universität wien

DISSERTATION

Titel der Dissertation

Mechanisms of chronic allergic inflammation

angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr. rer.nat.)

Verfasserin / Verfasser:	**Raffaela Campana**
Matrikel-Nummer:	0648318
Dissertationsgebiet (lt. Studienblatt):	441 Genetik-Mikrobiologie
Betreuerin / Betreuer:	**Univ.-Prof. Dr. Rudolf Valenta**

Wien, September 2009

Diese Arbeit wurde an der Medizinischen Universität Wien in der Forschungsgruppe von Prof. Dr. Rudolf Valenta, Abteilung für Immunpathologie, Institut für Pathophysiologie, Zentrum für Physiologie, Pathophysiologie und Immunologie, durchgeführt.

Acknowledgements

I acknowledge the people who have been involved and contributed to the formation of the understanding of the approach presented in this thesis. In particular, I wish to express my gratitude to my supervisor, Professor Dr. Rudolf Valenta for his encouragement and suggestions during this work.

I would like also to include my gratitude to my colleagues at Institute of Pathophysiology for sharing their valuable experiences, support, and insights. Finally, I want to thank my family for the encouragement and for a never-ending support.

Contents

Zusammenfassung

Typ I Allergie, eine genetisch determinierte Hypersensitivitätsreaktion, ist charakterisiert durch die Produktion von IgE Antikörpern gegen *per se* harmlose Antigene, auch Allergene (z.B.: inhalative Allergene) genannt. Die Sensibilisierung gegen respiratorische Allergene findet bereits früh in der Kindheit statt und führt zu allergischen Immunreaktionen. Nicht behandelt können milde Ausprägungen von Allergien (z.B.: Allergische Rhinokonjunktivitis) zu schweren Symptomen, wie Allergischem Asthma und Atopischer Dermatitis führen. Die zentrale Rolle von IgE Antikörpern in der Allergenerkennung und in der Aktivierung von Effektorzellen ist gut untersucht. Jedoch sind die immunologischen Mechanismen, die zu chronischen allergischen Manifestationen wie Atopischer Dermatitis, einer chronischen entzündlichen Hauterkrankung, führen, noch nicht vollständig geklärt. Einerseits scheinen IgE Antikörper an diesen chronischen Manifestationen beteiligt zu sein, da gezeigt werden konnte, dass IgE Antikörper Allergene T-Zellen präsentieren und dadurch eine starke T-Zell-Antwort induzieren. Andererseits wurde demonstriert, dass nicht-IgE-bindende Allergen-basierende Peptide T-Zellen aktivieren und dadurch Spätphasereaktionen hervorrufen können.

Anhand des Modellsystems Birkenpollenallergie, einer der häufigsten respiratorischen Allergien in Nord- und Mitteleuropa, und anhand des Birkenpollenhauptallergens, Bet v 1, liegt ein Schwerpunkt der Dissertation darin, die Beteiligung von IgE- und nicht-IgE-mediierten Effekten an chronischen allergischen Entzündungsreaktionen zu analysieren. Der zweite Teil der Arbeit untersucht Ansätze zur Verbesserung der Immuntherapie von Birkenpollenallergie.

Das erste Kapitel der Dissertation belegt die wichtige Rolle, die nicht-IgE-mediierte Reaktionen in chronischen allergischen Hautentzündungsreaktionen in Patienten mit Atopischer Dermatitis spielen. Das zweite Kapitel untersucht die biochemischen und immunologischen Eigenschaften eines „besonderen“ hypoallergenen Bet v 1 Derivats, nämlich des Bet v 1 Trimers. Das dritte Kapitel beschreibt die Anwendung einer neuen Strategie, die auf der molekularen Wiederzusammenfügung von Allergen Fragmenten beruht, zur Entwicklung eines hypoallergenen Bet v 1 Derivats. Dieses Derivat könnte zur verbesserten Immuntherapie von Birkenpollenallergie eingesetzt werden.

Summary

Type I allergy, a genetically determined hypersensitivity disease is characterized by the production of IgE antibodies against *per se* harmless antigens, called allergens (e.g., inhalant allergens). Allergic sensitization to respiratory allergens takes place early in life leading to an allergic immune response. Untreated, mild form of respiratory allergy (e.g., allergic rhinoconjunctivitis) can progress to severe manifestations such as allergic asthma or atopic dermatitis. The central role of IgE antibodies in the allergen-recognition and subsequent activation of effector cells is well established. However, the underlying pathomechanisms involved in chronic allergic manifestations such as atopic dermatitis, a chronic inflammatory skin disorder, are not completely understood. One the one hand, IgE antibodies seems to be involved in these chronic manifestations because it has been demonstrated that IgE facilitates the presentation of allergens to T cells resulting in a strong T cell activation. On the other hand it has also been shown that allergen-derived peptides without IgE binding capacity can activate T cells and induce late inflammatory responses.

Taking birch pollen allergy, one of the most common forms of respiratory allergy in northern and middle Europe, and the major birch pollen allergen, Bet v 1, as model systems, one part of this thesis focuses on the contribution of IgE- versus non-IgE-mediated effects to chronic allergic inflammation, and the second part investigates approaches for improving immunotherapy of birch pollen allergy.

The first chapter of the thesis provides evidence for an important role of non-IgE-mediated reactions in chronic allergic skin inflammation in patients with atopic dermatitis. The second chapter investigates the biochemical and immunological characteristics of an unusual hypoallergenic derivative of Bet v 1, i.e., a recombinant

Bet v 1 trimer, and the third part describes the application of a strategy based on rational molecular reassembly for conversion of Bet v 1 into hypoallergenic derivative molecules, which can represent potential candidates for immunotherapy of birch pollen allergy.

Aims of the thesis

Atopic dermatitis is a chronic inflammatory skin disorder affecting about 3-10% of individuals suffering from IgE-mediated allergies. It is well established that allergen-recognition by IgE antibodies initiates acute allergic manifestation by activation of mast cells and basophils. However the contribution of IgE-mediated versus non-IgE-mediated mechanisms to chronic allergic inflammation is a matter of controversy. A role of IgE-mediated mechanisms has been show by the fact that IgE-facilitated presentation of allergens leads to strong T cell activation. In contrast it has also been shown that non-IgE-reactive peptides can induce allergic inflammation by activation of T-cells and in that way can induce T cell-dependent late phase inflammatory responses.

The aim of this thesis was to use the defined recombinant Bet v 1 allergen and the non-IgE-reactive recombinant Bet v 1 fragments, produced by genetic engineering, to dissect the contribution of IgE- versus non-IgE-mediated mechanisms to chronic allergic inflammation. For this purpose, in **chapter 2** the fully IgE-reactive recombinant Bet v 1 (aa 1-160) molecule and the non-IgE-reactive rBet v 1 fragments (Bet v 1aF1: aa 1-73; Bet v 1aF2: aa 74-160) were used for *in vivo* skin prick testing and atopy patch testing in birch pollen allergic patients with and without atopic dermatitis. This study provides evidence for an important role of non-IgE-mediated reactions in chronic allergic skin inflammation in patients with atopic dermatitis, a finding that may have implications for the selection of therapy strategies.

Moreover, this thesis aimed for the generation, purification and characterization of hypoallergenic derivatives of the major birch pollen allergen, Bet v 1, for improvement of specific immunotherapy.

In **chapter 3**, the biochemical and immunological behavior of an unusual hypoallergenic derivative of Bet v 1, i.e., a recombinant Bet v 1 trimer, was investigated. The results revealed that in contrast to all other previously described hypoallergenic derivatives, where reduction of the allergic activity has been obtained by destruction of IgE epitopes, the Bet v 1 trimer represents a hypoallergenic allergen derivative due to an altered presentation and/or orientation of IgE epitopes.

Chapter 4 describes the application of a strategy based on rational molecular reassembly for conversion of Bet v 1 into hypoallergenic derivative molecules. We generated three hypoallergenic rBet v 1 derivatives and demonstrated that immunization with these reassembled molecules, which lacked IgE reactivity and inhibited allergenic activity, could induce IgG antibodies which recognized Bet v 1 and strongly inhibited patients' IgE binding to Bet v 1. These results demonstrated that our hypoallergenic rBet v 1 derivatives represent promising candidates for the treatment of birch pollen allergy. The strategy we used may not only contribute to the generation of new allergy vaccines but may also be applied for induction of mucosal tolerance, for gene therapy and genetic immunization protocols.

Declaration on the contribution to the manuscripts described in chapter 5 and chapter 6

I contributed to both manuscripts (see Chapter 5 and 6) with the measurement of levels of cytokines induced *in vitro* cell culture stimulation assays. Cytokine levels were measured using the xMAP Luminex fluorescent bead-based technology and fluorescence signals were read on Luminex 100 system. The xMAP Luminex technology is based on color-code beads, known as microspheres, with two fluorescent dyes. These microspheres are then read in a compact analyzer, the Luminex 100 System, using multiple lasers and high-speed digital-signal processors. I established this assay for the analysis of cytokines because of my interest in toxic T cell products that my be involved in chronic allergic inflammation.

Chapter 5 describes the principle of fusing nonallergenic allergen-derived peptides to viral carrier proteins for the generation of allergy vaccines, that may also protect against viral infections. The rhinovirus-derived protein VP1, which represents the surface protein involved in rhinovirus infections of respiratory cells, was used for the construction of a recombinant combination vaccine for allergy and rhinovirus infections. This study demonstrated that VP1-based vaccines may be useful for therapy of asthma caused by rhinovirus infections and allergens.

Chapter 6 investigated in a *in vitro* system the integrity and barrier function of the respiratory epithelium upon exposure to smoke. The study demonstrated that cigarette smoke reduced the barrier function of the respiratory epithelium and enabled penetration of allergens across respiratory epithelium. It was therefore concluded that cigarette smoke may contribute to the observed increase of allergic diseases.

1. Introduction

1.1 History of allergology

Although allergic diseases is described by some authors as “the epidemic of the 21st century”, allergic reactions and classical symptoms of allergies (i.e., swelling of the nasal mucosa, redness and itching of the conjunctiva, urticaria, abdominal pain, coughing and sneezing) are known for a very long time. It was in 1656 that the French physician Pierre Borel conducted the first scientific allergy test placing some egg material on the skin of one of his patient, which he suspected to be allergic to egg. Consequently blisters appeared on the skin of the patient. In 1819 a British pysician, John Bostock, described the affection of eyes and chest caused by fresh hay as *Catarrhus aestivus* and this later gave origin to the term hay fever. In 1873 another British doctor named Charles Blackley (Figure 1) could demonstrate that pollen plays a important role in the pathogenesis of allergy. To investigate his own hayfever, Blackley applied pollen through a small break in his skin and demonstrated that pollen extract could induce wheal and flare skin response. Moreover, he also demonstrated that pollen grains placed into the nose could induce rhinitis. In 1903 Portier and Richet found that immunization of dogs with increasing doses of the jellyfish *Physalia* toxin could sensitize them so that a second injection of the same protein would cause breathing difficulty, influx of fluid into the lungs and death. The term anaphylaxis was used by them to describe this opposite phenomenon of prophylaxis. Later it became clear that anaphylaxis occurs when allergens enter into the circulation of patients with immediate hypersensitivity.

It was in 1906 that the Austrian pediatrician Clemens von Pirquet (1874-1929) (Figure 2) introduced the term “allergy”, coined from the greek words “allos” meaning “changed” and “ergos” meaning “action”, as an “altered capacity of the body to react

to a foreign substance“ [1]. Currently, allergy is defined as a “hypersensitivity reaction initiated by specific immunologic mechanisms” [2]. In 1911, Leonard Noon and John Freeman developed the method of desensitisation defined as “consisting injecting increasing doses of an extract of pollen subcutaneously until the hypersensitivity reaction was diminished or abolished” [3], and helped to establish the basis for immunotherapy. In 1921, Prausnitz and Kuestner provided the first direct evidence that immediate skin hyperreactivity could be passively transferred by transferring serum from a fish allergic individual to a non-allergic person, which later presented skin reaction after administration of fish extract. Thus they postulated that an allergen-specific serum factor “reagin” exists. It took until 1967 for the factor referred as “reagin” to be biochemically characterized by Kimishige and Teruko Ishizaka (Figure 3) and to be classified as a new class of immunoglobulin, the immunoglobulin E (IgE). In 1937, Daniel Bovet demonstrated that antihistamines protect against symptoms of anaphylaxis and later in 1948 Philip Hench and Edward Kendall introduced corticosteroids in the treatment of asthma and both immediate and delayed allergic reactions, significantly improving the quality of live of allergic patients. In 1953, James Riley and Geoffrey West demonstrated that mast cell degranulation is the major source of histamine in the body and contributed to the understanding of inflammatory and allergic reactions.

In 1963, Coombs and Gell classified allergy into four types according to the pathophysiological mechanisms involved [4]:

Type I or immediate hypersensitivity: characterized by the production of IgE antibodies (see 1.2.1);

Type II or antibody-mediated hypersensitivity: occurs when IgG or IgM antibodies bind surface antigens present on cells of the body leading to the

destruction of the cell by activating complement or by antibody-dependent cytotoxicity;

Type III or immune complex disease: occurs when excess immune complexes are formed in the circulation that cannot be cleared by macrophages or other cells, resulting in inflammation and tissue destruction;

Type IV or delayed-type hypersensitivity: a T cell-mediated inflammatory response rather than antibody responses to antigens.

One of the recent important event in the history of allergy was the identification of leukotrienes by Begnt Samuelsson (Figure 4), that play key roles in allergy, asthma and inflammatory diseases.

The understanding of pathomechanisms underlying hypersensitivity reactions has increased significantly during the last decades [5] and its mechanisms are still extensively studied because many diseases related to allergy have increased in Western world over the past decades.

Figure 1. Charles Blackley performed allergy test by putting pollen directly into a cut in his skin.

Figure 2. Clemens von Pirquet coined the term “allergy”.

Figure 3. Kimishige Ishizaka (left) and Teruko Ishizaka (right) characterized immunoglobulin E in the serum of allergic patients.

Figure 4. Begnt Samuelsson discovered the leukotrienes.

1.2 Allergic disease

1.2.1 IgE-mediated Type I allergy

IgE-mediated allergies, a type I hypersensitivity with complex genetic background caused by IgE responses to harmless environmental antigens, affect approximately 25 % of the population in industrialized countries [5, 6]. In sensitized allergic patients IgE recognition of allergens, which *per se* are generally harmless antigens, leads to the formation of IgE allergen immune complexes that activate a variety of immune cells involved in allergic inflammation [7-9]. Allergic inflammation can occur in different target organs when an allergen crosslinks IgE antibodies bound to the high-affinity Fc receptor (FcεRI) on the mast cells resulting in the release of biological mediators (e.g., histamine) and pro-inflammatory cytokines, and causes symptoms of allergic rhinoconjunctivitis, asthma, dermatitis, food allergy and life-threatening systemic anaphylaxis, depending on the routes of allergen exposure and the type of allergens against which patients are sensitized. This immediate allergic reaction caused by mast-cell degranulation appears shortly after contact to the allergen and can be followed by late-phase responses, which may ensue some hours later and involve recruitment of other effector cells, in particularly Th2 lymphocytes, eosinophils and basophils (see 1.2.4). In addition, IgE also plays a important role in the pathogenesis of atopic dermatitis (see 1.2.5).

1.2.2 The role of allergens

An allergen is defined as an “antigen causing allergic disease” [2] and the question “What makes an antigen an allergen?” has not been satisfactorily answered yet. Allergens may be derived from different sources (e.g., pollen, food, animal dander, mites, moulds, venoms) and mainly represent highly water-soluble proteins or

glycoproteins with molecular masses between 5-80 kDa, however no common structure or biological properties could be identified which would predict their allergenic nature [7]. Much effort has been put into the characterization in terms of structural, molecular and immunological properties of some important allergens, for example Bet v 1 from birch pollen (*Betula verrucosa*), because pure and well characterized allergen preparations are prerequisites for allergy diagnosis and successful immunotherapy [10-12]. The induction of IgE responses by antigens is related to the concentration, the route of entry, or the particle size in case of aeroallergens [13, 14]. Additionally, epitope mapping of respiratory allergens (e.g., pollens) indicated that IgE recognizes mainly conformational epitopes [7].

Natural and recombinant allergens

Natural allergen extracts are usually used for allergy diagnosis and therapy. With the development of molecular biological techniques it became possible to produce allergens as recombinant proteins. Recombinant allergens should equal their purified natural counterparts, when used for diagnostics purposes. Recombinant allergens have the advantage over at the natural allergens that they can be produced as single and high purity proteins, on large scale and at reasonable costs [15].

1.2.3 Allergic sensitization and memory responses to allergens

Primary responses to allergens mainly develop during the first few years after birth and the respiratory tract seems to play an important role for sensitization to inhaled allergens (e.g., allergens from pollen) [16-18]. This primary response is known as sensitization (Figure 5a).

Mouse models indicate that professional antigen presenting cells (APC) (e.g., dendritic cells, B cells and macrophages) present in the airways, capture the antigens, process it into small peptides and present them on MHC molecules to T cells, and thus induce the primary response [19]. In case of B lymphocytes, the antigen is captured via specific membrane-bound immunoglobulin and presented directly to specific T cells. As a result of sensitization, specific-IgE antibodies to allergens are produced.

Allergic sensitization (primary response) involves the switch from allergen-specific IgM to IgE-producing B cells and leads to the establishment of allergen-specific IgE antibody response (memory response) [20]. Moreover, after sensitization allergic patients contain long-lived memory T cells.

1.2.4 Allergic reaction is divided into an immediate response and late-phase response

It is well established that acute as well as chronic inflammation is mediated by allergen-specific IgE antibodies [21]. Allergic inflammation is initiated when an allergen crosslinks preformed IgE antibodies bound to the high-affinity Fc receptors (FcεRI) on the mast cells. The inflammatory response after IgE-mediated mast-cell activation occurs as an immediate reaction (immediate type inflammation). The immediate reaction takes place within minutes of allergen provocation and is caused by mast cell degranulation, resulting in the release of mediators including histamine, serotonin, lipid mediators, proteases, chemokines, and cytokines [22] (Figure 5b).

This immediate reaction can be followed by a more severe inflammation known as a late phase response, which occurs 4-12 hours after antigen exposure and involves the recruitment of other effector cells, in particularly Th2 lymphocytes, eosinophils

and basophils [20]. These late phase reactions are mainly observed in certain allergic individuals that suffer from severe and chronic manifestations of allergy (e.g., atopic dermatitis and chronic allergic asthma), and are caused by the presentation of allergens to T cells leading to activation, proliferation and release of proinflammatory cytokines (e.g., IL-1, IL-6, IFN-gamma, TNF-alpha) [23] (Figure 5c).

The immediate and late phase reaction can be clinically distinguished. The immediate reaction is due to the activity of mediators (e.g., histamine, prostaglandines) that cause increase in vascular permeability and contraction of smooth muscles, whereas the late reaction is associated with a second phase of smooth muscle contraction, sustained edema, and cardinal features of allergic asthma [24].

Continuing allergen exposure can stimulate allergen-specific Th2 cells, which promote eosinophilia and further IgE production (see 1.2.5), and lead to chronic inflammatory response.

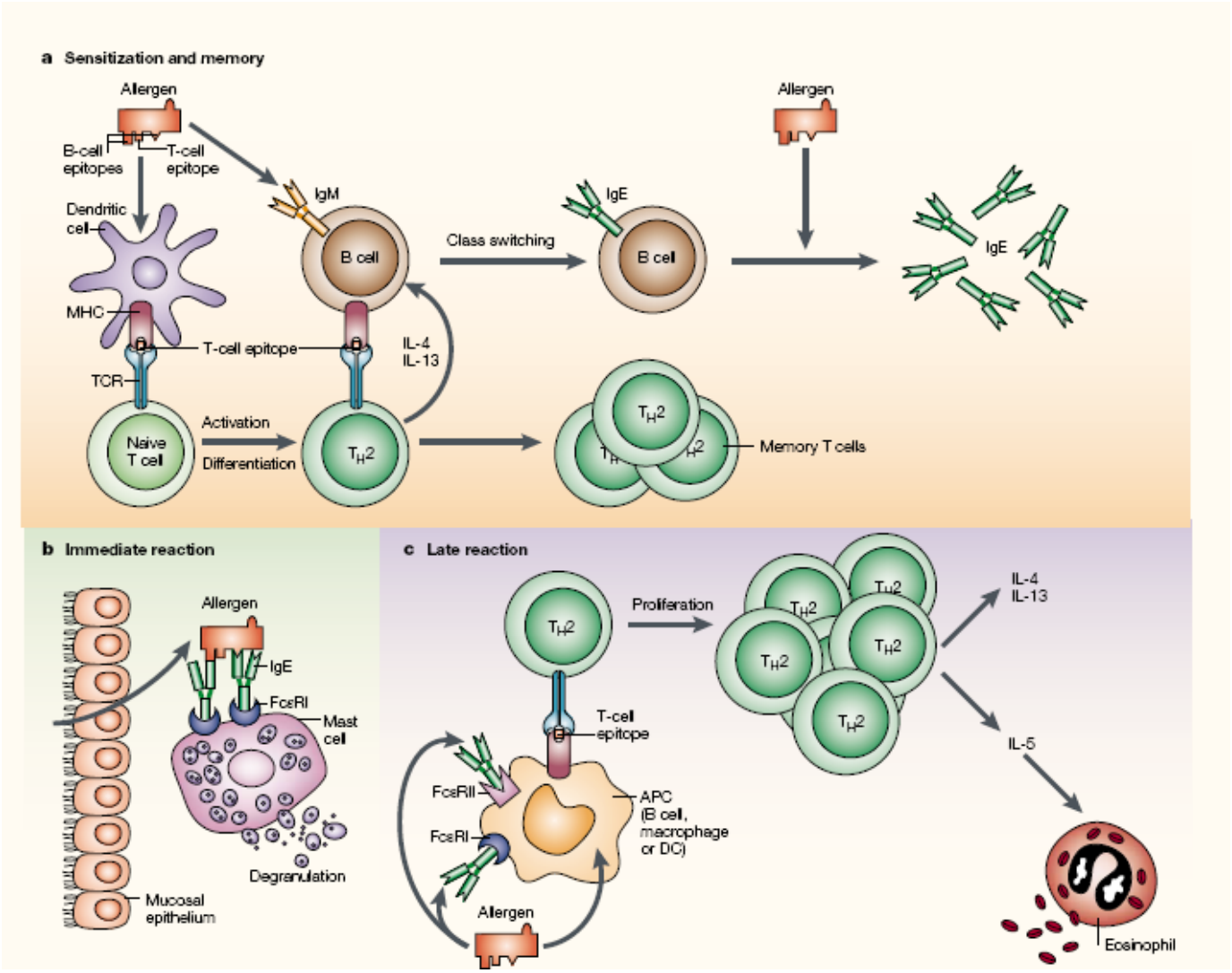


Figure 5. Induction of allergy by allergens. **(a)** Allergic sensitization and memory response, **(b)** immediate allergic reaction, **(c)** late phase allergic reaction [20].

1.2.5 Atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin disorder affecting about 3-10 % of individuals suffering from IgE-mediated allergies [25, 26]. The term atopic dermatitis was introduced by Hill and Sulzberger [27] due to the association between AD and respiratory allergy. The major clinical features of AD are appearance and distribution of eczematous lesions, dry skin, papules accompanied by intense pruritus, and cutaneous hyperreactivity (Table I) [28], and its histological characteristics are spongiosis, hypoerkeratosis, epidermal hyperplasia, and a perivascular infiltrate consisting mainly of T cells, macrophages, monocytes and antigen-presenting cells (APCs) [28, 29]. AD thus resembles many features of a delayed type hypersensitivity.

AD can be delineated in two forms: extrinsic or allergic form, affecting 70-90 % of AD patients, and intrinsic or nonallergic form accounting for 10-30 % of AD cases (Figure 6). The allergic subtype occurs in the context of sensitization towards environmental allergens and is accompanied by elevated serum IgE levels, whereas the nonallergic subtype is accompanied by low levels of serum IgE and the absence of detectable sensitization [30].

The observation that patients with T-cell immunodeficiency disorders frequently have elevated IgE levels and skin lesions that are indistinguishable from AD suggested a immunoregolatory basis in AD (Table II) [28] and today it is known that atopic dermatitis is characterized by an initial Th2 cytokines phase that switches to a secondary and more chronic Th1 eczematous phase [25].

Table I. Diagnostic features of AD [28]

Major features
• Pruritus and excoriations
• Typical appearance and distribution of skin lesions
Facial and extensor involvement in infancy and early childhood
Flexural involvement and lichenification by adolescence
• Chronic or frequently relapsing course (duration >6 weeks)
• Personal or family history of AD, allergic rhinoconjunctivitis, food allergy, or asthma
Minor features
• Increased susceptibility to skin infections, particularly *S. aureus*
• Xerosis (dryness of the skin)
• Early age of onset
• Multiple positive immediate prick skin test results
• Ichthyosis, keratosis pilaris, hyperlinearity of palms
• Nonspecific hand/foot dermatitis
• Scalp dermatitis (e.g., cradle cap)
• Elevated serum IgE levels

Table II. Immunologic features of AD [28]

• Increased IgE production
• Immediate skin test reactivity to multiple allergens
• Increased basophil spontaneous histamine release
• Decreased CD8 suppressor/cytotoxic number and function
• Increased expression of CD23 on mononuclear cells
• Chronic macrophage activation with increased secretion of GM-CSF, PGE_2, and IL-10
• Expansion of IL-4– and IL-5–secreting T_{H2}-like cells
• Decreased numbers of IFN-γ–secreting T_{H2}-like cells

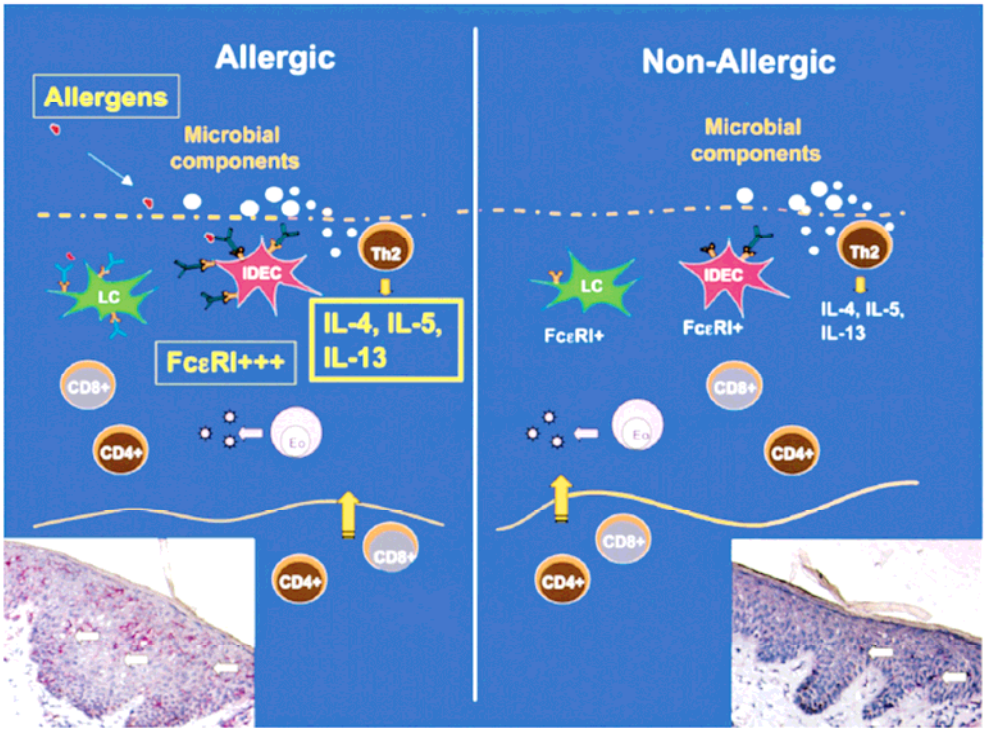


Figure 6. The differences between the allergic form of AD and the nonallergic form of AD [25].

Several family history and genetic-epidemiologic studies has demonstrated that AD has a high level of genetic background [31]. However, population studies showed that the prevalence of AD has been increasing over the last 20-30 years and thus, besides genetic factors environmental risk factors are also proposed in order to explain the rapid increase frequency of atopic disorders [32]. Several “candidate” genes (e.g., IL-4, IL-4R α-chain, IL-13 and CD14 genes) involved in Th1/Th2 cell balance have been identified and linked to the AD phenotype [33, 34, 25]. Moreover, according to the hygienic hypothesis, influences from the environment and exposure to microbial pathogens at the early years of life can protect against the development of AD by shifting the balance between Th1/Th2 cells towards a Th1 response [35]. Furthermore, factors of the modern lifestyle (i.e., the use of antibiotics and increase in adherence

to hygienic rules, what consequently leads to less bacterial contact) might limit Th1 response and support Th2 predominance in the immune system [36-38].

Although the basis of allergen-induced acute and late-phase responses is well understood, immune mechanisms regulating persistence of local tissue T cell activation and inflammation in chronic allergic skin diseases is not well established. Taken together, several studies indicate that "long-term allergen, microbial antigen or superantigen exposure, Th2 cell stimulation, allergen-specific IgE production, mast cell degranulation, eosinophil infiltration, and inflammation amplified by keratinocyte injury caused by scratching" [28] are involved and contribute to chronic allergic skin inflammation in individuals suffering from AD (Figure 7).

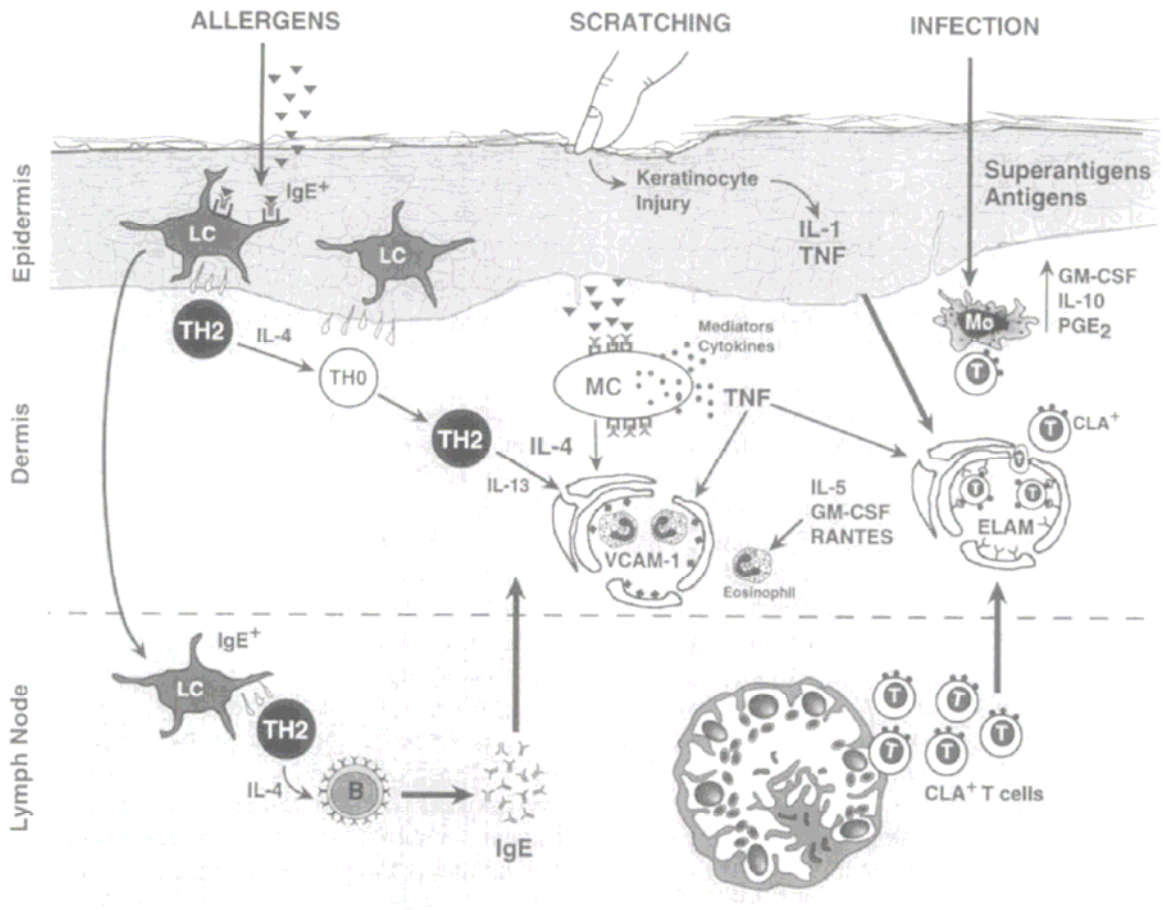


Figure 7. Pathogenesis of atopic dermatitis (AD) [30].

The role of IgE in AD is an important aspect for the understanding of pathomechanisms of chronic allergic skin inflammation. It is assumed that the penetration of allergens into the epidermis is facilitated because of the reduced skin barrier function in AD patients [39, 40]. It is well established that allergen-recognition by IgE antibodies initiates acute allergic manifestation by activation of mast cells and basophils [5]. However, the contribution of IgE-mediated versus non-IgE-mediated mechanisms to chronic allergic inflammation still remains a matter of controversy.

A role of IgE-mediated mechanisms in late-phase reaction has been suggested by the fact that IgE-facilitated presentation of allergens leads to strong T cell activation (Figure 8) [12, 41, 42]. Already in 1990 the importance of cell-bound IgE on epidermal Langerhans' cells for the presentation of allergens in patients with atopic dermatitis has been demonstrated [43]. Three years later, a study with house dust mite AD patients found that allergen presentation is mediated by IgE [44] and a further study showed in 1996, that a structural variant of the high affinity receptor for IgE (FcεRI), containing FcεRI-α and FcεRI-β chains, is expressed on peripheral blood dendritic cells population and that those cells use the FcεRI receptor for IgE-mediated allergen presentation [45]. Moreover, it has been shown that AD patients benefit from treatment with Omalizumab, an anti-human IgE antibody which binds free IgE without cross-linking IgE that is already bound to FcεRI receptors [46].

On the other hand, it has been shown that non-IgE-reactive peptides can induce allergic inflammation by activation of T-cells and can also induce T cell-dependent late phase inflammatory responses [47]. These studies demonstrated that allergens can activate T cells in an IgE-independent manner and that these allergen-specific T cells play a important role in the induction of late-phase symptoms (Figure 8) [12].

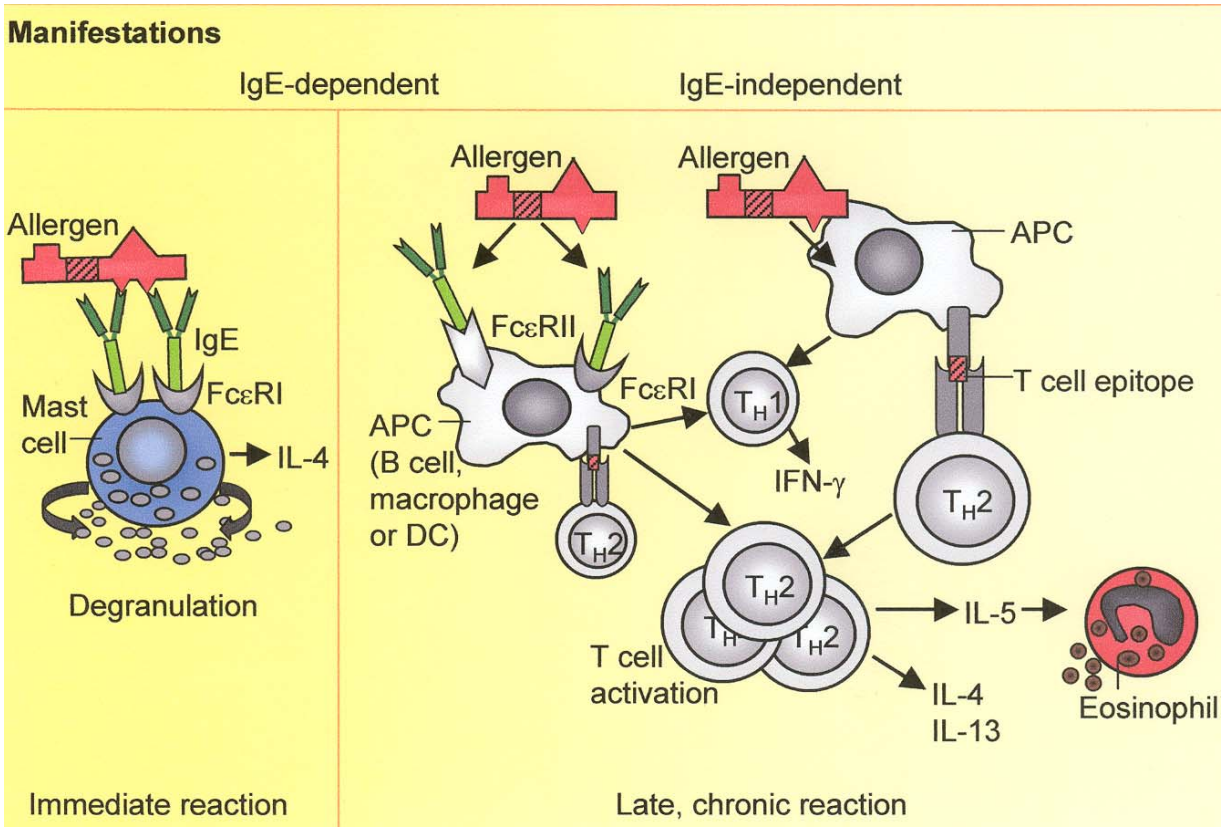


Figure 8. Mechanisms of allergic inflammation [12].

1.3 Allergy diagnosis and treatment

1.3.1 Diagnosis

The primary method for diagnosis of Type I hypersensitivity is based on the detection of serum allergen-specific IgE antibodies levels (*in vitro*) [48] and on the elicitation of immediate symptoms by *in vivo* provocation testing (e.g., skin testing, nasal provocation) [49].

Skin prick test (SPT) is the most common *in vivo* method used in clinical practice, because of its simplicity, safety and low costs. Moreover SPT is a rather quick method, taking 5-15 minutes to develop a skin response, which may persist for 30 minutes. The characteristic response is a wheal and flare formation. The wheal is caused by extravasation of serum from capilaries in the skin and it is accompanied by pruritus and large erythematous flare mediated by axon reflex [50]. The SPT is evaluated by the size of the wheal. A positive SPT indicates that the individual have specific-IgE antibodies on mast cells in their skin for the substance tested.

While immediate-type reactions can easily be identified by patients history, including the family history, by measurement of serum IgE levels, and by skin prick testing, the evaluation of the role of aeroallergens (e.g., pollens) and food allergy in patients with atopic dermatitis presents diagnostic difficulties. However, in recent years the atopy patch test (APT) has been established as a useful tool for the diagnosis of food allergy and evaluation of eczematous skin reactions because of its high predictive capacity for late phase reaction [51, 52]. APT is an epicutaneous patch test employing allergens known to elicit IgE-mediated reactions. According the European Task Force on Atopic Dermatitis (ETFAD), its reactions are read as: -, negative result; ?, only erythema, questionable; +, erythema, infiltration; ++, erythema, few papules (up to 3); +++, erythema, papules from 4 to < many; ++++, erythema, many or spreading

papules; +++++, erythema, vesicles [52]. A positive patch response induces macroscopic eczema, spongiosis and infiltrate of the cells into the dermis including basophils, eosinophils and lymphocytes. Biopsy of patch tests permit to analyse the presence of antigen-specific T cells in the skin after the challenge.

1.3.2 Allergen-specific immunotherapy

Allergen-specific immunotherapy (SIT) represents the only disease-modifying approach for IgE-mediated allergies and is based on the repeated administration of sensitizing allergen by subcutaneous or sublingual application [11, 53]. The allergens are usually adjuvant-adsorbed (e.g., aluminum hydroxide). Its mechanisms, which are not yet fully understood, include the induction allergen-specific IgG antibodies, which inhibit IgE recognition of allergens and thus block allergen-induced allergic inflammation and possibly affect immunoregulation [11, 53-55].

Although SIT is currently recognized as effective treatment for type I allergies, three major problems need to be overcome. First, immunotherapy is performed with allergen extracts containing mixtures of allergenic and non-allergenic which are difficult and in case of many allergen sources impossible to be standardize. Second, the administration of allergens can cause IgE-mediated side-effects including urticaria, allergic reactions, asthma and anaphylactic shock and third, because of the poor quality of the extracts and because of the side-effects therapeutically effective doses cannot be achieved [20, 54, 56, 57].

1.3.3 Recombinant allergens and hypoallergenic allergens for treatment

Although allergen extracts are currently used in allergy therapy, it has been shown that when used for vaccination they induce heterogenous immune responses due to

the different representation and immunogenicity of the specific allergens in the allergen extract [58]. Furthermore, the systemic administration of allergens during the course of the allergen-specific immunotherapy (SIT) can cause side effects.

Recombinant allergens have been shown to be useful tools for diagnosis of allergy once they maintain comparable immunological features to natural allergen [59]. Recombinant Bet v 1 has been recently used for vaccination of birch-allergic patients and demonstrated to be safe and effective [60].

In order to overcome IgE-mediated side effects several research groups have developed technologies for modifying the IgE reactivity of allergens by recombinant DNA technology [20], and hypoallergenic forms of allergens have been developed. Recombinant hypoallergenic allergen derivatives represent promising tools for diagnosis and therapy of type I allergy. Recombinant hypoallergenic molecules provide the advantage to lack IgE reactivity and should not cause immediate type symptoms, but they still have the ability to induce allergen-specific protective IgG antibody responses. Furthermore they preserve allergen-specific T cell epitopes and thus T cell modulatory activities are retained [61].

1.4 Birch pollen allergy

The white birch (*Betula verrucosa*) is considered as a major pollen-allergen-producing tree in northern Europe [62]. Birch is one of the main causes of Type I reactions, such as allergic bronchial asthma and allergic rhinoconjuctivitis, from spring to early summer also in middle Europe, North America, Australia and Asia.

Birch pollen allergy is mainly mediated by one major allergen, Bet v 1, which is recognized by 95 % of birch allergic individuals and up to 60 % of such patients react exclusively to Bet v 1 [63, 64]. In addition Bet v 1 is a cross-reactive allergen, which contains most of the IgE epitopes present in pollens of trees belonging to the *Fagales* order and in plant-derived food [65, 66]. The major birch pollen allergen, Bet v 1, comprises 160 amino acid residues [64] and is a 17 kDa protein.

With the availability of allergen-encoding cDNAs it became possible to express Bet v 1 in *Escherichia coli* as recombinant Bet v 1. Cloning, sequence analyses and expression of Bet v 1 as a recombinant protein have facilitated the characterization of this allergen [64, 67, 68]. Several studies showed that recombinant Bet v 1 presents molecular and immunological equivalence to natural Bet v 1. It has been demonstrated that Bet v 1 is equivalent to natural Bet v 1 in IgE-binding capacity, the ability to induce *in vitro* proliferation of allergen-specific T-cell clones, and the biological activity of Bet v 1 has been further demonstrated by skin prick testing and by the ability to release histamine from basophils derived from birch pollen-allergic individuals. Moreover, the recombinant Bet v 1 has been tested and compared to the Bet v 1 wild type in several *in vivo* studies including skin prick testing and vaccination for treatment of birch pollen allergic patients [59, 60, 69-71]. In a primate model study, recombinant Bet v 1 could induce type I allergy. The study demonstrated that monkeys immunized with recombinant Bet v 1 showed positive skin reactions to rBet

v 1 and allergen extracts from birch, alder and hazel pollen, which contain Bet v 1 homologous proteins [70]. Further *in vivo* characterization of potential allergy vaccines based on Aluminium hydroxide adsorbed rBet v 1 derivatives in mice showed that strong IgG1 and IgG2a/b response was induced by repeated vaccination with rBet v 1 [72].

Thus Bet v 1 represents a very well-characterized allergen, which could demonstrated its efficacy in diagnosis of birch sensitization and for allergen-specific immunotherapy.

Birch pollen specific immunotherapy

On the basis of recombinant-DNA technology it has become possible to modify important allergens, such as Bet v 1, and novel treatment strategies for specific immunotherapy (SIT) have been proposed. The development of allergy vaccines based on recombinant hypoallergenic allergen derivatives has been well advanced for birch pollen allergy because the major birch pollen allergen, Bet v 1, represents one of the first cloned allergens and because birch pollen allergy represents one of the most common forms of respiratory allergy in northern and middle Europe.

