE in latex-allergic subjects is well recognized[31] and may lead to multiple allergies. Alternatively, T cells that recognize cross-reactive epitopes may provide "intermolecular" help[32] and induce the production of specific antibodies to another allergen contributing to multiple clinical allergies.

Murine T-cell epitopes of Hev b 5 have been identified previously[33] in BALB/c mice with the same set of overlapping synthetic peptides. Mice were injected with rHev b 5/MBP and subsequent in vitro splenocyte proliferation assays were performed to identify T-cell determinants. The dominant murine T-cell determinants were Hev b 5 (1-20), (37-56), (73-92), (82-101), (109-128), (118-137), and (127-146). We have demonstrated that all 7 of these peptides also represent human T-cell determinants. There is precedent in the literature for shared murine and human allergenic T-cell epitopes.[34] Interestingly, the immunodominant human T-cell determinant Hev b 5 (46-65) recognized by all 5 subjects with IgE reactivity to rHev b 5/MBP in the current study was only a minor murine T-cell determinant.[33] This emphasizes the need to confirm T-cell epitope mapping performed in the mouse by human studies as a prelude to vaccine development for clinical use.

The cytokine data showing marked IL-5 and minimal IFN-γ production by oligoclonal latex-specific T cells after stimulation with proliferation-inducing peptides are consistent with the immunodominance of the Hev b 5 peptides identified. Peptide-specific T-cell proliferation and dominant T_H2-type cytokine profile (high IL-5/IFN-γ ratio) are compatible with the T-cell response supporting IgE synthesis to this allergen and eosinophil activation, which are necessary for the development of clinical allergy.

The full characterization of T- and B-cell epitopes of the Hev b 5 molecule might facilitate the design of specific immunotherapy regimens for latex hypersensitivity. Studies with mutant proteins of Phl p 5b from timothy grass[35] or T-cell epitope peptides of bee venom phospholipase A_2[36] have been encouraging. Earlier studies investigating peptide vaccines for the cat allergen Fel d 1 were disappointing, but these studies used large protein determinants rather than T-cell epitope peptides.[37] The retention of highly immunodominant T-cell epitopes in peptide epitope vaccines or mutant proteins with abrogated IgE binding would be desirable for therapeutic use.

REFERENCES

1. Slater JE. Latex allergy. J Allergy Clin Immunol 1994;94:139-49.
2. Sussman GL, Tarlo S, Dolovich J. The spectrum of IgE-mediated responses to latex. JAMA 1991;265:2844-7.
3. Grzybowski M, Ownby DR, Peyser PA, Johnson CC, Schork MA. The prevalence of anti-latex IgE antibodies among registered nurses. J Allergy Clin Immunol 1996;98:535-44.
4. Kibby T, Akl M. Prevalence of latex sensitization in a hospital employee population. Ann Allergy Asthma Immunol 1997;78:41-4.
5. Liss GM, Sussman GL, Deal K, Brown S, Cividino M, Siu S, et al. Latex allergy: epidemiological study of 1351 hospital workers. Occup Environ Med 1997;54:335-42.
6. Brown RH, Schauble JF, Hamilton RG. Prevalence of latex allergy among anesthesiologists: identification of sensitized but asymptomatic individuals. Anesthesiology 1998;89:292-9.
7. Douglas R, Morton J, Czarny D, O'Hehir RE. Prevalence of IgE-mediated allergy to latex in hospital nursing staff. Aust N Z J Med 1997; 27:165-9.
8. Michael T, Niggemann B, Moers A, Seidel U, Wahn U, Scheffner D. Risk factors for latex allergy in patients with spina bifida. Clin Exp Allergy 1996;26:934-9.
9. Brugnami G, Marabini A, Siracusa A, Abbritti G. Work-related late asthmatic response induced by latex allergy. J Allergy Clin Immunol 1995;96:457-64.
10. Ownby DR, Tomlanovich M, Sammons N, McCullough J. Anaphylaxis associated with latex allergy during barium enema examinations. AJR Am J Roentgenol 1991;156:903-8.
11. Kelly KJ. Management of the latex allergic patient. Immunol Allergy Clin North Am 1995;15:139-57.
12. Chen Z, Van Kampen V, Raulf-Heimsoth M, Baur X. Allergenic and antigenic determinants of latex allergen Hev b 1: peptide mapping of epitopes recognized by human, murine and rabbit antibodies. Clin Exp Allergy 1996;26:406-15.
13. Yeang HY, Cheong KF, Sunderasan E, Hamzah S, Chew NP, Hamid S, et al. The 14.6 kd rubber elongation factor (Hev b 1) and 24 kd (Hev b 3) rubber particle proteins are recognized by IgE from patients with spina bifida and latex allergy. J Allergy Clin Immunol 1996;98:628-39.
14. Sowka S, Wagner S, Krebitz M, Arija-Mad-Arifs S, Yusof F, Kinaciyan T, et al. cDNA cloning of the 43-kDa latex allergen Hev b 7 with sequence similarity to patatins and its expression in the yeast *Pichia pastoris*. Eur J Bioch 1998;255:213-9.
15. Scheiner O, Wagner B, Wagner S, Krebitz M, Crameri R, Niggeman B, et al. Cloning and molecular characterization of Hev b 3, a spina bifida-associated allergen from Hevea brasiliensis latex. Int Arch Allergy Immunol 1999;118:311-2.
16. Kostyal DA, Hickey VL, Noti JD, Sussman GL, Beezhold DH. Cloning and characterization of a latex allergen (Hev b 7): homology to patatin, a plant PLA2. Clin Exp Immunol 1998;112:355-62.
17. Akasawa A, Hsieh LS, Martin BM, Liu T, Lin Y. A novel acidic allergen, Hev b 5, in latex: purification, cloning and characterization. J Biol Chem 1996;271:25389-93.
18. Slater JE, Vedvick T, Arthur-Smith A, Trybul DE, Kekwick RG. Identification, cloning, and sequence of a major allergen (Hev b 5) from natural rubber latex (Hevea brasiliensis). J Biol Chem 1996;271:25394-9.
19. Chen Z, Posch A, Cremer R, Raulf-Heimsoth M, Baur X. Identification of hevein (Hev b 6.02) in Hevea latex as a major cross-reacting allergen

