Universität für Bodenkultur Wien

Investigation of antibody responses of birch pollen allergic individuals by using chimeric allergens

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Währinger Gürtel 18-20
1090 Wien

Betreuter: Univ.-Prof. Dr. Heimo Breiteneder

Eingereicht von

DI Barbara Gepp

Wien, März 2016

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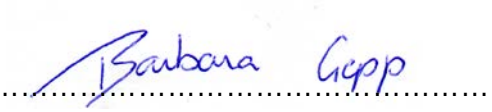
Wien, März 2016 

DI Barbara Gepp

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ABSTRACT

In Northern, Central, and Eastern Europe, the most allergenic pollen is produced by birch trees. Bet v 1 is the major birch pollen allergen and is recognized by serum IgE from more than 98% of birch pollen allergic patients. Seventy percent of birch pollen allergic individuals furthermore experience food allergies after eating fruits, vegetables, nuts, or legumes due to IgE-cross reactivity of Bet v 1 and homologous food allergens with high degrees of sequence similarity.

Knowledge of the B cell epitope distribution of Bet v 1 is important for the development of new concepts in diagnosis and for the design of safer allergen-based vaccines for allergen-specific immunotherapy (AIT). IgE epitopes of Bet v 1 are conformational and several methods have been applied to map such epitopes. However, the current knowledge of conformational B cell epitopes of Bet v 1 is far from complete.

Therefore, we designed four chimeric proteins by grafting four non-overlapping contiguous Bet v 1-specific surface areas onto the Bet v 1-related allergen from celeriac, Api g 1. The chimeras, Bet v 1 and Api g 1 were expressed in *Escherichia coli* and purified by chromatographic methods. These proteins were used to investigate the diversity of Bet v 1-specific IgE of 64 birch pollen allergic patients by ELISA. The minority of patients' sera (8%) showed binding to a single Bet v 1-specific surface area, whereas most of the sera recognized three (27%) or four (31%) Bet v 1-specific regions on the chimeras. A total of 12 different IgE recognition profiles with numbers of bound chimeras between 0 and 4 were observed. This indicates that the Bet v 1-specific IgE response is polyclonal, and the recognition profile is highly patient-specific. Furthermore, important IgE epitopes are distributed across the entire surface of Bet v 1 and are not limited to the P-loop.

The only specific and disease-modifying approach for the treatment of allergy is allergen-specific immunotherapy (AIT). During therapy, allergen-specific IgG antibodies are generated, which block the effect of disease-causing IgE antibodies. Although the behavior of immunoglobulins during AIT has been intensively studied, little is known about their epitope specificities.

Therefore, we investigated sera of 11 birch pollen allergic patients taken before, during, and after successful subcutaneous birch pollen immunotherapy. By performing ELISA experiments using our chimeras, we showed that the IgE and the early induced IgG1 and IgG4 repertoire remained unchanged during AIT in the majority of patients. The results for IgG4 were confirmed

by competitive immunoscreening of phage-displayed random peptides for 5 patients. Predicted IgG4 epitopes at 6 and 36 months of birch pollen immunotherapy overlapped by 37-63%.

Interestingly, we revealed by ELISA experiments that IgE of 7 sera bound to all 4 chimeras. However, only 2 showed IgG1 and 3 sera showed IgG4 binding to all chimeras. This indicates that mostly likely not all IgE binding epitopes could be blocked by induced IgG1 or IgG4 by direct epitope competition. Furthermore, we tested five immunotherapy-treated patients' sera for their activity to reduce the binding of Bet v 1-IgE complexes to CD23 on B cells. Notably, four of five sera showed 86-98% inhibition already after 6 months of AIT.

In summary, IgE is more diverse than IgG1 or IgG4 but still a high reduction of Bet v 1-IgE complex formation was observed already after 6 months of immunotherapy. This indicates that many mechanisms including inhibition of the IgE-facilitated allergen presentation and direct epitope competition of allergen-specific IgE and IgG antibodies synergistically lead to the improvement of allergic symptoms.

KEYWORDS

Bet v 1, Api g 1, chimeric proteins, IgE, IgG1, IgG4, epitope mapping, epitope repertoires, birch pollen immunotherapy

KURZFASSUNG

In Nord-, Mittel- und Osteuropa zählt die Birke zu den Hauptproduzenten von allergieauslösendem Pollen. Über 98% der Birkenpollenallergiker haben Serum-IgE gegen das Birkenpollen-Hauptallergen, Bet v 1. Weiters treten bei über 70% aller Birkenpollenallergiker nach dem Verzehr von Obst, Gemüse, Nüssen oder Hülsenfrüchten allergische Symptome auf. Ursache dafür ist die IgE-Kreuzreaktivität von Bet v 1 und homologen Nahrungsmittelallergenen mit hoher Sequenzähnlichkeit.

Das Wissen über die Verteilung von B-Zell-Epitopen auf der Oberfläche des Birkenpollen-Hauptallergens ist wichtig für die Entwicklung neuer Konzepte für die Diagnose und das Design von sicheren, auf Allergenen basierenden Vakzinen für die allergenspezifische Immuntherapie (AIT). IgE-Epitope von Bet v 1 sind Konformationsepitope und einige Methoden wurden angewandt, um diese zu charakterisieren. Das jetzige Wissen zu diesem Thema ist jedoch bei weitem nicht komplett.

Deswegen haben wir vier chimäre Proteine hergestellt, welche jeweils einen unterschiedlichen Bet v 1-spezifischen Oberflächenbereich besitzen. Diese Regionen wurden auf das Trägerprotein Api g 1, das Bet v 1-homologe Protein im Knollensellerie übertragen. Die chimären Proteine, Bet v 1 und Api g 1 wurden in *Escherichia coli* exprimiert und mittels chromatographischer Methoden gereinigt. Anschließend verwendeten wir die rekombinant hergestellten Proteine dazu, um die Diversität der IgE-Antwort auf Bet v 1 in 64 Birkenpollenallergikern mittels ELISA zu untersuchen.

Wir konnten zeigen, dass wenige Patientenseren (8%) eine spezifische IgE-Bindung an einen einzigen Oberflächenbereich von Bet v 1 zeigten, wogegen die meisten Seren drei (27%) oder alle vier (31%) Bet v 1-spezifischen Bereiche der Chimären erkannten. Insgesamt konnten zwölf verschiedene IgE-Erkennungsprofile beobachtet werden. Diese Ergebnisse zeigten, dass das Bet v 1-spezifische IgE-Epitoprepertoire polyklonal und sehr patientenspezifisch ist. Außerdem sind wichtige IgE-Epitope auf der gesamten Oberfläche von Bet v 1 verteilt und nicht auf die P-Loop Region beschränkt.

Die allergen spezifische Immuntherapie (AIT) ist bis heute die einzige Therapie, mit der allergischen Erkrankungen langfristig geheilt werden können. Während der Therapie entstehen IgG-Antikörper, welche die Wirkung von krankheitsauslösenden IgE-Antikörpern blockieren können. Obwohl die bei der AIT induzierten Immunglobuline bereits intensiv erforscht wurden, ist wenig darüber bekannt, ob sich die Spezifität der IgE- und IgG-Antikörper im Verlauf der Therapie verändert. Deswegen haben wir Serumproben von 11 Birkenpollenallergikern

untersucht, die vor, während und nach einer erfolgreichen subkutanen Birkenpollen-Immuntherapie entnommen wurden.

Durch ELISA-Experimente mit den Chimären konnten wir zeigen, dass sich die Epitop-Erkennung des allergen spezifischen IgE-, IgG1- und IgG4-Repertoires im Laufe der Therapie bei den meisten Patienten nicht verändert. Die IgG4-Ergebnisse konnten weiters durch kompetitives Immunscreening von an Phagen präsentierten Zufallspeptiden bestätigt werden. Bei den fünf getesteten Patienten wurde eine Überlappung der vorhergesagten IgG4-Epitope nach 6 und 36 Monaten Immuntherapie von 37-63% festgestellt.

Interessanterweise konnte außerdem durch ELISA-Experimente bei 7 Patientenseren die IgE-Bindung an alle vier chimären Proteinen detektiert werden. Im Gegensatz dazu wurde eine Binding von IgG1 und IgG4 an alle vier Chimäre nur bei 2 beziehungsweise 3 Seren beobachtet. Dies lässt die Schlussfolgerung zu, dass nicht alle IgE-Bindungsstellen von AIT-induzierten IgG-Antikörpern durch direkte Epitopkonkurrenz besetzt werden können. Außerdem haben wir mit fünf Patientenseren getestet, ob diese die Bindung von Bet v 1-IgE Komplexen an CD23 auf B-Zellen reduzieren können. Beeindruckend war das Ergebnis, dass 4 von 5 getesteten Seren bereits nach 6 Monaten Immuntherapie eine Inhibition von 86-98% aufwiesen.

Zusammenfassend zeigen Bet v 1-spezifische IgE Antikörper eine breitere Diversität als die bei der AIT induzierten IgG1 oder IgG4 Antikörper. Dennoch konnte eine starke Reduktion der Bet v 1-IgE Komplexbildung bereits nach 6 Monaten Immuntherapie beobachtet werden. Dies weist darauf hin, dass mehrere Mechanismen wie IgE-vermittelte Allergenpräsentation oder direkte Epitopkonkurrenz von IgE- und IgG-Antikörpern synergistisch zu der beobachteten Abschwächung der allergischen Symptome führen.

SCHLÜSSELWÖRTER

Bet v 1, Api g 1, chimäre Proteine, IgE, IgG1, IgG4, Epitopkartierung, Epitoprepertoire, Birkenpollen-Immuntherapie

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CHAPTER I:

General Introduction

1 IMMUNOLOGICAL BACKGROUND OF ALLERGY

1.1 The allergic response

The Austrian pediatrician Clemens von Pirquet first coined the term *allergy* in 1906 [1]. He described the unusual propensity of some individuals to develop symptoms or "hypersensitivity reactions" after exposure to certain substances. In 1963, Coombs and Gell described the four major types of allergic reactions based on their pathophysiology (Figure 1) [2].

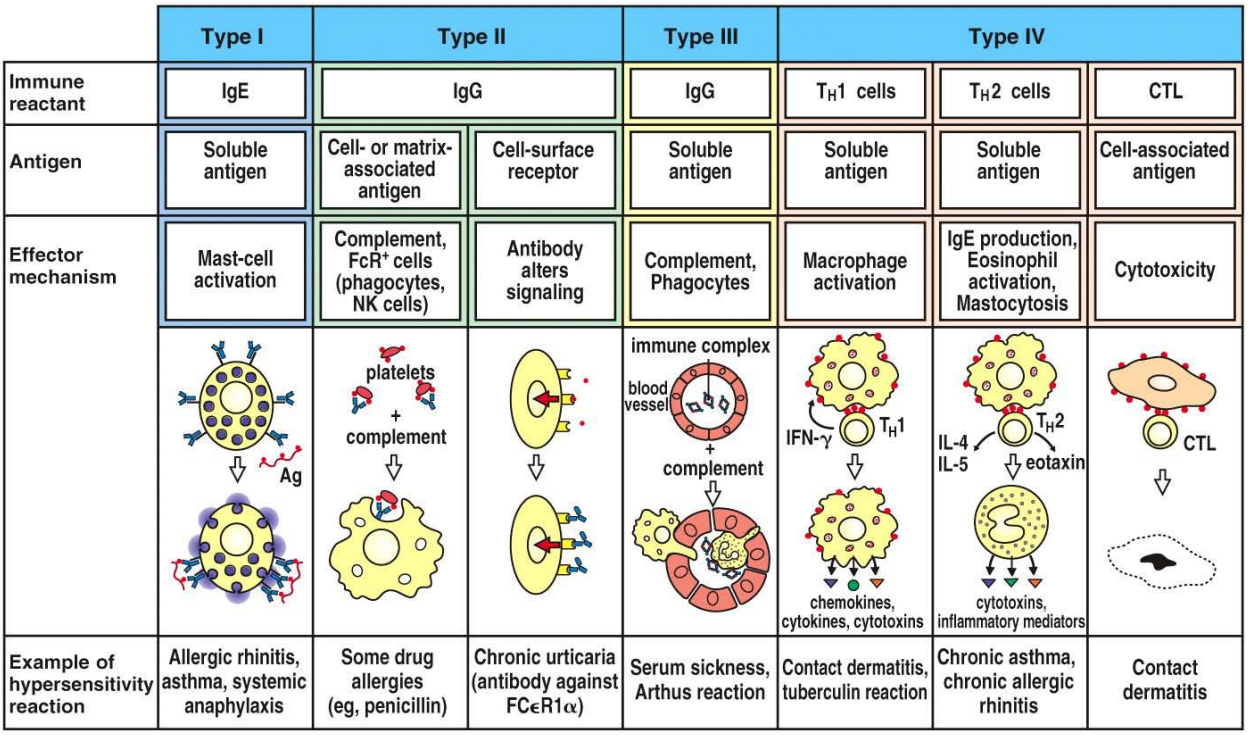

Figure 1 (Taken from Ref [3]): The four types of hypersensitivity reactions. Types I-III are antibody-mediated. Type I responses are immunoglobulin E (IgE)-mediated, whereas types II and III are mediated by IgG. Type II responses are directed against cell-surface or matrix antigens, whereas type III responses are directed against soluble antigens, and the tissue damage is caused by responses triggered by immune complexes. Type IV hypersensitivity reactions are T-cell mediated and can be subdivided into three groups.

However, the classical nomenclature for allergic reactions introduced by Gell and Coombs in 1963 has been regarded as not consistent with present knowledge. Hence, in 2001, a revised nomenclature for allergy was introduced by the EAACI nomenclature task force [4]. Very recently, Niggemann and Beyer suggested a new uniform grading system for allergic reactions [5].

1.2 Type I allergy

1.2.1 Sensitization

The primary effect that an allergen has on the immune system is called sensitization. In the case of pollen allergy, soluble allergens in the nanogram range are released from pollen on the mucosal surfaces of the respiratory tract. The airways are lined with a mucociliary blanket that is composed of airway epithelial cells which are connected by tight junctions [6]. This epithelial cell layer acts a barrier that excludes pathogens and inhaled antigens based on their molecular weight. In the airways, as well as in the gut, dendritic cells take up the allergen by extending their processes between epithelial cells directly into the lumen (Figure 2). This 'periscope' function is constitutively active in the airway mucosal dendritic cell population [6]. Dendritic cells are able to recognize antigens through expression of innate pathogen pattern recognition receptors, such as Toll-like receptors, C-type lectin receptors, and NOD-like receptors [7]. Dendritic cells take up, process and present antigens on their surface to promote the differentiation of CD4+ T cells to T helper 2 (Th2) cells during T-cell priming [8]. Hence, dendritic cells are central players in immune responses and essential to bridge innate and adaptive immunity.

However, the activation event can also occur indirectly through the activation of neighboring innate immune cells and epithelial cells [6]. Upon allergen stimulation, epithelial cells secrete thymic stromal lymphopoietin (TSLP), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 (IL-1), IL-25 and IL-33. The last two cytokines in turn have been shown to activate group 2 innate lymphoid cells (ILC2). ILC2s predominantly express IL-5, IL-9 and IL-13, and are likely involved in the early sensitization phase [9].

Th2 cells subsequently activate allergen-specific B cells by means of cognate T-cell-B-cell interactions (Figure 2). The immunoglobulin class switching of B cells to IgE is induced by the production of IL-4 and IL-13 by Th2 cells. The importance of IL-4 for class switching to IgE was shown by administration of an IL-4 antagonist during primary sensitization in mice, which resulted in a complete abrogation of the IgE response [10].

In the course of sensitization, allergen-specific long-lived memory T and B cells are generated which are strongly activated upon repeated allergen contact. Interestingly, allergen-specific IgE-producing B cells are present in the nasal [11] and bronchial mucosae [12], as well as in the peripheral blood of allergic patients.

The development of sensitization depends on many factors, including the host genotype, the type of allergen, the amount of allergen in the environment, and the exposure of the allergen

together with compounds which are able to enhance allergic sensitization (e.g. lipids [13], ligands of Toll-like receptors [14], chitin [15] and environmental pollutants [16]).

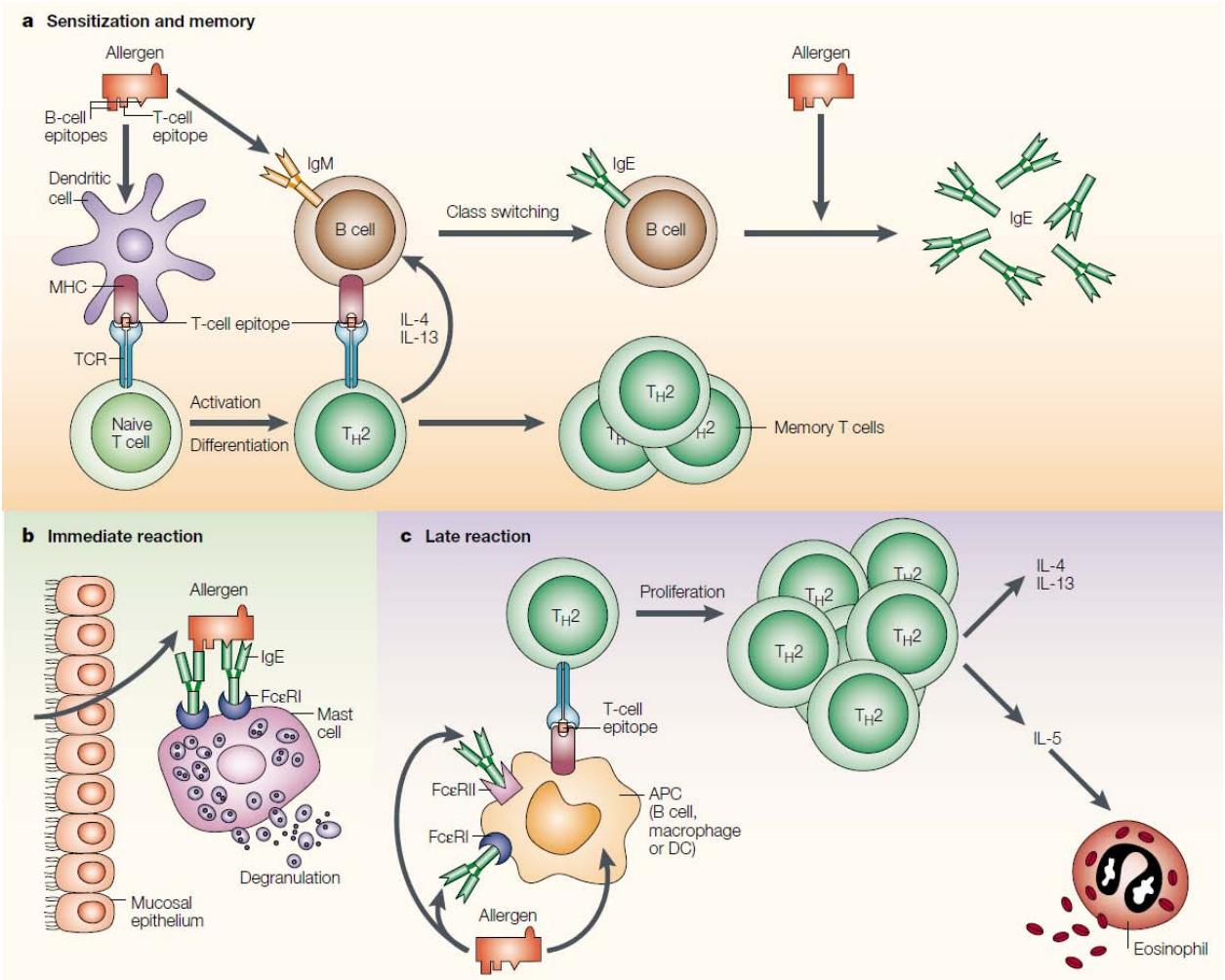

Figure 2 (Taken from Ref [17]): **Overview of the induction and maintenance of allergy. a)** Antigen-presenting cells take up the allergen and present it to naïve T cells (sensitization) or directly to T helper 2 cells. Cytokines such as IL-4 or IL-13 will be produced that favor immunoglobulin-class switching of specific B cells to IgE. Sensitization leads to the establishment of IgE^+ memory B cells and allergen-specific memory T cells. Subsequent repeated allergen contact will boost IgE^+ memory B cells. **b)** The cross-linking of effector-cell-bound IgE by allergens leads to the release of biologically active mediators (e.g. histamine) by means of degranulation. **c)** The late reaction is caused by the presentation of allergens to T cells, which become activated, proliferate and release proinflammatory cytokines (e.g. IL-4, IL-5, IL-13). This process might be enhanced by the IgE-mediated presentation of allergens to T cells. APC, antigen-presenting cell; DC, dendritic cell; TCR, T-cell receptor; MHC, Major histocompatibility complex.

1.2.2 The immediate phase reaction

IgE binds tightly to the high-affinity receptor FcεRI, which is present on mast cells and basophils (Figure 2) [18]. Cross-linking of adjacent IgE molecules by bivalent or multivalent allergens triggers a complex intracellular signaling process and leads to the degranulation of these cells and the release or the production of a range of inflammatory mediators, for example histamine, serine proteases, growth factors, leukotrienes, prostaglandins, and cytokines [19-24]. This, in turn, leads within minutes or even seconds to immediate allergic symptoms, such as rhinitis, conjunctivitis and asthma [25]. Furthermore, it was shown that higher levels of IgE upregulate the expression of FcεRI on mast cells and basophils.[26] Moreover, the survival of mast cells might be prolonged by prevention of apoptosis when monomeric IgE is bound to the cell surface [27, 28]. FcεRI was also shown to be expressed on other cell types such as epithelial cells [29], Langerhans cells [30], eosinophils [31], neutrophils [32] and thrombocytes [33].

1.2.3 The late phase reaction

Late phase reactions can be severe, especially in allergic individuals with chronic asthma or atopic dermatitis [17]. The clinical characteristics of the late-phase reactions are the local recruitment of Th2 cells, eosinophils, basophils and other leukocytes (Figure 2) [25]. The reactions occur after hours to days due to the activation of allergen-specific T cells or due to long-term consequences of the mediators released by mast cells during the early-phase reaction [24, 34, 35]. Mast-cell-derived products can have an impact on the biology of structural cells, including vascular endothelial cells, fibroblasts, epithelial cells, smooth muscle cells and nerve cells [20-22, 36-39].

1.3 Structure and function of immunoglobulins

Immunoglobulins consist of 2 heavy (H) and 2 light (L) chains (Figure 3), where the L chain can consist of either a κ or a λ chain. Each of the H and L chain contains one N-terminal variable domain (V). The H chains contain three or four C-terminal constant domains (C). H chains with 3 C domains contain a hinge region between the first (C_H1) and the second (C_H2) domain [40]. In the V region there are areas of increased variability called hypervariable regions or complementarity-determining regions (CDRs). Segments between the CDRs are invariable regions that are called framework residues. When the L and H chains are joined, the CDRs form a cleft that serves as the antigen-binding site. Therefore, mainly the amino acid sequences of the CDRs determine the shape and ionic properties of the antigen-binding site and define the specificity of the antibody [41].

Each type of antibody chain (κ light chains, λ light chains, and heavy chains) has a separate pool of gene segments and exons, which is located on a different chromosome and contains a large number of gene segments encoding the V region of an antibody chain. Each light-chain V region is encoded by a long variable (V) gene segment and a short joining (J) gene segment, whereas each heavy-chain V region is encoded by three gene segments, a V segment, a J segment, and a diversity (D) segment (Figure 3) [42]. Further diversity of the antibody repertoire arises from the insertion of non-coded nucleotides at the VDJ-junctions and the combination of initially created H chain diversity with diverse L chain V domain sequences. Moreover, antibody-encoding genes may diversify by somatic hypermutation following antigen encounter to select for antibodies with improved affinity for the antigen [43].

The paratope is the site of the immunoglobulin to which the antigen binds, whereas the epitope is the area on the antigen that is bound by the antibody. Immunoglobulins are mainly produced against intact antigens in soluble form and thus often bind to surface epitopes which represent conformational structures that are noncontiguous in the antigen's primary sequence. If antigens share similar or equivalent surface areas, the same antibody might bind to both of them, a phenomenon referred to as cross-reactivity [40].