Various different approaches have been developed for the generation of hypoallergenic variants of Bet v 1 and three have given promising results in SIT studies: fragmentation, oligomerization (rBet v 1 trimer) and chemical denaturation (Bet v 1 fold variant) [73-75]. The rBet v 1 fragments and rBet v 1 trimer have been extensively studied for their reduced allergenic nature in skin prick provocation testing in humans before they have been used for vaccination of birch pollen allergic patients in immunotherapy studies [73, 74, 76, 77].

The rBet v 1 fragments (F1: 1-74 and F2: 75-160), lacked IgE reactivity and exhibit strongly reduced or lacking allergenic activity (i.e., basophil activation and induction of immediate skin reactions) [73].

Treatment of birch pollen allergic patients with aluminium hydroxide adsorbates of Bet v 1 fragments and Bet v 1 trimer, could induced protective IgG antibodies which inhibited allergen-induced release of inflammatory mediators. The reduction of cutaneous sensitivity as well as the improvement of symptoms in actively treated patients were associated with the development of Bet v 1-specific IgG antibodies [78].

Although there were basically no IgE-mediated side effects observed in the patients who were treated with hypoallergenic Bet v 1 derivatives, late phase side effects were observed [79].

1.5 References

1. Von Pirquet C. Allergie. Munch Med Wochenschr 1906; **53**:1457.
2. Johansson SG, Bieber T, Dahl R, Friedmann PS, Lanier BQ, Lockey RF, Motala C, Ortega Martell JA, Platts-Mills TA, Ring J, Thien F, Van Cauwenberge P, Williams HC. Revised nomenclature for allergy for global use: Report of the nomenclature review committee of the world allergy organization, J Allergy Clin Immunol 2004; **113:**832-836.
3. Jackson M. John Freeman, hay fever and the origins of clinical allergy in Britain, 1900-1950. Stud Hist Phil Biol & Biomed Sci 2003; **34:**473-490.
4. Gell PGH, Coombs RRA. *Clinical Aspects of Immunology*. London: Blackwell Publishing, 1963.
5. Kay AB, Bousquet J, Holt PG and Kaplan AP. *Allergy and Allergic Diseases*. Blackwell Publishing, 2008.
6. Flöistrup H, Swartz J, Bergström A, Alm JS, Scheynius A, van Hage M, Waser M, Braun-Fahrländer C, Schram-Bijkerk D, Huber M, Zutavern A, von Mutius E, Ublagger E, Riedler J, Michaels KB, Pershagen G, The Parasifal Study Group. Allergic disease and sensitization in Steiner school children. J Allergy Clin Immunol 2006; **117:**59-66.
7. Valenta R, Kraft D. Recombinant allergen molecules: tools to study effector cell activation. Immunol Rev 2001; **179:**119-127.
8. Bischoff SC. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. Nat Rev Immunol 2007; **7:**93-104.
9. Holgate ST, Polosa R. Treatment strategies for allergy and asthma. Nat Rev Immunol 2008; **8:**218-230.

10. Valenta R, Kraft D. From allergen structure to new forms of allergen-specific immunotherapy. Curr Opin Immunol 2002; **14:**718-727.

11. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol 1998; **102:**558-562.

12. Valenta R, Ball T, Focke M, Linhart B, Mothes N, Niederberger V, Spitzauer S, Swoboda I, Vrtala S, Westritschnig K, Kraft D. Immunotherapy of allergic disease. Adv Immunol 2004; **82:**105-153.

13. Constant SL, Lee KS, Bottomly K. Site of antigen delivery can influence T cell priming: pulmonary environment promotes preferential Th2-type differentiation. Eur J Immunol 2000; **30:**840-847.

14. McCusker C, Chicoine M, Hamid Q, Mazer B. Site-specific sensitization in a murine model of allergic rhinitis: role of the upper airway in lower airways disease. J Allergy Clin Immunol 2002; **110:**891-898.

15. Kraft D, Ferreira F, Vrtala S, Breiteneder H, Ebner C, Valenta R, Susani M, Breitenbach M, Scheiner O. The importance of recombinant allergens for diagnosis and therapy of IgE-mediated allergies. Int Arch Allergy Immunol 1999; **118:**171-176.

16. Wahn U, Lau B, Bergmann R, Kulig M, Forster J, Bergmann K, Bauer CP, Guggenmoos-Holzmann I. Indoor allergen exposure is a risk factor for sensitization during the first three years of life. J Allergy Clin Immunol 1997; **99:**763-769.

17. Kulig M, Bergmann R, Klettke U, Wahn V, Tacke U, Wahn U. Natural course of sensitization to food and inhalant allergens during the first 6 years of life. J Allergy Clin Immunol 1999; **103**:1173-1179.

18. Niederberger V, Niggemann B, Kraft D, Spitzauer S, Valenta R. Evolution of IgM, IgE and IgG (1-4) antibody responses in early childhood monitored with recombinant allergen components: implications for class switch mechanisms. Eur J Immunol 2002; **32:**576-584.

19. Lambrecht BN. Allergen uptaken and presentation by dendritic cells. Curr Opin Allergy Clin Immunol 2001; **1:**51-59.

20. Valenta R. The future of antigen-specific immunotherapy of allergy. Nat Rev Immunol 2002; **2:**446-453.

21. Kinet JP. The high-affinity IgE receptor (FcεRI): from physiology to pathology. Annu Rev Immunol 1999; **17:**931-972.

22. Kawakami T, Galli SJ. Regulation of mast-cell and basophil function and survival by IgE. Nat Rev Immunol 2002; **2:**773-786.

23. Cieslewicz G, Tomkinson A, Adler A, Duez C, Schwarze J, Takeda K, Larson KA, Lee JJ, Irvin CG, Gelfand EW. The late, but not early, asthmatic response is dependent on IL-5 and correlates with eosinophil infiltration. J Clin Invest 1999; **104:**301-308.

24. Janeway CA, Travers P, Walport M, Shlomchik M. *Immunobiology 5 - The Immune system in health and disease*. Garland Publishing, 2001.

25. Novak N, Bieber T, Leung DYM. Immune mechanism leading to atopic dermatitis. J Allergy Clin Immunol 2003; **112:**128-139.

26. Wollenberger A, Kraft S, Oppel T, Bieber T. Atopic dermatitis: Pathogenetic mechanisms. Clin Exp Dermatol 2000; **25:**530-534.

27. Hill LW, Sulzberger MB. *Yearbook of dermatology and syphilology*. Chicago: Year Book Medical Publishers, 1933.

28. Leung DYM. Atopic dermatitis: The skin as a window into the pathogenesis of chronic allergic diseases. J Allergy Clin Immunol 1995; **96:**302-319.

29. Wollenberger A, Bieber T. Atopic dermatitis: from the genes to skin lesions. Allergy 2000; **55:**205-213.

30. Ring J, Przybilla B, Ruzicka T. *Handbook of Atopic Eczema*. Springer Publishing, 2006.

31. Coleman R, Trembath RC, Harper JI. Genetic studies of atopy and atopic dermatitis. Br J Dermatol 1997; **136:**1-5.

32. Ring J, Kramer U, Schafer T, Behrendt H. Why are allergies increasing?. Curr Opin Immunol 2001; **13:**701-708.

33. Wills-Karp M, Ewart SL. Time to draw breath:asthma-susceptibility genes are identified. Nat Rev Genet 2004; **5:**376-387.

34. Vercelli D. Genetic polymorphism in allergy and asthma. Curr Opin Immunol 2003; **15:**609-613.

35. Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: revisiting the hygiene hypothesis. Nat Rev Immunol 2001; **1:**69-75.

36. van Bever HP. Early events in atopy. Eur J Pediatr 2002; **161:**542-546.

37. Prescott SL, Macaubes C, Yabuhara A, Venaille TJ, Holt BJ, Habre W, Loh R, Sly PD, Holt PG. Developing patterns of T cell memory to enviromental allergens in the first two years of life. Int Arch Allergy Immunol 1997; **113:**75-79.

38. Prescott SL, Macaubes C, Holt BJ, Smallacombe TB, Loh R, Sly PD, Holt PG. Transplacental priming of the human immune system to enviromental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile. J Immunol 1998; **160:**4730-4737.

39. McGrath JA, Uitto J. The filaggrin story: novel insights into skin-barrier function and disease. Trends Mol Med 2008; **14:**20-27.

40. O'Regan GM, Sandilands A, McLean WH, Irvine AD. Filaggrin in atopic dermatitis. J Allergy Clin Immunol 2009; **124:**2-6.

41. Bieber T. FcεRI on antigen-presenting cells. Curr Opin Immunol 1996; **8:**773-777.

42. Stingl G, Maurer D. IgE-mediated allergen presentation via Fc epsilon on antigen-presenting cells. Int Arch Allergy Immunol 1997; **113:**24-29.

43. Mudde GC, Van Reijsen FC, Boland GJ, de Gast GC, Bruijnzeel PL, Bruijnzeel-Koomen CA. Allergen presentation by epidermal Langerhans' cells from patients with atopic dermatitis is mediated by IgE. Immunology 1990; **69:**335-341.

44. van der Heijden FL, Joost van Neerven RJ, van Katwijk M, Bos JD, Kapsenberg ML. Serum-IgE-facilitated allergen presentation in atopic desease. J Immunol 1993; **150:**3643-3650.

45. Maurer D, Fiebiger S, Ebner C, Reininger B, Fischer GF, Wichlas S, Jouvin MH, Schmitt-Egenolf M, Kraft D, Kinet JP, Stingl G. Peripheral blood dendritic cells express Fc epsilon RI as a complex composed of Fc epsilon RI alpha- and Fc epsilon RI gamma-chains and can use this receptor for IgE-mediated allergen presentation. J Immunol 1996; **157:**607-616.

46. Lane JE, Cheyney JM, Lane TN, Kent DE, Cohen DJ. Treatment of recalcitrant atopic dermatitis with omalizumab. J Am Acad Dermatol 2006; **54:**68-72.

47. Haselden BM, Kay AB, Larché M. Immunoglobulin E-independent Major Histocompatibility Complex-restricted T cell peptide epitope-induced late asthmatic reactions. J Exp Med 1999; **189:**1885-1894.

48. Wide L, Bennich H, Johansson SG. Diagnosis of allergy by an in vitro test for allergen antibodies. Lancet 1967; **2:**1105-1107.

49. Niederberger V, Stubner P, Spitzauer S, Kraft D, Valenta R, Ehrenberger K, Horak F. Skin test results but not serology reflect immediate type respiratory sensitivity: a study performed with recombinant allergen molecules. J Invest Dermatol 2001; **117:**848-851.

50. Roitt I, Brostoff J, Male D. *Immunology Sixth Edition*. Mosby Publishing, 2001.

51. Darsow U, Laifaoui J, Kerschenlohr K, Wollenberg A, Przybilla B, Wuthrich B, Borelli S Jr, Giusti F, Seidenari S, Drzimalla K, Simon D, Disch R, Borelli S, Devillers AC, Oranje AP, De Raeve L, Hachem JP, Dangoisse C, Blondeel A, Song M, Breuer K, Wulf A, Werfel T, Roul S, Taieb A, Bolhaar S, Bruijnzeel-Koomen C, Bronnimann M, Braathen LR, Didierlaurent A, Andre C, Ring J. The prevalence of positive reactions in the atopy patch test with aeroallergens and food allergens in subjects with atopic eczema: a European multicenter study. Allergy 2004; **59:**1318-1325.

52. Ring J, Darsow U, Gfesser M, Vieluf D. The atopy patch test in evaluating the role of aeroallergens in atopic eczema. Int Arch Allergy Immunol 1997; **113:**379-383.

53. Durham SR, Walker SM, Varga EM, Jacobson MR, O'Brien F, Noble W, Till SJ, Hamid QA, Nouri-Aria KT. Long-term clinical efficacy of grass-pollen immunotherapy. N Engl J Med 1999; **341:**468-475.

54. Larché M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol 2006; **6:**761-771.

55. Durham SR, Till SJ. Immunologic changes associated with allergen immunotherapy. J Allergy Clin Immunol 1998; **102:**157-164.

56. Winther L, Arnved J, Malling HJ, Nolte H, Mosbech H. Side-effects of allergen-specific immunotherapy: a prospective multi-centre study. Clin Exp Allergy 2006; **36:**254-260.

57. Ferreira F, Wallner M, Breiteneder H, Hartl A, Thalhamer J, Ebner C. Genetic engineering of allergens: Future therapeutic products. Int Arch Allergy Immunol 2002; **128:**171-178.

58. Mothes N, Heinzkill M, Drachenberg KJ, Sperr WR, Krauth MT, Majlesi Y, Semper H, Valent P, Niederberger V, Kraft D, Valenta R. Allergen-specific immunotherapy with a monophosphoryl lipd A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. Clin Exp Allergy 2003; **33:**1198-1208.

59. Pauli G, Oster JP, Deviller P, Heiss S, Bessot JC, Susani M, Ferreira F, Kraft D, Valenta R. Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: diagnostic value for birch pollen and associated allergies. J Allergy Clin Immunol 1996; **97:**100-1109.

60. Pauli G, Larsen TH, Rak S, Horak F, Patorello E, Valenta R, Purohit A, Arvidsson M, Kavina A, Schroeder J, Mothes N, Spitzauer S, Montagut A, Galvain S, Melac M, André C, Poulsen L, Malling HJ. Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. J Allergy Clin Immunol 2008; **122:**951-960.

61. Linhart B, Valenta R. Molecular design of allergy vaccines. Curr Opin Immunol 2005; **17:**646-655.

62. Eriksson NE, Holmen A. skin prick test with standardized extracts of inhalant allergens in 7099 adult patients with asthma or rhinitis cross-sensitizations and relationship to age, sex, month of birth and year of testing. J Invest Allergol Clin Immunol 1996; **6:**36-46.

63. Valenta R, Duchene M, Vrtala S, Birkner T, Ebner C, Hirschwehr R, Breitenbach M, Rumpold H, Scheiner O, Kraft D. Recombinant allergens for immunoblot diagnosis of tree-pollen allergy. J Allergy Clin Immunol 1991; **88:**889-894.

64. Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M. The gene coding for the major birch pollen allergen Bet v I, is highly homologous to a pea disease resistance response gene. EMBO J 1989; **8:**1935-1938.

65. Kazemi-Shirazi L, Pauli G, Purohit A, Spitzauer S, Fröschl R, Hoffmann-Sommergruber K, Breiteneder H, Scheiner O, Kraft D, Valenta R. Quantitative IgE inhibition experiments with purified recombinant allergens indicate pollen-derived allergens as the sensitizing agents responsible for many forms of plant food allergy. J Allergy Clin Immunol 2000; **105:**116-125.

66. Niederberger V, Pauli G, Grönlund H, Fröschl R, Rumpold H, Kraft D, Valenta R, Spitzauer S. Recombinant birch pollen allergens (rBet v 1 and rBet v 2) contain most of the OgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen: a quantitative IgE inhibition study with sera from different populations. J Allergy Clin Immunol 1998; **102:**579-591.

67. Ferreira F, Hoffmann-Sommergruber K, Breiteneder H, Pettenburgert K, Ebner C, Sommergruber W, Steiner R, Bohle B, Sperr W, Valent P, Kungl A, Breitenbach M, Kraft D, Scheiner O. Purification and characterization of recombinant Bet v 1, the major birch pollen allergen – Immunological equivalence to natural Bet v 1. J Biol Chem 1993; 268:19574-80.

68. Hoffmann-Sommergruber K, Susani M, Ferreira F, Jertschin P, Ahorn H, Steiner R, Kraft D, Scheiner O, Breiteneder H. High-level expression and purification of the major birch pollen allergen, Bet v 1. Protein Expr Purif 1997; 9:33-39.

69. Vrtala S, Mayer Peter, Ferreira F, Susani M, Sehon A, Kraft D, Valenta R. Induction of IgE antibodies in mice and rhesus monkeys with recombinant birch pollen allergens: Different allergenicity of Bet v 1 and Bet v 2. J Allergy Clin Immunol 1996; 98:913-21.

70. Ferreira F, Mayer P, Sperr WR, Valent P, Seiberler S, Ebner C, Liehl E, Scheiner O, Kraft D, Valenta R. Induction of IgE antibodies with predefined specificity in rhesus monkeys with recombinant birch pollen allergens, Bet v 1 and Bet v 2. J Allergy Clin Immunol 2000; 165:6653-6659.

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

72. Ferreira F, Mayer P, Sperr W, Valent P, Seiberler S, Ebner C, Liehl E, Scheiner O, Kraft D, Valenta R. Induction of IgE antibodies with predefined specificity in rhesus monkeys with recombinant Bet v 1 and Bet v 2.J Allergy Clin Immunol 1996; **97:**95-103.

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

74. Vrtala S, Hirtenlehner K, Susani M, Akdis M, Kussebi F, Akdis CA, Blaser K, Hufnagl P, Binder BR, Politou A, Pastore A, Vangelista L, Sperr WR, Semper H, Valent P, Ebner C, Kraft D, Valenta R. Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen, Bet v 1. FASEB J 2001; **15:**2045-2057.

75. Kahlert H, Suck R, Weber B, Nandy A, Wald M, Keller W, Cromwell O, Fiebig H. Characterization of hypoallergenic recombinant Bet v 1 variant as a candidate for allergen-specific immunotherapy. Int Arch Allergy Immunol 2008; **145:**193-206.

76. van Hage-Hamsten M, Kronqvist M, Zetterström O, Johansson E, Niederberger V, Vrtala S, Grönlund H, Grönneberg R, Valenta R. Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1: results obtained with a mix of two recombinant Bet v 1 fragments and recombinant Bet v 1 trimer in a Swedish population before the birch pollen season. J Allergy Clin Immunol 1999; **104:**969-977.

77. Pauli G, Purohit A, Oster JP, De Blay F, Vrtala S, Niederberger V, Kraft D, Valenta R. Comparison of genetically engeneered hypoallergenic rBet v 1 derivatives with rBet v 1 wild-type by skin prick and intradermal testing: results obtained in a French population. Clin Exp Allergy 2000; **30:**1076-1084.

78. Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P, Reisinger J, Pelzmann M, Hayek B, Kronqvist M, Gafvelin G, Grönlund H, Purohit A, Suck R, Fiebig H, Cromwell O, Pauli G, van Hage-Hamsten M, Valenta R. Vaccination with genetically engineered allergens prevents progression of allergy disease. Proc Natl Acad Sci U S A 2004; **101:**14677-14682.

79. Purohit A, Niederberger V, Kronqvist M, Horak F, Grönneberg R, Suck R, Weber B, Fiebig H, van Hage M, Pauli G, Valenta R, Cromwell O. Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives. Clin Exp Allergy 2008; **38:**1514-1525.

Non-IgE-mediated chronic allergic skin inflammation revealed with rBet v 1 fragments

Campana Raffaela, Mothes N, Rauter I, Vrtala S, Reininger R, Focke-Tejkl M, Lupinek C, Balic N, Spitzauer S, Valenta R

Published

Journal of Allergy and Clinical Immunology, 2008

alternative for MRI procedures in these patients. However, the predictive value of skin testing could be established only by the further use of these contrast media and demonstration of an acceptable tolerance. This has not been pursued in these patients because of lack of indication for another MRI examination as well as ethical reasons.

These 2 cases of IgE-mediated anaphylaxis to Gd-DOTA underline the importance of an appropriate allergy assessment, principally skin tests, to document the drug's involvement.

Frédéric Hasdenteufel, PharmD[a]
Samuel Luyasu, MD[b]
Jean-Marie Renaudin, MD[a]
Jean-Louis Paquay, MD[b]
Giuseppe Carbutti, MD[b]
Etienne Beaudouin, MD[a]
Denise Anne Moneret-Vautrin, MD[a]
Gisèle Kanny, MD, PhD[a]

From [a]Equipe d'accueil 3999, Allergy Diseases: Diagnosis and Therapeutics, Department of Internal Medicine, Clinical Immunology and Allergology, University Hospital of Nancy, Nancy, France; and [b]the Department of Internal Medicine and Intensive Care, Clinic of South Luxemburg, Arlon, Belgium. E-mail: frederic.hasdenteufel@wanadoo.fr.

Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

REFERENCES

1. Herborn CU, Honold E, Wolf M, Kemper J, Kinner S, Adam G, et al. Clinical safety and diagnostic value of the gadolinium chelate gadoterate meglumine (Gd-DOTA). Invest Radiol 2007;42:58-62.
2. Li A, Wong CS, Wong MK, Lee CM, Au Yeung MC. Acute adverse reactions to magnetic resonance contrast media: gadolinium chelates. Br J Radiol 2006;79:368-71.
3. Weiss KL. Severe anaphylactoid reaction after i.v. Gd-DTPA. Magn Reson Imaging 1990;8:817-8.
4. Rahman SL, Harbinson M, Mohiaddin R, Pennell DJ. Acute allergic reaction upon first exposure to gadolinium-DTPA: a case report. J Cardiovasc Magn Reson 2005;7:849-51.
5. Meuli RA, Maeder P. Life-threatening anaphylactoid reaction after iv injection of gadoterate meglumine. Am J Roentgenol 1996;166:729.
6. Beaudouin E, Kanny G, Blanloeil Y, Guilloux L, Renaudin JM, Moneret-Vautrin DA. Anaphylactic shock induced by gadoterate meglumine (DOTAREM). Allerg Immunol (Paris) 2003;35:382-5.
7. Brockow K, Romano A, Blanca M, Ring J, Pichler W, Demoly P. General considerations for skin test procedures in the diagnosis of drug hypersensitivity. Allergy 2002;57:45-51.
8. Baxter AB, Lazarus SC, Brasch RC. In vitro histamine release induced by magnetic resonance imaging and iodinated contrast media. Invest Radiol 1993;28:308-12.
9. Moneret-Vautrin DA, Laxenaire MC. The risk of allergy related to general anesthesia. Clin Exp Allergy 1993;23:629-33.

Available online October 8, 2007.
doi:10.1016/j.jaci.2007.08.027

Non–IgE-mediated chronic allergic skin inflammation revealed with rBet v 1 fragments

To the Editor:

Atopic dermatitis (AD) is a chronic inflammatory skin disorder affecting about 3% to 10% of patients with IgE-mediated allergies.[1] The cardinal features of AD are eczematous and erythematous skin lesions, which are characterized by spongiosis, epidermal hyperplasia, and perivascular infiltrates consisting of T cells, monocytes, macrophages, and antigen-presenting cells. AD thus resembles many features of delayed-type hypersensitivity. The symptoms of AD are triggered in the vast majority of patients with AD by exogenous exposure to allergens (ie, extrinsic AD), among them food and aeroallergens.[2] External application of allergens to the skin by means of atopy patch tests (APTs) can also be used to elicit the characteristic eczematous skin lesions in patients with AD for diagnostic purposes.[2]

The contribution of IgE-mediated versus non–IgE-mediated mechanisms to chronic inflammation in patients with AD is a matter of discussion. Evidence for an important role of IgE-mediated mechanisms comes from the following findings. Patients with AD with high levels of IgE express higher levels of FcεRI on monocytes and dendritic cells, and it has been demonstrated *in vitro* that IgE-facilitated antigen presentation activates allergen-specific T cells more efficiently than allergen presentation through non–IgE-mediated mechanisms.[3] Furthermore, there are reports that certain patients with AD benefit from treatment with anti-human IgE antibodies.[4] On the other hand, skin manifestations in patients with AD can be improved by using therapy strategies targeting T cells in a relatively selective manner.[5] In fact, there is also evidence for non–IgE-mediated inflammation in other chronic manifestations of allergy, such as asthma.[6]

To study the contribution of IgE-mediated versus non–IgE-mediated mechanisms in chronic allergic skin inflammation, we used a purified IgE-reactive major allergen (ie, the birch pollen allergen Bet v 1 [molecular weight, 17.4 kDa])[7] and 2 non–IgE-reactive hypoallergenic fragments thereof (F1: amino acids 1-74 [molecular weight, 8 kDa]; F2: amino acids 75-160 [molecular weight, 9.4 kDa),[8] which together comprise the full T-cell epitope repertoire of the Bet v 1 allergen but lack IgE reactivity and allergenic activity (ie, basophil activation and induction of immediate-type skin reactions) for APTs in patients with AD. We performed skin prick tests and APTs in 5 patients with AD and birch pollen allergy, a patient with birch pollen allergy without skin manifestations, an allergic person without birch pollen allergy, and 2 non-allergic individuals using rBet v 1 or a mix of the 2 rBet v 1 fragments (F1: amino acids 1-74; F2: amino acids 75-160).

The demographic, clinical, and serologic characterization of the individuals is summarized in Table I. IgE reactivity to rBet v 1 and rBet v 1 fragments (F1 and F2) was tested in a nondenaturing dot-blot assay[8] and showed that each of the 6 patients with birch pollen allergy (patients A-F) contained rBet v 1–specific IgE (Fig 1 and Table I: 13->100 kUA/L), but none of them contained IgE specific for the Bet v 1 fragments F1 and F2 (Fig 1 and Table I).

Skin prick testing performed on the backs of the patients confirmed the results of the dot-blot experiments because each of the 6 patients with birch pollen allergy but none of the individuals without birch pollen allergy showed immediate-type skin reactivity to rBet v 1 (20 and 40 μg/mL) after 20 minutes (Table I and Fig E1 in the Online Repository at www.jacionline.org). None of the 6 patients with birch pollen allergy had immediate-type skin reactions to equimolar mixes of the rBet v 1 fragments, which was in agreement with the negative result from the IgE dot-blot experiment (Table I and see Fig E1). In parallel to skin prick tests, APTs were performed with aluminum cups (Finn Chambers on Scanpor, Large, Epitest Ltd Oy, Tuusula, Finland) containing 160 μg of rBet v 1 or a mix containing 80 μg of each rBet v 1 fragment, as previously described.[9] When the APT result was read and photo documented after 48 hours, we found that rBet v 1 induced a positive eczematous reaction of varying intensity in each of the patients with birch pollen allergy and AD (subjects A-E)

TABLE I. Demographic, serologic, and clinical characterization of individuals

Subject	Sex/ Age (y)	Allergies	Symptoms	Total IgE (kU/L)	IgE CAP (kUA/L)		IgE			SPT (mm^2)					APT	
										rBet v 1		F1+F2				
					Birch	rBet v 1	rBet v 1	F1	F2	20 μg/mL	40 μg/mL	20 μg/mL	40 μg/mL	Histamine	rBet v 1	F1+F2
A	M/53	b, a, g, pf, npf, mo, mi	AD, RC, AS, OAS	860	15.7	14.6	+	−	−	100	132	−	−	16.6	+++	−
B	M/41	b, pf	RC, AD	69.3	27.4	27.1	+	−	−	56	121	−	−	8	++	+++
C	M/45	b, a, g, pf, mi	RC, AD, OAS	114	25.6	20.2	+	−	−	156	210	−	−	72	+	+
D	M/54	b, a, g, pf, npf, mo, mi, w	RC, AD	>5000	>100	>100	+	−	−	90	81	−	−	64	++++	++
E	M/37	b, a, g, w	RC, AD, AS	747	58.3	13	+	−	−	81	90	−	−	42	+++	+++
F	F/44	b, a, g	RC, AS, OAS	466	57.5	60.5	+	−	−	110	132	−	−	30	−	−
G	F/36	a, g	RC	25.7	<0.35	<0.35	−	−	−	−	−	−	−	15.5	−	−
H	F/26	No	−	<2.00	<0.35	<0.35	−	−	−	−	−	−	−	11.2	−	−
I	F/32	No	−	2.65	<0.35	<0.35	−	−	−	−	−	−	−	10.6	−	−

Subjects were characterized by sex, age, and clinical diagnosis of allergies. IgE reactivity for rBet v 1 and rBet v 1 fragments (F1 and F2) was analyzed in a dot-blot assay and by means of IgE CAP.
M, Male; *F*, female; *SPT*, skin prick test; *b*, birch; *a*, animals; *g*, grass; *pf*, plant food; *npf*, non–plant-derived food; *mo*, molds; *mi*, mites; *w*, weeds; *RC*, rhinoconjunctivitis; *AS*, asthma; *OAS*, oral allergy syndrome.

but not in the patient with birch pollen allergy without AD (subject F), the allergic patient without birch pollen allergy (subject G), and the 2 nonallergic persons (subjects H and I; Table I and see Fig E1). The non–IgE-reactive rBet v 1 fragment mix induced an eczematous reaction in 4 of the 5 patients with birch pollen allergy and AD but not in any of the other persons (subjects G-I; Table I and see Fig E1). Results of control APTs performed with Vaseline petroleum jelly (Unilever, London, United Kingdom) alone were negative in each of the 3 patients (data not shown).

We also studied the occurrence of IgE-bearing cells in the peripheral blood of the patients using an anti-IgE antibody that can recognize FcεRI-bound IgE antibodies but did not find an association between the presence of IgE-reactive cells and the presence or absence of IgE-mediated versus non–IgE-mediated APT reactions.

Our finding that Bet v 1 fragments containing only T-cell epitopes but no IgE epitopes could induce chronic allergic skin inflammation in 4 of the 5 patients with AD indicates that this reaction is not IgE mediated. The fact that 1 patient with AD (subject A) had an APT reaction only with IgE-reactive Bet v 1 but not with the Bet v 1 fragments and that subject D had a stronger reaction with rBet v 1 than with the fragments might be explained by the dominance of IgE-mediated allergen presentation mechanisms in these subjects, the loss of a critical T-cell epitope in the rBet v 1 fragments, or both.

In summary, our experiments provide evidence for an important role of non–IgE-mediated reactions in chronic allergen-induced skin inflammation in patients with AD. Our results also indicate that recombinant allergen derivatives lacking IgE epitopes but containing allergen-derived T-cell epitopes might be used as test reagents to reveal the contribution of IgE-mediated versus non–IgE-mediated mechanisms in chronic allergic inflammation. This result might have implications for the selection of therapy strategies that are either targeting IgE-mediated or purely T cell–mediated mechanisms. Should larger clinical studies confirm the results of our pilot studies, such tests might aid the clinician in optimizing therapy strategies for chronic allergic inflammation.

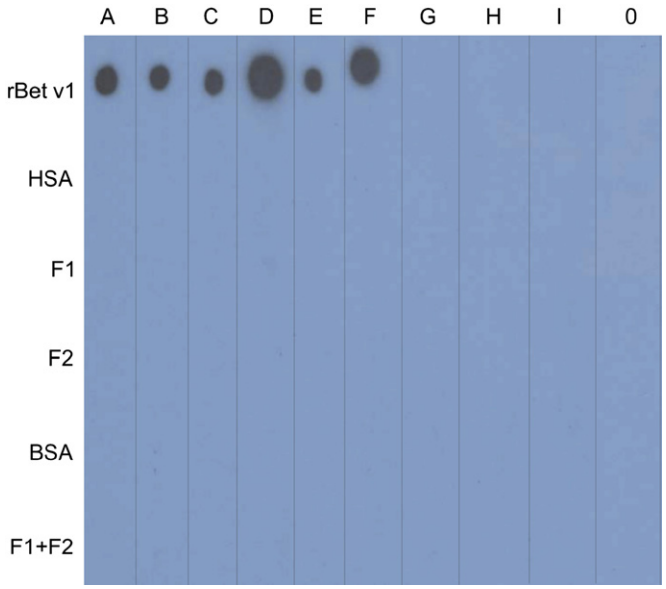


FIG 1. IgE reactivity. Dot-blotted antigens (rBet v 1, human serum albumin *[HSA]*, the rBet v 1 fragments F1 and F2, BSA, and an equimolar mix of rBet v 1 fragments *[F1+F2]*) were exposed to sera from the individuals listed in Table I *(A-I)* or buffer alone *(0)*. Bound IgE was detected and visualized by means of autoradiography.

Raffaela Campana, MSc[a]
Nadine Mothes, MD[a,b]
Ingrid Rauter, PhD[a]
Susanne Vrtala, PhD[a]
Renate Reininger, PhD[c]
Margarete Focke-Tejkl, PhD[a]
Christian Lupinek, MD[a]

Nadja Balic[c]
Susanne Spitzauer, MD[c]
Rudolf Valenta, MD[a]

From [a]the Division of Immunopathology, Department of Pathophysiology, Center of Physiology and Pathophysiology; [b]the Department of Dermatology; and [c]the Institute of Medical and Chemical Laboratory Diagnostics, Vienna General Hospital (AKH), Medical University of Vienna, Vienna, Austria. E-mail: rudolf.valenta@meduniwien.ac.at.

Supported by grants F1804 and F1815 of the Austrian Science Fund (FWF) and by the FFG, Vienna, Austria.

Disclosure of potential conflict of interest: R. Valenta has consulting arrangements with Biomay and Phadia. The rest of the authors have declared that they have no conflict of interest.

REFERENCES

1. Novak N, Bieber T, Leung DYM. Immune mechanisms leading to atopic dermatitis. J Allergy Clin Immunol 2003;112:128-39.
2. Darsow U, Laifaoui J, Kerschenlohr K, Wollenberg A, Przybilla B, Wuthrich B, et al. The prevalence of positive reactions in the atopy patch test with aeroallergens and food allergens in subjects with atopic eczema: a European multicenter study. Allergy 2004;59:1318-25.
3. Stingl G, Maurer D. IgE-mediated allergen presentation via Fc epsilon on antigen-presenting cells. Int Arch Allergy Immunol 1997;113:24-9.
4. Lane JE, Cheyney JM, Lane TN, Kent DE, Cohen DJ. Treatment of recalcitrant atopic dermatitis with omalizumab. J Am Acad Dermatol 2006;54:68-72.
5. Stuetz A, Baumann K, Grassberger M, Wolff K, Meingassner JG. Discovery of topical calcineurin inhibitors and pharmacological profile of pimecrolimus. Int Arch Allergy Immunol 2006;141:199-212.
6. Haselden BM, Kay AB, Larché M. Immunoglobulin E-independent major histocompatibility complex-restricted T cell peptide epitope-induced late asthmatic reactions. J Exp Med 1999;189:1885-94.
7. Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, et al. The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to a pea disease resistance response gene. EMBO J 1989;8:1935-8.
8. Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, et al. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments. J Clin Invest 1997;99:1673-81.
9. Ring J, Darsow U, Gfesser M, Vieluf D. The atopy patch test in evaluating the role of aeroallergens in atopic eczema. Int Arch Allergy Immunol 1997;113:379-83.

doi:10.1016/j.jaci.2007.09.014

Nasal nitric oxide as a noninvasive marker in the antibiotic treatment of acute bacterial sinusitis

To the Editor:

Acute bacterial sinusitis (ABS) is associated with inflammation of the sinuses. The resulting infection is from a bacterial growth in the fluid that accumulates when other conditions cause sinus blockage. Use of 2 major factors or 1 major and 2 minor factors for the clinical diagnosis of ABS is paramount.[1] Sampling with rhinoscopy from the maxillary sinuses for bacterial confirmation is the gold standard for confirming ABS,[2] but it is invasive and impractical at times.

Nitric oxide (NO) is a known noninvasive marker of inflammation that can be used to quickly measure airway inflammation. Sinus NO is found in the thousands range of parts per billion, which decreases to approximately half in the nose and by a factor of 100 in the lungs. NO is a product of constitutive and inducible NO synthase. Constitutive NO is protective against infection in respiratory airways. Although inducible NO levels are increased with inflammation in the lower airways, obstruction of the sinus ostia in sinusitis reduces the nasal NO level.[3,4]

We proposed to measure nasal NO levels in patients with ABS before and after antibiotic therapy. All patients were seen acutely for signs and symptoms of ABS with at least 3 major diagnostic factors. These patients presented to our allergy practice unscheduled and were not actively recruited. The mean age for the 15 patients (female patients, n = 12) was 33 years (range, 11-55 years). Seven of 15 patients have a history of chronic rhinosinusitis (CRS) defined by duration of their sinus disease, and 12 patients were atopic on the basis of history and skin prick test responses.

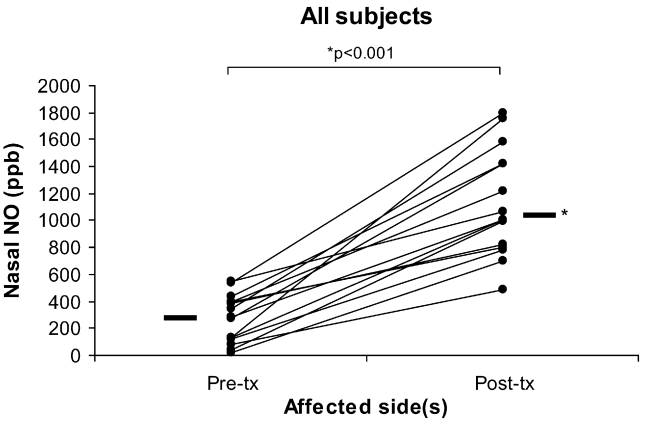


FIG 1. Pretreatment *(Pre-tx)* to posttreatment *(Post-tx)* NO levels in all subjects, with *black bars* denoting the median. *$P < .001$.

NO levels were measured online with an NO analyzer (NIOX, Aerocrine, Sweden) equipped with nasal NO software. Nasal NO levels were measured by means of chemiluminescence from a naris by using a nasal olive at a constant flow with the contralateral side open, as per American Thoracic Society guidelines.[3] The patient was instructed to inhale through the mouth to total lung capacity and breath hold for 20 seconds while humming.[5,6] Both sides were measured to compare differences from either side.

A computed tomographic (CT) scan of the sinuses was performed after the initial NO measurements and before initiation of antibiotic therapy. For bacterial coverage of their ABS, patients received, depending on their age and drug allergy history, oral amoxicillin-clavulanate, cephalosporin, or a quinolone for 5 to 14 days according to the prescribed course of therapy. NO levels were measured 2 weeks after the start of antibiotic therapy in the same manner. Patients were examined by the physician before and after treatment to confirm the diagnosis and response to therapy. Nasal medications that were being used before the ABS were continued, and no other new medications were initiated in addition to the antibiotic regimen.

The mean NO level was 275 ± 178 ppb before antibiotics and increased to 1126 ± 395 ppb after antibiotic treatment (Fig 1). In patients without CRS, the mean change of improvement in NO level was +1114 ppb after antibiotic therapy (mean postantibiotic NO value, 1400 ppb), although in patients with CRS, the mean change of improvement in NO was +550 ppb after antibiotic therapy (mean postantibiotic NO value, 811 ppb).

Ten of the 15 patients studied had lower levels of NO in the naris, which is consistent with their unilateral ABS symptoms and CT scan findings. The remaining 5 patients with bilateral ABS had bilateral NO levels decreased before treatment and later confirmed by means of CT scans. There was no significant difference between the naris in the bilateral ABS group. Nasal NO

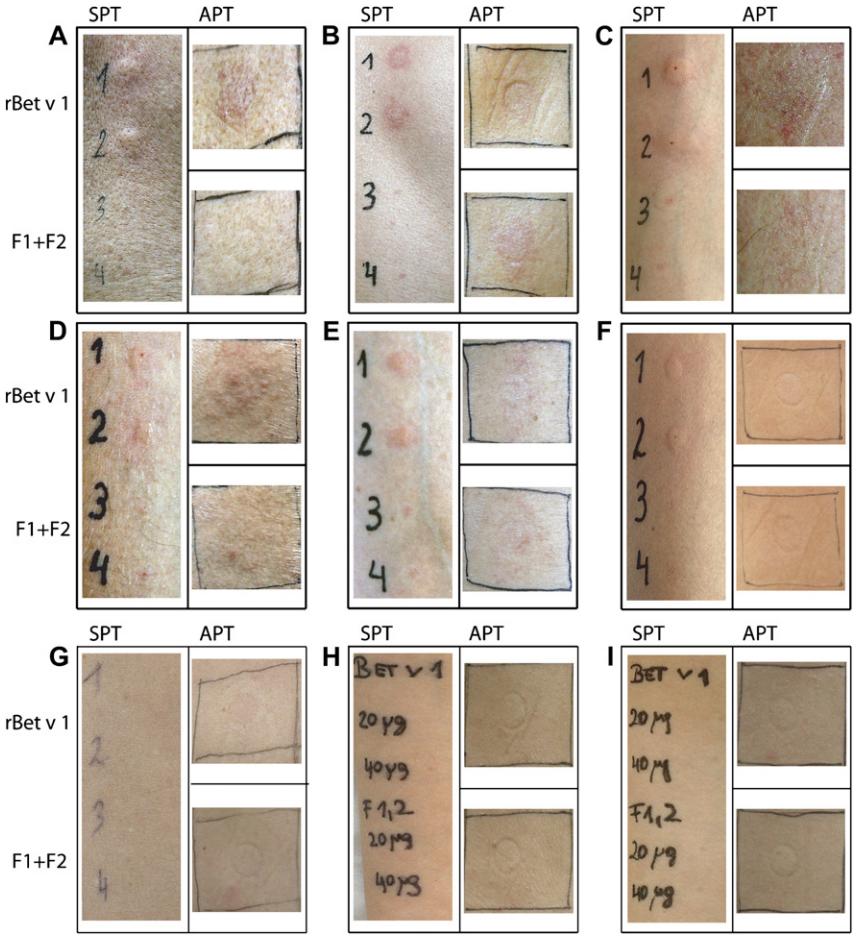


FIG E1. Immediate- and delayed-type skin reactions to rBet v 1 and rBet v 1 fragments. Skin prick tests *(SPT)* and APTs were performed with rBet v 1 (*1* and *2*) and rBet v 1 fragments (*3* and *4*) in 5 patients with AD (***A***-***E***) and in 1 individual with rhinoconjunctivitis (***F***) with birch pollen allergy, 1 allergic patient without birch pollen allergy (***G***), and 2 nonallergic individuals (***H*** and ***I***). Skin prick tests were performed with 2 antigen concentrations of 20 μg/mL (*1* and *3*) or 40 μg/mL (*2* and *4*). APTs were performed with 160 μg of rBet v 1 or with a mix containing 80 μg of each rBet v 1 fragment (F1+F2).

Altered IgE epitope presentation: A novel mechanism for hypoallergenicity revealed for Bet v 1 trimer

Campana Raffaela, Vrtala S, Maderegger B, Dall'Antonia Y, Blatt K, Focke-Tejkl M, Swoboda I, Scheiblhofer S, Gieras A, Neubauer A, Keller W, Valent P, Thalhamer J, Spitzauer S, Valenta R

Submitted

2009

Altered IgE epitope presentation: A novel mechanism for hypoallergenicity revealed for Bet v 1 trimer

R. Campana [1], S. Vrtala [1], B. Maderegger [2], Y. Dall'Antonia [3], K. Blatt [4], M. Focke-Tejkl [1,5], I. Swoboda [1,5], S. Scheiblhofer [6], A. Gieras [1], A. Neubauer [2], W. Keller [3], P. Valent [4], J. Thalhamer [6], S. Spitzauer [7], R. Valenta [1,5,*]

[1]Division of Immunopathology, Department of Pathophysiology, Center of Physiology and Pathophysiology, Vienna General Hospital (AKH), Medical University of Vienna, Austria; [2] Biomay AG, Vienna Competence Center, Austria; [3] Division of Structural Biology, Institute of Chemistry, Karl Franzens University of Graz, Graz, Austria; [4] Division of Hematology and Hemostaseology, Department of Internal Medicine I, Vienna General Hospital (AKH), Medical University of Vienna, Austria; [5] Christian Doppler Laboratory for Allergy Research, Vienna General Hospital (AKH), Medical University of Vienna, Austria; [6] Christian Doppler Laboratory for Allergy Diagnosis & Therapy, Department of Molecular Biology, University of Salzburg, Austria; [7] Institute of Medical and Chemical Laboratory Diagnostics, Vienna General Hospital (AKH), Medical University of Vienna, Austria

Key words: immunotherapy, protein aggregation, recombinant hypoallergenic molecules, rBet v 1

Abstract

Background: In contrast to other recombinant hypoallergenic allergen derivatives which showed reduced IgE reactivity, a recombinant trimer of the major birch pollen allergen Bet v 1 showed reduced allergenic activity despite preserved IgE reactivity.

Methods: We studied rBet v 1 trimer by SDS-PAGE, mass spectrometry, circular dichroism and gel filtration. Furthermore we investigated IgE and IgG reactivity of the rBet v 1 trimer in solid and liquid phase assays and compared its allergenic activity with that of rBet v 1 wildtype using basophil activation assays.

Results: In solid phase immunoassays rBet v 1 trimer exhibited even stronger IgE reactivity than the rBet v 1 monomer, whereas both proteins were equally well recognized by Bet v 1-specific IgG antibody probes. In fluid phase IgE experiments rBet v 1 trimer inhibited IgE reactivity to rBet v 1 wildtype but showed a more than 10-fold reduced allergenic activity compared to the rBet v 1 monomer. By analytical gel filtration it was demonstrated that, despite its monomeric appearance in SDS-PAGE the trimer occurred in fluid phase in the form of defined high molecular weight (>600 kDa) aggregates whereas rBet v 1 wildtype strictly appeared as monomeric protein.