with avocado fruit in patients with latex allergy. J Allergy Clin Immunol 1998;102:476-81.
20. Posch A, Chen Z, Raulf-Heimsoth M, Baur X. Latex allergens. Clin Exp Allergy 1998;28:134-40.
21. Breitender H. The allergens of Hevea brasiliensis. Allergy Clin Immunol Int 1998;10:101-9.
22. Brehler R, Theissen U, Mohr C, Luger T. "Latex-fruit syndrome": frequency of cross-reacting IgE antibodies. Allergy 1997;52:404-10.
23. Raulf-Heimsoth M, Chen Z, Rihs HP, Kalbacher H, Liebers V, Baur X. Analysis of T-cell reactive regions and HLA-DR4 binding motifs on the latex allergen (Hev b 1) rubber elongation factor. Clin Exp Allergy 1998;28:339-48.
24. Suphioglu C, Singh M, Simpson R, Ward L, Knox B. Identification of canary grass (Phalaris aquatica) pollen allergens by immunoblotting: IgE and IgG antibody binding studies. Allergy 1993;48:273-81.
25. O'Hehir RE, Young DB, Kay AB, Lamb JR. Cloned human T lymphocytes reactive with Dermatophagoides farinae (house dust mite): a comparison of T and B cell antigen recognition. Immunology 1987;62:635-40.
26. O'Hehir RE, Askonas BA, Lamb JR. Cell culture: lymphocyte clones. In: Albert WHW, Staines NA, editors. Methods of immunological analysis. Vol 3. Heidelberg: Springer-Verlag; 1993. p 120-38.
27. Beezhold DH, Hickey VL, Slater JE, Sussman GL. Human IgE-binding epitopes of the latex allergen Hev b 5. J Allergy Clin Immunol 1999;103:1166-72.
28. Sette A, Sidney J. HLA supertypes and supermotifs: a functional perspective on HLA polymorphism. Curr Opin Immunol 1998;10:478-82.
29. De Lalla C, Sturniolo T, Abbruzzese L, Hammer J, Siddoli A, Sinigaglia F, et al. Identification of novel T cell epitopes in Lol p 5a by computational prediction. J Immunol 1999;163:1725-9.
30. Ramachandram S, Thompson R, Gam A, Neva F. Recombinant cDNA clones for the immunodiagnosis of strongyloidiasis. J Infect Dis 1998;177:196-203.
31. Moller M, Kayma M, Viehof D, Paschke A, Stelnhart H. Determination and characterization of cross-reacting allergens in latex, avocado, banana and kiwi fruit. Allergy 1998;53:289-96.
32. Hoyne G, Jarnicki A, Thomas W, Lamb J. Characterization of the specificity and duration of T cell tolerance to intranasally administered peptides in mice: a role for intramolecular epitope suppression. Int Immunol 1997;9:1165-73.
33. Slater JE, Paupore EJ, O'Hehir RE. Murine B-cell and T-cell epitopes of the allergen Hev b 5 from natural rubber latex. Mol Immunol 1999;36:135-43.
34. O'Hehir RE, Hoyne GF, Thomas WR, Lamb JR. House dust mite allergy: from T cell epitopes to immunotherapy. Eur J Clin Invest 1993;23:763-72.
35. Schramm G, Kahlert H, Suck R, Weber B, Stuwe H, Muller W, et al. "Allergen engineering": variants of the timothy grass pollen allergen phl p 5b with reduced IgE-binding capacity but conserved T cell reactivity. J Immunol 1999;162:2406-14.
36. Muller U, Akdis C, Fricker M, Akdis M, Blesken T, Betens F, et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. J Allergy Clin Immunol 1998;101:747-54.
37. Simons FER, Imada M, Li Y, Hayglass KT. Fel d 1 peptides: effect on skin tests and cytokine synthesis in cat allergic human subjects. Int Immunol 1996;8:1937-45.