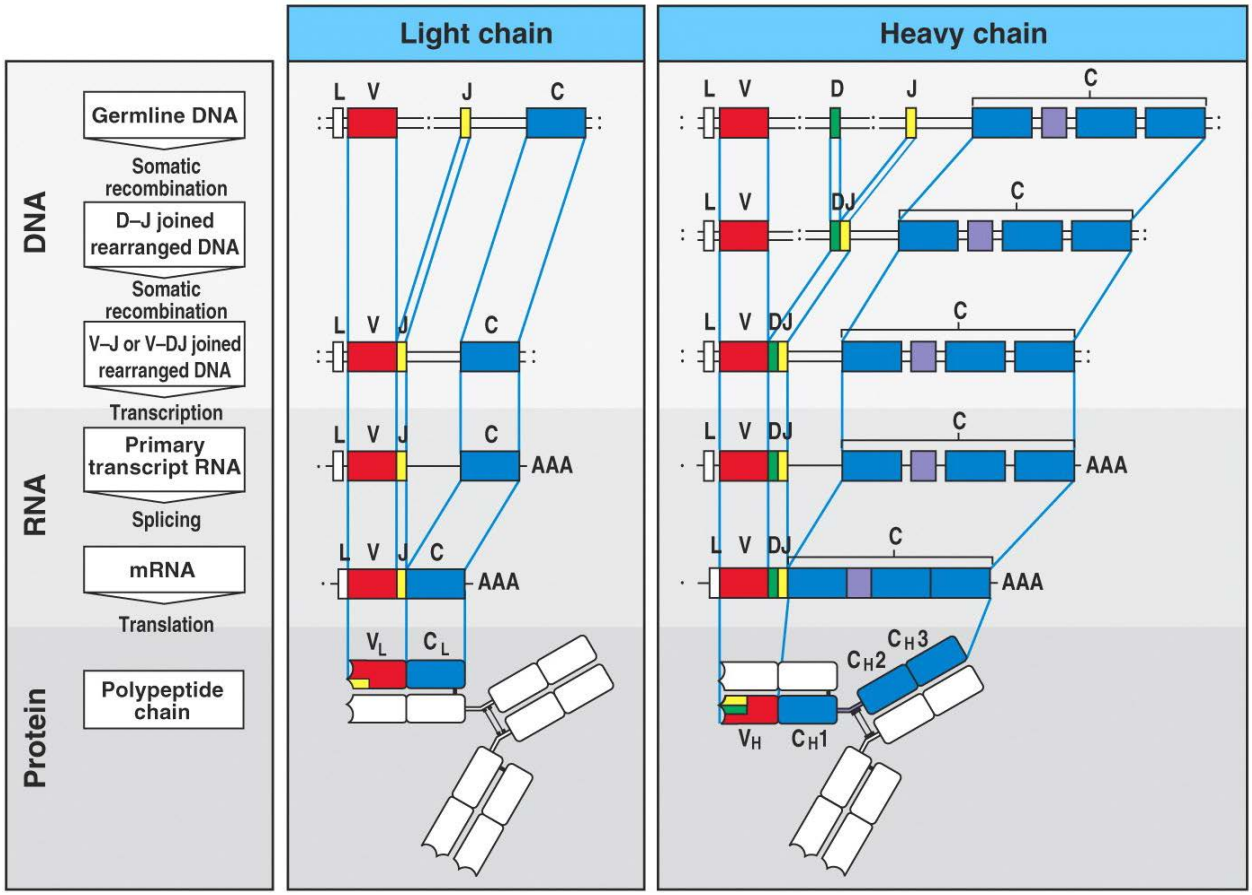

Figure 3 (Taken from Ref [3]): V-region genes are constructed from gene segments. Light-chain V-region genes are constructed from two segments. A variable (V) and a joining (J) gene segment are joined from a complete light-chain V-region exon. The V gene segment is preceded by an exon encoding a leader peptide (L), which directs the protein into the cell's secretory pathway and is then cleaved off. The light-chain C region is encoded in a separate exon and is joined to the V-region exon by splicing. Heavy-chain V regions are constructed from three gene segments. The diversity (D) and J gene segments join and then the V gene segment joins to the combined DJ sequence, forming the complete V_H exon. The heavy-chain C-region gene is encoded by several exons. The leader sequence is removed after translation and disulfide bonds are formed. The hinge region is shown in purple.

1.3.1 Immunoglobulins classes in humans

The five major classes of immunoglobulins in humans are IgM, IgD, IgG, IgA and IgE. They differ in their heavy chain structures and in their effector functions.

1.3.1.1 IgM

IgM is the first immunoglobulin expressed during B-cell development. Naïve B cells express monomeric IgM on their surface. Upon maturation and antigenic stimulation, multimeric IgM (usually pentameric) is secreted [40]. IgM has a mean serum level of 1.5 mg mL^{-1} and a half-life of 10 days in the serum [3]. The pentamer also contains a J-chain, which facilitates secretion at mucosal surfaces. IgM molecules have low affinity but high avidity due to their immaturity but pentameric nature. Therefore, IgM is very efficient in opsonizing antigens for destruction and complement fixation [40]. IgM is also associated with the primary immune response and therefore frequently used to diagnose acute exposure to an immunogen or pathogen [40].

1.3.1.2 IgD

In the serum, very low concentrations (0.04 mg mL^{-1}) of circulating IgD are found with a short half-life of 3 days [3]. This can be attributed to the sensitivity of the molecule, in particular the hinge region, to proteolysis [40]. It was suggested that a function of IgD is to deliver tolerogenic or apoptotic signals. Human B cells expressing more IgD than IgM show poor responsiveness to stimulation by antigens [44]. Furthermore, the fact that secreted IgD binds to respiratory antigens support the notion that secreted IgD enhances mucosal immunity. Secreted IgD also binds to an IgD receptor on basophils. In the presence of IgD cross-linking antigens, basophils migrate to systemic or mucosal lymphoid tissues, where they enhance immunity by releasing immunoactivating, proinflammatory and antimicrobial factors [44].

1.3.1.3 IgG

IgG is the most abundant isotype found in the body and has the longest serum half-life of 21 days for IgG1 and IgG4, 20 days for IgG2, and 7 days for IgG3 [3]. Based on the antigenic, structural, and functional differences in the constant region of the heavy chain, IgG was further subdivided. The subclasses were numbered according to the decreasing abundance in the blood of healthy individuals: IgG1 (9 mg mL^{-1}), IgG2 (3 mg mL^{-1}), IgG3 (1 mg mL^{-1}), and IgG4 (0.5 mg mL^{-1}) [3, 45]. Antibody flexibility and functional affinity of these subclasses is caused by the differences in the C_H domains. For clearance of opsonized pathogens, activation of the

complement cascade is essential. IgG4 is the only subclass which fails to fix complement. The affinity for C1q, the first component of the complement pathway which binds to the C_H2 domain of IgG, differs between the other IgG subclasses (IgG3 > IgG1 > IgG2). Furthermore, the IgG subclasses show differences in the affinity for the 3 FcγR classes. IgG1 and IgG3 bind to all 3 FcγR classes. IgG2 binds only to FcγRII. IgG4 binds to FcγRII and FcγRIII, although with significantly lower affinity than IgG1 [40].

IgG1 is primarily induced in antibody responses to soluble protein antigens and membrane proteins, mostly accompanied by low levels of IgG3 and IgG4 [46]. IgG2 is the most important antibody response to bacterial capsular polysaccharide antigens [46-49]. IgG3 is a very potent pro-inflammatory antibody and particular efficient in the induction of effector functions. Its shorter half-life may function in limiting the danger of excessive inflammatory responses [50]. IgG4 is often induced following repeated or long-term exposure to antigens in a non-infections setting and may become the dominant subclass. It is often induced by allergens in addition to IgE and IgG1. Antigen-specific IgG4 levels are high, for example, in bee keepers or in allergic individuals who have undergone immunotherapy [51-54].

1.3.1.4 IgA

IgA has a serum half-life of 6 days and the mean serum IgA levels (2.1 mg mL^{-1}) are considerably lower than the IgG levels [3, 55]. However, at mucosal surfaces and in secretions like saliva and breast milk, IgA levels are much higher than IgG levels [56]. Serum IgA is generally monomeric, however, IgA in the mucosa is a dimer, termed secretory IgA. The function of IgA is to protect mucosal surfaces from viruses, toxins, and bacteria by direct neutralization or prevention of binding to the mucosa [40].

1.3.1.5 IgE

IgE has the shortest half-life of approximately 2 days of all immunoglobulins and is present in very low concentrations (3×10^{-5} mg mL^{-1}) in the serum [3, 40]. The major part of IgE is bound by the FcεRI on mast cells and basophils with extremely high affinity, where it is present for the lifetime of the cell [40, 57]. In the absence of disease, the expression of IgE is tightly regulated. IgE levels increase throughout childhood with a peak between 10 to 15 years of age and decline throughout adulthood. IgE is associated with hypersensitivity and allergic reactions, but also with parasitic worm infections [40, 58].

2 BIRCH POLLEN ALLERGY

In Northern, Central, and Eastern Europe, the most allergenic tree pollen is produced by birch (*Betula*) [59]. Bet v 1 is the major birch pollen allergen and is recognized by serum IgE from more than 98% of Austrian, Finish or Swedish birch pollen allergic patients [60]. In Central and Eastern Europe, the main flowering period of birch trees usually starts from the beginning to mid-April. The pollen concentration in the air reaches its peak 1-3 weeks after the start of the season. The duration of the birch pollen season varies between 2 to 8 weeks, depending on the temperature. The amount of pollen production also varies by year [59].

Pollen is part of the male reproductive system of gymnosperms and angiosperms [61]. Most allergenic pollens are generated by wind-pollinated plants and have several features that have a major impact on allergy. The pollen grains are usually small with diameters ranging from 20-40 µm and can be distributed over a long distance. To increase the efficiency of fertilization, pollen is released in high quantities [61]. Furthermore, pollen grains are dry and require hydration to initiate germination [62]. Climate change has a major effect on airborne pollen, since the growth rate, the amount of pollen per plant, and the amount of protein per pollen grain has steadily increased with the rise in ambient temperature [63, 64]. Bet v 1 was shown to be mainly localized in the cytoplasm in close proximity to ribosome-rich regions. Upon rehydration, Bet v 1 is released within minutes from apertures in the pollen grain and is subsequently found on the whole pollen surface [65, 66].

2.1 The ubiquitous PR-10 family of plant pathogenesis-related proteins

In 1989, the cloning of Bet v 1.0101 was published and it turned out that its sequence was similar to a pea *pathogenesis-related proteins 10* (PR-10) [67]. Later, a variety of other Bet v 1 isoallergens only differing by a few amino acids were described to be present in birch pollen [68]. Currently, 27 Bet v 1 isoallergens are listed in the WHO/IUIS Allergen Nomenclature database (www.allergen.org;[69]): Bet v 1.0101-Bet v 1.0119, Bet v 1.0201-Bet v 1.0207 and Bet v 1.0301.

Birch belongs to the order Fagales [70]. Besides Bet v 1, other major Fagales pollen allergens, e.g. Aln g 1 (alder), Car b 1 (hornbeam), Ost c 1 (hop-hornbeam), Cor a 1 (hazel), Fag s 1 (beech), Cas s 1 (chestnut), and Que a 1 (oak) also belong to the PR-10 family and, therefore, show cross-reactivity (Figure 4) [70, 71].

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          1                                                   50
Bet_v_1   GVFNYETETT SVIPAARLFK AFILDGDNLF PKVAPQAISS VENIEGNGGP
Aln_g_1   GVFNYEAETP SVIPAARLFK AFILDGDKLL PKVAPEAVSS VENIEGNGGP
Car_b_1   GVFNYEAETP SVIPAARLFK SYVLDGDKLI PKVAPQVISS VENVGGNGGP
Ost_c_1   GVFNYEAETP SVIPAARLFK SYVLDGDKLI PKVAPQAISK VENVGGNGGP
Cor_a_1   GVFNYEVETP SVIPAARLFK SYVLDGDKLI PKVAPQAITS VENVEGNGGP
Fag_s_1   GVFTYESETT TVITPARLFK AFVLDADNLI PKVAPQAIKS SEIIEGSGGP
Cas_s_1   ~~~~~~~~ET SVIPPARLFK AFVLDSDNLI PKVLPQAIKS TEIIEGNGGP
Que_a_1   GVFTYESEDA SVIPPARLFK AFVLDSDNLI PKVVPQALKS TEIIEGNGGP

          51                                                  100
Bet_v_1   GTIKKISFPE GFPFKYVKDR VDEVDHTNFK YNYSVIEGGP IGDTLEKISN
Aln_g_1   GTIKKITFPE GSPFKYVKER VDEVDRVNFK YSFSVIEGGA VGDALEKVCN
Car_b_1   GTIKNITFAE GIPFKFVKER VDEVDNANFK YNYTVIEGDV LGDKLEKVSH
Ost_c_1   GTIKNITFAE GSPFKFVKER VDEVDNANFK YNYTVIEGDV LGDKLEKVSH
Cor_a_1   GTIKNITFGE GSRYKYVKER VDEVDNTNFT YSYTVIEGDV LGDKLEKVCH
Fag_s_1   GTIKKITFGE GSQFNYVKHR IDEIDNANFT YACTLIEGDA ISETLEKIAY
Cas_s_1   GTIKKITFGE ASKYKYSKHR IDALDPENCT YSFSVIEGDV LT.DIENVST
Que_a_1   GTIKKITFGE GSHLKHAKHR IDVIDPENFT YSFSVIEGDA LFDKLENVST

          101                                                 150
Bet_v_1   EIKIVATPDG GSILKISNKY HTKGDHEVKA EQVKASKEMG ETLLRAVESY
Aln_g_1   EIKIVAAPDG GSILKISNKF HTKGDHEINA EQIKIEKEKA VGLLKAVESY
Car_b_1   ELKIVAAPGG GSIVKISSKF HAKGYHEVNA EKMKGAKEMA EKLLRAVESY
Ost_c_1   ELKIVAAPGG GSILKISSKF HAKGDHEVNA EEMKGAKEMA EKLLRAVESY
Cor_a_1   ELKIVAAPGG GSILKISSKF HAKGDHEINA EEMKGAKEMA EKLLRAVETY
Fag_s_1   EIKLVASPDG GSILKSTSKY HTKGDHEIKE DQIKAGKEEA SGIFKAVEAY
Cas_s_1   ETKFVASPDG GTIMKSTTKY QTKGDFQLKE EQVQAAIEKA TGLFKAVEAY
Que_a_1   ETKIVASPDG GSIVKSTSKY QTKGDFQLTD ELIRGGKEKA SEVFKAVEAY