Conclusion: The results indicate that the hypoallergenic nature of the rBet v 1 trimer is due to formation of defined high molecular weight aggregates which may be responsible for an altered presentation of IgE epitopes in a form with reduced capacity to crosslink effector-cell bound IgE.

Introduction

Allergen-specific immunotherapy (SIT) represents the only antigen-specific and disease-modifying treatment approach for IgE-mediated allergy (1-3). However, the administration of allergens in the course of immunotherapy can induce local and systemic inflammation and in the worst case severe systemic and life-threatening side effects (4, 5).

In order to overcome the most severe type of side effects, i.e., systemic life-threatening anaphylaxis, which is caused by IgE-mediated mast cell and basophil degranulation, technologies have been developed for the reduction of IgE reactivity of allergy vaccines (6). Already forty years ago David Marsh and colleagues developed chemically modified allergen extracts which exhibited reduced IgE reactivity but at the same time could induce allergen-specific IgG antibodies and retained allergen-specific T cell epitopes (7). These chemically modified allergen preparations have been designated “allergoids” and until today these or similar preparations are the active ingredients of many routinely used allergy vaccines. Based on allergen-encoding sequences, several research groups have started to develop hypoallergenic allergen derivatives based on recombinant DNA technology (6, 8).

All hypoallergenic allergen derivatives exhibited reduced IgE reactivity and the reduced allergenic activity thus resulted from this reduction of IgE reactivity.

One exception to this rule is a recombinant trimer of the major birch pollen allergen, Bet v 1, which has been obtained by the expression of three copies of the cDNA coding for Bet v 1 (9). The recombinant Bet v 1 trimer exhibited a strongly reduced ability to induce IgE-mediated basophil activation, showed reduced induction of immediate type skin reactions and nasal reactivity and has been used in high doses for immunotherapy of birch pollen allergic patients (10, 11).

Surprisingly, despite its reduced allergenic activity, rBet v 1 trimer was found to exhibit IgE reactivity compared to the rBet v 1 wildtype allergen (9).

In this study we conducted a series of protein-chemical, structural and immunological experiments to re-investigate the unusual reduction of allergenic reactivity of the rBet v 1 trimer.

Methods

Plasmids, patients' sera and antibodies

The plasmids expressing rBet v 1 and rBet v 1 trimer are described (12, 9).

Birch pollen allergic patients (n = 11) were characterized by case history and skin prick testing. Specific IgE levels to birch pollen extract and rBet v 1 were determined by immuno CAP measurements (Phadia, Uppsala, Sweden) as described (13). Control serum was taken from a non-allergic volunteer with no history of birch pollen allergy, lack of skin reactivity and birch pollen-specific IgE. IgE reactivity testing and basophil activation experiments were done with serum samples and cells obtained from the same birch pollen allergic patients. Specific polyclonal rabbit Abs against the purified rBet v 1, rBet v 1 trimer and against two rBet v 1 fragments (F1 and F2) as described (9, 14). Monoclonal mouse IgG Abs against peptide 2 (mAb#2) comprising amino acids 30-59 of Bet v 1 and against peptide 6 (mAb#12) comprising amino acids 74-104 of Bet v 1 were obtained by immunization of mice using KLH-coupled synthetic peptides (peptide 2: LFPKVAPQAISSVENIEGNGGPPTIKKISF; peptide 6: EDVHTNFKYNYSVIEGGPIGDTLEKISNEIK). Monoclonal Bet v 1-specific antibody, Bip 1, is described (15). The mouse monoclonal antibody 4A6 was raised against purified recombinant birch pollen profilin (16). Anti-IgE mAb E-124.2.8 was obtained from Immunotech (Marseille, France). Chimeric Bip 1, an IgE monoclonal antibody with specificity for Bet v 1, was generated and purified as described (17).

Expression and purification of recombinant allergens

Recombinant Bet v 1 (batch # 21) and grass pollen allergen, Phl p 5 (batch # 04), were purchased from Biomay (Vienna, Austria).

Recombinant Bet v 1 trimer was expressed in *E.coli* BL 21 (DE3) (Stratagene, La Jolla, CA, USA) (Supplementary information 1).

SDS-PAGE and immunoblotting

Purified rBet v 1 and rBet v 1 trimer (5 μg protein/slot) were separated by 12.5 % SDS-PAGE in the presence or absence of 2-mercaptoethanol (18). Proteins were visualized by staining with Coomassie Brilliant Blue.

IgG and IgE-binding capacity of purified rBet v 1 and rBet v 1 trimer were also tested by Western blotting (Supplementary information 2).

Mass spectrometry and Circular dichroism

Mass spectrometry was done as described (Supplementary Information 3).

Circular dichroism (CD) measurements were done using a JASCO (Tokyo, Japan) J-810 spectropolarimeter. The CD spectra of purified rBet v 1 and Bet v 1 trimer were measured at room temperature at concentration of 0.1 mg/ml using a rectangular quartz cuvette with 0.2-cm path length. Far ultraviolet (UV) spectra were recorded in the wavelength ranges between 190 and 260 nm with a resolution of 0.5 nm at a scan speed of 50 nm/min. Data of three measurements were averaged. The final spectra were baseline-corrected and results were expressed as the mean residue ellipticity (Θ) at a given wavelength. The secondary structure content of rBet v 1 and rBet v 1 trimer was calculated using the secondary structure estimation program CDSSTR (19).

IgE and IgG reactivity of rBet v 1 and rBet v 1 trimer

Purified rBet v 1 and rBet v 1 trimer were tested for IgE reactivity in dot blot assays and by ELISA (Supplementary information 4).

Basophil activation by flow cytometry: CD203c assay

Peripheral blood samples were obtained from ten out of the eleven birch pollen allergic patients used for IgE reactivity testing. Blood was collected in heparinized tubes after informed consent was given. Blood aliquots (100 µl) were incubated (unique) with serial dilutions of rBet v1 (0.05 pM to 0.5 nM), rBet v1 trimer (0.05 pM to 0.5 nM) or anti-IgE mAb E-124.2.8 (Immunotech, Marseille, France) (1µg/ml), or PBS for 15 minutes at 37 °C, and CD203C expression was measured (20).

Gel Filtration

For gel filtration 300 µl aliquots of the proteins (rBet v 1 trimer: c=1.9mg/ml; rBet v 1: c=1mg/ml) were loaded onto a Superdex 200 10/300 GL column (GE Healthcare, Uppsala, Sweden) at 4ºC, equilibrated with 10 mM phosphate buffer pH 7.5 containing 150 mM NaCl. The flow rate was 0.5 ml/min and fractions of 0.5 ml were collected. The molecular masses (MM) of the fractions were calculated based on the gel filtration of standard proteins performed under identical conditions (BioRad: Thyroglobulin, 670 kDa; Bovine gamma globulin, 158 kDa; Chicken ovalbumin, 44 kDa; Equine myoglobin, 17 kDa; vitamin B12, 1.35 kDa; vitamin B12). The peak fractions were concentrated using a CENTRIVAP (Labconco Corp., Kansas City**,** MO, USA) vacuum concentrator without heating. Concentrated fractions were subjected to immunological investigations or for 5 days at 4ºC and reapplied to gelfiltration with the Superdex 200 10/300 GL column to check the stability of elution profile.

Rat basophil leukemia cells mediator-release assay

Rat basophil leukemia cells (RBL-2H3) transfected with the human high affinity IgE receptor FcεRI (clone RBL-703/21), kindly provided by S. Viehts (21), were loaded with different dilutions of the chimeric Bip 1 antibody (10 μg/ml, 1 μg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml, 10 pg/ml and 1 pg/ml), washed 3 times with Tyrode's buffer (Sigma-Aldrich, Vienna, Austria) and then exposed to different concentrations (10 μg/ml, 1 μg/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml) of rBet v 1, rBet v 1 trimer or a non-cross-reactive grass pollen allergen, Phl p 5. The release of β-hexosaminidase was measured as described (22).

Results

Expression, purification and physicochemical properties of rBet v 1 and rBet v 1 trimer

The Coomassie blue-stained SDS-PAGE shows that recombinant Bet v 1 trimer appears in SDS-PAGE under reducing as well as under non-reducing conditions mainly as a monomeric protein of approximately 52 kDa (Figs. 1A, +Met, -Met). Few very weak bands of lower and higher molecular weight than 52 kDa were observed in the recombinant trimer preparation. Recombinant Bet v 1 migrated as a single protein band at approximately 17 kDa (Fig. 1A, left).

MALDI-ToF analysis of purified rBet v 1 resulted in two prominant mass peaks of 17370.9 Da, corresponding to the monomeric form without methionine and another of 8654.9 Da, corresponding to the double charged molecule (Supplementary figure 1). MALDI-ToF analysis of rBet v 1 trimer resulted in a peak of 53068.1 Da. The mass determined by mass spectrometry for the trimer was slightly greater than the predicted mass which could be due to binding of urea (+ 43 Da), Na (+ 22 Da), K (+ 38 Da), oxidation of methionine (131 Da) and combinations of these modification (Supplementary figure 1). A peak corresponding to half of the molecule (double charged 26633.3 Da) was also observed. For measurement of the rBet v

1 trimer a higher laser intensity than for rBet v 1 had to be used (rBet v 1 trimer: 69%; rBet v 1: 52%), which resulted in a prominent matrix peak between 6000 to 14000 Da.

The Bet v 1 CD spectrum exhibits a shape typical for a mixed α/β fold, with a broad minimum at 215 nm and a maximum at 195 nm (Fig. 1B). In contrast, the CD spectrum of Bet v 1 trimer displays a shift of the minimum as well as the maximum towards shorter wavelengths, indicating a higher content of random-coil elements contributing to the overall structure (Fig. 1B). For rBet v 1 an α-helix content of 27 % and a β-sheet content of 30 % were calculated and for the trimer the values were 16 % and 28 %, respectively, indicating a loss of mainly α-helical secondary structure elements in the trimer as compared with the monomer structure.

Recombinant Bet v 1 trimer shows even stronger IgE reactivity than rBet v 1

Figure 2A shows the IgE reactivity from 11 birch pollen allergic individuals, one non-allergic person and buffer to rBet v 1 and rBet v 1 trimer. Each of the birch allergic patients sera displayed IgE reactivity to rBet v 1 trimer and to rBet v 1 (Fig. 2A, A-K).

Interestingly, rBet v 1 trimer showed always more intensive IgE reactivity than rBet v 1. No IgE reactivity was observed with serum from the non-allergic individual and with the buffer control (Fig. 2A, L and 0). Similar results were obtained in a Western blot assay. Each of the allergic patients showed stronger IgE reactivity to rBet v 1 trimer than to rBet v 1 (data not shown).

Also by ELISA we found that rBet v 1 trimer exhibited a much stronger IgE reactivity than rBet v 1 (Fig. 2B).

Similar recognition of solid-phase-bound rBet v 1 trimer and rBet v 1 by IgG antibodies

We then tested the binding of polyclonal rabbit antisera which had been raised against purified rBet v 1, rBet v 1 trimer and against two rBet v 1 fragments (F1 and F2) as well as mouse monoclonal IgG antibodies raised against peptide 2 (mAb#2) comprising amino acids

30-59 of Bet v 1 and against peptide 6 (mAb#12) comprising amino acids 75-104 of Bet v 1 or the Bip 1 mouse monoclonal antibody raised against natural, pollen-derived Bet v 1 to rBet v 1 and rBet v 1 trimer. Unlike allergic patients IgE, each of the IgG antibodies reacted equally well with the two proteins at each of the tested dilutions (Figs. 2C, A-G).

rBet v 1 trimer and rBet v 1 inhibit allergic patients IgE reactivity to each other equally well in fluid phase

Next we investigated the extent to which each of the two proteins can inhibit IgE reactivity to the other protein, by IgE ELISA competition assays. Sera from the very same 11 Bet v 1 allergic patients which had been tested for IgE reactivity before, were pre-incubated with different concentrations of either rBet v 1 trimer, rBet v 1 or BSA as control protein and then allowed to bind to ELISA plate-coupled rBet v 1 trimer or rBet v 1 (Fig. 2D). We found that rBet v 1 trimer and rBet v 1 inhibited patients IgE reactivity to rBet v 1 in a comparable manner at each of the four inhibitor concentrations (5 ng - 5 μg/ml) (Fig. 2D, left). Similar results were obtained when rBet v 1 trimer and rBet v 1 were tested for the inhibition of allergic patients IgE binding to rBet v 1 trimer (Fig. 2D, right).

Reduced allergenic activity of rBet v 1-trimer compared to rBet v 1 as determined by CD203c up-regulation on patients´ basophils

Heparinized blood samples from ten of the eleven allergic patients tested for IgE reactivity were incubated with different concentrations of rBet v 1 trimer or rBet v 1 (Fig. 3). For these experiments we used equimolar amounts of the proteins so that the amount of rBet v 1 equivalents in the rBet v 1 trimer preparation were approximately three-fold as high as in the rBet v 1 samples. Nevertheless, we found that rBet v 1 trimer exhibited a 10- to 100-fold reduced allergenic activity compared to rBet v 1 in almost each of the tested patients (Fig. 3).

In solution rBet v 1 trimer occurs in the form of defined aggregates whereas rBet v 1 is monomeric

Analytical gel filtration showed that the rBet v 1 trimer appeared in three distinct peaks in liquid phase. The first and most prominant peak eluted at 7.5ml, corresponding to approximately the 20-fold mass (i.e., 800-1000 kDa) of the protein. Thyroglobulin which has a molecular weight of 670 kDa eluted at 9.25 ml. A second peak of about half of the size, appeared at 10.5 ml corresponding to approximately the 10-fold mass of the trimer (i.e., 500 kDa) and a third small peak eluted at 12.5 ml corresponding to approximately the 2-fold of the trimer molecule (Fig. 4A). When the peaks of the trimer were re-run on a gel filtration column after storage at 4^{o}C (Fig. 4B) a similar profile was obtained. Most of the trimer appeared as high molecular weight aggregated form with a more than 10-fold molecular mass (Fig. 4B). When rBet v 1 was applied to analytical gel filtration, the protein eluted exclusively as a single peak at 16.9 ml corresponding to the monomeric form of the protein with a molecular mass of 17 kDa (Fig. 4C).

rBet v 1 trimer but not rBet v 1 induces β-hexosaminidase release from basophils loaded with monoclonal Bet v 1-specific IgE

RBL cells which had been transfected with human FcεRI were loaded with a rBet v 1-specific human monoclonal IgE antibody and subsequently exposed to different concentrations of rBet v 1 and rBet v 1 trimer. rBet v 1 trimer induced a dose-dependent release of β-hexosaminidase, starting at a concentration of 10 ng/ml with 14.86 % of total release and increased to 69.22 % when the RBL were exposed to the highest concentration of allergen tested (10 μg/ml) (Table 1). rBet v 1 failed to induce mediator release in sensitized RBL cells due to its monomeric nature (Table 1).

Discussion

In the last 10 years an increasing number recombinant or synthetic hypoallergenic allergen-derivatives has been engineered which are also characterized by a reduced reactivity with IgE antibodies (6, 8). Here we have investigated an unusual hypoallergenic allergen derivative, a recombinant trimeric form of the major birch pollen allergen, Bet v 1, which despite retained IgE reactivity was shown to exhibit reduced allergenic activity (9, 11).

Using SDS-PAGE performed under reducing and non-reducing conditions as well as mass spectrometry, rBet v 1 trimer appeared as a monomeric, soluble protein with a molecular weight corresponding to the molecular weight which was predicted based on its sequence. The comparison of the fold of the rBet v 1 trimer with that of the Bet v 1 monomer demonstrated that the trimer exhibited a similar fold as rBet v 1 with a reduction of alpha helical elements in its secondary structure. It has been shown for other hypoallergenic Bet v 1 derivatives such as recombinant fragments (23), a folding variant of Bet v 1 obtained by exposing rBet v 1 to NaOH (24) and for certain isoforms/mutants (25-27) that a loss of fold or altered fold is associated with reduced IgE reactivity. However, despite the altered fold, rBet v 1 trimer reacted with patients IgE antibodies as well as with several different IgG antibody probes recognizing sequential or conformational epitopes of Bet v 1 or certain Bet v 1 portions in a similar manner as rBet v 1. The IgE reactivity of rBet v 1 trimer was even higher than that of rBet v 1 wildtype in two different solid phase assays and comparable in fluid phase competition assays. The higher IgE reactivity in the solid phase assays may be explained by the fact that the immobilization procedure occupies more IgE epitopes on a small molecule (i.e., Bet v 1) as compared to the bigger trimer. Yet, both molecules showed identical IgE reactivity in fluid phase inhibition assays using different concentrations of each molecule for the inhibition of IgE reactivity.

Despite the fact that rBet v 1 trimer and rBet v 1 wildtype inhibited IgE binding to each other in a similar manner, we found that the trimeric form exhibited an a ten- to hundredfold

reduction of allergenic activity when exposed to basophils from allergic patients. This surprising reduction of allergenic activity cannot be explained by a variability of protein concentrations in the experiments because extra care was taken to determine the concentrations of trimer and rBet v 1 before each of the critical experiments in parallel and all experiments were performed using protein concentrations calculated to contain the same number of Bet v 1 molecules for IgE binding assays. For the basophil activation studies we even used equimolar amounts of trimer and rBet v 1. Thus the trimer preparation contained a threefold excess of Bet v 1 molecules compared to rBet v 1 but still the allergenic activity was 10-100 fold reduced. Furthermore, serum samples and cells were obtained from the very same patients for IgE reactivity testing and for the assessment of allergenic activity to assure that the results are not caused by a variability regarding patients materials.

Based on the first set of experiments we therefore had to conclude that the trimer despite maintained IgE reactivity exhibited a strongly reduced allergenic activity, presumably due to a less efficient cross-linking of basophil-bound IgE compared to the rBet v 1 wildtype.

Gel filtration/size exclusion experiments showed that the trimer despite its overall monomeric appearance in stained gels, occurred in the form of high molecular weight aggregates in solution. The majority of these aggregates resembled a peak of approximately the 20-fold mass of the trimer and two smaller fractions corresponding to the 10- and 2-fold mass, which even after collection and storage at 4°C remained stable. By contrast, rBet v 1 wildtype behaved strictly as monomeric protein in the gel filtration experiments. The monomeric appearance of rBet v 1 wildtype is in contrast to earlier published work which claimed that Bet v 1 and allergens in general exhibit their allergenic activity (i.e., cross-linking of effector cellbound IgE) due to oligomerization (28). We therefore performed another type of experiment in which we loaded rat basophil leukemia cells which had been transfected with the human FcεRI with Bet v 1-specific human monoclonal IgE antibodies and subsequently exposed them to the trimer and rBet v 1 wildtype allergen. The results of these experiments

confirmed that rBet v 1 wildtype behaves as monomeric protein in solution because it did not cross-link monoclonal IgE whereas the trimer induced cross-linking, albeit at relatively high concentrations which is in accordance with its low allergenic activity and in good agreement with earlier data which had been obtained with purified human mast cells (29).

Based on the obtained results we propose the following explanation for the hypoallergenic nature of the rBet v 1 trimer as shown in Figure 5. According to structural studies and site directed mutagenesis experiments there is evidence that Bet v 1 contains a major IgE epitope-containing region (26, 27) which is obviously accessible to IgE antibodies on the monomeric as well as on the trimeric form of Bet v 1 although the latter forms high molecular weight aggregates. Since Bet v 1 does not contain cysteins we assume that the formation of aggregates is due to an association of hydrophobic portions of the trimer within the aggregate leaving hydrophilic, IgE-reactive areas available outside. In this scenario both, the aggregated trimer and the monomers can bind a comparable number of IgE antibodies in solution (Fig. 5A-B), an assumption which is supported by the results of the IgE inhibition experiments. However, when the molecules are exposed to cell-bound IgE antibodies only the monomeric Bet v 1 molecules seem to be able to cause efficient cross-linking of IgE antibodies due to the fact that each of these molecules can present the IgE epitope containing areas well to cell-bound IgE (Fig. 5C-D). For the aggregated trimer we assume that only a certain percentage of the IgE epitopes which are available on the surface of this molecule assume a spatial arrangement which allows cross-linking of cell-bound IgE whereas a considerable number of the IgE epitopes is less well accessible to the cell-bound IgE antibodies (Fig. 5D).

Our hypothesis is in agreement with data obtained for other allergens indicating that the spatial arrangement of IgE epitopes on a given protein may have an important influence on the allergenic activity of this protein in terms of its capacity to induce allergic inflammation via cross-link of effector cell-bound IgE (30-32). It may thus be possible to convert highly

allergenic proteins into low allergenic proteins for therapeutic purposes by a hitherto unknown mechanism, i.e., a reorientation of IgE epitopes.

Acknowledgements

This study was supported by grant F1815, F1803, F1804, F1809 of the Austrian Science Fund (FWF), by the Christian Doppler Research Association Vienna, Austria and by a research grant from Biomay, Vienna, Austria.

References

1. Bousquet J, Lockey R, Malling HJ. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. J Allergy Clin Immunol 1998;**102:**558-62.
2. Durham SR, Till SJ. Immunologic changes associated with allergen immunotherapy. J Allergy Clin Immunol 1998;**102:**157-164.
3. Larché M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol 2006;**6:**761-771.
4. Winther L, Arnved J, Malling HJ, Nolte H, Mosbech H. Side-effects of allergen-specific immunotherapy: a prospective multi-centre study. Clin Exp Allergy 2006;**36:**254-260.
5. Bernstein DI, Wanner M, Orish L, Liss GM, Immunotherapy Committee, American Academy of Allergy, Asthma and Immunology. Twelve-year survey of fatal reactions to allergen injections and skin testing: 1990-2001. J Allergy Clin Immunol 2004;**113:**1129-1136.
6. Valenta R. The future of antigen-specific immunotherapy of allergy. Nat Rev Immunol 2002;**2:**446-453.

7. Marsh DG, Lichtenstein LM, Campbell DH. Studies on 'Allergoids' prepared from naturally occurring allergens. I. assay of allergenicity and antigenicity of formalinized rye group I component. Immunology 1970;**18:**705-722.
8. Linhart B, Valenta R. Molecular design of allergy vaccines. Curr Opin Immunol 2005;**17:**646-655.
9. Vrtala S, Hirtenlehner K, Susani M, Akdis M, Kussebi F, Akdis CA, et al. Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen, Bet v 1. FASEB J 2001;**15:**2045-2057.
10. van Hage-Hamsten M, Kronqvist M, Zetterström O, Johansson E, Niederberger V, Vrtala S, et al. Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1: results obtained with a mix of two recombinant Bet v 1 fragments and recombinant Bet v 1 trimer in a Swedish population before the birch pollen season. J Allergy Clin Immunol 1999;**104:**969-977.
11. Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P, et al. Vaccination with genetically engineered allergens prevents progression of allergic disease. Proc Natl Acad Sci USA 2004;**2:**14677-14682.
12. Hoffmann-Sommergruber K, Susani M, Ferreira F, Jertschin P, Ahorn H, Steiner R, et al. High-level expression and purification of the major birch pollen allergen, Bet v 1. Protein Expr Purif 1997;**9:**33-39.
13. Pauli G, Larsen TH, Rak S, Horak F, Pastorello E, Valenta R, et al. Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. J Allergy Clin Immunol 2008;**122:**951-960.
14. Vrtala S, Akdis CA, Budak F, Akdis M, Blaser K, Kraft D, et al. The cell epitope-containing hypoallergenic recombinant fragments of the major birch pollen allergen, Bet v 1, induce blocking antibodies. J Immunol 2000;**165:**6653-6659.

15. Laffer S, Vangelista L, Steinberger P, Kraft D, Pastore A, Valenta R. Molecular characterization of Bip 1, a monoclonal antibody that modulates IgE binding to birch pollen allergen, Bet v 1. J Immunol 1996;**157:**4953-4962.
16. Wiedemann P, Giehl K, Almo SC, Fedorov AA, Girvin M, Steinberger P, et al. Molecular and structural analysis of a continuous birch profilin epitope defined by a monoclonal antibody. J Biol Chem 1996;**271:**29915-29921.
17. Laffer S, Hogbom E, Roux KH, Sperr WR, Valent P, Bankl HC, et al. A molecular model of type I allergy: Identification and characterization of a nonanaphylactic anti-human IgE antibody fragment that blocks the IgE-FcεRI interaction and reacts with receptor-bound IgE. J Allergy Clin Immunol 2001;**108:**409-416.
18. Fling SP, Gregerson DS. Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. Anal Biochem 1986;**155:**83-88.
19. Whitmore L, Wallace BA. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res 2004;**32:**668-673.
20. Hauswirth AW, Natter S, Ghannadan M, Majlesi Y, Schernthaner GH, Sperr WR, et al. Recombinant allergens promote expression of CD203c on basophils in sensitized individuals. J Allergy Clin Immunol 2002;**110:**102-109.
21. Vogel L, Lüttkopf D, Hatahet L, Haustein D, Vieths S. Development of a functional *in vitro* assay as a novel tool for the standardization of allergen extracts in the human system. Allergy 2005;**60:**1021-1028.
22. Edlmayr J, Niespodziana K, Linhart B, Focke-Tejkl M, Westritschnig K, Scheiblhofer S, et al. A combination vaccine for allergy and rhinovirus infections based on rhinovirus-derived surface protein VPI and a nonallergenic peptide of the major timothy grass pollen allergen Phl p 1. J Immunol 2009;**182:**6298-6306.

23. Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, et al. 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 1997;**99:**1673-1681.
24. Kahlert H, Suck R, Weber B, Nandy A, Wald M, Keller W, et al. Characterization of hypoallergenic recombinant Bet v 1 variant as a candidate of allergen-specific immunotherapy. Int Arch Allergy Immunol 2008;**145:**193-206.
25. Ferreira F, Hirtenlehner K, Jilek A, Godnik-Cvar J, Breiteneder H, Grimm R, et al. Dissection of immunoglobulin E and T lymphocyte reactivity of isoforms of the major birch pollen allergen Bet v 1: potential use of hypoallergenic isoforms for immunotherapy. J Exp Med 1996;**183:**599-609.
26. Ferreira F, Ebner C, Kramer B, Casari G, Briza P, Kungl AJ, et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. FASEB J 1998;**12:**231-242.
27. Spangfort MD, Mirza O, Ipsen H, van Neerven RJ, Gajhede M, Larsen JN. 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 2003;**171:**3084-3090.
28. Schöll I, Kalkura N, Shedziankova Y, Bergmann A, Verdino P, Knittelfelder R, et al. Dimerization of the major birch pollen allergen Bet v 1 is important for its in vivo IgE-cross-linking potential in mice. J Immunol 2005;**175:**6645-6650.
29. Sellge G, Laffer S, Mierke C, Vrtala S, Hoffmann MW, Klempnauer J, et al. Development of an *in vitro* system for the study of allergens and allergen-specific immunoglobulin E and immunoglobulin G: Fcε receptor I supercross-linking is a possible new mechanism of immunoglobulin G-dependent enhancement of type I allergic reactions. Clin Exp Allergy 2005;**35:**774-781.

30. Fedorov AA, Ball T, Mahoney NM, Valenta R, Almo SC. The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin. Structure 1997;**15:**33-45.
31. Flicker S, Vrtala S, Steinberger P, Vangelista L, Bufe A, Petersen A, et al. A human monoclonal IgE antibody defines a highly allergenic fragment of the major timothy grass pollen allergen, Phl p 5: molecular, immunological, and structural characterization of the epitope-containing domain. J Immunol 2000;**165:**3849-3859.
32. Flicker S, Steinberger P, Ball T, Krauth MT, Verdino P, Valent P, et al. Spatial clustering of the IgE epitopes on the major timothy grass pollen allergen Phl p 1: importance for allergenic activity. J Allergy Clin Immunol 2006;**117:**1336-1343.

Figure and Tables

Figure 1. Physical-chemical characterization of rBet v 1 and rBet v 1 trimer by SDS-PAGE. **(A)** Comparison of purified recombinant Bet v 1 and rBet v 1 trimer (left). rBet v 1 trimer run in the presence (+Met) or absence (-Met) of 2-mercaptoethanol. M, molecular mass markers (kDa). **(B)** Circular dichroism analysis of rBet v 1 and rBet v 1 trimer. The mean residue ellipticities (*y*-axes) are shown for rBet v 1 trimer and for rBet v 1 at different wavelengths (*x*-axes).

Figure 2. IgE and IgG reactivity of rBet v 1 and rBet v 1 trimer. **(A)** IgE reactivity of dot-blotted rBet v 1 and rBet v 1 trimer. IgE reactivity of nitrocellulose-dotted rBet v 1, rBet v 1 trimer, human serum albumin (HSA) and bovine serum albumin (BSA) after incubation with sera from 11 birch pollen allergic patients (A-K), serum from one non-allergic person (L) or buffer (0). **(B)** IgE reactivity of ELISA plate-bound rBet v 1 and rBet v 1 trimer. IgE reactivities (OD values) of sera from 11 birch pollen allergic patients (A-K) to rBet v 1 and rBet v 1 trimer. **(C)** Reactivity of rBet v 1 and rBet v 1 trimer with specific IgG antibodies. IgG reactivities (y-axes: OD values) of different dilutions (x-axes) of rabbit anti-rBet v 1 (a), anti-F1 (b), anti-F2 (c), anti-Trimer (d), mAb#2 (e), mAb#12 (f) or mAb Bip 1 (g) with rBet v 1 (●), rBet v 1 trimer (■) and BSA (▲). **(D)** Inhibition of IgE binding to solid phase-bound rBet v 1 or rBet v 1 trimer by rBet v 1 or Bet v 1 trimer in fluid phase. The percentages of inhibition of IgE binding to solid phase-bound rBet v 1 (left) and rBet v 1 trimer (right) are shown on the (y-axes) after pre-incubation of sera from 11 birch pollen allergic patients with different concentrations (x-axes) of rBet v 1 or rBet v 1 trimer. Results are displayed as box plots with median values in the boxes, standard deviations and extremes (asterisks) and outliers (circle).

Figure 3. *In vitro* allergenic activity of rBet v 1 and rBet v 1 trimer as determined by CD203c up-regulation on allergic patients basophils. Blood samples from birch pollen allergic individuals were exposed to different concentrations of rBet v 1 (black bars) and rBet v 1 trimer (open bars), anti-IgE or buffer (0) (*x-axes*). The upregulation of CD203c expression is displayed in the form of stimulation indices (SI) (*y-axis*) for each of the 10 patients (A-J).

Figure 4. Aggregate formation of rBet v 1 trimer in solution. **(A)** Demonstration by gel filtration that rBet v 1 trimer occurs in fluid phase in the form of defined aggregates. Profile of rBet v 1 trimer eluted from a gel filtration column (x-axis: Elution volumes in ml; y-axis: absorbance at 280nm). Arrows indicate peaks of a protein standard consisting of thyroglobulin 670 kDa (9.25ml), bovine gamma globulin 158 kDa (12.35ml), chicken ovalbumin 44 kDa (15.17ml), equine myoglobin 17 kDa (17.23ml), Vitamin B 12 1.35 kDa (20.55ml). **(B)** Gel filtration re-run of the trimer peaks (blue: sample 14, ~ 8ml; pink: samples 16-17, ~ 9-9.5ml; green: samples 24-26, ~ 12-13.5ml) after 5 days of incubation at 4 ºC. **(C)** Elution profile of rBet v 1 (16.9 ml).

Table 1. Induction of ß-hexosaminidase release. RBL cells that had been transfected with human FcεRI were sensitized with different concentrations of human IgE mAb α Bet v 1 (Bip 1) and then stimulated with increasing concentrations of rBet v 1 and rBet v 1 trimer. The release of ß-hexosaminidase is shown as percentage of the total ß-hexosaminidase contents of the cells.

Figure 5. Model for the hypoallergenic behavior of rBet v 1 trimer. Bet v 1 molecules in their monomeric form (**A**: rBet v 1) or as aggregated trimer (**B**: Trimer) can bind a comparable number of IgE antibodies in solution. However, monomeric rBet v 1 molecules may be more efficient in cross-linking of effector cell-bound IgE (**C**) than the aggregated trimer (**D**). IgE

epitopes on monomeric forms of Bet v 1 can be well accessed by effector cell-bound IgE whereas only a certain portion of the IgE epitopes on the aggregated trimers comes into an optimal position for cross-linking of effector cell-bound IgE.

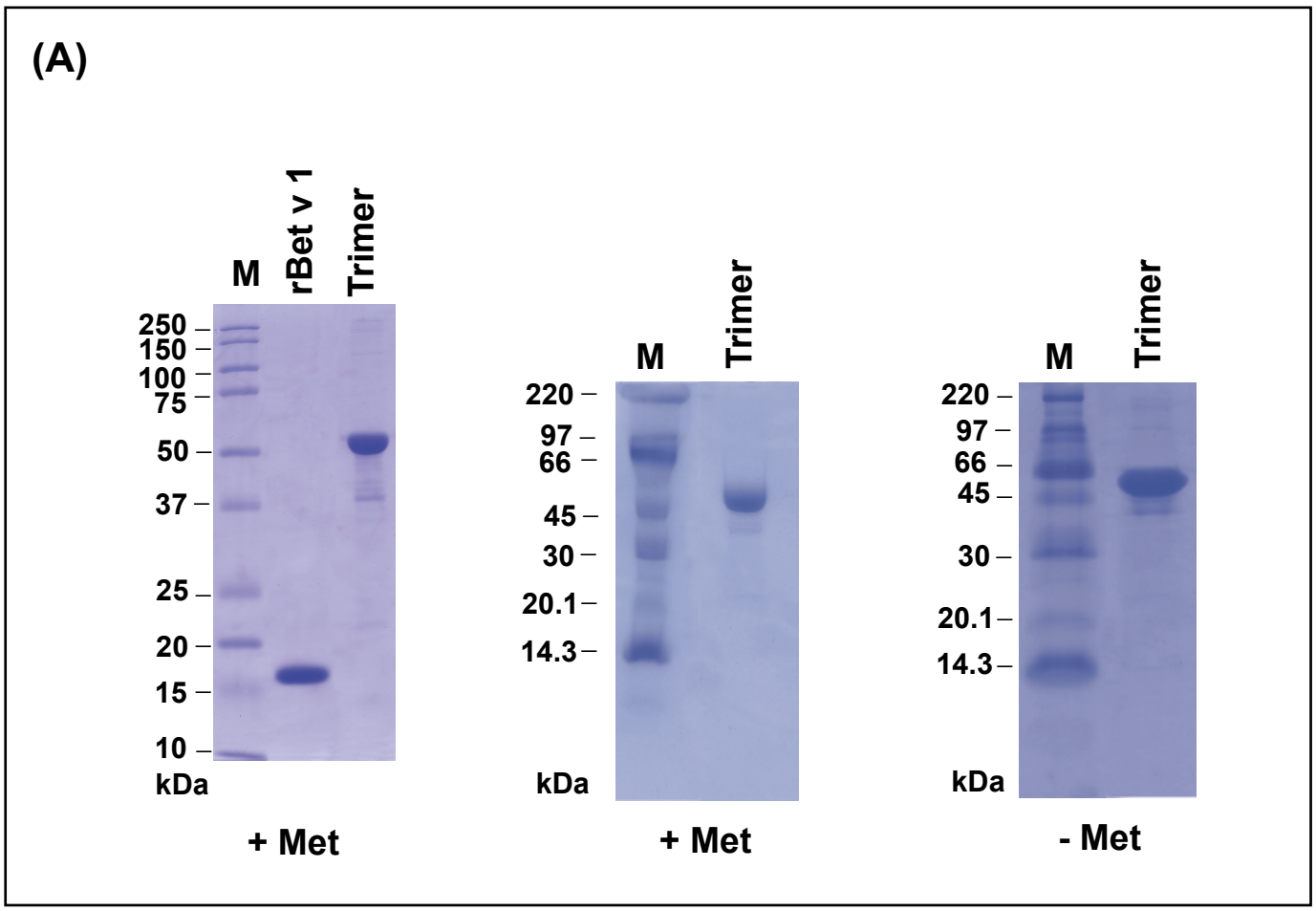

(A)
M
rBet v 1
Trimer
250
150
100
75
50
37
25
20
15
10
kDa
+ Met
M
Trimer
220
97
66
45
30
20.1
14.3
kDa
+ Met
M
Trimer
220
97
66
45
30
20.1
14.3
kDa
- Met

Figure 1A

(B)

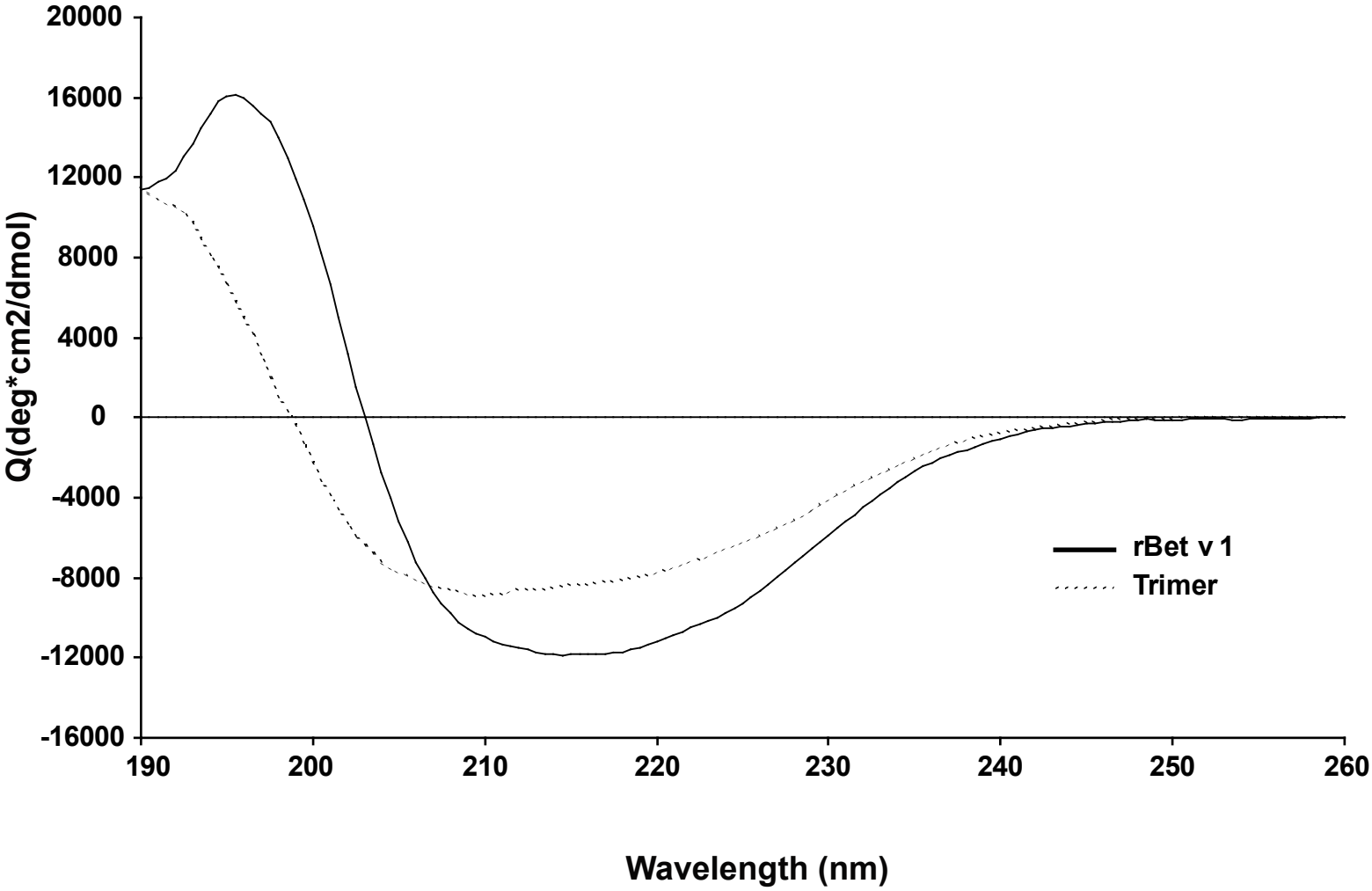

20000
16000
12000
8000
4000
0
-4000
-8000
-12000
-16000
Q(deg*cm2/dmol)
190
200
210
220
230
240
250
260
Wavelength (nm)
rBet v 1
Trimer

Figure 1B

(A)

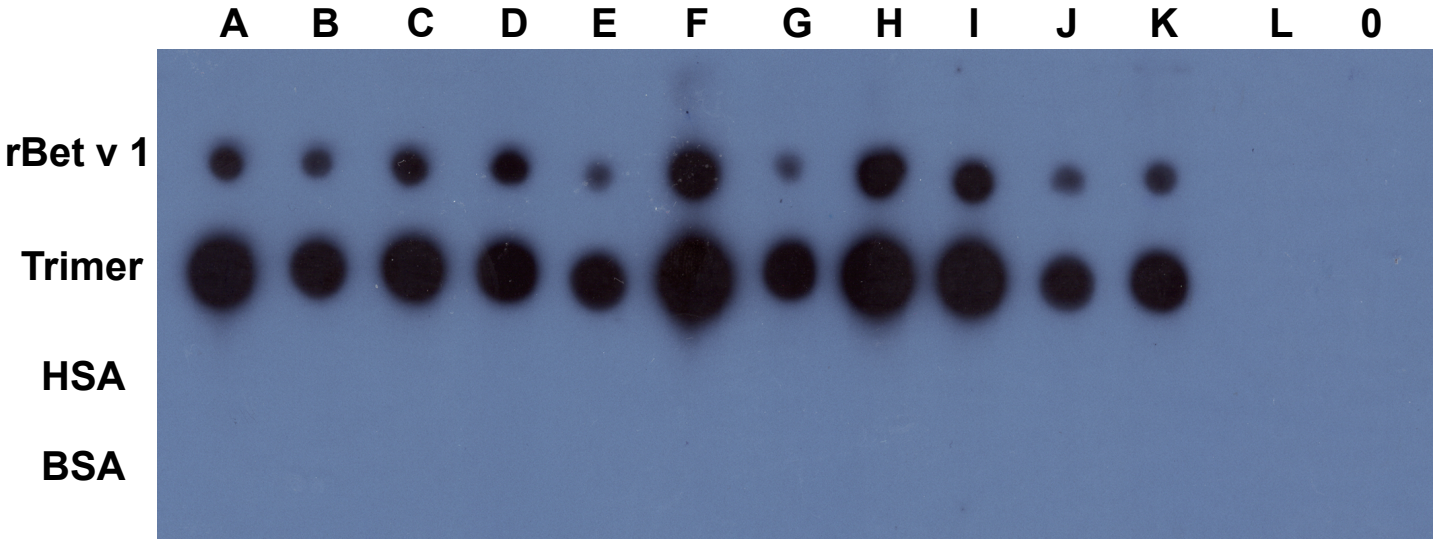


Figure 2A

(B)

	A	B	C	D	E	F	G	H	I	J	K
rBet v 1	0.389	0.065	0.191	0.094	0.134	0.51	0.327	0.268	0.328	0.182	0.908
rBet v 1 Trimer	2.47	0.661	1.958	0.628	0.978	3.608	3.114	2.244	3.148	1.367	3.567

