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Piia Karisola

Immunological Characterization and Engineering of the Major Latex Allergen, Hevein (Hev b 6.02)

UNIVERSITY OF JYVÄSKYLÄ

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Dum loquimur, fugerit invida aetas.
Carpe diem, quam minimum credula postero.

"While we are speaking, cruel time is running away.
Seize the day and trust least, if at all, to the future."

The poet Horatius is seeking to convince his friend, who has been reading horoscopes, not knowing about whether Jupiter has assigned many winters, or whether this is the last, but
instead to drink wine, enjoy this moment and not to think of the future.

ABSTRACT

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Immunological characterization and engineering of the major latex allergen, hevein (Hev b 6.02)
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Diss.

In IgE-mediated natural rubber latex (NRL) allergy, hevein (Hev b 6.02) is a major allergen, which is recognized by 70% of latex allergic patients. Hevein, which is extraordinarily stable, has an antifungal function in the rubber tree (*Hevea brasiliensis*). Homologous proteins with conserved hevein-like domains (HLD) are found in several plants and some of them are believed to participate in allergic cross-reactions in patients who suffer from so-called latex-fruit syndrome.

In this study, NRL allergy and hevein were used as a model pair to clarify the molecular mechanisms of an allergic reaction. In order to detect the conformational IgE-binding areas of hevein, parts of hevein and an immunologically non-reactive antimicrobial protein (AMP) of the amaranth (*Amaranthus caudatus*) were genetically combined using the evolutionary approach, which is based on the comparison of structural relatives. In addition, the potential IgE-binding amino acids were scanned with the guidance of sequential relatives and a total of 29 site-specific single-mutants of hevein were produced. Immunological tests with the sera from NRL-allergic patients identified six of the most important substitutions, and on the basis of this information three pilot molecules for immunotherapy in NRL allergy were constructed. Studies on the molecular reasons behind the IgE-cross-reactivity that exists between different HLDs in fresh fruits showed directly, for the first time, that isolated hevein (4.3 kDa) and homologous HLDs themselves, instead of whole endochitinases (31 kDa), are responsible of this allergic cross-reactivity.

Key words: Hevein (Hev b 6.02); hevein-like proteins; IgE antibody; immunotherapy; natural rubber latex allergy; protein engineering; rational design.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following scientific articles, which will be referred to in the text by their Roman numerals. In addition, some unpublished results are presented.

I Karisola, P., Alenius, H., Mikkola, J., Kalkkinen, N., Helin, J., Pentikäinen, O. T., Repo, S., Reunala, T., Turjanmaa, K., Johnson, M. S., Palosuo, T. & Kulomaa, M. S. 2002. The major conformational IgE-binding epitopes of hevein (Hev b6.02) are identified by a novel chimera-based allergen epitope mapping strategy. J. Biol. Chem. 277: 22656-22661.

II Karisola, P., Mikkola, J., Kalkkinen, N., Pentikäinen, O. T., Repo, S., Reunala, T., Turjanmaa, K., Johnson, M. S., Palosuo, T., Kulomaa, M. S. & Alenius, H. 2004. Construction of hevein (Hev b 6.02) with reduced allergenicity for immunotherapy of latex allergy by comutation of six amino acid residues on the conformational IgE epitopes. J. Immunol. 172: 2621-2628.

III Karisola, P., Kotovuori, A., Poikonen, S., Niskanen, E., Kalkkinen, N., Turjanmaa, K., Palosuo, T., Reunala, T., Alenius, H., Kulomaa, M. S. 2004. Isolated hevein-like domains but not 31 kDa endochitinases are responsible for IgE-mediated *in vitro* and *in vivo* reactions in latex-fruit syndrome. J. Allergy Clin. Immunol. (in press)

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RESPONSIBILITIES OF PIIA KARISOLA IN THE ARTICLES COMPRISING THIS THESIS

Articles I and II: I was chiefly responsible for the design, cloning, production and analysis of the chimeras and mutants. The majority of the immunological studies and molecular masses were done in the groups led by Docents Harri Alenius and Nisse Kalkkinen, Ph.D., respectively, but I also partially did them myself. The group led by Professor Mark Johnson did the molecular modeling at Åbo Akademi University.

Article III: I was mainly responsible for the planning, practical work and writing up of this study. Sanna Poikonen, M.D., performed the skin prick tests with NRL-allergic patients under the supervision of Docent Kristiina Turjanmaa and Professor Timo Reunala at the University hospital of Tampere. Einari Niskanen, M.Sc., did the molecular modeling.

All the above studies were carried out under the supervision of Professor Markku Kulomaa, Professor Timo Palosuo and Docent Harri Alenius, who all also participated in planning these studies and writing the articles.

ABBREVIATIONS

aa	amino acid
AMP	antimicrobial protein, originally from *Amaranthus caudatus*
APC	antigen-presenting cell
C_H	constant heavy chain
C_L	constant light chain
CD	cluster of differentiation
CDR	complementary determining region
C-terminal	carboxyl-terminal
3D	three dimensional
ELISA	enzyme-linked immunosorbent assay
Fab	fragment with specific antigen binding
Fc	fragment crystallizable
GlcNAc	N-acetyl-D-glucosamine
GMP	good manufacturing practice
RI/II	receptor I/II
HCW	health care workers
rHEV	recombinant hevein
HLD	hevein-like domain
Ig	immunoglobulin
IL	interleukin
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight
MBP	maltose-binding protein
MHC	major histocompatibility complex
NMR	nuclear magnetic resonance
NRL	natural rubber latex
N-terminal	amino-terminal
OAS	oral allergy syndrome
PCR	polymerase chain reaction
PR	pathogenesis-related
REF	rubber elongation factor
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf	*Spodoptera frugiperda*
SIT	specific immunotherapy
SPT	skin prick test
SRPP	small rubber particle protein
Th1	T-helper lymphocytes type 1
Th2	T-helper lymphocytes type 2
totRNA	total RNA
V_H	variable heavy chain
V_L	variable light chain
WGA	Wheat germ agglutinin
X-ray	roentgen crystallography

1 INTRODUCTION

In the industrialized countries more than 25% of the population suffers from immediate (type I) allergic reactions that are based on the IgE recognition of otherwise harmless antigens or allergens (Valenta 2002). One proposition for the increase in the prevalence of allergies is the "hygiene hypothesis", which states that a decrease in infectious diseases (mainly due to antibiotics and vaccinations) in early childhood is associated with allergic diseases later in life (Gern & Weiss 2000). Individuals with a familial atopic background and frequent exposure to allergens are susceptible to the development of hypersensitivity reactions to food or environmental antigens. The symptoms and even pathophysiology of immediate allergies are reasonably well characterized, but the principal reasons and molecular mechanisms underlying the allergies continue to be debated.

The detection and diagnosis of type I allergy is largely based on characteristic clinical symptoms and the presence of specific immunoglobulin E (IgE) antibodies in serum and in other body fluids (tears, nasal and bronchial secretions). Treatment options for allergic diseases are currently based mainly on allergen avoidance and the alleviation of symptoms. Conventional immunotherapy with allergen extracts is, however, one of the most promising approaches, althouhg dangerous side-effects including anaphylactic reactions have somewhat decreased its popularity. In order to understand the molecular basis of type I allergic immune responses, the identification of epitopes on the surface of allergen molecules is necessary. Conformational IgE-binding epitopes usually comprise 15-22 amino acid residues of which 5-10 have the strongest interactions and are essential for antibody binding. This knowledge is crucial, especially when designing tools and strategies for allergen-specific immunotherapy or for vaccination.

Natural rubber latex (NRL) allergy is used in this study as a model of the type I allergies. Independent, type IV hypersensitivity to latex is related to rubber chemicals and not to latex proteins (Nettis et al. 2002). Type I allergies can manifest themselves as a number of IgE-mediated clinical symptoms such as urticaria, allergic rhinitis, asthma and anaphylaxis. Today, over 200 proteins

and 13 different allergens (from Hev b 1 to Hev b 13) have been identified in the liquid latex of the rubber trees. A consensus exists that hevein (Hev b 6.02) is one of the most important NRL allergens. Hevein elicits positive skin prick test reactions to the great majority of NRL-allergic patients and more than 70% of NRL-allergic patients have IgE antibodies against it (Alenius et al. 1995, Chen et al. 1997a). Hevein is an advantageous model protein because of its small size (4.7 kDa, 43 aa), extraordinarily stable nature and resolved 3D-structure.

About 50% of latex-sensitized individuals are reported to suffer from allergic symptoms after eating tropical fruits, such as banana, avocado, papaya or kiwi (latex-fruit syndrome). It is suggested that endochitinases with conserved N-terminal HLD could be major candidates for cross-reacting allergens. However, this contention is based on clinical surveys and IgE-immunoblot studies against 31-kDa endochitinase molecules, no direct evidence having yet been found as to whether they are also able to induce clinical symptoms.

In this doctoral thesis, the principles of type I allergies are reviewed and the basic molecular interactions between antibody and antigen are studied with the major NRL allergen, Hev b 6.02. The objectives of these studies were to characterize conformational IgE-epitope areas of hevein (I), locate its key IgE-interacting amino acid residues (II) and, finally, to design and produce "hypoallergenic" hevein molecules that could be applied in immunotherapy for NRL allergy (II). In addition, the last study aimed to elucidate the clinical significance of 31-kDa endochitinases versus isolated about HLDs (4.3 kDa) and to find the molecular mechanisms behind these cross-responses by using HLD-containing proteins from rubber tree latex, avocado, banana, and WGA (III).

2 REVIEW OF THE LITERATURE

2.1 IgE-mediated allergy

IgE-antibodies were discovered in 1967 (Ishizaka & Ishizaka 1967, Johansson & Bennich 1967), but allergic hay fever or "rose fever" among aristocrats was described already in the 15th and 16th centuries (Wood 1986). The term "allergy" was introduced in 1906 by von Pirquet, who recognized that antigens (ability to **gen**erate **anti**bodies) induce changes in both protective immunity and hypersensitivity reactions (von Pirquet 1906). Nowadays the term is frequently used synonymously with the IgE-mediated or type I allergy that shows immediate responses to antigens. Among the classical four types (I-IV) of hypersensitivity reactions, free antibodies are not central components in type IV, where antigen-specific T-cells direct the delayed-type hypersensitivity reactions (Coombs & Gell 1963). Allergens are antigens that induce the immune system to produce IgE-antibodies (i.e. to sensitize) and to trigger symptoms in a sensitized individual (i.e. to elicit an allergic reaction) (Aalberse 2000). The properties of protein allergens are described in more detail in chapter 2.2.1. The term "atopy" (Greek atopos, meaning out of place) is often used to describe IgE-mediated diseases (Kay 2001). Persons with atopy have a hereditary predisposition to produce IgE-antibodies against common environmental antigens and to develop atopic diseases, such as allergic rhinitis, asthma and atopic eczema. In addition to genetic predisposition (Wills-Karp & Ewart 2004), environmental pollution, and changes in the daily diet seem to contribute to the increase in atopic allergies and asthma in economically advanced regions of the world (Black 2001, McGeady 2004).

2.1.1 Immunoglobulin E (IgE)

IgE-antibodies, evolved to defend the host against large parasites, are nowadays more often connected to both the early and the late phases of allergic

inflammation (Yazdanbakhsh et al. 2001, Novak et al. 2003). IgE-class antibodies are Y-shaped glycoproteins (188 kDa) composed of two identical heavy chains and two identical light chains (Fig. 1) (Ishizaka & Ishizaka 1967, Johansson & Bennich 1967). Variable domains at the N terminus of all chains form loop structures that together constitute the antigen-binding site (complementary determining regions, CDR). When an immunoglobulin molecule is digested with papain, two Fab-fragments (**F**ragments for **a**ntigen **b**inding) and a C-terminal constant Fc-domain (**F**ragment **c**rystallizable) are formed (Janeway et al. 2001). Recombinant Fab-fragments are widely used as immunological tools, and the Fc-domain that determines the antibody isotype (IgE) is a natural anchor for effector molecules and cells.

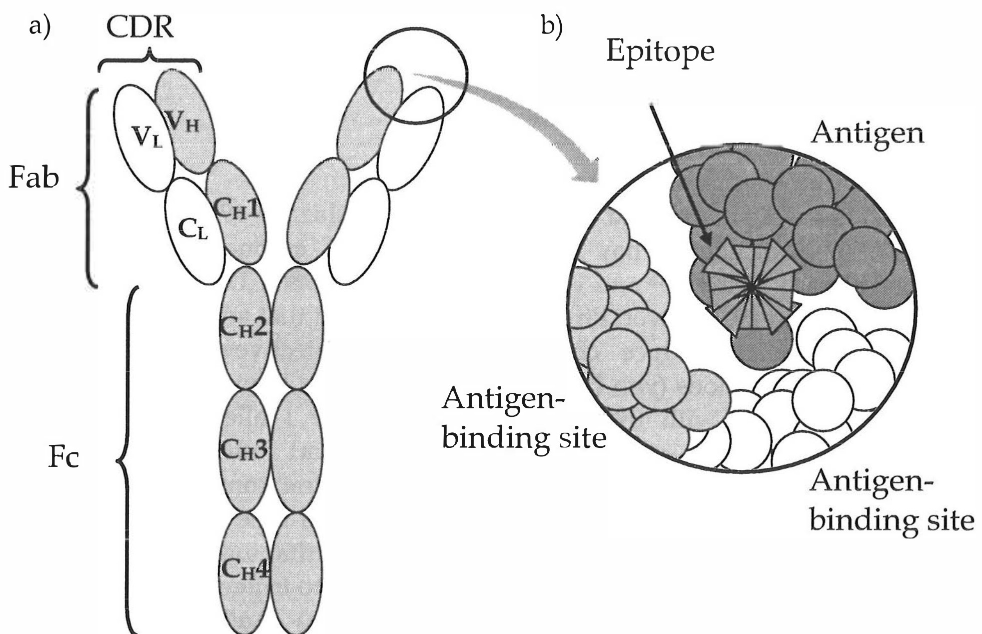

FIGURE 1 (a) The IgE antibody is composed of two identical heavy chains (grey) that contain four constant (C_H1-4) and one variable chain (V_H) whereas light chains (white) contain one constant (C_L) and one variable chain (V_L). (b) Complementary determining regions (CDRs) are located at the N terminus of the variable region of the heavy and light chains. In the close-up view an antigen-binding site is bound with the antigen.

IgE is one of the five major classes of immunoglobulins (IgA, IgD, IgE, IgG and IgM) in humans and it has certain individual characteristics. Its half-life in the body is only 2 days and it is present in serum at a very low concentration, ranging usually from 50 to 300 ng/ml, as compared to 21 days and 10 mg/ml for IgG (Sutton & Gould 1993). Biological activity of IgE is mediated by the high-affinity (K_d = 10^{-9} - 10^{-10} M) IgE-receptors (FcεRI) (Garman et al. 2000), which capture most IgEs on the surface of mast cells, basophils and activated eosinophils. The interaction of an antigen with these complexes initiates the

immediate allergic reaction. The interaction of allergens, allergen-specific IgE and FcεRI seem to be retained in the immunological memory *in vivo* for long periods of time (years) and therefore they together represent a key to the pathomechanism in allergies (Kubo et al. 2003).

The second, low-affinity IgE-binding Fc-receptor FcεRII, known also as CD23, is present on the surface of many different cell types including B-cells, activated T-cells, monocytes, eosinophils, platelets and follicular dendritic cells (Kehry & Yamashita 1989). According to studies in knockout and transgenic mice, trimeric CD23 enhances the capture and presentation of the antigen on APCs (Yu et al. 1994, Payet et al. 1999). As a negative regulator, it represses the IgE-synthesis when the same antigen reappears later (Kilmon et al. 2004). Mac-2 (CPB35, εBP) is the third IgE-binding molecule on the cell surface, but it does not have connections with allergies (Truong et al. 1993).

2.1.2 Induction of IgE response

The immune system has evolved to mount two different types of responses to antigens: humoral immunity resulting in the production of antibodies, and cell-mediated immunity that directs the action of lymphocytes, rather than antibodies. Humoral immunity is mainly a defence against free bacteria and viruses in body fluids, whereas cell-mediated immunity targets microbes inside the host's own cells.

After antigen stimulation, of the many circulating white blood cells, T-lymphocytes (T-cells) and B-lymphocytes (B-cells) develop into T-effector cells and plasma cells, respectively. A specialized subset of effector T-cells called T-helper lymphocytes (Th), carrying CD4-molecules on their surface, play a pivotal role in stimulating both humoral and cell-mediated immunity against antigens. Naïve Th-cells (Th0) can differentiate into Th1 or Th2 subsets depending on the prevailing cytokine milieu (Fig. 2). In the presence of IL-12, T-bet and STAT4 transcription factors play a crucial role in expanding the Th0-cells to effector Th1-cells that then begin to secrete IFN-γ (Geha et al. 2003). This leads to cell-mediated immunity and to the production of IgG-class antibodies from plasma cells. In the presence of IL-4, the optional activation of Th0-cells leads to activation of GATA-3 and STAT6 transcription factors, and differentiation of the effector Th2-cells. The latter cells secrete IL-4, IL-5 and IL-13, which participate in the mounting of humoral immune responses and enhance the release of inflammatory antibodies, IgE, IgG4.

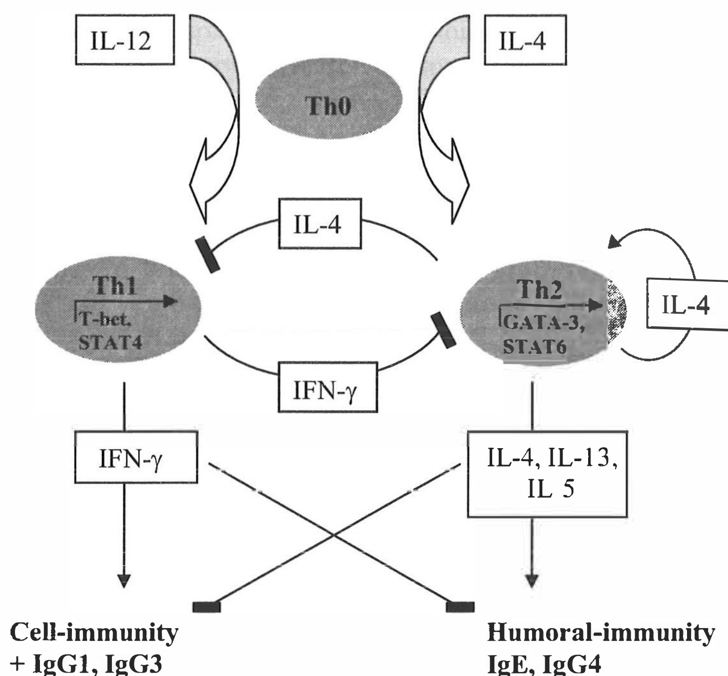

FIGURE 2 Th1/Th2 balance. Depending on the cytokine milieu Th0-cells may develop to Th1 or Th2-cells that inhibit each other and drive cell-mediated immunity or humoral immunity responses. Modified from McGeady (2004).

In normally functioning immune system, minute amounts of available antigens are non-specifically recognized, internalized and processed by dendritic cells, monocytes or magrophages, which are all antigen-presenting cells (APCs) in skin, blood and lymph. They migrate to germinal centers located at regional lymph nodes and attach a fragment of the processed antigen to major histocompatibility complex (MHC) I or II on their surface (Janeway et al. 2001). When naïve T-helper cells (Th0) specific to the formed fragment/receptor-combination on APCs recognize and interact with this complex, they become activated and differentiate into effector cells (Fig. 3 a). In the case of allergy, this "conversation" leads to the proliferation of Th2-cells and memory-Th2-cells, and to the secretion of cytokines, such as IL-4, IL-5 and IL-13.

Activated Th2-cells interact with allergen-specific B-cells bearing still IgM-antibodies on their surfaces (Fig. 3 a). Direct class switching in B-cells is a two-step process known as sensitization when it leads to the differentiation of plasma cells and production of IgE. It needs the binding of IL-4 and IL-13 to B-cell surface receptors (IL-4Rα and IL-13Rα), and co-stimulation where CD40 interacts with the CD40 ligand on the T-cell surface (Jaiswal & Croft 1997, Bacharier & Geha 2000). The allergen-specific IgE-antibodies produced then bind to high-affinity IgE-receptors (FcεRI) on the surface of effector cells, including B-cells, basophils, mast cells, monocytes, eosinophils and dendritic cells (Parronchi et al. 1991, Dombrowicz et al. 1993, Gauchat et al. 1993, Gounni et al. 1994,). In the elicitation process, when the sensitizing allergen is again

present, it directly cross-links IgEs on the B-cell surface and leads to enhanced production of free IgE-antibodies. In the absence of the allergen, monomeric IgEs can regulate the surface expression of FcεRI on these cells and influence cell survival, proliferation or apoptosis, depending on the prevailing circumstances (Saini & MacGlashan 2002, Kitaura et al. 2003).

Immediate allergic reactions occur when the allergen again crosses the body barrier and cross-links two specific IgE-FcεRI complexes on the surface of mast cells or basophils, and preformed chemical mediators (histamine, tryptase, cytokines) are released (Fig. 3 b). The inflammatory cascade continues with the synthesis and release of histamine, leukotrienes and prostaglandins (Geha et al. 2003). Depending on the allergen dose and the route of entry, this response promotes the inflammatory reactions seen on the vasculature, smooth muscle, connective tissue, mucous glands and on inflammatory cells (Janeway et al. 2001). Additionally, mast cells also produce cytokines such as TNF-β, IL-4, IL-5 and IL-13, which have various functions in the continuation and enhancement of the inflammation (Williams & Galli 2000). In addition to mediators released from mast cells, new interaction between the APC (macrophage, B-cell or dendritic cell) and memory-Th2-cells causes their activation and proliferation, and the release of Th2-cytokines (especially IL-5), thus leading to chronic, delayed manifestation of the disease. The blocking of these amplification processes is one of the central goals in current allergen and asthma therapies (Laffer et al. 2001).

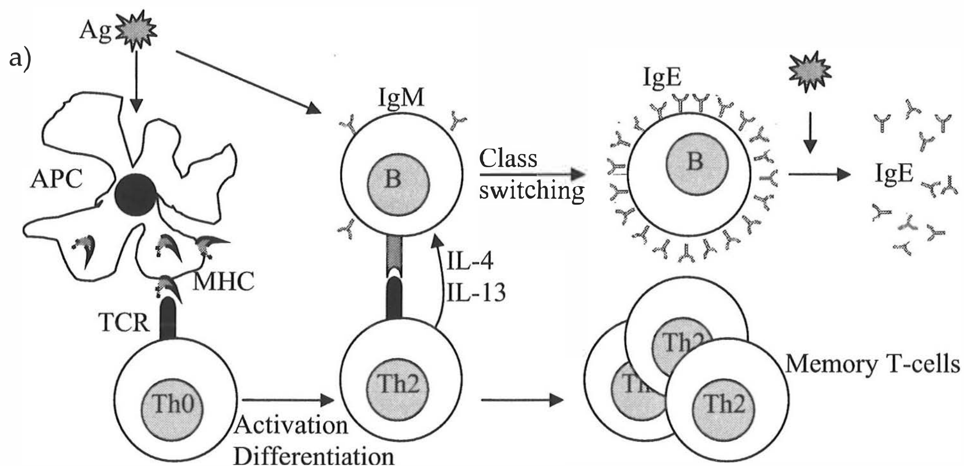

FIGURE 3 (continues)

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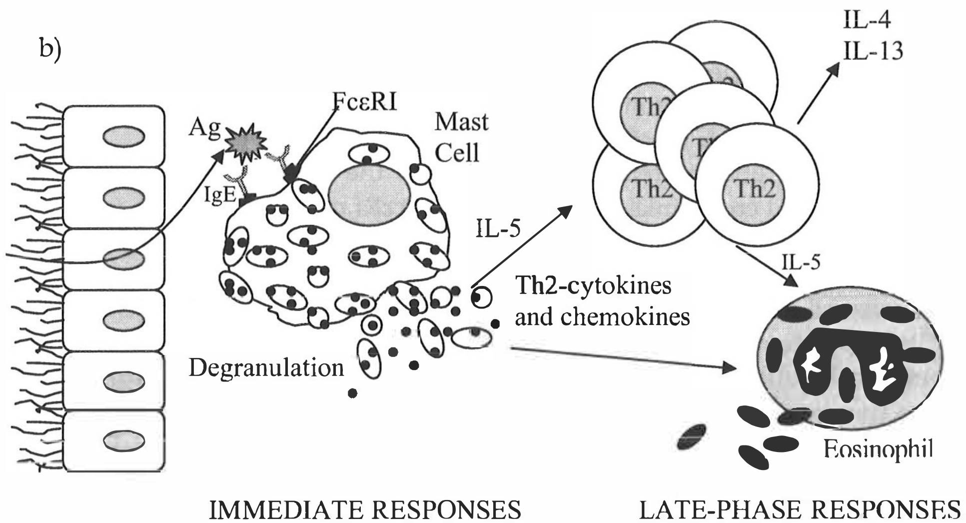


FIGURE 3 Schematic and simplified presentation of induction and maintenance of allergy by allergens. (a) Initial contact with intact allergen favors allergen uptake by APC and/or Ig-mediated capture by specific B-cells with the help of Th2-cells. B-cells mature to plasma cells and produce IgE, and allergen-specific memory T-cells are established. Subsequent allergen contact boosts B-cells to produce IgEs. (b) The allergen driven cross-linking of the effector-cell-bound IgEs leads to release of mediators and the immediate symptoms of allergy. Mediators activate Th2-cells that begin to drive the late-phase responses. Activated Th2-cells, as well as mast cells release cytokines and chemokines that induce tissue eosinophilia to secrete the inflammatory mediators and cause late-phase responses. Figures modified from Valenta (2002).