          151
Bet_v_1   LLAHSDAYN
Aln_g_1   LLAHSDAYN
Car_b_1   LLAHTAEYN
Ost_c_1   LLAH~~~~~
Cor_a_1   LLAHSAEYN
Fag_s_1   LLAN~~~~~
Cas_s_1   LLAN~~~~~
Que_a_1   LVA~~~~~~
```

Figure 4: Multiple sequence alignment of Bet v 1 and other major Fagales pollen allergens. The alignment was generated using Clustal Omega (1.2.1) [72].

Furthermore, PR-10 family members are present in many foods, such as fruits of Rosaceae (e.g. Mal d 1 in apple [73], Pru av 1 in cherry [74], Pyr c 1 in pear [75]), vegetables of Apiaceae (e.g. Api g 1 in celeriac [76], Dau c 1 in carrot [77]), seeds of the Fabaceae (Gly m 4 in soybean [78], Vig r 1 in mungbean [79], and Ara h 8 in peanut [80] as well as in tree nuts (Cor a 1 in hazelnut [81]). These proteins share high degrees of sequence similarities with Bet v 1 and, therefore, are the main causes of pollen-related food allergies, which occur in more than 70% of birch pollen allergic individuals (Figure 5) [82]. Bet v 1-specific IgE antibodies are able to bind to these dietary proteins and thus cause allergic reactions predominantly in the oropharynx, termed the oral allergy syndrome (OAS) [83].

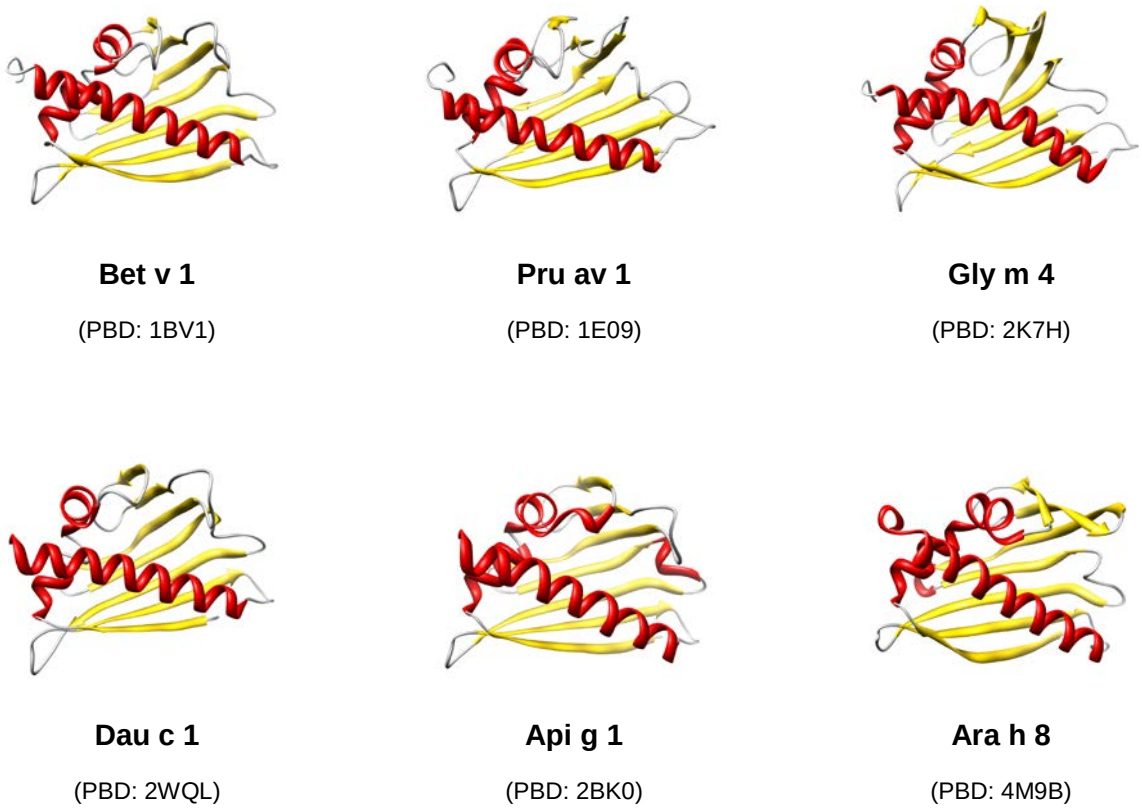


Figure 5: **Ribbon representations of known crystal structures of Bet v 1 and cross-reactive Bet v 1-homologues from plant foods.** Ribbon models were generated by using UCSF Chimera [84].

The PR-10 family is ubiquitously distributed among dicotyledonous plants [71] and also present in monocots [85] and gymnosperms [86]. In contrast to most PR families, PR-10 proteins are primarily cytosolic [87, 88]. They are acidic proteins of 15-18 kDa and were first discovered in peas and parsley [89, 90]. PR-10 proteins are members of small gene families of differentially expressed isoforms. In white birch, at least 13 gene loci were found, seven of which encoded a mixture of Bet v 1 isoforms expressed specifically in pollen [91]. Until now, the role of PR-10 proteins is poorly understood but it is believed that they have a protective role since they are expressed when plants encounter environmental or pathogenic stress. However, some PR-10 proteins are also constitutively expressed in high amounts in certain tissues such as pollens, fruits, seeds, or tubers [92]. Although their role in the plant cell is not known, their conserved sequence motifs and their distribution throughout the plant kingdom suggest that they fulfill an indispensable function [92]. Recently, it has been argued that the role of Bet v 1 and its isoforms in pollen is to protect pollen DNA from UV-damage, and that the mixture of different isoforms was suggested to provide an individual fingerprint to prevent self-pollination [93, 94].

The PR-10 family is a member of a large family of plant proteins termed the Bet v 1 family [88]. In recent years, a number of diverse plant proteins with low sequence similarities to Bet v 1 were identified. A classification by sequence similarity yielded several subfamilies related to the PR-10 subfamily [88]. For example, the sequence of a cytokinin-specific binding protein (CSBP) from mung bean showed 31% identity and 45% similarity to Bet v 1 [95]. (S)-Norcoclaurine synthases (NCS) from poppy and meadow rue (*Thalictrum flavum*) displayed sequence identities to PR-10 proteins between 28% and 38% [96]. Even more distantly related to the PR-10 subfamily are the major latex proteins/ripening-related proteins, whose members were first identified in the latex of opium poppy [97] and later found in ripe fruits such as strawberry and cucumber [98].

2.2 Bet v 1-like superfamily

The Bet v 1 family is part of the Bet v 1-like superfamily, also termed StAR-related superfamily after the lipid binding domain of the steroidogenic acute regulatory protein (StAR), a mammalian steroid binding protein. The Bet v 1-like superfamily is currently divided into 14 families in version 29.0 of the Pfam protein family database (pfam.xfam.org [99]). These families show no significant sequence similarities but they share the typical Bet v 1 fold. Members of the Bet v 1-like superfamily are found in all three domains of life, archaea, bacteria, and eukaryotes [88]. Their biochemical functions cover a wide spectrum mostly involving binding of plant hormones, lipids, antibiotics and other larger ligands.

2.3 Structure of Bet v 1

The typical Bet v 1 fold has the secondary structure arrangement β-α_2-β_6-α with a seven-stranded anti-parallel β-sheet wrapped around a 25 residues long C-terminal α-helix. The most striking feature of the Bet v 1 structure is the presence of a forked hydrophobic cavity which is 30 Å long and has a volume of approximately 1500 $Å^3$ [100]. It is located between residues lining the interior surface of the β-sheet and the three α-helices, and functions as ligand-binding site [101, 102]. The cavity contains only a few residues with side chains that can be charged or that can form hydrogen bonds [100].

Amino acid sequence alignments of Bet v 1 and other Fagales pollen allergens and PR-proteins revealed another particular feature which is the clustering of surface-exposed conserved polar residues in close vicinity to the cavity. A glycine-rich loop formed by four conserved glycine residues, 46, 48, 49 and 51, connects the β-strands 2 and 3 [100]. The sequence of this loop shows similarity to the P-loop motif (-G-X-G-G-X-G-), found in many nucleotide binding proteins

[103]. Indeed, RNase activity was the first biochemical activity proposed for PR-10 proteins. It was described first for PR-10 proteins from ginseng [104] and later also for other PR-10 family members including Bet v 1 [105-107]. However, ribonucleolytic activity has been detected only in a few PR-10 proteins and the biological significance of the in vitro RNase activity remains controversial [92].

2.4 Ligand binding of Bet v 1

The similarities of PR-10 proteins with the StAR domain, which is associated with the transfer of lipids [108], prompted researches to search for ligands fitting into the hydrophobic cavity of Bet v 1 and its homologues. First, it was shown that Pru av 1, the Bet v 1 homologue from cherry, interacts with the plant steroid hormones brassinosteroids [109].

Later, Mogensen et al. [110] revealed that Bet v 1 is promiscuous with respect to ligand binding activity because the protein is able to bind a wide range of fatty acids, flavonoids, cytokinins and sterols with moderate to high affinity. It was suggested that Bet v 1 contains two ligand binding sites. The authors further speculated that Bet v 1 might play a role in transporting lipids and flavonoids to the stigmatic surface, which may be necessary for proper hydration and germination.

A hypoallergenic isoallergen of Bet v 1 was crystallized in complex with deoxycholate, a compound with is not found as a metabolite in plants [101]. However, deoxycholate shares striking structural similarity with brassinosteroids, a family of ubiquitous plant steroid hormones which have been already shown to bind to Pru av 1 [109]. Kofler et al. [111] crystallized Bet v 1 variants in complex with an array of ligands. They identified different binding modes of Bet v 1 depending on the isoforms or the presence of other ligands. It was furthermore reported that ligand binding to Bet v 1 generally increased the volume of the hydrophobic pocket, hence altering the protein surface [112].

In 2014, Seutter von Loetzen et al. discovered the physiological ligand of Bet v 1, the glycosylated flavonol quercetin-3-O-sophoroside (Q3OS) [93]. Flavonoids, which are a main class of pigments for coloration in plants [113] act as signals for pollinators, participate in the facilitation of pollen tube germination, are involved in plant hormone signaling, and protect plants from UV radiation [114, 115]. The authors speculate that flavonoids are stored as glycosylated precursors and probably are converted into its active deglycosylated form during rehydration. The lipid binding capacity of Bet v 1 may play an important role in this process because Q3OS binding to the hydrophobic cavity might be displaced by lipids and thus make it accessible for deglycosylation.

3 B CELL EPITOPES OF BET V 1-RELATED ALLERGENS

In 1984, Benjamin et al. [116] described that the whole surface of a protein was antigenic, but this did not mean that every part of the surface was equally important. Some epitopes were argued to be recognized much more often than others. Especially, information about B cell epitopes could help to elucidate special features of the IgE immune response and could be translated into the improvement of prevention and treatment of allergy [117]. Furthermore, knowledge of an allergen's epitopes is important to predict cross-reactivity [117]. In principle, if the IgE binding epitopes of an allergen are known, it should be possible to determine which proteins will cross-reactive with a given allergen. Moreover, prediction of potential cross-reactivity is essential for the risk assessment of novel foods. Until now, this prediction is based rather on sequence similarity than on known epitopes. In addition, knowledge about B cell epitopes of major allergens is crucial for the design of safer vaccines. Based on these data, new hypoallergenic variants of an allergen can be developed to provide a patient-tailored specific immunotherapy with reduced anaphylactic side effects [118].

IgE antibodies may bind to continuous stretches of the primary sequence or to discontinuous epitopes of an allergen, meaning that surface residues belonging to the binding region come into close proximity due to the three-dimensional folding of the protein [119]. IgE binding epitopes of Bet v 1 are conformation-dependent, which was, among others, shown by Vrtala et al. [120]. In this study, the three-dimensional structure of Bet v 1 was disrupted by expressing it in two parts containing the start methionine plus amino acid residues 1-74 and 75-159 of the mature protein. Both fragments were unable to bind IgE. For this reason, overlapping peptides containing linear epitopes cannot be applied for mapping of the conformational B cell epitopes of Bet v 1.

3.1 IgE epitope mapping using Bet v 1 isoforms

Detection of IgE-binding to Bet v 1 isoforms, only differing in few amino acids, was one of the first attempts to characterize B cell epitopes on Bet v 1 for polyclonal IgE antibodies (Figure 6) [121]. Surprisingly, IgE-binding properties to the nine examined isoforms showed striking differences. Interestingly, all 30 birch pollen-allergic patients tested revealed the same binding patterns. According to the results, the Bet v 1 isoforms were grouped into high IgE-binding proteins (isoforms a, e and j), proteins with intermediate IgE-binding capacity (isoforms b, c and f) and low/non IgE-binders (isoforms d, g and l).

Similar results were obtained by Wagner et al. [122], who confirmed that Bet v 1.0102 (formerly Bet v 1d) and Bet v 1.0107 (formerly Bet v 1l) induced only a minimal IgE response in contrast to Bet v 1.0101 (former Bet v 1a), which most likely acts as the sensitizing agent.

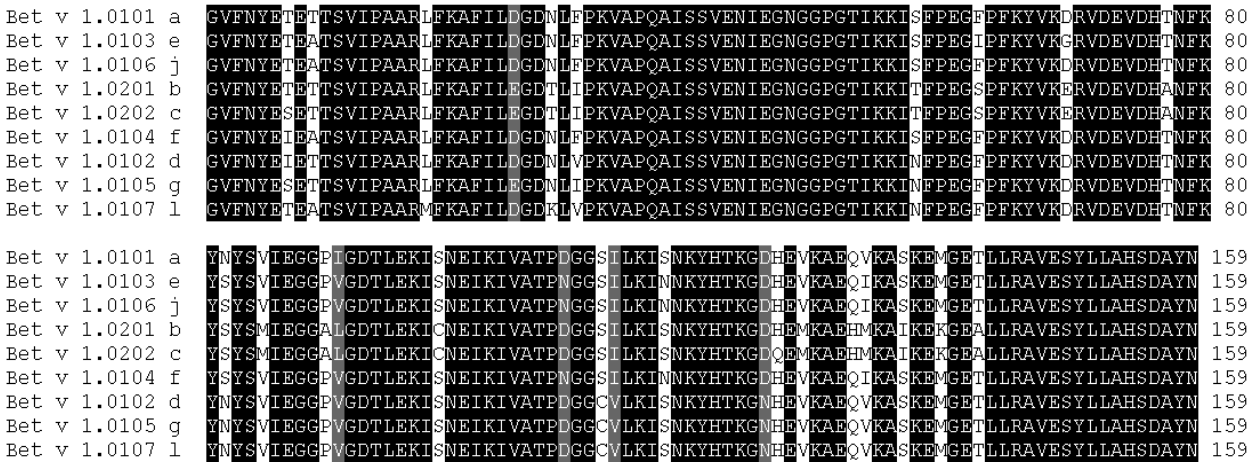


Figure 6: **Multiple sequence alignment of Bet v 1 isoforms**. The alignment was generated by using the Sequence Manipulation Suite [123]. Residues identical in all sequences are shaded black, position containing conservative exchanges are shaded grey.

3.2 IgE epitope mapping using site-directed mutagenesis

Another approach for the determination of IgE-binding epitopes on the surface of Bet v 1 was performed by Ferreira et al. using site-directed mutagenesis [124]. The point mutations Thr10Pro, Phe30Val, Ser57Asn, Ser112Cys, Ile113Val, and Asp125Asn were introduced into the sequence of Bet v 1 (Figure 7). This design was based on sequence comparison of Cor a 1 and the high and low IgE binding isoforms of Bet v 1 (Figure 6). The resulting molecule was recognized by allergen-specific T cells but displayed a significantly reduced IgE binding capacity. By testing each of the six single mutants, a reduction of IgE binding was observed for some but not all patients, which illustrates the diversity of individual patients' IgE antibodies.

In a similar study performed by Ma et al. [125], the same 6 point mutations were introduced into the sequence of Mal d 1, the Bet v 1-homologue from apple. The integrity of the secondary structure of the resulting recombinant protein was confirmed. The mutant showed a reduced capacity to bind specific IgE as compared to the wild-type protein.

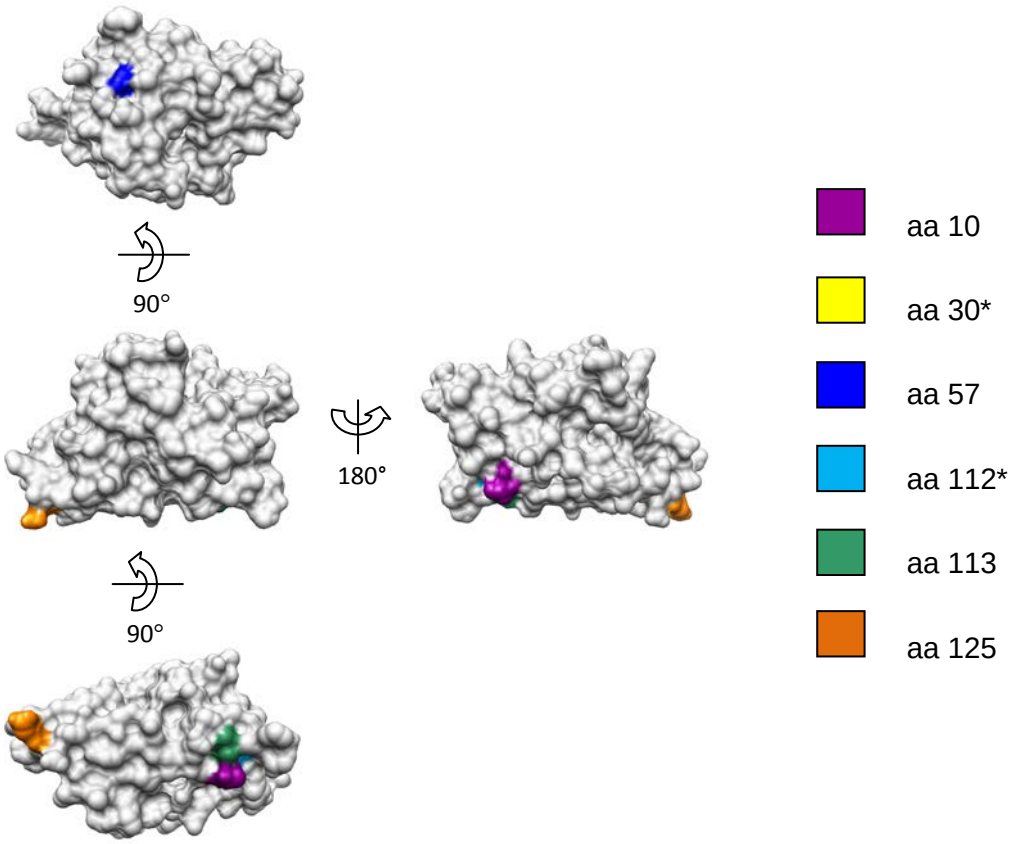


Figure 7: **Mutated amino acid residues in the studies of Ferreira et al. [124] and Ma et al. [125].** Bet v 1.0112 (PDB: 1BV1) showing the six amino acid residues (aa) that were mutated. *Not visible because not surface-exposed. The models were prepared with UCSF Chimera [84].

Spangfort et al. [126] produced a series of mutations in surface-exposed amino acid residues expected to be important for binding of the monoclonal antibody BV16 to Bet v 1.0112 (Figure 17; page 26). These Bet v 1 mutants have mutations at positions Glu45Ser, Pro108Gly and Asn28Thr + Lys32Gln (double mutant), respectively (Figure 9). Glu45 is located one residue N-terminally from the highly conserved P-loop of Bet v 1 (amino acid residues 46-52; Figure 8). IgE binding to the Glu45Ser mutant was significantly reduced for all individual sera and for a serum pool. Mutations located in surface areas conserved throughout the Fagales group 1 allergens, Asn28Thr + Lys32Gln, Glu45Ser and Pro108Gly, reduced binding of the serum pool IgE. The mutation Glu60Ser, although conserved throughout the Fagales, and Asn47Ser did not significantly reduce IgE binding.

Figure 8: Multiple sequence alignment of Bet v 1 and homologous proteins. The sequences of Bet v 1.0101 (high IgE-binding isoform), Bet v 1.0102 (low IgE-binding isoform), Pru av 1.0101, Mal d 1.0101, Api g 1.0101 (high IgE-binding isoform) and Api g 1.0201 (low IgE-binding isoform) were aligned. The P-loop (amino acids 46-52) is boxed. The alignment was generated using Clustal Omega (1.2.1) [72].

Wiche et al. [127] generated the monoclonal antibody mP16, which reacted with Bet v 1.0101, Pru av 1, Mal d 1, and Cor a 1.0401 (hazelnut). It did not bind to Bet v 1.0107, Cor a 1.0101 (pollen), Dau c 1.0104, Api g 1.0101, Api g 1.0201, and the hypoallergenic Bet v 1 mutant described by Spangfort et al. [126]. Sequence alignment revealed that the two amino acids Asn28 and Pro108 seemed to be critical for the binding of the monoclonal antibody to Pru av 1 (Figure 9). Reduced binding of patients' IgE was observed for a Pru av 1 Asn28Lys mutant and the double mutant Asn28Lys/Pro108Ala compared to the wildtype protein. However, the difference of IgE binding to the Pru av 1 wildtype and the Pru av 1 Pro108Ala mutant was not significant.

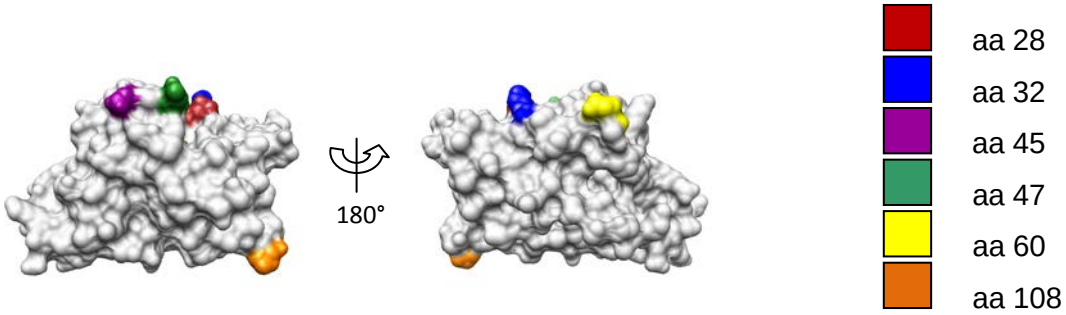


Figure 9: **Mutated amino acid residues in the studies of Spangfort et al. [126] and Wiche et al. [127].** Bet v 1.0112 (PDB: 1BV1) showing the amino acid residues (aa) that were mutated in the studies of Spangfort et al. (aa 28, 32, 45, 47, 60, and 108) and Wiche et al. (aa 28, 108). The models were prepared with UCSF Chimera [84].

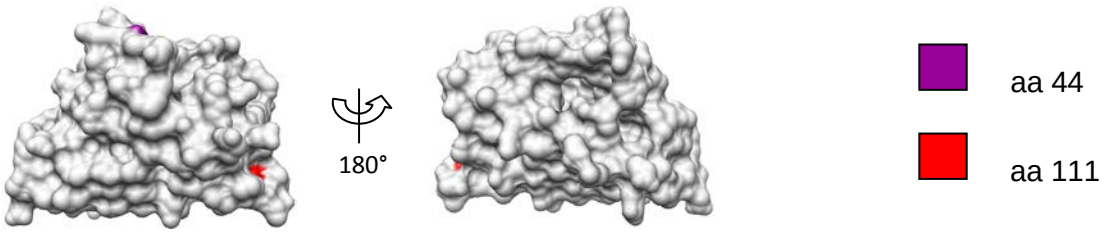
The importance of the P-loop region as a major IgE-binding epitope for Bet v 1-specific IgE was moreover confirmed by studies of Scheurer et al. [128] and Neudecker et al. [129]. All Pru av 1 mutants with the single amino acid substitutions Glu45Trp, Gly46Pro and the amino acid deletion Thr52 revealed a lower IgE binding capacity for IgE from a subgroup of allergic patients (Figure 10).

Point mutation analysis of two isoforms of the Bet v 1-homologue in celeriac, Api g 1, was performed by Wangorsch et al. [130]. The two isoforms Api g 1.0101 and Api g 1.0201 differ in their amino acid sequence in the region of P-loop (Figure 8). Api g 1.0101 has a gap at position 44 and the following amino acid residues is a positively charged lysine, whereas Api g 1.0201 has, similar to Bet v 1, a negatively charged glutamic acid residue at the corresponding position. Despite this fact, the IgE-binding frequency and capacity of Api g 1.0101 were clearly higher than those of Api g 1.0201. The higher IgE-binding capacity of Api g 1.0101 could be further increased by substitution of Lys for Glu at position 44 (position 45 in Bet v 1).

The same substitution of Lys44 in Api g 1.0101 by Glu was investigated by Neudecker et al. (Figure 10) [129]. One third of the patients' sera showed clearly enhanced IgE binding to the mutant, one third revealed reduced IgE binding to the mutant compared to the wildtype and IgE binding of the remaining third of the sera was unaffected.

Api g 1.0101 compared to Bet v 1, Pru av 1 and Api g 1.0201 is shorter at the C-terminus by five amino acid residues (Figure 8). To investigate Bet v 1-specific IgE binding to the C-terminus, a Pru av 1 mutant lacking amino acids 155-159 was produced [129]. However, IgE binding to the mutant was comparable to the wildtype protein.

Api g 1:



Pru av 1:

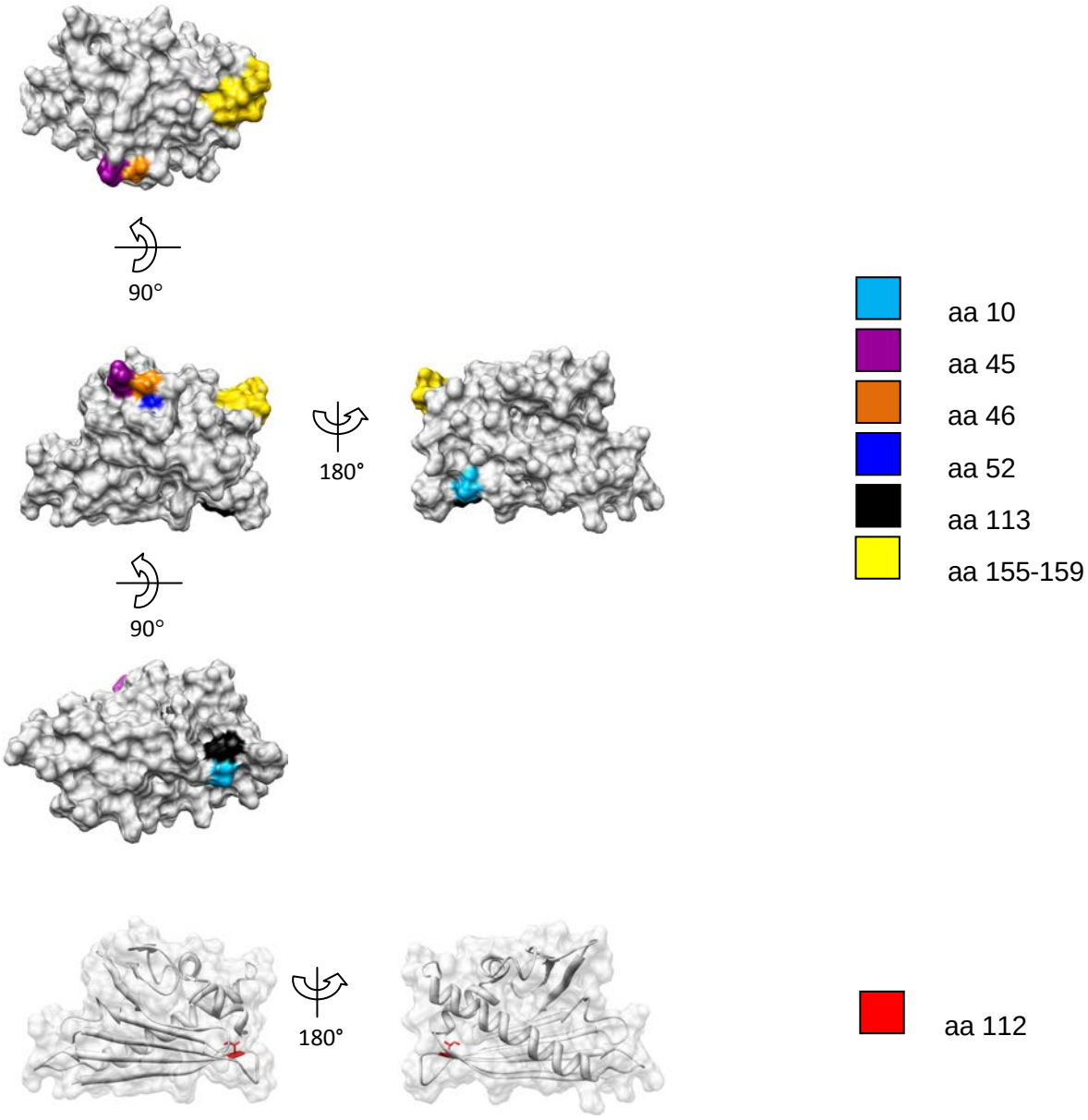


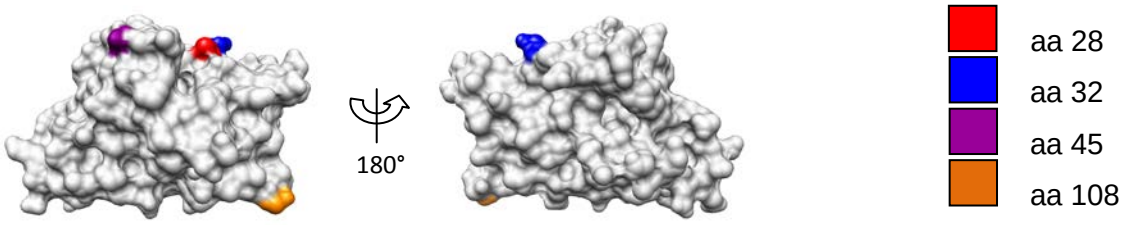
Figure 10: Mutated amino acid residues in the studies of Wangorsch et al. [130], Neudecker et al. [129] and Scheurer et al. [128]. Api g 1.0101 (PDB: 2BK0) and Pru av 1.0101 (PDB: 1E09) showing the amino acid residues (aa) mutated in the studies of Wangorsch et al. (aa 44, aa 111 of Api g 1), Neudecker et al. (aa 45, 112, 155-159 of Pru av 1 and aa 44 of Api g 1), and Scheurer et al. (aa 10, 46, 52, 112, 113 of Pru av 1). The models were prepared with UCSF Chimera [84].