Figure 2B

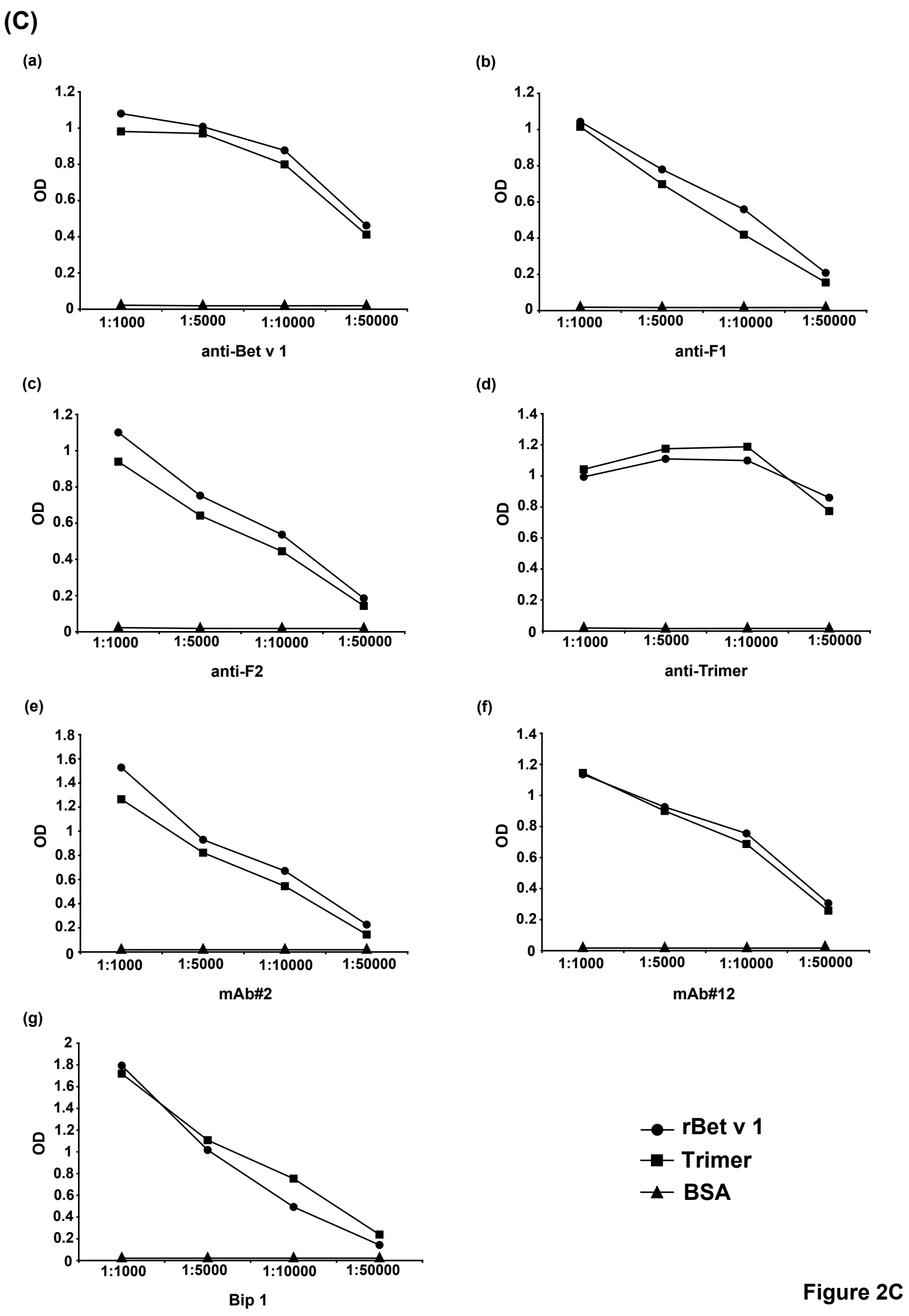

(C)
(a)
OD
1.2
1
0.8
0.6
0.4
0.2
0
1:1000
1:5000
1:10000
1:50000
anti-Bet v 1
(b)
anti-F1
(c)
anti-F2
(d)
1.4
anti-Trimer
(e)
1.8
1.6
mAb#2
(f)
mAb#12
(g)
2
Bip 1
rBet v 1
Trimer
BSA

Figure 2C

(D)

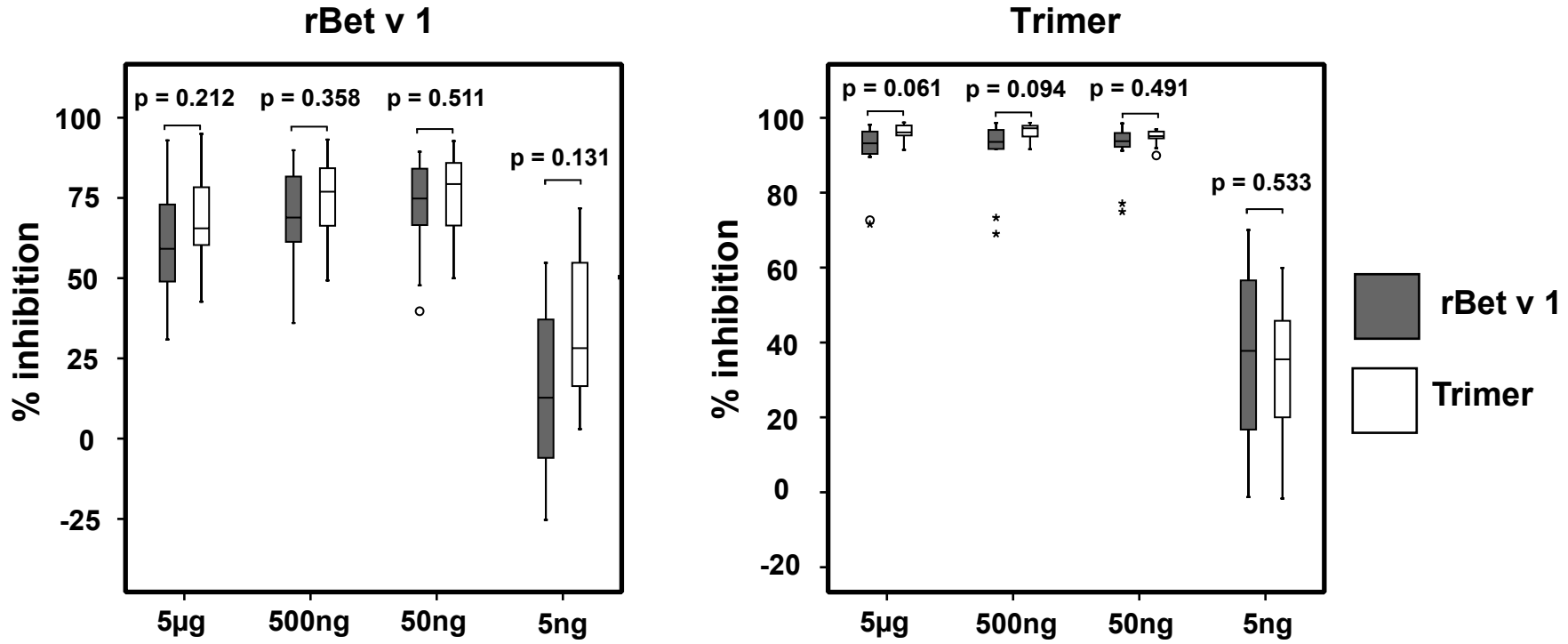

rBet v 1
Trimer
% inhibition
p = 0.212
p = 0.358
p = 0.511
p = 0.131
p = 0.061
p = 0.094
p = 0.491
p = 0.533
100
75
50
25
0
-25
80
60
40
20
-20
5µg
500ng
50ng
5ng
rBet v 1
Trimer

Figure 2D

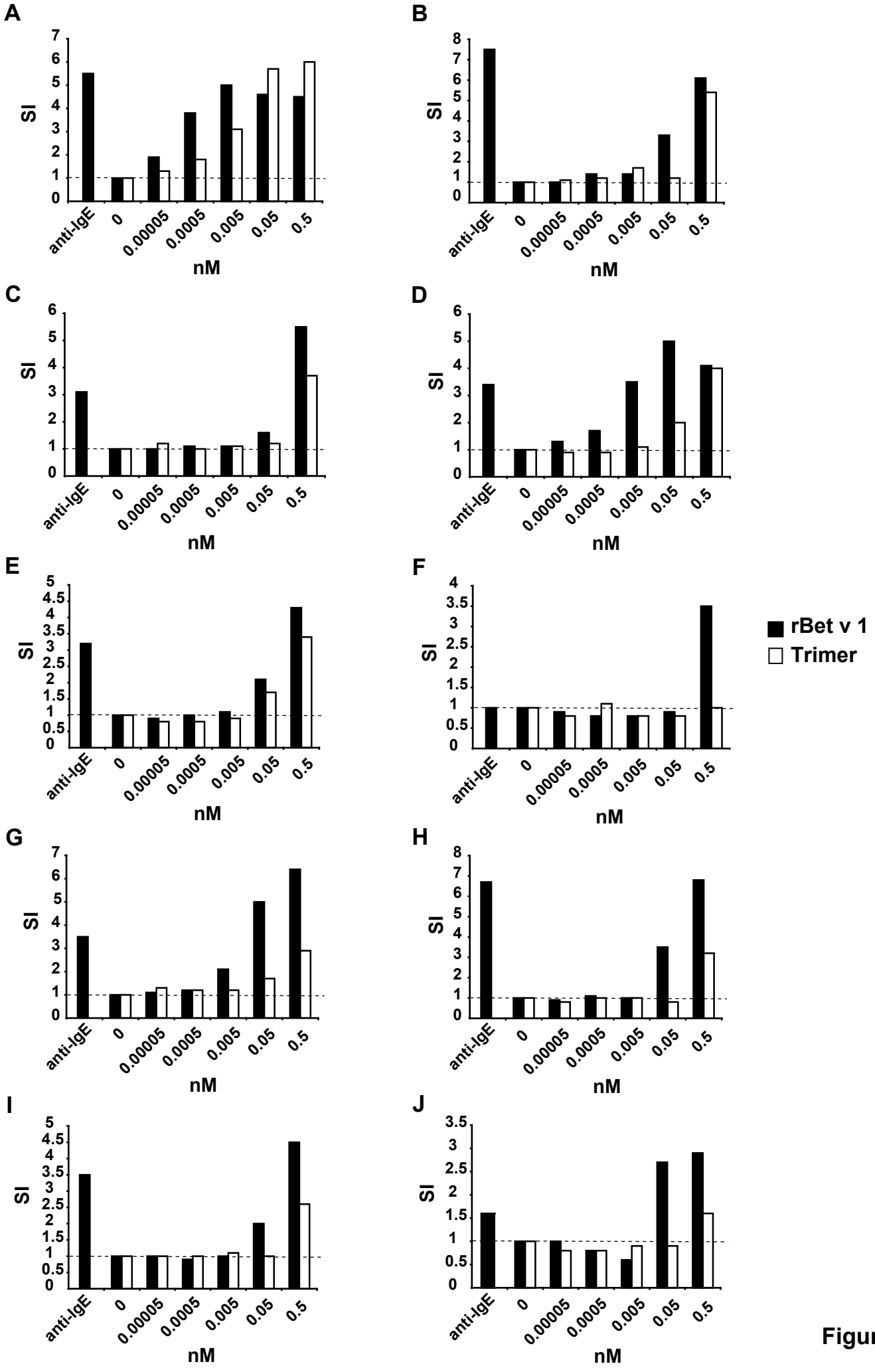
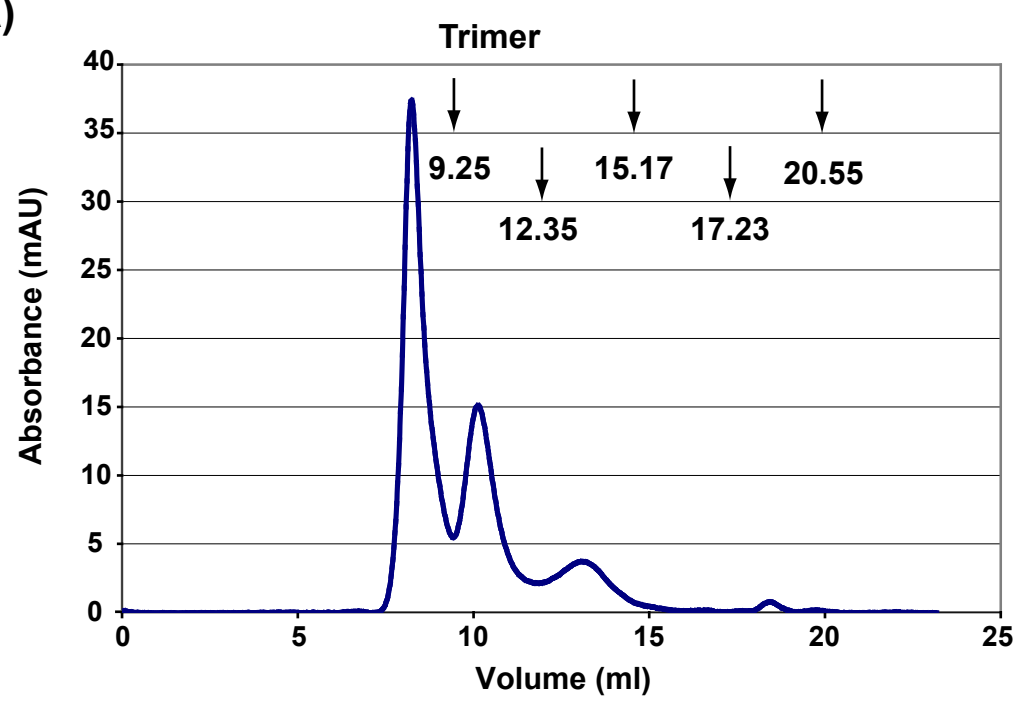
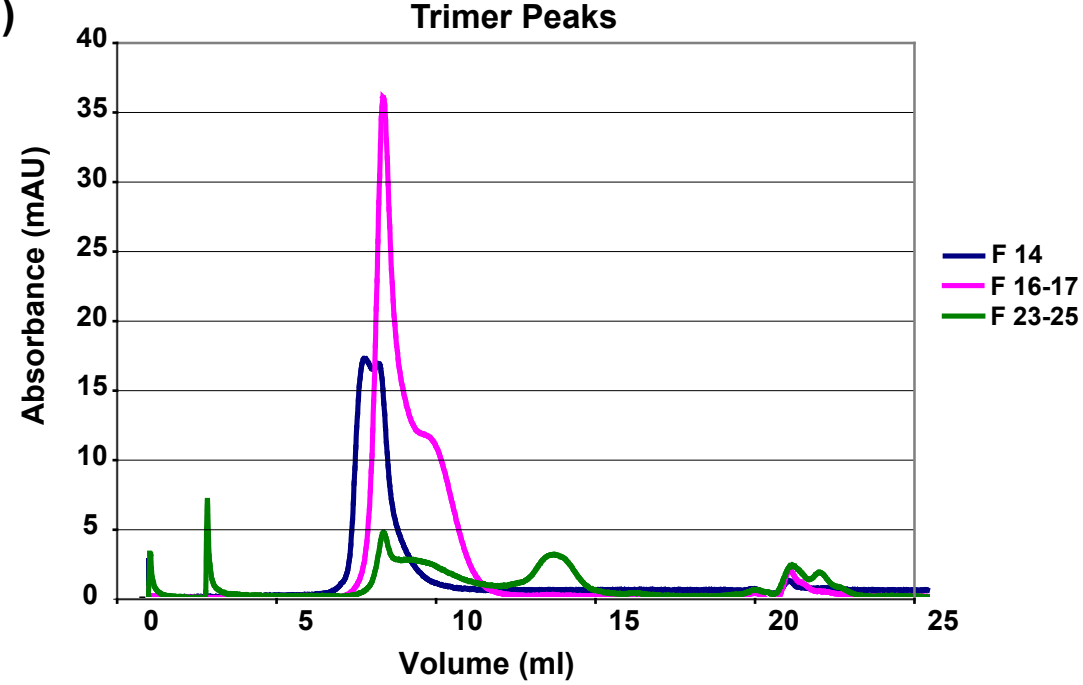

A
B
C
D
E
F
G
H
I
J
SI
anti-IgE
0
0.00005
0.0005
0.005
0.05
0.5
nM
rBet v 1
Trimer

Figure 3

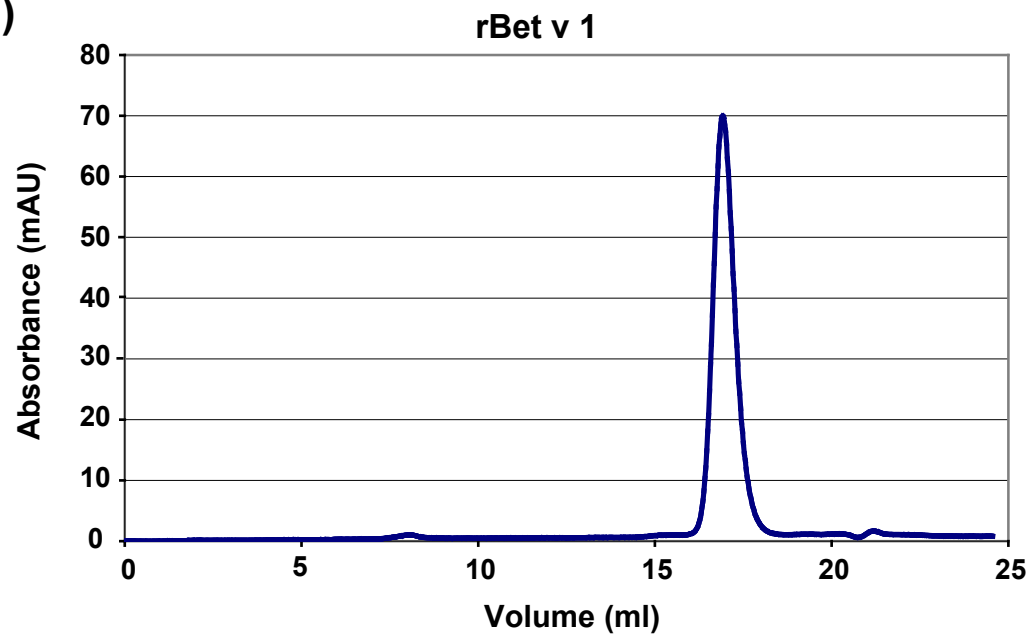
(A)



(B)



(C)



Figures 4A-C

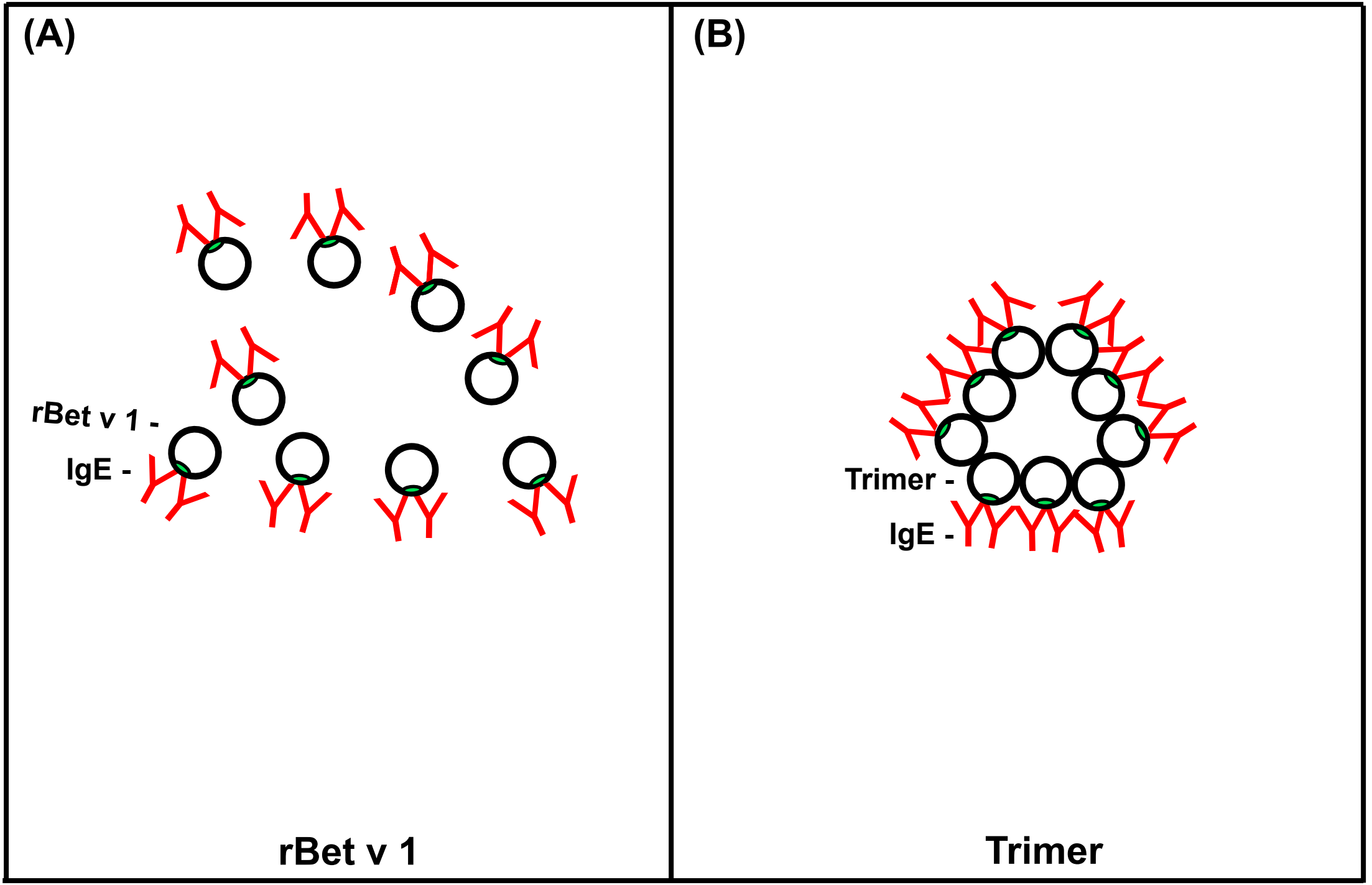


Figure 5

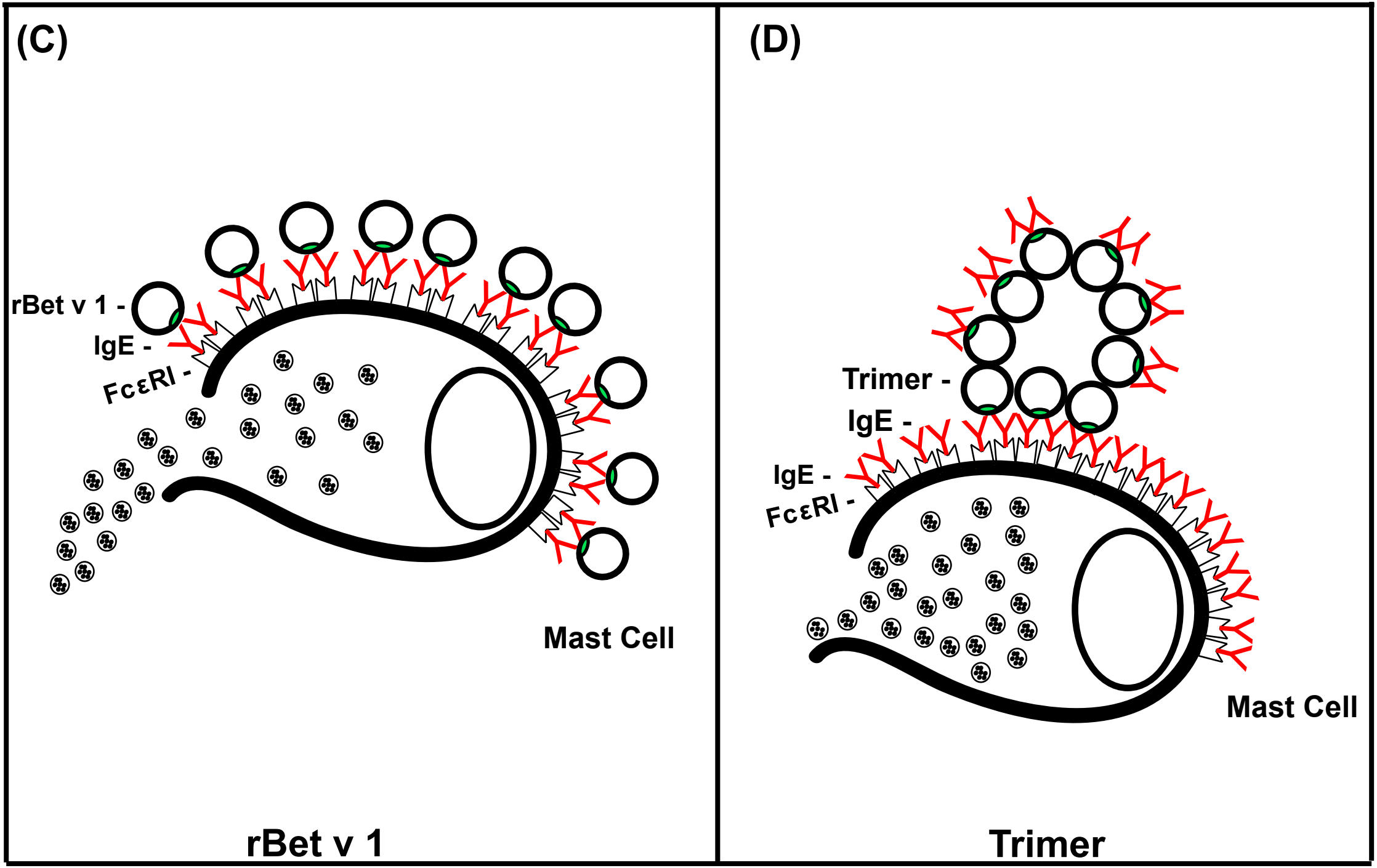
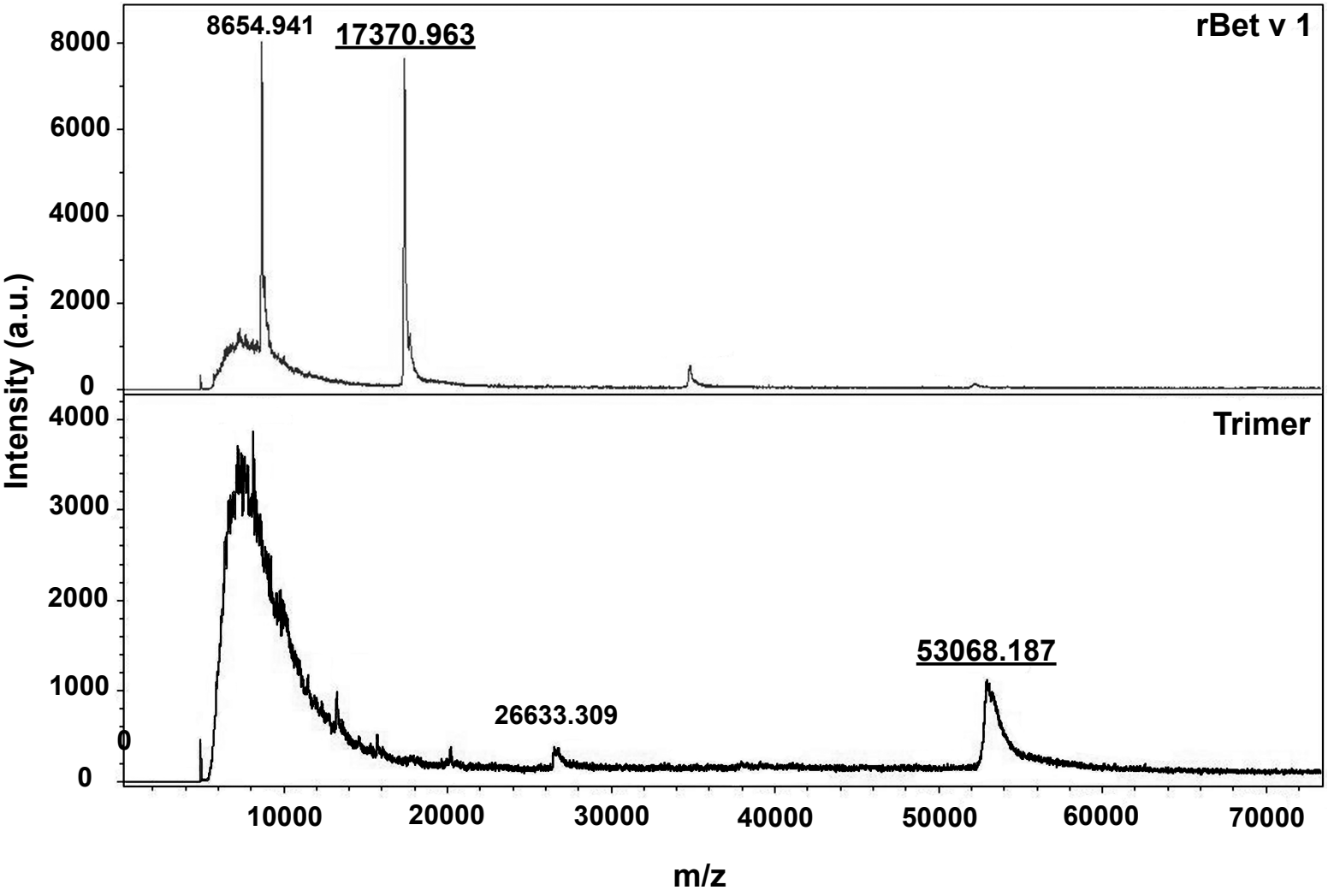

(C)
rBet v 1 -
IgE -
FcεRI -
Mast Cell
rBet v 1
(D)
Trimer -
IgE -
IgE -
FcεRI -
Mast Cell
Trimer

Figure 5

Table 1. Induction of ß-hexosaminidase release.

	rBet v 1				Trimer			
Bip 1dilution	**10 µg/ml**	**1 µg/ml**	**100 ng/ml**	**10 ng/ml**	**10 µg/ml**	**1 µg/ml**	**100 ng/ml**	**10 ng/ml**
10 µg/ml	4.0	2.8	2.1	1.4	69.2	65.3	14.9	0.3
1 µg/ml	1.8	1.5	1.2	0.8	69.1	58.5	9.6	0.1
100 ng/ml	0.4	0.3	0.3	0.3	50	41.8	1.8	0.5



Supplementary Figure 1: Mass spectrometric analysis of rBet v 1 and rBet v 1 trimer. the mass/charge ratios are shown on the *x-axes*, and the signal intensities are displayed on the *y-axis* as absolute value in the investigated mass range.

Supplementary Information 1: Expression and purification of rBet v 1 trimer

Batch fermentation of *E.coli* BL 21 (DE3)/pET-17b-Bet v 1 trimer was carried out in a 10 L Bioflow 3000 fermenter (New Brunswick Scientific, New Jersey, USA) in LB medium with the addition of 0.05 % (v/v) glycerol, 0.25 % (w/v) $MgSO_4$. 7 H_2O, and 0.18 % Na_2HPO_4 . 2 H_2O for 8 h at 37°C, until a cell density (OD_{600nm}) of 7 was reached. As soon as OD_{600nm} reached 1, expression of Bet v 1 trimer and formation of inclusion bodies was induced by adding isopropyl-β-thiogalactopyranoside (IPTG) (Calbiochem, Merck KgaA, Darmstadt, Germany) to a final concentration of 0.5 mM. Inclusion body fractions containing rBet v 1 trimer were isolated by an enzymatic treatment using lysozyme (0.1 mg/g cells) (Sigma-Aldrich, St. Louis, MO, USA) and benzonase (6 U/g cells) (Merck KgaA, Darmstadt, Germany), followed by repetitive freezing and thawing in a buffer containing 50 mM Trisbase pH 8.0, 1 mM EDTA and 0.1 % v/v Triton X-100 (5 ml/ g cells). After the freezing and thawing, NaCl and EDTA were added to a final concentration of 200 mM and 2 mM, respectively, and the suspension was centrifuged (10.000 g for 30 min at 4°C) leaving Bet v 1 trimer containing inclusion bodies in the pellet. After washing the pellet (3 times with 1% v/v Triton, 2 mM EDTA, 2 mM ß-mercaptoethanol, 20 mM Tris/HCl pH 8.0 and 2 times with 50% ethanol, 20mM Tris/HCl pH 8.0), inclusion bodies were suspended and stirred for 15 min in buffer A (6 M urea, 10 mM Tris, 1 mM EDTA, pH 8.0). After centrifugation (10.000 g for 30 min at 4°C), the protein was applied to a DEAE sepharose column (Amersham Biosciences, Uppsala, Sweden) and equilibrated with buffer A. The protein was eluted with a linear gradient from 0-500 mM NaCl in buffer A. Fractions containing Bet v 1 trimer as the major component were identified by SDS-PAGE, pooled, and dialyzed against buffer B (6M urea, 10 mM NaH_2PO_4, 1mM EDTA, pH 4.8).

The Bet v 1 trimer was rechromatographed on an SP sepharose (Amersham Biosciences, Uppsala, Sweden) and equilibrated with buffer B. Protein was eluted with a linear gradient from 0-500 mM NaCl in buffer B. Fractions containing pure Bet v 1 trimer as the major

component were identified by SDS-PAGE, pooled, and dialyzed stepwise against 5 mM sodium phosphate buffer pH 7.4 containing decreasing concentrations (6-0 M) of urea. Finally, the protein was subjected to 0.2 µm filtration.

The purity of the protein preparations was checked by SDS-PAGE and Coomassie Blue staining and the presence of endotoxins was assessed by endotoxin testing (QCL-1000^{R} Chromogenic LAL Endpoint Assay, Bio-Whittaker, Walkersville, USA). The concentration of rBet v 1 and rBet v 1 trimer was checked by colorimetric detection and quantification of total protein using bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}(Micro BCA^{TM} Protein Assay Kit, Thermo Scientific, Pierce, Rockford, USA). Tests were performed in triplicates. Diluted protein samples (0.5-20 µg /ml) were incubated for 1 hour at 60°C and the absorbance was measured at 562 nm according to the assay protocol. Determinations of protein concentrations were performed for all of the proteins in parallel in order to ensure that the concentrations of the different proteins can be directly compared. Determinations of the protein concentrations were also repeated for each of the proteins shortly before experiments were performed (e.g., IgE antibody reactivity testing, IgE inhibition assays, CD analysis, basophil activation experiments, gel filtration), in order to exclude that results are influenced by alterations in the protein concentrations.

Supplementary Information 2: Immunoblottting:

For this Western blotting, 5µg of each protein/slot was separated by SDS-PAGE (18) and blotted onto nitrocellulose. Nitrocelluloses containing blotted proteins were incubated either with a 1:2000 dilution of a rabbit anti-rBet v 1 fragment 1 or fragment 2 specific anti-serum, or with 1:10 dilutions of sera from eleven birch pollen allergic individuals or serum from one non-allergic individual. Bound IgG and IgE antibodies were detected with a 1:1000 dilution of ^{125}I-labeled donkey anti-rabbit antibodies and a 1:20 dilution of ^{125}I-labeled anti-human IgE antibodies (RAST RIA, Demeditec Diagnostics, Germany), respectively. Bound ^{125}I-labeled antibodies were visualized by autoradiography (9).

Supplementary Information 3: Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectra were acquired in a linear mode on a Microflex mass spectrometer (Bruker, Billerica, MA, USA) equipped with a 337 nm ultraviolet laser (150 μJ at 337 nm). Hundred single laser shots were averaged for each mass spectrum. Samples were dissolved in 10 % acetonitrile (0.1 % trifluoroacetic acid), and dihydroxybenzoic acid (dissolved in 60 % acetonitrile, 0.1 % trifluoroacetic acid) was used as a matrix. For sample preparation a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

Supplementary Information 4: IgE and IgG reactivity of rBet v 1 and rBet v 1 trimer by dot blot, ELISA and ELISA inhibition

For dot blot experiments, two µL aliquots containing 1µg of purified rBet v 1, rBet v 1 trimer, bovine serum albumin (BSA) and human serum albumin (HSA) (negative controls) (Roth, Karlsruhe, Germany) were dotted on nitrocellulose membranes. Nitrocellulose membranes containing dot-blotted proteins were incubated with a 1:10 dilution, in PBS, of sera from eleven birch pollen allergic individuals, one non-allergic individual or buffer without addition of serum. Bound IgE antibodies were detected with a 1:20 dilution of ^{125}I-labeled anti-human IgE antibodies (RAST RIA, Demeditec Diagnostics, Germany) and visualized by autoradiography (9).

ELISA plates (Greiner, Kremsmünster, Austria) were coated with rBet v 1, rBet v 1 trimer or BSA (5 µg in100 µl/well diluted in PBS) at 4°C overnight. Plates were blocked with 2 % w/v bovine serum albumin (BSA) (Roth, Karlsruhe, Germany) in PBS-T (PBS + 0.05 % v/v Tween 20) for 6 hours, and incubated either with rabbit anti-Bet v 1, rabbit anti-Bet v 1 fragment 1, rabbit anti-Bet v 1 fragment 2, rabbit anti-Bet v 1 trimer, or pre-immune sera in four different dilutions (1:1000, 1:5000, 1:10000 and 1:50000), in PBS 0.5 % w/v BSA/ 0.05 % v/v Tween, at 4°C overnight. Bound rabbit IgG antibodies were detected with a 1:1000 diluted anti-rabbit IgG, Horseradish Peroxidase-linked whole antibody from donkey (GE Healthcare, UK Limited) for 1 hour at 37°C and for 1 hour at 4°C.

Detection of rBet v 1 trimer and rBet v 1 with mouse monoclonal antibodies was performed as follows. Plates were coated with rBet v 1, rBet v 1 trimer or BSA (5 µg in100 µl/well, diluted in PBS) at 4°C overnight, and then incubated either with mouse monoclonal IgG antibodies against peptide 2 (mAb#2) (aa 30-59) or peptide 6 (mAb#12) (aa 74-104) from Bet v 1, with mouse monoclonal antibody (4A6), with Bip 1 or with mouse monoclonal IgG1 (murine anti-IgE) in four different dilutions (1:1000, 1:5000, 1:10000 and 1:50000), in PBS

0.5 % BSA/ 0.05 % Tween, at 4°C overnight. Bound mouse monoclonal IgG antibodies were detected with a 1:1000 diluted anti-mouse IgG, Horseradish Peroxidase-linked whole antibody from sheep (GE Healthcare, UK Limited) for 1 hour at 37°C and 4°C.

Plates were washed with PBS-T (PBS + 0.05 % Tween 20) between incubation steps and color development was performed by addition of staining solution ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; Sigma-Aldrich, St.Louis, Missouri, USA) (100µl/well). The optical density was measured in an ELISA Reader (Dynatech, Denkendorf, Germany) at 405 nm. Results represent means of duplicate determination with a variation of ≤ 10 %.

IgE ELISA inhibition assay

ELISA plates (Greiner, Kremsmünster, Austria) were coated with 100µl of rBet v 1, rBet v 1 trimer or BSA (5 µg in 100 µl/well, diluted in PBS) for six hours at room temperature. Plates were blocked with 2% bovine serum albumin (BSA) (Roth, Karlsruhe, Germany) in PBS-T (PBS 0.05% Tween 20) at 4˚C overnight. Sera from eleven birch allergic individuals (1:4 diluted in PBS) were preincubated overnight at 4˚C with increasing concentrations (0.5 ng - 5 µg) of the rBet v 1, rBet v 1 trimer or BSA before they were applied to the plates (100 µl/well). Bound human IgE was detected using a 1:2500 diluted AP-conjugated (alkaline phosphatase) mouse monoclonal anti-human IgE antibody (BD Pharmingen, San Diego, California, USA). Plates were washed with PBS-T (PBS + 0.05 % Tween 20) between incubation steps and color development was performed by addition of staining solution ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; Sigma-Aldrich, St.Louis, Missouri, USA) (100µl/well). The optical density was measured in an ELISA Reader (Dynatech, Denkendorf, Germany) at 405 nm.

Hypoallergenic derivatives of the major birch pollen allergen Bet v 1 obtained by rationale sequence reassembly

Campana Raffaela, Vrtala S, Maderegger B, Jertschin P, Stegfellner G, Swoboda I, Focke-Tejkl M, Blatt K, Gieras A, Neubauer A, Valent P, Spitzauer S, Valenta R

Submitted

2009

Hypoallergenic derivatives of the major birch pollen allergen Bet v 1 obtained by rationale sequence reassembly

Raffaela Campana, MSc,[a] Susanne Vrtala, PhD,[a] Bernhard Maderegger, PhD,[b] Peter Jertschin, PhD,[b] Gottfried Stegfellner, PhD,[b] Ines Swoboda, PhD,[a,c] Margarete Focke-Tejkl, PhD,[a,c] Katharina Blatt, [d] Anna Gieras, MSc,[a,c] Angela Neubauer, PhD,[b] Peter Valent, MD,[d] Susanne Spitzauer, MD,[e] Rudolf Valenta, MD,[a,c]*

[a] Division of Immunopathology, Department of Pathophysiology, Center of Physiology and Pathophysiology, Vienna General Hospital (AKH), Medical University of Vienna, Austria; [b] Biomay AG, Vienna Competence Center, Austria; [c] Christian Doppler Laboratory for Allergy Research, Vienna General Hospital (AKH), Medical University of Vienna, Austria; [d] Division of Hematology and Hemostaseology, Department of Internal Medicine I, Vienna General Hospital (AKH), Medical University of Vienna, Austria; [e] Institute of Medical and Chemical Laboratory Diagnostics, Vienna General Hospital (AKH), Medical University of Vienna, Austria

Abstract

Background: Approximately 100 million patients suffer from birch pollen allergy.

Objective: Rational design of recombinant derivatives of the major birch pollen allergen, Bet v 1, characterized by reduced IgE reactivity, preservation of sequences relevant for the induction of allergen-specific blocking IgG and maintenance of T cell epitopes for immunotherapy of birch pollen allergy.

Methods: Three recombinant mosaic proteins derived from Bet v 1 were generated by re-assembly of codon-optimized genes coding for Bet v 1 fragments containing the elements for the induction of allergen-specific blocking IgG antibodies and the major T cell epitopes. The proteins were expressed in *Escherichia coli* as recombinant mosaic molecules and compared with the Bet v 1 wild-type protein by chemical and structural methods, regarding IgE- and IgG-binding capacity, in basophil activation assays and tested for the *in vivo* induction of IgG responses which block patients‘ IgE recognition of the Bet v 1 allergen.

Results: Three rBet v 1 mosaic proteins with strongly reduced IgE reactivity and allergenic activity were expressed and purified. Immunization with the recombinant hypoallergens induced IgG antibodies which inhibited allergic patients IgE reactivity to Bet v 1 stronger than those induced with the rBet v 1 wildtype allergen.

Conclusion: We report the generation and preclinical characterization of three hypoallergenic rBet v 1 derivatives with favorable properties for immunotherapy of birch pollen allergy.

Introduction

Allergy to pollen from birch and related trees (e.g., alder, hazel) is one of the most important allergies in Northern and Middle Europe, North America and in certain parts of Australia and Asia [1]. The cDNA coding for the culprit allergen in birch pollen allergy, Bet v 1, has been cloned and recombinant Bet v 1 assembling the structural, immunological and allergenic properties of natural Bet v 1 has been expressed in *Escherichia coli* [2-6]. In a recently reported double-blind placebo-controlled immunotherapy trial it was shown that rBet v 1 treatment was as effective as treatment with birch pollen extract or purified natural Bet v 1 demonstrating that rBet v 1 can replace a complex birch pollen extract [7]. In attempts to eliminate the risk of IgE-mediated side effects, several hypoallergenic derivatives of Bet v 1 have been made by recombinant technology [8]. Two hypoallergenic rBet v 1 derivatives (i.e., two rBet v 1 fragments, rBet v 1 trimer) [9, 10] have been also used for immunotherapy of birch pollen allergic patients [11]. The clinical outcome of the study performed with the hypoallergenic derivatives was less successful than the study conducted with rBet v 1 which may have been due to factors related to the study design and the lack of a pollen season in one of the study centers [12]. However, patients treated with the recombinant hypoallergenic Bet v 1 derivatives tolerated more than the five-fold maintenance dose compared to the doses given in the rBet v 1 study and showed a blunting of the rises of the systemic IgE responses caused by seasonal allergen exposure [11]. Moreover, there was a clear reduction of nasal symptoms in a subgroup of patients when objective nasal provocation testing was performed [13]. Further clinical studies performed with a hypoallergenic derivative made by chemical denaturation of rBet v 1 yielded similar immunological effects as the study performed with the recombinant hypoallergenic Bet v 1 derivatives and demonstrated clinically efficacy up to phase III studies demonstrating the therapeutic usefulness of recombinant hypoallergenic molecules [14-16].

Here we report the rational design, structural, immunological and preclinical characterization of three recombinant hypoallergenic Bet v 1 derivatives omitting chemical denaturation steps.

These derivatives were made by re-assembly of the complete Bet v 1 sequence within single molecules using codon-optimízed synthetic genes in which the sequences relevant for the induction of blocking IgG antibodies and the dominant T cell epitopes were maintained.

Material and Methods

Patients' sera, plasmids and recombinant allergen

Patients suffering from birch pollen allergy were characterized by case history and positive skin prick testing. Serum IgE Abs specific to birch pollen extract and rBet v 1 were determined by immuno CAP measurements (Phadia, Uppsala, Sweden) as described [3]. Control sera were taken from two non-allergic volunteers.

The plasmid pET 17b (Novagen Inc., Madison, WI, USA), used for the expression of rBet v1 and of the rBet v 1 derivatives is described [17]. The recombinant *Escherichia coli*-expressed (BL 21-DE3) (Stratagene, La Jolla, CA, USA) birch pollen allergen Bet v 1 (batch # 21), was obtained from Biomay (Vienna, Austria).