2.1.3 Clinical manifestations

Specific immediate reactions of type I allergy often begin within seconds after exposure, depending on the dose of the antigen and route of its entry. Histamine, prostaglandins and other preformed mediators cause a rapid increase in vascular permeability and the contraction of smooth muscle. Inhaled allergens cause swelling (edema), mucus secretion and irritation at the respiratory tract and itching of eyes. Local urticaria, dermatitis and hives are typical acute allergic reactions after allergen contact with mucous membranes or skin. Sensitivity varies from person-to-person and symptoms range from the irritating sniffles of hay fever to the life-threatening circulatory collapse that occurs in systemic anaphylaxis (Janeway et al. 2001).

Immediate responses may proceed to the development of late-phase responses 8-12 hours after exposure to the antigen. IgE-mediated activation of the mast cells during and after the immediate response leads to an inflammation cascade that is mediated by the already produced prostaglandins, cytokines (IL-5) and chemokines. They recruit eosinophils, basophils and Th2-cells to the site of reaction and cause a second phase of smooth muscle

contraction, leading to edema and blockage of airways (asthma). Continued exposure to the allergen can evolve into chronic inflammation, clearly seen in atopic dermatitis and in chronic allergic asthma (Pearlman 1999, Leung et al. 2004).

2.1.4 Therapeutic possibilities

As mentioned above, treatment options for allergic diseases are currently based mainly on allergen avoidance and the use of corticosteroids, antihistamines and other drugs to control inflammation and asthma (Horner & Raz 2002). New anti-IgE-based drugs (Omalizumab, TNX-901) include humanized mouse monoclonal IgG-antibodies that hinder the binding of IgEs to FcεRI on the mast cells or basophils, and decrease the total IgE concentration in serum to less than 1% of pretreated levels (Chang 2000, Finn et al. 2003, Leung et al. 2003). Allergy signaling molecules are also potential therapeutic targets (Gorska & Alam 2003, Seki et al. 2003), but at the moment immunotherapy is the only antigen-specific treatment for allergic diseases.

The current form of allergen specific immunotherapy (SIT) has developed slowly since its debut in 1911, when Leonard Noon immunized patients suffering from pollen-induced hayfever with subcutaneous injections of pollen extracts (Noon 1911). The administration of increasing doses of the disease-eliciting allergens (or their derivatives) induces a state of unresponsiveness toward the applied allergens. During SIT, IL-10 and allergen-specific IgGs (most notably the IgG4 isotype) are usually increased in serum, although allergen-specific IgE decreases only slightly (Till et al. 2004). At the same time effector cells, including the eosinophils, mast cells, and basophils, are reduced at allergic mucosal sites and allergen-induced mediator secretions are suppressed. Recent studies show that conventional immunotherapy or the oral administration of the allergen leads to the development of allergen-specific T-regulatory cells (Tr, meaning Tr1, Th3 and $CD4^{+}CD25^{+}$ T-cells) that suppress most of the further allergen-specific Th2-responses by producing TGF-β, IL-10 and/or IL-4 at the site of naïve T-cell priming (Bellinghausen et al. 2003, Francis et al. 2003, Jutel et al. 2003). However, the precise mechanisms operating in SIT are still not completely understood (Durham & Till 1998, Wachholz & Durham 2004).

Systemic and late asthmatic side-effects or fever may appear during SIT, because natural allergen extracts contain complex mixtures of proteins from various sources such as pollen, mites, bacteria, moulds and animal epithelia (Malling 1999, van der Veen et al. 2001, Gastaminza et al. 2003, Trivedi et al. 2003). Natural extracts used in conventional immunotherapy can nowadays be replaced with pure, genetically engineered allergens, produced under conditions of good manufacturing practice (GMP) (Chapman et al. 2000). Recombinant allergens are easy to standardize and optimize (homogenous protein, precise concentration), but the risk of anaphylactic reactions remains.

In the first trials to enhance tolerance to protein allergens, chemical modification of allergen extracts (allergoids) or proteolytic digestion of

allergens, were used to destroy their native 3D-structure or IgE-binding epitopes (del Val et al. 1999, Kahlert et al. 2000). Later, when the cDNA of an allergen had been cloned but its tertiary structure and epitopes remained unknown, partial deletion of the allergen or destruction of its disulphide bridges was accomplished as has been described in various studies on the house dust mite (Der f 1-2, Der p 1-2) (Smith & Chapman 1996, Takai et al. 1997, Smith et al. 1998, Takai et al. 1999), *Parietaria judaica* pollen (Par j 1) (Bonura et al. 2001) and cow dander (Bos d 2) (Kauppinen et al. 1999). Even in cases where epitopes are not precisely known, site-directed mutagenesis may result in potential "hypoallergens" that maintain efficient allergen uptake by APCs but have highly reduced risk of anaphylaxis (Ferreira et al. 1998). And in some cases deletions and point mutations have been successfully combined (Schramm et al. 1999). Throughout this thesis the term "hypoallergen" is used to describe protein molecules whose IgE-binding ability has been reduced.

2.2 Protein allergens

Originally, it is thought that immediate-type hypersensitivity reaction and IgE-antibodies protected the human body against helminthic parasites secreting proteolytic enzymes while invading the host (Maizels & Yazdanbakhsh 2003). However, most allergens are not enzymes and virtually any protein, complex of proteins, carbohydrate or small organic compound may function as an allergen. Carbohydrate determinants are usually weak allergens and cause mainly non-specific cross-reactions, whereas most of the major allergens are proteins (Aalberse 1998, Ebo et al. 2004). The term major allergen signifies an antigen that is recognized by more than 50% of allergic patients.

In general, many (food) allergens are small in size (< 70 kDa), have good solubility, and are highly resistant to proteases, heat and denaturing agents (Aalberse 2000, Maleki et al. 2000). Strong protein allergens are often also weak immunogens, meaning that although they induce allergic reactions they have poor capacity to elicit adaptive immune responses. This is most likely due to the fact that processed allergen peptides share their homology with endogenous host peptides, thereby preventing T-cell responses against these allergens (Kinnunen et al. 2003).

Many plant allergens are homologous to PR-type (pathogenesis-related) proteins, whose expression is enhanced during environmental or biological stress, like plant wounding, or they belong to a small number of function-based protein classes (Breiteneder & Ebner 2000, Hoffmann-Sommergruber 2002). Fruit and vegetables with high water content are prone to fungal attack, and their chitinases, proteases as well as antifungal proteins are their predominant allergens. On the other hand, the allergens in hard and dry seeds fall into two classes; the seed storage proteins and the enzyme inhibitors that prevent the endosperm from being digested by insects or fungi.

Lack of knowledge of the natural functions and 3D-structures of allergens have complicated their classification, and currently several different systems are in use. The World Health Organization/International Union of Immunological Societies (WHO/IUIS) administers an official database of all identified allergens and their isoallergens at http://www.allergen.org. They suggest that allergens should be named according to their source, where the first three letters of the name indicate the genus, the fourth is the first letter of the species, and an Arabic numeral signals the order of identification of the allergen. The same numeral is generally used to designate homologous allergens of related species. According to this nomenclature Hev b 6.02 (hevein), indicates the ***Hevea brasiliensis*** tree and protein, which was the sixth allergen found in the rubber tree, and shares a common gene with differently modified allergen proteins (Hev b 6.01, Hev b 6.02 and Hev b 6.03).

2.2.1 Structural properties

The purification and molecular cloning of allergens during the last decade have had an important impact on structural and immunological studies, enabling the description of allergen folds, which are the usual targets of IgE-antibody cross-reactivity (Stanley & Bannon 1999). The first 3D-structures of plant allergens were resolved by X-ray crystallography and NMR and published in 1996. Progress in the structural analyses after that was, however, somewhat disappointing since it was not possible to identify structural motifs or determinants that could unambiguously indicate potential allergenicity (Gajhede et al. 1996, Fedorov et al. 1997). In spite of this, five structure-based protein families have since been grouped as allergic folds according to protein proximity plots (Table 1) (Aalberse 2000).

The first class contains immunoglobulin-like proteins, serine proteases and trypsin inhibitors that have antiparallel β-strands. The second class, characterized by antiparallel β-sheets intimately associated with one or more α-helices is composed of lipocalins, cystatin, profilins and aspartate proteases. The third class contains (α+β) structures, in which the α-helix and β-structural elements are not intimately associated. Different proteins like lysozymes, mite group 1, vespid group 5, ovotransferrin, cyclophilin and phospholipase A2 belong to this group. The fourth class contains albumins, calmodulins and hemoglobins that have α-helical structures. All of the other identified allergens are assigned to a fifth class that contains β-helical structures (pectate lyases, serine proteases, GSTs, enolases, amylases), coiled-coil structures (tropomyosins) and small, single-domain proteins like ovomucoids or hevein (Aalberse 2000).

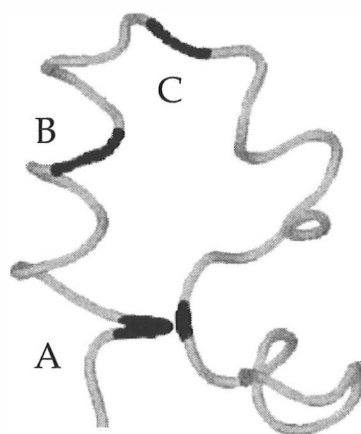
TABLE 1 Classification of protein folds in allergens. Table modified from (Aalberse 2000).

Fold	Allergens
(1) Antiparallel β-strands	The Ig-fold family
	Grass group 2 (1BMW, 1WHO, 1WHP)
	Grass group 1 (C terminus), Grass group 3
	Mite group 2 (1A9V, 1AHK, 1AHM)
	Serine proteases (example: 1DPO, trypsin)
	Mite group 3, 6 and 9
	Soybean Kunitz-type trypsin inhibitor (1AVW)
	Ole e 1, Grass group 11
	Fruits group 2: thaumatin (1AUN)
	Vicilin: peanut Ara h 1 (1CAW, 1DGR, 1DGW)
(2) Antiparallel β-sheets with α-helices	Tree group 1 (1BTV, 1BV1)
	Lipocalin, Milk -lactoglobulin (1BLG)
	Mouse (1MUP) and rat urinary protein (2A2G, 2A2U)
	Dog Can f 1, Can f 2, Bovine Bos d 2,
	Horse Equ c 1 (1BJ7), Cockroach Bla g 4
	Cystatin: cat allergen 430 (1A67, 1CEW)
	Profilin (1CQA)
	Aspartate protease (2REN), Cockroach Bla g 2
(3) (α+β) structures	Mite group 1 (2ACT, 1CSB)
	Lysozyme (1HEL)/lactalbumin (1HFZ)
	Vespid group 5 (1CFE)
	Ovotransferrin = conalbumin (1OVT)
	Cyclophilin (2CYH): Grass group 4, Tree group 7
	Phospholipase A2 (1POC)
(4) α-Helical	Nonspecific lipid transfer protein (1BWO)
	Seed 2S albumin (1PNB)
	Insect hemoglobin (1ECO)
	Fish parvalbumin (1CPD, 5CPV)
	Calmodulin (1OSA): Bet v 4, Jun o 2, Phl p 7
	Mellitin from bee venom (1MLT)
	Fel d 1 chain 1 (2UTG)
	Serum albumin (1UOR)
(5) Other structures	
β-helix:	Pectate lyase (1AIR, 2PEC), Amb e 1-2, Cry j 1
	Serpin-family, Ovalbumin (1OVA)
	PLA1 (1LPA)
	Glutathione S-transferase (1HNB, 1GTA), Cockroach group 5, Mite group 8, Schistosomal glutathione S-transferase
	Mitogillin: Asp f 1 (1AQ2)
	MnSOD Asp f 6 (1MNG)
	Enolase (1NEL)
	Amylase (1JAE)
	Ovotransferrin (1OVT)
Coiled coil:	Tropomyosin (1C1G, 1TMZ, 2TMA): Shrimp group 1, Mite group 10, Cockroach
Small proteins:	Ovomucoid (third domain only) (1OMU, 1OVO, 1CT4)
	Hevein (**1HEV**)
	Amb e 5 (1BBG, 2BBG, 3BBG)

2.2.2 Epitopes

In 1960 Niels Jerne coined the term epitope when he proposed that "an antigen particle carries several epitopes, meaning surface configurations, single determinants, structural themes, immunogenic elements, haptenic groups, antigenic patterns and specific areas" (Jerne 1960). In this conception epitopes or antigenic determinants occur on the surface of a native protein (foldon), whereas epitopes that become immunologically available only after decomposition or denaturation of the antigen are called cryptotopes (Anderton et al. 2002). T-cell epitopes represent hidden epitopes of this kind because T-cells recognize processed peptides (13-17 aa) associated with MHC molecules on the surface of APCs (Rudensky et al. 1991). Traditionally, epitopes are divided into linear T-cell epitopes and linear or structural (conformational) B-cell epitopes (Fig. 4). Debate continues, however, over the linear or conformational nature of IgE-binding-B-cell epitopes and their possible influence on the development of allergies.

a)



b)

FIGURE 4 (a) A conformational (A) and two linear (B and C) epitopes in folded protein are scattered over the protein primary sequence (b) in denatured protein.

IgE-binding epitopes of allergens are still today usually identified by synthetic peptides, 8-15 amino acid residues long, that overlap with 2-3 residues and are recognized in the pool of serum obtained from allergic individuals (Kurup et al. 2003). The important amino acid residues on the epitope are mapped by a new set of peptides containing single alanine substitutions (Rabjohn et al. 1999). An analogous idea is used in many other approaches, including random peptide libraries (Coley et al. 2001), in bacterial or phage-based systems (Davies et al.

2000, Surman et al. 2001) and in computer-assisted algorithms (Bian et al. 2003). These techniques are restricted to short linear sequences within the unfolded protein and are suitable for certain allergens such as water-salt-insoluble wheat ω-5-gliadin that causes allergic responses after enzymic digestion in the intestine (Reese et al. 2001, Matsuo et al. 2004). However, linear peptide techniques are not believed to recognize the same epitopes as antibodies elicited during natural exposure to correctly folded antigens (Crameri 2003).

The structural studies of allergens do not support the dichotomy of linear versus conformational, but in all cases protein epitopes seem to be conformational (Gershoni et al. 1997). The first 3D-structure of an antigen-antibody complex was resolved in 1985 (Amit et al. 1985). It revealed a highly complementary but irregular, flat surface with protrusions and valleys at the antibody interface (Amit et al. 1986). Even T-cells seem to recognize the specific structure of a peptide-MHC-complex on an APC instead of a short peptide (Haque et al. 2001, Maeurer et al. 2001). It is estimated that the B-cell epitope area on the antigen encompasses 60-90 nm^2 (600-900 $Å^2$) and contains 75-120 hydrogen bonds (Laver et al. 1990, Cauerhff et al. 2004,). Complementary determining regions (CDRs) of Fab-fragments seem to form reversible, noncovalent interactions (electrostatic, hydrogen bonding, van der Waals, hydrophobic) with 15-22 residues on an antigen. In more detailed studies, however, only 5-6 of these residues contribute to the binding energy and form a so-called "energetic or functional epitope", whereas the surrounding residues merely complement the interaction (Laver et al. 1990).

2.2.3 IgE cross-reactivity

If one antibody (or T-cell receptor) binds to two or more structurally and functionally conserved proteins they are said to be cross-reactive (Aalberse et al. 2001a). Aalberse and coworkers (Aalberse et al. 2000) have proposed that biologically active allergens can be divided into complete (most relevant) and incomplete (cross-reactive) allergens according to their ability to induce IgE-antibodies. Complete allergens induce the activation of pro-inflammatory T-cells, production of high-affinity antibodies from plasma cells and activation of sensitized mast cells, whereas incomplete allergens are able only to trigger mast cells. Many IgE-antibodies cross-react "by chance" with closely related antigens and patients with cat allergy often also present symptoms from dog dander (Spitzauer et al. 1997). On occasion, some antibodies recognize and bind antigens having no clear relationship to the immunogen. The cross-responses are then explained by a highly conserved primary (identity > 70%) and tertiary allergen structure (Ferreira et al. 2004).

In addition to similarities in the structural and physicochemical properties of proteins, valency and affinity are the major factors determining the immunological consequences of antigen-antibody binding by hydrogen-bonds and electrostatic interactions. In this context, valency means the number of available IgE-binding sites on the allergen, and binding affinity reflects the

quotient of association rate and dissociation rate of the antigen-antibody complex, which has typically a half-life of 1 min – 1 day (Aalberse et al. 2001b). Instead of weak association and quick dissociation the rapid reassociation of antibody Fab-fragments with a multivalent allergen enhances the presentation and processing of the antigen in T-cells, causing diverse allergic responses (Guermonprez et al. 1998, Wensing et al. 2002).

The phenomenon known as determinant spreading may partly explain why patients with one allergy become easily sensitized to other allergens later in life. Subsequent contacts with same antigen or antigenic variants increase the polyclonality of the immune response to common epitopes on different proteins, as in some autoimmunity diseases (Mohapatra 1994). In affinity maturation antigen-antibody interaction is enhanced up to thousand times due to structural changes and an increase in additional interfacial hydrogen bonds and van der Waal contacts (Cauerhff et al. 2004). In addition to a broadened and enhanced antibody army, a specific antibody molecule may adopt different binding-site conformations and interact with slightly different antigens and increase the possibility of cross-responses (James et al. 2003).

2.3 NRL allergy

IgE-mediated allergy to NRL has been seen as an important occupational health issue for the past 15 years. This allergy poses a particular threat to individuals with high workplace exposure (health care workers (HCW), rubber workers), or individuals with congenital malformations and need of multiple surgical interventions. NRL allergy can lead to occupational asthma, anaphylaxis and even death. The quantification and standardization of NRL proteins in latex goods has significantly improved during the last few years, but still diagnosis awaits similar development (Palosuo et al. 2002, Tomazic-Jezic & Lucas 2002). The only currently available treatment, which is based on the avoidance of latex, can ultimately mean either a change of workplace and subsequent loss of career or, alternatively costly strategies like installing "latex-free" zones in hospitals (Zucker-Pinchoff & Stadtmauer 2002).

2.3.1 History, risk groups and prevalence

Archaeologists have found that rubber balls made from the latex of rubber trees were used as early as 1600 BC in Mayan games (Ownby 2002). The first reported case of immediate hypersensitivity (type I) reaction to NRL, however, was not described until 1927 (Stern 1927). A woman was suffering from continuous urticaria and oral angioedema, but when her NRL-containing dental prosthesis was removed, the symptoms disappeared. Skin testing and oral challenge with a piece of rubber confirmed her allergy to rubber. At the beginning of the 1980s additional urticaria cases (Nutter 1979, Forström 1980,)

and the first intraoperative anaphylaxis reactions after latex glove exposure were described (Turjanmaa et al. 1984). In 1983, NRL allergy was shown to be an IgE-mediated disease and its association with spina bifida patients was later documented (Köpman & Hannuksela 1983, Slater 1989). Turjanmaa and her coworkers revealed the importance of the inhalation route, as allergens could be absorbed in glove powder (Turjanmaa et al. 1990). In the early 1990s, Sussman and coworkers (Sussman et al. 1991) also connected contact urticaria to skin exposure, and allergic rhinitis, conjunctivitis and asthma to inhaled allergens. As the most severe consequences of latex allergy, 15 anaphylactic deaths were reported in the USA after exposure to barium enemas containing natural rubber (Ownby et al. 1991, Tomazic et al. 1992). The first link to fruit allergy was established in 1991 when a woman suffering from latex allergy got symptoms after eating bananas (M'Raihi et al. 1991).

Concerns about AIDS and other transmissible infectious diseases have increased the use of latex gloves. HCWs comprise the largest single groups at risk for NRL allergy due to their frequent exposure to natural rubber products in their occupation (Tarlo et al. 1990). Two years ago, latex-induced asthma was described as the fourth-most-common cause of occupational asthma among all exposed workers (Vandenplas et al. 2002). Likewise, children with spina bifida and other congenital anomalies are at high risk of developing allergy to NRL because of the need of multiple surgical operations and frequent exposure to various medical latex devices (gloves and catheters). Today, NRL products in the general environment are considered as an important source of sensitization. NRL antigens have been detected in urban air and in debris deposited near freeways. It has been suggested that it is not the exposure to road traffic per se but to latex particles from car tires that is associated with NRL allergy in children (Williams et al. 1995, Hirsch et al. 2000). The identity of the putative allergens in hard rubber particles has not, however, been verified and it has not been shown that these particles are associated with clinical allergy. Other recently identified risk groups are the latex glove users among airport personnel and food service employees. In fact, any device containing so-called soft latex (balloons, condoms, pacifiers, hair glue, hot-water bottles) may cause immediate allergic reactions to sensitized individuals (Axelsson et al. 1988, Wrangsjö et al. 1988, Levy et al. 1992, Wakelin 2002) and in the future more attention should be paid to general house-hold devices.

Estimates of the prevalence of NRL allergy in the general population vary widely, from 1% to 12% whereas 1-22% of HCW are reported to be sensitized to NRL (Sussman et al. 1998, Poley & Slater 2000). However, when reliable and validated methods are used in diagnosis, less than one per cent of the general population can be shown to suffer from latex allergy (Turjanmaa et al. 2002). The largest occasional group suffering from latex allergy has been children with spina bifida, of whom as many as 24% to 60% have been reported to have latex allergy (Poley & Slater 2000). Indeed, 6-20% of total population may possibly be sensitized to latex, but only one in 20 sensitized individuals experiences clinical latex allergy symptoms (Sosovec et al. 1998). Currently, the number of NRL

allergy cases seems to be decreasing in developed countries, but increasing in developing countries where people are just beginning to consume increasing amounts of disposable latex products.

2.3.2 Symptoms, diagnosis and treatment

The symptoms of hypersensitivity reactions to NRL allergy vary from local reactions to severe systemic reactions. The most common immediate reaction, urticaria, appears within 15 minutes and disappears in 30 to 60 minutes after allergen exposure. It manifests itself as itching, redness, wheal and flare reactions on the skin or on the mucous membranes in oral, vaginal and rectal areas (Turjanmaa & Reunala 1989). Also, systemic reactions like angioedema and anaphylactic shock may occur after exposure through mucous membranes (Leynadier et al. 1989, Laurent et al. 1992). Allergic rhinitis and asthma may ensue after inhalation of NRL allergens bound to glove powder (Carrillo et al. 1986, Baur & Jäger 1990, Tarlo et al. 1990).

Diagnosis of NRL allergy is primarily based on the clinical history of the patient. The most reliable method of diagnosis is the skin prick test (SPT) where the forearm of the patient is pricked with a lancet dipped into NRL extract. In the presence of NRL-specific IgEs, degranulation of mast cells and basophils leads to the release of histamine and other allergic mediators that cause immediate wheal and flare reactions on the site of puncture. Additional information supporting the diagnosis can be obtained from tests *in vitro* such as RAST, AlaSTAT or ELISA that show the presence of circulating IgE to NRL. Uncertain findings can be confirmed by challenge/provocation tests (Turjanmaa 2001).

Treatment options for NRL allergy are limited. Repeated injections of monoclonal anti-IgE injections (Omalizumab) and even music therapy have been successfully tested, but avoiding exposure to NRL allergens continues to remain the mainstay against latex allergy (Kimata 2003, Leynadier et al. 2004). The current classification of non-latex products to "latex-free" and low-latex protein-containing products to "latex-safe", helps the prevention of sensitization and reduces allergic reactions (Zucker-Pinchoff & Stadtmauer 2002). The most sensitive patients are counseled to wear a medic-alert bracelet and to carry medication (Epi-pen) with them at all times (Zucker-Pinchoff & Stadtmauer 2002).

2.3.3 Major allergens

Most of the natural rubber in the world is obtained from the latex of the rubber trees, *Hevea brasiliensis*. Latex is the cytoplasm of specialized tube-like cells, known as laticifers, and its natural function is to seal damaged sites on the surface of the tree, like resin in cornifers (Ko et al. 2003). Sealing is a coagulation process involving the aggregation of rubber particles by hevein (Hev b 6.02) and rubber elongation factor (Hev b 1) (Dennis & Light 1989). Other proteins

such as prenyltransferase and Hev b 3 (Oh et al. 1999) link short isoprene units into cis-1,4-polyisoprene chains, which are responsible for the structural integrity of latex. NRL contains a vast number of additional potential allergens, such as proteins or polypeptides whose specific function is unclear (Table 2).

TABLE 2 Natural rubber latex allergens. Complete or partially sequenced latex antigens.

Nomenclature	Trivial name	Mr [kDa]	pI	Homologues /Cross-reactivity
Hev b 1	Rubber elongation factor	14.5	4.9	Papain
Hev b 2	Endo-1,3-β-glucanase	34-36	9.5	Other glucanases
Hev b 3	REF-like particle prot. Small rubber particle prot	23	4.8	
Hev b 4	Microhelix protein	50-57	4.5	
Hev b 5	Acidic protein	16	3.5	Kiwi acidic protein
Hev b 6.01	Prohevein (tobacco)	21	5.6	CBP 20 and PRP 4A
Hev b 6.02	Hevein	4.7	4.96	
Hev b 6.03	Prohevein C-domain	14	6.4	Win1, win2
Hev b 7	Patatin-like protein	46	4.8	Patatin (Solanaceae)
Hev b 8	Profilin	15.7	4.9	Pollen-food panallergen
Hev b 9	Enolase	47.7	5.6	Mold enolases
Hev b 10	Superoxide dismutase	22.9	6.3	Mold SO-dismutases
Hev b 11	Class I chitinase	33	5.1	Class I chitinases
Hev b 12	Lipid transfer protein	9.3		Food lipid transfer proteins
Hev b 13	formerly Hev b 7.01	42		Patatin
-	Hevamine (class III chitinase)			Lysozyme
-	Prenyltransferase			

Debate continues on the clinical significance of the major NRL allergens. Yeang and coworkers suggest that Hev b 2, Hev b 6, Hev b 13 and possibly also Hev b 4 are the major latex allergens (Yeang 2004). According to studies by our group (Turjanmaa et al. 2002) and by others (Nel & Gujuluva 1998, Posch et al. 1998), the most important latex allergens for healthy adult subjects are Hev b 5 (Akasawa et al. 1996, Slater et al. 1996) and Hev b 6.02 (Alenius et al. 1996, Chen et al. 1997a), whereas Hev b 1 and Hev b 3 are the major allergens for patients with spina bifida and other congenital anomalies. Discrepancies between findings may owe to differences in protein purification techniques, the immunological methods used in analysis and patient cohorts, who come from widely geographically distributed areas where people may simply be sensitized to different proteins. In addition, rubber proteins may be modified, aggregated or fragmented during manufacturing procedures while allergy tests are usually performed with extracts of "raw" latex or with purified proteins.