The amino acid at position 112 differs between Bet v 1.0101 and the low IgE binding isoform Bet v 1.0102 (Figure 8). Therefore, this position was selected for mutagenesis in Bet v 1 (Ser112), Mal d 1 (Ser111) [131], Pru av 1 (Ser112) [128, 129] as well as in Api g 1.0101 (Ser111) and Api g 1.0201 (Ser111) [130]. In all cases, the exchange of serine to proline destabilized the structure and led, with the exception of Api g 1.0201, to decreased IgE binding capacities of these mutants. The exchange of Ser112 to Ala in Pru av 1, however, exhibited an IgE-binding capacity similar to that of the wildtype protein [129]. Substitution of the adjacent amino acid Ile113 in Pru av 1 also did not have an effect on the IgE binding capacity (Figure 10) [128].

In Pru av 1, the amino acid position 10 was furthermore investigated by Scheurer et al. [128]. It was proposed to be a putative important residue for the IgE binding of the major hazel allergen, Cor a 1 [132]. Pru av 1 and Cor a 1 have a high degree of sequence identity (58%), however, no reduction of IgE binding was observed when Thr10 was changed to Pro in Pru av 1 (Figure 10).

Based on the results of Spangfort et al. [126] (Figure 9), two genetically engineered forms of Bet v 1 were designed which harbored either four or nine point mutations located on their surface (Figure 11) [133]. The rationale was to change the surface topography of three (four point mutant) or five (nine point mutant) different surface areas to reduce IgE binding but retain the correct folding of the protein. Both mutants exhibited a reduced binding of patients' pooled specific serum IgE. The four point mutant was furthermore crystallized (PDB: 1QMR) and the overall structure was comparable with the structure of the native Bet v 1.

Four point mutant:



Nine point mutant:

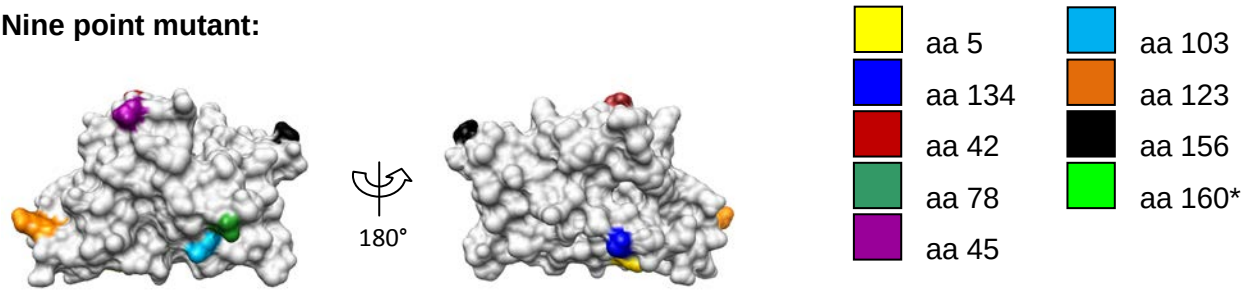


Figure 11: Mutated amino acid residues in the study of Holm et al. (2004) [133]. The four point mutant of Bet v 1.0112 (PDB: 1QMR) and Bet v 1.0112 (PDB: 1BV1) with nine point mutations are depicted. *This amino acid is not present in natural Bet v 1 and is a C-terminal extension and not a substitution. The models were prepared with UCSF Chimera [84].

3.3 IgE epitope mapping using murine Bet v 1-specific monoclonal antibodies

Another attempt to map IgE binding hot spots on the surface of Bet v 1 was done by Lebecque et al. [134], who immunized mice with a birch pollen extract to obtain a panel of mouse monoclonal anti-Bet v 1 antibodies. Four of those antibodies strongly inhibited IgE binding to Bet v 1 and recognized three overlapping Bet v 1-derived synthetic dodecapeptides that corresponded to the region of amino acid residues 49 to 66 (Figure 12).

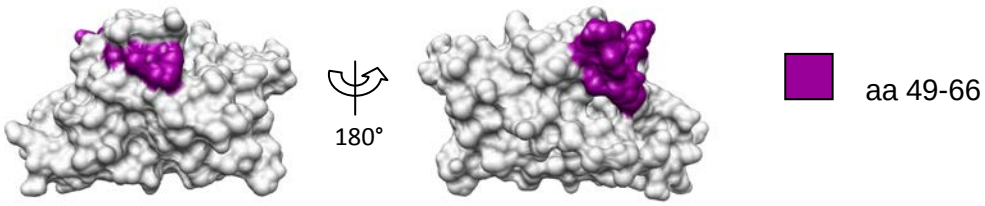


Figure 12: IgE-epitope area of Bet v 1 described by Lebecque et al. [134]. Bet v 1.0112 (PDB: 1BV1) showing the amino acid residues (aa) 49-66. The models were prepared with UCSF Chimera [84].

Gieras et al. [135] mapped IgE epitopes on Bet v 1 with peptide-specific monoclonal antibodies obtained by immunization of mice with two synthetic Bet v 1-derived peptides comprising amino acid residues 29-58 and 73-103. The obtained monoclonal antibodies strongly inhibited IgE binding to Bet v 1 (52-75%). Using truncated peptides, the binding sites for the mAbs were then mapped to amino acids 49-58, 73-88 and 88-103, respectively (Figure 13).

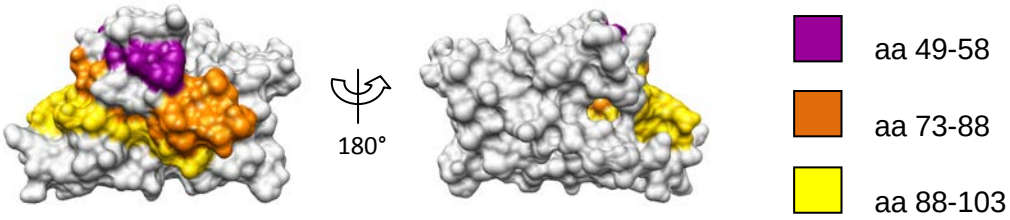


Figure 13: IgE-epitope area of Bet v 1 described by Gieras et al. [135]. Bet v 1.0112 (PDB: 1BV1) showing the binding sites for the monoclonal antibodies. The models were prepared with UCSF Chimera [84].

3.4 IgE epitope mapping using human Bet v 1-specific IgE antibodies

Hecker et al. [136] used single-chain variable fragments (scFv) composed of V_L and IgE-derived V_H epsilon domains from human IgE from birch pollen-allergic patients' lymphocytes to assign the corresponding Bet v 1-specific IgE binding epitope. This was performed by generating IgE-derived phage libraries to acquire the epitope specificity of allergen-specific antibodies. The epitope of a Bet v 1-specific antibody selected from the library could be mapped to the C-terminal helix of Bet v 1 comprising amino acid residues 132-154 (Figure 14).

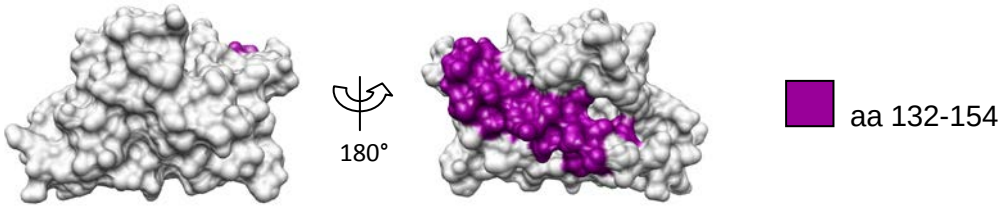


Figure 14: **Epitope area of a Bet v 1-specific antibody described by Hecker et al. [136].** Bet v 1.0101 (PDB: 1BV1) showing amino acid residues (aa) 132-154. The models were prepared with UCSF Chimera [84].

A similar approach was carried out by Levin et al. [137] The authors isolated four Bet v 1-specific antibody fragments from a combinatorial antibody fragment library derived from IgE heavy chain variable domains from the human IgE repertoire and characterized their specificities. Two antibody single-chain fragments showed reactivity to a peptide containing residues Ile56 to Asp69. A detailed evaluation of the antibody fragment with the higher affinity revealed that residues Pro59, Phe62, Pro63, and Lys65 were most critical for binding; Ile56, Gly61 and Tyr66 were of intermediate importance and the remaining residues were of low significance for the interaction. The other two antibody fragments were mapped to a peptide covering residues Gly26 to Ser39 (Figure 15). Moreover, in this study, the first high-resolution structure of a human allergen-specific IgE fragment in the scFv format (M0418) was published.

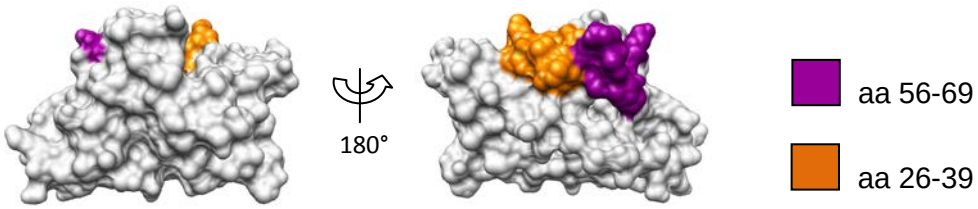


Figure 15: **Epitope areas of Bet v 1-specific IgE antibodies described by Levin et al. [137].** Bet v 1.0112 (PDB: 1BV1) showing amino acid residues (aa) 56-69 in purple or residues 26-39 in orange. The models were prepared with UCSF Chimera [84].

3.5 IgE epitope mapping by crystallization of a Bet v 1-antibody fragment complex

X-ray analysis of antigen-IgE antibody complexes represents the most direct technique to define IgE binding epitopes on proteins. Similar information can be obtained by NMR spectroscopy [117]. However, such approaches require homogenous reagents (e.g. monoclonal antibodies) and cannot be performed using polyclonal human serum IgE. Monoclonal IgE is difficult to obtain. Therefore, Mirza et al. obtained Fab fragments of a purified anti-Bet v 1 monoclonal murine IgG1 (BV16) for the use as a model system [138]. They showed that BV16 inhibited ~40% of the binding of a pool of birch pollen-allergic patients' serum IgE to Bet v 1. This finding supports the concept of dominating IgE epitopes. The BV16 Fab was crystallized in complex with Bet v 1.0112 (Figure 16) and was shown to bind to the P-loop of Bet v 1 (residues 46-52). The residues 42-52 constituted ~80% of the contact surface, where Glu45 was located in the center of the epitope defined by BV16. The other contact residues were Arg70, Asp72, His76, Ile86, Glu87 and Lys97.

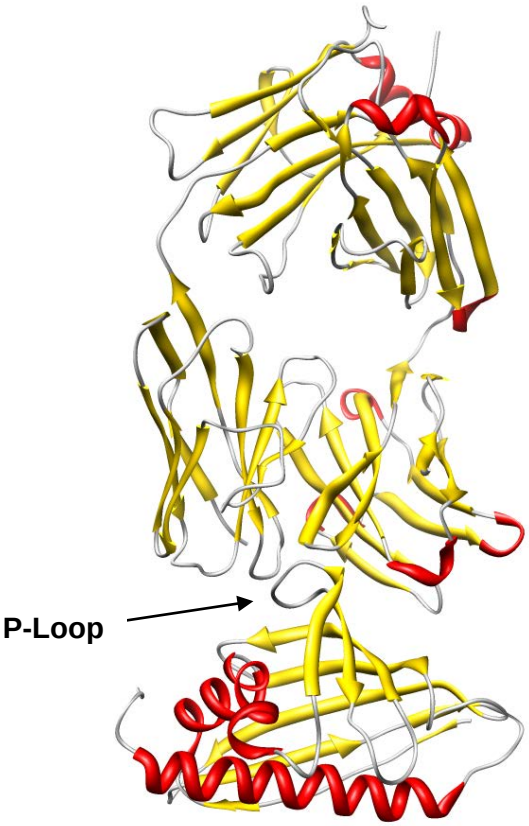


Figure 16: Bet v 1.0112 in complex with the BV16 Fab (PDB: 1FSK). Bet v 1 is located at the bottom.

Binding of BV16 to a Bet v 1 mutant with a single amino acid substitution Glu45Ser was completely abolished and IgE binding to this mutant was significantly reduced for four individual patients' sera as well as for a serum pool up to 50% [126]. Furthermore, other surface-exposed residues expected to be important for the binding of BV16 to Bet v 1 were exchanged (Figure 17). The mutants carried the point mutations Asn28Thr + Lys32Gln (double mutant), Glu45Ser, Lys55Asn, Glu60Ser, Thr77Ala, and Pro108Gly, respectively (Figure 9; page 19). In agreement with the structure of the complex, substitution of residues in positions 28, 32, 60, 77, and 108 did not significantly affect the binding of the monoclonal antibody [126].

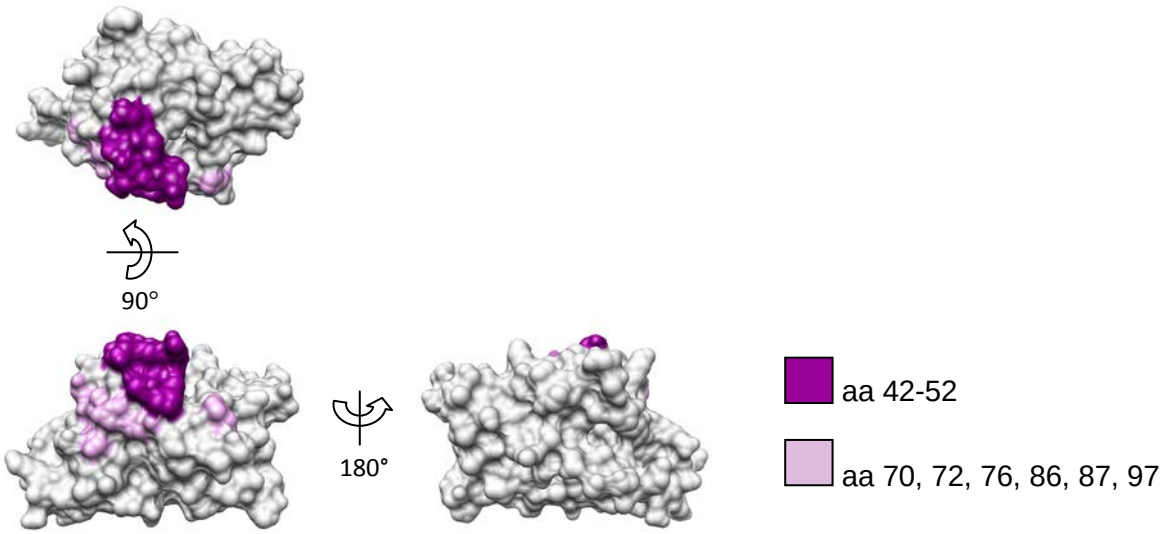


Figure 17: Contact surface of Bet v 1.0112 (PDB: 1BV1) and the antibody BV16 as described by Mirza et al. [138]. Amino acid residues 42-52 constitute the main binding region (depicted in purple). The other contact residues are shown in lilac. The models were prepared with UCSF Chimera [84].

3.6 IgE epitope mapping using chimeric proteins

The generation of chimeric proteins by grafting surface exposed amino acids onto a scaffold protein is an elegant approach to study IgE binding. Holm et al. [139] successfully grafted the conformational epitope defined by the murine anti-Bet v 1 monoclonal antibody BV16 (Figure 17) onto the Bet v 1 homologous scaffold molecule from apple, Mal d 1, by introducing 5 point mutations (Ile43Asn, Leu44Ile, Asp47Asn, Lys70Arg, Glu76His). Following the grafting process, no loss of epitope functionality was observed. The authors further revealed that increasing the surface similarity of Mal d 1 to Bet v 1 by substituting six (Ile43Asn, Leu44Ile, Asp47Asn, Gly65Lys, Lys70Arg, Glu76His) or eight (Glu12Val, Pro16Ala, Ser107Thr, Gly108Pro, +109Asp, Ser110Gly, Lys152Leu, Pro155Ser) amino acid residues in the sequence of Mal d 1 increased the protein's potency to activate basophils from birch pollen allergic patients who would not respond to natural Mal d 1 (Figure 18). However, the results varied from patient to patient, indicating that the individual patient's IgE recognition patterns differed.

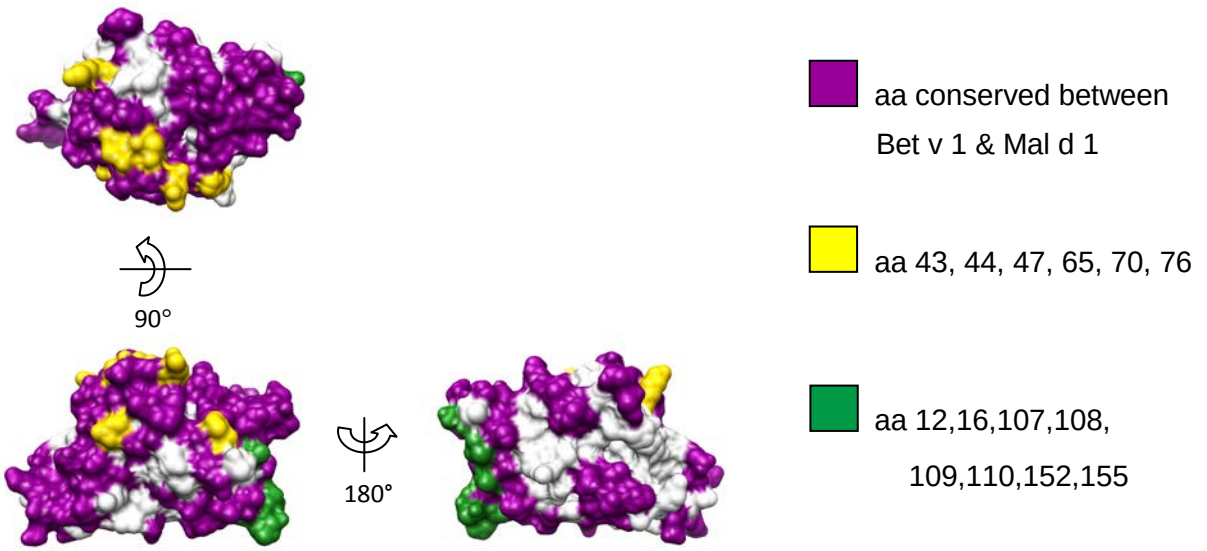


Figure 18: Mutated amino acid residues in the study of Holm et al. (2011) [139]. Bet v 1.0112 (PDB: 1BV1) showing the amino acid residues (aa) that were mutated. Conserved amino acids are depicted in purple. The models were prepared with UCSF Chimera [84].

Berkner et al. [140] grafted single amino acid residues which are part of the epitope defined by the mAb BV16 onto a low-IgE binding scaffold protein with the typical Bet v 1 fold. For this purpose, the enzyme (S)-norcoclaurine synthase (NCS) from meadow rue (*Thalictrum flavum)* was chosen. By inserting Asn57 and exchanging the amino acids Ile58, Asp60, Val63 or in addition also Asp68 in the sequence of NCS to the corresponding ones of Bet v 1 (Asn43, Glu45, Asn47, Pro50 and Lys55), IgE binding to both chimeras was observed for 25/70 (36%) and 50/70 (71%) of birch pollen allergic patients, respectively (Figure 19). In contrast, only 4/77 (6%) of the sera showed IgE binding to the scaffold protein.

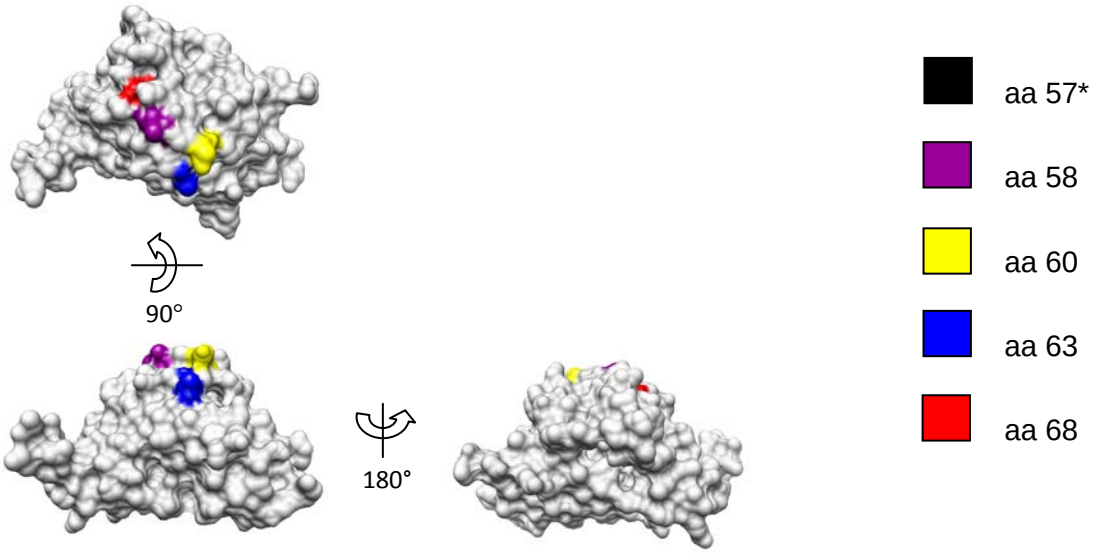


Figure 19: Mutated amino acid residues in the study of Berkner et al. [140]. (S)-Norcoclaurine synthase (PDB: 2VNE) showing the mutated amino acid residues Ile58Glu, Asp60Asn, Val63Pro, and Asp68Lys. *Asn57 does not exist in the sequence of NCS and was inserted into the mutants. The models were prepared with UCSF Chimera [84].

Klinglmayr et al. [141] used this technique to graft five Mal d 1 stretches onto Bet v 1, each comprising 7 or 8 amino acids. The grafting regions were defined by addition of 3 amino acid residues from Mal d 1 to the N- and C-terminal flanking regions of Thr10, Phe30, Ser57, Ser112/Ile113, and Asp125 in the Bet v 1.0101 sequence, respectively, which were previously shown to be essential for IgE binding to Bet v 1 (Figure 7; page 17) [124]. Four of the chimeras still displayed IgE reactivity whereas the chimera with the grafted Mal d 1 stretch around the Bet v 1 amino acid residues Ser112 and Ile113 lost the IgE binding capacity most probably due to a change in the conformation. The four other mutations were combined on one molecule which was named BMC. IgE reactivity to BMC was significantly reduced in patients not suffering from the birch pollen-food syndrome compared with birch pollen allergic patients with clinical reactivity to apple.

3.7 IgE epitope mapping using mimotopes

The use of phage display libraries to select peptides that mimic natural epitopes, called mimotopes, is another technique to help predict the localization of IgE binding epitopes. During biopanning, allergen-specific antibodies are incubated with a phage display library. Phages that bind specifically to the antibodies are amplified during several repeated panning rounds. Colony

screening is then used to identify phage clones which specifically bind to the allergen-specific antibodies. A strong signal in these tests predicts good mimicry of the respective antibody's epitope. This has to be additionally proven by competition assays with the original allergen. After sequencing of clones, computational mapping of the peptide sequence to the surface of the antigen has to be performed [142].

Ganglberger et al. [143] purified IgE from a serum pool of birch pollen allergic patients and used this approach to identify IgE binding areas of Bet v 1. By using a self-developed 3-dimensional coarse-grained epitope search, the IgE binding regions were predicted to be located at residues 9-22 and 104-113 (Figure 20). Furthermore, the localization of a mimotope of the monoclonal murine anti-Bet v 1 antibody BIP 1 was predicted and mapped to position 58-67.

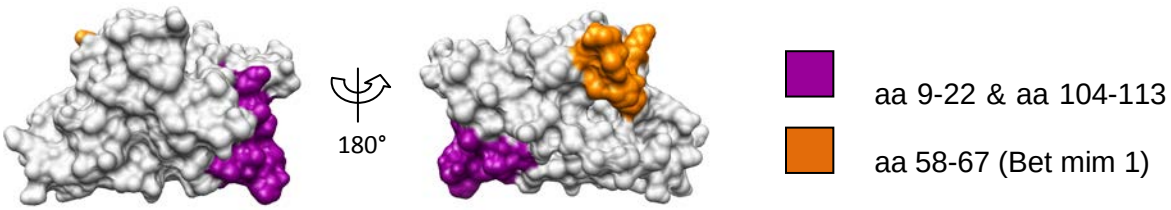


Figure 20: Predicted epitope areas described by Ganglberger et al. [143]. Bet v 1.0112 (PDB: 1BV1) showing the IgE binding areas predicted. In orange, the localization of Bet mim 1 is depicted. The models were prepared with UCSF Chimera [84].

The same technique was used by Mittag et al. [144]. The authors purified polyclonal IgE from individual sera to perform competitive immunoscreening of a phage-displayed random 7-mer peptide library. The obtained peptides were localized on the protein surface using a computer-based algorithm (Epitope mapping tool (EMT), Novozymes A/S). The individual IgE binding patterns of five birch pollen allergic patients to Bet v 1 and to the homologous food allergens Gly m 4 (soy), Ara h 8 (peanut) and Pru av 1 (cherry) were investigated. A strong patient to patient variation was observed, however, three defined antigenic surface areas were predicted to bind IgE from all or most of the patients (Figure 21).

Epitope area 1: 1-7, 84-99, 120-129

Epitope area 2: 141-152, 153-159, 8-15, 16-24, 25-38

Epitope area 3: 25-38, 39-53, 54-67

Figure 21: Predicted epitope areas described by Mittag et al. [144]. Bet v 1.0112 (PDB: 1BV1) showing the three predicted epitope areas. The models were prepared with UCSF Chimera [84].

3.8 *In silico* prediction of IgE epitopes

SPADE which stands for Surface comparison-based Prediction of Allergenic Discontinuous Epitopes is a software which is able to predict the IgE binding epitopes of a group of allergens on the basis of at least 2 structural models and the IgE cross-reactivity data [145]. The *in silico* prediction of cross-reactive IgE epitopes on the surface of Bet v 1 identified two neighboring patches. The larger area is predominantly linear and spans parts of the C-terminal alpha helix including residues 127-132 plus 135 (Figure 22). The smaller patch contains the two amino acid residues 92 and 93. By comparing Bet v 1.0101 with its hypoallergenic isoform Bet v 1.0107, another IgE epitope was predicted. It comprises amino acid residues 59 to 64, 89 to 94, and 123 to 133 (Figure 22).

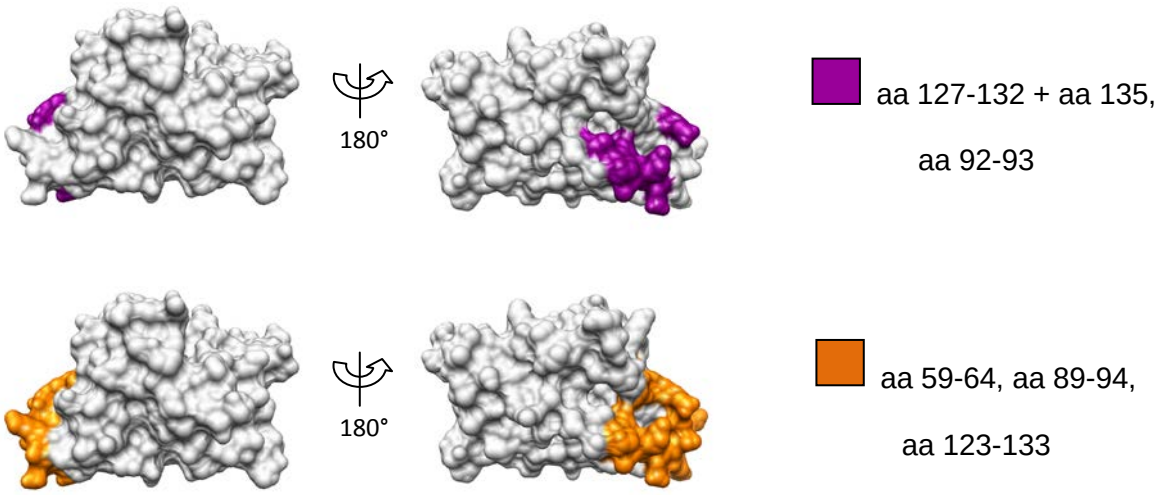


Figure 22: Predicted epitope areas described by Dall'Antonia et al. [145]. Bet v 1.0101 (1BV1) showing *in silico* predicted IgE-binding areas. The models were prepared with UCSF Chimera [84].

4 ALLERGEN-SPECIFIC IMMUNOTHERAPY

In 1911, Leonard Noon published the first documentation of the effectiveness of subcutaneous immunotherapy in grass-allergic patients with hay fever [146]. A key feature of allergen-specific immunotherapy (AIT) is to change the course of disease by altering the underlying pathology. AIT is defined as a method to administer increasing amounts of specific allergens to patients with clinical symptoms, caused by those allergens based on a specific IgE-mediated sensitization to modulate the immune system to tolerate this specific allergen again (Figure 23) [147, 148].

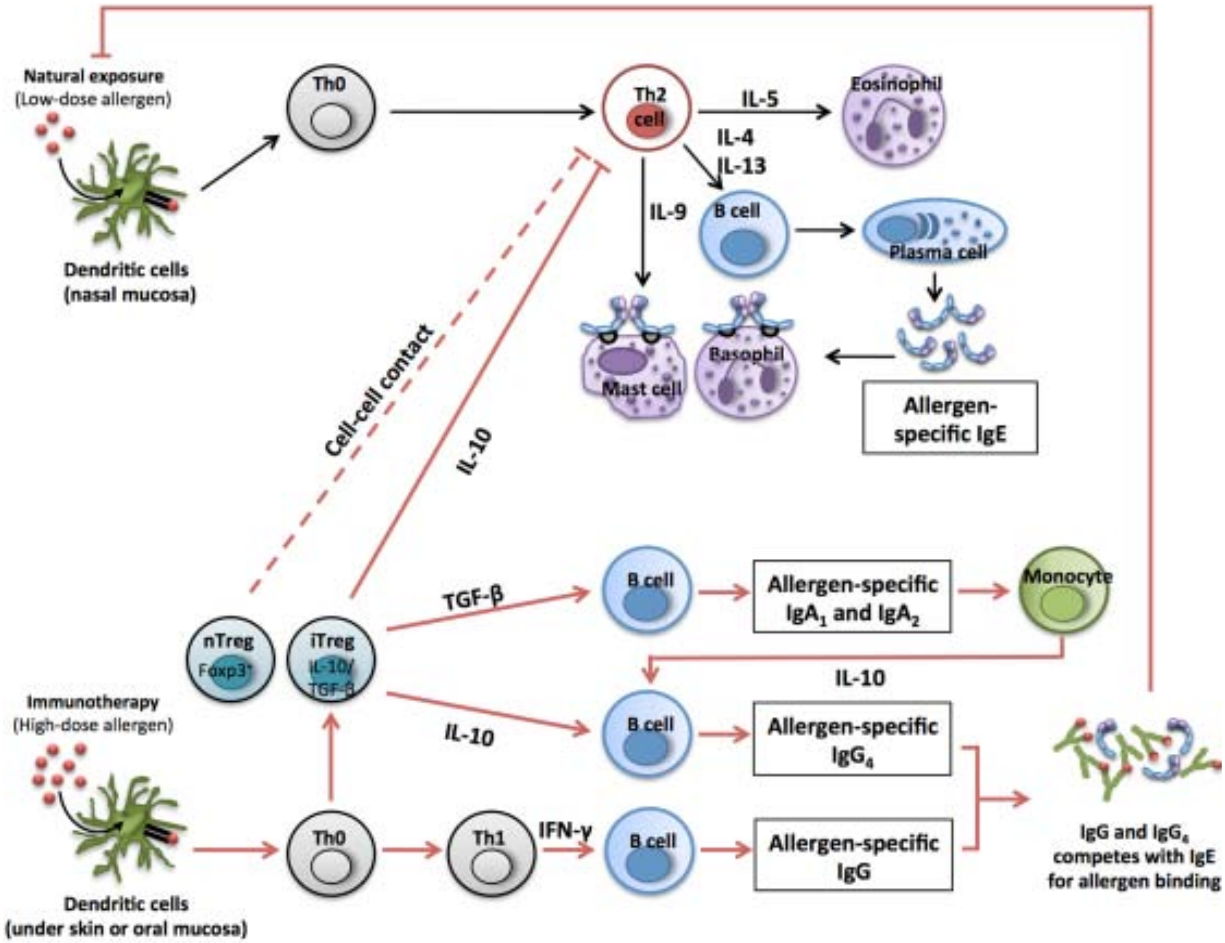


Figure 23 (Taken from Ref [149]): Differences between the immunological mechanisms after low- or high-dose expose to the allergen. Low-dose exposure of allergen drives the type I IgE-mediated allergic response in atopic individuals. High-dose exposure provokes a shift of T cell polarization from a Th2 to a Th1 response. This is accompanied by an increase in Th1 cytokines (IL-12, IFN-γ). Furthermore, T regulatory cells [natural Treg cells (nTreg) and inducible Treg cells (iTreg)] and cytokines such as IL-10 and TGF-β are induced which are important to suppress Th1 and Th2 responses and contribute to the induction of allergen-specific IgG4, IgA1 and IgA2.

Currently, subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT) are the two types of AIT used in clinical practice, and several novel AIT approaches are under evaluation in clinical trials [147, 150]. In case of SCIT, incremental allergen doses are given for a period of 8-16 weeks, followed by monthly maintenance injections for a period of 3-5 years [151]. SLIT uses an effective alternative route whereby a tablet is kept under the tongue for 1-2 min and then swallowed [148]. Both types of immunotherapy are suitable for children and adults for a variety of allergens, such as pollen, house dust mite, cat, dog, and insect venoms [152]. Immunotherapy was reported to affect symptoms of allergic inflammation but also to alter the disease pathology in the long-term, resulting in reduced disease severity, less drug usage, a long-term curative effect after completing the treatment, and prevention of sensitization to additional allergens [147]. Although many mechanisms of AIT are not fully understood, they include changes in the characteristics of allergen-specific memory B and T cells, the production of specific antibody isotypes to skew the immune response towards tolerance, as well as decreased activation, mediator release and tissue migration of mast cells, basophils and eosinophils [147].

4.1 Mechanisms of immunotherapy