Monoclonal antibodies

Bip 1, a monoclonal antibody with specificity for the major birch pollen allergen Bet v 1, is described [18]. The mouse mAb 4A6 was raised against purified recombinant birch pollen profilin [19]. Anti-IgE mAb E-124.2.8 was purchased from Immunotech (Marseille, France).

Mouse IgG mAbs against peptide 2 (mAb#2) (aa 30-59) and against peptide 6 (mAb#12) (aa 74-104) of Bet v 1 were obtained by immunization of mice using KLH-coupled synthetic peptides (peptide 2: LFPKVAPQAISSVENIEGNGGPPTIKKISF; peptide 6: EDVHTNFKYNYSVIEGGPIGDTLEKISNEIK).

Construction of hypoallergenic Bet v 1 derivatives

Based on the Bet v 1 sequence [2], synthetic genes were generated giving rise to three different recombinant Bet v 1 derivatives: Restructured Bet v 1 #1 (Bet v 1-rs1) comprising amino acids 75-160 + 1-74, Restructured Bet v 1 #2 (Bet v 1 rs2) comprising amino acids 110-160 + 1-109, and Bet v 1 mosaic comprising amino acids 110-160 + 60-109 + 1-59 (Fig. 1A).

The synthetic genes of each of the recombinant Bet v 1 derivatives were cloned into the pET17b (Novagen Inc., Madison, WI, USA) cloning vector via *Nde*I and *Eco*Rl restriction sites. The coding sequences were optimized for expression in *E.coli* and the correct sequence of the derivative molecules was confirmed by double stranded DNA sequencing (Eurofins Medigenomix GmbH, Ebersberg, ATG Biosynthetics GmbH, Merzhausen, Germany).

Expression and purification of the recombinant hypoallergenic Bet v 1 derivatives

The plasmids containing the reassembled constructs were transformed into the *E.coli* BL 21 (DE3) expression strain (Stratagene, LA Jolla, CA, USA). Batch fermentation of *E.coli* BL 21 (DE3) transformed with pET-17b-Bet v 1-rs1, -rs2 or mosaic was carried out at 37°C in a 10 L fermenter (New Brunswick, Bioflow 3000) in LB medium with the addition of 0.05 % (v/v) glycerol, 0.25 % (w/v) $MgSO_4$. 7 H_2O, and 0.18 % Na_2HPO_4 . 2 H_2O for 8 h at 37°C until a cell density (OD_{600nm}) of 0.4 to 0.6 was reached. Protein expression was induced by adding 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG) (Calbiochem, Merck, Darmstadt, Germany). Recombinant proteins were produced as described in supplementary information #1.

Detection of IgG binding capacity of rBet v 1 and rBet v 1 derivatives

Purified recombinant Bet v 1 and rBet v 1 derivative molecules were tested for reacting with specific antibodies. Five μg of each protein/slot was separated by SDS-PAGE [20] and blotted onto nitrocellulose [21]. Nitrocellulose blotted proteins were incubated either with a 1:2000

dilution of a rabbit anti-rBet v 1 or the corresponding pre-immune serum, or with a 1:1000 dilution of mouse monoclonal IgG antibodies. Bound IgG antibodies were detected with a 1:1000 dilution of ^{125}I-labeled goat anti-rabbit antibodies or with a 1:1000 dilution of ^{125}I-labeled goat anti-mouse antibodies (NEN Life Science Products, Inc., Boston, Massachusetts, USA) and visualized by autoradiography [9].

IgE reactivity of dot-blotted rBet v 1 and Bet v 1 derivatives

IgE reactivity of purified rBet v 1 derivatives was tested and compared to rBet v 1 by dot blot assays. Two µL aliquots containing 1µg of purified rBet v 1, each of the rBet v 1 derivatives, bovine serum albumin (BSA) and human serum albumin (HSA) (negative control proteins) (Roth, Karlsruhe, Germany) were dotted onto nitrocellulose. Nitrocellulose were incubated with sera from nineteen birch pollen allergic individuals, two non-allergic individuals or buffer without addition of serum. Bound IgE antibodies were detected with a 1:20 dilution of ^{125}I-labeled anti-human IgE antibodies (RAST RIA, Demeditec Diagnostics, Germany). The presence of rBet v 1 and rBet v 1 derivatives on the nitrocellulose membrane was shown with rabbit anti-rBet v 1 antibodies, which were detected with 1:1000 dilution of ^{125}I-labeled donkey anti-rabbit antibodies (RAST RIA, Demeditec Diagnostics, Germany).

Immunization of rabbits and determination of IgG antibody levels

Rabbits were immunized twice, at study day 0 and at study day 28, with 200 µg of purified rBet v 1, rBet v 1-rs1, rBet v 1-rs2, or rBet v 1-mosaic initially adsorbed to CFA (Complete Freund's adjuvant) and followed by booster injection using IFA (Incomplete Freund's adjuvant). Pre-immune sera were obtained from the rabbits before immunization (Charles River Breeding Laboratories, Kisslegg, Germany).

ELISA plates (Greiner, Kremsmünster, Austria) were coated with rBet v 1, rBet v 1-rs1, rBet v 1-rs2, rBet v 1-mosaic or BSA (negative control) (5 µg/ml diluted in PBS) at 4°C overnight.

After washing three times with PBS-T (PBS + 0.05 % Tween 20) and blocking with 2 % bovine serum albumin (BSA) (Roth, Karlsruhe, Germany) in PBS-T for 6 hours, plates were incubated either with rabbit anti-Bet v 1, rabbit anti-Bet v 1-rs1, rabbit anti-Bet v 1-rs2, or with rabbit anti-Bet v 1-mosaic in five different dilutions (1:1000, 1:5000, 1:10000, 1:100000 and 1:1000000), made in 0.5 w/vol % BSA in PBS-T, at 4°C overnight. Controls were performed with normal rabbit antibodies. Plates were washed five times with PBS-T and bound rabbit IgG antibodies were detected with a 1:1000 diluted anti-rabbit IgG Horseradish Peroxidase linked whole antibody from donkey (GE Healthcare, UK Limited) for 1 hour at 37°C and 4°C. After washing with PBS-T (5 times) the color development was performed by addition of staining solution ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; Sigma-Aldrich, St.Louis, Missouri, USA) (100µl/well). The optical density was measured using an ELISA Reader (Dynatech, Denkendorf, Germany) at 405 nm.

Allergenic activity of allergen derivatives

The allergenic activity of allergen derivatives was compared with that of the Bet v 1 wildtype allergen using CD203c assays as described in supplementary information #2.

Inhibition of allergic patients' IgE binding to Bet v 1 by IgG antibodies

The inhibition of allergic patients' IgE binding to Bet v 1 by IgG antibodies was shown as described in supplementary information #3.

Results

Rationale construction of hypoallergenic rBet v 1 derivatives

It has been shown that two recombinant fragments of Bet v 1 comprising amino acids 1-74 and 75-160 preserved the Bet v 1-specific T cell epitopes but exhibited an approximately 100 fold reduced allergenic activity compared to rBet v 1 as shown *in vitro* by basophil activation

testing and in several *in vivo* provocation studies [9, 22-25]. Each of these fragments contains a peptide defined by two monoclonal antibodies mAb#2 (aa 30-59) and mAb#12 (aa 74-104) which induced Bet v 1-specific IgG antibodies inhibiting the binding of birch pollen allergic patients IgE to Bet v 1 [26] (Fig. 1A). The first Bet v 1 derivative, designated Bet v 1-rs1, was made by re-assembling the hypoallergenic fragments aa 1-74 and aa 75-160 within one molecule as a tail-to-head construct as described for a Phl p 12 derivative [27] (Fig. 1A). The second Bet v 1 derivative, designated Bet v 1-rs2, was prepared by re-assembling aa 1-109 which contains three peptides (P2: aa 30-59; P3: aa 50-79; and P6: aa 74-104) that had induced strong blocking IgG antibody responses against Bet v 1 [26] at the C-terminus and a portion comprising aa 110-160 at the N-terminus. Because of the possibility that the fragment aa 1-109 might regain allergenic activity, it was broken into two pieces, aa 60-109 containing P6 and aa 1-59 containing P2, and a mosaic was constructed from these pieces. This mosaic, designated Bet v 1-mosaic, consists of Bet v 1 portions aa 110-160, aa 60-109 and aa 1-59 from the N- to the C-terminus (Fig. 1A).

High level expression, purification, structural and immunochemical characterization of rBet v 1 derivatives

Using the plasmids containing the cDNAs coding for the three Bet v 1 derivative molecules (Bet v 1-rs1, Bet v 1-rs2 and Bet v 1-mosaic) high level expression of the recombinant proteins yielding more than 20% of the total *E. coli* proteins was obtained. Each of the recombinant proteins could be purified from the inclusion body fraction of the bacteria via several chromatography steps to more than 90 % purity (Fig. 1B). There was no sign of aggregation of the proteins which yielded single bands both under reducing as well as non-reducing conditions in SDS-PAGE (data not shown). The experimentally determined mass was in good agreement with the mass calculated from the deduced amino acid sequence (data not shown). The overall secondary structure contents of the three recombinant Bet v 1

derivatives were compared with that of rBet v 1 by CD. In contrast to rBet v 1, which exhibited the typical fold of a mixed α helical and ß sheet containing protein, all three rBet v 1 derivatives were unfolded (data not shown).

Next we used Bet v 1-specific antibody probes to test IgG reactivity of the Bet v 1 derivatives. Nitrocellulose-blotted rBet v 1 and rBet v 1 derivatives reacted with the polyclonal rabbit antibodies which had been raised against rBet v 1 (Fig. 2A). Interestingly, the monoclonal antibody Bip 1 which recognizes conformational epitopes of Bet v 1 reacted only with the folded rBet v 1 wild type protein but not with the unfolded rBet v 1 derivatives (Fig. 2B). The mAbs specific for P2 (mAb#2) (aa 30-59) and for P6 (mAb#12) (aa 74-104) reacted with rBet v 1 and each of the three rBet v 1 derivatives (Figs. 2C-D). The rabbits pre-immune serum and an isotype-matched mouse monoclonal antibody without specificity for Bet v 1 did not show any binding (data not shown).

Comparison of the IgE reactivity of rBet v 1 and rBet v 1 derivatives

Aliquots of rBet v 1, rBet v 1-rs1, rBet v 1-rs2 or rBet v 1-mosaic were dotted under non-denaturing conditions onto nitrocellulose membranes. We found that none of the 19 birch pollen allergic patients tested exhibited any detectable IgE reactivity to the rBet v 1 derivatives whereas they showed IgE-binding to rBet v 1 (Fig. 3, lanes 1-19). No IgE reactivity to the control proteins HSA and BSA was found. Serum IgE from non-allergic individuals and buffer showed no reactivity to any of the proteins (Fig. 3, lanes 20-21, 0).

The presence of rBet v 1 and rBet v 1 derivatives on the membrane was confirmed by testing with rabbit anti-rBet v 1 antibodies (Fig. 3, lane 22).

Reduced allergenic activity of rBet v 1 derivatives

In a first set of experiments, we compared rBet v 1-rs1 with rBet v 1 and an equimolar mix of rBet v 1 fragments for their allergenic activity using basophils from birch pollen allergic

patients. rBet v 1-rs1 did not cause any upregulation of the CD203c expression up to the top concentration (i.e., 50 pM) tested in the six patients whereas rBet v 1 started to induce basophil activation already at 0.5 pM and each of the patients responded to 50 pM. rBet v 1-rs1 exhibited even lower allergenic activity than the rBet v 1 fragment mix which induced CD203c upregulation at 5pM in one patient and at 50 pM in two of the 6 patients (Fig. 4A). We then compared the three rBet v 1 derivatives with rBet v 1 regarding allergenic activity using basophils from additional 4 patients (Fig. 4B). In three patients, rBet v 1 derivatives did not induce any CD203c upregulation up to the top concentration of 50 pM, whereas rBet v 1 caused activation at 5 pM. In the fourth patient, the rBet v 1 derivatives showed a 100-fold reduction of allergenic activity compared to rBet v 1 (Fig. 4B).

Immunization with rBet v 1 derivatives induces IgG antibodies which recognize Bet v 1 and strongly inhibit patients IgE binding to Bet v 1

As shown in Figure 5, rabbits immunized with the rBet v 1 derivatives showed an almost comparable IgG response to rBet v 1 wild type as the rabbit immunized with rBet v 1 (Fig. 5A). Rabbit anti-rBet v 1 antibodies reacted with the rBet v 1 derivatives albeit somewhat weaker than the anti-rBet v 1 derivative antibodies (Figs. 5B-D).

We then investigated whether rBet v 1 derivative-induced rabbit IgG antibodies can inhibit the binding of patients' serum IgE to the wild-type rBet v 1 in an ELISA competition assay. The anti-rBet v 1-rs1 antiserum inhibited the binding of birch pollen allergic patients' IgE to rBet v 1 between 56.5 % and 98 % (85 % mean inhibition). The anti-rBet v 1-rs2 antiserum showed inhibition rates between 59.5 % and 98.5 % (87 % mean inhibition) and the anti-rBet v 1-mosaic inhibited between 58 % and 99.5 % (82 % mean inhibition). Interestingly, the rabbit antiserum against rBet v 1 derivatives showed higher average inhibition rates than the anti-rBet v 1 antibodies which yielded only 62 % mean inhibition of IgE binding to rBet v 1 (Table 1).

Discussion

In this study we constructed by rational molecular reassembly three recombinant hypoallergenic derivatives of the major birch pollen allergen, Bet v 1 for immunotherapy. The recombinant Bet v 1 fragments which have been re-assembled as a tail-to-head construct for the first derivative, Bet v 1-rs1, have been evaluated regarding their allergenic activity in several skin test and provocation test studies in birch pollen allergic patients showing a consistent reduction of allergenic activity [5, 9, 23, 11, 7, 24]. This first hypoallergenic derivative also exhibited a strongly reduced allergenic activity. Similar results were obtained for the second re-assembled molecule although we maintained in Bet v 1-rs2 the portions necessary for the induction of IgG antibodies against the IgE binding sites of Bet v 1 in a large fragment comprising aa 1-109 which was preceded by a smaller portion comprising aa 110-160 containing the major T cell epitope of Bet v 1 [28]. The third derivative, designated Bet v 1-mosaic was made similar as Bet v 1-rs2. Again the C-terminal portion aa 110-160 carrying the major T cell epitope was placed at the N-terminus but the portion aa 1-109 responsible for the induction of blocking antibodies was disrupted again to eliminate possible IgE reactivity to such a large fragment. The fragment aa 1-109 was therefore re-assembled by two fragments containing two peptides which have been shown to induce blocking IgG responses [26].

All three rBet v 1 derivatives could be expressed at high levels in an *E. coli* expression system and purified via several chromatography steps close to homogeneity. They showed no signs of aggregation, and were recognized by antibodies raised against those peptides which induced blocking antibody responses against Bet v 1. A monoclonal antibody, Bip 1, which recognizes a conformational epitope on Bet v 1 did not react with the three derivatives indicating that their three-dimensional structure is altered compared to Bet v 1. The latter assumption was confirmed by CD measurements demonstrating that the three derivatives represented unfolded molecules.

The three derivatives lacked relevant IgE reactivity as already observed for the unfolded rBet v 1-fragments and exhibited an even greater reduction of allergenic activity than a mix of the two fragments [9]. The three recombinant Bet v 1 derivatives therefore show important advantages over the previously characterized rBet v 1 fragments. They combine both molecules within one protein which can be easily expressed in large amounts in *E. coli*. Furthermore, their allergenic activity seems to be even lower than that of the fragments.

Several *in vitro* results as well as immunotherapy studies indicate that blocking IgG antibodies are involved in protection against allergic immune responses. It has been shown that allergen-specific blocking IgG inhibits immediate and late-phase responses. Moreover, allergen-specific blocking IgG antibodies inhibit the boosts of IgE production induced by allergen contact [11, 31].

In fact, a recent immunotherapy study performed with rBet v 1 demonstrated that the reduction of clinical symptoms and skin sensitivity was associated with the production of allergen-specific IgG responses [7].

Therefore immunization experiments with the three rBet v 1 derivatives were performed and it was shown that the derivatives induced comparable IgG responses to the Bet v 1 wild-type allergen as Bet v 1 wildtype itself.

Even more important we found that the IgG antibodies induced with each of the three rBet v 1 derivatives inhibited the binding of birch pollen allergic patients' IgE to the Bet v 1 allergen better than those induced with the folded rBet v 1 wildtype molecule. This result was quite unexpected but may be attributed to the fact that we have maintained those peptides in the rBet v 1 derivatives, which were involved in the induction of blocking antibodies.

We believe that the strategy of allergen fragmentation and consecutive systematic recombination in the form of mosaic molecules has advantages over the use of small hypoallergenic allergen fragments because small protein fragments may exhibit lower immunogenicity and induce low allergen-specific IgG antibodies. In addition, the generation

of one single reassembled molecule may facilitate the production and purification steps [8]. We did not have the chance to compare the three rBet v 1 hypoallergens with the recently described rBet v 1 derivative obtained by the shuffling of DNA sequences from Bet v 1, Aln g 1 and Cor a 1 but it is likely that their allergenic activity is lower because the IgE reactivity of the shuffled constructs was reduced only 8-20 fold [29] whereas we could not detect any IgE binding to the three derivatives at least in those patients which we tested.

The three recombinant derivatives characterized here may also have several advantages over a hypoallergenic rBet v 1 folding variant which has been successfully used for immunotherapy of patients [14, 16]. The folding variant exhibited residual IgE reactivity and needs to be produced by chemical modification [14] of the rBet v 1 molecules whereas the hypoallergenic derivatives described here can be obtained directly by recombinant expression. The mosaic approach used by us also seems to have the advantage to be generally applicable to allergenic molecules from different sources [30, 31].

In conclusion, the hypoallergenic Bet v 1 derivatives described here may be considered promising candidate molecules for active vaccination but also for the induction of tolerance [32, 33]. Moreover, their genes may be useful for gene therapy and genetic immunization protocols [34, 35] because the resulting proteins exhibit reduced allergenic activity.

Acknowledgments

This study was supported by grants F1803, F1804, F1815, F1809 and L214-B13 of the Austrian Science Fund (FWF), and by a research grant from Biomay, Vienna, Austria.

References

1. Mothes N, Horak F, Valenta R. Transition from a botanical to a molecular classification in tree pollen allergy: Implications for diagnosis and therapy. Int Arch Allergy Immunol 2004; 135:357-73.
2. Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rulpold H, et al. The gene coding for the major birch pollen allergen Bet v I, is highly homologous to a pea disease resistance response gene. EMBO J 1989; 8:1935-8.
3. Niederberger V, Pauli G, Grönlund H, Fröschl R, Rumpold H, Kraft D, et al. Recombinant birch pollen allergens (rBet v 1 and rBet v 2) contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen: a quantitative IgE inhibition study with sera from different populations. J Allergy Clin Immunol 1998; 102:579-91.
4. Kazemi-Shirazi L, Pauli G, Purohit A, Spitzauer S, Fröschl R, Hoffmann-Sommergruber K, et al. Quantitative IgE inhibition experiments with purified recombinant allergens indicate pollen-derived allergens as the sensitizing agents responsible for many forms of plant food allergy. J Allergy Clin Immunol 2000; 105:116-25.
5. Pauli G, Oster JP, Deviller P, Heiss S, Bessot JC, Susani M, et al. Skin testing with recombinant allergens rBet v 1 and birch profillin, rBet v 2: diagnostic value for birch pollen and associated allergies. J Allergy Clin Immunol 1996; 97:1100-9.
6. Movérare R, Westritschnig K, Svensson M, Hayek B, Bende M, Pauli G, et al. Different IgE reactivity profiles in birch pollen-sensitive patients from six European populations revealed by recombinant allergens: an imprint of local sensitization. Int Arch Allergy Immunol 2002; 128:325-35.

7. Pauli G, Larsen TH, Rak S, Horak F, Patorello E, Valenta R, et al. Efficacy of recombinant birch pollen vaccine for the treatment of birch-allergic rhinoconjunctivitis. J Allergy Clin Immunol 2008; 122:951-960.
8. Linhart B, Valenta R. Molecular design of allergy vaccines. Curr Opin Immunol 2005; 17:646-55.
9. Vrtala S, Hirtenlehner K, Vangelista L, Pastore A, Eichler HG, Sperr WR, et al. 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 1997; 99:1673-81.
10. Vrtala S, Hirtenlehner K, Susani M, Akdis M, Kussebi F, Akdis CA, et al. Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen, Bet v 1. FASEB J 2001; 15:2045-57.
11. Niederberger V, Horak F, Vrtala S, Spitzauer S, Krauth MT, Valent P, et al. Vaccination with genetically engineered allergens prevents progression of allergy disease. Proc Natl Acad Sci U S A 2004; 101:14677-82.
12. Purohit A, Niederberger V, Kronqvist M, Horak F, Grönneberg R, Suck R, et al. Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives. Clin Exp Allergy 2008; 38:1514-25.
13. Reisinger J, Horak F, Pauli G, van Hage M, Crommwell O, König F, et al. Allergen-specific nasal IgG antibodies induced by vaccination with genetically modified allergens are associated with reduced nasal allergen sensitivity. J Allergy Clin Immunol 2005; 116:347-54.
14. Kahlert H, Suck R, Weber B, Nandy A, Wald M, Keller W, et al. Characterization of hypoallergenic recombinant Bet v 1 variant as a candidate for allergen-specific immunotherapy. Int Arch Allergy Immunol 2008; 145:193-206.

15. Klimek L, Bachert C, Doerner C, Meyer H, Narkus A. Specific immunotherapy with recombinant birch pollen allergen rBet v 1-FV is clinically efficacious (abstract). Allergy Clin Immunol Int 2005; (suppl 1):15.
16. Rak S. Clinical results with an hypoallergenic recombinant birch pollen allergen derivative (abstract). Congress of European Academy of Allergy and Clinical Immunology 2009.
17. Hoffmann-Sommergruber K, Susani M, Ferreira F, Jertschin P, Ahorn H, Steiner R, et al. High-level expression and purification of the major birch pollen allergen, Bet v 1. Protein Expr Purif 1997; 9:33-39.
18. Laffer S, Vangelista L, Steinberger P, Kraft D, Pastore A, Valenta R. Molecular characterization of Bip 1, a monoclonal antibody that modulates IgE binding to birch pollen allergen, Bet v 1. J Immunol 1996; 157:4953-62.
19. Wiedemann P, Giehl K, Almo SC, Fedorov AA, Girvin M, Steinberger P, et al. Molecular and structural analysis of a continuous birch profilin epitope defined by a monoclonal antibody. J Biol Chem 1996; 271:29915-21.
20. Fling SP, Gregerson DS. Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. Anal Biochem 1986; 155:83-88.
21. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; 76:4350-54.
22. Vrtala S, Akdis CA, Budak F, Akdis M, Blaser K, Kraft D, Valenta R. T cell epitope-containing hypoallergenic recombinant fragments of the major birch pollen allergen, Bet v 1, induce blocking antibodies. J Immunol 2000; 165:6653-59.
23. Pauli G, Purohit A, Oster JP, De Blay F, Vrtala S, Niederberger V, et al. Comparison of genetically engineered hypoallergenic rBet v 1 derivatives with rBet v 1 wild-type

by skin prick and intradermal testing: results obtained in a French population. Clin Exp Allergy 2000; 30: 1076-84.

24. van Hage-Hamsten, Kronqvist M, Zetterström O, Johansson E, Niederberger V, Vrtala S, et al. Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1: Results obtained with a mix of two recombinant Bet v 1 fragments and recombinant Bet v 1 trimer in a Swedish population before the birch pollen season. J Allergy Clin Immunol 1999; 104:969-77.
25. Nopp A, Halldén G, Lundahl J, Johansson E, Vrtala S, Valenta R, et al. Comparison of inflammatory responses to genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1 and to recombinant Bet v 1 wild type in skin chamber fluids collected from birch pollen-allergic patients. J Allergy Clin Immunol 2000; 106:101-9.
26. Focke M, Linhart B, Hartl A, Wiedermann U, Sperr WR, Valent P, et al. Non-anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for preventive vaccination. Clin Exp Allergy 2004; 34:1525-33.
27. Westrischnig K, Linhart B, Focke-Tejkl M, Pavkov T, Keller W, Ball T, et al. A hypoallergenic vaccine obtained by tail-to head restructuring of timothy grass pollen profilin, Phl p 12, for the treatment of cross-sensitization to profilin. J Immunol 2007; 179:7624-34.
28. Jahn-Schmid B, Radakovics A, Lüttkopf D, Scheurer S, Vieths S, Ebner C, Bohle B. Bet v 1 $_{142-153}$ is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. J Allergy clin Immunol 2005; 116:213-19.
29. Wallner M, Stöcklinger A, Thalhamer T, Bohle B, Vogel L, Briza P, Breiteneder H, Vieths S, Hartl A, Mari A, Ebner C, Lackner P, Hammerl P, Thalhamer J, Ferreira F.

Allergy multivaccines created by DNA shuffling of tree pollen allergens. J Allergy Clin Immunol 2007; 120:374-80.

30. Ball T, Linhart B, Sonneck K, Blatt K, Herrmann H, Valent P, et al. Reducing allergenicity by altering allergen fold: a mosaic protein of Phl p 1 for allergy vaccination. Allergy 2009; 64:569-580.
31. Mothes-Luksch N, Stumvoll S, Linhart B, Focke M, Krauth MT, Hauswirth A, Valent P, Verdino P, Pavkov T, Keller W, Grote M, Valenta R. Disruption of allergenic activity of the major grass pollen allergen Phl p 2 by reassembly as a mosaic protein. J Immunol 2008; 181:4864-73.
32. Valenta R. The future of antigen-specific immunotherapy of allergy. Nat Rev Immunol 2002; 2:446-53.
33. Wiedermann U. Prophylaxis and therapy of allergy by mucosal tolerance induction with recombinant allergens or allergen constructs. Curr Drug Targets Inflamm Allergy 2005; 4:577-83.
34. Weiss R, Scheiblhofer S, Thalhamer J. DNA vaccines for allergy treatment. Methods Mol Med 2006; 127:253-67.
35. Barany U, Linhart B, Pilat N, Gattringer M, Bagley J, Muehlbacher F, et al. Tolerization of a type I allergic immune response through transplantation of genetically modified hematopoietic stem cells. J Immunol 2008; 180:8168-75.

Figures and Table

Figure 1. Hypoallergenic rBet v 1 derivatives. (A) Construction scheme of the rBet v 1 derivatives (Bet v 1-rs1, Bet v 1-rs2, Bet v 1-mosaic). The amino acids at the borders of the protein segments and the binding sites of two monoclonal antibodies mAb#2 and mAb#12 are indicated. (B) SDS-PAGE of purified rBet v 1 and rBet v 1 derivatives. Comparison of purified recombinant Bet v 1 and rBet v 1 derivatives. M, molecular mass markers (kDa).

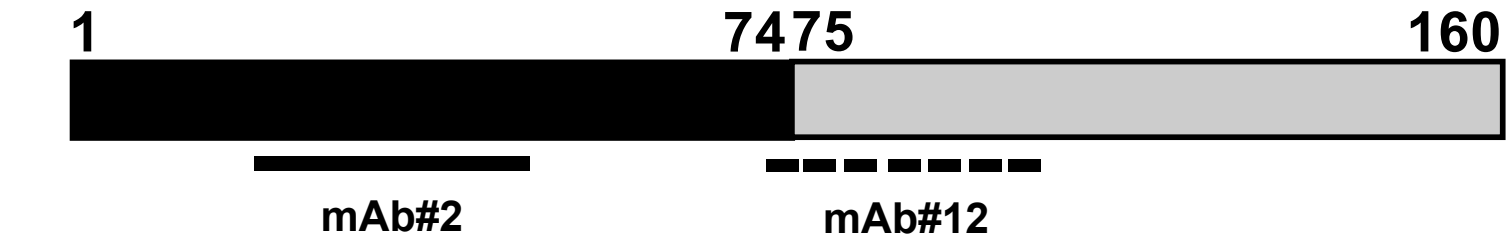
Figure 2. IgG reactivity of rBet v 1 and rBet v 1 derivatives with Bet v 1-specific antibodies. Nitrocellulose blotted rBet v 1 and rBet v 1 derivatives were probed with rabbit anti-Bet v 1 antibodies (rαBet v 1) (A), mAb Bip 1 (B), mAb#2 (C) or mAb#12 (D). Molecular weights are displayed on the right margins in kilo Daltons (kDa).

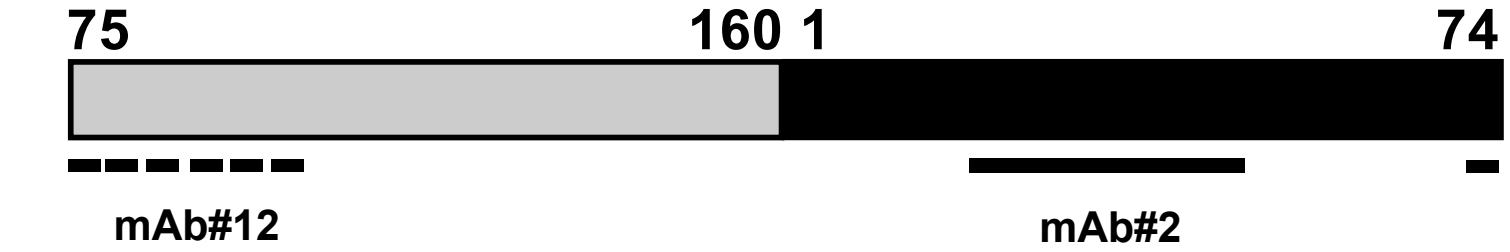
Figure 3. IgE-reactivity of rBet v 1 and rBet v 1derivatives. Nitrocellulose-dotted rBet v 1, rBet v 1- rs1, rBet v 1- rs2 and rBet v 1-mosaic as well as HSA and BSA were exposed to sera from 19 birch pollen allergic patients (lanes 1-19), two non-allergic individuals (lanes 20-21) buffer (0) or rabbit anti-rBet v 1 antibodies (lane 22). Bound IgE and rabbit IgG antibodies were detected and visualized by autoradiography.

Figure 4. Allergenic activity of rBet v 1 and rBet v 1 derivatives as determined by CD203c upregulation on allergic patients basophils. (A) Blood samples from six birch-allergic patients (A-F) were exposed to increasing concentrations (0.005 to 50 pM) of rBet v 1, an equimolar mixture of rBet v 1 fragments (F1+F2) or rBet v 1- rs1 (x-axes). (B) Incubation of blood samples from additional four birch pollen allergic individuals (G-J) with increasing concentrations (0.005 to 50 pM) of rBet v 1, rBet v 1-rs1, rBet v 1-rs2, rBet v 1-mosaic (x-axes). Anti-IgE served as a positive control. The stimulation indices (SI) (y-axis) reflect the up-regulation of CD203c expression compared to buffer (0).

Figure 5. Reactivity of rabbit anti-rBet v 1 or anti-rBet v 1 derivative antibodies with rBet v 1 and rBet v 1 derivatives. Different dilutions (x-axes) of rabbit anti-rBet v 1 (anti-Bet v 1: ●), anti-rBet v 1derivative antibodies (anti-Bet v 1- rs1: ■; anti-Bet v 1-rs 2: ▲; anti-Bet v 1-mosaic: **x**) or antibodies from a normal rabbit (▬) were reacted with rBet v 1 (A), rBet v 1-rs1 (B), rBet v 1-rs2 (C) or rBet v 1-mosaic (D). Optical density (OD) values (y-axes) correspond to the amounts of bound antibodies.

Table 1. Inhibition of allergic patients' IgE-binding to rBet v 1 by IgG antibodies. rBet v 1 was pre-incubated with rabbit IgG antibodies raised against rBet v 1, rBet v 1-rs1, rBet v 1-rs2, rBet v 1-mosaic or the corresponding rabbit pre-immune sera (nrs) and then exposed to sera from 18 birch pollen allergic patients. The OD values correspond to the amounts of bound IgE antibodies. Percentages (%) inhibition of patients' IgE-binding to rBet v 1 obtained with each antiserum versus the pre-immune sera as well as the mean inhibition values are displayed.


rBet v 1
1
74
75
160
mAb#2
mAb#12


Bet v 1- rs 1
75
160
1
74
mAb#12
mAb#2


Bet v 1- rs 2
110
160
1
109
mAb#2
mAb#12


Bet v 1- mosaic
110
160
60
109
1
59
mAb#12
mAb#2

Figure 1A

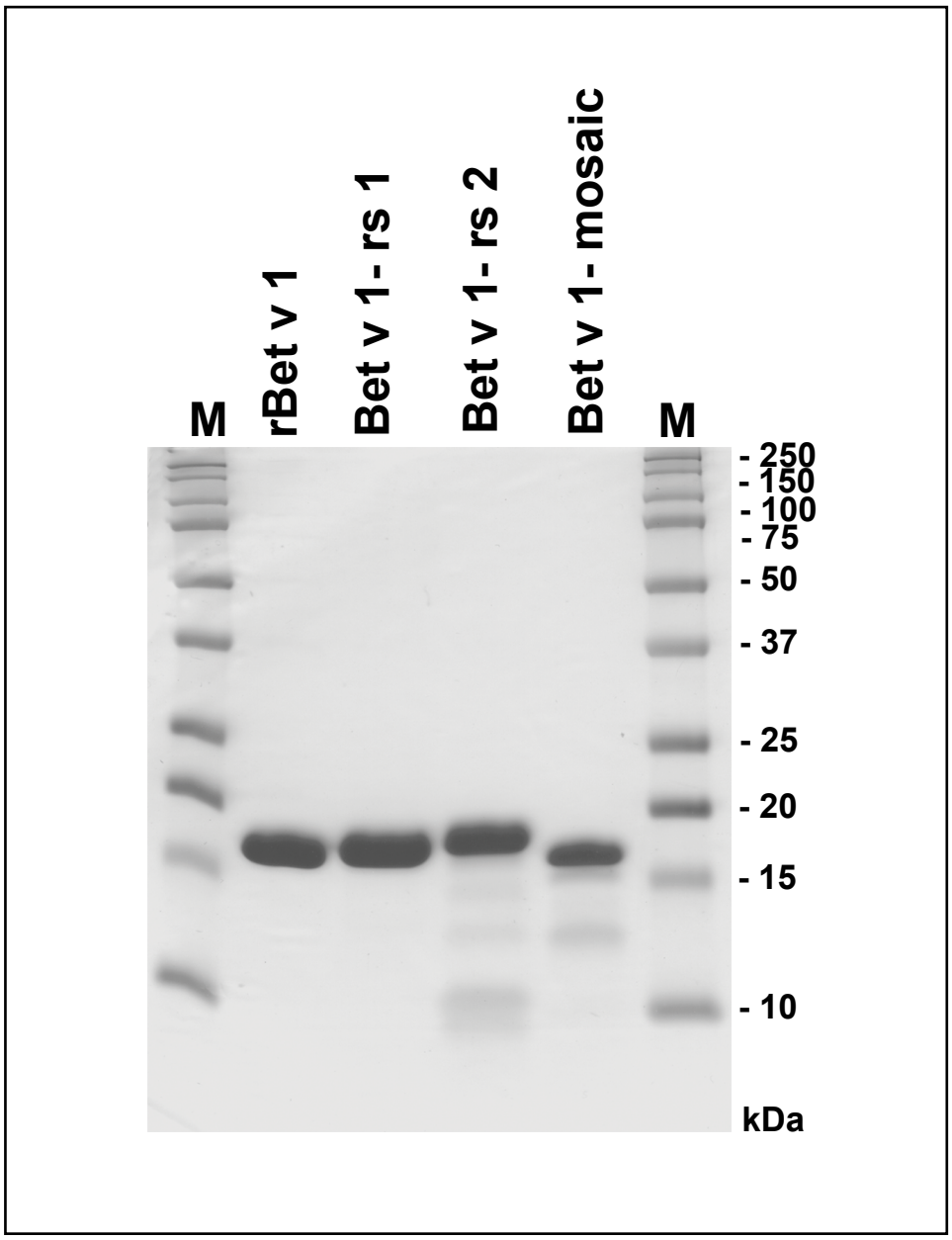

M
rBet v 1
Bet v 1- rs 1
Bet v 1- rs 2
Bet v 1- mosaic
M
- 250
- 150
- 100
- 75
- 50
- 37
- 25
- 20
- 15
- 10
kDa

Figure 1B

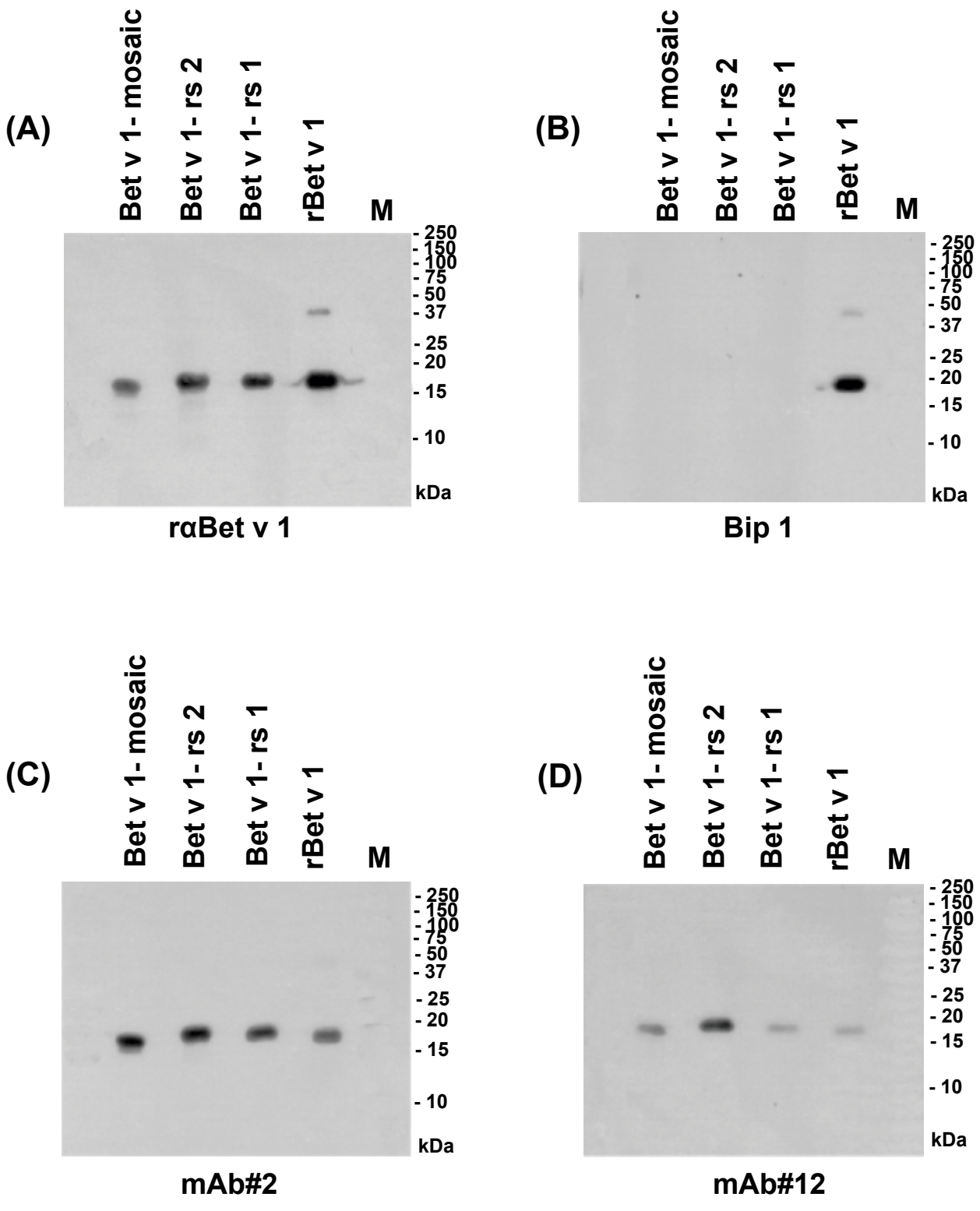

(A)
Bet v 1- mosaic
Bet v 1- rs 2
Bet v 1- rs 1
rBet v 1
M
250
150
100
75
50
37
25
20
15
10
kDa
rαBet v 1
(B)
Bip 1
(C)
mAb#2
(D)
mAb#12

Figure 2

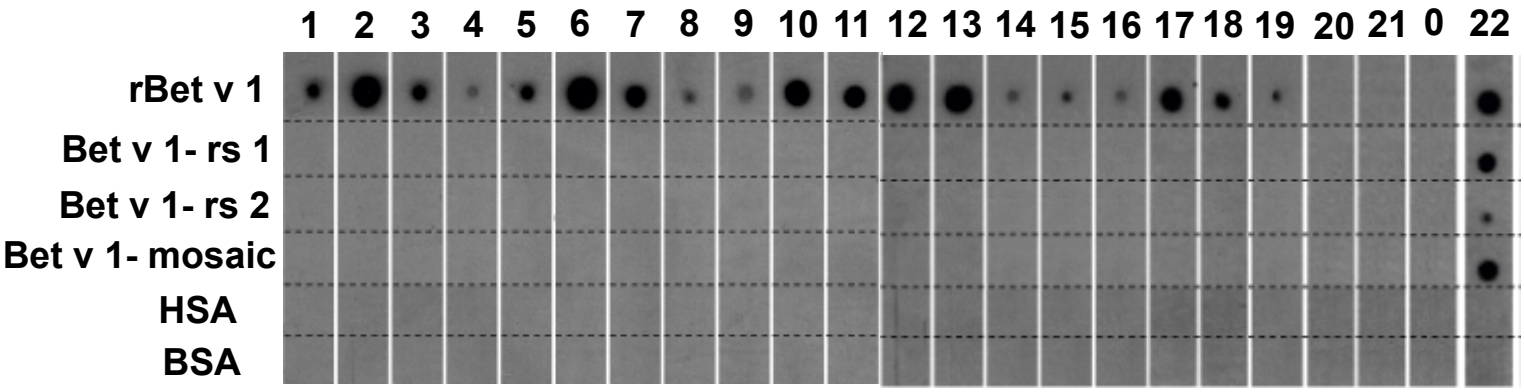

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 0 22
rBet v 1
Bet v 1- rs 1
Bet v 1- rs 2
Bet v 1- mosaic
HSA
BSA

Figure 3

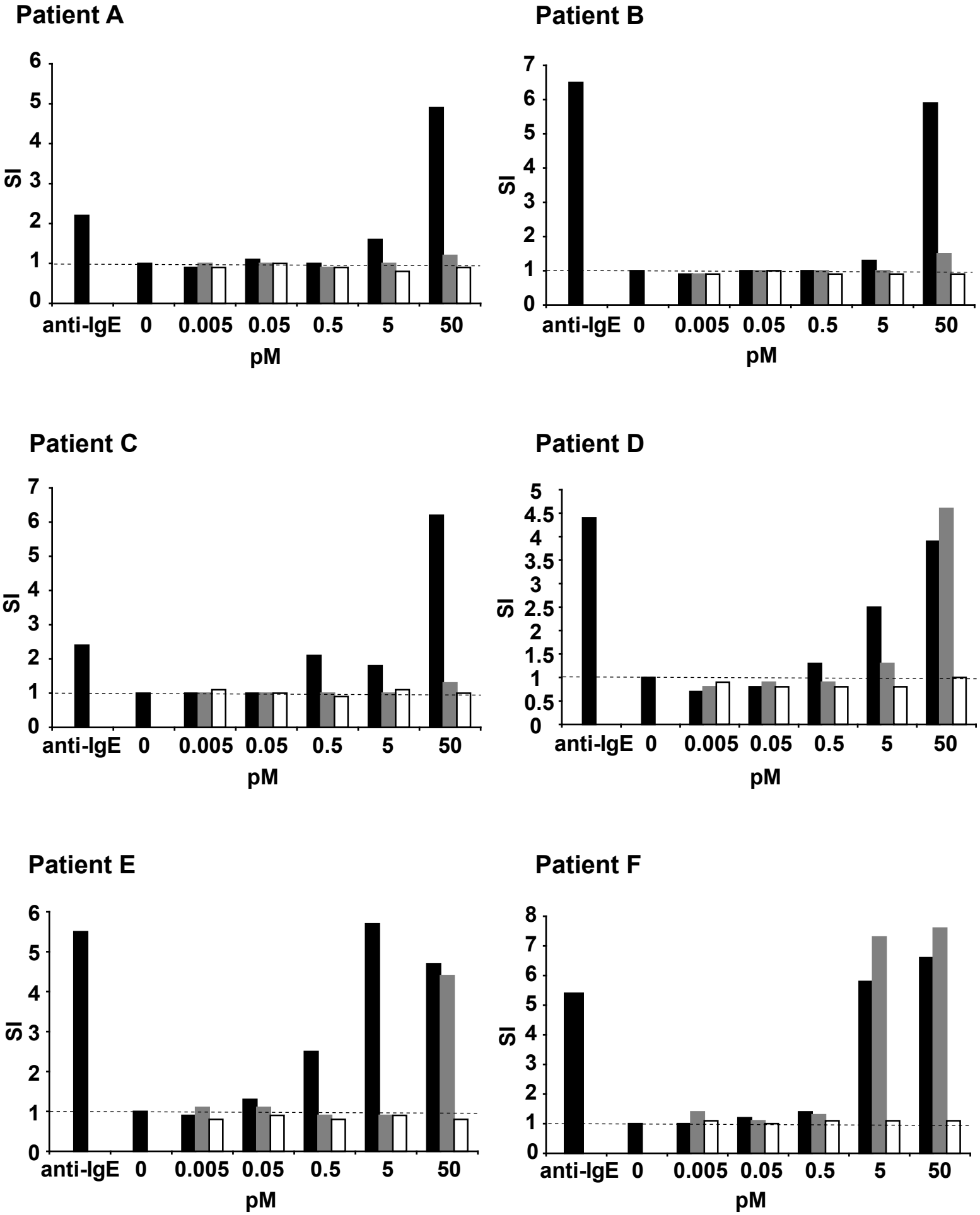

Patient A
Patient B
Patient C
Patient D
Patient E
Patient F
SI
anti-IgE
0
0.005
0.05
0.5
5
50
pM
rBet v 1
F1 + F2
Bet v 1- rs 1