Hev b 1 and Hev b 3. Hev b 1, the first purified latex antigen (Czuppon et al. 1993), was shown to have the ability to induce lymphocyte proliferation in 52% of latex sensitized patients (Raulf-Heimsoth et al. 1996). Possible cross-reaction with papain was reported, and it was thought to be due to the

homologous areas in their primary sequence (Baur et al. 1995). Linear-IgE epitopes of Hev b 1 were mapped by Chen et al. (1996), and Raulf-Heimsoth et al. (1998) identified the T-cell epitopes. Hev b 1 is reported to contribute especially in the sensitization of spina bifida patients to NRL allergens (Chen et al. 1997b, Rihs et al. 1998). Also, a non-soluble small rubber particle protein (SRPP), Hev b 3, has shown a close connection with medical personnel and spina bifida patients of whom 3-9% and 29-72%, respectively, are allergic to this protein (Yeang et al. 1996, Wagner et al. 1999, Bohle et al. 2000). Yeang et al. (1998) suggested that latex devices can come into direct contact with the mucosal membranes of spina bifida patients without the need for the allergen to be substantially present in a dissolved state. This property may also open possibilities for the development of mucosal tolerance to Hev b 1 and Hev b 3 allergens (Hufnagl et al. 2003). In reverse, water (sweat) soluble allergens are more easily transmitted to adults through glove powder and just the brief use of examination or surgical gloves may sensitize to user.

Hev b 5 and Hev b 6.01. During glove manufacture, highly acidic (pI 3.5) and proline rich Hev b 5 may degrade and form aggregates and interact with other latex proteins that may be abundantly present in powdered NRL gloves (Sutherland et al. 2002). This major allergen was reported to be recognized by sera from 56% of spina bifida patients and 92% of HCW suffering from latex allergy (Slater et al. 1996). Basic studies were done by Slater (1999) and by de Silva (2000) and their coworkers who mapped the B-cell and T-cell epitopes of Hev b 5. The genetically modified Hev b 5 has served as a potential pilot immunotherapy molecule with altered conformation but retained T-cell epitopes (Beezhold et al. 2001, Hardy et al. 2003). Later on, Slater also suggesfully pioneered a specific immunotherapy vaccine by cloning cDNA of Hev b 5 to engineered DNA plasmid and introduced it to mouse with and without bacterial base adjuvants (Slater et al. 1998a, Slater et al. 1998b).

Hevein (Hev b 6.02) at the N terminus of prohevein (Hev b 6.01) is a major latex allergen recognized by 70-80% of latex allergic patients, and by approximately one fourth of spina bifida patients (Alenius et al. 1995, Chen et al. 1997a). Hevein, identified as an IgE-binding domain of prohevein by inhibition-ELISA (Alenius et al. 1996), is extraordinarily stable, and it is abundant in many latex glove extracts. The immunological role of hevein has been widely characterized, Banerjee et al. (1997) and Beezhold et al. (1997) identified the linear IgE-epitopes of hevein. The group led by Baur (Rihs et al. 2002) established the relationship between the immune response to hevein and HLA class II antigens DQ8 and DR4. Furthermore, in 2004 they reported results of B-cell and T-cell responses to hevein (Raulf-Heimsoth et al. 2004). In addition to data presented in the present thesis, Reyes-Lopez et al. (2004) have recently identified the B-cell epitopes of hevein using modified hevein molecules, and de Silva et al. (2004) have identified the T-cell epitopes of hevein by specific T-cell clones. In a murine model of NRL allergy, prohevein was shown to be the major sensitizing allergen (Xia et al. 2001).

Other allergens. The immunological role of Hev b 7 and its cross-reactivity with homologous potato patatins remains unclear (Sowka et al. 1999, Seppälä et al. 2000). In most studies it is, however, classified as a minor latex allergen that, in addition to Hev b 1 and Hev b 3, may also sensitize spina bifida patients (Sowka et al. 1998, Kurup et al. 2000, Wagner et al. 2001). Latex profilin, Hev b 8, and cross-reacting enolase Hev b 9 are also minor proteins in NRL. Hev b 8 is recognized by 20% of HCWs and by 12% of sera from spina bifida patients (Rihs et al. 2000) whereas 14.5% of NRL allergic patients recognize Hev b 9 (Wagner et al. 2000). Hev b 11 was recently cloned and shown to be a hevein-like protein that belongs to class I chitinases. According to previous results, Hev b 6.02-MBP fusion could inhibit IgE binding to solid-phase-bound Hev b 11-MBP from 0-92% (O'Riordain et al. 2002). Also the role of early nodule-specific protein homologue, Hev b 13, is controversial (Arif et al. 2004, Palosuo et al. 2004).

2.3.4 Hevein

Hevein protein (Hev b 6.02) was identified already in 1960 and cloned in 1975 (Archer 1960, Waljuno et al. 1975), but it was almost another 20 years before it was recognized to be an important allergen (Broekaert et al. 1990, Alenius et al. 1995, Rozynek et al. 1998). Its extraordinary stability, natural functions and allergenicity have been widely substantiated, but the details of the molecular mechanisms in the immunological interactions between hevein and its counterparts are still only partially understood. In addition to rubber trees, hevein-like domains (HLDs) with chitin-binding activity (chitinases) are found in almost every plant. Different HLDs have highly conserved sequences and they might have evolved from a common ancestor gene by genetic transposition (Shinshi et al. 1990).

2.3.4.1 Biosynthesis

One tapping of a moderately productive rubber tree exudes about 100 ml of latex, consisting mainly of the cytoplasm of specialized cells known as laticifers (Soedjanaatmadja et al. 1995). About 15% of their volume consists of lutoid-bodies which are organelles of vacuolar origin containing several proteins. The collected latex is completely regenerated within three days and during that time 0.1-0.2 grams of hevein are synthesized, an amount which corresponds to only 1% of the total proteins in latex. Hevein-derivatives are enriched in lutoids, which contain equal quantities of prohevein (Hev b 6.01, 20-21 kDa) and its C-terminal domain (Hev b 6.03, 14 kDa), which are present in 1:30 molar ratio to hevein (Hev b 6.02, 4.7 kDa) (Soedjanaatmadja et al. 1995). In addition to mature hevein, the purified hevein samples seem to contain pseudo-hevein (about ten percent), which has six amino acid substitutions as compared to Hev b 6.02 (Soedjanaatmadja et al. 1994). In the rubber tree, hevein is synthesized in the leaves and stems (but not in roots) according to wounding experiments or

exogenous application of stress-related hormones like abscisic acid and ethylene (Broekaert et al. 1990).

Mature hevein seems to be the result of three proteolytic cleavages of the preprohevein *in vivo* (Fig. 5). The first cleavage involves removal of the N-terminal signal peptide (17 aa) which is necessary for cotranslational translocation into the lumen of the rough endoplasmic reticulum. The C-terminal truncation of hevein was originally thought to be an artefact produced during the purification procedure but it is most probably a vacuolar sorting signal (174-204 aa) that directs mature prohevein (Hev b 6.01, 1-173 aa) to lutoid bodies (Soedjanaatmadja et al. 1995). A subsequent post-translational processing could give rise to the formation of the N-terminal Hev b 6.02 (43 aa) with hinge region (6 aa) and C-terminal Hev b 6.03 (144 aa). As a final step the linker region is removed from the mature hevein (43 aa) and the C-terminal domain is degraded (Lee et al. 1991, Soedjanaatmadja et al. 1995). At least in trees suffering from dry bark disease or wounding, hevein is released from lutoid particles *in planta*, and in the presence of Ca^{2+} the latex coagulation is thought to inhibit the invasion of microbes (Gidrol et al. 1994).

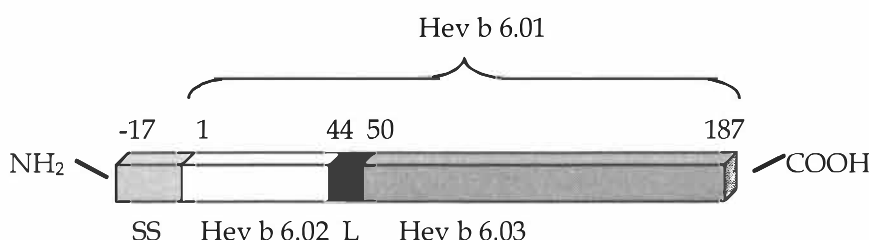


FIGURE 5 Composition of prohevein (Hev b 6.01). N-terminal signal sequence (SS) is cleaved off from hevein (Hev b 6.02), which is linked with a short linker (L) to the large C-terminal domain (Hev b 6.03).

2.3.4.2 Structural and functional properties

In order to exist as an allergen in rubber products, hevein has to resist vulcanization during latex manufacturing when NRL is treated with ammonia at temperatures up to 160 °C. This extraordinary stability is mainly due to the compact structure and four intramolecular disulfide bridges that are highly conserved in all HLDs. Also pH has an important effect: even 75% of denaturated hevein can renaturate in pH range 1.82-3.67 (90 °C) but above pH 4 reversibility is drastically decreased. At higher pH values hevein is unfolded, intermolecular disulfide bridges are formed and aggregation follows (Hernandez-Arana et al. 1995).

The 3D-structure of hevein was first determined by X-ray crystallography (Rodriguez-Romero et al. 1991) and defined further by NMR (1HEV) (Andersen et al. 1993). Very recently, further X-ray crystallographic studies have been published (Reyes-Lopez et al. 2004). Hydrophobic clusters, typical to small proteins, are missing in hevein, and its fold is fixed by the closure of the

disulfides. Local secondary structures form a helical conformation called the toxin-agglutinin fold, also found in WGA and in snake and scorpion toxins, and having dramatic effects on in living organisms (Fig. 6). Homologous neurotoxins from snake interrupt nerve-muscle communication, leading to paralysis or even death (Drenth et al. 1980). WGA stimulates human lymphocytes to divide and inhibits fungal growth (Brown et al. 1976, Drenth et al. 1980).

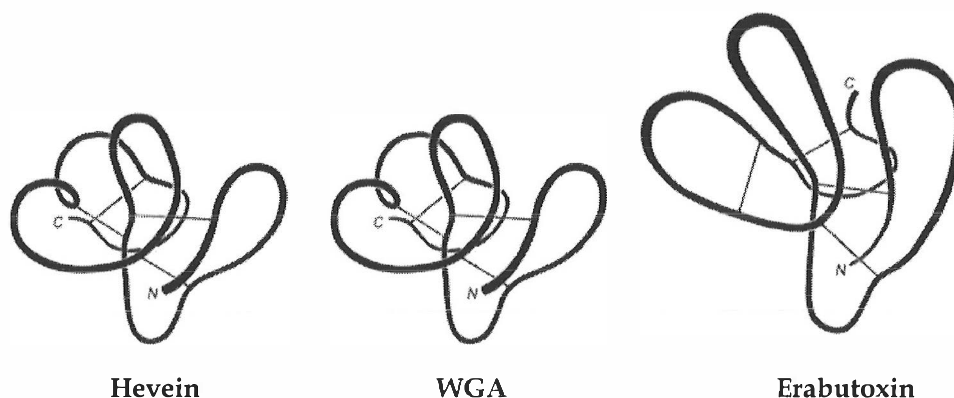


FIGURE 6 Schematic presentation of polypeptide backbone of toxin-agglutinin fold of hevein (left), one domain of WGA (middle) and a neurotoxin, erabutoxin (right).

Prohevein does not exhibit any catalytic activity, but hevein itself is notably antifungal and also shows antinutrient activity against insects (Van Parijs et al. 1991). It is classified as belonging to the family of pathogenesis-related proteins (PR-3) which include plant self-defense proteins and enzymes, known as phytoalexins, necessary for the biosynthesis of plant antibiotics (Yagami 1998).

Hevein recognizes and binds chitin, a β(1->4)-linked N-acetyl glucosamine (GlcNAc) (Asensio et al. 1995). The aromatic residues S19, W21, W23 and Y30 on the hevein molecule bind to $(GlcNAc)_{2\text{-}4}$ with millimolar affinity. The binding becomes stronger when the residues P13, L16, S19, C24 of hevein form an extended binding site to longer chitin chains (Asensio et al. 2000). Substantially similar binding modes are suggested by NMR-studies with WGA (Espinosa et al. 2000) and by computer-based simulations between hevein and chitin oligomers (Colombo et al. 2004).

2.3.5 Hevein-like domains (HLDs)

Plant proteins containing at least one HLD are grouped into chitin-binding lectins according to their carbohydrate-binding activity. This superfamily comprises merolectins consisting of a single HLD (Hev b 6.02, Prs a 1) or a slightly truncated HLD (AMP from *Amaranthus caudatus*) at the N terminus of a the corresponding protein (Broekaert et al. 1992). Chitin-binding hololectins are composed of two (UDA, *Urtica dioica*), three (PL-C, *Phytolacca americana*), four

(WGA, *Triticum aestivum*) or seven (PL-B, *Phytolacca americana*) tandemly arrayed HLDs (Van Damme et al. 1999,Hayashida et al. 2003). Depending on the number of cysteines, HLDs have from three to five intramolecular disulfide bridges with variable constellations (Fig. 7) (Huang et al. 2004).

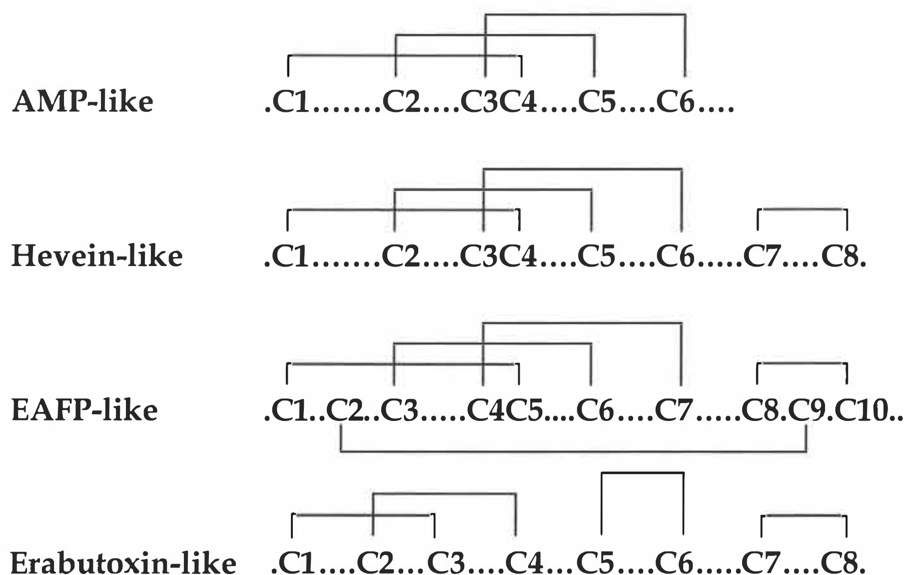


FIGURE 7 Comparison of disulfide bridge formations in hevein-like domains in antimicrobial protein (AMP), hevein, erabutoxin and *Eucommia* antifungal peptide (EAFP). Modified from Huang et al. (2004) and from Drenth et al. (1980).

The presence of an N-terminal HLD is also the hallmark of type-I plant chitinases (EC 3.2.1.14, family 19) that hydrolyze internal β-1,4-glycosidic linkages of chitin (Fukamizo 2000). Chitin is a key structural constituent of the cell walls of fungi and exoskeleton of invertebrates, such as insects and nematodes (Muzzarelli 1977). The existence of an N-terminal HLD enhances the endochitinase action by a threefold increase in its affinity and activity in acidic pH, at high temperatures or in dilute substrate concentrations (Iseli et al. 1993). In these cases, the HLD is thought to anchor the C-terminal enzyme near to its substrate (Iseli et al. 1993). Small hevein-like monomers or dimers (in UDA), without a large C-terminal sorting signal, penetrate through the fungal cell wall to reach the plasma membrane. Once inside, they are thought to inhibit cell-wall morphogenesis by binding or cross-linking newly synthesized chitin chains (Van Parijs et al. 1991). On the other hand, type-II chitinases lacking a HLD at their N terminus seem to have lower activation energy, although they are more active in high substrate concentrations (Iseli et al. 1993).

2.3.6 Latex-fruit syndrome

About 50% of latex allergic patients are also sensitized to certain plant foods, especially fresh tropical fruits such as banana, avocado, papaya or kiwi (Table 3) (Lavaud et al. 1995, Beezhold et al. 1996, Blanco et al. 1999). With respect to clinical manifestations, the typical presentation is oral allergy syndrome, but as many as half of the adverse reactions may be systemic (Blanco 2003). Often an anaphylactic reaction is the initial manifestation of a food hypersensitivity and the spectrum of food allergies seems to broaden within time (Blanco 2003).

The original sensitizing agent is not always clearly identifiable (Condemi 2002). Several latex allergens like Hev b 2, Hev b 6.01, Hev b 7, Hev b 8 and Hevb b 9 have homologous proteins in other plants but class I chitinases with a HLD are the most potential candidates operating in latex-fruits syndrome (Diaz-Perales et al. 1998, Mikkola et al. 1998, Yagami et al. 1998, Diaz-Perales et al. 1999). In contrast to so-called complete food allergens that are stable to digestion, class I (endo)chitinases (30-35 kDa) in vegetables and fruits are easily inactivated by heat and proteases (Yagami et al. 2000).

The N-terminal chitin-binding hevein-like domains (HLDs) in rubber latex, chestnut, avocado and banana chitinases show 70-80% identity (Wagner & Breiteneder 2002). Structurally and sequentially similar proteins are also found in wheat, stinging nettle, potato, white bean mistletoe and pineapple, but there are no reported cases of allergic reactions or even of IgE-binding to these proteins (Sánchez-Monge et al. 2000, Stoeva et al. 2001). Homologous class-II chitinases lacking N-terminal HLDs are also reported to be a reason for allergic cross-reactions, although usually IgE from patients bind only weakly to them (Subroto et al. 1999).

In addition, IgE reactivity to tree (birch) or grass (mugwort) pollen has been reported to cause allergy to latex (Díez-Gómez et al. 1999, Levy et al. 2000), and carbohydrate determinants have also been identified as cross-reactive agents in the pollen-latex syndrome (Fuchs et al. 1997). The clinical significance of cross-reactive carbohydrate determinants (CCDs) has not yet been fully established (Aalberse 1998).

TABLE 3 Food hypersensitivities associated with latex-allergy (Wagner et al. 2002).

Group	Definition	Foods
I	Frequent and significant associations	Banana, avocado, kiwi, chestnut
II	Other significant associations, but only described in certain studies	Potato, tomato, shellfish
III	Common association, but number of cases not enough to reach significant levels	Papaya, pineapple, passion fruit, mango, fig, nuts, stone fruits, pe-arch, cherry, apricot, melon, apple
IV	Less common associations (continuously growing list)	Guava, fish, carrot, strawberry, peanut, pepper, grape, oregano, dill, coconut, condurango, bark, milk, pear, spinach, beet, lychee

3 OBJECTIVE OF THE STUDY

Objective of the present study was to characterize in detail IgE-binding epitopes on the surface of the hevein molecule, and use this information in the design of hypoallergenic hevein mutants for use in immunotherapy for NRL allergy. In addition, the molecular basis for latex-fruit syndrome was explored. The more detailed aims of this study were:

1. To identify the IgE-binding regions of hevein (Hev b 6.02) with the "gain in function" chimera-based allergen epitope mapping strategy.

2. To locate the IgE-binding amino acid residues of hevein by site-directed mutagenesis and the production of recombinant proteins.

3. To construct hypoallergenic pilot molecules for immunotherapy that have native-like fold and intact T-cell epitopes but reduced IgE-binding ability.

4. To characterize the molecular reasons for latex-fruit syndrome with HLDs that are found in selected plants causing cross-reactions in NRL-allergic patients.

4 SUMMARY OF MATERIALS AND METHODS

The materials and methods used in this study are described in more detail in the original publications (I-III).

4.1 Programs used in protein design and modeling

HLDs in the Swiss-Prot Protein Sequence Database were identified using the advanced BLAST search (Altschul et al. 1997) service provided by the National Center for Biotechnology Information (I, II and III). Protein multiple sequence alignments were carried out using CLUSTAL X (Thompson et al. 1997) and DNAMAN 4.11 (Lynnon Biosoft, Vaudreil, Canada). PAWS (ProteoMetrix, New York, NY, USA) was used to calculate the theoretical molecular weights, and the GCG package (Genetic Computer Group, Madison, WISC, USA) or DNAMAN were used to estimate the theoretical pI values for mutants, chimeras and homologous proteins (I-III).

The resolved 3D-structures of hevein (I-III), AMP (I) and WGA (III) were verified and the most representative structures were selected using criteria from WHATCHECK (Wilson et al. 1998). Hevein (PDB: 1HEV) was used as a template structure, and the homology-based structural models in studies I and II were performed with VERTAA and MODELLER 4.0 programs in the BODIL Modeling Environment (Lehtonen JV, Still D-J, Rantanen V-V, Gyllenberg M and Johnson MS, unpublished; www.abo.fi/fak/mnf/bkf/research/johnson/bodil.html). Chimera models in study I were energy-minimized using the GROMOS96 43a1 force field and deepest descent method in GROMACS2.0 (Berendsen et al. 1995). All substituents in the 29 hevein mutants (II) were tested with side chains found in the rotamer library of the WHATIF modeling program. Homology-based models for HLDs of avocado and banana (III) were modelled with MODELLER (Sali & Blundell 1993).

The figures in studies I and II were rendered with the help of InsightII 98.0 (Molecular Simulations Inc, San Diego, USA), Molscript 2.1 (Avatar, Stockholm, Sweden) and Raster3D (University of Washington, Seattle, WA, USA). All representations of protein structures in study III were prepared with PyMOL (DeLano, W.L. The PyMOL Molecular Graphics System (2002) on World Wide Web http://www.pymol.org).

4.2 Synthetic peptides and synthesis for cDNAs

Synthetic peptides (I) containing the N terminus of hevein in linN (aa 1-11), the core region of hevein in lincore (12-32) and the C terminus of hevein in linC (33-43) were purchased from MedProbe (Oslo, Norway). To ensure the peptide linearity, a similar set of peptides was purchased but each of their cysteine residues was replaced with serine residues.

Because the cDNA for AMP (I) was not available, it was constructed using PCR by multiplying two annealed oligonucleotides whose product corresponded to AMP sequence X72641 in the NCBI databank. In order to achieve cDNA for banana HLD (III), totRNA was extracted from the fruit, leaf and peel of banana and reverse transcribed with a cDNA synthesis kit (Enhanced avian RT-PCR Kit, Sigma, USA). The resultant cDNA from banana leaf was finally cloned into pBacAvs+C vector. Both of the constructed cDNAs were sequenced by dideoxynucleotide sequencing with a Li-Cor DNA sequencer (Li-Cor Biotechnology Division, Lincoln, NE, USA).

4.3 Construction of expression vectors

Either the QuickChange™ Site-Directed Mutagenesis system (Stratagene, La Jolla, CA USA) or the megaprimer method (Sarkar & Sommer 1990) were used for site-specific mutations of hevein or AMP cDNAs. After PCR amplifications, the fragments, with a thrombin cleavage site, were subcloned into a pBacAvs+C vector, which was digested with *BamH*I/*Hind*III (Airenne et al. 1999). The constructs were transformed into *E. coli* JM109 cells and the constructs were always confirmed by sequencing. The recombinant baculoviruses were produced using the Bac-to-Bac baculovirus expression system (GIBCO BRL, Life Technologies, Gaithersburg, MD, USA). The recombinant baculoviruses were constructed by recombination, when the gene of interest was attached to the bacmid genome of DH10Bac *E. coli* cells. Purified bacmids were used to transfect Sf9 cells (line IPLB-Sf21-AE, Gibco), and functional baculoviruses were produced in three days. They were used for the large-scale production of recombinant or mutated proteins.

4.4 Production and purification of hevein-like domains (HLDs)

Recombinant, chimeric and mutated hevein and HLDs were produced as avidin fusion proteins in Sf9 insect cells (in the absence of biotin) to ensure the correct folding and formation of the requisite four disulphide bridges. After protein purification with 2-iminobiotin-agarose, fusion proteins were eluted to 50 mM Tris-HCl (pH 8) with an excess of biotin (I, II) or to 25/50 mM ammonium acetate (pH 4) (II, III). Eluted fractions were separated by SDS-PAGE and analyzed by immunoblotting (rabbit antiserum against avidin or hevein), and fractions containing the fusion protein were selected for more detailed studies. The cleavage buffer was changed with a FastDesalting gel filtration column (Amersham Biosciences, Uppsala, Sweden) and concentrated fractions were digested overnight with the excess of thrombin and separated with 5/2 reverse phase chromatography (Amersham Biosciences, Uppsala, Sweden) through the Shimadzu (Shimadzu Corp.,Kyoto, Japan) or Waters (Waters Corp., Milford, MA, USA) HPLC systems.

HLD-containing proteins from the rubber latex, avocado and banana (III) were attached to a chitin affinity column and further purified by anion-exchange chromatography or by reverse phase chromatography.

4.5 Protein analysis and characterization

For the characterization of each of the cleaved and RP-separated proteins, their molecular masses were measured with a time-of-flight (MALDI-TOF) Bruker Biflex II instrument (Bruker-Daltonic, Bremen, Germany) (I-III). In studies I and III the N-terminal amino acid sequences were also obtained (ABI 494 A Procise™ Sequencer, Perkin Elmer Life Sciences, Boston, MA, USA).

To test the stability of intact endochitinases (31 kDa) from avocado and banana (III), 50 µg of protein was digested with 2.4 µg pepsin (Sigma Aldrich, St Louis, MO, USA) for 0, 30, 60 and 120 minutes in 1 M HCl. After each time point, the pH of the reaction solution was raised above six with 1M NaOH in order to inactivate pepsin. Protein digests were analyzed by HPLC on a Vydac 0.1 x 15 cm reversed phase column (LC Packings, Amsterdam, The Netherlands).