Cellular and molecular events that occur during the course of AIT can be classified into four groups (Figure 24) [147]. After the first administration of AIT, an early desensitization effect including decreases of mast cell and basophil degranulation can be observed. Furthermore, a decrease of the tendency for systemic anaphylaxis starts to take place within hours. Allergen-specific regulatory T and B cells are generated during the course of AIT and allergen-specific effector T cells are suppressed. The regulation of antibody isotypes manifests itself as an early increase in specific IgE levels followed by a later decrease, and an early and continuous increase in specific IgG4 levels. After several months of AIT, a decrease of tissue mast cells and eosinophils and a reduced release of their mediators is accompanied by a decrease of type I skin test reactivity.

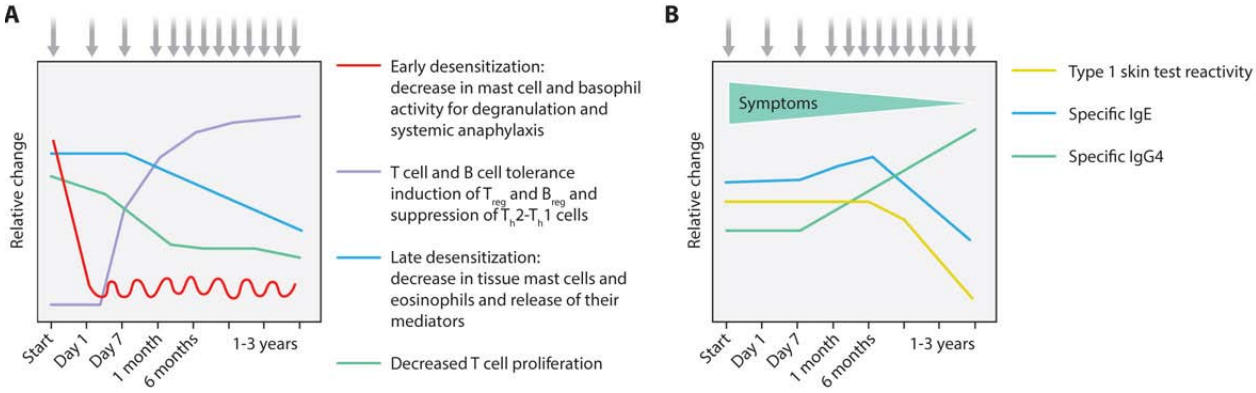


Figure 24 (Taken from reference [147]): Immunological changes during AIT. Already after the first injection, decreases in mast cell and basophil activation and degranulation can be observed. This is followed by generation of allergen-specific T cell tolerance with the development of specific Tregs and Bregs and inhibition of allergen-specific Th1 and Th2 cells. Specific IgE shows an early increase and a relatively late decrease. Specific IgG4 continuously increases as long as the therapy continues. In parallel to clinical improvement, decreases in tissue mast cells and eosinophils and release of their mediators and decreased skin late-phase responses take place.

4.1.1 Rapid desensitization of mast cells and basophils by allergens

After the first injection, a decrease of the susceptibility of basophils and mast cells to degranulation can be observed [153, 154]. Similar effects were described during rapid drug desensitization (RDD) [155], a technique that induces temporary tolerance to a drug. Through RDD, patients with IgE- and non-IgE-mediated hypersensitivity reactions can safely be administered essential medications while minimizing or completely preventing adverse reactions.

The main inflammatory mediator released on FcεRI triggering of mast cells and basophils is histamine, which acts through histamine receptors (HRs). H1R possesses proinflammatory properties, whereas H2R was reported to be associated with tolerogenic immune responses [156]. In patients undergoing insect venom immunotherapy, it was shown that the H2R might be involved in the very early desensitization effect. Within the first 6 hours of the build-up phase of immunotherapy, rapid upregulation of H2R was observed. This resulted in suppression of the FcεRI-induced activation and mediator release of basophils [157].

4.1.2 Regulatory T and B cells in AIT

Peripheral T cell tolerance is essential for normal immune responses and for a successful AIT [158]. Increased IL-10 secretion which particularly originates from activated and antigen-specific Treg and Breg cell populations during AIT and natural allergen exposure is the key factor for the tolerant state of specific cells [159-163]. High IL-10-producing Treg and Breg subsets are called

IL-10-secreting regulatory T (T_R1) cells and IL-10-secreting regulatory B (B_R1) cells, respectively. Allergen-specific $CD4^+$ T cells which predominantly produce IFN-γ (Th1), IL-4 (Th2) and IL-10 (T_R1-like cells) are present in healthy and allergic individuals, although in different proportions [162]. The dominant subsets against environmental allergens in healthy individuals are IL-10 secreting T_R1 cells, whereas in allergic patients, allergen-specific IL-4-secreting T cells exist in high frequency [162]. The investigation of high-dose allergen exposure of healthy subjects, for example non-allergic bee keepers and cat owners, has provided interesting insights into the nature of Treg responses in tolerance [164, 165]. Apparently, the T- and B-cell subsets that become dominant during AIT or natural antigen exposure are IL-10 secreting regulatory T_R1 cells and IL-10 secreting regulatory B_R1 cells. In addition to IL-10, peripheral tolerance involves multiple suppressive factors, such as TGF-β, and the T cell surface receptors CTLA-4 (cytotoxic T lymphocyte antigen 4), and PD-1 (programmed death 1) [162].

4.1.3 AIT and allergen-specific antibody responses

4.1.3.1 The IgE response during AIT

Natural exposure to a specific allergen is often associated with an increase in IgE synthesis. Similarly, it can be observed that AIT induces a transient early rise of allergen-specific IgE levels followed by a decrease over months or years of continued treatment [166]. Successful treatment is associated with blunting of seasonal increases in allergen-specific IgE levels [149]. However, changes of IgE levels cannot account for the reduced responsiveness to specific allergens that are caused by AIT as the decrease of serum IgE levels occurs only later, is relatively minor and poorly correlated with the observed clinical improvements.

4.1.3.2 The IgG response during AIT

Instead, allergen-specific IgG antibodies seem to contribute more directly to the efficacy of AIT. Measurements of IgG subtype levels during AIT revealed specific increases in the range of 10-100 fold of the concentrations of IgG1 and especially of IgG4 [167]. In contrast, changes of IgG2 and IgG3 levels during AIT were not significant [168]. Despite the increases in allergen-specific IgG1 and IgG4 antibodies, many studies have failed to show a correlation between absolute antibody levels and clinical efficacy [149].

During immunotherapy, IgG1 levels were described to rise earlier than IgG4 levels with a plateau after 12 months [169]. This early IgG1-dominated response has been reported to be necessary to induce the suppression of the IgE antibody production [170].

IgG4 is an unusual immunoglobulin with several features that may contribute to its non-inflammatory role. Although AIT induces high IgG4 levels, these antibodies are non-precipitating [171]. IgG4 is the only antibody class with this capacity because the two arms of IgG4 have the ability to separate and recombine by means of dynamic Fab arm exchange [172]. This bi-specificity turns the antibody functionally monovalent, hence preventing it from forming complexes and activating B cells [171].

4.1.3.3 Possible inhibitory functions of IgG antibodies induced during AIT

Many possible mechanisms has been described which contribute to the inhibitory function of IgG1 and IgG4 induced during AIT. Both antibodies are thought to capture the allergen before it reaches the effector cell-bound IgE, thus preventing the activation of mast cells and basophils [147]. Allergen-specific IgG may be directed against the same epitopes as IgE, which leads to a direct competition for allergen binding and results in a blocking effect. Moreover, steric inhibition by binding of IgG to sites adjacent to IgE-epitopes could also contribute to IgE-blocking [173].

IgG antibodies that recognize different epitopes than IgE antibodies and hence are unable to compete with IgE for allergen binding protect against IgE-induced mast cell activation by triggering inhibitory signals through the low-affinity Fcγ receptor IIB (FcγRIIB). In a murine model of allergy, FcγRIIB was shown to be involved in the inhibitory activities of IgG. Co-aggregation of FcεRI and FcγRIIB by IgE and IgG antibodies simultaneously bound to an allergen results in the suppression of mast cell degranulation [174]. This inhibitory effect was also observed in human mast cells [175] and could be mimicked by Fcγ-Fcε fusion proteins [176] or an allergen-Fcγ fusion protein [177].

Recently, a novel mechanism of IgG-mediated mast cell desensitization was described. Uermösi and colleagues revealed that IgG antibodies were actively involved in the down-regulation of FcεRI-bound IgE. Cross-linking of FcγRIIB with FcεRI enhanced IgE internalization while the mast cell activation was inhibited [178].

Blocking antibodies do not only have an effect on the inhibition of mast cell and basophil degranulation, but can also interfere with T-cell activation. For the development and perpetuation of the allergic immune response, T-cell activation is crucial and is also directly linked to the late-phase response and to chronic inflammation of the airways (Figure 2; page 4). The low annual exposure to aeroallergens suggests that IgE-mediated allergen presentation via

CD23 or FcεRI, which are expressed on cells with antigen-presenting properties, is the most likely *in vivo* mechanism for T-cell activation in allergic patients [179]. Van Neerven et al. [180] demonstrated that AIT-induced serum IgG antibodies inhibited IgE-facilitated allergen presentation at low allergen concentrations, resulting in reduced T cell proliferation and decreased production of IL-4, IL-5, IL-10 and IFN-γ. The authors furthermore revealed that 100- to 1000-fold higher allergen doses were needed to activate birch-allergen-specific T cells after AIT [181]. These findings also provided an explanation for the reduced T cell activation and cytokine production as well as for the decreased allergen-induced late-phase reactions which have been described after successful immunotherapy [167].

Using a simplified flow cytometric assay that determines binding of allergen-IgE complexes to CD23 on EBV-transformed B cells, Wachholz et al. [182] revealed that increases in allergen-specific IgG in sera collected during grass pollen immunotherapy were associated with inhibition of allergen-IgE binding. In addition, the authors confirmed that the inhibitory activity was present in the IgG fraction and was associated with an improvement of clinical symptoms.

4.1.3.4 Epitope diversity and specificity of allergen-specific antibodies and their modulation during AIT

Although the levels of antibody production over time of IgE, IgG1 and IgG4 production during immunotherapy have been intensively studied, little is known about the development of epitope diversities and specificities of these antibodies during AIT. The first study which deals with this topic was published in 2013 [183]. Vickery et al. investigated the longitudinal effects of peanut oral immunotherapy (OIT) on the diversity and specificity of the antibody repertoires directed against the three major peanut allergens Ara h 1 to 3. They used a library of peptides, consisting of 15 amino acids overlapping by 12, which correspond to the primary sequences of the allergens. The authors reported that the antibody repertoire undergoes dynamic and individualized changes during OIT. The clonality of the IgE repertoire remained mainly unchanged. However, roughly one third of the patients generated new IgE specificities, although an overall reduction of allergen-specific IgE levels occurred in each of them. Furthermore, they showed that OIT induced a polyclonal expansion of peanut-specific IgG4 including novel specificities. The binding affinity of peanut-specific IgE and IgG4 remained unchanged over time.

Savilahti et al. [184] investigated IgE and IgG4 binding profiles of children with cow's milk allergy to cow's milk protein-derived peptides with a microarray-based immunoassay before and after oral immunotherapy (OIT). In 26 children who successfully completed immunotherapy, IgE binding to cow's milk peptides declined and IgG4 binding increased. Six children who failed to

complete the therapy due to adverse reactions developed increased quantities and affinities of peptide-specific IgE and higher epitope diversities of IgE and IgG4 but little overlap of IgE and IgG4 binding to cow's milk peptides.

4.1.4 Suppression of late-phase responses of effector cells during AIT

Long-term AIT results in a reduction of immediate and late phase responses after allergen provocation. After successful immunotherapy, higher allergen amounts are required to induce immediate or late phase reactions. Furthermore, a reduction of nasal, bronchial and conjunctival hyperreactivity to non-specific stimuli was observed and correlated with clinical improvement [185, 186]. AIT also reduces eosinophil activation and chemotactic factors for eosinophils and neutrophils which correlates with reduced bronchial hyperactivity and clinical improvement. Immunotherapy further inhibits eosinophil priming during the allergen season and modulates the thresholds for basophil and mast cell activation which results in a decreased histamine release [187-189]. IL-10 downregulates the proinflammatory mediator release of mast cells, it reduces eosinophil activity by suppressing IL-5, GM-CSF and CD40 expression and it enhances eosinophil cell death [190, 191].

5 AIMS

Knowledge of the location and distribution of IgE epitopes on the surface of Bet v 1 is important for several reasons. Epitope information is useful for elucidating the characteristics of the IgE response, for predicting cross-reactivity and for making allergen vaccines safer. IgE epitopes of Bet v 1 are conformational [120] and several methods have been applied to map such epitopes. However, the current knowledge of conformational B cell epitopes of Bet v 1 is far from complete.

Therefore, we designed four chimeric proteins composed of Bet v 1.0101 and its homologue in celeriac, Api g 1.0101. The latter protein was chosen as the scaffold protein because it shows a low sequence identity of 41% to Bet v 1 and its crystal structure is known [192]. Each chimera was generated by exchanging 10-13 Api g 1-specific surface residues by Bet v 1-specific residues. Hence, each of the chimeric proteins contains a contiguous Bet v 1-specific area which comprises approximately one quarter of the surface. With these chimeras the following points should be elucidated:

- Where are the locations of IgE epitopes on the surface of Bet v 1?
- Is there a spatial clustering of important Bet v 1-specific IgE epitopes?
- Do all birch pollen individuals recognize the same IgE epitopes?

Allergen-specific immunotherapy is the only specific and disease-modifying approach for the treatment of allergy. However, only 70% of the patients who are allergic to major allergens reveal ameliorations of allergic symptoms after AIT [193]. Furthermore, several disadvantages have limited its broad applicability. Due to the allergenic activity of natural allergen extracts, the dose has to be increased gradually up to a clinically effective maintenance dose, which requires multiple injections. In addition, treatment for periods of less than 2 years was found to be less effective [194]. Therefore, a major aim is to understand the complex mechanisms of immunotherapy and to develop new tools in order to improve the efficacy of AIT.

It is well known that the induction of allergen-specific IgG is crucial for a successful immunotherapy [147]. Both, IgG1 and especially IgG4 are considered as blocking antibodies. However, the development of the antibody repertoires during AIT is little investigated on the epitope level. Therefore, we planned to evaluate whether the IgG4 antibody diversity expands, decreases or remains constant during immunotherapy by comparing an early and a late point of birch pollen AIT using phage display and the chimera-based approach. In addition, the IgE-blocking activity at those time points should be assessed by IgE-facilitated allergen-binding assays.

Furthermore, we aimed to monitor the epitope repertoires of Bet v 1-specific IgE, IgG1 and IgG4 at the beginning and after 1, 3, 6, 12, 18, 24, 30 and 36 months of immunotherapy by using the chimera-based approach.

The major questions to be answered are:

- Are the epitope diversities and specificities of Bet v 1-specific IgE and AIT-induced Bet v 1-specific IgG1 and IgG4 similar?
- Do the Bet v 1-specific IgE, IgG1 and IgG4 repertoires change during immunotherapy?

CHAPTER II:

Manuscript 1

Research Article

Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy

Barbara Gepp[1], Nina Lengger[1], Merima Bublin[1], Wolfgang Hemmer[2], Heimo Breiteneder[1], Christian Radauer[1]

[1]Department of Pathophysiologie and Allergy Research, Medical University of Vienna, Austria

[2]Floridsdorfer Allergiezentrum, Vienna, Austria.

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Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy

Barbara Gepp, MSc,[a] Nina Lengger,[a] Merima Bublin, PhD,[a] Wolfgang Hemmer, PhD,[b] Heimo Breiteneder, PhD,[a] and Christian Radauer, PhD[a] *Vienna, Austria*

Background: Characterization of IgE-binding epitopes of allergens and determination of their patient-specific relevance is crucial for the diagnosis and treatment of allergy.
Objective: We sought to assess the contribution of specific surface areas of the major birch pollen allergen Bet v 1.0101 to binding IgE of individual patients.
Methods: Four distinct areas of Bet v 1 representing in total 81% of its surface were grafted onto the scaffold of its homolog, Api g 1.0101, to yield the chimeras Api-Bet-1 to Api-Bet-4. The chimeras were expressed in *Escherichia coli* and purified. IgE binding of 64 sera from Bet v 1–sensitized subjects with birch pollen allergy was determined by using direct ELISA. Specificity was assessed by means of inhibition ELISA.
Results: rApi g 1.0101, Api-Bet-1, Api-Bet-2, Api-Bet-3, and Api-Bet-4 bound IgE from 44%, 89%, 80%, 78%, and 48% of the patients, respectively. By comparing the amount of IgE binding to the chimeras and to rApi g 1.0101, 81%, 70%, 75%, and 45% of the patients showed significantly enhanced IgE binding to Api-Bet-1, Api-Bet-2, Api-Bet-3, and Api-Bet-4, respectively. The minority (8%) of the sera revealed enhanced IgE binding exclusively to a single chimera, whereas 31% showed increased IgE binding to all 4 chimeras compared with rApi g 1.0101. The chimeras inhibited up to 70% of IgE binding to rBet v 1.0101, confirming the specific IgE recognition of the grafted regions.
Conclusion: The Bet v 1–specific IgE response is polyclonal, and epitopes are spread across the entire Bet v 1 surface. Furthermore, the IgE recognition profile of Bet v 1 is highly patient specific. (J Allergy Clin Immunol 2014;134:188-94.)

Key words: *Bet v 1.0101, Api g 1.0101, patient-specific IgE repertoire, chimera-based technology, IgE epitope mapping, birch pollen allergy*