Figure 4A

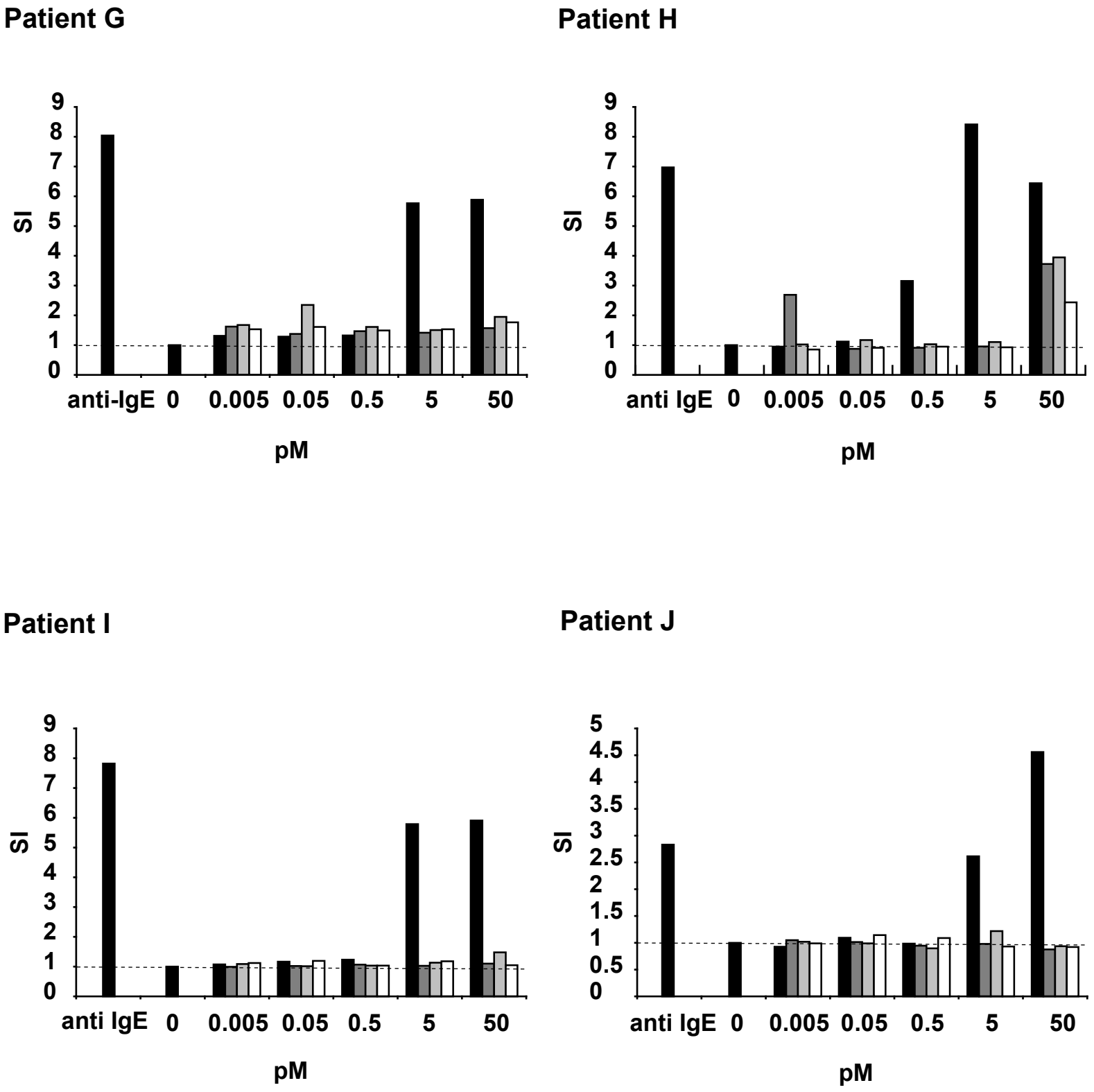

Patient G
Patient H
Patient I
Patient J
SI
pM
anti-IgE
anti IgE
0
0.005
0.05
0.5
5
50
rBet v 1
Bet v 1- rs 1
Bet v 1- rs 2
Bet v 1- mosaic

Figure 4B

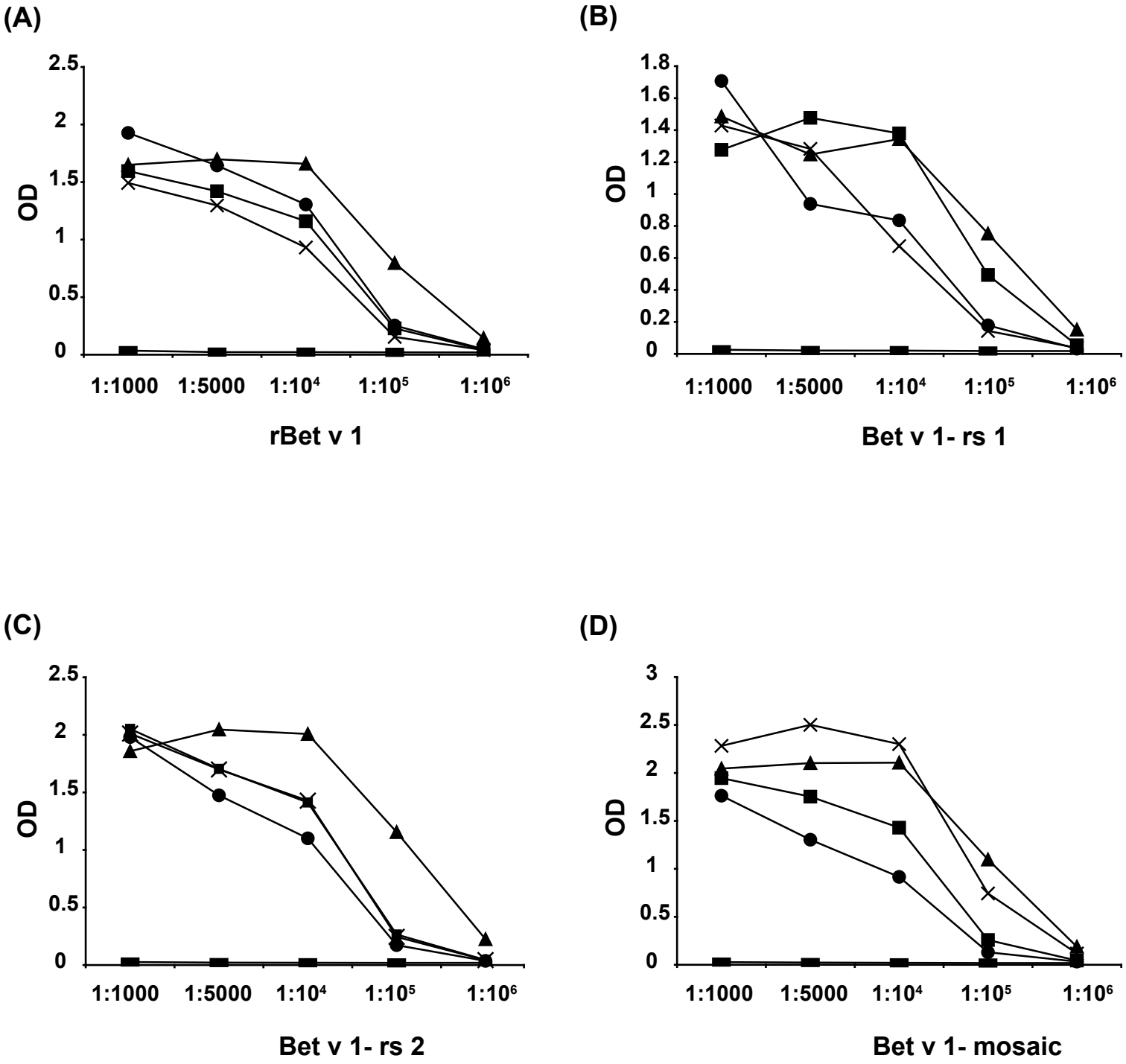

(A)
OD
2.5
2
1.5
1
0.5
0
1:1000
1:5000
1:10⁴
1:10⁵
1:10⁶
rBet v 1
(B)
OD
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1:1000
1:5000
1:10⁴
1:10⁵
1:10⁶
Bet v 1- rs 1
(C)
OD
2.5
2
1.5
1
0.5
0
1:1000
1:5000
1:10⁴
1:10⁵
1:10⁶
Bet v 1- rs 2
(D)
OD
3
2.5
2
1.5
1
0.5
0
1:1000
1:5000
1:10⁴
1:10⁵
1:10⁶
Bet v 1- mosaic

Figure 5

Table 1. Inhibition of allergic patients' IgE-binding to rBet v 1 by IgG antibodies

	OD values		% inhibition	OD values		% inhibition	OD values		% inhibition	OD values		% inhibition
Patient	nrs	rα Bet v 1	rα Bet v 1	nrs	α Bet v 1rs 1	α Bet v 1rs1	nrs	α Bet v 1rs 2	α Bet v 1rs2	nrs	α Bet v 1mosaic	α Bet v 1mosaic
1	0.111	0.066	40.5	0.121	0.037	69.5	0.13	0.036	72	0.131	0.044	66.5
2	0.353	0.15	57.5	0.404	0.082	79.7	0.433	0.071	83.5	0.477	0.117	75.5
3	1.055	0.269	74.5	1.193	0.06	95	1.248	0.049	96	1.445	0.085	94
4	0.306	0.093	69.6	0.336	0.038	89	0.357	0.035	90	0.391	0.047	88
5	0.508	0.14	72.5	0.612	0.052	91.5	0.657	0.049	92.5	0.674	0.061	91
6	3.211	0.466	85.5	3.335	0.095	98	3.471	0.047	98.5	3.601	0.408	89
7	1.662	0.483	71	1.902	0.074	96	2.026	0.063	97	2.098	0.154	93
8	0.125	0.086	31	0.14	0.033	76.5	0.142	0.033	77	0.149	0.045	70
9	0.991	0.374	62	0.442	0.128	71	0.452	0.092	79.6	0.466	0.171	64
10	1.571	0.315	80	1.546	0.063	96	1.817	0.04	98	1.977	0.219	89
11	0.582	0.245	58	0.655	0.039	94	0.689	0.027	96	0.704	0.074	89.5
12	1.32	0.154	88.5	1.641	0.063	96	1.833	0.048	97	2.051	0.109	95
13	1.424	0.361	74.7	1.573	0.048	97	1.682	0.038	98	1.705	0.12	99.5
14	0.158	0.127	19.6	0.16	0.06	62.5	0.177	0.061	65.5	0.198	0.068	66
15	0.098	0.057	41.8	0.106	0.046	56.5	0.111	0.045	59.5	0.111	0.047	58
16	0.617	0.073	88	0.738	0.049	93	0.805	0.047	94	0.805	0.086	89
17	0.523	0.134	74.4	0.546	0.048	91	0.586	0.041	93	0.602	0.071	88
18	0.118	0.083	29.7	0.126	0.035	72	0.135	0.033	75.5	0.144	0.04	72
% mean inhibition			**62**			**85**			**87**			**82**

Supplementary Table I. Demographic, serologic, and clinical characterization of the individuals

Allergic Subjects	Sex/Age	Birch pollen related symptoms	Type of treatment	IgE CAP (kUA/L)		Total IgE (kU/L)	Other allergies
				Birch	rBet v 1		
1	M/56	RC, AD, AS, OAS	AH, TCt	15.7	14.6	860	a, g, pf, npf, mo, mi
2	M/44	RC, AD	AH	27.4	27.1	69.3	pf
3 (C)	M/28	RC, AD	AH, TCt, SIT	76.4	16.84	>5000	a, g, pf, npf, mo, mi, w
4 (H)	M/27	RC, AD	AH, TCt	15.4	10.1	281	a, pf, mi
5	M/47	RC, AD, OAS	no	25.6	20.2	114	a, g, pf, mi
6	M/38	RC, AD	AH, TCt	>200	>200	>10000	a, g, pf, npf, mo, mi, w
7	F/46	RC, AD	TCt	>100	26	>5000	a, g, pf, npf, mo, mi, w
8	M	RC, AD	AH, TCt, UV	12	13.66	3674	a, g, pf, npf, mi, w
9	F/42	RC, AD, AS	AH, SIT	145.4	47.8	>10000	a, g, pf, npf, mi, w
10 (F)	M/29	RC	AH	98.1	>100	760	a, g, pf, npf, mo, mi, w
11	F	RC, AD	no	170.6	85.2	6192	a, g, pf, npf, mo, mi, w
12	F/46	RC, AS, OAS	no	57.5	60.5	466	a, g, pf, mi
13 (A)	F/30	RC, OAS	AH	>100	>100	>5000	a, g, pf, npf, mo, mi, w
14 (B)	M/29	RC	no	12.4	10.4	285	pf
15 (E)	M/42	RC	no	1.01	1	36.1	mi
16 (D)	F/30	RC	no	4.87	3.41	144	a, g, pf, npf, mi
17	M/47	RC, OAS	no	37.1	28.7	144	a, g, pf, mo, w
18	M/52	RC	AH, BD, TCt	36.9	36	115	pf
19	M/51	RC	AH, TCt	8.45	7.41	29.7	g, pf, mi
(G)	M/32	RC	AH, SIT	6.61	3.88	102	a, g, pf, npf, mi,mo, w
(I)	M/23	RC	AH	24.8	21.7	233	a, g, pf, npf, mo, mi, w
(J)	F/30	RC, AD,AS, OAS	AH, BD	58.7	47	4886	a, pf, mo, w
Non-Allergic Subjects							
20	F/28	-	no	<0.35	<0.35	<2.00	0
21	F/34	-	no	<0.35	<0.35	2.65	0

- (A-H) represent the subjects studied by CD203c expression experiments
- M, Male; F, Female; RC, rhinoconjunctivitis; AD, atopic dermatitis; AS, asthma; OAS, oral allergy syndrome
- AH, antihistaminicos; BD, bronchodilator; TCt, topical corticosteroids, SIT, specific-immunotherapy; UV, ultraviolet-light therapy; no, no therapy at the time of analysis
- a, animals; g, grass; pf, plant food; npf, non-plant-derived food; mo, molds; mi, mites; w, weeds; 0, no allergy

Supplementary Information # 1: Purification and characterization of recombinant hypoallergens

Inclusion bodies were isolated from the cells using lysozyme (0.1 mg/ g cell wet weight) (Sigma-Aldrich, St. Louis, MO, USA) and repetitive freezing and thawing in buffer I (50 mM Tris base, 1 mM EDTA and 0.1 % Triton X-100) for Bet v 1-rs1 and Bet v 1-mosaic, or buffer II (25 mM NaH_2PO_4, pH 7.4 and 0.1 % Triton X-100) for Bet v 1-rs 2 (5 mL/ g cell wet weight). NaCl was added to a final concentration of 200 mM, and the suspensions were centrifuged (10000 g for 30 min. at 4°C) leaving the proteins containing inclusion bodies in the pellet.

Bet v 1-rs1 and Bet v 1-rs2 pellets were washed with 1% Triton X-100, 2 mM EDTA, 2 mM ß-mercaptoethanol, 20 mM Tris/HCl pH 8.0 (3 times) and afterwards with 50% ethanol, 20 mM Tris/HCl pH 8.0 (2 times). The Bet v 1-mosaic pellet was washed once with 1% Triton X-100, 20 mM NaH_2PO_4 pH 7.4 and once with 25% ethanol, 20 mM NaH_2PO_4 pH 7.0. Inclusion bodies were suspended and stirred for 30 min in buffer A (6 M urea, 10 mM Tris/HCl, 1 mM EDTA, pH 7.0) in case of Bet v 1-rs1, or in buffer B (5 M urea in 20 mM sodium acetate buffer, pH 5.0) in case of Bet v 1-rs2 and mosaic. The suspensions were centrifuged (10000 g for 30 min. at 4°C) and the final supernatant used for purification.

Recombinant Bet v 1-rs1 was first purified by anion exchange chromatography (AIEC) on a Q-Sepharose FF column (GE Healthcare, UK Limited) by applying a linear gradient from 0-250 mM NaCl in buffer A. Fractions containing Bet v 1-rs1 were dialyzed against buffer C (6 M urea, 20mM NaH_2PO_4, 1.5 M NaCl, pH 4.5) and subjected to hydrophobic interaction chromatography (HIC) using a Phenyl Sepharose FF column (GE Healthcare, UK Limited) equilibrated with buffer C. Purified Bet v 1-rs1 was eluted with a linear gradient from 0 % to 100 % buffer D (6 M urea, 20mM Tris base, pH 9.3) and pure fractions were dialyzed first

against 6M urea and then against 1mM acetic acid. Finally, the protein was subjected to 0.2 µm filtration and stored at -20°C.

Recombinant Bet v 1-rs2 and -mosaic were purified by cation exchange chromatography (CIEC) using a SP-Sepharose FF column (GE Healthcare, UK Limited) equilibrated with buffer B and eluted with a linear gradient from 0-400 mM NaCl in the same buffer. To the fractions containing Bet v 1- rs2 NaCl was added to a final concentration of 1.7 M. Protein was centrifuged (10000 g for 30 min at 4°C) and the supernatant subjected to hydrophobic interaction chromatography (HIC) using a Phenyl Sepharose FF column (GE Healthcare, UK Limited), equilibrated with buffer E (6 M urea, 1.7 M NaCl in a 20 mM sodium acetate buffer, pH 5.0). Bet v 1-rs2 was then eluted with a linear gradient from 1.7 - 0 M NaCl in buffer E and fractions containing > 90 % pure rBet v 1-rs2 were dialyzed against buffer F (6 M Urea, 20 mM NaH_2PO_4 pH 7.0) and subjected to anion exchange chromatography (AIEC) using a Q-Sepharose FF column (GE Healthcare, UK Limited) equilibrated with the buffer F. To the fractions containing Bet v 1- mosaic urea and NaCl were added to a final concentration of 6.5 M and 3.5 M respectively. The protein was centrifuged (10000 g for 30 min at 4°C) and the supernatant subjected to hydrophobic interaction chromatography (HIC) using a Phenyl Sepharose FF column (GE Healthcare, UK Limited), equilibrated buffer G (6.5 M urea, 3.5 M NaCl in a 20 mM sodium acetate buffer, pH 5.0). Bet v 1- mosaic was eluted with a linear gradient from 3.5 - 0 M NaCl in buffer G.

Finally, Bet v 1-rs2 or -mosaic fractions containing > 90 % purity were pooled, dialyzed against 5 mM NaH_2PO_4 pH 7.4 and subjected to 0.2 µm filtration and stored at -20°C.

Purified rBet v 1 and each of the rBet v 1 derivatives (5 µg protein/slot) were resolved on 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the presence or absence of 2-mercaptoethanol [1]. Proteins were visualized by staining with Coomassie brilliant blue. The presence of endotoxin was determined in a limulus amebocyte lysate (LAL)

chromogenic assay (QCL-1000^{R} Chromogenic LAL Endpoint Assay, Bio-Whittaker, Walkersville, USA).

Laser desorption mass spectra of the proteins were acquired in a linear mode with a MALDI-ToF instrument (Microflex, Bruker, Billerica, MA, USA). Samples were dissolved in 10 % acetonitrile (0.1 % trifluoroacetic acid), and sinapinic acid (dissolved in 60 % acetonitrile, 0.1 % trifluoroacetic acid) was used as a matrix. For sample preparation a 1:1 mixture of protein and matrix solution was deposited onto the target and air-dried.

CD spectra were acquired on a JASCO (Tokyo, Japan) J-810 spectropolarimeter. CD measurements were performed with purified rBet v 1 and rBet v 1 derivatives at room temperature, at protein concentrations of 0.1 mg/ml using a rectangular quartz cuvette with 0.1-cm path length. Far ultraviolet (UV) spectra were recorded in the wavelength ranges between 190 and 260 nm with a resolution of 0.5 nm at a scan speed of 50 nm/min and resulted from averaging of three measurements. The final spectra were baseline-corrected and results were expressed as the mean residue ellipticity (Θ) at a given wavelength. The secondary structure content of rBet v 1 and Bet v 1 derivative molecules was calculated using the secondary structure estimation program CDSSTR [2].

References

1. Fling SP, Gregerson DS. Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. Anal Biochem 1986; 155:83-88.
2. Whitmore L, Wallace BA. DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. Nucleic Acids Res 2004; 32:668-73.

Supplementary Information # 2: The allergenic activity of allergen derivatives

Heparinized peripheral blood samples were obtained from birch pollen allergic individuals after informed consent was given. Blood aliquots (100 µl) from six patients were incubated (unique) with serial dilutions (0.005 to 50 pM) of rBet v1, an equimolar mix of the rBet v 1 fragments (F1+F2), or rBet v 1-rs 1 for 15 minutes at 37°C. Blood aliquots (100 µl) from additional four patients were incubated (unique) with serial dilutions (0.005 to 50 pM) of rBet v1, rBet v 1-rs1, rBet v 1-rs2, or rBet v 1-mosaic as described above. A monoclonal anti-IgE antibody E-124.2.8 (1µg/ml) (Immunotech, Marseille, France) and PBS (control buffer) were used as controls. Thereafter, samples were washed in PBS containing 20 mM EDTA (Gibco, Carlsbad, California, USA) and cells were incubated with 10 µl of PE-conjugated CD203c mAb 97A6 (Immunotech, Marseille, France) for 15 minutes at room temperature. After erythrocyte lysis using FACS^{TM} Lysing Solution (Becton Dickinson Biosciences, San Jose, California, USA), cells were washed and resuspended in PBS and then analyzed by two-color flow cytometry on a FACSScan (Becton Dickinson Biosciences, San Jose, California, USA) using Flowjo Software (Tree Star Inc., Ashland, Oregon, USA). Anti-IgE-induced up-regulation of CD203c was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated ($MFI_{control}$) cells, and is expressed as stimulation index (MFI_{stim} : $MFI_{control}$) [1].

Reference:

1. Hauswirth AW, Natter S, Ghannadan M, Majlesi Y, Schernthaner GH, Sperr WR, et al. Recombinant allergens promote expression of CD203c on basophils in sensitized individuals. J Allergy Clin Immunol 2002; 110:102-09.

Supplementary Information # 3: ELISA competition assay

In order to analyze the ability of rBet v 1 derivatives-induced rabbit IgG to inhibit the binding of birch pollen allergic patients' IgE to rBet v 1, an ELISA competition assay was performed. ELISA plates (Greiner, Kremsmünster, Austria) were coated with 100µl of rBet v 1 (5 µg/ml diluted in PBS) overnight at 4˚C. Plates were blocked with 2% bovine serum albumin (BSA) (Roth, Karlsruhe, Germany) in PBS-T (PBS 0.05% Tween 20) for 6 hours at 4˚C overnight and then preincubated overnight at 4˚C with 1:50 dilutions (in PBS 0.5 % BSA/ 0.05 % Tween) of the rabbit sera anti-Bet v 1, anti-Bet v 1-rs1, anti-Bet v 1-rs2 or anti-Bet v 1-mosaic, and for control purposes by using the corresponding rabbit pre-immune sera. Plates were washed three times with PBS-T and incubated with 1:10 diluted sera from 18 birch pollen allergic patients sensitized to Bet v 1. Bound human IgE antibodies were detected using a 1:2500 diluted AP-conjugated (alkaline phosphatase) mouse monoclonal anti-human IgE antibody (BD Pharmingen, San Diego, California, USA). Color development was performed by addition of staining solution ABTS (2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; Sigma-Aldrich, St.Louis, Missouri, USA) (100µl/well) and the optical density was measured in an ELISA Reader (Dynatech, Denkendorf, Germany) at 405 nm. The percentage of inhibition of IgE-binding was calculated using the OD values obtained, as follows: percent inhibition of IgE binding = 100 – (ODs/ODp) x 100. ODs, extinction coefficient after preincubation with the rabbit serum. ODp, extinction coefficient after preincubation with the pre-immune serum.

A combination vaccine for allergy and rhinovirus infections based on rhinovirus-derived surface protein VP1 and a nonallergenic peptide of the major timothy grass pollen allergen Phl p 1

Edlmayr J, Niespodziana K, Linhart B, Focke-Tejkl M, Westritschnig K, Scheiblhofer S, Stoecklinger A, Kneidinger M, Valent P, **Campana R**, Thalhamer J, Popow-Kraupp T, Valenta R.

Published

Journal of Immunology, 2009

A Combination Vaccine for Allergy and Rhinovirus Infections Based on Rhinovirus-Derived Surface Protein VP1 and a Nonallergenic Peptide of the Major Timothy Grass Pollen Allergen Phl p 1[1]

Johanna Edlmayr,* Katarzyna Niespodziana,* Birgit Linhart,* Margarete Focke-Tejkl,* Kerstin Westritschnig,* Sandra Scheiblhofer,[§] Angelika Stoecklinger,[§] Michael Kneidinger,[†] Peter Valent,[†] Raffaela Campana,* Josef Thalhamer,[§] Theresia Popow-Kraupp,[‡] and Rudolf Valenta[2]*

Allergens and rhinovirus infections are among the most common elicitors of respiratory diseases. We report the construction of a recombinant combination vaccine for allergy and rhinovirus infections based on rhinovirus-derived VP1, the surface protein which is critically involved in infection of respiratory cells, and a nonallergenic peptide of the major grass pollen allergen Phl p 1. Recombinant hybrid molecules consisting of VP1 and a Phl p 1-derived peptide of 31 aa were expressed in *Escherichia coli*. The hybrid molecules did not react with IgE Abs from grass pollen allergic patients and lacked allergenic activity when exposed to basophils from allergic patients. Upon immunization of mice and rabbits, the hybrids did not sensitize against Phl p 1 but induced protective IgG Abs that cross-reacted with group 1 allergens from different grass species and blocked allergic patients' IgE reactivity to Phl p 1 as well as Phl p 1-induced basophil degranulation. Moreover, hybrid-induced IgG Abs inhibited rhinovirus infection of cultured human epithelial cells. The principle of fusing nonallergenic allergen-derived peptides onto viral carrier proteins may be used for the engineering of safe allergy vaccines which also protect against viral infections. ***The Journal of Immunology,*** **2009, 182: 6298–6306.**

According to the World Health Organization, asthma belongs to one of the most severe and disabling diseases (1). Allergens and respiratory viruses are among the most common environmental factors implicated in the pathogenesis of asthma (2). More than 25% of the population suffers from IgE-mediated allergies and ~30% of patients suffering from persistent allergic rhinitis also suffer from asthma (3). The link between upper and lower airway diseases is also underlined by the fact that patients suffering from untreated allergic rhinoconjunctivitis frequently develop asthma bronchiole (4). In this context, it has been shown that allergen exposure via the nasal and respiratory mucosa induces strong rises of allergen-specific IgE levels, which are responsible for increased allergen sensitivity in the target organs of allergy (5). In addition, several studies highlight the importance of rhinovirus infections in the context of allergic asthma (6, 7).

Human rhinoviruses (HRV)[3] have been identified in the late 1950s and early 1960s as the cause of the common cold in the upper respiratory tract. With the use of PCR-based technology, HRV have been identified in 60–90% of acute exacerbations of asthma in children and adults (6). Rhinoviruses are not only a major cause of asthma exacerbations (8) but allergic individuals suffer also more often and prolonged from rhinovirus infections in the lower respiratory tract (9). Furthermore, there is evidence for a deficient innate immune response (i.e., a deficient-type III IFN-λ production) to rhinovirus infections in asthmatic individuals (10).

To date, no effective vaccines or antiviral therapies have been approved for either the prevention or the treatment of HRV infection. However, for allergic diseases, allergen-specific immunotherapy is available as an allergen-specific and disease- modifying form of allergy treatment (11). It is effective for the treatment of allergic asthma and prevents the progression of allergic rhinoconjunctivitis to allergic asthma (12). Due to the rapid progress made in the field of allergen characterization, several new forms of allergen-specific immunotherapy have been developed and entered in clinical trials in allergic patients (reviewed in Refs. 11, 13, and 14).

According to the sequences of major allergens, synthetic peptides containing T cell epitopes without IgE reactivity have been identified with the aim to induce tolerance in allergen-specific T

*Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology, [†]Department of Internal Medicine I, Division of Hematology and Hemostaseology, [‡]Department of Virology, Medical University of Vienna, Vienna, Austria; and [§]Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology, University of Salzburg, Salzburg, Austria

Received for publication November 27, 2007. Accepted for publication March 4, 2009.

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[1] This study was supported by Grants L214-B13, F1815, and DK-IAI of the Austrian Science Fund (Fonds zur Förderung der Wissenschaftlichen Forschung) and in part by the Christian Doppler Research Foundation.

[2] Address correspondence and reprint requests to Dr. Rudolf Valenta, Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology, Medical University of Vienna, AKH Ebene 3Q, Währinger Gürtel 18-20, 1090 Vienna, Austria. E-mail address: rudolf.valenta@meduniwien.ac.at

[3] Abbreviations used in this paper: HRV, human rhinovirus; KLH, keyhole limpet hemocyanin; HSA, human serum albumin; MFI, mean fluorescence intensity; SI, stimulation index; $TCID_{50}$, 50% tissue culture infectious dose; RBL, rat basophil leukemia cell.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0713622

cells (15). DNA-based vaccination has been shown to redirect allergic immune responses in experimental animal studies and CpG-coupled allergens have been used to modulate the immune responses and were used for immunotherapy of ragweed allergic patients (16–18). Furthermore, recombinant allergens and genetically engineered allergen derivatives with reduced allergenic activity have been used for allergy vaccination of allergic patients (reviewed in Ref. 19).

In this study, we report the development of a novel type of vaccine for the combined treatment of allergen- and rhinovirus-induced asthma. The active ingredient of this vaccine is a fusion protein consisting of the rhinovirus-derived VP1 surface protein (20, 21) and a hypoallergenic peptide selected from the major grass pollen allergen Phl p 1 (22). VP1 contains the motifs required for HRV binding to the target cells and is recognized by HRV-neutralizing Abs (20, 21). The Phl p 1 peptide is located at the Phl p 1 C terminus which contains the majority of IgE epitopes recognized by patients' IgE but lacks allergenic activity (22, 23). The fusion protein was thus expected to induce IgG Abs which block allergic patients' IgE recognition of the Phl p 1 allergen and to inhibit HRV infection.

In this study, we report the construction, expression, and purification of the VP1 fusion protein. We demonstrate the lack of allergenic activity of the fusion protein using allergic patients' IgE and their blood basophils. Furthermore, we show that IgG Abs obtained after immunization of animals with the fusion protein inhibit allergic patients' IgE binding to the Phl p 1 allergen, allergen-induced basophil degranulation, and protect against HRV infection of cultured human cells.

Materials and Methods

Allergic patients, allergen extracts, recombinant allergens, synthetic peptides, and peptide conjugates

Sera were obtained from patients allergic to grass pollen as confirmed by case history, skin prick testing, and measurement of specific IgE Abs and are numbered consistently in the manuscript (22). Pollen from different grass and corn species (*Phleum pratense*: Timothy grass; *Secale cereale*: rye*; Poa pratensis*: Kentucky bluegrass; *Phragmites australis*: Australian reed; *Triticum sativum*: cultivated wheat; *Lolium perenne*: rye grass; *Avena sativa*: cultivated oat; *Anthoxanthum*: sweet vernal grass) were purchased from Allergon. Natural grass pollen extracts were prepared as described previously (24). Purified recombinant rPhl p 1 was obtained from Biomay.

The Phl p 1-derived peptide P5 CVRYTTEGGTKTEAEDVIPEG WKADTAYESK was synthesized and coupled to keyhole limpet hemocyanin (KLH) as described previously (22).

Construction of vector pVP1

Virus stocks of strains HRV89 and 14 were obtained from the collection at the Institute of Virology, Medical University of Vienna. Viral RNA was prepared from cell culture supernatants using the QIAamp viral RNA kit (Qiagen) and RNase inhibitor (Boehringer Mannheim) was added to a final concentration of 0.01 U/μl. The VP1 cDNA was amplified by RT-PCR using a SuperScript One-Step RT-PCR kit from Invitrogen using the following primers: 5′-CGGAATTCATTAATATGAACCCAGTTGAAAAT TATATAGATAGTGTATTA-3′ and 5′-CGATTAATTCAGTGGTGGTG GTGGTGGTGGACGTTTGTAACGGTAA-3′. The restriction sites (*Eco*RI, *Ase*I) are underlined. The VP1 cDNA was subcloned into the *Nde*I and *Eco*RI sites of the plasmid pET 17b and transformed into *Escherichia coli* BL21 DE3 from Novagen (Merck Bioscience) for protein expression. The DNA sequence of the construct was confirmed by nucleotide sequencing (MWG) (25).

To allow the insertion of cDNAs coding for unrelated peptides at the 5′ end of the VP1-encoding cDNA, the plasmid construct was modified by changing the CATAAT site which resulted from the subcloning of the *Ase*I ends of the cDNA into the *Nde*I site of the vector into an *Afl*II site (CTT AAG) by site-directed mutagenesis using a Quick Change Site Mutagenesis kit (Stratagene) and the primers *Afl*II forward, 5′-CTTTAAGAAGGA GATATACTTAAGATGAACCCAGTTG-3′ and *Afl*II reverse, 5′-CAA CTGGGTTCATCTTAAGTATATCTCCTTCTTAAAG-3′. Next, the primers *Age*I forward, 5′-CCTGATGTTTTTACCGGTACAAACGTCC ACCAC-3′ and *Age*I reverse, 5′-GTGGTGGACGTTTGTACCGGTAAA AACATCAGG-3′ were used to introduce an *Age*I site before the last three codons of the VP1-encoding cDNA to allow the insertion of cDNAs coding for peptides at the 3′ end. The resulting plasmid was designated pVP1 (Fig. 1*a* and supplemental Fig. 1[4]).

Construction of plasmids expressing a VP1-P5 and a VP1-2xP5 hybrid protein

A recombinant hybrid protein (VP1-P5) consisting of VP1 and the Phl p 1-derived peptide P5 fused to the VP1 N terminus was obtained by PCR amplification of a P5-encoding cDNA using forward 5′- CGCGCTTAAG ATGGTCCGCTACACCACCGAGGGC-3′ and reverse 5′-CGCG CTTAAGCTTGGACTCGTAGGCGGTGTCGGC-3′ primers and the Phl p 1-encoding cDNA as a template.

The P5-encoding cDNA was inserted into the *Afl*II restriction site of the pVP1 vector. The resulting construct was further modified by insertion of a P5-encoding cDNA into the *Age*I site at the 3′ end of the VP1-encoding cDNA to yield a hybrid consisting of VP1 with a N-terminal and a C-terminal P5 peptide designated VP1-2xP5.

Expression and purification of VP1, VP1-P5, and VP1-2xP5

Recombinant VP1, VP1-P5, and VP1-2xP5 were expressed in *E. coli* BL21(DE3) and purified from the inclusion body fraction after solubilization in 6 M guanidinium hydrochloride, 100 mM NaH_2PO_4, and 10 mM Tris (pH 8) using a Ni-NTA affinity matrix (Qiagen). Elution was performed at pH 3.5. Protein preparations were dialyzed against H_2O_{dd} and checked for purity by SDS-PAGE and Coomassie blue staining Membranes containing purified VP1, VP1-P5, and VP1-2xP5 were exposed to a monoclonal mouse anti-His tag Ab (Dianova). Bound Abs were detected with alkaline phosphatase-coupled rabbit anti-mouse Abs (BD Pharmingen).

Allergic patients' IgE reactivity to the fusion proteins and basophil activation

Allergic patients' IgE reactivity to Phl p 1, VP1-P5, VP1-2xP5, or human serum albumin (HSA) was measured by ELISA as described elsewhere (26). Sera from three nonallergic individuals were tested as negative controls. The coating of the test Ags to the ELISA plates was confirmed with specific Ab probes. The allergenic activity of the VP1 fusion proteins was compared with that of the Phl p 1 allergen by in vitro basophil activation tests. For this purpose, peripheral blood was obtained from three grass pollen allergic patients after informed consent was obtained. Basophil activation in heparinized whole blood samples and flow cytometric measurement of CD203c expression was performed as previously described (27). In brief, blood aliquots (100 μl) were incubated with serial dilutions of rPhl p 1, VP1-2xP5 (0.05–50 pM), anti-IgE Ab (1 μg/ml, E-124-2-8 D ε; Immunotech), or buffer alone (PBS). Allergen-induced up-regulation of CD203c as determined by flow cytometry with PE-conjugated mAb 97A6 (CD203c) was calculated from mean fluorescence intensities (MFIs) obtained with stimulated (MFI_{stim}) and unstimulated ($MFI_{control}$) cells and was expressed as stimulation index ($SI = MFI_{stim}:MFI_{control}$) (27).

Immunization of mice and rabbits, reactivity of mouse and rabbit Abs with VP1 and Phl p 1, and demonstration of reaginic activity of mouse Abs

Groups of five mice each were immunized three times s.c. with 5 μg of P5, rPhl p 1, VP1, VP1-P5, or VP1-2xP5 adsorbed to aluminum hydroxide in 3-wk intervals and bled from the tail veins. Animals were maintained in the animal care unit of the Department of Pathophysiology (Medical University of Vienna) according to the local guidelines for animal care (28). The mouse immunization experiments were repeated three times. Rabbit Abs specific for VP1, KLH-P5, VP1-P5, and VP1-2xP5 were obtained by immunizing rabbits (Charles River). ELISA plates (Nunc Maxisorb) were coated with 5 μg/ml of the Ags and incubated with mouse sera diluted 1/500 as previously described (28, 29). Bound mouse IgG1, IgG2a, or IgG2b were detected with monoclonal rat anti-mouse IgG1, IgG2a, or IgG2b Abs (BD Pharmingen) diluted 1/1000, respectively, and then with goat anti-rat IgG HRP-coupled Abs (Amersham Biosciences) diluted 1/2000. Bound rabbit IgG Abs were detected with 1/2000 diluted donkey anti-rabbit IgG HRP-coupled Abs (Amersham Biosciences). OD was measured at 405 and 490 nm in an ELISA reader (Dynatech).

The induction of Phl p 1-specific IgE Abs with allergenic activity was determined in sera from mice that had been immunized with Phl p 1, VP1-P5,

[4] The online version of this article contains supplemental material.

VP1-2xP5, or VP1 using RBL-2H3 cells. Rat basophil leukemia cells (RBLs) were loaded with mouse sera and timothy grass pollen extract containing natural Phl p 1 allergen (~5 μg/ml) or VP1 as described elsewhere (30).

Proliferation of mouse spleen cells and cytokine analysis

Spleen cells were prepared from mice that had been immunized four times with P5, VP1, VP1-P5, KLH-P5, or PBS 10 days after the last immunization. Spleen cell cultures from each mouse were stimulated with P5 (0.26 μg/100 μl), VP1 (2 μg/100 μl), Con A (2 μg/100 μl) (positive control), or medium alone and after 4 days [3H]thymidine uptake was measured and displayed as SI as described previously (30). Cytokines were measured after stimulation of spleen cells with Phl p 1, KLH, VP1, or Con A (15 μg/50 μl each). IL-4, IL-5, IL-10, TGF-ß, and IFN-γ levels were measured by xMAP Luminex fluorescent bead-based technology. The assay was performed according to the manufacturer's introduction (R&D Systems) and fluorescence signal was read on a Luminex 100 System (Luminex). Cytokine measurements were done for splenocytes from each mouse for a particular cytokine. From these values, the assay cutoff level for the given cytokine was subtracted and this value was normalized to the proliferation results (SI) according to the formula: (mean cytokine level − cytokine cutoff)/SI = factor. The mean values of the factors were calculated for each group of mice and each cytokine to allow a comparison among the groups.

Statistics

Differences in Ab and cytokine levels induced by immunization of mice were determined by the Mann-Whitney *U* test using SPSS software. A value of $p < 0.05$ was considered as significant.

Cross-reactivity of anti-VP1-2xP5 Abs with natural group 1 grass pollen allergens

Grass pollen extracts from eight grass species were separated on a 12.5% SDS-PAGE and blotted onto a nitrocellulose membrane. Identically prepared membranes were incubated with rabbit anti-VP1-2xP5 Abs or the corresponding preimmune Ig overnight at 4°C and bound IgG Abs were detected with ^{125}I-labeled donkey anti-rabbit IgG as previously described (22).

Inhibition of allergic patients' IgE binding to Phl p 1 and of Phl p 1-induced basophil degranulation with vaccine-induced IgG Abs

The inhibition of allergic patients' IgE reactivity to Phl p 1 by IgG Abs which had been raised by immunization of rabbits with the rPhl p 1 allergen, VP1-P5, VP1-2xP5, and a KLH-coupled peptide P5 (22) was measured by ELISA as described elsewhere (28, 29). In brief, ELISA plates were coated with 1 μg/ml rPhl p 1, washed, and blocked. Then plate-bound Phl p 1 was preincubated with 1/250 dilutions of specific rabbit Ig or, for control purposes, of the preimmune Ig. After washing, plates were incubated with 1/3 diluted sera from allergic patients with a RAST class of >3 to timothy grass pollen allergens and bound IgE Abs were detected with 1/1000 diluted alkaline phosphatase-coupled mouse monoclonal anti-human IgE Abs (BD Pharmingen). The OD corresponding to bound IgE was measured at 405 and 450 nm. The percentage of inhibition of IgE binding achieved by preincubation with the anti-peptide antisera was calculated as previously described (28).

The inhibition of Phl p 1-induced basophil degranulation by vaccine-induced IgG Abs was investigated as follows: RBL-2H3 cells transfected with human high-affinity IgE receptor (FcεR1) (31) were loaded with serum IgE from Phl p 1-allergic patients and then exposed to 100 ng/ml Phl p 1 that had been preincubated with increasing concentrations (0, 2.5, 5, or 10% v/v) of rabbit Ig raised against Phl p 1 or VP1-2xP5 or the corresponding preimmune Ig. The release of β-hexosaminidase was measured as described previously (31) and is expressed as percentage of total cellular β-hexosaminidase.

HRV neutralization test

The virus stock titer used in the experiment was determined by 50% tissue culture infectious dose ($TCID_{50}$) titration on HeLa cells according to the Spearman-Kaerber method (32).

For the neutralization tests, 300-μl aliquots containing a 100 $TCID_{50}$ of HRV89 or 14 were preincubated for 2 h at 37°C with 300-μl aliquots (undiluted, 1/2–1/32) of the anti-VP1-P5 or anti-VP1-2xP5 antiserum before addition to HeLa cells. Infection of HeLa cells with 100 $TCID_{50}$ of HRV89 or 14 was performed for control purposes. For control purposes, cells were incubated with medium alone or immune serum without virus. The cytotoxic effect of the virus was visualized with crystal violet after 3 days (33).