4.6 Patients' sera used in the studies I-III

A total of 57 serum samples were obtained from NRL-allergic patients (52 females, five males, mean age 46 years), who all showed positive SPTs to a NRL glove extract (Triflex glove eluate or Stallergenes, Fresnes, France).

The control sera (I-II) were obtained from 19 atopic individuals with no latex or banana allergies. In study III, eleven atopic, but non-allergic patients were used as negative controls, and nine banana, but not latex allergic patients were used as controls for hevein-specificity.

4.7 Tests *in vitro*: direct-ELISA and inhibition-ELISA

ELISA 96-well plates (Nunc, Roskilde, Denmark) were coated with purified hevein at a concentration of 240 pmol/ml (1 µg/ml, I), 720 pmol/ml (3 µg/ml, II) or 100 pmol/ml of the native endochitinases (III) in 50 mM carbonate buffer. The proteins were applied to a polystyrene microtiter plate (100 µl/well; Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The wells were emptied and the remained protein binding sites were blocked with 1% human serum albumin (HSA) in a carbonate buffer at room temperature (RT, 20-23 °C) for 1 hour. After rinsing the plates three times, 100 µl of serum (diluted 1/10 with PBS-Tween-HSA; PBS containing 0.02% Tween 20 and 0.2% HSA) solution was added to the wells and incubated at RT for 2 hours. In the control wells serum was replaced with an equal volume of PBS-Tween-HSA. After the washes, 100 µl of biotinylated goat anti-human IgE (Vector, USA; diluted 1/1000 with PBS-Tween-HSA) was added to the wells and incubated at RT for 1 hour. After washing again, 100 µl of streptavidin-conjugated alkaline phosphatase (Bio-Rad; diluted 1/1000) was incubated in the wells for 1 hour. Substrate (*p*-nitrophenyl phosphate, Sigma Aldrich, St Louis, MO, USA) was added over the washed pellets and the absorbance was assayed at 405 nm by an automated ELISA reader (Multiskan Ascent, Thermolabsystems, Helsinki, Finland).

Inhibition-ELISA plates were coated with the relevant proteins at concentrations of 480 pmol/ml (2 µg/ml, I), 720 pmol/ml (3 µg/ml, II) or 100 pmol/ml (III). Patient sera (diluted 1:5) were preincubated with an equal volume of inhibitor solutions (diluted to ten-fold or hundred-fold series) for one hour before applying them to the microtiter plate. Chimeras, synthetic peptides and hevein mutants were used as inhibitors in PBS-Tween-HSA, concentrations ranging between 10 - 0.0001 µg/ml and for peptides up to 100 µg/ml. In study III, the binding of IgE to 31 kDa endochitinases was inhibited with HLDs at concentrations of 500, 50, 5 and 0.05 pmol/ml. All other steps were performed as described above for direct-ELISA.

4.8 Test *in vivo*: skin prick tests (SPT)

The samples for SPT were prepared from reversed phase (RP) or ion-exchange purified proteins, whose buffer was changed to water by a gel filtration column.

To ensure the exclusion of ACN and other contaminants, SPT samples were always concentrated to half volume by evaporation and then diluted to PBS.

SPTs were performed on the volar surface of the forearm with a sterile one-peak lancet (ALK Abello, Horsholm, Denmark). Evaporated and diluted RP-fractions were used as negative controls (I, II) and histamine at a concentration of 50 µg/ml was used as a positive control (I-III).

5 REVIEW OF THE RESULTS

5.1 Conformational IgE-binding areas of hevein (I)

5.1.1 Design and modeling of AMP chimeras

In order to design chimeras, hevein-based sequence alignments were performed. The central area of hevein was extensively conserved in all of the 92 homologs. Hevein homologs with resolved 3D-structure were possible target proteins with which to study the conformational epitopes of hevein. The antimicrobial protein (AMP, 30 aa) from the amaranth (*Amaranthus caudatus*) was selected as an adaptor protein (I, Fig. 1 and Fig. 2). In the immunological tests native AMP, extracted from seeds of the amaranth, had no IgE-binding ability to the sera of NRL-allergic patients. In the structural alignment, AMP and hevein shared highly conserved core regions (aa 12-31 in hevein), but the N termini were divergent and the C terminus was totally lacking in AMP. Hevein was thus divided into three parts, i.e., the N terminus (aa 1-11 in AMPN), core-region (12-31 in AMPcore) and C terminus (32-43 in AMPC). They were replaced in the chimeric molecules with the corresponding stretches of AMP.

Before the production of these chimeras, their 3D-structures were modeled, energy-minimized and evaluated to identify possible poor geometry or bad contacts in interactions between side chains (I, Fig. 3). No problems with either packing or side chain interactions were seen when the four chimeras were compared to structures of hevein and AMP. Major differences occurred at the N-terminal area of chimeras, but these differences were not expected to interfere with the stable folding or packing of these constructs (AMPN, AMPN+C). The C-terminal part of hevein contains ten extra amino acid residues and an additional disulphide bridge as compared to AMP, but it formed no internal interactions that would be interrupted in the chimeras (AMPC and AMPN+C). In each of the models, it was possible to build the disulphide bridges in a way similar to those in the original NMR structures.

5.1.2 Production and purification of recombinant proteins

AMP and AMPcore cDNAs were constructed by annealing two long primers using PCR, whereas the other three chimeras were synthesized with terminal primers from AMP cDNA. After subcloning, the recombinant proteins and chimeras were produced in Sf9 insect cells using the Bac-to-Bac™ Baculovirus Expression System that has previously been shown to be a reliable method for the production of recombinant hevein (Airenne et al. 1999).

On the basis of avidin-biotin interaction, affinity-purified protein fractions were routinely analyzed by SDS-PAGE and immunoblotting. After protease cleavage of the avidin tag, the hevein chimeras were separated in an RP column and their molecular masses were always confirmed by mass spectrometry and, in some cases, also by N-terminal sequencing. The protein yields for chimeric proteins varied between 175 µg - 2000 µg per litre.

5.1.3 IgE-binding of chimeras

The IgE-binding ability of hevein-AMP chimeras was studied by direct-ELISA and in inhibition-ELISA. ELISA tests were performed with the native and recombinant hevein and AMP, with all of the chimeras and mutants, and with the synthetic, linear peptides of hevein. These studies indicated that the IgE-binding abilities of the recombinant hevein and the recombinant AMP were virtually identical to that of their native counterparts.

Only two of the 16 patients studied recognized the hevein core region within the AMP termini (I, Fig. 4), indicating that the hevein core region is an improbable IgE-epitope area. In contrast, the chimeric AMPN with the N terminus of hevein was recognized by 14 out of 16 patients and the chimeric AMPC with C-terminal residues from hevein was recognized by six out of 16 patients. However, when both the N-terminal and C-terminal regions of hevein were fused with AMP core (HEVN+C), all 16 patients showed IgE binding against the chimera. A similar test with corresponding sequences was also performed with synthetic peptides but none of the patients recognized any of them.

Inhibition-ELISA analyses were performed with six patients whose sera recognized the N terminus and/or C terminus of hevein in the direct-ELISA. The results confirmed the conformational nature of the IgE-epitopes, since none of the linear peptides exhibited any inhibition in IgE-binding, even at high concentrations (100 µg/ml). Also the pool of linear peptides was unable to inhibit IgE-binding to the solid-phase AMPN+C chimera, while native hevein exhibited total inhibition against all the chimeras.

ELISA inhibitions were performed against solid-phase hevein to compare IgE-binding affinities (II, Fig. 5). Chimeric protein HEVN+C notably inhibited the binding of IgEs to hevein in a dose-dependent manner. Patient-specific variation was evident and average inhibition in the case of five patients was 60-97%, although in the case of one patient the inhibition was only 20%. In

contrast, HEVC was able to inhibit only slightly (maximally 30%) and HEVN was unable to compete with native hevein at all. Inhibitions were also performed with all three linear peptides, but even at the highest peptide concentration (100 μg/ml), no significant inhibition was detected.

5.2 IgE-binding amino acid residues of hevein (II)

In the more detailed study of the epitope areas (II), the hevein sequence was mapped by replacing its amino acid residues with substituents that are similar in size and present in analogous sites in natural hevein homologs. The 29 hevein mutants produced showed variable IgE-binding abilities in inhibition-ELISA. In addition, the serum samples from 26 NRL-allergic individuals had large patient-specific variation. Because of the vast number of different inhibition results, the scanning data were summarized in columns that indicated the amount of the reduction in inhibitions (II, Fig. 2). According to the threshold of 25% in inhibition, six single mutations (R5A, K10A, E29A, Y30F, H35A and Q38V) had the strongest influence on the IgE binding.

These six mutants were carefully studied in five reference patients with variable IgE-binding patterns. They were selected to represent all the NRL-allergic patients used in the scanning experiments. As much as ten to hundred fold higher amounts of the mutants were required to inhibit IgE-binding by 50% as compared to native hevein. The mutants R5A and K10A caused the weakest inhibitions (about 40%), revealing the disintegration or modification of the IgE-epitope of hevein (II, Fig. 3).

5.3 Hypoallergenic molecules (II)

5.3.1 Modeling

On the basis of the immunological studies and IgE-binding abilities, three pilot molecules (HevΔ3A, HevΔ3B and HevΔ6) with three or six of the most important substitutions were designed for immunotherapy. In modeling, they showed a largely retained fold and the possibility to form all four disulfide bridges of native hevein (II, Fig. 4). However, the charge distribution on the surface of HΔ3A changed quite drastically due to the elimination of three charged residues, R5, E29 and H35. The R5A mutation, especially, destroyed several main interactions between positively charged arginine (R5) and polar glutamines (Q6, Q20, Q38) and asparagine (N36) in that area. The other two mutations in HΔ3A (E29 and H35) caused less severe effects by eliminating the interactions between Q29, T27 and H35. The other polar residues, however, continued to interact with each other and stabilized the overall structure. The

surface topology of HΔ3B was less altered; its sole major change was a mutation of K10A that destroyed the hydrogen bonding between K10 and, the main chain carbonyl of Q2, while the N-terminal disulphide bridge (C3-C18) continued to stabilize the structure.

Six simultaneous mutations (R5A, K10A, E29A, Y30F, H35A and Q38V) in HΔ6 induced a clear change in the shape and the charge (less charged) distribution of the surface near the corresponding positions. The surface charge distribution was markedly changed when three charged residues (R5, K10 and E29) were substituted with alanine (II, Fig. 4). Evidently, the mutation R5A resulted in similar and even stronger structural changes, as seen in HΔ3A, because Q38V was co-mutated. The mutation K10A destroyed an important interaction near the N terminus by substantially changing the shape and the charge distribution of the surface. The other two mutations in HΔ6, E29A and H35A, led to structural changes similar to those in the HΔ3A structure. However, E29A was located relatively close to the phenyl ring of Y30F, enabling hydrophobic interactions between E29A and Y30F.

5.3.2 IgE-binding of chimeras

The synergic effect of substitution of the most important amino acid residues to IgE-binding was studied in three combinatory mutants. They contained one amino acid from the N terminus, one from the C terminus and one from the hevein core region (HΔ3A and HΔ3B), or all of the six substitutions were combined (HΔ6). All these combinatory mutants inhibited the binding of IgE to solid-phase hevein in a dose-dependent manner. As predicted by the modeling, the triple mutants (HΔ3A and HΔ3B) showed fewer radical surface or structural changes when compared to the hexa mutant, as was also seen in the inhibitions (II, Fig. 5).

In two patients (patients 19 and 22), the 50% level of inhibition in IgE binding was reached with all the combinatory mutants at concentrations 10 to 100-fold higher than with the recombinant hevein. In two other patients (patients 2 and 8) a 10 000-fold higher amount of mutants was needed to achieve the same, 50% level of inhibition. In one patient (patient 26), HΔ3B and HΔ6 caused dramatic reduction in IgE-binding, but hardly any effect was seen with HΔ3B. Despite the variation between patients, the most consistent and strongest reduction in IgE-binding was obtained with HΔ6, which contained all the six mutations.

5.3.3 Skin prick tests

The importance of IgE-binding amino acid residues on hevein were proven by skin prick tests (SPT) *in vivo*. Four NRL-allergic patients previously shown to respond positively to hevein were tested with the combinatory mutants. SPT reactivity was abolished in three patients to the triple mutant HΔ3A and

reduced in the fourth patient. Mutant HΔ3B was the most allergenic molecule, as in one patient the SPT reactivity resembled that of native hevein and in two of patients a reduction was noticed. Only one patient showed a totally negative SPT response to H3ΔB. However, with mutant HΔ6 the SPT reactivity was totally abolished in all four patients.

5.4 HLDs in latex-fruit syndrome (III)

5.4.1 Proteins

Native hevein (4.7 kDa) was extracted from B-serum of rubber latex by chitin-based affinity chromatography and separated in RP chromatography. Anion exchange chromatography was used to purify prohevein (18.5 kDa) from the latex B-serum. HLD-containing proteins were purified from fresh avocado and banana fruits. After robust chitin-affinity purification, RP-chromatogram of avocado and banana extracts revealed several fractions that were collected and analyzed by SDS-PAGE. Fractions that were detected with rabbit anti-hevein antibodies in direct-ELISA were selected to further studies and were further characterized in more detail by protein chemical techniques. Their molecular masses were measured with mass spectrometry and two forms (4.3 kDa and 31.1 kDa) of HLD-containing proteins were detected in avocado, but only the larger form (31.2 kDa) was detected in banana. Both of the avocado-derived proteins showed an identical N-terminal sequence (EQCGRQAGGALCPGGLCCSQ...), and the peptide mass fingerprints confirmed that the extracted proteins were endochitinases from avocado and banana. It proved impossible to isolate mature HLD from banana; therefore, totRNA was extracted from banana peel, fruit and leaf after which cDNA was obtained from leaf extract (III). The recombinant HLD-proteins from banana and from WGA were produced and purified as hevein chimeras and mutants in previous studies (I, II).

5.4.2 Skin prick tests

In order to test the immunological cross-reactivity of phylogenetically unrelated plant species within NRL-allergic patients, tests *in vivo* with 15 latex-allergic and nine banana-allergic patients were performed. The purified and characterized HLDs and 31 kDa endochitinase proteins from latex, avocado, banana and wheat, were used as test samples in SPTs (III, Table 2). When 31 kDa endochitinases and mature HLDs (4.3 kDa) were compared, the latter turned out to be superior in SPT reactivity. As many as 73% of the latex-allergic patients reacted positively to HLDs of avocado and banana whereas the 31 kDa endochitinases, which contain a large C-terminal domain in addition to HLD, produced weak reactions. Only one patient reacted positively to these 31 kDa

endochitinases from banana and avocado. All the 15 NRL-allergic patients tested had been shown previously to have IgE against hevein, and now these patients showed SPT-positive reactions to hevein and 13 of them also reacted to prohevein. In addition, seven (47%) and six (40%) of the 15 NRL-allergic patients reacted to fresh banana and avocado fruit, respectively. Moreover, three of them showed positive reactivity to 18-kDa WGA, too. The nine banana-allergic patients in the control group had negative SPTs to NRL, and none showed SPT reactivity to any of the large or small HLD proteins.

5.4.3 ELISA studies

The IgE-binding efficiencies of isolated HLDs (4.3 kDa) as well as 31-kDa endochitinases from avocado, banana and latex were examined in inhibition-ELISA. In addition to strong inhibition of prohevein and hevein, HLDs from avocado and banana inhibited IgE binding to prohevein on average by 59% and 38%, respectively (III, Fig. 1 A). The corresponding 31-kDa proproteins inhibited IgE-binding only weakly (17% and 20%), at equimolar concentrations (III, Fig. 1 A). The importance of HLDs was further confirmed, since the HLD of avocado inhibited IgE binding to solid-phase endochitinases from avocado and banana on average by 77% and 73%, respectively (III, Fig. 1 B and C). Although patient-specific variation exists, the patients were categorized into two or three different groups according their IgE-recognition patterns against prohevein and 31-kDa endochitinases from avocado and banana.

5.4.4 HLDs in WGA

After the clinical role of HLD-containing proteins from different plants had been established, the next task was to clarify the reasons for their importance via the structurally known WGA protein. Four HLDs from WGA were produced and purified and their ability to inhibit IgE-binding to hevein was tested (III, Fig. 2). Two of the WGA domains (the first and the fourth) showed a 1 000 – 10 000-fold reduction on average in their ability to inhibit IgE-binding to solid-phase hevein at the highest concentrations, whereas the other two WGA domains and the 18-kDa WGA itself exhibited no inhibition (III, Fig. 2).

5.4.5 Sequences and structures

In order to assess the molecular basis for the different immunological responses in latex-allergic patients, a multiple sequence alignment to hevein and HLDs from avocado, banana and wheat was performed (III, Fig. 3). Significant amounts of conserved small (Gly, Ser) or aromatic amino acid residues (Trp, Tyr) in addition to eight disulfide bridge-forming cysteines were detected. The typical extra loop (His35 - Asn36) of hevein was, however, absent in all the other HLD proteins.

In the absence of coordinates for fruit 31-kDa endochitinases, HLDs from avocado and banana were modeled, and they showed high sequence identity (76% and 63%, respectively) with hevein. As anticipated, the 3D-structure of hevein and the performed models were also highly similar (III, Fig. 4A), showing exceptions only at sites that also differed in their primary sequence (R5, S32-N36). When the "key amino acid residues" on the surface of our previously identified IgE-epitope of hevein (R5, K10, E29, Y30, H35 and Q38) were compared with those on the modeled HLDs, they seemed to remain in the conserved position (III, Fig. 4 B – 4 D).

The four isolated HLDs of WGA (PBD: WGA9) were also superimposed almost identically throughout their C-alpha traces with hevein (Fig. 5 A). Major differences were observed at the terminal parts, especially between hevein residues 34-37. Notably, charged amino acid residue R5 on hevein pointed in a slightly different direction from the corresponding residues on the WGA subunits. Moreover, only the fourth HLD in WGA had a positively charged amino acid residue (Lys) in the corresponding position. When all the six IgE-binding residues of hevein were compared to corresponding residues on the four HLDs in WGA structure, they were again found to possess a highly solvent-exposed conformation (Fig. 5 B).

6 DISCUSSION

Atopic diseases are common in the Western world where even every fourth person suffers from allergies. In this thesis, the basic molecular mechanisms of type I allergies and possible protein tools for future treatments were approached. NRL allergy was used as a model, because most of the NRL allergens have been well characterized and latex-sensitized patients frequently suffer from allergic cross-reactions after eating fresh fruits (latex-fruit syndrome). The major latex allergen, hevein (Hev b 6.02), is a protein with characteristic features, offering the researcher an intriguing target for detailed studies on its immunology and structural biology. Animal and human studies have shown that denatured allergens or linear peptides of allergens do not induce immediate allergic reactions in individuals who are sensitized to the native allergen, although such peptides do stimulate allergen-specific T-cell responses (Takatsu et al. 1975, Norman et al. 1980). Recombinant DNA technology and protein engineering were used to keep the overall structure of hevein as native as possible, while identifying the IgE-epitopes and designing immunotherapy tools.

One major goal of this study was to investigate the antigen-antibody interaction of hevein and IgE molecules at the molecular level. A strategy of rational design was used to partially rearrange the conformational epitopes by transferring parts of hevein to an immunologically silent adaptor protein (AMP) from the amaranth (I). The IgE-binding residues were scanned one by one and, finally, the IgE-hevein interaction was interfered by mutating a minimal number of amino acids (the six most important) on the surface of hevein (II). Three hypoallergenic immunotherapy tools were designed and produced and their allergenicity and immunogenicity were studied *in vitro* and *in vivo* (II).

Clinical observations with NRL-allergic patients led us to focus on highly conserved hevein-like domains (HLDs) from different plant sources. NRL-allergic patients are known often to produce allergic reactions after eating HLD-containing avocado and banana, but not after WGA, although this wheat protein contains four HLDs per molecule. Results from studies addressing the

hevein-related latex-fruit syndrome deepened the knowledge of epitopes and IgE-cross-reactivity in NRL-allergic patients (III).

6.1 Production of hevein and HLDs (I-III)

The plant protein databases currently available offer about a hundred known evolutionary-evolved HLDs, which were individually considered when selecting the scaffold protein for chimeras and reliable target residues for each site-specific substitution. After the design and modeling, the modified hevein molecules were produced. Other possible methods such as alanine scanning, random mutagenesis or substitution with all the other amino acids were considered to be too unreliable or laborious. Directed evolution within gene-shuffling systems has been shown to be extremely efficient, and basically it can offer all the possible combinations of amino acid residues for proteins, whose activity, folding or function are optimized (Stemmer 1994). If the selection is based on phenotype (i.e. fluorescent color) or on biological function, such as ligand-binding, as many as tens of thousands of new mutants may be achieved, with an improvement in enzyme activity of up to 500-fold in a few rounds of shuffling (Reetz 2004, Zahnd et al. 2004). However, in the absence of reliable methods of selection, this mimicking of natural processes and screening of large libraries is wasted. Moreover, similarly the most common way of expressing randomized proteins using the filamentous phage-display technique would not have been optimal in this case. Hevein has four intramolecular disulfide bridges that usually pair incorrectly in bacteria, and only 10% of expressed hevein molecules have been shown to be produced as active proteins in the bacterial periplasm (Halme 2003). Because of their small size and the demanding nature of making post-translational modifications, hevein and its modified forms were produced in insect cells (*Spodoptera frugiperda*) known to have diverse protein processing capabilities comparable to those in plant and mammalian cells (Richardson 1995).

For enhanced production and efficient purification, an N-terminal avidin tag was used in all the hevein constructs (Airenne et al. 1999). In the initial studies, enterokinase was used to cleave the fusion proteins. However, the protease seemed to have nonspecific activity, and to avoid inclusion of four extra N-terminal residues (DDDK), the few first hevein mutant constructs were recloned and converted to forms suitable for thrombin, which cleaved fusions as expected. This modification also avoided precipitation of the mutant H35A. Low protein yield and the possible precipitation of mutants Q20A and Q38V were bypassed by changing the pH of the elution buffer from 4 to ~7 and by eluting the proteins from solid-phase 2-iminobiotin with an excess of free biotin.

The native-like folding of the modified hevein and HLDs was always confirmed by measuring their molecular masses and checking their

immunological recognition with anti-hevein antibodies. In addition, it would have been advantageous to test certain functional properties such as chitin-binding ability, at least with those molecules in which chitin-interacting residues were intact. However, the hevein and chitin molecules were too small to be measured by an optical biosensor (IASYS). In order to confirm the retained 3D-structure, other techniques such as circular dichroism spectroscopy were attempted but given up because of the high amount of protein needed. The immunological properties of the hevein-derived proteins were mainly tested by *in vitro* and *in vivo* studies.

6.2 IgE-binding regions of hevein (I)

Few years ago, partial deletion of a given protein was a technique commonly used to identify the functional regions and amino acid residues of an allergen (Tamborini et al. 1997, Tang et al. 2000). Accordingly, in the present approach, deletions were also employed to make the first hevein mutants. However, removing the hevein-specific loop (H35-N36) or deleting the last disulfide bridge by C-terminal truncation (aa 33-43) led to precipitation of the proteins, thereby rendering them impossible to handle (data not shown). Later, protein databases were found to contain tens of naturally existing HLDs with different domain organization and variability in length. The antimicrobial protein (AMP) from the amaranth with its known 3D-structure seemed to be a good natural analogue of the deletion mutants, and in keeping with the present study concept, IgEs from NRL-allergic patients did not recognize it. According to the rational design concept, the availability of the 3D-structures (1MMC and 1HEV) enabled the transfer of parts between hevein and the immunologically silent homologue, AMP.

After sequential and structural comparisons, the hevein sequence was divided into three parts. Using this novel chimera-based allergen epitope mapping strategy, we designed four chimeras containing an N terminus (AMPN, aa 1-11), core-region (AMPcore, aa 12-32) and C terminus (AMPC, aa 33-43) or both termini (AMPN+C) from hevein fused within corresponding areas of AMP. In the light of modeling, all the disulphide bridges seemed to be formed in these hypoallergenic molecules and no problems with packing or side chain interactions were detected. The constructed proteins were produced and purified in a soluble form.

In parallel to our studies (I) King et al. (2001) published a similar constellation of two poorly cross-reacting allergens Ves v 5 (23 kDA) from the yellow jacket (*Vespula vulgaris*) and Pol a 5 (23 kDa) the from paper wasp (*Polistes annularis*). Although the 3D-structure of Pol a 5 was unknown, sequentially homologous segments of Ves v 5 in variable length (9-155 aa) were cloned to corresponding sites in Pol a 5. Nine hybrids were produced in yeast and their native-like structures were shown by circular dichroism spectroscopy

and by mouse monoclonal antibodies that were unable to bind to denatured Ves v 5.

The ELISA experiments showed that sera from most patients (88%) bound to the N terminus of hevein, whereas the hevein core region had only a minimal influence on IgE binding. In contrast, sera from some patients (38%) bound to the C terminus of hevein. However, all patients recognized the combination of both hevein termini (AMPN+C) with AMP core in direct-ELISA. It was therefore concluded that the spatial structure plays an important role in the formation of the hevein epitope. Interestingly, none of the patients recognized any of the linear peptides corresponding to the termini and core regions of hevein. Due to the variation in recognition of the hevein terminal parts, these results supported the idea that two different or two shared conformational epitopes are present in hevein. An essentially similar conclusion was obtained in a study where a phage display library of human single-chain antibodies showed closely related sequences in the two ultimate hevein-specific IgE-antibodies (Laukkanen et al. 2003).

In order to estimate the affinity with which IgE binds to the reconstructed epitopes, AMPN, AMPC and AMPN+C were tested in inhibition-ELISA. Again, the strongest binding efficiencies were seen with AMPN+C which inhibited ~60% of total IgE-binding when compared to native hevein (100%). In contrast, IgE bound to the AMPN and AMPC chimeras so weakly that they were unable to compete with the native hevein at all. IgE seemed to bind so weakly to the terminal parts of hevein that the formation of epitopes resembling the native ones was expected only when both hevein termini were simultaneously attached to the AMP core region.