Birch is one of the main elicitors of pollinosis in Europe.[1] More than 98% of patients with birch pollen allergy from Austria, Finland, and Sweden are sensitized to the major birch pollen allergen Bet v 1,[2] which belongs to the pathogenesis-related 10 family of plant pathogenesis-related proteins.[3] Additionally, more than 70% of patients with birch pollen allergy have adverse reactions to certain plant foods.[4] This cross-reactivity is caused by sensitization to Bet v 1 and binding of Bet v 1–specific IgE to homologous plant food allergens. Bet v 1–related proteins have been identified as major allergens in apple (Mal d 1), celery (Api g 1), cherry (Pru av 1), and carrot (Dau c 1), among others.[5-8]

Until now, little has been known about the nature of IgE-binding epitopes of Bet v 1 and related plant food allergens. Epitope mapping is crucial to understand immune responses to allergens and allergen cross-reactivity among homologous proteins. Furthermore, knowledge about pivotal IgE-binding regions provides the basic information required for the design of safe and effective reagents used for allergen-specific immunotherapy, the only curative and specific approach in the treatment of allergy.[9]

It was shown that IgE binding to Bet v 1 was highly dependent on the protein's native conformation.[10,11] Thus the analysis of IgE-binding epitopes of Bet v 1 represents a challenging task. Thus far, only 1 epitope was indirectly determined by means of the cocrystallization of Bet v 1.0112 and the Fab fragment of a murine mAb capable of blocking IgE binding to Bet v 1 by 40%.[11] This epitope covered the P-loop, a highly conserved region among pathogenesis-related 10 family members.[12] Furthermore, the contribution of the P-loop to IgE binding of Bet v 1 was proved by means of site-directed mutagenesis of Bet v 1 and Pru av 1. Exchange of Glu45 in both proteins reduced IgE binding for most patients' sera.[13,14] The existence of high and low IgE binding isoallergens of Bet v 1 and the generation of hypoallergenic mutants led to the definition of further key residues important for IgE binding to Bet v 1 and Mal d 1.[15-17] Another strategy to identify epitopes is based on mimicking the epitope in its interaction with IgE by short peptides selected from random-peptide libraries. This so-called mimotope technology was applied to identify preferred IgE-binding regions of Bet v 1.[18,19] Engineering of chimeric proteins of Bet v 1 and homologous proteins represents a further approach for investigating B-cell epitopes. By using epitope grafting, 3 IgE-binding regions important for cross-reactivity between Bet v 1 and Mal d 1 were examined.[20-22]

Because the diversity of the IgE response to Bet v 1 among individual patients with birch pollen allergy has never been investigated in detail, we aimed to determine the patient-specific IgE recognition profile of a large group of patients. We generated 4 chimeras of Bet v 1.0101 and its low-allergenic, nonsensitizing homolog Api g 1.0101 from celeriac.[23-25] On the basis of the known crystal structures of Bet v 1[26] and Api g 1,[27] 4 selected Bet v 1–specific portions covering the major part of the molecular surface were grafted onto the Api g 1 scaffold.

From [a]the Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, and [b]Floridsdorfer Allergiezentrum, Vienna.

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Corresponding author: Heimo Breiteneder, PhD, Department of Pathophysiology and Allergy Research, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria. E-mail: heimo.breiteneder@meduniwien.ac.at.

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METHODS

Patients and control subjects

In a retrospective study 64 residual serum samples of Austrian Bet v 1–sensitized patients with birch pollen allergy drawn during routine diagnosis at the Floridsdorfer Allergiezentrum, Vienna, Austria, were included (see Table E1 in this article's Online Repository at www.jacionline.org). The patients underwent no interventions related to the study. The use of anonymized serum samples and clinical records without obtaining written consent of the patients was approved by the ethics committee of the Medical University of Vienna (approval no. 718/2010).

Patients were selected on the basis of a typical case history of birch pollen allergy, positive skin prick test responses to birch pollen, and/or *in vitro* IgE detection to rBet v 1 or birch pollen extract (≥0.35 kU_A/L; ImmunoCAP, Thermo-Fisher, Uppsala, Sweden). The average age of the patients was 34 years (range, 7-79 years). The sex distribution was balanced, with 56% female and 44% male patients. History of food allergy to common birch pollen–associated plant foods was assessed based on questioning by an experienced allergist. Fifty-two percent (n = 33) had allergic symptoms after ingestion of plant foods, with a single patient reporting celery allergy. Twenty-two percent (n = 14) did not report food allergies, and for the rest (n = 17), these data were not available. As a negative control, sera from 7 nonallergic patients without histories of type I allergy to common allergen sources were included.

Design of the Api g 1–Bet v 1 chimeras

Chimeric proteins of Bet v 1.0101 and its homolog Api g 1.0101 were generated to investigate IgE binding to defined Bet v 1.0101–specific surface areas. Grafting of Bet v 1–specific surface areas onto the Api g 1.0101 scaffold was achieved by replacing Api g 1.0101–specific solvent-accessible (>20%) residues by corresponding Bet v 1.0101–specific residues (Fig 1). We generated the chimeric protein Api-Bet-1 by grafting Glu45, the central residue of the previously identified P-loop epitope, and surrounding residues, identified by using UCSF Chimera,[28] onto Api g 1.0101. The region opposite the P-loop (Api-Bet-2), the C-terminus and surrounding residues (Api-Bet-3), and the C-terminal α-helix (Api-Bet-4) of Bet v 1.0101 were grafted in the same manner to generate spatially well-distributed Bet v 1.0101–specific surface areas on Api g 1.0101.

Cloning, expression and purification, and physicochemical analysis of the recombinant proteins

Production and analysis of the recombinant proteins was performed as described in the Methods section in this article's Online Repository at www.jacionline.org.

IgE ELISA

For direct ELISA, microtiter plates (Maxisorp; Nalge Nunc International, Roskilde, Denmark) were coated overnight at 4°C with 1 μg/mL individual chimeric proteins, a mixture of all chimeras (1 μg/mL each), and rApi g 1.0101 or rBet v 1.0101, respectively, in 50 mmol/L sodium carbonate buffer, pH 9.6. After blocking of nonspecific binding sites, sera (1:10 dilution) were incubated in duplicates overnight at 4°C. Specific IgE was detected by using an alkaline phosphatase–conjugated mouse anti-human IgE mAb (BD Pharmingen, San Jose, Calif), followed by color development with Sigma FAST p-nitrophenyl phosphate tablets (Sigma-Aldrich, St Louis, Mo) and measurement of the absorbance at 405 nm.

OD values were measured at several time points. For each serum, the measurement with an OD of approximately 1.0 for Bet v 1.0101 was normalized to a 1-hour substrate incubation period after subtracting the OD values of the buffer controls (see Table E2 in this article's Online Repository at www.jacionline.org). Comparison of measurements at different times proved that the OD values increased with time in a linear fashion (data not shown). Hence normalized OD values were roughly proportional to allergen-specific IgE concentrations.

Individual sera from 7 nonallergic donors were included as negative controls. Normalized OD values exceeding the mean negative control value by more than 3 SDs were considered positive.

For each serum, specific IgE binding to the grafted regions of each chimera was assessed by calculating the difference of the OD values of the chimera and rApi g 1.0101. The difference was considered positive if it exceeded 3 times the SD of the negative control value.

ELISA inhibition

For inhibition ELISA, coating, blocking, and detection were performed, as described above. Either Bet v 1.0101 or the chimeras were coated to the solid phase. In inhibition assays, in which rBet v 1.0101 was coated, IgG was removed in advance by means of incubation of prediluted sera on an anti-human IgG (BD Pharmingen)–coated plate. Sera were diluted 30- to 100-fold. Inhibition was performed by preincubating diluted sera with 10-fold serial dilutions from 0.01 to 100 μg/mL of the individual chimeric proteins, a mixture of all chimeras, and rApi g 1.0101 or rBet v 1.0101, respectively, before they were applied to the plates.

For cross-inhibition between the chimeras, all chimeras, rApi g 1.0101, and rBet v 1.0101 (1 μg/mL) were coated to the solid phase and incubated with patients' sera (diluted 10- to 60-fold). The supernatants were transferred to a second plate, which was coated with all 4 chimeras or buffer only. IgE binding to the second plate was detected, as described above.

Inhibition values were calculated as follows:

$$\text{Inhibition } [\%] = (1 - \text{OD}_{\text{inhibited}}/\text{OD}_{\text{noninhibited}}) \times 100.$$

ELISA with Bet v 1–specific mAbs

Binding of Bet v 1–specific mAbs to rBet v 1, rApi g 1, and the chimeras was tested by using ELISA, as described in the Methods section in this article's Online Repository.

Statistical analyses

The Friedman test (α = .05) was performed to test whether the amount of IgE binding to each of the 4 chimeras differed significantly from that to rApi g 1.0101. The relationship between the number of chimeras recognized better than rApi g 1.0101 and the amount of rBet v 1–specific IgE present in patients' sera was analyzed by performing Spearman correlation (α = .05).

RESULTS

Biochemical characterization of the recombinant proteins

The structural integrity of the recombinant proteins was confirmed by means of circular dichroism spectroscopy, mass spectrometry, and ELISA with Bet v 1–specific mAbs, as described in the Results section in this article's Online Repository at www.jacionline.org.

IgE-binding profiles of Bet v 1 are highly patient specific

IgE-binding capacities of rBet v 1.0101, rApi g 1.0101, and the chimeras were determined by means of ELISA. All 64 tested sera displayed rBet v 1–specific IgE, whereas only 44% of the sera bound to rApi g 1 (Table I). Total IgE-binding capacities of all 4 chimeras were significantly higher than that of rApi g 1 ($P < .001$; median OD for rApi g 1, 0.028; median ODs for the chimeras, 0.076-0.222; see Fig E3 in this article's Online Repository at www.jacionline.org).

For each serum, specific IgE binding to the grafted regions of each chimera was assessed by calculating the difference of the ELISA OD values of the chimera and the template rApi g 1.0101. The grafted regions of Api-Bet-1, Api-Bet-2, and Api-Bet-3 were recognized by 70% to 81% of the sera, whereas only 45% recognized Api-Bet-4 (Table I).

Patients were categorized according to their binding patterns to evaluate individual IgE recognition profiles (Fig 2). Interestingly,

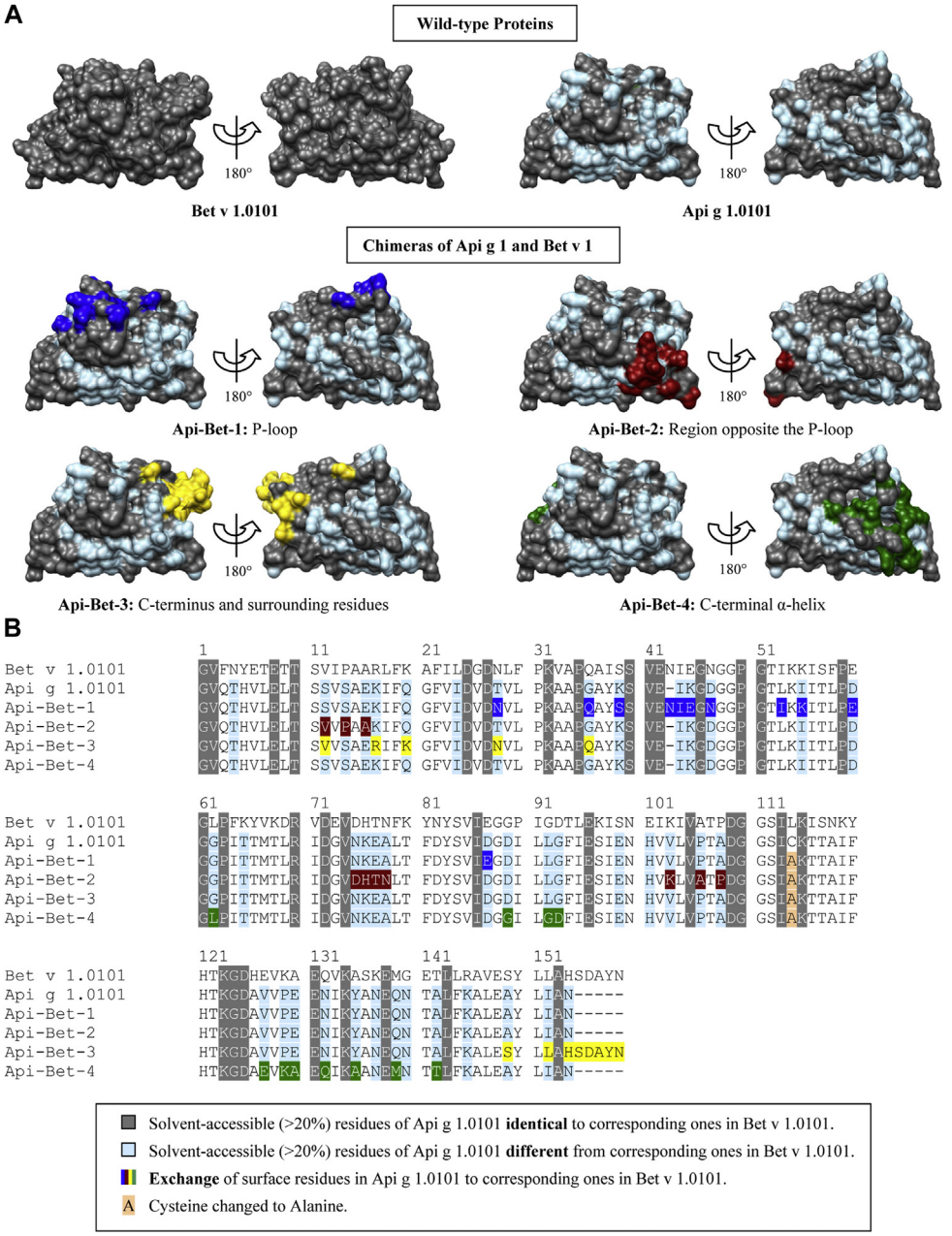

FIG 1. Sequence and structural comparison of the chimeras. **A,** Front and back views (rotated by 180° around a vertical axis) of the parent molecules and the chimeras are depicted. *Colors* indicating mutated residues were mapped onto the Api g 1 surface. The models were prepared with UCSF Chimera.[28] **B,** Multiple sequence alignment of proteins.

only 5 (8%) of 64 sera exclusively recognized the grafted regions of a single chimeric protein. Furthermore, 19 and 17 (30% and 27%, respectively) sera showed increased IgE binding to 2 or 3 chimeras, respectively, compared with rApi g 1.0101. The highest number of patients possessed IgE directed to all 4 grafted regions (20/64 [31%]). Only 3 (5%) sera did not bind to any grafted region.

Inhibition ELISA confirms IgE specificity for the grafted areas

Data of 4 representative sera are depicted in Fig 3. Direct ELISA data (Fig 3, *A*) show the highly patient-specific IgE-binding patterns to the chimeric proteins. The percentage of Bet v 1–specific IgE that bound to the chimeras was tested by using an inhibition ELISA (Fig 3, *B*) in which rBet v 1.0101 was coated to the solid phase. Self-inhibition of rBet v 1.0101 was complete at inhibitor concentrations of 10 μg/mL. In most cases the chimeras did not reach saturating inhibition, even at 100 μg/mL. Maximum inhibition values ranged from 7% to 71%, depending on the serum and the inhibitor protein. A mixture of all 4 chimeras inhibited IgE binding to Bet v 1 by 55% to 77%. In contrast, rApi g 1 inhibited IgE binding to rBet v 1 by only 2% to 27%.

The 4 chimeras were coated to the solid phase to examine the percentage of chimera-specific IgE that bound to the grafted areas

TABLE I. Frequencies of IgE binding among patients with birch pollen allergy (n = 64) to rBet v 1, rApi g 1, and the chimeras determined by means of ELISA

	Frequencies of recognition	Frequencies of IgE binding to grafted regions*
rBet v 1	100%	100%
rApi g 1	44%	—
Api-Bet-1	89%	81%
Api-Bet-2	80%	70%
Api-Bet-3	78%	75%
Api-Bet-4	48%	45%

*Percentage of patients with IgE binding significantly increased compared with rApi g 1.

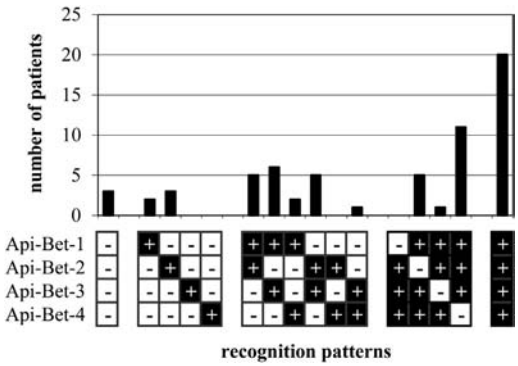

FIG 2. Patient-specific patterns of IgE binding to the grafted regions of chimeric proteins. IgE binding of Bet v 1–sensitized patients' sera (n = 64) was determined by means of IgE ELISA. The OD values obtained with rApi g 1 were subtracted, and significantly positive values were counted.

(Fig 3, *C*). Inhibition was performed with rBet v 1.0101, rApi g 1.0101, and the immobilized chimeras. In all cases almost complete inhibition of IgE binding to the chimeras by rBet v 1.0101 was observed. The extent to which rApi g 1.0101 inhibited IgE binding to the chimeras showed large differences between the tested sera. Inhibitions with 3 sera (4, 20, and 31) yielded percentages between 10% and 68%, with the exception of a single high value. In contrast, inhibitions with serum 2 resulted in high extents of inhibition (82% to 91%) for all 4 chimeras, indicating low percentages of IgE binding to the grafted areas.

Furthermore, ELISA inhibitions were performed in which IgE binding to each chimera was inhibited by all other chimeras (see Fig E5, *B*, in this article's Online Repository at www.jacionline.org). We observed partial cross-reactivity between Api-Bet-1 and Api-Bet-4, as well as between Api-Bet-2 and Api-Bet-3, for some sera.

The amount of Bet v 1–specific IgE correlates with the number of chimeras recognized

The number of chimeras to which IgE binding to the grafted region was detected and the amount of Bet v 1–specific IgE in patients' sera showed a significant correlation ($r = 0.35$, $P = .01$; see Fig E4 in this article's Online Repository at www.jacionline.org). Comparing the OD values of the Bet v 1–specific IgE ELISA after 1 hour yielded a median OD of 1.03 for sera that recognized no chimeras, whereas this value was 1.98 and 2.39 for sera binding to 3 or all 4 chimeras, respectively.

DISCUSSION

Thus far, little is known about the distribution of IgE-binding epitopes on the surface of the major birch pollen allergen Bet v 1. We grafted defined Bet v 1 surface areas onto the structurally homologous celery allergen Api g 1.0101, which has a much lower capacity to bind IgE from patients with birch pollen allergy. We then used these chimeric proteins to analyze IgE binding to the grafted areas for a large group of patients with birch pollen allergy. A similar approach was used to investigate IgE binding to the P-loop[20] and other relevant single amino acid residues of Bet v 1[21,22] or other allergens.[29-31] However, we are the first to analyze IgE binding to a large portion of the solvent-exposed surface area of Bet v 1.0101.

The combined mutated residues of all 4 chimeric allergens, including residues conserved between Bet v 1.0101 and Api g 1.0101, comprised more than 80% of the molecular surface of Bet v 1.0101. To obtain reliable data, we ensured that the recombinant proteins folded correctly. We checked the secondary structures of the chimeras using circular dichroism spectroscopy and obtained spectra highly similar to that of rApi g 1 (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). Furthermore, we performed an ELISA with 2 Bet v 1–specific mAbs that bound to rBet v 1 and Api-Bet-1 but not to the other chimeras and rApi g 1 (see Fig E2 in this article's Online Repository at www.jacionline.org). Moreover, a Bet v 1–specific, recombinant, human single-chain variable antibody fragment (manuscript in preparation) exclusively bound to rBet v 1 and Api-Bet-3. These experiments proved that Api-Bet-1 and Api-Bet-3 contained single Bet v 1–like regions on their surfaces, which were responsible for their specific antibody-binding abilities. The correct fold of the chimeras was further substantiated by the fact that the amounts of IgE binding to all chimeras were equal or greater than the amounts of rApi g 1–specific IgE in 62 of 64 sera (see Table E3 in this article's Online Repository at www.jacionline.org).

Several studies aiming to map IgE-binding epitopes on Bet v 1.0101 were performed. The first IgE-binding epitope on Bet v 1 was located by means of crystallization of an antibody-antigen complex and comprised an area covering the P-loop.[11] The crucial role of this conserved region for IgE binding was confirmed in several studies.[11,20,32] Furthermore, the fact that an mAb binding to this epitope was able to inhibit specific IgE binding by approximately 40% supported the concept that a few epitopes dominated the IgE response to Bet v 1.[14]