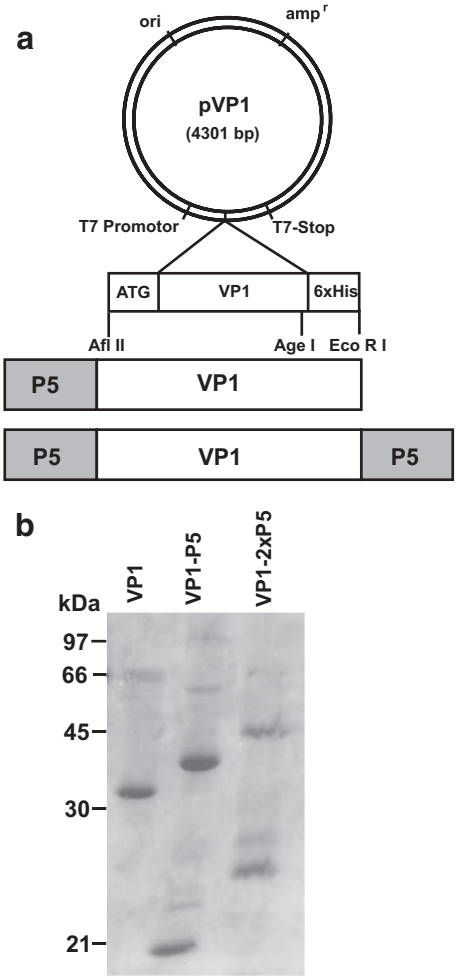


FIGURE 1. Representation of pVP1, expression and purification of VP1, VP1-P5, and VP1-2xP5. *a*, Construction of an expression plasmid (pVP1) containing the cDNA coding for VP1. The VP1-encoding cDNA was inserted into the multiple cloning site of plasmid pET17b and an *Afl*II and *Age*I restriction sites were introduced at the 5′ and 3′ end of the VP1-encoding cDNA, respectively. *b*, SDS-PAGE containing purified VP1, VP1-P5, and VP1-2xP5. Molecular masses in kDa are indicated.

Results

Expression and purification of VP1 and of a fusion protein consisting of VP1 and one or two grass pollen allergen peptides

Plasmid pVP1 (Fig. 1*a*) was constructed by replacing the *Nde*I/*Eco*RI fragment of the multiple cloning site of pET-17b with the cDNA sequence encoding the complete VP1 protein of human rhinovirus strain 89. The *Nde*I and *Eco*RI restriction sites (of pET-17b) were used for insertion of the VP1 cDNA containing *Ase*I and *Eco*RI sites at the 5′ and 3′ end, respectively. An *Afl*II (5′ end) and an *Age*I (3′ end) restriction site were generated at the ends of the VP1-encoding cDNA by mutagenesis to allow fusion with foreign cDNAs. The *Age*I restriction site was placed between the 3′ end of the VP1 cDNA and a cDNA coding for a C-terminal hexahistidine tag (Fig. 1*a* and supplemental Fig. 1). Plasmid VP1 thus allows the expression of complete VP1 with a C-terminal hexahistidine tag. In addition, allergen-derived peptides can be fused to the N and/or C terminus of VP1.

Recombinant VP1 was expressed in *E. coli* with a C-terminal hexahistidine tag and purified by nickel affinity chromatography in a single-step procedure yielding ~5 mg of protein/L of culture. Recombinant VP1 migrates at ~34 kDa in SDS-PAGE (Fig. 1*b*). Next, we expressed and purified two fusion proteins consisting of

Table I. *IgE reactivity to HSA, Phl p 1, VP1-P5, and VP1-2xP5*[a]

Patient	IgE Reactivity of Allergic Patients' Sera			
	HSA	Phl p 1	VP1-P5	VP1-2xP5
1	0.056	0.235	0.077	0.087
2	0.046	0.586	0.061	0.066
3	0.049	0.9	0.059	0.057
4	0.07	1.031	0.061	0.059
5	0.016	0.889	n.d.	0.178
6	0.044	0.308	0.044	0.051
7	0.054	0.605	0.05	0.046
8	0.047	0.476	0.052	0.055
9	0.045	0.151	0.045	0.045
10	0.061	0.288	0.048	0.047
11	0.048	1.879	0.059	0.061
12	0.059	1.638	n.d.	0.092
13	0.051	2.098	0.048	0.046
14	0.064	0.166	0.044	0.046
15	0.05	0.697	0.059	0.048
16	0.058	1.864	0.079	0.069
17	0.053	1.39	0.053	0.048
18	0.046	1.062	0.045	0.045
19	0.045	0.845	0.059	0.056
20	0.008	0.4	n.d.	0.099
21	0.048	0.397	0.058	0.057
22	0.045	0.24	n.d.	0.02
23	0.051	1.117	0.056	0.058
Mean	**0.048**	**0.837**	**0.056**	**0.062**
24	0.048	0.052	0.051	0.047
25	0.042	0.043	0.05	0.044
26	0.043	0.048	0.048	0.044

[a] Serum IgE reactivity to the purified Ags was determined by ELISA and is displayed as mean OD values of duplicate measurements for sera from 23 grass pollen allergic patients. Mean OD values for the 23 sera and each antigen are indicated in bold.

VP1 and the nonallergenic peptide (P5) derived from the major Timothy grass pollen allergen Phl p 1. In the fusion protein VP1-P5, the peptide is fused to the N terminus of VP1 and in the fusion protein VP1-2xP5 one peptide is attached to the VP1 N and one to the C terminus. Recombinant VP1-P5 and VP1-2xP5 exhibited a molecular mass of ~37 and 41 kDa, respectively, in SDS-PAGE (Fig. 1*b*). Bands below 30 kDa in the recombinant protein preparations reacted with an anti-His tag Ab and therefore represent degradation products of the purified recombinant proteins.

VP1-P5 and VP1-2xP5 are nonallergenic fusion proteins

Recombinant VP1-P5 and VP1-2xP5 were compared with the complete recombinant Phl p 1 allergen regarding IgE reactivity using sera from 23 grass pollen allergic patients by ELISA (Table I). Each of the grass pollen allergic patients showed IgE reactivity to rPhl p 1 (mean OD, 0.837). However, no relevant IgE reactivity to VP1-P5 or VP1-2xP5 was found. Likewise, no relevant IgE binding was observed when three nonallergic individuals were tested for IgE reactivity to the fusion proteins (Table I). No IgE reactivity to HSA (mean OD, 0.048) could be detected (Table I).

The lack of allergenic activity of VP1-2xP5 was confirmed by experiments using basophils from grass pollen allergic patients (Fig. 2). rPhl p 1 and anti-IgE induced the up-regulation of CD203c on basophils from each of the three grass pollen allergic patients at concentrations of 0.05 pM, whereas VP1-2xP5 did not induce any response up to a concentration of 50 pM (Fig. 2).

VP1-P5 and VP1-2xP5 induce a VP1- and grass pollen-specific Th1-like immune response with lowered Th2 activation

To determine the immunogenicity of VP1 and its ability to act as a carrier for allergen-derived peptides, groups of mice were immunized with the following Ags: P5, rPhl p 1, VP1, VP1-P5, and VP1-2xP5. VP1- and Phl p 1- specific IgG1 Ab levels were determined by ELISA (Fig. 3). VP1-specific IgG1 Abs were detected already 3 wk after immunization in mice that had received VP1 or VP1- containing allergen-derived peptides, but not in mice that had been immunized with P5 or Phl p 1 (Fig. 3*a*).

The fusion of the Phl p 1-derived peptide (P5) to VP1 strongly increased the immunogenicity of P5 because no relevant Phl p 1-specific IgG1 responses were found in mice immunized with P5 alone, whereas VP1-P5 and VP1-2xP5-immunized mice showed Phl p 1-specific IgG1 responses after 3 wk, which continued to increase after 6 and 9 wk (Fig. 3*b*). The Phl p 1-specific IgG1 responses in the latter two groups of mice were even of comparable magnitude to the IgG1 responses induced by immunization with the complete Phl p 1 allergen. Immunization with VP1 alone did not induce any Phl p 1-specific immune response (Fig. 3*b*).

VP1-P5 and VP1-2xP5 induced IgG2a and IgG2b responses to Phl p 1. No Phl p 1-specific IgG2 responses were found in P5- and VP1-immunized mice (Fig. 3*b*). Interestingly, VP1-specific IgG2a and IgG2b responses were significantly stronger ($p < 0.05$) in the VP1-immunized mice as compared with the mice having received VP1-P5 or VP1-2xP5, indicating that VP1 contributes to the Th1 component of the immune responses whereas the allergen-derived peptides seemed to reduce this activity (Fig. 3*a*). To determine the specificity of T cell responses, spleen cells from immunized mice were exposed to P5, VP1, or to culture medium alone (Fig. 4, *x*-axes). Spleen cells from VP1-immunized mice showed proliferation (SI > 6) to VP1 but no relevant proliferation to P5 or to medium alone. Spleen cells from VP1-P5-immunized mice that had developed IgG responses against Phl p 1 did not show relevant proliferation to P5 but responded strongly (SI > 6) to VP1, indicating that the Phl p 1-specific IgG responses had received VP1-specific T cell help (Fig. 4). P5-immunized mice did not show any relevant proliferation similar to cells from a nonimmunized mouse (Fig. 4). Spleen cells from VP1-P5-immunized mice did not release relevant levels of IL-5, whereas a stronger secretion of IL-5 was found in culture supernatants from

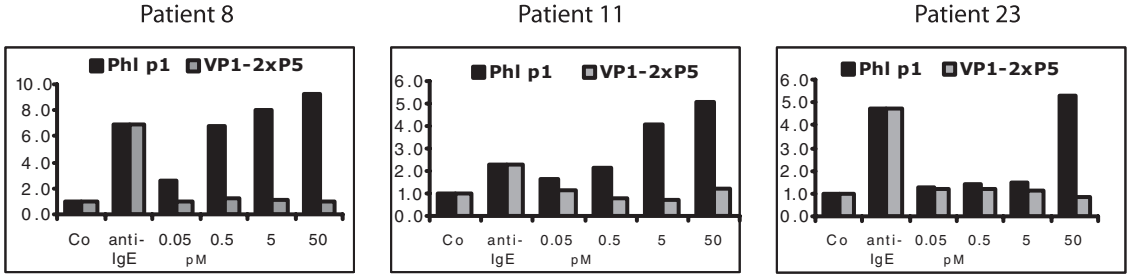


FIGURE 2. Activation of human basophils. Blood of three grass pollen allergic patients (patients 8, 11, and 23) were stimulated with PBS (Co), anti-IgE, serial dilutions (0.05, 0.5, 5, and 50 pM) of Phl p 1 (■) or VP1–2xP5 (▨) (*x*-axes). CD203c expression is displayed as SI (*y*-axes).

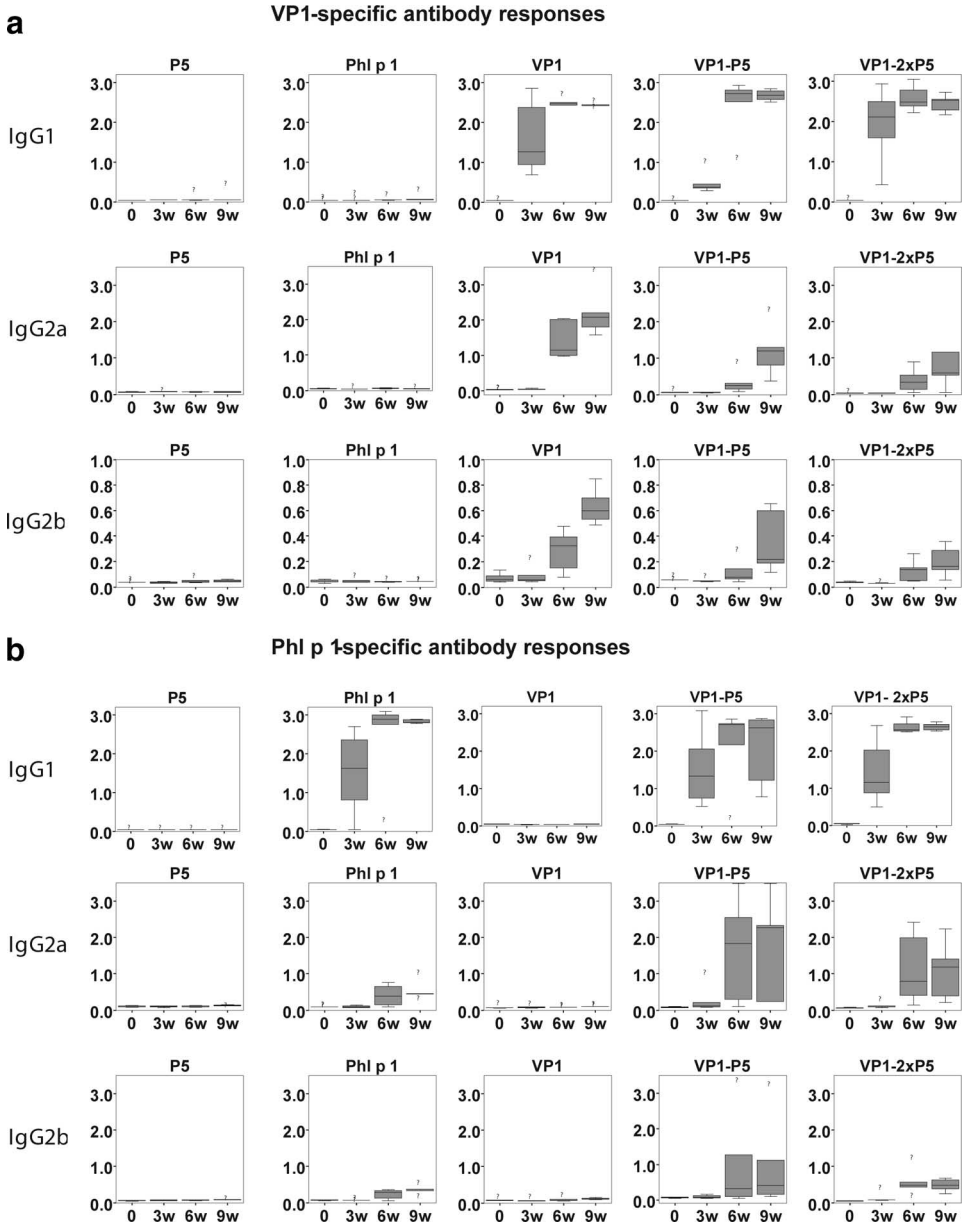
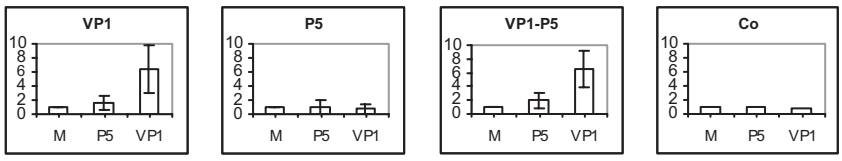


FIGURE 3. Immune responses of immunized mice. *a–f*, Phl p 1- and VP1-specific IgG1 and IgG2 responses. Mice were immunized with P5, Phl p 1, VP1, VP1-P5, and VP1-2xP5 (*top boxes*). Serum samples were taken on the day of the first immunization (0) and in 3-wk intervals (3w–9w) (*x*-axes). IgG1 and IgG2 reactivities are displayed for each mouse group as box plots, where 50% of the values are within the boxes and nonoutliers between the bars. The lines within the boxes indicate the median values. IgG1, IgG2a, and IgG2b levels specific for VP1 (*a*) and Phl p 1 (*b*) are displayed as OD values (*x*-axes). IgG2a (*c*) and IgG2b (*d*) levels specific for Phl p 1 and IgG2a (*e*) and IgG2b (*f*) levels specific for VP1 are displayed as OD values (*y*-axes).

spleen cells of mice that had been immunized with KLH-P5 (Fig. 4). No relevant differences regarding the production of IL-10, IL-4, and TGF-ß were found between the different groups (Fig. 4).

VP1-2xP5 does not induce an allergenic immune response against Phl p 1 or against VP1

To study whether immunization with the various Ags (Phl p 1, VP1, VP1-P5, VP1-2xP5) induces reaginic IgE Abs, RBLs were loaded with sera from immunized mice and exposed to pollen-derived Phl p 1 or VP1 (Fig. 5). Immunization with Phl p 1 but not with VP1 or the VP1-P5 constructs induced reaginic Abs which lead to degranulation of RBLs in response to timothy grass pollen-derived Phl p 1 (Fig. 5, Timothy grass pollen extract). Interestingly, neither immunization with VP1, VP1-P5, nor VP1-2xP5 induced IgE Abs which caused degranulation of RBLs in responses to the VP1 protein (Fig. 5, VP1).



Immunogen	IL-10	IL-4	IL-5	IFN-γ	TGF-ß
KLH-P5	3.3	0	30.2	0	0.4
VP1-P5	1.7	0	0	0	0.2
PBS	7.1	0	0.4	0	0.8

FIGURE 4. T cell responses of immunized mice. The lymphoproliferative responses (*y*-axes: SI ± SD) of immunized mice (*top* of the boxes: immunogens) and a nonimmunized (Co) mouse stimulated with medium alone (M), P5, or VP1 (*x*-axes) are displayed. The table shows the levels of IL-10, IL-4, IL-5, IFN-γ, and TGF-ß in Phl p 1-stimulated spleen cell cultures after normalization to the proliferations results (i.e., cytokine level/SI) in groups of mice immunized with different immunogens (KLH-P5, VP1-P5) or buffer (PBS).

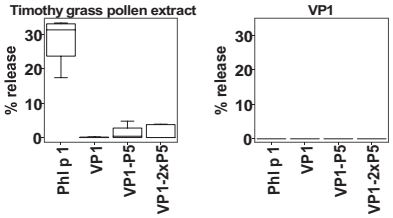


FIGURE 5. Reduced allergenicity of VP1-P5 and VP1-2xP5 vs Phl p 1. RBLs were loaded with sera from immunized mice (Immunogens: *bottom* of the boxes) and incubated either with natural Phl p 1 (Timothy grass pollen extract) or VP1 (*top* of the boxes). The ß-hexosaminidase releases are displayed as percentages of total releases for each mouse group as box plots, where 50% of the values are within the boxes and nonoutliers between the bars. The lines within the boxes indicate the median values.

Anti-VP1-2xP5 Abs cross-react with natural group 1 pollen allergens from several grass species

To study whether IgG Abs induced with the hybrid proteins cross-react with natural group 1 allergens from several grass species, immunoblot experiments were performed. Fig. 6*a* shows that rabbit anti-VP1-2xP5 Abs strongly bound to natural Phl p 1 in *P. pratense* pollen and to natural group 1 allergens in pollen from several other grass species including *P. pratensis*, *L. perenne*, and *Anthoxanthum odoratum*. A distinct but weaker reactivity to group 1 allergens from *P. australis*, A. *sativa*, *T. aestivum*, *and S. cereale* were found (Fig. 6*a*). No IgG reactivity to the pollen allergens was found when an identically prepared blot was exposed to the rabbit's preimmune serum (Fig. 6*b*).

VP1-2xP5-specific Abs inhibit allergic patients' IgE binding to Phl p 1 and Phl p 1-induced basophil degranulation more efficiently than Abs raised against the complete allergen

Serum IgE Abs from 18 grass pollen allergic patients were allowed to bind to Phl p 1 which had been preincubated with anti-Phl p 1, anti-KLH-P5, anti-VP1-P5, or anti-VP1-2xP5 Abs (Table II). Abs raised against the complete Phl p 1 allergen showed a rather weak inhibition of IgE reactivity ranging from 0 to 45%. A stronger

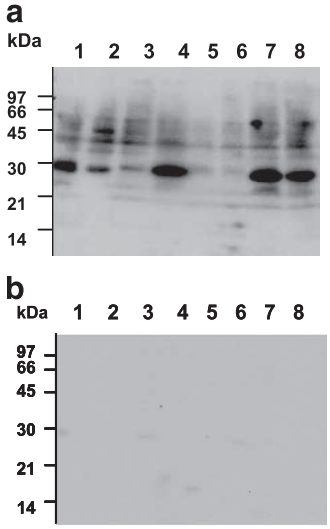


FIGURE 6. Cross-reactivity of anti-VP1-2xP5 Abs with natural group 1 grass pollen allergens. Nitrocellulose-blotted pollen extracts from eight grasses (nos. 1–8: *P. pratense*, *P. australis*, *A. sativa*, *P. pratensis*, *T. sativum*, *S. cereale*, *L. perenne*, *A. odoratum*) were incubated with rabbit anti-VP1-2xP5 Abs (*a*) or the corresponding preimmune serum (*b*). Molecular masses are displayed on the *left margin* in kDa.

Table II. *Inhibition of grass pollen allergic patients' IgE binding to Phl p 1 with IgG Abs*[a]

	Inhibition of IgE binding (%)			
Patient	Anti-Phl p 1	Anti-KLH-P5	Anti-VP1-P5	Anti-VP1-2xP5
1	1	13	13	31
2	0	51	19	66
3	27	49	36	65
4	16	46	40	71
5	14	55	39	57
6	13	35	24	55
7	18	37	26	61
8	29	55	24	72
9	17	51	41	64
10	9	46	31	65
11	0	64	50	67
12	0	55	28	88
13	14	55	50	91
14	0	7	32	29
15	11	56	58	58
16	45	70	68	74
17	28	58	55	72
18	24	41	50	36
Mean	15	47	39	63

[a] The inhibitions of allergic patients' (nos. 1–18) IgE binding by anti-Phl p 1, anti-KLH-P5, anti-VP1-P5, or anti-VP1-2xP5 Abs to ELISA plate-bound Phl p 1 are expressed as percentages of inhibition using the preimmune Abs as reference. Mean inhibitions for the group of 18 patients are displayed in the bottom line.

inhibition of IgE reactivity was obtained with Abs raised against VP1-P5 (13–68%; mean inhibition 39%) and Abs raised against KLH-coupled P5 (7–70%; mean inhibition 47%; Table II). The strongest inhibition of allergic patients' IgE reactivity was obtained with Abs raised against the VP1-2xP5 construct ranging from 29 to 91%, with a mean inhibition of 63% (Table II).

The much stronger inhibition of patients' IgE reactivity of Phl p 1 by anti-VP1-2xP5 Abs compared with anti-Phl p 1 Abs may be explained by the higher titer of the anti-VP1-2xP5 IgG compared with the anti-Phl p 1 IgG (Table III).

Similar results were obtained when anti-VP1-2xP5 Abs were studied for their capacity to inhibit Phl p 1-induced degranulation of RBLs that had been loaded with IgE from four grass pollen allergic patients (Fig. 7). The anti-VP1-2xP5 antiserum started to inhibit Phl p 1-induced basophil degranulation already at a concentration of 2.5% and caused a >50% inhibition of degranulation at a concentration of 10% (Fig. 7). The Phl p 1-specific Abs caused a much weaker inhibition of Phl p 1-induced degranulation, which became detectable only at a concentration of 10%.

VP1-specific Abs inhibit HRV89 infection of HeLa cells

Next, we were interested to investigate whether VP1-specific IgG Abs can inhibit human rhinovirus infection of HeLa cells. Results

Table III. *Titration of rabbit anti-Phl p 1 and anti-VP1-2xP5 IgG Abs*[a]

	IgG Reactivity to HSA, Phl p 1, and VP1-2xP5					
	Anti-Phl p 1			Anti-VP1-2xP5		
Serum Dilution	HSA	Phl p 1	VP1-2xP5	HSA	Phl p 1	VP1-2xP5
1/10,000	0.003	0.493	0.116	0.000	0.914	1.438
1/20,000	0.000	0.454	0.063	0.000	0.835	1.476
1/50,000	0.000	0.075	0.026	0.044	0.332	0.903
1/100,000	0.000	0.117	0.020	0.018	0.281	0.885

[a] Different serum dilutions of rabbit anti-Phl p 1 and rabbit anti-VP1-2xP5 were tested for reactivity to HSA, Phl p 1, or VP1-2xP5. OD values correspond to bound IgG Abs.

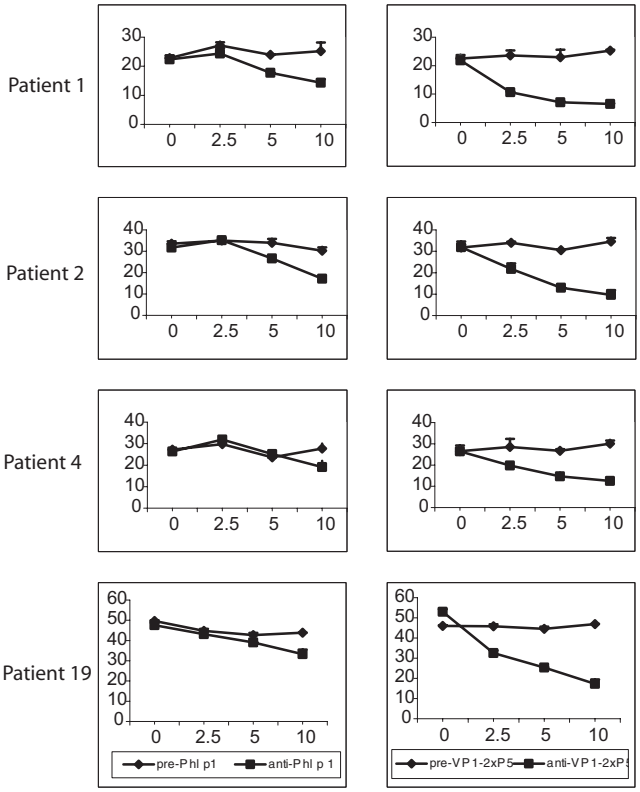


FIGURE 7. Inhibition of IgE-mediated basophil degranulation by VP1-2xP5-specific IgG Abs. RBLs transfected with the human FcεRI were loaded with IgE from different grass pollen allergic patients (*left margin*, nos. 1–4 and 19) and exposed to Phl p 1 along with increasing concentrations of rabbit anti-Phl p 1 or anti-VP1–2xP5 antiserum or the preimmune serum in the presence of Phl p 1 (X-axes: percentages, v/v, of rabbit antisera). ß-Hexosaminidase releases are displayed as percentages of total ß-hexosaminidase contents of the cells (*y*-axes).

from a representative experiment performed with a HRV89 strain are shown in Fig. 8. When cells were infected with of 100 $TCID_{50}$ of HRV89, a complete cytopathic effect was observed (Fig. 8, *row C*, well 1). Addition of VP1-P5- as well as VP1-2xP5-specific Abs prevented HRV-induced cell death up to a dilution of the VP1-specific antiserum of 1/32 (Fig. 8, *rows A* and *B*). Cells incubated in medium without virus were fully alive (Fig. 8, *row C*, wells 2 and 3). Addition of antisera to the noninfected cells showed no relevant effects (Fig. 8, *row C*, wells 4–6). Similar results with anti-VP1 Abs were obtained when another rhinovirus strain (HRV14) was used instead of HRV89 (data not shown).

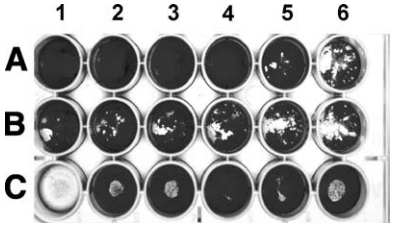


FIGURE 8. VP1-specific Abs inhibit HRV89 infection of HeLa cells. HeLa cells were seeded in a tissue culture plate at equal density per well. In rows *A* and *B*, cells were infected with the 100-fold dose of HRV89 that had left 50% of cells intact (100 $TCID_{50}$). Viral infection was performed in the presence of serial dilutions of anti-VP1-P5 Abs (undiluted, 1/2–1/32 wells 1–6) (*row A*) or serial dilutions of anti-VP1–2xP5 Abs (*row B*). Well 1 in *row C* was incubated with 100 $TCID_{50}$, wells 2 and 3 with medium alone, and wells 4–6 with the Abs alone (well 4, anti-VP1; well 5, anti-VP1-P5; well 6, anti-VP1-2xP5). Intact cells were stained with a violet dye.

Discussion

Allergens and rhinovirus infections are among the most common triggers of asthma. To develop a vaccine which would confer protective immunity against rhinovirus infections as well as against allergies, we constructed an expression plasmid that allows the expression and purification of chimeric proteins consisting of rhinovirus-derived VP1 and allergen-derived peptides. VP1 was selected because it is the HRV-derived surface protein that is critically involved in rhinovirus infection of human cells, shows a high degree of sequence homology among various rhinovirus strains, and is recognized by Abs neutralizing rhinovirus infections (34, 35).

The VP1-based allergy vaccine was designed to minimize the risk of inducing IgE- and T cell-mediated side effects during therapy. For the reduction of IgE-mediated side effects, allergen-derived peptides without IgE reactivity, as represented by the Phl p 1-derived peptide can be selected according to B cell epitope mapping data, prediction of surface-exposed areas using computer programs, or according to the three-dimensional allergen structures (22, 29). When coupled to a foreign carrier molecule and used for immunization, these peptides induce robust allergen-specific IgG responses, which block IgE recognition of the allergen and IgE-mediated allergic inflammation. The peptides can be further selected to minimize the presence of frequently recognized allergen T cell epitopes to reduce T cell-mediated side effects as have been described in clinical trial performed with non-IgE-reactive T cell epitope peptides (36). The carrier principle is based on classical work performed by Benacerraf and colleagues (37–39) who studied the mechanisms underlying Ab production against small haptens that have been coupled to carriers. Their work has demonstrated that Ab responses can be obtained against haptens when they are coupled to unrelated carrier molecules that are recognized by T lymphocytes.

The VP1-based vaccine for the major timothy grass pollen allergen exemplifies the possible beneficial features of a combined vaccine for allergy and rhinovirus infections that is based on small allergen-derived peptides that are coupled to carrier molecules derived from viruses.

Recombinant VP1-P5 and rVP1-2xP5 lack IgE reactivity and allergenic activity. Therefore, the vaccine should induce little or no IgE-mediated side effects in allergic patients. Immunization of mice and rabbits showed that the proteins induced allergen-specific IgG responses which inhibited allergic patients' IgE binding to the complete Phl p 1 allergen and Phl p 1-induced immediate allergic inflammation as demonstrated by the inhibition of basophil degranulation. Interestingly, VP1-2xP5-induced IgG Abs inhibited allergic patients' IgE recognition of Phl p 1 much stronger than those IgG Abs induced by immunization with the complete Phl p 1 allergen, suggesting that the VP1-based vaccine should be more effective than currently existing vaccines containing the Phl p 1 allergen. IgG Abs induced with the VP1-based vaccine cross-reacted with group 1 allergens from several common grasses, indicating that the vaccine should be also useful to treat allergies to various grasses. A National Center for Biotechnology Information BLAST search with the P5 amino acid sequence demonstrated in fact a close relation of the timothy grass-derived peptide with homologous peptides from *L. perenne*, *P. pratensis*, *T. aestivum*, and *A. odoratum* which also reacted strongly with the VP1-2xP5-induced IgG Abs. Vaccination of one healthy volunteer has shown that the VP1-based vaccine also induces allergen-specific and rhinovirus-specific immune responses in humans (data not shown).

Unfortunately, the efficacy of prophylactic or therapeutic application of our vaccine in a purely mouse-based model could not be studied because the murine IgE response is directed against different IgE epitopes than that of Phl p 1-allergic patients. However, the VP1-based allergy vaccine has induced in animals IgG Abs that blocked allergic patients' IgE recognition of the allergen. Thus, similar data have been obtained for the VP1-based vaccine as for other hypoallergenic allergen derivatives that have already been successfully applied in patients in clinical trials (40, 41).

Grass pollen also include other important allergens besides the group 1 allergen represented by Phl p 1, but it has been shown that a mixture of only four allergens, i.e., Phl p 1, Phl p 2, Phl p 5, and Phl p 6, was sufficient to treat grass pollen allergy (42). At present, we have identified hypoallergenic peptides from the latter three allergens and it should therefore be feasible to construct a VP1-based grass pollen vaccine using the approach described in this study.

As another possibly advantageous feature of the VP1-based vaccine, we noted that the VP1 portion of the hybrid vaccine seemed to drive the immune response toward Th1 because we observed higher Phl p 1-specific IgG2 Ab levels and lower levels of IL-5 in VP1-P5- and VP1-2xP5- immunized mice compared with Phl p 1-immunized mice.

The VP1-based grass pollen vaccine induced mainly VP1-specific T cell responses but no allergen-specific T cell responses could be detected. We therefore assume that the T cell help for the allergen-specific IgG response induced by the vaccine comes from VP1-specific T cells. It is therefore likely that the VP-based allergy vaccines will induce little or no side effects through stimulation of allergen-specific T cells and thus be superior to recombinant hypoallergenic allergen derivatives which still can elicit T cell activation (43) and also induced late-phase side effects when injected into patients (44).

Furthermore, it may be expected that the VP1-based vaccine does not induce allergen-specific T cell responses or allergic sensitization and therefore should be suited for prophylactic applications. Support for this assumption comes from our finding that the VP1-based vaccines did not induce reaginic Phl p 1-specific IgE Abs upon immunization, whereas the complete natural Phl p 1 allergen primed strong reaginic Phl p 1-specific IgE responses.

Finally, we found that the VP1-based vaccine gave rise to VP1-specific Abs which inhibited the rhinovirus infection of human HeLa cells. In fact, we have demonstrated that Abs induced with HRV89-derived VP1 not only protected against HRV89 infections but also against infections caused by a distantly related rhinovirus strain such as HRV14 (data not shown).

VP1-based allergy vaccines may therefore be useful for therapy and prevention of asthma caused by allergens and rhinovirus infections.

Disclosures

R.V. is a consultant for Phadia (Uppsala, Sweden) and for Biomay (Vienna, Austria).

References

1. Bousquet, J., P. J. Bousquet, P. Godard, and J. P. Daures. 2005. The public health implications of asthma. *Bull. World Health Organ.* 83: 548–554.
2. Heymann, P. W., T. A. Platts-Mills, and S. L. Johnston. 2005. Role of viral infections, atopy and antiviral immunity in the etiology of wheezing exacerbations among children and young adults. *Pediatr. Infect. Dis. J.* 24: 217–222.
3. Demoly, P., and J. Bousquet. 2006. The relation between asthma and allergic rhinitis. *Lancet* 368: 711–712.
4. Simons, F. E. 1999. Allergic rhinobronchitis: the asthma-allergic rhinitis link. *J. Allergy Clin. Immunol.* 104: 534–540.
5. Niederberger, V., J. Ring, J. Rakoski, S. Jager, S. Spitzauer, P. Valent, F. Horak, M. Kundi, and R. Valenta. 2007. Antigens drive memory IgE responses in human allergy via the nasal mucosa. *Int. Arch. Allergy Immunol.* 142: 133–144.
6. Holgate, S. T. 2006. Rhinoviruses in the pathogenesis of asthma: the bronchial epithelium as a major disease target. *J. Allergy Clin. Immunol.* 118: 587–590.
7. Papadopoulos, N. G., and S. Psarras. 2003. Rhinoviruses in the pathogenesis of asthma. *Curr. Allergy Asthma Rep.* 3: 137–145.
8. Mallia, P., and S. L. Johnston. 2006. How viral infectious cause exacerbation of airway diseases. *Chest* 130: 1203–1210.
9. Corne, J. M., C. Marshall, S. Smith, J. Schreiber, G. Sanderson, S. T. Holgate, and S. L. Johnston. 2002. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. *Lancet* 359: 831–834.
10. Contoli, M., S. D. Message, V. Laza-Stanca, M. R. Edwards, P. A. Wark, N. W. Bartlett, T. Kebadze, P. Mallia, L. A. Stanciu, H. L. Parker, et al. 2006. Role of deficient type III interferon-λ production in asthma exacerbations. *Nat. Med.* 12: 1023–1026.
11. Larche, M., C. A. Akdis, and R. Valenta. 2006. Immunological mechanism of allergen-specific immunotherapy. *Nat. Rev. Immunol.* 6: 761–771.
12. Moller, C. S. Dreborg, H. A. Ferdousi, S. Halken, A. Host, L. Jacobsen, A. Koivikko, D. Y. Koller, B. Niggemann, L. A. Norberg, et al. 2002. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT study). *J. Allergy Clin. Immunol.* 109: 251–256.
13. Valenta, R. 2002. The future of antigen-specific immunotherapy of allergy. *Nat. Rev. Immunol.* 2: 446–453.
14. Linhart, B., and R. Valenta. 2005. Molecular design of allergy vaccines. *Curr. Opin. Immunol.* 17: 646–655.
15. Larche, M., and D. C. Wraith. 2005. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat. Med.* 11: 69–76.
16. Weiss, R., S. Scheiblhofer, M. Gabler, F. Ferreira, W. W. Leitner, and J. Thalhamer. 2006. Is genetic vaccination against allergy possible? *Int. Arch. Allergy Immunol.* 139: 332–345.
17. Kanzler, H., F. J. Barrat, E. M. Hessel, and R. L. Coffman. 2007. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat. Med.* 13: 552–559.
18. Creticos, P. S., J. T. Schroeder, R. G. Hamilton, S. L. Balcer-Whaley, A. P. Khattignavong, R. Lindblad, H. Li, R. Coffman, V. Seyfert, J. J. Eiden, and D. Broide. 2006. Immunotherapy with ragweed-Toll-like receptor 9 agonist vaccine for allergic rhinitis. *N. Engl. J. Med.* 355: 1445–1455.
19. Valenta, R., and V. Niederberger. 2007. Recombinant allergens for immunotherapy. *J. Allergy Clin. Immunol.* 119: 826–830.
20. Stanway, G., P. J. Hughes, R. C. Mountford, P. D. Minor, and J. W. Almond. 1984. The complete nucleotide sequence of a common cold virus: human rhinovirus 14. *Nucleic Acids Res.* 12: 7859–7875.
21. Rossman, M. G., E. Arnold, J. W. Erickson, E. A. Frankenberger, J. P. Griffith, H. J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, et al. 1985. Structure of a human common cold virus and functional relationship to other picornaviruses. *Nature* 317: 145–153.
22. Focke, M., V. Mahler, T. Ball, W. R. Sperr, Y. Majlesi, D. Kraft, and R. Valenta. 2001. Nonanaphylactic synthetic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1, for allergy vaccination. *FASEB J.* 15: 2042–2044.
23. Flicker, S., P. Steinberger, T. Ball, M. T. Krauth, P. Verdino, P. Valent, S. Almo, and R. Valenta. 2006. Spatial clustering of the IgE epitopes on the major timothy grass pollen allergen Phl p 1: importance for allergenic activity. *J. Allergy Clin. Immunol.* 117: 1336–1343.
24. Vrtala, S., M. Grote, M. Duchene, R. van Ree, D. Kraft, O. Scheiner, and R. Valenta. 1993. Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *Int. Arch. Allergy Immunol.* 102: 160–169.
25. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
26. Vrtala, S., M. Susani, P. Valent, S. Laffer, C. Dolecek, D. Kraft, and R. Valenta. 1996. Immunologic characterization of purified recombinant timothy grass pollen (*Phleum pratense*) allergens (Phl p 1, Phl p 2, Phl p 5). *J. Allergy Clin. Immunol.* 97: 781–787.
27. Hauswirth, A. W., S. Natter, M. Ghannadan, Y. Majlesi, G. H. Schernthaner, W. Sperr, H.-J. Bühring, R. Valenta, and P. Valent. 2002. Recombinant allergens promote expression of CD203c on basophils in sensitized individuals. *J. Allergy Clin. Immunol.* 110: 102–109.
28. Vrtala, S., T. Ball, B. Pandjaitan, C. Suphioglu, B. Knox, W. R. Sperr, P. Valent, D. Kraft, and R. Valenta. 1998. Immunization with purified natural and recombinant allergens induce mouse IgG1 antibodies that recognize similar epitopes as human IgE and inhibit the human IgE-allergen interaction and allergen-induced basophil degranulation. *J. Immunol.* 160: 6137–6144.
29. Focke, M., B. Linhart, A. Hartl, U. Wiedermann, W. R. Sperr, P. Valent, J. Thalhamer, D. Kraft, and R. Valenta. 2004. Non-anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for preventive vaccination. *Clin. Exp. Allergy* 34: 1525–1533.
30. Linhart, B., S. Bigenzahn, A. Hartl, C. Lupinek, J. Thalhamer, R. Valenta, and T. Wekerle. 2007. Costimulation blockade inhibits allergic sensitization but does not affect established allergy in a murine model of grass pollen allergy. *J. Immunol.* 178: 3924–3931.
31. Kaul, S., D. Lüttkopf, B. Kastner, G. Hoeltz, S. Vieths, and A. Hoffmann. 2007. Mediator release assays based on human or murine immunoglobulin E in allergen standardization. *Clin. Exp. Allergy* 37: 141–150.
32. Kaerber, G. 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch. Exp. Pathol. Pharmakol.* 162: 480–483.

33. Moser, R., L. Snyers, J. Wruss, J. Angulo, H. Peters, and D. Blaas. 2005. Neutralization of a common cold virus by concatemers of the third ligand binding module of the VLDL-receptor strongly depends on the number of modules. *Virology* 338: 259–269.

34. Laine, P., S. Blomqvist, C. Savolainen, K. Andries, and T. Hovi. 2006. Alignment of capsid protein VP1 sequences of all human rhinovirus prototype strains: conserved motifs and functional domains. *J. Gen. Virol.* 87: 129–138.

35. Sherry, B., A. G. Mosser, R. J. Colonno, and R. R. Rueckert. 1986. Use of monoclonal antibodies to identify four neutralization immunogens on a common cold picornavirus human rhinovirus 14. *J. Virol.* 57: 246–257.

36. Oldfield, W. L., A. B. Kay, and M. Larche. 2001. Allergen-derived T cell peptide-induced late asthmatic reactions precede the induction of antigen-specific hyporesponsiveness in atopic allergic asthmatic subjects. *J. Immunol.* 167: 1734–1739.

37. Siskind, G. W., W. E. Paul, and B. Benacerraf. 1965. Studies on the effect of carrier molecule on antihapten antibody synthesis: I. Effect of carrier on the nature of the antibody synthesized. *J. Exp. Med.* 123: 673–688.

38. Paul, W. E., D. H. Katz, E. A. Goidl, and B. Benacerraf. 1970. Carrier function in anti-hapten immune responses: II. Specific properties of carrier cells capable of enhancing anti-hapten antibody responses. *J. Exp. Med.* 132: 283–299.

39. Rock, K. L., B. Benacerraf, and A. K. Abbas. 1984. Antigen presentation by hapten-specific B lymphocytes: I. Role of surface immunoglobulin receptors. *J. Exp. Med.* 160: 1102–1113.

40. Vrtala, S., C. A. Akdis, F. Budak, M. Akdis, K. Blaser, D. Kraft, and R. Valenta. 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.

41. Niederberger, V., F. Horak, S. Vrtala, S. Spitzauer, M. T. Krauth, P. Valent, J. Reisinger, M. Pelzmann, B. Hayek, M. Krongvist, et al. 2004. Vaccination with genetically engineered allergens prevents progression of allergic disease. *Proc. Natl. Acad. Sci. USA* 101(Suppl. 2): 14677–14682.

42. Jutel, M., L. Jäger, R. Suck, H. Meyer, H. Fiebig, and O. Cromwell. 2005. Allergen-specific immunotherapy with recombinant grass pollen allergens. *J. Allergy Clin. Immunol.* 116: 608–613.

43. Campana, R., N. Mothes, I. Rauter, S. Vrtala, R. Reininger, M. Focke-Tejkl, C. Lupinek, N. Balic, S. Spitzauer, and R. Valenta. 2008. Non-IgE-mediated chronic allergic skin inflammation revealed with rBet v 1 fragments. *J. Allergy Clin. Immunol.* 121: 528–530.

44. Purohit, A., V. Niederberger, M. Kronqvist, F. Horak, R. Grönneberg, R. Suck, B. Weber, H. Fiebig, M. van Hage, G. Pauli, et al. 2008. Clinical effects of immunotherapy with genetically modified recombinant birch pollen Bet v 1 derivatives. *Clin. Exp. Allergy.* 38: 1514–1525.

Cigarette smoke facilitates allergen penetration across respiratory epithelium

Gangl K, Reininger R, Bernhard D, **Campana R**, Pree I, Reisinger J, Kneidinger M, Kundi M, Dolznig H, Thurnher D, Valent P, Chen KW, Vrtala S, Spitzauer S, Valenta R, Niederberger V.