King and coworkers (King et al. 2001) have similarly shown that immunization of mice with hybrids containing long N-terminal parts from Ves v 5 stimulate more production of Ves v 5 specific antibodies than Pol a 5 specific antibodies. Comparison of the antibody concentrations in these mice showed that of the three N-terminal hybrids, PV_{1-155} was as immunogenic as the recombinant Ves v 5 (aa 1-204), whereas PV_{1-46} and PV_{1-18} were only half and one-ninth as immunogenic, respectively (King et al. 2001). According to histamine release studies, the N-terminal chimera PV_{1-155} showed no decrease in allergenicity with basophils of patients sensitive to yellow jacket bees. In contrast, hybrids PV_{1-46} and PV_{1-18} showed 126-fold and 583-fold reduced allergenicity, respectively. In addition to higher IgG/IgE-ratio against N-terminal parts of Ves v 5 in stimulated mice, these results were believed to reflect a loss of epitopes in the N-terminal hybrids. The authors concluded that the yellow jacket allergen, Ves v 5, contains discontinuous B-cell epitopes that are mainly located at its N-terminal region (King et al. 2001).

In spite of the similar approaches to mapping the discontinuous epitope regions of proteins, certain differences exist in these two studies. In our study, regions were transferred between structurally known proteins, from an allergen to a virtually nonallergenic adaptor protein, to produce "gain in function" chimeras. These molecules indeed verified the existence of functionally active

B-cell epitopes better than "loss of function" hybrids. In addition, direct IgE-binding reactivity was assessed using human sera (IgE) instead of mouse monoclonal antibodies. Finally, on the basis of the inhibition studies it was concluded that relatively small conformational regions of hevein contain the major IgE-epitopes and that the N-terminal amino acid residues have the most significant influence on the affinity of IgE antibodies.

6.3 IgE-interacting amino acids on hevein (II)

After the identification of the conformational IgE-binding regions of hevein, it was essential to learn the precise location of the IgE-interacting amino acid residues to be able to design potential immunotherapy molecules. Immunization with modified hevein molecules was suspected of hampering the immediate IgE-driven reactions and, instead, of activating specific T-cells to divert the cell-mediated reactions. However, at the time when these studies were begun, the epitopes of hevein were only partially known and it was not sure if they were retained in hevein-AMP chimeras. Thus, in order to design hypoallergenic molecules with minimal modifications, the hevein sequence was scanned and the IgE-binding key residues were identified by site-directed mutagenesis. All the residues in hevein, except for disulfide bonding cysteines, are somehow water-exposed and therefore potential IgE-interacting amino acid residues. In addition to cysteines, all glycines and alanines were excluded from mutagenesis because of their unlikely role in IgE binding.

Before the final selection of the single-mutations to be incorporated into hevein, the consequences of different mutation approaches were tested. Two of the hevein amino acid residues (R5, N40) were replaced with two evolutionary-tested substitutions. The aim of the first two substitutions (R5A, N40A) was to dampen down the effect of the original residue and the second ones (R5E, N40R) reversed their charge. As expected, substitutions that changed the surface charge distribution showed highly decreased inhibition, whereas alanine substitutions resulted in less drastic effects (data not shown). The R5E mutant showed the most dramatic reduction in inhibition of all and therefore other concurrent substitutions (R5E+H35A, R5E+delH35-N36, R5E+D34G and R5E+K10A) were performed. Unfortunately, the latter substitutions seemed to result in conformational changes or mispairing of disulfide bonds since correct molecular masses were not detected.

In the following experiments, substituting amino acid residues that were neutral and equal in size were selected to eliminate the effect of the original hevein residues. Altogether 29 single-residue substitutions of hevein were engineered and tested in ELISA-inhibition with the sera of NRL-allergic patients. At least in the case of lysozyme and cutinase, the mutation of surface residues resulted in only minor changes in the overall protein structure (Matthews 1995, Petersen et al. 1998). Substituted residues usually adapt easily

to variable environments due to their biophysical properties, and water molecules are reported to fill the extra space when a large residue is substituted by a smaller one (De Filippis et al. 1994). In reverse, improper substitution, such as S112P in a cherry allergen (Pru av 1), may bring about a dramatic decrease in the allergenicity that it is likely to be a consequence of the disrupted native tertiary structure (Neudecker et al. 2003). As expected, 66% of the single hevein mutants showed only moderately (less than 10%) reduced allergenicity. Fortunately, none of the produced single mutants precipitated or showed signs of misfolding upon measuring molecular masses.

Structural studies have shown that Fab fragments cover an irregular but flat area on an antigen in the company of 15-22 residues, of which the key residues (5-6 aa) are responsible for stringent binding (Laver et al. 1990). In our studies, six of the substitutions (R5A, K10A, E29A, Y30F, H35A and Q38V) showed the most pronounced decrease in inhibition assays (>25%), and they were studied in more detail with five selected patients who showed variable recognition patterns. The largest reductions (up to 60%) in inhibition were seen with the single mutant R5A, but substitutions K10A and E29A also reduced IgE-binding by 40% and 25%, respectively, in some patients. In contrast, Y30F, H35A and Q38V showed smaller changes in IgE-binding (10-20%) in these five patients.

When the 3D-structure of hevein was compared with all other resolved structures of HLDs (UDA, WGA, AMP), positively charged residue R5 seemed to be specific to latex hevein. It protrudes from the N terminus of hevein and occupies a large area on its surface. In spite of variable research methods and proteins, several authors have repeatedly concluded that hydrophobic amino acids followed by polar and (positively) charged residues are both typical of and important for IgE-epitopes (He et al. 2002, Mine & Rupa 2003) .

The issue of the T-cell epitopes of hevein has only recently been raised in the literature. Poor immunogenicity and stimulation activity by peripheral blood mononuclear cells from 27 HCWs suggested that there are no T-cell epitopes in the hevein sequence (Raulf-Heimsoth et al. 2004). Conversely, short-term oligoclonal T-cell lines derived from 10 NRL-allergic patients located the epitopes mostly in the core region of hevein, which contains residues 10-29 and 19-38, depending on the different T-cell clones used (de Silva et al. 2004). With hindsight, it can be concluded that the design and production of single site-specific mutants was suggessful. Only six point-mutations caused a high reduction in IgE-binding to the molecules, while the possible T-cell epitope sequences were minimally disturbed.

6.4 Structural epitopes (I, II)

The debate about the role and significance of continuous and discontinuous epitopes appears to be gradually resolving in form of the view according to

which IgE-binding epitopes are discontinuous and conformational. The first glues of structural epitopes were discovered already in 1985, when some of the structures of antigen-antibody complexes between hen egg-white lysozyme and monoclonal antibodies (IgG) were resolved for the first time, using X-ray crystallography (Amit et al. 1985). In further crystallographic studies, monoclonal antibodies and mutants of lysozyme confirmed that only from two to five specific residues of the epitope areas (containing altogether about 20 aa) maintain the critical interactions with different monoclonal antibodies (Dall'Acqua et al. 1996, Dall'Acqua et al. 1998, Li et al. 2000).

Pioneering studies on allergen epitopes have been performed with Bet v 1 from birch pollen. After the identification of linear T-cell epitopes of Bet v 1 (Ebner et al. 1993, Ebner et al. 1995), its 3D-structure and three discontinuous B-cell epitope areas interacting with mouse monoclonal IgG1 Fab-fragments were resolved (Gajhede et al. 1996, Mirza et al. 2000). Peptide mapping and the phage-displayed mimotopes have indicated (by implication) the absense of linear IgE-binding epitopes (Lebecque et al. 1997, Jensen-Jarolim et al. 1998). On the basis of multiple sequence analyses of natural hypoallergenic protein variants (homologous pollen proteins and isoforms of Bet v 1), site-directed mutagenesis was used to located the three structural IgE-binding regions. All six single-point mutants made in Bet v 1 displayed full IgE-binding activity in more than one allergic patient, suggesting that the single substitutions caused minimal perturbations to the overall folding and structural characteristics of the protein. The combination of these six mutations, however, led to significantly reduced IgE-binding activity although 8/9 of the specific T-cell clones were able to proliferate (Ferreira et al. 1998).

The present findings in studies I and II clearly support the idea that the IgE-epitopes on hevein are conformational. The AMP-chimera containing both N terminus and C terminus (AMPN+C) of hevein strongly inhibited IgE-binding to hevein, whereas the corresponding linear peptides were totally unreactive. Detailed site-specific scanning revealed no linear stretches, but six critical residues with the strongest binding ability to IgEs were identified. The discontinuous epitope areas of hevein may contain more amino acid residues than the six so far identified, but they probably have moreof a supporting than major role.

With regard to linear B-cell epitopes of hevein, Banerjee et al. (1997) identified continuous IgE-binding epitopes with short linear peptides to amino acids 19-24 and 25-37, and essentially similar results were reported by Beezhold et al. (1997) (aa 13-24 and 29-36). However, our studies on peptides in the hevein core showed contrary results. A synthetic peptide from the core region of hevein did not bind any IgE from NRL-allergic patients, and was thus unable to compete with native hevein. In addition, the use of linear synthetic peptides come in for considerable criticism because of the uncontrollable, nonspecific binding of charged peptides used at high concentrations as well as usage of denatured assay environments (SDS-PAGE and immunoblotting) (Aalberse 2000).

Recently, the crystallographic structure of hevein was defined (resolution 1.5 Å), and the importance of the central parts for antibody recognition once again highlighted (Reyes-Lopez et al. 2004). The conserved carbohydrate binding tryptophans (W21 and W23) of hevein were oxidized with BNPS-skatole (3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole), and a serum pool obtained from ten NRL-allergic children showed on average only 20% inhibition of IgE-binding. It was suggested, therefore, that the tryptophans constitute a conformational epitope of hevein. Mutations W21F and W23Y were performed, but only 17% and 10% of the patients had reduced IgE-binding ability with these mutants. Different results may be explained by reference to differences in patient cohorts and in the techniques used. A summary of the studies on hevein B-cell epitopes and T-cell epitopes are shown in Fig. 8.

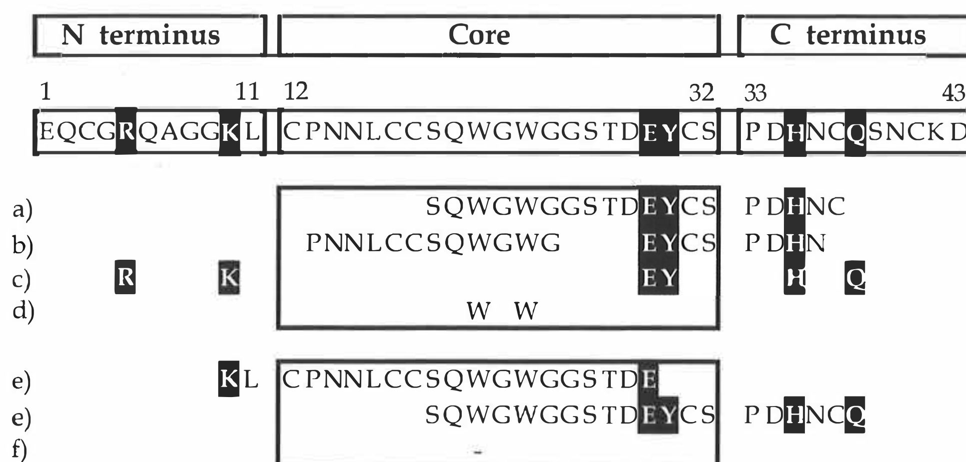


FIGURE 8 The B-cell epitopes of hevein (a-d) were reported by (a) Banerjee et al. (1997, aa 19-24, 25-37), (b) Beezhold et al. (1997, aa 13-24, 29-36), (c) Karisola et al. (2004, aa 5, 10, 29, 30, 35, 38), and (d) Reyes-Lopez et al. (2004, aa 21, 23). The T-cell epitopes of hevein (e-f) were reported by (e) de Silva et al. (2004, aa 10-29, 19-38) and (f) Raulf-Heimsoth et al. (2004, no epitopes).

6.5 Hevein-like domains as targets for allergic cross-reactions (III)

Cross-responses have been recognized for some decades now in allergic patients, but ideas as to their underlying causes have only recently been mooted. It has been proposed that identical conformational epitopes in proteins from unrelated plant species "fool" allergen-specific IgEs to cross-react with other similar proteins and induce allergic symptoms in patients (Aalberse et al. 2001b). The most common presentation of cross-reactivity is the so-called "pollen-food allergy syndrome", known also as oral allergy syndrome (OAS),

where fresh apple, hazelnuts, cherry, celery or carrot cause symptoms in up to 70% of patients allergic to birch pollen (Neudecker et al. 2001, Bohle et al. 2003). Up to 95% of birch allergic patients recognize the major allergen, Bet v 1, whose conserved α/β-structure with a steroid-binding cavity is found in virtually identical proteins occurring in many fruits and plants, such as the apple (Mal d 2), cherry (Pru av 1) and celery (Api g 1) (Krebitz et al. 2003, Neudecker et al. 2003).

Also ~50% of NRL-allergic patients have latex-fruit syndrome characterized by allergic symptoms after eating avocado, banana, chestnut, kiwi or papaya. The N-terminal hevein-like domains (HLDs) in the 31-kDa plant endochitinases are thought to be the key structures underlying these cross-reactions detected by immunoblots or by SPTs. The Hev b 6.02 recognized by 70% of NRL-allergic patients shows a highly similar primary and tertiary structure to that found in other plant HLDs studied. To our knowledge the present (III) investigation is the first to study IgE-binding to isolated HLDs and corresponding 31 kDa endochitinases directly, using SPTs and ELISA tests.

IgE antibodies with specificity to hevein in sera from NRL-allergic patients showed surprisingly strong reactions to isolated HLDs from avocado and banana in SPTs and in ELISA tests, whereas whole 31-kDa endochitinases were recognized only weakly. In SPTs 73% of patients (13/15) showed positive responses to isolated HLDs, whereas only one patient reacted against whole proteins from avocado and banana. Studies *in vitro* with the same proteins revealed that the binding of IgE to hevein was efficiently inhibited by HLDs of avocado and banana 59% and 39%, respectively, confirming the importance of small-sized HLDs in these cross-reactions. Moreover, IgEs in sera from NRL patients had weak binding efficiency to entire 31-kDa endochitinase molecules of avocado and banana, since inhibitions with the endochitinases were only 17% and 20%, respectively. These results immediately raised the question: why HLDs are superior in their reactivity as compared to intact endochitinases? It is known that many strong allergens can be stable and small in size but these properties cannot be directly linked to allergenicity. In all likelihood the conserved HLDs carry out antifungal functions in plants, but so far only a few of them have been reported as giving rise to allergic reactions.

The present results strongly suggest that HLDs induce allergic symptoms mostly as separate molecules. The large, unconserved C-terminal domain in the whole endochitinase (31 kDa) may hide the conformational epitopes of the N-terminal HLDs and thereby sterically hinder their interactions with IgEs. According to current knowledge, HLD-containing endochitinases are processed stepwise, which separates the C-terminal domain and HLDs during ripening of the fruits. Therefore all material containing HLD proteins should in theory cause allergic symptoms. However, this is not the case and the reason why only some of the endochitinases evoke allergenic symptoms despite sharing highly similar primary and tertiary sequences of HLDs remains unknown.

No studies dealing with allergen properties in the different domains of a protein have been reported. Therefore, this issue was approached through

wheat germ agglutinin. WGA is composed of four intermolecularly connected HLDs in one molecule which are not cleaved to separate HLDs in nature. Individual HLDs of WGA were produced and tested, and the results were compared to whole WGA. Unexpectedly, only two of the four HLDs had slight IgE-binding ability, and the other HLDs as well as the whole WGA were virtually unable to bind IgE. The results of the inhibition experiments supported the contention that latex hevein was the primary sensitizer. When the IgE-binding amino acid residues of hevein (on the epitope area) were compared to amino acid residues on the HLDs of WGA, several substitutions were identified. This indicates that amino acid residue substitutions, in addition to domain constellation, affect the affinity of IgE to an allergen.

Aalberse and coworkers (2001a) have emphasized the importance of antibody affinity and valency in the antigen-antibody interaction. They showed that if the dose required for 50% inhibition by the cross-reacting allergen in ELISA is less than five times the dose required for the primary allergen, the cross-reactivity should be classified as high affinity. On the other hand, if the relative efficiency in the inhibition test is more than 25-fold lower, the cross-reactivity should be classified as low affinity. When our results are compared with this definition, HLDs from avocado, banana and latex cause high affinity cross-reactions, whereas corresponding intact endochitinases show minimal cross-reactivity presumably due to differences in their structural constellation. In accordance with earlier reports (Diaz-Perales et al. 2002, Wagner et al. 2002), it can be concluded that patients with latex-fruit syndrome are primarily sensitized to latex and have later started to experience symptoms from avocado and banana. Reactions against WGA are, however, rare.

At the moment, SPTs are almost exclusively performed with natural extracts of allergenic material and the same types of reagents are also used in immunotherapy for allergic diseases. Such extracts are heterogenous complexes of both allergenic and non-allergenic proteins and polypeptides whose relative and absolute amounts are unknown, thereby representing a substantial risk for anaphylactic reactions (Turjanmaa et al. 1997, Hamilton & Adkinson 2003, Sastre et al. 2003). The recent progress in molecular allergology and biochip technology may allow the use of allergen microarrays in the diagnosis of allergies and in identification of patient-specific IgE-reactivity profiles (Hiller et al. 2002, Harwanegg et al. 2003, Deinhofer et al. 2004). Detailed knowledge of allergens at the molecular level is essential to enhance the specificity and accuracy of SPTs and materials used in immunotherapy. It should be kept in mind also that individuals can become sensitized to proteins that are processed and altered during manufacturing, cooking or in gastrointestinal digestion. Therefore, optimal testing for a suspected allergy should eventually utilize purified allergens or materials that are comparably processed.

6.6 Production of hypoallergenic molecules for immunotherapy (II)

It has been suggested that so-called hypoallergens are unable to cross-link IgEs on the surface of mast cells, but instead they are processed by APCs, and their (mostly) intact T-cell epitopes are possibly presented to Tr-cells that favour the production of IgGs instead of anaphylactic IgE in plasma cells (Nouri-Aria et al. 2004, Westritschnig et al. 2004). Different approaches to the production of hypoallergenic molecules have also been tested with Bet v 1, the forerunner in these studies. First, it was expressed as two fragments (residues 1-74 and 75-160) that exhibited random coil structure and minimal allergenicity (Vrtala et al. 1997, Vrtala et al. 2000). Later these fragments proved to be suitable also for induction of mucosal tolerance (Wiedermann et al. 2001), but even shorter fragments (residues 9-22 and 104-123) were subsequently produced and tested. Naturally, their IgE-binding ability was again abolished, but due to the resolution of their epitopes, the T-cell response was retained and they induced the production of blocking IgG-class antibodies (Ganglberger et al. 2000). In a structure-based design of the Bet v 1 hypoallergen a single point mutation (E45S) was introduced to the dominant epitope region. This caused a reduction of up to 50% in the binding of human IgEs while retaining its structure (Spangfort et al. 2003). The authors estimated that about 10% of the surface on Bet v 1 is included in the IgE-binding epitopes, and suggested that several mutations are probably needed to achieve a molecule with substantially reduced anaphylactic potential in a larger patient population.

The combinatory hevein mutants (HΔ3A, HΔ3B HΔ6) contained either three or six of the most important substitutions that caused the largest reductions in IgE-binding in the epitope scanning experiments. For HΔ3A, the surface charge distribution was tested by modeling, as its substitutions R5A, E29A and H35A removed wide charged areas on hevein. The mutation R5A destroyed the main interactions between positively charge arginine, polar glutamines (Q6, Q20, Q38) and asparagine (N36) in that region. Although the interactions in this mutant were slightly modified, the inhibitions were highly reduced only in two of the patients. Similar results were obtained with the mutant HΔ3B in which only K10A interfered the main chain hydrogen bonding between K10 and Q2. The greatest changes in charge distribution and surface topology were already seen in modeling with mutant HΔ6, and its inhibition capacity was highly reduced with four of the five patients. The inhibition in the last patient was also reduced by ~30%.

In the present study, the inhibition pattern of one patient (patient 26) deviated clearly from that of all the others. The chimera HΔ3A inhibited the binding of his IgEs as perfectly as native hevein, whereas mutants HΔ3B and HΔ6 inhibited only 45-50%. This clearly shows the individual nature of different patients' epitopes, which depend on the antigens to which the person has been sensitized during his or her life. The variation in SPTs was, however, less

noticeable. With one exception, all patients gave positive SPT responses to both HΔ3A and HΔ3B mutants, and all of them showed negative response to the mutant HΔ6. It has been suggested that upon prolonged immunization, patients enhance their IgE-binding affinity by antibody maturation and eventually all patients will recognize substantially the same epitopes irrespective of the sensitization route, allergen amount or geographical distribution (Pierson-Mullany et al. 2000, Jackola et al. 2002).

It is obviously difficult to design a hypoallergen by mutating only a few amino acid residues on the surface of the allergen, even for selected patients. In this study, six was the minimum number of substitutions that led to reduced IgE-binding to hevein in all of the studied NRL-allergic patients. Taking advantage of the known 3D-structure and using site-directed mutagenesis, the IgE-binding epitopes of the major mite allergen, Der f 2 (129 aa), were identified to contain ten, mostly charged or hydrophobic amino acid residues (D7, A9, N10, K15, D19, N71, A72, M76, K77 and A120) (Nishiyama et al. 1995, Ichikawa et al. 1998). In later studies, Takai and coworkers (2001) mutated two lysines (K15 and K77) at the epitope hot spots but only a slight reduction was noted in IgE-binding and in basophil stimulation. The authors also concluded that more concurrent substitutions are needed to destroy a sufficient number of epitopes to lead, eventually, to significant changes in reactivity in a large cohort of patients.

Instead of deleting or destroying the B-cell epitopes, increased immunogenicity and reduced allergenicity may be reached by a reverse method in which allergens are covalently combined. Murine studies with Bet v 1 showed that two fragments (1-74 and 75-160 aa) as well as a trimeric form of native-like Bet v 1 protein display reduced allergenicity and ability to induce IgG1 type antibodies (Mahler et al. 2004). The trimeric Bet v 1 retained essentially the same secondary structures as the monomeric Bet v 1 as well as intact B-cell and T-cell epitopes, but its IgE-reactivity was substantially reduced (Vrtala et al. 2001). The reasons for the hypoallergenic nature of the trimeric Bet v 1 have not been elucidated, but steric hindrance of the IgE-binding sites seems a likely explanation. A somewhat similar approach to inhibit allergenicity can be used with two or more different allergens, as shown by dimers containing the group 5 and 6 allergens of *Phaleum pratense* (Linhart et al. 2002). Furthermore, DNA vaccination has also been tested with the two Bet v 1 fragments and a combinatory Bet v 1 mutant containing six substitutions. They prevented the production of antigen-specific IgG2a and IgE and induced efficient production of IgG1-type antibodies (Hochreiter et al. 2003).

6.7 Future prospects

The availability of hypoallergenic variants for the major allergens would be of great help both in reducing exposure to allergens and in the design of safer

forms of immunotherapy. With regard to NRL allergy, a theoretical possibility could be the engineering of transgenic rubber trees that produce hypoallergenic latex (hevein). However, for several reasons, this approach does not appear realistic. As is known, any protein may operate as an allergen (Taylor & Hefle 2001); hence in contrast to reducing the number of allergies, the consequence could be the birth of new allergens and increased numbers of allergic patients. In fact, genes coding HLDs have already been transferred to tobacco and tomato to increase resistance to pathogenic fungi (Koo et al. 2002, Lee et al. 2003), but given the documented allergenic potential of HLDs, this kind of approach may carry considerable risks.

Animal models are important tools for the preclinical evaluation of new approaches to prophylaxis and therapy against type I allergic diseases. In order to illuminate the mechanisms involved and to evaluate the impact of environmental stimuli, time and genetic predisposition, various models have been proposed. An ideal murine model for human type I allergic disease should closely resemble the characteristic features of the human disease. It should include the production of high amounts of antigen-specific immunoglobulins, development of immediate-type hypersensitivity with early and late phase responses, and pertinent immunopathologic changes in the target organs. Thus far, murine models have been developed for allergies to birch and grass pollen, house-dust mite, latex, cat, and bee/wasp venom (Herz et al. 2004).

At least in mouse models, immunotherapy seems to be a potential technique to direct the Th1/Th2 balance towards Th1 dominance. Injection of modified major allergens into sensitized animals is not expected to provoke immediate allergic responses, but T-cell-mediated responses are activated and IgG class antibodies will be produced instead of IgE. However, site-directed mutagenesis eliminating selected IgE-binding epitopes is unlikely to offer substantial help in this regard. We are dealing with hundreds of different allergen structures and, more importantly, with an unknown but certainly much larger number of highly patient-specific IgE-binding epitopes. Therefore, from a practical point of view, site-directed mutagenesis will not be the method of choice for the development of allergy vaccines, although it is useful in theoretical studies and in occasional specific cases. Covalent linking of a few or multiple allergens on one hand and, and DNA-vaccination on the other hand, are expected to be valuable tools in future studies. It is too early as yet, however, to evaluate their eventual impact on allergy treatments. Irrespective of the currently still unresolved questions, the future immunotherapy molecule should be safe, easy to standardize, and cheap to produce. It should help large numbers of allergic individuals without the risk of anaphylaxis.

In addition to symptomatic treatments, one may use immunomodulation as an option in the fight against allergic diseases. It should be possible to vaccinate animals, and possibly also humans, against the development of allergic disorders (Horner et al. 2001, Horner et al. 2002). These procedures might protect atopy-prone neonates or young children from developing allergen-specific Th2-type immune responses. However, before these

techniques have moved from bench to bedside, several more years of research are needed. Owing to the complex nature of atopic disorders, it is unreasonable to expect to see immunomodulation procedures as magic weapons capable of totally inhibiting the development of or curing allergic disorders. However, this form of therapy might result in a significant reduction in the severity of the disease.