The P-loop of Api g 1.0101 is different because it harbors a positively charged lysine instead of a negatively charged glutamic acid at the corresponding position in Bet v 1.0101. We investigated in detail IgE binding to the P-loop of Bet v 1.0101 by replacing 11 amino acids of Api g 1.0101 by the corresponding Bet v 1.0101–derived residues (Fig 1). The Api g 1 derivative generated was termed Api-Bet-1, and 81% of the patients with birch pollen allergy showed higher IgE binding to this chimera than to rApi g 1.0101 (Table I). Surprisingly, only 2 of 64 patients exclusively recognized Api-Bet-1, indicating that the area around the P-loop is by far not the only region important for IgE binding to Bet v 1.0101 (Fig 2). This prediction is corroborated by the fact that, contrary to Api g 1.0101, the isoallergen Api g 1.0201, which comprises a P-loop similar to Bet v 1.0101,[23] has a low IgE-binding capacity.

In contrast to Bet v 1.0101, Api g 1.0101 has a C-terminus shortened by 5 residues. In Api-Bet-3 these residues were added and another 8 amino acids were mutated to obtain a Bet v

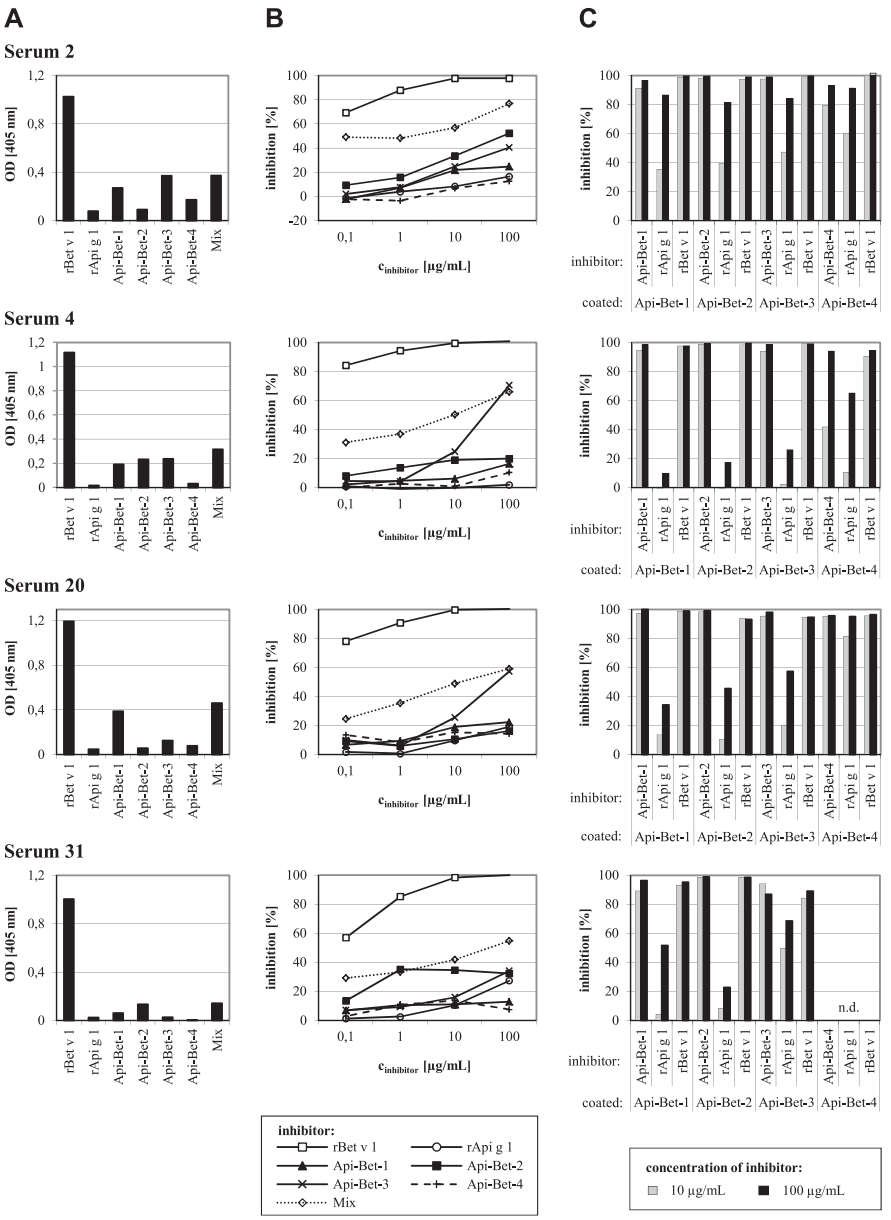

FIG 3. ELISA data of 4 representative patients. **A,** Direct ELISA showing IgE binding to rBet v 1.0101, rApi g 1.0101, the chimeras, and a mix of all chimeras (nonnormalized OD values). **B** and **C,** Inhibition of IgE binding to immobilized rBet v 1.0101 (Fig 3, *B*) or the chimeras (Fig 3, *C*) by means of preincubation with rBet v 1.0101 (positive control), rApi g 1.0101, and the chimeras. *n.d.*, Not done.

1–specific area around the C-terminus (Fig 1). Contrary to Neudecker et al,[13] who showed that removing the C-terminal residues 155-159 from Pru av 1.0101 did not affect IgE reactivity, we observed an increased recognition of Api-Bet-3 compared with rApi g 1.0101 for 75% of the sera (Table I). Furthermore, Api-Bet-3 exhibited the highest IgE binding capacity (see Fig E3) and was able to inhibit IgE binding to rBet v 1 by up to 70% (Fig 3, *B*).

For Api-Bet-4, 11 amino acids were mutated in Api g 1.0101 to create a Bet v 1–specific area around the C-terminal α helix (Fig 1). Compared with the other chimeras, Api-Bet-4 bound the lowest amount of IgE, and only 45% of the patients showed enhanced IgE binding to this chimera compared with rApi g 1.0101. In a recent study[22] an rBet v 1–specific IgE antibody was selected from a phage library constructed from IgE-encoding cDNAs isolated from Bet v 1–sensitized patients. This antibody bound to the C-terminal helix of Bet v 1, but not to Mal d 1, which was also proved by grafting the C-terminal helix of Bet v 1 onto Mal d 1. However, the significance of this epitope was not tested with patients' sera.

In our study we showed that all the chimeras, each of which was bearing a distinct Bet v 1–specific surface area, bound IgE from a high percentage of patients' sera. In line with our data, previous studies showed that the entire surface of a protein is potentially antigenic.[33] Interestingly, 31% of patients with birch pollen allergy recognized all 4 grafted areas, indicating that the immune response to Bet v 1.0101 is highly polyclonal. Nevertheless, we observed a total of 12 different recognition profiles in our patient

sample, with the number of bound chimeras between 0 and 4 (Fig 2).

Consistent with our results, a high patient-to-patient variation was also observed when investigating IgE binding to the P-loop mutants of Pru av 1 and Api g 1.0101.[13,23,34] Furthermore, Holm et al[20] observed patient-specific IgE repertoires by grafting of a Bet v 1–specific epitope containing the P-loop onto Mal d 1. Moreover, IgE binding to various Bet v 1 mutants displayed divergent recognition patterns,[16,35] and high heterogeneity of IgE specificity could also be observed by comparing IgE binding to Bet v 1 and homologous food proteins.[19] However, most of these studies were performed with small patient groups.

In a recent study of allergen-specific IgE from patients sensitized to the major house dust mite allergen Der p 2, it was shown that the complexity of the allergic patients' IgE repertoire correlated with the serum concentration of allergen-specific IgE.[36] In accordance with this finding, we revealed that the amount of Bet v 1–specific IgE present in patients' sera correlated with the number of chimeras recognized, a value representing the complexity of the IgE epitope repertoire (see Fig E4).

Reduction of IgE cross-linking on the surfaces of mast cells or basophils by vaccine components during specific immunotherapy is crucial for preventing severe side effects. Therefore characterization of IgE-binding epitopes is of paramount importance for developing artificial hypoallergens or peptide vaccines for safer and more effective immunotherapy. Mapping of IgE epitopes by using a chimera-based approach offers the possibility of analyzing a defined area (eg, a single IgE epitope) of an allergen with polyclonal patients' sera. Thus this technology might be used as diagnostic tool to determine the patient-specific response to defined epitopes of a major allergen or to cross-reactive homologs. In addition, this will pave the way for a patient-tailored epitope-based therapy.

Taken together, this study demonstrates that it is possible to graft defined areas of a major allergen onto a low IgE-binding homolog to evaluate IgE binding to the grafted region. Furthermore, investigation of a large group of patients with birch pollen allergy showed that the repertoire of Bet v 1–specific IgE is highly patient specific and polyclonal. A single major epitope on Bet v 1.0101 important for all patients with birch pollen allergy does not exist, and relevant IgE-binding epitopes are located across the entire surface of Bet v 1.0101.

Key messages

- **The Bet v 1–specific IgE response is polyclonal, and the recognition profile is highly patient specific.**
- **The existence of a single major IgE epitope on Bet v 1 can be excluded.**
- **The IgE epitopes are distributed across the entire surface of Bet v 1.**

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ONLINE REPOSITORY

Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in birch pollen-allergic patients

Barbara Gepp MSc[1], Nina Lengger[1], Merima Bublin PhD[1], Wolfgang Hemmer PhD[2], Heimo Breiteneder PhD[1], Christian Radauer PhD[1]

[1]Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria
[2]Floridsdorfer Allergiezentrum, Vienna, Austria

Corresponding author:
Heimo Breiteneder PhD
Department of Pathophysiology and Allergy Research, Medical University of Vienna,
Währinger Gürtel 18-20, 1090 Vienna, Austria.
Tel.: +43 1 40400 5102; Fax: +43 1 40400 5130.
E-mail address: heimo.breiteneder@meduniwien.ac.at

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METHODS

Cloning, Expression and Purification of Recombinant Proteins

Expression constructs were generated by inserting synthetic codon-optimized DNA molecules (Eurofines MWG Operon, Ebersberg, Germany) into the *Escherichia coli* vectors pET28a(+) (Novagen, Madison, WI, USA) for Bet v 1.0101, Api g 1.0101, Api-Bet-1 and Api-Bet-4 (NcoI/EcoRI used) and pET32a(+) (Novagen) for Api-Bet-2 and Api-Bet-3 (BglII/EcoRI used). Proteins expressed in the latter vector yield fusion proteins with an N-terminal 6x histidine tag and thioredoxin. For removal of the His-tagged thioredoxin an oligonucleotide encoding a PreScission Protease (GE Healthcare, Little Chalfont, UK) cleavage site (5'-LeuGluValLeuPheGln/GlyPro-3') was inserted directly upstream of the Api-Bet-2 and -3 genes between the NcoI and BglII restriction sites.

All chimeras, Bet v 1.0101 and Api g 1.0101 were expressed in freshly transformed *Escherichia coli* BL21 [DE3] (Novagen, Madison, WI) and grown at 30° C in LB-medium supplemented with 25 mg/L kanamycin (Bet v 1.0101, Api g 1.0101, Api-Bet-1 and Api-Bet-4) or at 20°C in LB-medium supplemented with 100 mg/L ampicillin (Api-Bet-2 and -3). Protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside at an OD_{600} of 0.8. After overnight expression, the cells were harvested by centrifugation. The pellets were resuspended in 50 mM Tris/HCl pH 8.0, 10 mM EDTA, 10 mM DTT supplemented with protease inhibitor tablets (Roche Diagnostics, Mannheim, Germany) for rBet v 1.0101, rApi g 1.0101, Api-Bet-1 and -4 or 50 mM Tris/HCl pH 8.0, 300 mM NaCl supplemented with protease inhibitor tablets for Api-Bet-2 and -3 and lysis was performed using a French Pressure Cell (SLM Aminco, Rochester, NY, USA).

Purification of rBet v 1.0101, rApi g 1.0101, Api-Bet-1 and -4 was achieved by hydrophobic interaction chromatography (Phenyl Sepharose; GE Healthcare, Little Chalfont, UK), anion exchange chromatography (Q-Sepharose; GE Healthcare) and size exclusion chromatography (Sephacryl S-200; GE Healthcare). For Api-Bet-2 and -3, the major part of the fusion proteins remained in the pellet. The soluble fraction was directly loaded onto a Ni^{2+}-based immobilized metal ion affinity chromatography column (IMAC-Sepharose; GE Healthcare). The insoluble fraction was denatured with 50 mM Tris/HCl pH 8.0, 8 M Urea, 300 mM NaCl, loaded onto the IMAC-column and renatured in 50 mM Tris/HCl, 300 mM NaCl, 5% glycerol by decreasing the urea concentration from 8 M to 0 M within 120 minutes at 0.8 mL/min. Elution was achieved with 100 mM EDTA pH 8,0. Purification of Api-Bet-2 and -3 was obtained after cleavage of the fusion proteins with PreScission Protease (GE Healthcare), removal of thioredoxin with IMAC-Sepharose (GE Healthcare) and removal of PreScission Protease with affinity (GSTrap, GE Healthcare) or size exclusion chromatography (Sephacryl S-200; GE

Healthcare). The purified proteins were dialyzed against 10 mM sodium phosphate buffer pH 7.4 and stored at -20°C.

Physicochemical analysis of the recombinant proteins

Purity of the recombinant proteins was analyzed by SDS-PAGE and Coomassie Brilliant Blue R-250 staining. The identity was checked by Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany). Secondary structures were checked by circular dichroism spectroscopy and recorded in 10 mM sodium phosphate buffer pH 7.4 at 0.2 mg/mL in a 0.1-cm quartz cuvette using a J-810 spectopolarimeter (Jasco, Easton, MD, USA).

ELISA with monoclonal anti-Bet v 1 antibodies

Microtiter plates (Maxisorp, Nalge Nunc International, Roskilde, Denmark) were coated overnight at 4°C using 2 µg/mL recombinant protein in 50 mM sodium carbonate buffer, pH 9.6. After blocking of non-specific binding sites, cell culture supernatants of BIP 1 and BIP 4[1] (1:10 dilution) were incubated in duplicates overnight at 4°C. Bound antibodies were detected by a 1:5000 diluted alkaline phosphatase-conjugated rabbit anti-mouse IgM+IgG antibody (Jackson ImmunoResearch, Suffolk, UK) followed by color development using Sigma FAST p-nitrophenyl phosphate tablets (Sigma-Aldrich, St. Loise, MO, USA) and measurement of the absorbance at 405nm.

RESULTS

Biochemical characterization of the recombinant proteins

To confirm structural integrity of the recombinant proteins, the presence of secondary structure elements was analyzed by CD-spectroscopy. rBet v 1.0101, rApi g 1.0101 and the chimeric proteins showed nearly identical CD-spectra typical for folded proteins with mixed α-β structures (Fig. E1A). Api-Bet-2 and Api-Bet-3 were obtained after denaturing purification and in vitro refolding. Nevertheless, their CD-spectra were identical to those of protein preparations obtained after native purification (Fig. E1B).

Furthermore, MALDI-TOF mass spectrometry was used to show the integrity of the recombinant proteins. The molecular masses of the chimeric proteins were measured as follows (theoretical mass is given in parentheses): rBet v 1.0101 (17439) 17439, rApi g 1.0101 (16189) 16190, Api-Bet-1 (16358) 16359, Api-Bet-2 (16461) 16465, Api-Bet-3 (17157) 17154, Api-Bet-4 (16115) 16113. These values confirm the correct cleavage of Api-Bet-2 and Api-Bet-3 from thioredoxin and prove that the initial methionine of Api-Bet-1 and Api-Bet-4 was removed.

In order to confirm the structural integrity of the chimeric proteins, we performed an ELISA with the Bet v 1-specific monoclonal antibodies BIP 1 and BIP 4[1] (Fig. E2). Both antibodies bound to rBet v 1 and Api-Bet-1, but not to rApi g 1 and the other chimeras.

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TABLES

TABLE E1: Clinical data of the patients included in this study.

#	Age	Sex	Food allergies	Positive skin prick test[a]	Total IgE [kU/l]	Bet v 1 CAP [kU/l]
1	17	m	n.k.	bi ash gr hdm	96	35.4
2	34	m	apple, pear, nuts	bi hdm	310	77.5
3	51	f	n.k.	bi gr hdm ca do	29	11.2
4	24	m	apple, nuts, kiwi	bi ash gr	605	82.5
5	7	m	kiwi	bi ash gr	290	>100
6	25	m	n.k.	bi ash gr rag pla	85	14.0
7	13	f	n.k.	bi gr mug ash hdm ca do alt	1100	35.4
8	25	m	no	bi gr hdm ca do	57	4.2
9	42	m	n.k.	bi hdm	66	45.1
10	62	f	no	bi	82	4.2
11	46	m	apple	bi ash gr	102	11.2
12	34	m	no	bi ash gr pla hdm	135	14.0
13	29	f	no	bi gr	82	8.4
14	16	m	apple, citrus	bi gr ca alt	245	14.0
15	40	f	no	bi gr mug rag hdm	315	>100
16	16	m	n.k.	bi ash gr	300	92.5
17	61	f	n.k.	bi ca	860	>100
18	43	f	n.k.	bi mug pla	21	7.0
19	34	m	no	bi ash gr rag pla ca do alt	250	45.1
20	55	f	apple, pear, nuts, soja	bi	154	87.5
21	50	f	no	bi hdm	130	16.8
22	24	m	n.k.	bi ash	31	22.4
23	36	m	n.k.	bi ash pla	120	19.1
24	38	f	n.k.	bi gr mug ca ho	925	87.5
25	33	f	n.k.	bi alt	14	7.0
26	10	m	n.k.	bi ash mug gr hdm ca	157	12.6
27	8	m	apple, cherry	bi gr hdm alt	66	35.4
28	34	f	apple, pear, stone fruit, nuts	bi gr hdm ca	246	41.9
29	47	f	apple, peach, kiwi	bi gr ca do hdm	272	>100
30	18	f	no	bi gr mug hdm ca	148	9.8
31	79	m	apple	bi ash gr mug ca do pla	810	45.1
32	30	f	no	bi gr	98	25.6
33	30	f	spinach	bi gr ash rap pla mug	240	35.4
34	29	f	apple, carrot	bi gr pla mug hdm	63	25.6
35	41	f	n.k.	bi	50	22.4
36	15	f	apricot, peanut	bi gr ash pla hdm	803	25.6
37	38	m	n.k.	bi gr ca do	1330	32.1
38	13	f	n.k.	bi ash gr mug rag	910	>100

#	Age	Sex	Food allergies	Positive skin prick test[a]	Total IgE [kU/l]	Bet v 1 CAP [kU/l]
39	36	m	apple	bi ash gr hdm	92	52.5
40	34	f	n.k.	bi ash gr hdm	165	62.5
41	27	f	apple, pear, stone fruit, nuts	bi ash gr mug rag	380	>100
42	22	f	no	bi	56	25.6
43	37	f	nuts	bi	420	>100
44	24	f	apple	bi gr pla hdm	345	15.4
45	21	m	apple, nuts	bi gr hdm ca ho	435	87.5
46	35	m	apple, nuts, carrot	n.k.	1700	>100[b]
47	30	f	nuts	n.k.	386	17.51-50[b]
48	30	f	apple, nuts	bi gr alt	38	3.51-17.5
49	28	m	apple, peach	n.k.	36	5.05[b]
50	32	f	apple, peach	n.k.	70	16
51	31	f	apple, nuts, peach	bi	4155	47.7
52	30	f	apple, nuts, peach	n.k.	31	8.57[b]
53	38	m	no	n.k.	444	76.4
54	28	m	no	bi alt	47	8.15[b]
55	28	f	apple, peach, cherry, carrot, nuts, jackfruit, tempeh	n.k.	256	11.0
56	24	f	no	n.k.	92	1.37[b]
57	41	m	apple, peach, cherry, carrot, jackfruit	n.k.	57	2.5
58	40	m	no	n.k.	83	9.74[b]
59	34	m	nuts, apple	n.k.	217	50[b]
60	49	f	apple, pear, apricot, peach, cherry, nuts	n.k.	50	14.0
61	40	f	apple, pear	n.k.	70	3.7
62	63	f	apple, celery, carrot, citrus, egg white	n.k.	158	2.3
63	67	f	apple, nuts, peach, apricot	n.k.	34	12.3
64	43	m	apple, nuts, melon, banana	n.k.	559	25.1

[a] **bi:** birch, **gr**: grass, **mug**: mugwort, **pla**: plantain, **rag**: ragweed, **rap**: rape, **ca**: cat, **do**: dog, **ho**: horse, **hdm**: house dust mite, **alt**: Alternaria

[b] CAP to birch pollen

n.k.: not known

TABLE E2: Raw and normalized IgE-ELISA OD-values of sera from all allergic patients' (1-64) and non-allergic individuals' (NHS1-NHS7) sera included in this study.

	Measured ELISA OD values (raw data) after subtracting buffer control values							Normalized ELISA OD/h					
Serum #	rBet v 1	rApi g 1	Api-Bet-1	Api-Bet-2	Api-Bet-3	Api-Bet-4	Substrate incubation period [min]	rBet v 1	rApi g 1	Api-Bet-1	Api-Bet-2	Api-Bet-3	Api-Bet-4
1	0,856	0,039	0,204	0,060	0,183	0,113	10	5.134	0.237	1.222	0.362	1.097	0.677
2	1,250	0,083	0,308	0,210	0,423	0,198	15	5.001	0.334	1.231	0.840	1.693	0.793
3	0,936	-0,001	0,014	0,118	0,308	0,018	30	1.872	-0.001	0.028	0.237	0.616	0.036
4	1,617	0,006	0,303	0,277	0,322	0,050	15	6.469	0.024	1.211	1.110	1.288	0.198
5	0,897	0,008	0,048	0,253	0,481	0,050	7	7.690	0.072	0.409	2.165	4.123	0.432
6	0,820	0,086	0,280	0,076	0,113	0,165	30	1.641	0.172	0.560	0.153	0.226	0.330
7	0,913	0,007	0,189	0,113	0,107	0,014	15	3.651	0.029	0.757	0.451	0.429	0.055
8	0,675	0,024	0,038	0,044	0,056	0,076	60	0.675	0.024	0.038	0.044	0.056	0.076
9	0,878	0,118	0,328	0,148	0,293	0,319	25	2.106	0.283	0.787	0.354	0.703	0.767
10	0,893	0,003	0,337	0,073	0,141	0,013	60	0.893	0.003	0.337	0.073	0.141	0.013
11	1,016	0,014	0,093	0,098	0,021	0,025	30	2.032	0.028	0.187	0.195	0.041	0.051
12	1,812	0,054	0,332	0,037	0,113	0,112	7	15.532	0.461	2.844	0.316	0.970	0.964
13	0,856	0,018	0,015	0,090	0,029	0,033	40	1.284	0.028	0.023	0.135	0.043	0.049
14	0,929	0,067	0,068	0,076	0,161	0,200	30	1.857	0.134	0.137	0.152	0.322	0.400
15	0,915	0,006	0,010	0,072	0,125	0,012	10	5.489	0.037	0.060	0.434	0.752	0.069
16	1,145	0,017	0,389	0,067	0,119	0,132	10	6.868	0.105	2.334	0.403	0.714	0.792
17	1,045	0,038	0,224	0,048	0,121	0,084	7	8.953	0.330	1.918	0.409	1.039	0.718
18	0,817	0,039	0,127	0,127	0,186	0,100	45	1.090	0.052	0.169	0.170	0.249	0.134
19	0,974	0,003	0,173	0,046	0,015	0,003	20	2.922	0.009	0.519	0.138	0.046	0.010
20	1,067	0,018	0,327	0,113	0,124	0,106	15	4.270	0.070	1.309	0.451	0.495	0.423
21	0,915	0,035	0,158	0,137	0,248	0,108	25	2.195	0.083	0.380	0.328	0.596	0.260
22	0,901	-0,003	-0,004	0,127	0,085	-0,001	20	2.702	-0.009	-0.013	0.381	0.255	-0.002
23	0,939	0,002	0,056	0,059	0,011	-0,002	25	2.254	0.005	0.133	0.142	0.026	-0.005
24	0,754	0,028	0,125	0,103	0,244	0,134	20	2.263	0.083	0.376	0.308	0.731	0.401
25	0,771	0,021	0,018	0,004	0,034	0,006	45	1.027	0.028	0.024	0.006	0.046	0.008
26	0,897	0,014	0,024	0,092	0,180	0,030	35	1.538	0.024	0.041	0.158	0.308	0.052
27	0,981	0,009	0,162	0,115	0,207	0,068	25	2.355	0.022	0.388	0.275	0.498	0.162
28	1,045	0,019	0,113	0,166	0,276	0,045	20	3.135	0.057	0.339	0.498	0.828	0.136