Published

Allergy, 2009

Allergy 2009: 64: 398–405

DOI: 10.1111/j.1398-9995.2008.01861.x

Original article

Cigarette smoke facilitates allergen penetration across respiratory epithelium

Background: The association between cigarette smoke exposure and allergic airway disease is a matter for debate. We sought to investigate in an *in vitro* system whether active smoking reduces the integrity and barrier function of the respiratory epithelium and thus facilitates allergen penetration.
Methods: We cultured the human bronchial epithelial cell line 16HBE14o− in a transwell culture system as a surrogate for the intact respiratory epithelium. The cell monolayer was exposed to standardized cigarette smoke extract (CSE). The extent and effects of trans-epithelial allergen penetration were measured using ^{125}I-labelled purified major respiratory allergens (rBet v 1, rPhl p 5 and rDer p 2) and histamine release experiments.
Results: Exposure of cells to concentrations of CSE similar to those found in smokers induced the development of para-cellular gaps and a decrease in trans-epithelial resistance. CSE exposure induced a more than threefold increase in allergen penetration. Increased subepithelial allergen concentrations provoked a substantial augmentation of histamine release from sensitized basophils.
Conclusions: Our results indicate that cigarette smoke is a potent factor capable of reducing the barrier function of the respiratory epithelium for allergens and may contribute to increased allergic inflammation, exacerbation of allergic disease and boosting of IgE memory.

K. Gangl[1], R. Reininger[2], D. Bernhard[3], R. Campana[4], I. Pree[1], J. Reisinger[1], M. Kneidinger[5], M. Kundi[6], H. Dolznig[7], D. Thurnher[1], P. Valent[5], K.-W. Chen[4], S. Vrtala[5], S. Spitzauer[2], R. Valenta[4], V. Niederberger[1]

[1]Department of Otorhinolaryngology, Vienna General Hospital, Medical University of Vienna, Vienna; [2]Clinical Institute for Medical and Chemical Laboratory Diagnostics, Medical University of Vienna, Vienna; [3]Cardiac Surgery Research Laboratory, Department of Cardiac Surgery, Medical University of Innsbruck, Innsbruck; [4]Christian Doppler Laboratory for Allergy Research, Division of Immunopathology, Department of Pathophysiology, Center for Physiology and Pathophysiology, Medical University of Vienna, Vienna; [5]Department of Internal Medicine I, Division of Hematology and Hemostaseology, Medical University of Vienna, Vienna; [6]Institute of Environmental Health, Center for Public Health, Medical University of Vienna, Vienna; [7]Department of Pathology, Medical University of Vienna, Vienna, Austria

Key words: allergen penetration; cigarette smoke; permeability; recombinant allergens; respiratory epithelium.

Verena Niederberger, MD
Department of Otorhinolaryngology
Vienna General Hospital, AKH, Medical University of Vienna
Waehringer Guertel 18-20
A-1090 Vienna
Austria

Accepted for publication 7 July 2008

Smoking is one of the environmental factors thought to have an impact on allergic diseases (1). Both allergic disease and smoking have an extremely high prevalence: it has been estimated that more than 25% of the population of industrialized countries suffer from IgE-mediated allergies, with a continuous tendency to increase (2). The prevalence of smoking is 20.9% in the adult population in the USA (3) and even higher in many European countries (4). Allergic patients suffering from airway disease tend to quit smoking in order to improve airway function (5, 6), but nevertheless there is a huge number of allergic individuals who are also cigarette smokers. Whether cigarette smoking has an impact on the development and course of allergy is a controversial matter.

Several epidemiological studies suggest a positive association between smoking and allergy. For example,

Abbreviations: CSE, cigarette smoke extract; PI, propidium iodide; r, recombinant; SEM, standard error of the mean; TER, trans-epithelial resistance.

elevated levels both of total and specific IgE have been found in smokers and in asthmatic smokers (7–9). Smoking has also been identified as a risk factor for sensitization to house dust mite allergens (10) and for the development of occupational sensitization towards several environmental allergens (11, 12). However, several other epidemiological studies report a negative association between active smoking and atopy (2, 5, 13).

Yet, passive smoke exposure in children through parental smoking has been shown to be associated with an increased risk of allergic sensitization later in life (14, 15). A recent meta-analysis of studies on occupational exposure indicated that smoking may promote IgE-mediated sensitization and development of airway diseases (16). The latter observation gains support from experimental studies performed in mice which show that smoke-exposed mice mount higher allergen-specific IgE levels and develop more severe allergen-induced lung inflammation than mice without smoke exposure (17–19). In already sensitized allergic patients inhalation of tobacco smoke induced an increase in allergen-specific IgE in nasal lavage fluids when patients were exposed to respiratory allergens (20).

In this study, we investigated whether cigarette smoke can directly impair the barrier function of the respiratory epithelium for allergens which would provide an explanation for the observations that cigarette smoke promotes sensitization to allergens, allergen-induced allergic inflammation and boosting of IgE memory responses. It has been shown that cigarette smoke has a direct influence on respiratory epithelial and alveolar cells (21–25). In this study we investigated how smoking affects the penetration of defined allergens through the respiratory epithelium.

For this purpose, we used a bronchial epithelial cell line as a surrogate for the intact respiratory epithelium and several purified important respiratory allergens in a two-chamber transwell system to investigate the impact of cigarette smoke on respiratory barrier function for allergens as well as on allergen-induced release of inflammatory mediators from co-cultured basophils.

Methods

Culture of human bronchial epithelial cell layers

An epithelial cell line (16HBE14o−) derived from human bronchial epithelial cells was grown in cell layers (26). These cells can be used as a surrogate for the respiratory epithelium because they grow in polarized monolayers and retain properties of differentiated airway epithelial cells, form tight junctions, apical microvilli and cilia and exhibit regulated ion transport.

16HBE14o− cells were grown in minimal essential medium (Gibco, Carlsbad, CA, USA) containing 10% foetal calf serum (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 2 mM glutamine (Gibco) and were passaged when reaching approximately 75–85% confluence. For barrier function experiments, cells were plated at a density of 2×10^5 per 500 μl of medium to the upper chamber of 12-mm-diameter transwell inserts (polyester membranes, 0.4 μm pore size; Costar, Cambridge, MA, USA). The upper chamber was filled with 500 μl of medium and the lower chamber was filled with 1500 μl of medium.

Preparation of aqueous cigarette smoke extract

A cigarette smoking machine which was previously described was used for the production of aqueous cigarette smoke extract (CSE) (27, 28). In short, CSE was produced by consecutively bubbling the smoke of two commercially available filter cigarettes (Marlboro; Philip Morris International Inc., New York, NY, USA; nicotine: 0.8 mg; tar 10 mg) through 8 ml of minimal essential medium (Gibco) containing 100 U/ml penicillin (Gibco), 100 μg/mL streptomycin (Gibco) and 2 mM glutamine (Gibco) (27, 28). Cigarettes were machine smoked at a rate of 15 ml/s for 2 s followed by a pause of 28 s, mimicking the smoking habits of an average smoker (29). The contents of the CSE produced in this setting have been standardized to contain a nicotine concentration of 44 ng nicotine/ml CSE (28) which is comparable with plasma nicotine concentrations of smokers (43.7 ± 38 ng/ml) (30). The dilutions used in this study (2–32% of the CSE produced in the machine) were in the range of 14.1 (32%) to 0.9 (2%) ng nicotine/ml.

Examination of cell monolayers by using phase contrast microscopy

Cells were cultured in 25-cm^2 tissue culture flasks. When cells were confluent, medium was changed and cells were incubated with different concentrations of CSE (2%, 4%, 8%, 16% and 32%) or medium alone at 37°C in a humidified atmosphere containing 5% CO_2. After 3 h of incubation, cells were examined with a phase contrast microscope (Zeiss Axiovert 40C, Zeiss, Oberkochen, Germany) and photographed with a camera mounted on the microscope.

Measurement of trans-epithelial resistance under the influence of different concentrations of cigarette smoke extract

Epithelial barrier function was determined by measuring trans-epithelial resistance (TER) using an ohmvoltmeter (Millipore, Bedford, MA, USA). Baseline resistance across polyester membranes without cellular monolayers was 100 Ω/cm^2 on average. The respective values were subtracted from TER measurements. Experiments investigating the effect of CSE on epithelial barrier function were started when TER had reached at least 1000 Ω/cm^2.

Aliquots of minimal essential medium (Gibco) supplemented with 100 U/ml penicillin (Gibco), 100 μg/mL streptomycin (Gibco) and 2 mM glutamine (Gibco), containing different concentrations of CSE were added either to the upper chamber (addition of 160 μl) or to both the upper (addition of 160 μl) and lower chambers (addition of 480 μl) of the transwell system, resulting in the exposure of 16HBE14o− monolayers to increasing doses of aqueous CSE (2%, 4%, 8%, 16% and 32%). These experiments were conducted once in duplicate and once in quadruplicate wells and were compared with duplicate/quadruplicate control wells containing culture medium without CSE. The experiments were then repeated with a lower concentration range of CSE (2%, 4%, 6%, 8%, 10% and 12%) three times in triplicate wells.

Quantification of cell death by using flow cytometry

The proportion of viable 16HBE14o− cells after incubation with different concentrations of CSE (0%, 2%, 4%, 8%, 16% and 32%) was determined by using flow cytometry (Beckman Coulter,

Fullerton, CA, USA) using nuclear propidium iodide fluorescence dye (PI) (Alexis Biochemicals, Carlsbad, CA, USA). Cells were incubated with different concentrations of CSE for 3 h at 37°C in a humidified atmosphere containing 5% CO_2. Cells were then trypsinized to detach them from the cell culture flask bottom, washed with PBS and stained with PI according to the manufacturer's instructions. Necrotic cells are permeable for PI. Cell debris and small matter were excluded from the analysis.

Immunocytochemistry

Cells were cultured on tissue culture slides (BD, Franklin Lakes, NJ, USA) and exposed to different concentrations of CSE (4%, 8% and 16%) for 3 h. After fixation with acetone/methanole (1 : 1), they were stained with a polyclonal rabbit IgG antibody against ZO-1 (Atlas Antibodies, Stockholm, Sweden) and an FITC-marked secondary antibody (Alexa Fluor 546, Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions and were examined with a confocal laser scanning microscope (LSM 5 Exciter Laser Scanning Microscope; Zeiss, Oberkochen, Germany).

Quantification of trans-epithelial allergen migration

Recombinant Bet v 1 (17 kDa), the major birch pollen allergen, and recombinant Phl p 5 (28 kDa), a major timothy grass pollen allergen, were obtained from Biomay (Vienna, Austria). Recombinant house dust mite allergen Der p 2 (14 kDa) was produced as described (31). Purified allergens were ^{125}I labelled by using the chloramin T method (32). ^{125}I-labelled allergens (250 000 cpm/well) were added to the upper chamber of the transwell system after incubation of cells with CSE (2%, 4%, 8%, 16% and 32%) or medium alone for 1 h added to either the upper or both the upper and lower chambers. Two hours thereafter aliquots from the upper and lower chambers were analysed in a gamma counter (Wallac, Turku, Finland). Cells were removed from the membrane and lysed for determination of intracellular radioactivity. Counts per minute were determined for both chambers and the cell layer. All experiments and measurements were performed in duplicates.

Basophil histamine release experiments

Transwell cell culture monolayers were incubated with increasing dilutions of CSE (2%, 4%, 8%, 16% and 32%) or medium alone for 1 h, added to either the upper or both the upper and lower chambers. Subsequently, 50 ng of rBet v 1 was added to the upper chamber. After 3 h, aliquots were removed from the lower chamber for basophil histamine release experiments. Basophils from two different patients with sensitization to Bet v 1 were isolated using dextran sedimentation and exposed to 100 μl of culture medium from the lower chamber (33). For control purposes, basophils were incubated with various concentrations of rBet v 1 (0.1–0.01 μg/ml) and with aliquots of lower chambers that had been incubated with CSE without the addition of Bet v 1 or with medium alone. Release of histamine was measured with RIA (Immunotech, Marseille, France) and is expressed as a percentage of total histamine release. Transwell experiments to study the effects of rBet v 1 penetration on basophil release were performed in duplicate wells. Histamine release experiments and measurements were performed in triplicates.

Cytokine measurements

Transwell cell culture monolayers were incubated with increasing dilutions of CSE (2%, 4%, 8%, 16% and 32%) or medium alone for 24 h, added to both the upper and lower chambers. Before the addition of CSE, 3 and 24 h after the addition of CSE, aliquots of cell culture medium (100 μl) were taken and stored at −70°C. The concentrations of the cytokines, IL-1β, IL-2, IL-4, IL-5, IL- 6, Il-8, IL-10, IL-12, IL-13, IFN-γ, TNF-α and GM-CSF, secreted into the upper and lower compartments by the HBE cell layer before and after the addition of CSE were measured using the xMAP Luminex fluorescent bead-based technology (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and fluorescence signal was read on Luminex 100 System (Luminex Corp., Austin, TX, USA).

Statistical analysis

Endpoints were analysed by an analysis of variance with linear contrasts for comparison of CSE concentrations against controls. Data were arcsine transformed to obtain homogeneity of variance. For histamine release, patients and wells were considered random factors. For all statistical analyses, values of $P < 0.05$ were considered significant.

Results

Cigarette smoke extract decreases trans-epithelial resistance of human respiratory epithelial cells

We examined whether exposure to CSE affects the epithelial barrier function of human 16HBE14o− bronchial epithelial cell monolayers. In Fig. 1A, the development of TER after the addition of different CSE concentrations to the upper and lower chambers is displayed. After 3 h of incubation with 8%, 16% and 32% of CSE, the TER dropped sharply and significantly (8%, 16% and 32% of CSE: $P < 0.01$). Lower CSE concentrations (2% and 4%) did not have a relevant influence on TER when compared with incubation with medium alone. A similar effect was seen after incubation for 7 and 24 h. In Fig. 1B, the development of TER between 2% and 12% of CSE is shown in greater detail. It can be seen that the observed drop in TER after 3 h of incubation already starts at a CSE concentration of 6%.

In a similar experiment, different CSE concentrations were added exclusively to the upper chamber of the transwell system. A drop in TER was seen after 3 h of incubation with 16% (61% reduction) and 32% of CSE (complete loss of TER) but not with 8% of CSE. This effect was consistently seen also after 7 and 24 h of incubation (data not shown).

In order to determine whether 16HBE14o− cells were viable at the CSE concentrations used in these experiments, flow cytometric analyses were performed using propidium iodide as a marker for cell necrosis. A slight increase in the proportion of necrotic cells was seen after 3 h of incubation with 8% of CSE (Table 1). After incubation with 16% and 32% of CSE, 55.5% and 74.4% of the cells stained positive with propidium iodide respectively (Table 1). A repetition of this experiment yielded similar results.

Similar levels of various cytokines (IL-1β, IL-2, IL-4, IL-5, IL- 6, Il-8, IL-10, IL-12, IL-13, IFN-γ, TNF-α and

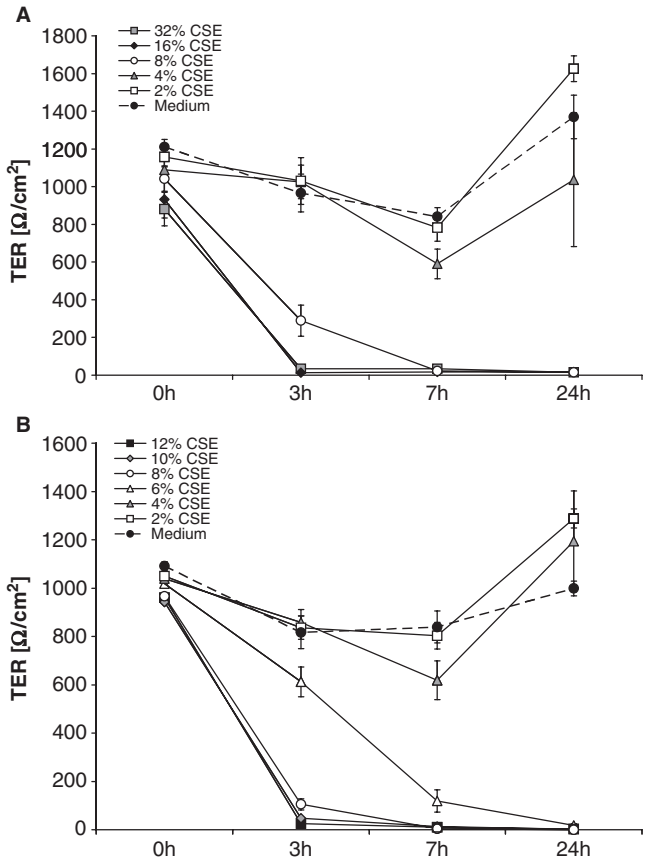


Figure 1. Exposure of an epithelial cell monolayer to cigarette smoke extract (CSE) induces a decrease in trans-epithelial resistance (TER). Increasing concentrations of CSE (2%, 4%, 8%, 16% and 32%) were added to the upper and lower chambers of the transwell system containing confluent monolayers of 16HBE14o− cells. Changes in TER (Ω/cm^2; *y*-axis) are shown for different time points (*x*-axis). (A) The same experiment was also repeated in a lower CSE concentration range (2%, 4%, 6%, 8%, 10% and 12%). (B) SEM are displayed.

GM-CSF) were measured in the supernatants of 16HBE14o− cells 3 h after the addition of 2%, 4% and 8% of CSE or of culture medium alone. However, lower levels of TNF-α, GM-CSF, IL-1β, IL-6 and IL-8 were detected 3 h after the addition of 16% and 32% of CSE when compared with the addition of culture medium alone.

CSE induces morphological changes in 16HBE14o− cell monolayers

After 3 h of incubation with increasing concentrations of CSE, cell contraction and a widening of intercellular gaps were seen in the cell monolayer starting from a CSE concentration of 4% (Fig. 2) as determined by using phase contrast microscopy. Cell monolayers remained unchanged upon incubation with cell culture medium alone (Fig. 2) and with 2% of CSE. Immunostaining for ZO-1 after 3 h of incubation with increasing

Table 1. Induction of cell necrosis by cigarette smoke extract

	% of propidium iodide-positive cells
Medium	5.3
2% CSE	5.9
4% CSE	6.4
8% CSE	15.7
16% CSE	55.5
32% CSE	74.4

Cells were incubated with different concentrations of cigarette smoke extract (2%, 4%, 8%, 16% and 32%) or medium alone for 3 h and were then analysed with propidium iodide (PI) staining and flow cytometric analysis. The percentages of necrotic cells are displayed.

concentrations of CSE (4%, 8% and 16%) showed only a slight alteration in the staining pattern (i.e. a slight loss of continuity in the ZO-1 staining with increasing CSE concentrations) when compared with the medium control (data not shown).

A decrease in TER caused by CSE exposure is associated with increased epithelial permeability for allergens

We measured the permeability of the respiratory epithelial cell layer for three different allergens (rBet v 1, rPhl p 5 and rDer p 2) after incubation of cell monolayers with various concentrations of CSE. Results from experiments in which CSE was added to both the upper and lower chambers are shown in Fig. 3. Incubation with 4% of CSE did not substantially increase the permeability for the allergens when compared with incubation with medium alone. Permeability for Der p 2 and Phl p 5 was significantly increased after incubation with 8% of CSE (Der p 2 and Phl p 5; Fig. 3). Permeability for Bet v 1 was enhanced after incubation with 16% of CSE (Fig. 3). A similar but less pronounced effect on allergen permeability was seen when different CSE concentrations were added exclusively to the upper chamber (data not shown). No radiolabelled allergens were detected in the cell layer, indicating paracellular penetration (data not shown).

CSE exposure increases subepithelial allergen concentrations and leads to augmented histamine release from sensitized basophils

We investigated whether the CSE-induced increase in allergen concentrations in the lower chambers of transwell cultures affects immediate type allergic inflammation. Transwell cultures of 16HBE14o− monolayers were incubated with increasing concentrations of CSE in the upper and lower chambers of the transwell system before 50 ng of rBet v 1 was added to the upper chambers. Histamine release experiments were performed by incubating basophil granulocytes from two different birch pollen allergic patients (Fig. 4A and B) with allergen-containing aliquots of culture medium from lower chambers. Incubation of cell monolayers with 8% of CSE and above led to an increase in

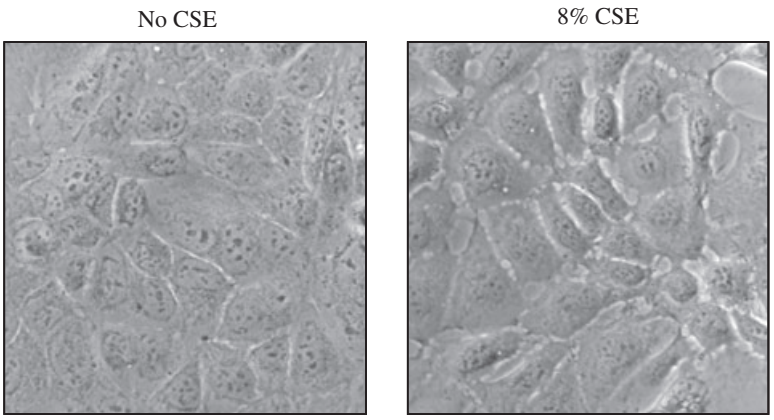


Figure 2. Cigarette smoke extract (CSE) induces morphological changes in confluent monolayers of 16HBE14o− cells. Intercellular gaps are seen in the 16HBE14o− monolayer after incubation with 8% of CSE for 3 h (right), but not after incubation with cell culture medium (left).

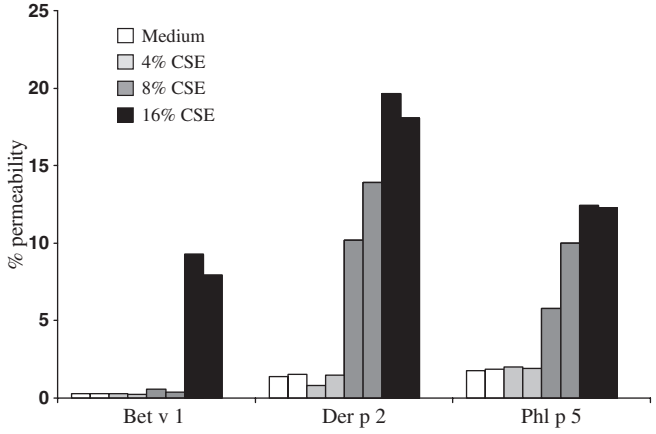


Figure 3. Cigarette smoke extract facilitates allergen penetration across epithelial cell layers. 16HBE14o− monolayers were incubated with three different concentrations of CSE (4%, 8%, 16%, different shades of grey) in the upper and lower chambers or medium alone (white bars) for 1 h. Subsequently, ^{125}I-labelled recombinant Bet v 1, Phl p 5 or Der p 2 were added to the upper chamber. The percentage of allergen that had penetrated to the lower chamber after 2 h is displayed on the *y*-axis. Experiments performed in duplicate wells are shown.

the Bet v 1 concentration in the lower chambers which induced an increase in histamine release from effector cells in both experiments ($P < 0.01$; Fig. 4A and B). Incubation with 2% and 4% of CSE yielded similar histamine release as incubation with cell culture medium alone. No histamine release was observed after incubation of basophils with cell culture medium containing 16% of CSE without the addition of allergen (Fig. 4).

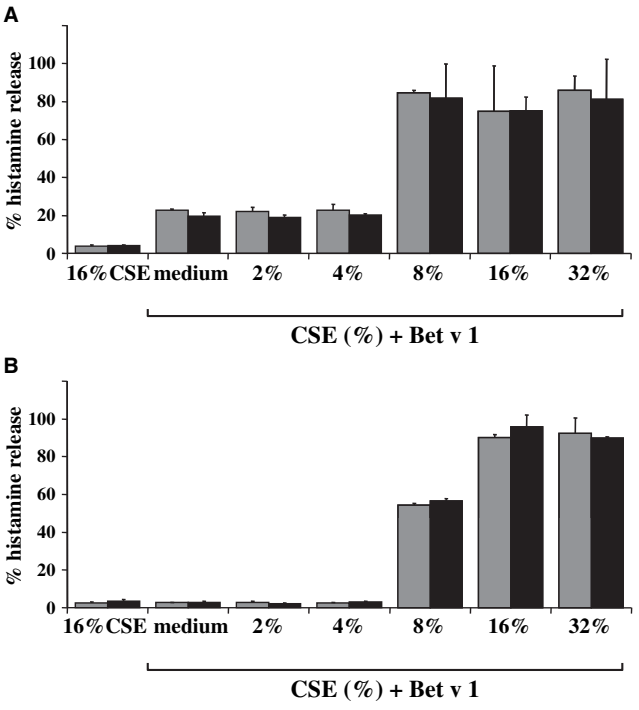


Figure 4. Enhanced trans-epithelial allergen penetration caused by cigarette smoke extract (CSE) exposure leads to an increase in histamine release from sensitized basophils. Epithelial cell layers were incubated for 1 h with different concentrations of CSE (2%, 4%, 8%, 16% and 32%; *x*-axis) or medium alone (0%) before 50 ng of rBet v 1 was added to the upper chambers. Media obtained from the lower chambers 3 h later were added to sensitized basophils from two different birch pollen allergic patients (A,B). The percentage of total histamine release is displayed on the *y*-axis. A control experiment without the addition of rBet v 1 is shown on the left-hand side of the graphs. Black and grey bars indicate the results obtained with supernatants from duplicate wells of the transwell system. Histamine release was measured in triplicates (error bars).

Discussion

In this study, we used three major respiratory allergens from birch pollen, grass pollen and the house dust mite to

investigate the impact of cigarette smoke exposure on the permeability of a respiratory epithelial cell layer. We found that CSE led to a substantial impairment of the barrier function of the respiratory epithelium and an increased permeability for each of the three respiratory allergens tested. The increased subepithelial allergen concentration resulted in an augmented release of inflammatory mediators from basophil granulocytes which were used as a surrogate for subepithelial effector cells. This result suggests that cigarette smoking damages the respiratory epithelial barrier and allows allergens to intrude more efficiently into the subepithelial tissues where they may cause increased allergic inflammation through activation of various immune cells, among them mast cells, T cells and eosinophils.

Our *in vitro* data thus suggest that cigarette smoke-enhanced allergen penetration may be a factor for the increased lung inflammation observed in mice which had been exposed to allergen in conjunction with cigarette smoke (18).

Cigarette smoke-enhanced allergen penetration through the respiratory epithelium would also explain observations that mice which are exposed to smoke become more easily sensitized to respiratory allergens, although a possible adjuvant activity may also play a role (19).

We have recently found that respiratory allergen contact strongly boosts the secondary IgE response to allergens and allergen-specific sensitivity (34). Another recent study reported that nasal challenge with environmental tobacco smoke together with allergen increased allergen-specific IgE production in the nasal lavage fluids of allergic patients and exacerbated allergic airway disease (20). This finding may also be explained by our data because cigarette smoke-enhanced allergen penetration may lead to increased stimulation of allergen-specific IgE- and cytokine-producing cells in addition to a possible adjuvant effect of cigarette smoke.

It is likely that the inconsistent epidemiology of smoking and the development and severity of type I allergy mentioned in the introduction may be explained by the fact that smoking can impact on allergic disease via two main different mechanisms: first, a modulatory effect of smoking on the immune response was described (35). This is illustrated for example by the decreased prevalence of hypersensitivity pneumonitis in smokers when compared with that in nonsmokers (36). Second, cigarette smoke was shown to have a direct influence on respiratory epithelial and alveolar cells (21–25). In hypersensitivity pneumonitis, higher airway permeability and damage to the air–blood barrier were found (37, 38) and smoking was linked to a chronic progression of the disease (39). Our own data show that smoking can reduce the barrier function of the epithelium and may thus allow substantially increased allergen penetration, thus resulting in higher IgE levels and a higher incidence of allergic sensitization in certain populations (10–12, 14–16).

Our model allows to test directly the effects of cigarette smoke on the penetration of defined allergens through the respiratory epithelium and is not influenced by nonallergenic components, such as proteases which may be present in certain allergen sources (e.g. house dust mite extracts) but not in others. In this context, several allergen source-derived factors were identified which either promote the migration of allergens through the epithelium (23) or bias the development of immune responses towards Th2 (40).

Our experiments were performed with dilutions of CSE containing nicotine concentrations of 14.1 (32% of CSE) to 0.9 ng/ml (2% of CSE), which is comparable with plasma nicotine concentrations of smokers (30). Given the fact that nicotine and other components of cigarette smoke have to diffuse into the blood from the airways, it is unlikely that respiratory mucus contains lower concentrations of smoke components than the blood. Furthermore, it has been shown that the saliva of smokers contains even higher nicotine concentrations after consumption of two cigarettes (41). The CSE concentrations we used may therefore be expected *in vivo*. It is quite possible that at higher CSE concentrations the damage in the respiratory epithelium may be less pronounced due to the clearing function of the cilial activity *in vivo* compared with the *in vitro* cultured epithelial cells; still the necrotic effect in medium is approximately 5.3% and increases only to 15.7% at 8% of CSE and thus is actually quite small. We found only a slight loss of continuity in the ZO-1 staining after CSE exposure suggesting that the perturbation of tight junctions does not play a major role in CSE-enhanced allergen penetration.

Another advantage of the *in vitro* system used by us is that it takes into account the covering protective lining of aqueous mucus present in the human airways where cigarette smoke may be enriched.

In conclusion, we have demonstrated that concentrations of cigarette smoke comparable with those found in smokers impair the barrier function of the respiratory epithelium which allows greater concentrations of allergens to penetrate. This may contribute to the exacerbation of allergic disease by aggravating airway symptoms and by boosting the IgE memory response.

Acknowledgments

This study was supported by Grant: SFB 1803, 1804, 1809, 1815 and 1818 of the Austrian Science Fund (FWF); Medizinischer Forschungsfonds Innsbruck (MFI project number: 4302). We thank Prof. D. Kerjaschki for his support regarding the immunohistochemistry experiments.

References

1. Frew AJ. Advances in environmental and occupational diseases 2003. J Allergy Clin Immunol 2004; **113**:1161–1166.
2. Wüthrich B, Schindler C, Medici TC, Zellweger JP, Leuenberger P. IgE levels, atopy markers and hay fever in relation to age, sex and smoking status in a normal adult Swiss population. SAPALDIA (Swiss Study on Air Pollution and Lung Diseases in Adults) Team. Int Arch Allergy Immunol 1996;**111**:396–402.
3. Centers for Disease Control and Prevention. Tobacco use among adults: United States, 2005. MMWR Morbid Mortal Wkly Rep 2006;**55**:1145–1148. Available at: http://www.cdc.gov/tobacco/data_statistics/MMWR/2006/index.htm.
4. Thompson NC, Chaudhuri R, Livingston E. Asthma and cigarette smoking. Eur Respir J 2004;**24**:822–833.
5. Barbee RA, Kaltenborn W, Lebowitz MD, Burrows B. Longitudinal changes in allergen skin reactivity in a community population sample. J Allergy Clin Immunol 1987;**79**:16–24.
6. Becklake MR, Lalloo U. The 'healthy smoker': a phenomenon of health selection? Respiration 1990;**57**:137–144.
7. Barbee RA, Halonen M, Kaltenborn W, Lebowitz M, Burrows B. A longitudinal study of serum IgE in a community cohort: correlations with age, sex, smoking, and atopic status. J Allergy Clin Immunol 1987;**79**:919–927.
8. Sherrill DL, Halonen M, Burrows B. Relationships between total serum IgE, atopy, and smoking: a twenty-year follow-up analysis. J Allergy Clin Immunol 1994;**94**:954–962.
9. Oryszczyn MP, Annesi-Maesano I, Charpin D, Paty E, Maccario J, Kauffmann F. Relationships of active and passive smoking to total IgE in adults of the Epidemiological Study of the Genetics and Environment of Asthma, Bronchial Hyperresponsiveness, and Atopy (EGEA). Am J Respir Crit Care Med 2000;**161**:1241–1246.
10. Jarvis D, Chinn S, Luczynska C, Burney P. The association of smoking with sensitization to common environmental allergens: results from the European Community Respiratory Health Survey. J Allergy Clin Immunol 1999;**104**:934–940.
11. Mapp CE, Boschetto P, Maestrelli P, Fabbri LM. Occupational asthma. Am J Respir Crit Care Med 2005;**172**:280–305.
12. Siracusa A, Marabini A, Folletti I, Moscato G. Smoking and occupational asthma. Clin Exp Allergy 2006;**36**:577–584.
13. Linneberg A, Nielsen NH, Madsen F, Frolund L, Dirksen A, Jorgensen T. Smoking and the development of allergic sensitization to aeroallergens in adults: a prospective population-based study. The Copenhagen Allergy Study. Allergy 2001;**56**:328–332.
14. Kulig M, Luck W, Wahn U, The Multicenter Allergy Study Group, Germany. The association between pre- and postnatal tobacco smoke and allergic sensitization during early childhood. Hum Exp Toxicol 1999; **18**:241–244.
15. Krämer U, Lemmen CH, Behrendt H, Link E, Schäfer T, Gostomzyk J et al. The effect of environmental tobacco smoke on eczema and allergic sensitization in children. Br J Dermatol 2004; **150**:111–118.
16. Nielsen GD, Olsen O, Larsen ST, Lovik M, Poulsen LK, Glue C et al. IgE-mediated sensitisation, rhinits and asthma from occupational exposures. Smoking as a model for airborne adjuvants? Toxicology 2005;**216**:87–105.
17. Rumold R, Jyrala M, Diaz-Sanchez D. Secondhand smoke induces allergic sensitization in mice. J Immunol 2001; **167**:4765–4770.
18. Moerloose KB, Pauwels RA, Joos GF. Short-term cigarette smoke exposure enhances allergic airway inflammation in mice. Am J Respir Crit Care Med 2005;**172**:168–172.
19. Moerloose KB, Robays LJ, Maes T, Brusselle GG, Tournoy KG, Joos GF. Cigarette smoke exposure facilitates allergic sensitization in mice. Respir Res 2006;**7**:49.
20. Diaz-Sanchez D, Rumold R, Gong H Jr. Challenge with environmental tobacco smoke exacerbates allergic airway disease in human beings. J Allergy Clin Immunol 2006;**118**: 441–446.
21. Jones JG, Minty BD, Lawler P, Hulands G, Crawley JC, Veall N. Increased alveolar epithelial permeability in cigarette smokers. Lancet 1980;**1**: 66–68.
22. Li XY, Rahman I, Donaldson K, MacNee W. Mechanisms of cigarette smoke induced increased airspace permeability. Thorax 1996;**51**:465–471.
23. Rusznak C, Sapsford RJ, Devalia JL, Justin John R, Hewitt EL, Lamont AG et al. Cigarette smoke potentiates house dust mite allergen-induced increase in the permeability of human bronchial epithelial cells *in vitro*. Am J Respir Cell Mol Biol 1999;**20**:1238–1250.
24. Mason GR, Uszler JM, Effros RM, Reid E. Rapidly reversible alterations of pulmonary epithelial permeability induced by smoking. Chest 1983;**83**:6–11.
25. Kennedy SM, Elwood RK, Wiggs BJ, Paré PD, Hogg JC. Increased airway mucosal permeability of smokers. Relationship to airway reactivity. Am Rev Respir Dis 1984;**129**:143–148.
26. Wan H, Winton HL, Soeller C, Stewart GA, Thompson PJ, Gruenert DC et al. Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o−. Eur Respir J 2000;**15**:1058–1068.
27. Bernhard D, Huck CW, Jakschitz T, Pfister G, Henderson B, Bonn GK et al. Development and evaluation of an in vitro model for the analysis of cigarette smoke effects on cultured cells and tissues. J Pharmacol Toxicol Methods 2004;**50**:45–51.
28. Bernhard D, Pfister G, Huck CW, Kind M, Salvenmoser W, Bonn GK et al. Disruption of vascular endothelial homeostasis by tobacco smoke – impact on atherosclerosis. FASEB J 2003; **17**:2302–2304.
29. Djordjevic MV, Stellman SD, Zang E. Doses of nicotine and lung carcinogens delivered to cigarette smokers. J Natl Cancer Inst 2000;**92**:106–111.
30. Zuccaro P, Altieri I, Rosa M, Passa AR, Pichini S, Ricciarello G et al. Determination of nicotine and four metabolites in the serum of smokers by high-performance liquid chromatography with ultraviolet detection. J Chromatogr 1993;**621**:257–261.
31. Hales BJ, Hazell LA, Smith W, Thomas WR. Genetic variation of Der p 2 allergens: effects on T cell responses and immunoglobulin E binding. Clin Exp Allergy 2002;**32**:1461–1467.
32. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbour, NY: Cold Spring Harbour Laboratory Press, 1989.
33. Valenta R, Sperr WR, Ferreira F, Valent P, Sillaber C, Tejkl M et al. Induction of specific histamine release from basophils with purified natural and recombinant birch pollen allergens. J Allergy Clin Immunol 1993;**91**:88–97.

34. Niederberger V, Ring J, Rakoski J, Jager S, Spitzauer S, Valent P et al. Antigens drive memory IgE responses in human allergy via the nasal mucosa. Int Arch Allergy Immunol 2007;**142**: 133–144.
35. Sopori M. Effects of cigarette smoke on the immune system. Nat Rev Immunol 2002;**2**:372–377.
36. McSharry C, Banham SW, Boyd G. Effect of cigarette smoking on the antibody response to inhaled antigens and the prevalence of extrinsic allergic alveolitis among pigeon breeders. Clin Allergy 1985;**15**:487–494.
37. Thomeer MJ, Dehaes B, Mortelmans L, Demedts M. Pertechnegas lung clearance in different forms of interstitial lung disease. Eur Respir J 2002;**19**:31–36.
38. Planès C, Valeyre D, Loiseau A, Bernaudin JF, Soler P. Ultrastructural alterations of the air–blood barrier in sarcoidosis and hypersensitivity pneumonitis and their relation to lung histopathology. Am J Respir Crit Care Med 1994;**150**:1067–1074.
39. Ohtsuka Y, Munakata M, Tanimura K, Ukita H, Kusaka H, Masaki Y et al. Smoking promotes insidious and chronic farmer's lung disease, and deteriorates the clinical outcome. Intern Med 1995;**34**:966–971.
40. Mariani V, Gilles S, Jakob T, Thiel M, Mueller MJ, Ring J et al. Immunomodulatory mediators from pollen enhance the migratory capacity of dendritic cells and license them for Th2 attraction. J Immunol 2007;**178**:7623–7631.
41. Feyerabend C, Higenbottam T, Russell MA. Nicotine concentrations in urine and saliva of smokers and non-smokers. Br Med J (Clin Res Ed) 1982;**285**:1002–1004.

Curriculum vitae

Raffaela Campana

Personal Data

Date of Birth: 1980-09-26

Place of birth: Sao Paulo

Nationality: Italian

Education

2001-2004 Study of Biology at the University of Santo Amaro

1985-1998 Primary and Grammar school

Career History

Since May 2005 PhD thesis at the Department of Pathophysiology – Medical University of Vienna, under supervision of Prof. Rudolf Valenta

2001-2004 Diploma thesis at Laboratory of Microesferes and Liposomes, Biotechnology Center – Institute Butantan, Brasil, under supervision of Prof. Dr. Maria Helena Bueno da Costa

July 2004 Trainee at Group of RNA Folding and Catalysis, Vienna Biocenter – University of Vienna, under supervision of Prof. Renée Schroeder

Membership

European Academy of Allergology and Clinical Immunology (EAACI) – Junior Member

Austrian Society of Allergology and Immunology (ÖGAI)

Awards

FAPESP (Brazilian Scientific Foundation), 2002- 2004

Scientific Education

2006 Training in Luminex Technology Fact Sheet, Institute of Pathophysiology, Medical University of Vienna

Abstracts - Poster and Oral Presentation

1. CAMPANA R, Vrtala S, Maderegger B, Dall'Antonia Y, Blatt K, Focke-Tejkl M, Swoboda I, Scheiblhofer S, Gieras, A, Neubauer, A, Keller, W, Valent P, Thalhamer J, Spitzauer S, Valenta R. "Studies on the hypoallergenic nature of a recombinant trimer of the major birch pollen allergen Bet v 1". 2nd European Congress of Immunology, Berlin, Germany, September 2009.

2. CAMPANA R., Mothes N, Rauter I, Vrtala S, Reininger R, Focke-Tejkl M, Lupinek C, Balic N, Spitzauer S and Valenta R. "Non-IgE mediated chronic allergic skin inflammation revealed with rBet v 1 fragments". XXVII Congress of the European Academy of Allergology and Clinical Immunology (EAACI), Barcelona, Spain, June 2008.

3. CAMPANA R., Mothes N, Rauter I, Vrtala S, Reininger R, Focke-Tejkl M, Lupinek C, Balic N, Spitzauer S and Valenta R. "Non-IgE mediated chronic allergic skin inflammation revealed with rBet v 1 fragments". 27th Symposium Collegium Internationale Allergologicum (CIA), Curacao, Netherlands Antilles, May 2008.

4. 6th EAACI-GA^{2}LEN Davos Meeting, Pichl, Austria, Jan 31-Feb 03 2008 – CAMPANA R., "Non-IgE mediated chronic allergic skin inflammation revealed with rBet v 1 fragments".

5. CAMPANA R., Mothes N, Rauter I, Vrtala S, Reininger R, Focke-Tejkl M, Lupinek C, Balic N, Spitzauer S and Valenta R. "Non-IgE mediated chronic allergic skin

inflammation revealed with rBet v 1 fragments". Annual Meeting of the Austrian Society of Allergology and Immunology (ÖGAI), Alpbach, Austria, Dec 2007.

6. CAMPANA R, Namur JAM, Takata CS, Araujo PS and Costa MHB. „How electrostatic and hydrophobic interactions contribute to the adsorption of proteins on $Al(OH)_3$". XXXIII Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology (SBBq), 15-18th May, 2004 at Caxambú, MG, Brazil.

7. CAMPANA R, Namur JAM, Schuch V, Takata CS, Araújo PS and Costa MHB. „A Simple Method to Obtain Purified Diphtheria Toxoid". V Annual Meeting of the Institute Butantan, 01-05th December, 2003 at Sao Paulo, SP, Brazil.

8. CAMPANA R, Namur JAM, Schuch V, Takata CS, Araújo PS and Costa MHB. „A Simple Method to Obtain Purified Diphtheria Toxoid". XXXII Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology (SBBq), 17-20th May, 2003 at Caxambú, MG, Brazil.

9. Namur JAM, CAMPANA R, Quintilio W, Esteves MI, Takata CS, Higashi HG, Alario AF, Moro AM, Araújo PS and Costa MHB. „Microencapsulation of diphtheric toxoid within biomimicking systems". XXXI Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology (SBBq) 18-21st May, 2002 at Caxambú, MG, Brazil.

10. Costa MHB, Quintilio W, Namur JAM, CAMPANA R, Sant'Anna OA, Yamaguchi IK, Guidolin R, Alario AF, Araújo PS, Schwendener RA and Gregoriadis G. „Rational design to encapsulate snake venoms within soybean phosphatidylcholine liposomes". XXXI Annual Meeting of the Brazilian Society of Biochemistry and Molecular Biology (SBBq), 18-21st May, 2002 at Caxambú, MG, Brazil.

Diploma Thesis

R. CAMPANA: "Interaction between Diphtheric toxoid and ionic interface", 2004. Instituto Butantan, Sao Paulo, Brazil.

Publications

1. **R. Campana**, N. Mothes, I. Rauter, S. Vrtala, R. Reininger, M. Focke-Tejkl, C. Lupinek, N. Balic, S. Spitzauer, R. Valenta. Mechanisms of IgE-and non-IgE-mediated chronic allergic skin inflammation revealed with recombinant hypoallergenic Bet v 1 fragments. J Allergy Clin Immunol, 2008; 121: 528-530.

2. **R. Campana**, JAM. Namur, C.Takata, P.Araujo and MHB. Costa. 2004. Ionic interfaces and diphtheria toxoid interactions. Prot. Expression and Purification, 2004; 33: 161-165.

3. J. Tinhofer, K. Niespodziana, B. Linhart, M. Focke-Tejkl, K. Westritschnig, S. Scheiblhofer, **R. Campana**, A. Stoecklinger, M. Kneidinger, P. Valent, J. Thalhamer, T. Popow-Kraupp, R. Valenta. A combination vaccine for allergy and rhinovirus infections based on rhinovirus-derived surface protein VP1 and a non-allergic peptide of the major timothy grass pollen allergen, Phl p 1. J Immunology, 2009; 182: 6298-62306.

4. K. Gangl, R. Reininger, D. Bernhard, **R. Campana,** I. Pree, J. Reisinger, M. Kneidinger, M. Kundi, P. Valent, K. W. Chen, S. Vrtala, R. Valenta, V. Niederberger. Cigarette smoke faciliates allergen penetration across respiratory epithelium and increases sub-epithelial allergic inflammation. Allergy, 2009; 64: 398-405.