7 CONCLUSIONS

The studies on hevein and latex allergy presented in this dissertation are part of a larger project set up to further our understanding of the molecular basis of the reaction between an antigen (allergen) and antibody (IgE) in NRL allergies and the factors affecting the development of allergy. Furthermore, the knowledge obtained by modifying structural epitopes of major allergens can be used to create new tools for the prophylaxis, diagnosis and cure of allergies. The main conclusions of this study are:

1. A novel chimera-based technique in which part of the allergen molecule is combined with an analogous but non-allergenic protein produces gain-in-function proteins that have the IgE-binding regions of an allergen. This technique may also be used in locating of other functional sites in proteins.

2. The IgE-binding epitopes of hevein (Hev b 6.02) are highly conformational and the most important IgE-binding amino acid residues are located at its N-terminal region.

3. Six of the most important amino acids (R5, K10, E29, Y30, H35 and Q38) on the hevein epitope are scattered over the whole primary sequence of hevein.

4. Co-mutation of these six single amino acid substitutions produced hevein hypoallergens showing minimal allergenicity due to the destruction of their IgE-epitopes. These molecules are potential immunotherapy tools in the treatments of NRL allergy.

5. Stable hevein and homologous hevein-like domains (HLDs) at the N terminus of plant endochitinases (31 kDa) are the cross-reacting agents in latex-fruit syndrome. In addition to its structural constellation, the amount of substituted amino acid residues on the conformational epitope defines whether its IgE-binding affinity is strong enough to cause cross-reactions.

6. Patients suffering from latex-fruit syndrome seem to be sensitized primarily to latex hevein and be susceptible to developing cross-reactions to avocado and banana but rarely to WGA.

7. Hevein and NRL allergy can serve as a model pair in the study of the molecular basics of allergies and antigen-antibody interactions (epitopes).

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YHTEENVETO (Résumé in Finnish)

Luonnonkumiallergian pääallergeenin, heveiinin (Hev b 6.02), immunologisten ominaisuuksien karakterisointi ja muokkaus

Teollistuneiden maiden väestöstä yli 25% kärsii välittömän tyypin I allergiasta, jossa elimistön IgE-vasta-aineet tunnistavat tavallisesti harmittoman antigeenin (eli allergeenin). Allergioiden yleistymistä on selitetty "hygieniahypoteesilla", minkä mukaan lapsen luonnollinen altistus erilaisille mikrobeille ja tulehduksille suojaa myöhemmin allergioilta ja astmalta. Perinnöllinen atopia tai toistuva kosketus allergeenien kanssa voi kehittyä yliherkkyysreaktioksi ruokaa ja/tai ympäristön antigeeneja kohtaan. Allergian oireet on kartoitettu laajalti, mutta sairauden perimmäiset syyt ja molekyylitason mekanismit ovat intensiivisen tutkimuksen ja keskustelun kohteena.

Tyypin I allergioiden diagnosointi ja hoito perustuvat pääasiassa seerumissa ja muissa elimistön nesteissä olevan immunoglobuliini-E:n (IgE) mittaamiseen ja vähentämiseen. Yhä edelleen allergiatautien paras hoitomenetelmä on allergeenien välttäminen ja kortikosteroidien sekä antihistamiinien käyttö oireiden lievittämisessä. Perinteinen immunoterapia, jossa elimistöä siedätetään alati kasvavalla allergeenimäärällä, edustaa yhtä allergioiden harvoista hoitokeinoista. Sen haittapuolena ovat kuitenkin vakavat sivuvaikutukset, jopa anafylaktinen shokki. Entistä tehokkaampia ja tarkoituksenmukaisempia hoitokeinoja voidaan kehittää allergioihin, mutta ensin on kartoitettava immunologian perusmekanismit ja allergeenimolekyylien ominaisuudet.

Viimeisten 15 vuoden aikana erityisesti työterveysongelmana tunnettua luonnonkumiallergiaa (engl. natural rubber latex allergy, NRL) käytettiin tässä tutkimuksessa mallina, joka edustaa tyyppi I:n allergioita. Se aiheuttaa useita IgE-välitteisiä kliinisiä sairauksia, kuten astmaa, allergista nuhaa, nokkosrokkoa ja anafylaksiaa. Nykyään kumipuun lateksista on tunnistettu yli 200 valkuaisainetta, joista ainakin 13 toimii allergioihin osallistuvana antigeeninä (Hev b 1 – Hev b 13). Tässä työssä keskityttiin heveiiniin (Hev b 6.02), jota vastaan 70% luonnonkumiallergisista potilaista on IgE-luokan vasta-aineita. Tämä hyvin pienikokoinen (4.7 kDa, 43 aminohappotähdettä) molekyyli on edustava malliproteiini, sillä se on äärimmäisen kestävä ja sen kolmiulotteinen rakenne on selvitetty.

Ennen kuin tyypin I allergioiden immuunivasteen molekyylitason tapahtumia voidaan ymmärtää, on välttämätöntä tuntea allergeenin pinnalla olevat rakenteelliset tunnistus- ja sitoutumiskohdat eli epitoopit. Yhdistämällä mallitukseen perustuva suunnittelu ja proteiinien muokkausmenetelmät, heveiinin rakenteelliset epitooppealueet paikannettiin. Heveiinin todennäköisiä IgE:n sitomiskohtia siirrettiin rakenteellisesti ja sekventiaalisesti hyvin samankaltaiselle, mutta ei IgE:tä sitovalle vastaanottajaproteiinille, AMP:lle (engl. antimicrobial protein) (I). Muodostettujen kimera-proteiinien avulla pystyttiin selvittämään IgE-epitooppialueet, mutta IgE:n kanssa vuorovaikuttavia amino-

happotähteitä ei vielä voitu määrittää. Rakenteellinen B-soluepitooppi koostuu yleensä 15-22 aminohappotähteestä, joista 5-10 ovat tärkeimpiä ja muodostavat tiukimmat sidokset IgE-molekyylin kanssa. Näiden aminohappojen löytämiseksi teimme evoluutioon perustuvia paikkaspesifisiä pistemutaatioita (29 kpl) koko heveiinin sekvenssin alueelle (II). Lopulta yhdistimme kolme tai kuusi potentiaalisinta mutaatiota samaan molekyyliin, ja näiden "hypoallergeenisten" heveiinien kliininen merkitys tutkittiin sekä NRL allergisten potilaiden seerumilla *in vitro* että ihopistokokeilla *in vivo* (II).

Luonnonkumiallergisilla potilailla on usein lateksi-hedelmä -oireyhtymä, missä he saavat välittömiä allergiaoireita tuoreista hedelmistä, kuten avokadosta ja banaanista. Potilaiden IgE:n tiedetään ristiinreagoivan kasvien endokitinaasi-proteiinien kanssa, joiden N-terminaalisessa päässä on heveiinin kaltainen domaini (engl. **h**evein-**l**ike **d**omain, HLD). Hyvin konservoitunut, yksittäinen tai muutamasta HLD:stä koostuva endokitinaasi löytyy useimmista kasvilajeista, mutta suurempien C-terminaalisten alueiden sekvenssi ja toiminta näyttävät vaihtelevan enemmän. Väitöskirjan kolmannessa osatyössä (III) tutkittiin, aiheuttavatko kokonaiset endokitinaasit vastaavanlaisia allergisia reaktioita *in vivo* kuin eristetyt HLD:t yksinään ja onko HLD:ien erilainen ryhmittely syy mahdollisiin immunologisiin eroihin.

Lopullisena tarkoituksena on kehittää täsmähoitomenetelmä NRL allergian hoitoon. Tämän työn osatöiden (I-III) perusteella on jo suunniteltu ja tuotettu sarja DNA-rokotteita, joiden tehoa tullaan arvioimaan ja testaamaan lateksiallergiaa varten kehitetyssä hiirimallissa.

REFERENCES

Aalberse, R. C. 1998. Clinical relevance of carbohydrate allergen epitopes. Allergy 53 (45 Suppl), 54-57.

Aalberse, R. C. 2000. Structural biology of allergens. J. Allergy Clin. Immunol. 106, 228-238.

Aalberse, R. C., Akkerdaas, J. & Van Ree, R. 2001a. Cross-reactivity of IgE antibodies to allergens. Allergy 56, 478-490.

Aalberse, R. C., Kleine Budde, I., Stapel, S. O. & Van Ree, R. 2001b. Structural aspects of cross-reactivity and its relation to antibody affinity. Allergy 56 (Suppl. 67), 27-29.

Airenne, K. J., Laitinen, O. H., Alenius, H., Mikkola, J., Kalkkinen, N., Arif, S. A., Yeang, H. Y., Palosuo, T. & Kulomaa, M. S. 1999. Avidin is a promising tag for fusion proteins produced in baculovirus-infected insect cells. Protein Expr. Purif. 17, 139-145.

Akasawa, A., Hsieh, L. S., Martin, B. M., Liu, T. & Lin, Y. 1996. A novel acidic allergen, Hev b 5, in latex. Purification, cloning and characterization. J. Biol. Chem. 271, 25389-25393.

Alenius, H., Kalkkinen, N., Lukka, M., Reunala, T., Turjanmaa, K., Mäkinen-Kiljunen, S., Yip, E. & Palosuo, T. 1995. Prohevein from the rubber tree (Hevea brasiliensis) is a major latex allergen. Clin. Exp. Allergy 25, 659-665.

Alenius, H., Kalkkinen, N., Reunala, T., Turjanmaa, K. & Palosuo, T. 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, 1618-1625.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Amit, A. G., Mariuzza, R. A., Phillips, S. E. & Poljak, R. J. 1985. Three-dimensional structure of an antigen-antibody complex at 6 A resolution. Nature 313, 156-158.

Amit, A. G., Mariuzza, R. A., Phillips, S. E. & Poljak, R. J. 1986. Three-dimensional structure of an antigen-antibody complex at 2.8 Å resolution. Science 233, 747-753.

Andersen, N. H., Cao, B., Rodriguez-Romero, A. & Arreguin, B. 1993. Hevein: NMR assignment and assessment of solution-state folding for the agglutinin-toxin motif. Biochemistry 32, 1407-1422.

Anderton, S. M., Viner, N. J., Matharu, P., Lowrey, P. A. & Wraith, D. C. 2002. Influence of a dominant cryptic epitope on autoimmune T cell tolerance. Nat. Immunol. 3, 175-181.

Archer, B. L. 1960. The proteins of Hevea brasiliensis Latex. 4. Isolation and characterization of crystalline hevein. Biochem J. 75, 236-240.

Arif, S. A., Hamilton, R. G., Yusof, F., Chew, N. P., Loke, Y. H., Nimkar, S., Beintema, J. J. & Yeang, H. Y. 2004. Isolation and characterization of the

early nodule-specific protein homologue (Hev b 13), an allergenic lipolytic esterase from Hevea brasiliensis latex. J. Biol. Chem. 279, 23933-23941.

Asensio, J. L., Canada, F. J., Bruix, M., Rodriguez-Romero, A. & Jimenez-Barbero, J. 1995. The interaction of hevein with N-acetylglucosamine-containing oligosaccharides. Solution structure of hevein complexed to chitobiose. Eur. J. Biochem. 230, 621-633.

Asensio, J. L., Canada, F. J., Siebert, H. C., Laynez, J., Poveda, A., Nieto, P. M., Soedjanaamadja, U. M., Gabius, H. J. & Jimenez-Barbero, J. 2000. Structural basis for chitin recognition by defense proteins: GlcNAc residues are bound in a multivalent fashion by extended binding sites in hevein domains. Chem. Biol. 7, 529-543.

Axelsson, I. G., Eriksson, M. & Wrangsjö, K. 1988. Anaphylaxis and angioedema due to rubber allergy in children. Acta Paediatr Scand 77, 314-316.

Bacharier, L. B. & Geha, R. S. 2000. Molecular mechanisms of IgE regulation. J. Allergy Clin. Immunol. 105, S547-558.

Banerjee, B., Wang, X., Kelly, K. J., Fink, J. N., Sussman, G. L. & Kurup, V. P. 1997. IgE from latex-allergic patients binds to cloned and expressed B cell epitopes of prohevein. J. Immunol. 159, 5724-5732.

Baur, X. & Jäger, D. 1990. Airborne antigens from latex gloves. Lancet 335, 912.

Baur, X., Chen, Z., Rozynek, P., Duser, M. & Raulf-Heimsoth, M. 1995. Cross-reacting IgE antibodies recognizing latex allergens, including Hev b 1, as well as papain. Allergy 50, 604-609.

Beezhold, D. H., Sussman, G. L., Liss, G. M. & Chang, N. S. 1996. Latex allergy can induce clinical reactions to specific foods. Clin. Exp. Allergy 26, 416-422.

Beezhold, D. H., Kostyal, D. A. & Sussman, G. L. 1997. IgE epitope analysis of the hevein preprotein; a major latex allergen. Clin. Exp. Immunol. 108, 114-121.

Beezhold, D. H., Hickey, V. L. & Sussman, G. L. 2001. Mutational analysis of the IgE epitopes in the latex allergen Hev b 5. J. Allergy Clin. Immunol. 107, 1069-1076.

Bellinghausen, I., Klostermann, B., Knop, J. & Saloga, J. 2003. Human CD4+CD25+ T cells derived from the majority of atopic donors are able to suppress TH1 and TH2 cytokine production. J. Allergy Clin. Immunol. 111, 862-868.

Bian, H., Reidhaar-Olson, J. F. & Hammer, J. 2003. The use of bioinformatics for identifying class II-restricted T-cell epitopes. Methods 29, 299-309.

Black, P. 2001. Why is the prevalence of allergy and autoimmunity increasing? Trends Immunol. 22, 354-355.

Blanco, C. 2003. Latex-fruit syndrome. Curr. Allergy Asthma Rep. 3, 47-53.

Blanco, C., Diaz-Perales, A., Collada, C., Sanchez-Monge, R., Aragoncillo, C., Castillo, R., Ortega, N., Alvarez, M., Carrillo, T. & Salcedo, G. 1999. Class I chitinases as potential panallergens involved in the latex-fruit syndrome. J. Allergy Clin. Immunol. 103, 507-513.

Bohle, B., Wagner, B., Vollmann, U., Buck, D., Niggemann, B., Szepfalusi, Z., Fischer, G., Scheiner, O., Breiteneder, H. & Ebner, C. 2000. Characterization of T cell responses to Hev b 3, an allergen associated with latex allergy in spina bifida patients. J. Immunol. 164, 4393-4398.

Bohle, B., Radakovics, A., Jahn-Schmid, B., Hoffmann-Sommergruber, K., Fischer, G. F. & Ebner, C. 2003. Bet v 1, the major birch pollen allergen, initiates sensitization to Api g 1, the major allergen in celery: evidence at the T cell level. Eur. J. Immunol. 33, 3303-3310.

Bonura, A., Amoroso, S., Locorotondo, G., Di Felice, G., Tinghino, R., Geraci, D. & Colombo, P. 2001. Hypoallergenic variants of the Parietaria judaica major allergen Par j 1: a member of the non-specific lipid transfer protein plant family. Int. Arch. Allergy Immunol. 126, 32-40.

Breiteneder, H. & Ebner, C. 2000. Molecular and biochemical classification of plant-derived food allergens. J. Allergy Clin. Immunol. 106 (1 Pt 1), 27-36.

Broekaert, I., Lee, H. I., Kush, A., Chua, N. H. & Raikhel, N. 1990. Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (Hevea brasiliensis). Proc. Natl. Acad. Sci. U S A 87, 7633-7637.

Broekaert, W. F., Marien, W., Terras, F. R., De Bolle, M. F., Proost, P., Van Damme, J., Dillen, L., Claeys, M., Rees, S. B., Vanderleyden, J. & Et Al. 1992. Antimicrobial peptides from Amaranthus caudatus seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. Biochemistry 31, 4308-4314.

Brown, J. M., Leon, M. A. & Lightbody, J. J. 1976. Isolation of a human lymphocyte mitogen from wheat germ with N-acetyl-D-glucosamine specificity. J. Immunol. 117, 1976-1980.

Carrillo, T., Cuevas, M., Munoz, T., Hinojosa, M. & Moneo, I. 1986. Contact urticaria and rhinitis from latex surgical gloves. Contact Dermatitis 15, 69-72.

Cauerhff, A., Goldbaum, F. A. & Braden, B. C. 2004. Structural mechanism for affinity maturation of an anti-lysozyme antibody. Proc. Natl. Acad. Sci. U S A 101, 3539-3544.

Chang, T. W. 2000. The pharmacological basis of anti-IgE therapy. Nat. Biotechnol. 18, 157-162.

Chapman, M. D., Smith, A. M., Vailes, L. D., Arruda, L. K., Dhanaraj, V. & Pomes, A. 2000. Recombinant allergens for diagnosis and therapy of allergic disease. J. Allergy Clin. Immunol. 106, 409-418.

Chen, Z., Van Kampen, V., Raulf-Heimsoth, M. & Baur, X. 1996. Allergenic and antigenic determinants of latex allergen Hev b 1: peptide mapping of epitopes recognized by human, murine and rabbit antibodies. Clin. Exp. Allergy 26, 406-415.

Chen, Z., Posch, A., Lohaus, C., Raulf-Heimsoth, M., Meyer, H. E. & Baur, X. 1997a. Isolation and identification of hevein as a major IgE-binding polypeptide in Hevea latex. J. Allergy Clin. Immunol. 99, 402-409.

Chen, Z., Cremer, R., Posch, A., Raulf-Heimsoth, M., Rihs, H. P. & Baur, X. 1997b. 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, 684-693.

Coley, A. M., Campanale, N. V., Casey, J. L., Hodder, A. N., Crewther, P. E., Anders, R. F., Tilley, L. M. & Foley, M. 2001. Rapid and precise epitope mapping of monoclonal antibodies against Plasmodium falciparum AMA1 by combined phage display of fragments and random peptides. Protein Eng. 14, 691-698.

Colombo, G., Meli, M., Canada, J., Asensio, J. L. & Jimenez-Barbero, J. 2004. Toward the understanding of the structure and dynamics of protein-carbohydrate interactions: molecular dynamics studies of the complexes between hevein and oligosaccharidic ligands. Carbohydr. Res. 339, 985-994.

Condemi, J. J. 2002. Allergic reactions to natural rubber latex at home, to rubber products, and to cross-reacting foods. J. Allergy Clin. Immunol. 110 (2 Suppl), S107-110.

Coombs, R. R. A. & Gell, P. G. H. 1963. The classification of allergic reactions underlying disease. In: Gell, P. G. H. & Coombs, R. R. A. (eds), Clinical aspects of Immunology: 317-337. Blackwell Scientific Publications, Oxford.

Crameri, R. 2003. Correlating IgE reactivity with three-dimensional structure. Biochem. J. 376, e1-2.

Czuppon, A. B., Chen, Z., Rennert, S., Engelke, T., Meyer, H. E., Heber, M. & Baur, X. 1993. The rubber elongation factor of rubber trees (Hevea brasiliensis) is the major allergen in latex. J. Allergy Clin. Immunol. 92, 690-697.

Dall'acqua, W., Goldman, E. R., Eisenstein, E. & Mariuzza, R. A. 1996. A mutational analysis of the binding of two different proteins to the same antibody. Biochemistry 35, 9667-9676.

Dall'acqua, W., Goldman, E. R., Lin, W., Teng, C., Tsuchiya, D., Li, H., Ysern, X., Braden, B. C., Li, Y., Smith-Gill, S. J. & Mariuzza, R. A. 1998. A Mutational Analysis of Binding Interactions in an Antigen-Antibody Protein-Protein Complex. Biochemistry 37, 7981-7991.

Davies, J. M., O'hehir R, E. & Suphioglu, C. 2000. Use of phage display technology to investigate allergen-antibody interactions. J. Allergy Clin. Immunol. 105 (6 Pt 1), 1085-1092.

De Filippis, V., Sander, C. & Vriend, G. 1994. Predicting local structural changes that result from point mutations. Protein Eng. 7, 1203-1208.

de Silva, H. D., Sutherland, M. F., Suphioglu, C., Mclellan, S. C., Slater, J. E., Rolland, J. M. & O'hehir R, E. 2000. Human T-cell epitopes of the latex allergen Hev b 5 in health care workers. J. Allergy Clin. Immunol. 105, 1017-1024.

de Silva, H. D., Gardner, L. M., Drew, A. C., Beezhold, D. H., Rolland, J. M. & O'hehir, R. E. 2004. The hevein domain of the major latex-glove allergen

Hev b 6.01 contains dominant T cell reactive sites. Clin. Exp. Allergy 34, 611-618.

Deinhofer, K., Sevcik, H., Balic, N., Harwanegg, C., Hiller, R., Rumpold, H., Mueller, M. W. & Spitzauer, S. 2004. Microarrayed allergens for IgE profiling. Methods 32, 249-254.

del Val, G., Yee, B. C., Lozano, R. M., Buchanan, B. B., Ermel, R. W., Lee, Y. M. & Frick, O. L. 1999. Thioredoxin treatment increases digestibility and lowers allergenicity of milk. J. Allergy Clin. Immunol. 103, 690-697.

Dennis, M. S. & Light, D. R. 1989. Rubber elongation factor from Hevea brasiliensis. Identification, characterization, and role in rubber biosynthesis. J. Biol. Chem. 264, 18608-18617.

Diaz-Perales, A., Collada, C., Blanco, C., Sanchez-Monge, R., Carrillo, T., Aragoncillo, C. & Salcedo, G. 1998. Class I chitinases with hevein-like domain, but not class II enzymes, are relevant chestnut and avocado allergens. J. Allergy Clin. Immunol. 102, 127-133.

Diaz-Perales, A., Collada, C., Blanco, C., Sanchez-Monge, R., Carrillo, T., Aragoncillo, C. & Salcedo, G. 1999. Cross-reactions in the latex-fruit syndrome: A relevant role of chitinases but not of complex asparagine-linked glycans. J. Allergy Clin. Immunol. 104, 681-687.

Diaz-Perales, A., Sanchez-Monge, R., Blanco, C., Lombardero, M., Carillo, T. & Salcedo, G. 2002. What is the role of the hevein-like domain of fruit class I chitinases in their allergenic capacity? Clin. Exp. Allergy 32, 448-454.

Díez-Gómez, M. L., Quirce, S., Cuevas, M., Sanchez-Fernandez, C., Baz, G., Moradiellos, F. J. & Martinez, A. 1999. Fruit-pollen-latex cross-reactivity: implication of profilin (Bet v 2). Allergy 54, 951-961.

Dombrowicz, D., Flamand, V., Brigman, K. K., Koller, B. H. & Kinet, J. P. 1993. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor alpha chain gene. Cell 75, 969-976.

Drenth, J., Low, B. W., Richardson, J. S. & Wright, C. S. 1980. The toxin-agglutinin fold. A new group of small protein structures organized around a four-disulfide core. J. Biol. Chem. 255, 2652-2655.

Durham, S. R. & Till, S. J. 1998. Immunologic changes associated with allergen immunotherapy. J. Allergy Clin. Immunol. 102, 157-164.

Ebner, C., Szepfalusi, Z., Ferreira, F., Jilek, A., Valenta, R., Parronchi, P., Maggi, E., Romagnani, S., Scheiner, O. & Kraft, D. 1993. Identification of multiple T cell epitopes on Bet v I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. J. Immunol. 150, 1047-1054.

Ebner, C., Hirschwehr, R., Bauer, L., Breiteneder, H., Valenta, R., Ebner, H., Kraft, D. & Scheiner, O. 1995. Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). J. Allergy Clin. Immunol. 95, 962-969.

Ebo, D. G., Hagendorens, M. M., Bridts, C. H., De Clerck, L. S. & Stevens, W. J. 2004. Sensitization to cross-reactive carbohydrate determinants and the ubiquitous protein profilin: mimickers of allergy. Clin. Exp. Allergy 34, 137-144.

Espinosa, J. F., Asensio, J. L., Garcia, J. L., Laynez, J., Bruix, M., Wright, C., Siebert, H. C., Gabius, H. J., Canada, F. J. & Jimenez-Barbero, J. 2000. NMR investigations of protein-carbohydrate interactions binding studies and refined three-dimensional solution structure of the complex between the B domain of wheat germ agglutinin and N, N', N''-triacetylchitotriose. Eur. J. Biochem. 267, 3965-3978.

Fedorov, A. A., Ball, T., Mahoney, N. M., Valenta, R. & Almo, S. C. 1997. The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin. Structure 5, 33-45.

Ferreira, F., Ebner, C., Kramer, B., Casari, G., Briza, P., Kungl, A. J., Grimm, R., Jahn-Schmid, B., Breiteneder, H., Kraft, D., Breitenbach, M., Rheinberger, H. J. & Scheiner, O. 1998. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. Faseb J. 12, 231-242.

Ferreira, F., Hawranek, T., Gruber, P., Wopfner, N. & Mari, A. 2004. Allergic cross-reactivity: from gene to the clinic. Allergy 59, 243-267.

Finn, A., Gross, G., Van Bavel, J., Lee, T., Windom, H., Everhard, F., Fowler-Taylor, A., Liu, J. & Gupta, N. 2003. Omalizumab improves asthma-related quality of life in patients with severe allergic asthma. J. Allergy Clin. Immunol. 111, 278-284.

Forström, L. 1980. Contact urticaria from latex surgical gloves. Contact Dermatitis 6, 33-34.

Francis, J. N., Till, S. J. & Durham, S. R. 2003. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. J. Allergy Clin. Immunol. 111, 1255-1261.

Fuchs, T., Spitzauer, S., Vente, C., Hevler, J., Kapiotis, S., Rumpold, H., Kraft, D. & Valenta, R. 1997. Natural latex, grass pollen, and weed pollen share IgE epitopes. J. Allergy Clin. Immunol. 100, 356-364.

Fukamizo, T. 2000. Chitinolytic enzymes: catalysis, substrate binding, and their application. Curr. Protein Pept. Sci. 1, 105-124.

Gajhede, M., Osmark, P., Poulsen, F. M., Ipsen, H., Larsen, J. N., Joost Van Neerven, R. J., Schou, C., Lowenstein, H. & Spangfort, M. D. 1996. X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. Nat. Struct. Biol. 3, 1040-1045.

Ganglberger, E., Grunberger, K., Sponer, B., Radauer, C., Breiteneder, H., Boltz-Nitulescu, G., Scheiner, O. & Jensen-Jarolim, E. 2000. Allergen mimotopes for 3-dimensional epitope search and induction of antibodies inhibiting human IgE. Faseb J. 14, 2177-2184.

Garman, S. C., Wurzburg, B. A., Tarchevskaya, S. S., Kinet, J. P. & Jardetzky, T. S. 2000. Structure of the Fc fragment of human IgE bound to its high-affinity receptor Fc epsilonRI alpha. Nature 406, 259-266.

Gastaminza, G., Algorta, J., Audicana, M., Etxenagusia, M., Fernandez, E. & Munoz, D. 2003. Systemic reactions to immunotherapy: influence of composition and manufacturer. Clin. Exp. Allergy 33, 470-474.

Gauchat, J. F., Henchoz, S., Mazzei, G., Aubry, J. P., Brunner, T., Blasey, H., Life, P., Talabot, D., Flores-Romo, L., Thompson, J. & Et Al. 1993. Induction of human IgE synthesis in B cells by mast cells and basophils. Nature 365, 340-343.

Geha, R. S., Jabara, H. H. & Brodeur, S. R. 2003. The regulation of immunoglobulin E class-switch recombination. Nat. Rev. Immunol. 3, 721-732.

Gern, J. E. & Weiss, S. T. 2000. Protection against atopic diseases by measles--a rash conclusion? JAMA 283, 394-395.

Gershoni, J. M., Stern, B. & Denisova, G. 1997. Combinatorial libraries, epitope structure and the prediction of protein conformations. Immunol. Today 18, 108-110.

Gidrol, X., Chrestin, H., Tan, H. L. & Kush, A. 1994. Hevein, a lectin-like protein from Hevea brasiliensis (rubber tree) is involved in the coagulation of latex. J. Biol. Chem. 269, 9278-9283.

Gorska, M. M. & Alam, R. 2003. Signaling molecules as therapeutic targets in allergic diseases. J. Allergy Clin. Immunol. 112, 241-250; quiz 251.

Gounni, A. S., Lamkhioued, B., Ochiai, K., Tanaka, Y., Delaporte, E., Capron, A., Kinet, J. P. & Capron, M. 1994. High-affinity IgE receptor on eosinophils is involved in defence against parasites. Nature 367, 183-186.

Guermonprez, P., England, P., Bedouelle, H. & Leclerc, C. 1998. The rate of dissociation between antibody and antigen determines the efficiency of antibody-mediated antigen presentation to T cells. J. Immunol. 161, 4542-4548.

Halme, K. 2003. The production and downstream processing of recombinant natural rubber latex allergens of hevea brasiliensis. Pro gradu -study in biotechiniques.

Hamilton, R. G. & Adkinson, N. F., Jr. 2003. 23. Clinical laboratory assessment of IgE-dependent hypersensitivity. J. Allergy Clin. Immunol. 111 (2 Suppl), S687-701.

Haque, M. A., Hawes, J. W. & Blum, J. S. 2001. Cysteinylation of MHC class II ligands: peptide endocytosis and reduction within APC influences T cell recognition. J. Immunol. 166, 4543-4551.

Hardy, C. L., Kenins, L., Drew, A. C., Rolland, J. M. & O'hehir, R. E. 2003. Characterization of a mouse model of allergy to a major occupational latex glove allergen Hev b 5. Am. J. Respir. Crit. Care Med. 167, 1393-1399.

Harwanegg, C., Laffer, S., Hiller, R., Mueller, M. W., Kraft, D., Spitzauer, S. & Valenta, R. 2003. Microarrayed recombinant allergens for diagnosis of allergy. Clin. Exp. Allergy 33, 7-13.

Hayashida, M., Fujii, T., Hamasu, M., Ishiguro, M. & Hata, Y. 2003. Similarity between protein-protein and protein-carbohydrate interactions, revealed by two crystal structures of lectins from the roots of pokeweed. J. Mol. Biol. 334, 551-565.

He, X. L., Grigg, M. E., Boothroyd, J. C. & Garcia, K. C. 2002. Structure of the immunodominant surface antigen from the Toxoplasma gondii SRS superfamily. Nat. Struct. Biol. 9, 606-611.

Hernandez-Arana, A., Rojo-Dominguez, A., Soriano-Garcia, M. & Rodriguez-Romero, A. 1995. The thermal unfolding of hevein, a small disulfide-rich protein. Eur. J. Biochem. 228, 649-652.

Herz, U., Renz, H. & Wiedermann, U. 2004. Animal models of type I allergy using recombinant allergens. Methods 32, 271-280.

Hiller, R., Laffer, S., Harwanegg, C., Huber, M., Schmidt, W. M., Twardosz, A., Barletta, B., Becker, W. M., Blaser, K., Breiteneder, H., Chapman, M., Crameri, R., Duchene, M., Ferreira, F., Fiebig, H., Hoffmann-Sommergruber, K., King, T. P., Kleber-Janke, T., Kurup, V. P., Lehrer, S. B., Lidholm, J., Muller, U., Pini, C., Reese, G., Scheiner, O., Scheynius, A., Shen, H. D., Spitzauer, S., Suck, R., Swoboda, I., Thomas, W., Tinghino, R., Van Hage-Hamsten, M., Virtanen, T., Kraft, D., Muller, M. W. & Valenta, R. 2002. Microarrayed allergen molecules: diagnostic gatekeepers for allergy treatment. Faseb J. 16, 414-416.

Hirsch, T., Neumeister, V., Weiland, S. K., Von Mutius, E., Hirsch, D., Grafe, H., Duhme, H. & Leupold, W. 2000. Traffic exposure and allergic sensitization against latex in children. J. Allergy Clin. Immunol. 106, 573-578.

Hochreiter, R., Ferreira, F., Thalhamer, J. & Hammerl, P. 2003. TH1-promoting DNA immunization against allergens modulates the ratio of IgG1/IgG2a but does not affect the anaphylactic potential of IgG1 antibodies: no evidence for the synthesis of nonanaphylactic IgG1. J. Allergy Clin. Immunol. 112, 579-584.

Hoffmann-Sommergruber, K. 2002. Pathogenesis-related (PR)-proteins identified as allergens. Biochem. Soc. Trans. 30, 930-935.

Horner, A. A., Datta, S. K., Takabayashi, K., Belyakov, I. M., Hayashi, T., Cinman, N., Nguyen, M. D., Van Uden, J. H., Berzofsky, J. A., Richman, D. D. & Raz, E. 2001. Immunostimulatory DNA-based vaccines elicit multifaceted immune responses against HIV at systemic and mucosal sites. J. Immunol. 167, 1584-1591.

Horner, A. A. & Raz, E. 2002. Immunostimulatory sequence oligodeoxynucleotide-based vaccination and immunomodulation: two unique but complementary strategies for the treatment of allergic diseases. J. Allergy Clin. Immunol. 110, 706-712.

Huang, R. H., Xiang, Y., Tu, G. Z., Zhang, Y. & Wang, D. C. 2004. Solution structure of Eucommia antifungal peptide: a novel structural model distinct with a five-disulfide motif. Biochemistry 43, 6005-6012.

Hufnagl, K., Wagner, B., Winkler, B., Baier, K., Hochreiter, R., Thalhamer, J., Kraft, D., Scheiner, O., Breiteneder, H. & Wiedermann, U. 2003. Induction of mucosal tolerance with recombinant Hev b 1 and recombinant Hev b 3 for prevention of latex allergy in BALB/c mice. Clin. Exp. Immunol. 133, 170-176.

Ichikawa, S., Hatanaka, H., Yuuki, T., Iwamoto, N., Kojima, S., Nishiyama, C., Ogura, K., Okumura, Y. & Inagaki, F. 1998. Solution structure of Der f 2, the major mite allergen for atopic diseases. J. Biol. Chem. 273, 356-360.

Iseli, B., Boller, T. & Neuhaus, J. M. 1993. The N-terminal cysteine-rich domain of tobacco class I chitinase is essential for chitin binding but not for catalytic or antifungal activity. Plant Physiol. 103, 221-226.

Ishizaka, K. & Ishizaka, T. 1967. Identification of gamma-E-antibodies as a carrier of reaginic activity. J. Immunol. 99, 1187-1198.

Jackola, D. R., Pierson-Mullany, L. K., Liebeler, C. L., Blumenthal, M. N. & Rosenberg, A. 2002. Variable binding affinities for allergen suggest a 'selective competition' among immunoglobulins in atopic and non-atopic humans. Mol. Immunol. 39, 367-377.

Jaiswal, A. I. & Croft, M. 1997. CD40 ligand induction on T cell subsets by peptide-presenting B cells: implications for development of the primary T and B cell response. J. Immunol. 159, 2282-2291.

James, L. C., Roversi, P. & Tawfik, D. S. 2003. Antibody multispecificity mediated by conformational diversity. Science 299, 1362-1367.

Janeway, C. A., Travers, P., Walport, M. & Shlomchik, M. 2001. Immunobiology: The immune system in health & disease. Garland Science Publishing, New York.

Jensen-Jarolim, E., Leitner, A., Kalchhauser, H., Zurcher, A., Ganglberger, E., Bohle, B., Scheiner, O., Boltz-Nitulescu, G. & Breiteneder, H. 1998. Peptide mimotopes displayed by phage inhibit antibody binding to bet v 1, the major birch pollen allergen, and induce specific IgG response in mice. Faseb J. 12, 1635-1642.

Jerne, N. K. 1960. Immunological speculations. Annu. Rev. Microbiol. 14, 341-358.

Johansson, S. G. & Bennich, H. 1967. Immunological studies of an atypical (myeloma) immunoglobulin. Immunology 13, 381-394.

Jutel, M., Akdis, M., Budak, F., Aebischer-Casaulta, C., Wrzyszcz, M., Blaser, K. & Akdis, C. A. 2003. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. Eur. J. Immunol. 33, 1205-1214.

Kahlert, H., Grage-Griebenow, E., Stuwe, H. T., Cromwell, O. & Fiebig, H. 2000. T cell reactivity with allergoids: influence of the type of APC. J. Immunol. 65, 1807-1815.

Kauppinen, J., Zeiler, T., Rautiainen, J., Rytkonen-Nissinen, M., Taivainen, A., Mantyjarvi, R. & Virtanen, T. 1999. Mutant derivatives of the main respiratory allergen of cow are less allergenic than the intact molecule. Clin. Exp. Allergy 29, 989-996.

Kay, A. B. 2001. Allergy and allergic diseases. First of two parts. N. Engl. J. Med. 344, 30-37.

Kehry, M. R. & Yamashita, L. C. 1989. Low-affinity IgE receptor (CD23) function on mouse B cells: role in IgE-dependent antigen focusing. Proc. Natl. Acad. Sci. U S A 86, 7556-7560.

Kilmon, M. A., Shelburne, A. E., Chan-Li, Y., Holmes, K. L. & Conrad, D. H. 2004. CD23 trimers are preassociated on the cell surface even in the absence of its ligand, IgE. J. Immunol. 172, 1065-1073.

Kimata, H. 2003. Listening to Mozart reduces allergic skin wheal responses and in vitro allergen-specific IgE production in atopic dermatitis patients with latex allergy. Behav. Med. 29, 15-19.

King, T. P., Jim, S. Y., Monsalve, R. I., Kagey-Sobotka, A., Lichtenstein, L. M. & Spangfort, M. D. 2001. Recombinant allergens with reduced allergenicity but retaining immunogenicity of the natural allergens: hybrids of yellow jacket and paper wasp venom allergen antigen 5s. J. Immunol. 166, 6057-6065.

Kinnunen, T., Buhot, C., Närvänen, A., Rytkönen-Nissinen, M., Saarelainen, S., Pouvelle-Moratille, S., Rautiainen, J., Taivainen, A., Maillere, B., Mäntyjärvi, R. & Virtanen, T. 2003. The immunodominant epitope of lipocalin allergen Bos d 2 is suboptimal for human T cells. Eur. J. Immunol. 33, 1717-1726.

Kitaura, J., Song, J., Tsai, M., Asai, K., Maeda-Yamamoto, M., Mocsai, A., Kawakami, Y., Liu, F. T., Lowell, C. A., Barisas, B. G., Galli, S. J. & Kawakami, T. 2003. Evidence that IgE molecules mediate a spectrum of effects on mast cell survival and activation via aggregation of the FcepsilonRI. Proc. Natl. Acad. Sci. U S A 100, 12911-12916.

Ko, J. H., Chow, K. S. & Han, K. H. 2003. Transcriptome analysis reveals novel features of the molecular events occurring in the laticifers of Hevea brasiliensis (para rubber tree). Plant Mol. Biol. 53, 479-492.

Koo, J. C., Chun, H. J., Park, H. C., Kim, M. C., Koo, Y. D., Koo, S. C., Ok, H. M., Park, S. J., Lee, S. H., Yun, D. J., Lim, C. O., Bahk, J. D., Lee, S. Y. & Cho, M. J. 2002. Over-expression of a seed specific hevein-like antimicrobial peptide from Pharbitis nil enhances resistance to a fungal pathogen in transgenic tobacco plants. Plant Mol. Biol. 50, 441-452.

Köpman, A. & Hannuksela, M. 1983. Contact urticaria to rubber. Duodecim 99, 221-224.

Krebitz, M., Wagner, B., Ferreira, F., Peterbauer, C., Campillo, N., Witty, M., Kolarich, D., Steinkellner, H., Scheiner, O. & Breiteneder, H. 2003. Plant-based heterologous expression of Mal d 2, a thaumatin-like protein and allergen of apple (Malus domestica), and its characterization as an antifungal protein. J. Mol. Biol. 329, 721-730.

Kubo, S., Nakayama, T., Matsuoka, K., Yonekawa, H. & Karasuyama, H. 2003. Long term maintenance of IgE-mediated memory in mast cells in the absence of detectable serum IgE. J. Immunol. 170, 775-780.

Kurup, V. P., Yeang, H. Y., Sussman, G. L., Bansal, N. K., Beezhold, D. H., Kelly, K. J., Hoffman, D. R., Williams, B. & Fink, J. N. 2000. Detection of immunoglobulin antibodies in the sera of patients using purified latex allergens. Clin. Exp. Allergy 30, 359-369.

Kurup, V. P., Vijay, H. M., Kumar, V., Castillo, L. & Elms, N. 2003. IgE binding synthetic peptides of Alt a 1, a major allergen of Alternaria alternata. Peptides 24, 179-185.

Laffer, S., Hogbom, E., Roux, K. H., Sperr, W. R., Valent, P., Bankl, H. C., Vangelista, L., Kricek, F., Kraft, D., Gronlund, H. & Valenta, R. 2001. A molecular model of type I allergy: identification and characterization of a nonanaphylactic anti-human IgE antibody fragment that blocks the IgE-FcepsilonRI interaction and reacts with receptor-bound IgE. J. Allergy Clin. Immunol. 108, 409-416.

Laukkanen, M. L., Mäkinen-Kiljunen, S., Isoherranen, K., Haahtela, T., Söderlund, H. & Takkinen, K. 2003. Hevein-specific recombinant IgE antibodies from human single-chain antibody phage display libraries. J. Immunol. Methods 278, 271-281.

Laurent, J., Malet, R., Smiejan, J. M., Madelenat, P. & Herman, D. 1992. Latex hypersensitivity after natural delivery. J. Allergy Clin. Immunol. 89, 779-780.

Lavaud, F., Prevost, A., Cossart, C., Guerin, L., Bernard, J. & Kochman, S. 1995. Allergy to latex, avocado pear, and banana: evidence for a 30 kd antigen in immunoblotting. J. Allergy Clin. Immunol. 95, 557-564.

Laver, W. G., Air, G. M., Webster, R. G. & Smith-Gill, S. J. 1990. Epitopes on protein antigens: misconceptions and realities [published erratum appears in Cell 1990 Aug 10;62(3):following 608]. Cell 61, 553-556.

Lebecque, S., Dolecek, C., Laffer, S., Visco, V., Denepoux, S., Pin, J. J., Guret, C., Boltz-Nitulescu, G., Weyer, A. & Valenta, R. 1997. Immunologic characterization of monoclonal antibodies that modulate human IgE binding to the major birch pollen allergen Bet v 1. J. Allergy Clin. Immunol. 99, 374-384.

Lee, H. I., Broekaert, W. F., Raikhel, N. V. & Lee, H. 1991. Co- and post-translational processing of the hevein preproprotein of latex of the rubber tree (Hevea brasiliensis). J. Biol. Chem. 266, 15944-15948.

Lee, O. S., Lee, B., Park, N., Koo, J. C., Kim, Y. H., Prasad, D. T., Karigar, C., Chun, H. J., Jeong, B. R., Kim, D. H., Nam, J., Yun, J. G., Kwak, S. S., Cho, M. J. & Yun, D. J. 2003. Pn-AMPs, the hevein-like proteins from Pharbitis nil confers disease resistance against phytopathogenic fungi in tomato, Lycopersicum esculentum. Phytochemistry 62, 1073-1079.

Leung, D. Y., Sampson, H. A., Yunginger, J. W., Burks, A. W., Jr., Schneider, L. C., Wortel, C. H., Davis, F. M., Hyun, J. D. & Shanahan, W. R., Jr. 2003. Effect of anti-IgE therapy in patients with peanut allergy. N. Engl. J. Med. 348, 986-993.

Leung, D. Y., Boguniewicz, M., Howell, M. D., Nomura, I. & Hamid, Q. A. 2004. New insights into atopic dermatitis. J. Clin. Invest. 113, 651-657.

Levy, D. A., Charpin, D., Pecquet, C., Leynadier, F. & Vervloet, D. 1992. Allergy to latex. Allergy 47, 579-587.

Levy, D. A., Mounedji, N., Noirot, C. & Leynadier, F. 2000. Allergic sensitization and clinical reactions to latex, food and pollen in adult patients. Clin. Exp. Allergy 30, 270-275.

Leynadier, F., Pecquet, C. & Dry, J. 1989. Anaphylaxis to latex during surgery. Anaesthesia 44, 547-550.

Leynadier, F., Doudou, O., Gaouar, H., Le Gros, V., Bourdeix, I., Guyomarch-Cocco, L. & Trunet, P. 2004. Effect of omalizumab in health care workers with occupational latex allergy. J. Allergy Clin. Immunol. 113, 360-361.

Li, Y., Li, H., Smith-Gill, S. J. & Mariuzza, R. A. 2000. Three-dimensional structures of the free and antigen-bound Fab from monoclonal antilysozyme antibody HyHEL-63. Biochemistry 39, 6296-6309.

Linhart, B., Jahn-Schmid, B., Verdino, P., Keller, W., Ebner, C., Kraft, D. & Valenta, R. 2002. Combination vaccines for the treatment of grass pollen allergy consisting of genetically engineered hybrid molecules with increased immunogenicity. Faseb J. 16, 1301-1303.

Maeurer, M., Hohn, H., Castelli, C., Salter, R., Necker, A., Reichert, T., Knuth, A. & Jager, E. 2001. Antigen recognition by T cells: a strong sense of structure. Trends Immunol. 22, 599-601.

Mahler, V., Vrtala, S., Kuss, O., Diepgen, T. L., Suck, R., Cromwell, O., Fiebig, H., Hartl, A., Thalhamer, J., Schuler, G., Kraft, D. & Valenta, R. 2004. Vaccines for birch pollen allergy based on genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1. Clin. Exp. Allergy 34, 115-122.

Maizels, R. M. & Yazdanbakhsh, M. 2003. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat. Rev. Immunol. 3, 733-744.

Maleki, S. J., Kopper, R. A., Shin, D. S., Park, C. W., Compadre, C. M., Sampson, H., Burks, A. W. & Bannon, G. A. 2000. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. J. Immunol. 164, 5844-5849.

Malling, H. J. 1999. Allergen-specific immunotherapy. Present state and directions for the future. Allergy 54 (Suppl 50), 30-33.

Matsuo, H., Morita, E., Tatham, A. S., Morimoto, K., Horikawa, T., Osuna, H., Ikezawa, Z., Kaneko, S., Kohno, K. & Dekio, S. 2004. Identification of the IgE-binding epitope in omega-5 gliadin, a major allergen in wheat-dependent exercise-induced anaphylaxis. J. Biol. Chem. 279, 12135-12140.

Matthews, B. W. 1995. Studies on protein stability with T4 lysozyme. Adv. Protein Chem. 46, 249-278.

McGeady, S. J. 2004. Immunocompetence and allergy. Pediatrics 113 (4 Suppl), 1107-1113.

Mikkola, J. H., Alenius, H., Kalkkinen, N., Turjanmaa, K., Palosuo, T. & Reunala, T. 1998. Hevein-like protein domains as a possible cause for allergen cross-reactivity between latex and banana. J. Allergy Clin. Immunol. 102, 1005-1012.

Mine, Y. & Rupa, P. 2003. Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken egg ovalbumin. Protein Eng. 16, 747-752.

Mirza, O., Henriksen, A., Ipsen, H., Larsen, J. N., Wissenbach, M., Spangfort, M. D. & Gajhede, M. 2000. Dominant epitopes and allergic cross-reactivity: complex formation between a Fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. J. Immunol. 165, 331-338.

Mohapatra, S. S. 1994. Determinant spreading: implications in allergic disorders. Immunol. Today 15, 596-597.

M'Raihi, L., Charpin, D., Pons, A., Bongrand, P. & Vervloet, D. 1991. Cross-reactivity between latex and banana. J. Allergy Clin. Immunol. 87, 129-130.

Muzzarelli, R. A. 1977. Chitin. Pergamon Press, Ancona.

Nel, A. & Gujuluva, C. 1998. Latex antigens: identification and use in clinical and experimental studies, including crossreactivity with food and pollen allergens. Ann. Allergy Asthma Immunol. 81, 388-396.

Nettis, E., Assennato, G., Ferrannini, A. & Tursi, A. 2002. Type I allergy to natural rubber latex and type IV allergy to rubber chemicals in health care workers with glove-related skin symptoms. Clin. Exp. Allergy 32, 441-447.

Neudecker, P., Schweimer, K., Nerkamp, J., Scheurer, S., Vieths, S., Sticht, H. & Rosch, P. 2001. Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1. J. Biol. Chem. 276, 22756-22763.

Neudecker, P., Lehmann, K., Nerkamp, J., Haase, T., Wangorsch, A., Fotisch, K., Hoffmann, S., Rosch, P., Vieths, S. & Scheurer, S. 2003. Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry (Prunus avium) and celery (Apium graveolens): correlating IgE reactivity with three-dimensional structure. Biochem. J. 376, 97-107.

Nishiyama, C., Fukada, M., Usui, Y., Iwamoto, N., Yuuki, T., Okumura, Y. & Okudaira, H. 1995. Analysis of the IgE-epitope of Der f 2, a major mite allergen, by in vitro mutagenesis. Mol. Immunol. 32, 1021-1029.

Noon, L. 1911. Prophylactic inoculation against hayfever. Lancet 1, 1572-1573.

Norman, P. S., Ishizaka, K., Lichtenstein, L. M. & Adkinson, N. F., Jr. 1980. Treatment of ragweed hay fever with urea-denatured antigen E. J. Allergy Clin. Immunol. 66, 336-341.

Nouri-Aria, K. T., Wachholz, P. A., Francis, J. N., Jacobson, M. R., Walker, S. M., Wilcock, L. K., Staple, S. Q., Aalberse, R. C., Till, S. J. & Durham, S. R. 2004. Grass pollen immunotherapy induces mucosal and peripheral IL-10 responses and blocking IgG activity. J. Immunol. 172, 3252-3259.

Novak, N., Kraft, S. & Bieber, T. 2003. Unraveling the mission of FcepsilonRI on antigen-presenting cells. J. Allergy Clin. Immunol. 111, 38-44.

Nutter, A. F. 1979. Contact urticaria to rubber. Br. J. Dermatol. 101, 597-598.

Oh, S. K., Kang, H., Shin, D. H., Yang, J., Chow, K. S., Yeang, H. Y., Wagner, B., Breiteneder, H. & Han, K. H. 1999. Isolation, characterization, and functional analysis of a novel cDNA clone encoding a small rubber particle protein from Hevea brasiliensis. J. Biol. Chem. 274, 17132-17138.

O'Riordain, G., Radauer, C., Hoffmann-Sommergruber, K., Adhami, F., Peterbauer, C. K., Blanco, C., Godnic-Cvar, J., Scheiner, O., Ebner, C. & Breiteneder, H. 2002. Cloning and molecular characterization of the Hevea brasiliensis allergen Hev b 11, a class I chitinase. Clin. Exp. Allergy 32, 455-462.

Ownby, D. R., Tomlanovich, M., Sammons, N. & Mccullough, J. 1991. Anaphylaxis associated with latex allergy during barium enema examinations. Am. J. Roentgenol. 156, 903-908.

Ownby, D. R. 2002. A history of latex allergy. J. Allergy Clin. Immunol. 110 (Suppl), S27-32.

Palosuo, T., Alenius, H. & Turjanmaa, K. 2002. Quantitation of latex allergens. Methods 27, 52-58.

Palosuo, T., Lehto, M., Kotovuori, A., Kalkkinen, N., Alenius, H., Reunala, T. & Turjanmaa, K. 2004. Prevalence of IgE antibodies to extensively purified Hev b13 and Hev b2 in natural rubber latex (NRL) allergic patients. J. Allergy Clin. Immunol. 113 (Suppl), S77.

Parronchi, P., Macchia, D., Piccinni, M. P., Biswas, P., Simonelli, C., Maggi, E., Ricci, M., Ansari, A. A. & Romagnani, S. 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, 4538-4542.

Payet, M. E., Woodward, E. C. & Conrad, D. H. 1999. Humoral response suppression observed with CD23 transgenics. J. Immunol. 163, 217-223.

Pearlman, D. S. 1999. Pathophysiology of the inflammatory response. J. Allergy Clin. Immunol. 104, S132-137.

Petersen, S. B., Jonson, P. H., Fojan, P., Petersen, E. I., Petersen, M. T., Hansen, S., Ishak, R. J. & Hough, E. 1998. Protein engineering the surface of enzymes. J. Biotechnol. 66, 11-26.

Pierson-Mullany, L. K., Jackola, D. R., Blumenthal, M. N. & Rosenberg, A. 2000. Characterization of polyclonal allergen-specific IgE responses by affinity distributions. Mol. Immunol. 37, 613-620.

Poley, G. E., Jr. & Slater, J. E. 2000. Latex allergy. J. Allergy Clin. Immunol. 105, 1054-1062.

Posch, A., Chen, Z., Raulf-Heimsoth, M. & Baur, X. 1998. Latex allergens. Clin. Exp. Allergy 28, 134-140.

Rabjohn, P., Helm, E. M., Stanley, J. S., West, C. M., Sampson, H. A., Burks, A. W. & Bannon, G. A. 1999. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. J. Clin. Invest. 103, 535-542.

Raulf-Heimsoth, M., Chen, Z., Liebers, V., Allmers, H. & Baur, X. 1996. Lymphocyte proliferation response to extracts from different latex materials and to the purified latex allergen Hev b 1 (rubber elongation factor). J. Allergy Clin. Immunol. 98, 640-651.

Raulf-Heimsoth, M., Chen, Z., Rihs, H. P., Kalbacher, H., Liebers, V. & Baur, X. 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, 339-348.

Raulf-Heimsoth, M., Rozynek, P., Bruning, T. & Rihs, H. P. 2004. Characterization of B- and T-cell responses and HLA-DR4 binding motifs of the latex allergen Hev b 6.01 (prohevein) and its post-transcriptionally formed proteins Hev b 6.02 and Hev b 6.03. Allergy 59, 724-733.

Reese, G., Ayuso, R., Leong-Kee, S. M., Plante, M. J. & Lehrer, S. B. 2001. Characterization and identification of allergen epitopes: recombinant peptide libraries and synthetic, overlapping peptides. J. Chromatogr. B. Biomed. Sci. Appl. 756, 157-163.

Reetz, M. T. 2004. Controlling the enantioselectivity of enzymes by directed evolution: practical and theoretical ramifications. Proc. Natl. Acad. Sci. U S A 101, 5716-5722.

Reyes-Lopez, C. A., Hernandez-Santoyo, A., Pedraza-Escalona, M., Mendoza, G., Hernandez-Arana, A. & Rodriguez-Romero, A. 2004. Insights into a conformational epitope of Hev b 6.02 (hevein). Biochem. Biophys. Res. Commun. 314, 123-130.

Richardson, C. D. 1995. Baculovirus expression protocol. Richardson, C. D. (ed.), Met. Mol. Biol. 39, Humana Press, Totowa NJ.

Rihs, H. P., Cremer, R., Chen, Z. & Baur, X. 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, 175-180.

Rihs, H.-P., Rozynek, P., Baur, X., Lundberg, M. & Cremer, R. 2000. PCR-based cloning, isolation, and IgE-binding properties of recombinant latex profilin (rHev b 8). Allergy 55, 712-717.

Rihs, H. P., Chen, Z., Rüeff, F., Cremer, R., Raulf-Heimsoth, M., Baur, X., Moneret-Vautrin, D. A. & Brüning, T. 2002. HLA-Dq8 and the HLA-DQ8-DR4 haplotype are positively associated with the hevein-specific IgE immune response in health care workers with latex allergy. J. Allergy Clin. Immunol. 110, 507-514.

Rodriguez-Romero, A., Ravichandran, K. G. & Soriano-Garcia, M. 1991. Crystal structure of hevein at 2.8 A resolution. FEBS Lett. 291, 307-309.

Rozynek, P., Posch, A. & Baur, X. 1998. Cloning, expression and characterization of the major latex allergen prohevein. Clin. Exp. Allergy 28, 1418-1426.

Rudensky, A., Preston-Hurlburt, P., Hong, S. C., Barlow, A. & Janeway, C. A., Jr. 1991. Sequence analysis of peptides bound to MHC class II molecules. Nature 353, 622-627.

Saini, S. S. & MacGlashan, D. 2002. How IgE upregulates the allergic response. Curr. Opin. Immunol. 14, 694-697.

Sali, A. & Blundell, T. L. 1993. Comparative protein modelling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779-815.

Sánchez-Monge, R., Blanco, C., Perales, A. D., Collada, C., Carrillo, T., Aragoncillo, C. & Salcedo, G. 2000. Class I chitinases, the panallergens

responsible for the latex-fruit syndrome, are induced by ethylene treatment and inactivated by heating. J. Allergy Clin. Immunol. 106, 190-195.

Sarkar, G. & Sommer, S. S. 1990. The "megaprimer" method of site-directed mutagenesis. Biotechniques 8, 404-407.

Sastre, J., Fernandez-Nieto, M., Rico, P., Martin, S., Barber, D., Cuesta, J., De Las Heras, M. & Quirce, S. 2003. Specific immunotherapy with a standardized latex extract in allergic workers: a double-blind, placebo-controlled study. J. Allergy Clin. Immunol. 111, 985-994.

Schramm, G., Kahlert, H., Suck, R., Weber, B., Stuwe, H. T., Muller, W. D., Bufe, A., Becker, W. M., Schlaak, M. W., Jager, L., Cromwell, O. & Fiebig, H. 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, 2406-2414.

Seki, Y., Inoue, H., Nagata, N., Hayashi, K., Fukuyama, S., Matsumoto, K., Komine, O., Hamano, S., Himeno, K., Inagaki-Ohara, K., Cacalano, N., O'garra, A., Oshida, T., Saito, H., Johnston, J. A., Yoshimura, A. & Kubo, M. 2003. SOCS-3 regulates onset and maintenance of T(H)2-mediated allergic responses. Nat. Med. 9, 1047-1054.

Seppälä, U., Palosuo, T., Kalkkinen, N., Ylitalo, L., Reunala, T. & Turjanmaa, K. 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, 266-273.

Shinshi, H., Neuhas, J. M., Ryals, J. & Meins, F., Jr. 1990. Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. Plant Mol. Biol. 14, 357-368.

Slater, J. E. 1989. Rubber anaphylaxis. N. Engl. J. Med. 320, 1126-1130.

Slater, J. E., Vedvick, T., Arthur-Smith, A., Trybul, D. E. & Kekwick, R. G. 1996. Identification, cloning, and sequence of a major allergen (Hev b 5) from natural rubber latex (Hevea brasiliensis). J. Biol. Chem. 271, 25394-25399.

Slater, J. E., Paupore, E., Zhang, Y. T. & Colberg-Poley, A. M. 1998a. 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, 469-475.

Slater, J. E., Paupore, E. J., Elwell, M. R. & Truscott, W. 1998b. Lipopolysaccharide augments IgG and IgE responses of mice to the latex allergen Hev b 5. J. Allergy Clin. Immunol. 102, 977-983.

Slater, J. E., Paupore, E. J. & O'hehir, R. E. 1999. Murine B-cell and T-cell epitopes of the allergen Hev b 5 from natural rubber latex. Mol. Immunol. 36, 135-143.

Smith, A. M. & Chapman, M. D. 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, 399-405.

Smith, A. M., Chapman, M. D., Taketomi, E. A., Platts-Mills, T. A. & Sung, S. S. 1998. Recombinant allergens for immunotherapy: a Der p 2 variant with reduced IgE reactivity retains T-cell epitopes. J. Allergy Clin. Immunol. 101, 423-425.

Soedjanaatmadja, U. M., Hofsteenge, J., Jeronimus-Stratingh, C. M., Bruins, A. P. & Beintema, J. J. 1994. Demonstration by mass spectrometry that pseudo-hevein and hevein have ragged C-terminal sequences. Biochim. Biophys. Acta 1209, 144-148.

Soedjanaatmadja, U. M., Subroto, T. & Beintema, J. J. 1995. Processed products of the hevein precursor in the latex of the rubber tree (Hevea brasiliensis). FEBS Lett. 363, 211-213.

Sosovec, D., Bourne, G. & Davis, D. 1998. Clinical assessment and effective management of latex allergy. Surg. Serv. Manag. 4, 11-15.

Sowka, S., Wagner, S., Krebitz, M., Arija-Mad-Arif, S., Yusof, F., Kinaciyan, T., Brehler, R., Scheiner, O. & Breiteneder, H. 1998. 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. Biochem. 255, 213-219.

Sowka, S., Hafner, C., Radauer, C., Focke, M., Brehler, R., Astwood, J. D., Arif, S. A., Kanani, A., Sussman, G. L., Scheiner, O., Beezhold, D. H. & Breiteneder, H. 1999. Molecular and immunologic characterization of new isoforms of the Hevea brasiliensis latex allergen hev b 7: evidence of no cross-reactivity between hev b 7 isoforms and potato patatin and proteins from avocado and banana. J. Allergy Clin. Immunol. 104, 1302-1310.

Spangfort, M. D., Mirza, O., Ipsen, H., Van Neerven, R. J., Gajhede, M. & Larsen, J. N. 2003. Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. J. Immunol. 171, 3084-3090.

Spitzauer, S., Pandjaitan, B., Muhl, S., Ebner, C., Kraft, D., Valenta, R. & Rumpold, H. 1997. Major cat and dog allergens share IgE epitopes. J. Allergy Clin. Immunol. 99, 100-106.

Stanley, J. S. & Bannon, G. A. 1999. Biochemistry of food allergens. Clin. Rev. Allergy Immunol. 17, 279-291.

Stemmer, W. P. 1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature 370, 389-391.

Stern, G. 1927. Überempfindlichkeit gegen Kautschuk als Ursache von Urticaria und Quinckeschem Ödem. Klin. Wochenschr. 6, 1096-1097.

Stoeva, S., Franz, M., Wacker, R., Krauspenhaar, R., Guthohrlein, E., Mikhailov, A., Betzel, C. & Voelter, W. 2001. Primary structure, isoforms, and molecular modeling of a chitin-binding mistletoe lectin. Arch. Biochem. Biophys. 392, 23-31.

Subroto, T., Sufiati, S. & Beintema, J. J. 1999. Papaya (Carica papaya) lysozyme is a member of the family 19 (basic, class II) chitinases. J. Mol. Evol. 49, 819-821.

Surman, S., Lockey, T. D., Slobod, K. S., Jones, B., Riberdy, J. M., White, S. W., Doherty, P. C. & Hurwitz, J. L. 2001. Localization of CD4+ T cell epitope

hotspots to exposed strands of HIV envelope glycoprotein suggests structural influences on antigen processing. Proc. Natl. Acad. Sci. U S A 98, 4587-4592.

Sussman, G. L., Tarlo, S. & Dolovich, J. 1991. The spectrum of IgE-mediated responses to latex. JAMA 265, 2844-2847.

Sussman, G. L., Liss, G. M., Deal, K., Brown, S., Cividino, M., Siu, S., Beezhold, D. H., Smith, G., Swanson, M. C., Yunginger, J., Douglas, A., Holness, D. L., Lebert, P., Keith, P., Waserman, S. & Turjanmaa, K. 1998. Incidence of latex sensitization among latex glove users. J. Allergy Clin. Immunol. 101, 171-178.

Sutherland, M. F., Drew, A., Rolland, J. M., Slater, J. E., Suphioglu, C. & O'hehir, R. E. 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, 583-589.

Sutton, B. J. & Gould, H. J. 1993. The human IgE network. Nature 366, 421-428.

Takai, T., Yokota, T., Yasue, M., Nishiyama, C., Yuuki, T., Mori, A., Okudaira, H. & Okumura, Y. 1997. Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. Nat. Biotechnol. 15, 754-758.

Takai, T., Mori, A., Yuuki, T., Okudaira, H. & Okumura, Y. 1999. Non-anaphylactic combination of partially deleted fragments of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. Mol. Immunol. 36, 1055-1065.

Takai, T., Hatanaka, H., Ichikawa, S., Yokota, T., Inagaki, F. & Okumura, Y. 2001. Effects of double mutation at two distant IgE-binding sites in the three-dimensional structure of the major house dust mite allergen Der f 2 on IgE-binding and histamine-releasing activity. Biosci. Biotechnol. Biochem. 65, 1601-1609.

Takatsu, K., Ishizaka, K. & King, T. P. 1975. Immunogenic properties of modified antigen E. III. Effect of repeated injections of modified antigen on immunocompetent cells specific for native antigen. J. Immunol. 115, 1469-1476.

Tamborini, E., Faccini, S., Lidholm, J., Svensson, M., Brandazza, A., Longhi, R., Groenlund, H., Sidoli, A. & Arosio, P. 1997. Biochemical and immunological characterization of recombinant allergen Lol p 1. Eur. J. Biochem. 249, 886-894.

Tang, B., Banerjee, B., Greenberger, P. A., Fink, J. N., Kelly, K. J. & Kurup, V. P. 2000. Antibody binding of deletion mutants of Asp f 2, the major Aspergillus fumigatus allergen. Biochem. Biophys. Res. Commun. 270, 1128-1135.

Tarlo, S. M., Wong, L., Roos, J. & Booth, N. 1990. Occupational asthma caused by latex in a surgical glove manufacturing plant. J. Allergy Clin. Immunol. 85, 626-631.

Taylor, S. L. & Hefle, S. L. 2001. Will genetically modified foods be allergenic? J. Allergy Clin. Immunol. 107, 765-771.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876-4882.

Till, S. J., Francis, J. N., Nouri-Aria, K. & Durham, S. R. 2004. Mechanisms of immunotherapy. J. Allergy Clin. Immunol. 113, 1025-1034.

Tomazic, V. J., Withrow, T. J., Fisher, B. R. & Dillard, S. F. 1992. Latex-associated allergies and anaphylactic reactions. Clin. Immunol. Immunopathol. 64, 89-97.

Tomazic-Jezic, V. J. & Lucas, A. D. 2002. Protein and allergen assays for natural rubber latex products. J. Allergy Clin. Immunol. 110 (2 Suppl), S40-46.

Trivedi, B., Valerio, C. & Slater, J. E. 2003. Endotoxin content of standardized allergen vaccines. J. Allergy Clin. Immunol. 111, 777-783.

Truong, M. J., Gruart, V., Kusnierz, J. P., Papin, J. P., Loiseau, S., Capron, A. & Capron, M. 1993. Human neutrophils express immunoglobulin E (IgE)-binding proteins (Mac-2/epsilon BP) of the S-type lectin family: role in IgE-dependent activation. J. Exp. Med. 177, 243-248.

Turjanmaa, K. 2001. Diagnosis of latex allergy. Allergy 56, 810-812.

Turjanmaa, K. & Reunala, T. 1989. Condoms as a source of latex allergen and cause of contact urticaria. Contact Dermatitis 20, 360-364.

Turjanmaa, K., Reunala, T., Tuimala, R. & Kärkkäinen, T. 1984. Severe IgE-mediated allergy to surgical gloves. Allergy 39 (Suppl 2), 2.

Turjanmaa, K., Reunala, T., Alenius, H., Brummer-Korvenkontio, H. & Palosuo, T. 1990. Allergens in latex surgical gloves and glove powder. Lancet 336, 1588.

Turjanmaa, K., Palosuo, T., Alenius, H., Leynadier, F., Autegarden, J. E., Andre, C., Sicard, H., Hrabina, M. & Tran, T. X. 1997. Latex allergy diagnosis: in vivo and in vitro standardization of a natural rubber latex extract. Allergy 52, 41-50.

Turjanmaa, K., Alenius, H., Reunala, T. & Palosuo, T. 2002. Recent developments in latex allergy. Curr. Opin. Allergy Clin. Immunol. 2, 407-412.

Valenta, R. 2002. The future of antigen-specific immunotherapy of allergy. Nat. Rev. Immunol. 2, 446-453.

Van Damme, E. J., Charels, D., Roy, S., Tierens, K., Barre, A., Martins, J. C., Rouge, P., Van Leuven, F., Does, M. & Peumans, W. J. 1999. A gene encoding a hevein-like protein from elderberry fruits is homologous to PR-4 and class V chitinase genes. Plant Physiol. 119, 1547-1556.

van der Veen, M. J., Jansen, H. M., Aalberse, R. C. & Van Der Zee, J. S. 2001. Der p 1 and Der p 2 induce less severe late asthmatic responses than native Dermatophagoides pteronyssinus extract after a similar early asthmatic response. Clin. Exp. Allergy 31, 705-714.

Van Parijs, J., Broekaert, W. F., Goldstein, I. J. & Peumans, W. J. 1991. Hevein: an antifungal protein from rubber-tree (Hevea brasiliensis) latex. Planta 183, 258-264.

Vandenplas, O., Jamart, J., Delwiche, J. P., Evrard, G. & Larbanois, A. 2002. Occupational asthma caused by natural rubber latex: outcome according to cessation or reduction of exposure. J. Allergy Clin. Immunol. 109, 125-130.

von Pirquet, C. 1906. Allergie. Munchen Med. Wchnschr. 53, 1457.

Vrtala, S., Hirtenlehner, K., Vangelista, L., Pastore, A., Eichler, H. G., Sperr, W. R., Valent, P., Ebner, C., Kraft, D. & Valenta, R. 1997. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. J. Clin. Invest. 99, 1673-1681.

Vrtala, S., Akdis, C. A., Budak, F., Akdis, M., Blaser, K., Kraft, D. & Valenta, R. 2000. T cell epitope-containing hypoallergenic recombinant fragments of the major birch pollen allergen, Bet v 1, induce blocking antibodies. J. Immunol. 165, 6653-6659.

Vrtala, S., Hirtenlehner, K., Susani, M., Akdis, M., Kussebi, F., Akdis, C. A., Blaser, K., Hufnagl, P., Binder, B. R., Politou, A., Pastore, A., Vangelista, L., Sperr, W. R., Semper, H., Valent, P., Ebner, C., Kraft, D. & Valenta, R. 2001. Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1. Faseb J. 15, 2045-2047.

Wachholz, P. A. & Durham, S. R. 2004. Mechanisms of immunotherapy: IgG revisited. Curr. Opin. Allergy Clin. Immunol. 4, 313-318.

Wagner, B., Krebitz, M., Buck, D., Niggemann, B., Yeang, H. Y., Han, K. H., Scheiner, O. & Breiteneder, H. 1999. Cloning, expression, and characterization of recombinant Hev b 3, a Hevea brasiliensis protein associated with latex allergy in patients with spina bifida. J. Allergy Clin. Immunol. 104, 1084-1092.

Wagner, B., Buck, D., Hafner, C., Sowka, S., Niggemann, B., Scheiner, O. & Breiteneder, H. 2001. Hev b 7 is a Hevea brasiliensis protein associated with latex allergy in children with spina bifida. J. Allergy Clin. Immunol. 108, 621-627.

Wagner, S. & Breiteneder, H. 2002. The latex-fruit syndrome. Biochem. Soc. Trans. 30, 935-940.

Wagner, S., Breiteneder, H., Simon-Nobbe, B., Susani, M., Krebitz, M., Niggemann, B., Brehler, R., Scheiner, O. & Hoffmann-Sommergruber, K. 2000. Hev b 9, an enolase and a new cross-reactive allergen from hevea latex and molds. Purification, characterization, cloning and expression. Eur. J. Biochem. 267, 7006-7014.

Wakelin, S. H. 2002. Contact anaphylaxis from natural rubber latex used as an adhesive for hair extensions. Br. J. Dermatol. 146, 340-341.

Waljuno, K., Scholma, R. A., Beintema, J., Mariono, A. & Hahn, A. M. 1975. Amino acid sequence of hevein. Proc. Int. Rubber Conf. 2, 518-531.

Wensing, M., Akkerdaas, J. H., Van Leeuwen, W. A., Stapel, S. O., Bruijnzeel-Koomen, C. A., Aalberse, R. C., Bast, B. J., Knulst, A. C. & Van Ree, R. 2002. IgE to Bet v 1 and profilin: cross-reactivity patterns and clinical relevance. J. Allergy Clin. Immunol. 110, 435-442.

Westritschnig, K., Focke, M., Verdino, P., Goessler, W., Keller, W., Twardosz, A., Mari, A., Horak, F., Wiedermann, U., Hartl, A., Thalhamer, J., Sperr, W. R., Valent, P. & Valenta, R. 2004. Generation of an allergy vaccine by disruption of the three-dimensional structure of the cross-reactive calcium-binding allergen, Phl p 7. J. Immunol. 172, 5684-5692.

Wiedermann, U., Herz, U., Baier, K., Vrtala, S., Neuhaus-Steinmetz, U., Bohle, B., Dekan, G., Renz, H., Ebner, C., Valenta, R. & Kraft, D. 2001. Intranasal treatment with a recombinant hypoallergenic derivative of the major birch pollen allergen Bet v 1 prevents allergic sensitization and airway inflammation in mice. Int. Arch. Allergy Immunol. 126, 68-77.

Williams, C. M. & Galli, S. J. 2000. The diverse potential effector and immunoregulatory roles of mast cells in allergic disease. J. Allergy Clin. Immunol. 105, 847-859.

Williams, P. B., Buhr, M. P., Weber, R. W., Volz, M. A., Koepke, J. W. & Selner, J. C. 1995. Latex allergen in respirable particulate air pollution. J. Allergy Clin. Immunol. 95, 88-95.

Wills-Karp, M. & Ewart, S. L. 2004. Time to draw breath: asthma-susceptibility genes are identified. Nat. Rev. Genet. 5, 376-387.

Wilson, K. S., Butterworth, S., Dauter, Z., Lamzin, V. S., Walsh, M., Wodak, S., Pontius, J., Richelle, J., Vaguine, A., Sander, C., Hooft, R. W. W., Vriend, G., Thornton, J. M., Laskowski, R. A., Macarthur, M. W., Dodson, E. J., Murshudov, G., Oldfield, T. J., R., K. & Rullmann, J. A. C. 1998. Who checks the checkers? Four validation tools applied to eight atomic resolution structures. EU 3-D Validation Network. J. Mol. Biol. 276, 417-436.

Wood, S. F. 1986. Review of hay fever. 1. Historical background and mechanisms. Fam. Pract. 3, 54-63.

Wrangsjö, K., Wahlberg, J. E. & Axelsson, I. G. 1988. IgE-mediated allergy to natural rubber in 30 patients with contact urticaria. Contact Dermatitis 19, 264-271.

Xia, J.-Q., Rickaby, D. A., Hebderson, J. D., Kelly, K. J., Dawson, C. A., Fink, J. N., Skrinska, V. & Kurup, V. P. 2001. The Major Latex Allergen Hev B 6 Elicit Allergic Response in a Mouse Model of Latex Allergy. J. Allergy Clin. Immunol. 107 (Suppl), S320.

Yagami, T. 1998. Plant defense-related proteins and latex allergy. Environ. Dermatol. 5 (suppl. 2) 31-39.

Yagami, T., Sato, M., Nakamura, A., Komiyama, T., Kitagawa, K., Akasawa, A. & Ikezawa, Z. 1998. Plant defense-related enzymes as latex antigens. J. Allergy Clin. Immunol. 101, 379-385.

Yagami, T., Haishima, Y., Nakamura, A., Osuna, H. & Ikezawa, Z. 2000. Digestibility of allergens extracted from natural rubber latex and vegetable foods. J. Allergy Clin. Immunol. 106, 752-762.

Yazdanbakhsh, M., Van Den Biggelaar, A. & Maizels, R. M. 2001. Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. Trends Immunol. 22, 372-377.

Yeang, H. Y. 2004. Natural rubber latex allergens: new developments. Curr. Opin. Allergy Clin. Immunol. 4, 99-104.

Yeang, H. Y., Cheong, K. F., Sunderasan, E., Hamzah, S., Chew, N. P., Hamid, S., Hamilton, R. G. & Cardosa, M. J. 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, 628-639.

Yeang, H. Y., Ward, M. A., Zamri, A. S., Dennis, M. S. & Light, D. R. 1998. Amino acid sequence similarity of Hev b 3 to two previously reported 27- and 23-kDa latex proteins allergenic to spina bifida patients. Allergy 53, 513-519.

Yu, P., Kosco-Vilbois, M., Richards, M., Kohler, G. & Lamers, M. C. 1994. Negative feedback regulation of IgE synthesis by murine CD23. Nature 369, 753-756.

Zahnd, C., Spinelli, S., Luginbuhl, B., Amstutz, P., Cambillau, C. & Pluckthun, A. 2004. Directed in vitro evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. J. Biol. Chem. 279, 18870-18877.

Zucker-Pinchoff, B. & Stadtmauer, G. J. 2002. Latex allergy. Mt Sinai J. Med. 69, 88-95.

ORIGINAL PAPERS

I

THE MAJOR CONFORMATIONAL IGE-BINDING EPITOPES OF HEVEIN (HEV B6.02) ARE IDENTIFIED BY A NOVEL CHIMERA-BASED ALLERGEN EPITOPE MAPPING STRATEGY

by

Karisola, P., Alenius, H., Mikkola, J., Kalkkinen, N., Helin, J., Pentikäinen, O. T., Repo, S., Reunala, T., Turjanmaa, K., Johnson, M. S., Palosuo, T. & Kulomaa, M. S. 2002

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II

CONSTRUCTION OF HEVEIN (HEV B 6.02) WITH REDUCED ALLERGENICITY FOR IMMUNOTHERAPY OF LATEX ALLERGY BY COMUTATION OF SIX AMINO ACID RESIDUES ON THE CONFORMATIONAL IGE EPITOPES

by

Karisola, P., Mikkola, J., Kalkkinen, N., Pentikäinen, O. T., Repo, S., Reunala, T., Turjanmaa, K., Johnson, M. S., Palosuo, T., Kulomaa, M. S. & Alenius, H.
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III

ISOLATED HEVEIN-LIKE DOMAINS BUT NOT 31 KDA ENDOCHITINASES ARE RESPONSIBLE FOR IGE-MEDIATED *IN VITRO* AND *IN VIVO* REACTIONS IN LATEX-FRUIT SYNDROME

by

Karisola, P., Kotovuori, A., Poikonen, S., Niskanen, E., Kalkkinen, N., Turjanmaa, K., Palosuo, T., Reunala, T., Alenius, H., Kulomaa, M. S.

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