29	1,508	0,035	0,325	0,086	0,132	0,047	15	6.0324	0.139	1.3008	0.3456	0.5272	0.1898
30	0,837	0,024	0,044	0,284	0,058	0,039	30	1.673	0.048	0.089	0.567	0.117	0.077
31	1,167	0,007	0,038	0,212	0,028	0,010	15	4.666	0.027	0.152	0.848	0.111	0.038
32	1,023	0,015	0,023	0,029	0,050	0,018	15	4.094	0.059	0.090	0.117	0.200	0.073
33	0,843	0,014	0,094	0,028	0,051	0,007	25	2.023	0.035	0.225	0.067	0.122	0.018
34	0,723	0,127	0,349	0,037	0,034	0,204	45	0.965	0.169	0.465	0.049	0.045	0.272
35	1,035	0,008	0,119	0,025	0,023	0,007	20	3.105	0.025	0.357	0.074	0.070	0.022
36	0,844	0,002	0,000	0,134	0,173	0,002	40	1.266	0.003	0.000	0.202	0.260	0.003
37	0,937	0,014	0,146	0,087	0,155	0,133	30	1.875	0.029	0.291	0.175	0.310	0.265
38	1,031	0,020	0,073	0,200	0,171	0,119	10	6.189	0.119	0.436	1.198	1.026	0.715
39	0,825	-0,002	0,060	0,207	0,296	0,032	25	1.979	-0.004	0.145	0.496	0.710	0.076
40	0,805	0,002	0,094	0,147	0,166	0,050	20	2.416	0.007	0.283	0.441	0.498	0.151

	Measured ELISA OD values (raw data) after subtracting buffer control values							Normalized ELISA OD/h					
Serum #	rBet v 1	rApi g 1	Api-Bet-1	Api-Bet-2	Api-Bet-3	Api-Bet-4	Substrate incubation period [min]	rBet v 1	rApi g 1	Api-Bet-1	Api-Bet-2	Api-Bet-3	Api-Bet-4
41	0,985	0,034	0,212	0,095	0,054	0,258	15	3.940	0.136	0.848	0.380	0.217	1.031
42	0,949	0,167	0,428	0,154	0,295	0,548	25	2.277	0.400	1.028	0.370	0.709	1.315
43	0,857	0,014	0,009	0,027	0,016	0,026	15	3.429	0.057	0.038	0.108	0.064	0.105
44	0,681	-0,009	0,088	-0,003	-0,004	-0,001	50	0.818	-0.011	0.106	-0.004	-0.005	-0.001
45	0,976	0,023	0,270	0,026	0,062	0,185	25	2.343	0.056	0.649	0.063	0.148	0.443
46	0,872	0,023	0,045	0,079	0,126	0,061	10	5.231	0.137	0.269	0.475	0.754	0.363
47	0,981	-0,008	0,151	0,103	0,238	0,002	35	1.681	-0.013	0.258	0.176	0.408	0.003
48	0,787	0,024	0,122	0,064	0,091	0,094	75	0.630	0.019	0.097	0.051	0.073	0.075
49	0,850	-0,008	0,017	0,029	0,040	0,025	45	1.133	-0.011	0.022	0.039	0.054	0.033
50	0,835	0,009	0,146	0,066	0,017	0,004	45	1.113	0.012	0.195	0.087	0.022	0.005
51	0,909	0,028	0,138	0,076	0,195	0,200	40	1.364	0.043	0.207	0.114	0.292	0.300
52	0,786	-0,016	0,025	0,052	0,062	-0,008	50	0.943	-0.020	0.030	0.062	0.075	-0.009
53	1,073	0,009	0,050	0,024	0,043	0,026	25	2.576	0.022	0.120	0.057	0.104	0.061
54	0,985	-0,003	0,012	0,064	0,135	0,048	40	1.478	-0.005	0.019	0.096	0.202	0.072
55	0,331	-0,012	-0,010	0,001	0,001	-0,011	75	0.265	-0.010	-0.008	0.001	0.001	-0.009
56	0,437	-0,010	0,076	0,001	0,049	-0,009	75	0.349	-0.008	0.061	0.001	0.040	-0.007
57	0,393	0,023	0,078	0,041	0,091	0,096	75	0.314	0.018	0.062	0.033	0.073	0.077
58	0,861	-0,013	-0,008	0,081	0,116	0,016	45	1.148	-0.017	-0.011	0.108	0.155	0.021
59	1,060	-0,006	0,055	0,397	0,463	0,248	20	3.179	-0.018	0.166	1.191	1.388	0.743
60	0,874	-0,001	0,010	0,088	0,099	0,033	40	1.311	-0.001	0.015	0.131	0.148	0.049
61	0,586	-0,011	-0,013	0,017	0,010	-0,004	75	0.468	-0.009	-0.011	0.013	0.008	-0.003
62	0,383	-0,012	-0,019	0,010	-0,004	-0,009	75	0.307	-0.010	-0.015	0.008	-0.003	-0.007
63	0,952	0,005	0,047	0,207	0,144	0,011	40	1.428	0.008	0.070	0.310	0.216	0.017
64	0,889	0,011	0,025	0,036	0,060	0,035	40	1.333	0.016	0.038	0.054	0.091	0.053
NHS 1	-0.001	0.008	0.000	-0.011	0.011	0.012	60	-0.001	0.008	0.000	-0.011	0.011	0.012
NHS 2	-0.005	0.007	-0.002	-0.011	0.016	0.000	60	-0.005	0.007	-0.002	-0.011	0.016	0.000
NHS 3	-0.006	0.008	-0.003	-0.016	0.006	0.007	60	-0.006	0.008	-0.003	-0.016	0.006	0.007
NHS 4	0.017	0.008	0.003	0.001	0.022	0.030	60	0.017	0.008	0.003	0.001	0.022	0.030
NHS 5	-0.010	0.003	-0.004	-0.015	0.005	-0.003	60	-0.010	0.003	-0.004	-0.015	0.005	-0.003
NHS 6	-0.010	0.014	-0.002	-0.017	0.001	0.004	60	-0.010	0.014	-0.002	-0.017	0.001	0.004
NHS 7	-0.008	0.002	-0.002	-0.020	-0.005	-0.001	60	-0.008	0.002	-0.002	-0.020	-0.005	-0.001

TABLE E3: Amounts of allergen-specific IgE from all allergic patients' (1-64) and non-allergic individuals' (NHS1-NHS7) sera included in this study. OD values of the IgE ELISAs specific for rBet v 1.0101, rApi g 1.0101 and the four chimeras were normalized to a substrate incubation period of 1 hour after subtraction of the buffer control values.

	Normalized ELISA OD/h						OD/h chimeras – OD/h Api g 1			
Serum	**rBet v 1**	**rApi g 1**	**Api-Bet-1**	**Api-Bet-2**	**Api-Bet-3**	**Api-Bet-4**	**Api-Bet-1**	**Api-Bet-2**	**Api-Bet-3**	**Api-Bet-4**
1	**5.134**	**0.237**	**1.222**	**0.362**	**1.097**	**0.677**	**0.985**	**0.125**	**0.860**	**0.441**
2	**5.001**	**0.334**	**1.231**	**0.840**	**1.693**	**0.793**	**0.897**	**0.506**	**1.359**	**0.459**
3	**1.872**	-0.001	**0.028**	**0.237**	**0.616**	0.036	**0.029**	**0.238**	**0.618**	0.037
4	**6.469**	0.024	**1.211**	**1.110**	**1.288**	**0.198**	**1.187**	**1.086**	**1.264**	**0.174**
5	**7.690**	0.072	**0.409**	**2.165**	**4.123**	**0.432**	**0.337**	**2.093**	**4.050**	**0.360**
6	**1.641**	**0.172**	**0.560**	**0.153**	**0.226**	**0.330**	**0.388**	-0.019	0.054	**0.158**
7	**3.651**	0.029	**0.757**	**0.451**	**0.429**	0.055	**0.728**	**0.422**	**0.400**	0.026
8	**0.675**	**0.024**	**0.038**	**0.044**	**0.056**	**0.076**	**0.014**	0.020	**0.031**	**0.052**
9	**2.106**	**0.283**	**0.787**	**0.354**	**0.703**	**0.767**	**0.504**	**0.071**	**0.420**	**0.484**
10	**0.893**	0.003	**0.337**	**0.073**	**0.141**	0.013	**0.334**	**0.071**	**0.139**	0.010
11	**2.032**	0.028	**0.187**	**0.195**	0.041	0.051	**0.159**	**0.167**	0.013	0.023
12	**15.532**	**0.461**	**2.844**	**0.316**	**0.970**	**0.964**	**2.383**	*-0.146*	**0.509**	**0.502**
13	**1.284**	**0.028**	**0.023**	**0.135**	0.043	0.049	-0.005	**0.108**	0.016	0.021
14	**1.857**	**0.134**	**0.137**	**0.152**	**0.322**	**0.400**	0.002	0.018	**0.187**	**0.266**
15	**5.489**	0.037	0.060	**0.434**	**0.752**	0.069	0.023	**0.398**	**0.715**	0.032
16	**6.868**	**0.105**	**2.334**	**0.403**	**0.714**	**0.792**	**2.230**	**0.298**	**0.610**	**0.688**
17	**8.953**	**0.330**	**1.918**	**0.409**	**1.039**	**0.718**	**1.588**	0.080	**0.709**	**0.388**
18	**1.090**	**0.052**	**0.169**	**0.170**	**0.249**	**0.134**	**0.117**	**0.118**	**0.197**	**0.082**
19	**2.922**	0.009	**0.519**	**0.138**	0.046	0.010	**0.509**	**0.129**	0.037	0.001
20	**4.270**	**0.070**	**1.309**	**0.451**	**0.495**	**0.423**	**1.239**	**0.381**	**0.424**	**0.353**
21	**2.195**	**0.083**	**0.380**	**0.328**	**0.596**	**0.260**	**0.297**	**0.245**	**0.513**	**0.177**
22	**2.702**	-0.009	-0.013	**0.381**	**0.255**	-0.002	-0.003	**0.391**	**0.265**	0.008
23	**2.254**	0.005	**0.133**	**0.142**	0.026	-0.005	**0.128**	**0.137**	0.021	-0.010
24	**2.263**	**0.083**	**0.376**	**0.308**	**0.731**	**0.401**	**0.293**	**0.225**	**0.648**	**0.317**
25	**1.027**	**0.028**	**0.024**	0.006	0.046	0.008	-0.004	-0.022	0.018	-0.020
26	**1.538**	**0.024**	**0.041**	**0.158**	**0.308**	0.052	0.017	**0.133**	**0.284**	0.028
27	**2.355**	0.022	**0.388**	**0.275**	**0.498**	**0.162**	**0.366**	**0.253**	**0.475**	**0.140**
28	**3.135**	**0.057**	**0.339**	**0.498**	**0.828**	**0.136**	**0.282**	**0.441**	**0.771**	0.079
29	**6.0324**	**0.139**	**1.3008**	**0.3456**	**0.5272**	**0.1898**	**1.162**	**0.207**	**0.388**	0.051
30	**1.673**	**0.048**	**0.089**	**0.567**	**0.117**	0.077	**0.041**	**0.520**	**0.069**	0.030
31	**4.666**	0.027	**0.152**	**0.848**	0.111	0.038	**0.124**	**0.820**	0.084	0.011
32	**4.094**	0.059	**0.090**	0.117	**0.200**	0.073	**0.031**	0.058	**0.141**	0.014
33	**2.023**	0.035	**0.225**	0.067	**0.122**	0.018	**0.190**	0.033	**0.088**	-0.017
34	**0.965**	**0.169**	**0.465**	0.049	0.045	**0.272**	**0.296**	*-0.120*	*-0.124*	**0.103**
35	**3.105**	0.025	**0.357**	0.074	0.070	0.022	**0.332**	0.050	0.045	-0.003
36	**1.266**	0.003	0.000	**0.202**	**0.260**	0.003	-0.004	**0.198**	**0.257**	0.000
37	**1.875**	0.029	**0.291**	**0.175**	**0.310**	**0.265**	**0.263**	**0.146**	**0.281**	**0.236**
38	**6.189**	**0.119**	**0.436**	**1.198**	**1.026**	**0.715**	**0.317**	**1.079**	**0.907**	**0.596**

39	**1.979**	-0.004	**0.145**	**0.496**	**0.710**	0.076	**0.148**	**0.500**	**0.713**	0.080
40	**2.416**	0.007	**0.283**	**0.441**	**0.498**	**0.151**	**0.276**	**0.434**	**0.491**	**0.144**
41	**3.940**	**0.136**	**0.848**	**0.380**	**0.217**	**1.031**	**0.712**	**0.244**	0.081	**0.895**
42	**2.277**	**0.400**	**1.028**	**0.370**	**0.709**	**1.315**	**0.628**	-0.030	**0.309**	**0.915**
43	**3.429**	0.057	**0.038**	0.108	0.064	0.105	-0.019	0.051	0.007	0.048
44	**0.818**	-0.011	**0.106**	-0.004	-0.005	-0.001	**0.117**	0.008	0.006	0.010
45	**2.343**	**0.056**	**0.649**	0.063	**0.148**	**0.443**	**0.593**	0.008	**0.092**	**0.387**
46	**5.231**	**0.137**	**0.269**	**0.475**	**0.754**	**0.363**	**0.132**	**0.338**	**0.617**	**0.226**
47	**1.681**	-0.013	**0.258**	**0.176**	**0.408**	0.003	**0.272**	**0.190**	**0.422**	0.016
48	**0.630**	**0.019**	**0.097**	**0.051**	**0.073**	**0.075**	**0.078**	**0.032**	**0.054**	**0.056**
49	**1.133**	-0.011	**0.022**	0.039	**0.054**	0.033	**0.033**	0.050	**0.065**	0.044
50	**1.113**	0.012	**0.195**	**0.087**	0.022	0.005	**0.184**	**0.076**	0.011	-0.006
51	**1.364**	**0.043**	**0.207**	**0.114**	**0.292**	**0.300**	**0.164**	**0.071**	**0.250**	**0.258**
52	**0.943**	-0.020	**0.030**	**0.062**	**0.075**	-0.009	**0.049**	**0.081**	**0.094**	0.010
53	**2.576**	0.022	**0.120**	0.057	**0.104**	0.061	**0.098**	0.035	**0.082**	0.040
54	**1.478**	-0.005	**0.019**	**0.096**	**0.202**	**0.072**	**0.023**	**0.101**	**0.207**	**0.076**
55	**0.265**	-0.010	-0.008	0.001	0.001	-0.009	0.002	0.011	0.011	0.001
56	**0.349**	-0.008	**0.061**	0.001	**0.040**	-0.007	**0.069**	0.009	**0.048**	0.001
57	**0.314**	**0.018**	**0.062**	**0.033**	**0.073**	**0.077**	**0.044**	**0.015**	**0.055**	**0.059**
58	**1.148**	-0.017	-0.011	**0.108**	**0.155**	0.021	0.006	**0.125**	**0.172**	0.039
59	**3.179**	-0.018	**0.166**	**1.191**	**1.388**	**0.743**	**0.184**	**1.209**	**1.407**	**0.762**
60	**1.311**	-0.001	**0.015**	**0.131**	**0.148**	0.049	**0.016**	**0.132**	**0.149**	0.050
61	**0.468**	-0.009	-0.011	**0.013**	0.008	-0.003	-0.002	**0.022**	0.017	0.006
62	**0.307**	-0.010	-0.015	**0.008**	-0.003	-0.007	-0.006	**0.018**	0.007	0.003
63	**1.428**	0.008	**0.070**	**0.310**	**0.216**	0.017	**0.062**	**0.303**	**0.208**	0.009
64	**1.333**	0.016	**0.038**	0.054	**0.091**	0.053	**0.022**	0.038	**0.075**	0.037
NHS 1	-0.001	0.008	0.000	-0.011	0.011	0.012				
NHS 2	-0.005	0.007	-0.002	-0.011	0.016	0.000				
NHS 3	-0.006	0.008	-0.003	-0.016	0.006	0.007				
NHS 4	0.017	0.008	0.003	0.001	0.022	0.030				
NHS 5	-0.010	0.003	-0.004	-0.015	0.005	-0.003				
NHS 6	-0.010	0.014	-0.002	-0.017	0.001	0.004				
NHS 7	-0.008	0.002	-0.002	-0.020	-0.005	-0.001				

bold: significantly positive values

italics: ODs significantly lower than Api g 1-specific ODs

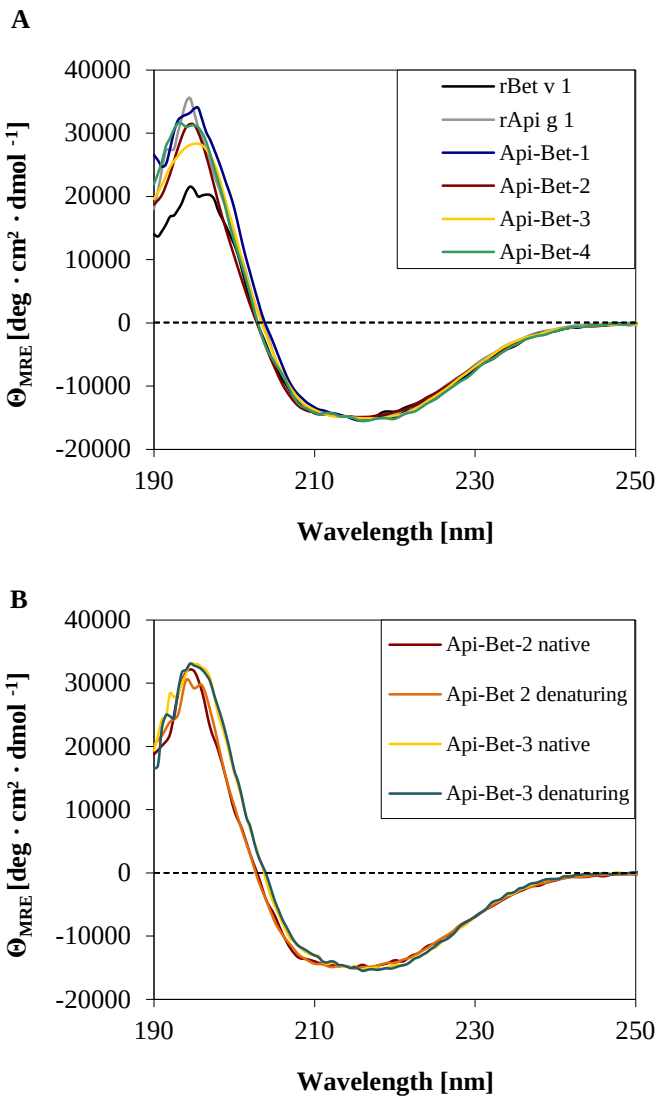

Figure E1. A. CD-spectra of rBet v1.0101, rApi g 1.0101 and the four chimeric proteins. Spectra are presented as mean residue molar ellipticity. **B.** Comparison of the CD-spectra of Api-Bet-2 and 3 purified at native and denaturing conditions.

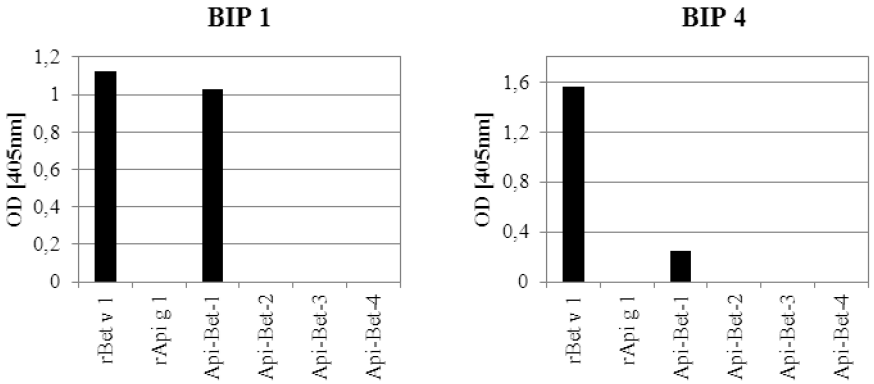

Figure E2. Binding of the Bet v 1-specific monoclonal antibodies BIP 1 and BIP 4 to rBet v 1.0101, rApi g 1.0101 and the chimeras. Antibody binding was measured by ELISA and detected by an alkaline phosphatase-labeled anti-mouse antibody.

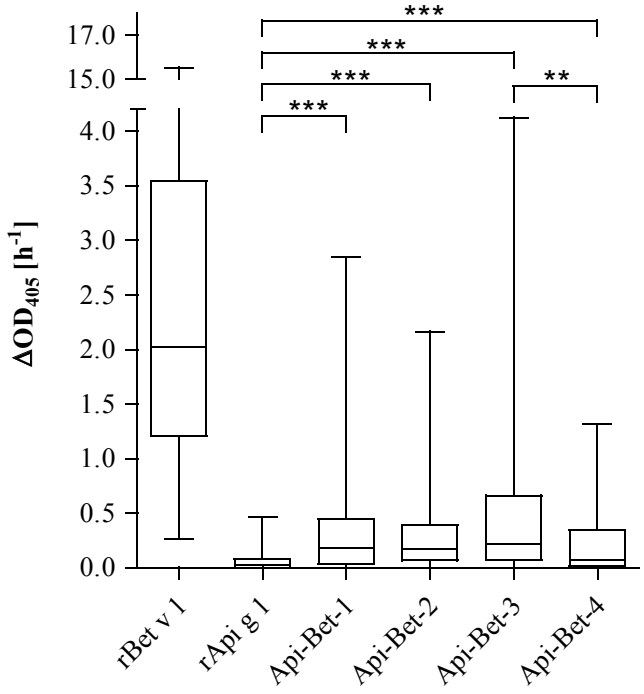

Figure E3. IgE binding of 64 sera to rBet v 1.0101, rApi g 1.0101 and the chimeras obtained by ELISA. Horizontal bars, boxes and whiskers indicate medians, quartiles and total ranges, respectively. The distributions of OD-values were compared using the Friedman test (*** $P < 0.001$, ** $P<0.01$).

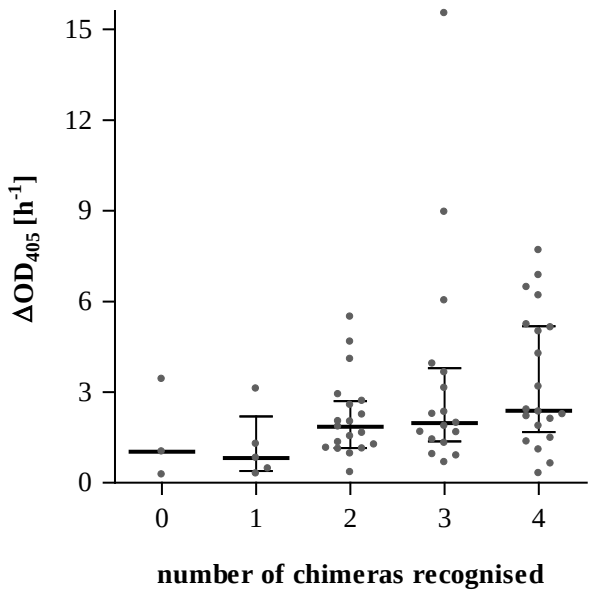

Figure E4. Correlation of the amount of Bet v 1-specific IgE with the number of chimeras recognized. OD values obtained by IgE-ELISA were normalized to serum dilutions of 1:10 and alkaline phosphatase substrate incubation times of 1 hour. Bars and whiskers represent medians and quartiles.

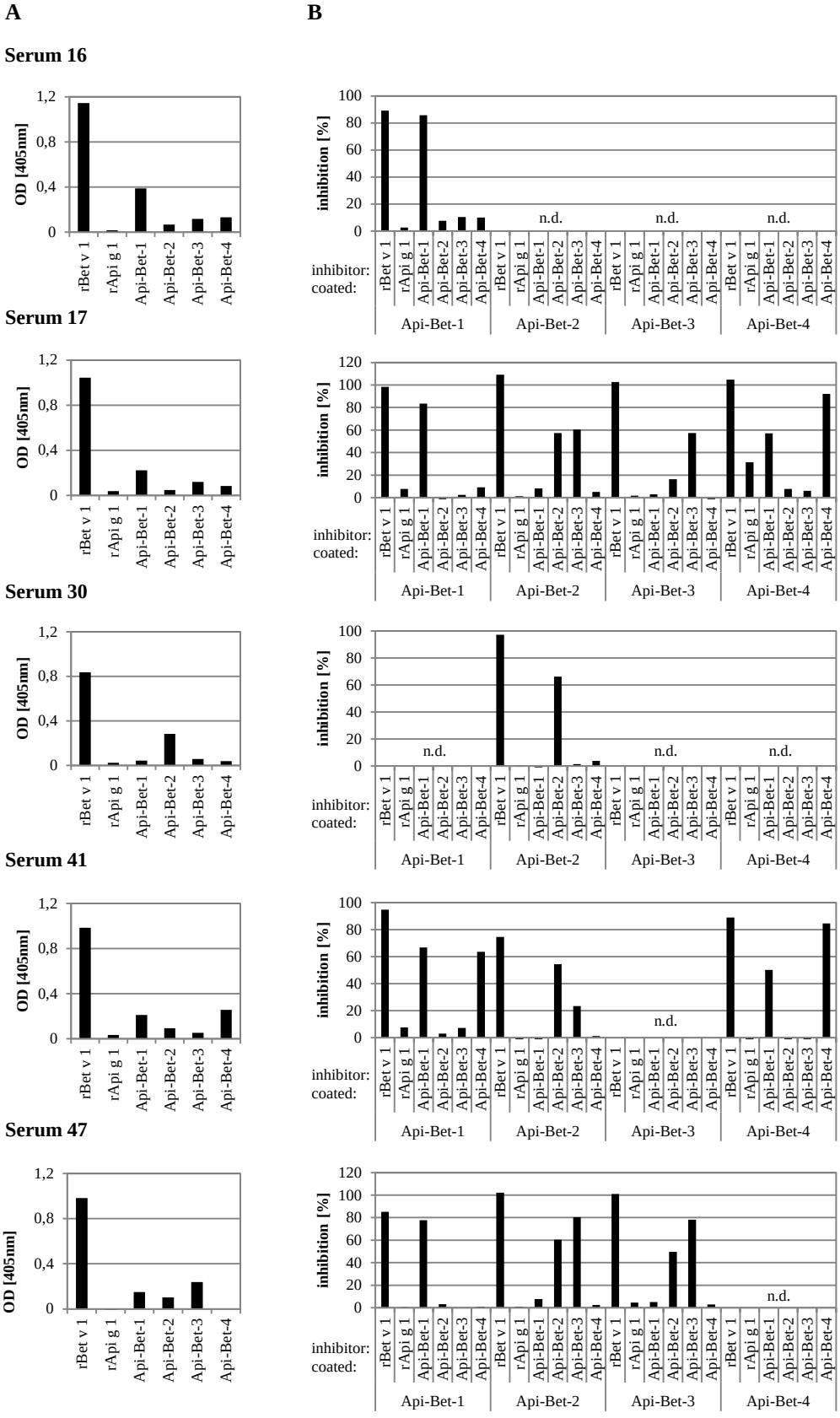

Figure E5. IgE cross-inhibition between the chimeric allergens. **A.** Direct ELISA; **B.** Inhibition ELISA. Inhibiting chimeras were coated to the solid phase at 1 µg/ml and incubated with patients' sera. The supernatants were transferred to a second plate with all four chimeras immobilized, and bound IgE was detected by an alkaline phosphatase-labeled anti-human IgE antibody. n.d.: not done

CHAPTER III:

Manuscript 2

Letter to the editor

The diversity of Bet v 1-specific IgG4 antibodies remains mostly constant during the course of birch pollen immunotherapy

Brinda Subbarayal[1,2], Dirk Schiller[3], Christian Möbs[4], Wolfgang Pfützner[4], Beatrice Jahn-Schmid[1], Barbara Gepp[1], Heimo Breiteneder[1], Stefan Vieths[3], Barbara Bohle[1,2]

[1]Department of Pathophysiologie and Allergy Research, Medical University of Vienna, Austria

[2]Christian Doppler Laboratory for Immunomodulation, Medical University of Vienna, Austria

[3]Division of Allergology, Paul-Ehrlich-Institut, Langen, Germany

[4]Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany

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The diversity of Bet v 1–specific IgG_4 antibodies remains mostly constant during the course of birch pollen immunotherapy

To the Editor:

It is well documented that allergen-specific immunotherapy (AIT) induces high titers of allergen-specific IgG antibodies, which may block IgE-mediated effector mechanisms by preventing IgE binding to allergens. Both IgG_1 and IgG_4 antibodies have been considered as blocking antibodies; however, the functional role of allergen-specific IgG_4 antibodies has been most intensely studied so far. Still, only limited data on the epitope specificity and diversity of AIT-induced allergen-specific IgG_4 antibodies exist. Recently, the antibody repertoire after a median time period of 41 months of oral AIT has been studied in a high-throughput peptide microarray by using linear peptides of major peanut allergens.[1] The authors concluded that the peanut-specific IgG_4 repertoire broadly expanded in a polyclonal fashion and included novel specificities that recognized epitopes different from IgE antibodies.[1] In a different type of AIT, namely, subcutaneous treatment with birch pollen extract (BP-AIT) containing 5% to 10% of the sole major allergen Bet v 1, we have reported that most Bet v 1–specific IgG_4 antibodies induced after 36 months recognized IgE epitopes and only a minority represented *de novo* specificities.[2] In the present longitudinal study, we sought to investigate whether the diversity of Bet v 1–specific IgG_4 antibodies expands, decreases, or remains similar in the course of BP-AIT.

Because antibody epitopes on Bet v 1 are conformational,[3-5] we performed competitive immunoscreening of phage-displayed peptides to predict IgG_4-binding areas[2,6] using sera collected after 6 and 36 months of BP-AIT from 5 BP-allergic patients with significantly improved respiratory symptoms to natural BP exposure and reduced skin prick test reactivity to BP extract.[7] Briefly, each serum sample was incubated with antihuman IgG_4-coated magnetic beads that were then used to screen a phage-displayed peptide library (Ph.D.-12 Phage Display Peptide Library, New England BioLabs, Beverly, Mass). Bound phages were specifically eluted by competition with Bet v 1. The amino acid (aa) sequences of the phage-displayed 12-mer peptides were determined. This procedure was repeated until at least 4 peptides for each serum sample were obtained (see Table E1 in this article's Online Repository at www.jacionline.org). Each peptide sequence was mapped onto the molecular surface of Bet v 1 (PDB code 1BV1) with the "EpiSearch" algorithm to predict conformational regions.[8] In total, 36 and 40 IgG_4 epitopes, each comprising 10 to 23 aa, were predicted at month 6 and 36 of BP-AIT, respectively (see Table E1). Next, we assessed the percentage of identical aa in the same IgG_4-binding Bet v 1–surface areas predicted at both time points. For patient 1, 44.7% aa were identical; for patient 2, 61.5%; for patient 3, 62.5%; for patient 4, 53.8%; and for patient 5, 36.8%. Fig 1 illustrates the entire surface area predicted to be recognized by IgG_4 antibodies on Bet v 1 at each time point and their overlap for each individual. The remarkable overlap of predicted IgG_4 epitopes at 6 and 36 months of BP-AIT in 4 of the 5 patients (80%) indicated that the early established IgG_4 repertoire did not expand further with prolonged treatment.

To complement the *in silico* data, we assessed the IgE-blocking activity by facilitated antibody-binding assays as described.[2] Briefly, each serum sample was preincubated with Bet v 1 and the same indicator serum containing more than 100 kU/L of Bet v 1–specific IgE with a presumed highly diverse IgE repertoire. Thereafter, the activity of each serum to reduce the binding of Bet v 1-IgE complexes to $CD23^+$ B cells was determined. Bet v 1–specific $IgG_{1\text{-}4}$ levels in the sera were assessed by ELISA. None of the patients developed Bet v 1–specific IgG_2 and IgG_3 antibodies (data not shown), and only patients 1, 3, and 4 developed Bet v 1–specific IgG_1 antibodies (see Fig E1 in this article's Online Repository at www.jacionline.org). Bet v 1–specific IgG_4 antibody levels were also quantified by ImmunoCAP (Thermo Fisher Scientific, Uppsala, Sweden). Table I shows that the IgE-blocking activity of sera from patient 2 had reached 89% already after 6 months of BP-AIT and was not markedly enhanced after 36 months despite further increase in Bet v 1–specific IgG_4 antibody levels. The finding that the IgE-blocking activity did not rise in parallel to the quantity of specific IgG_4 antibodies suggested that the sera at both time points contained comparable IgG_4 specificities able to block most IgE epitopes recognized by the indicator serum. These data accorded with the large overlap of the predicted IgG_4-binding regions at both time points (Fig 1). However, patient 4 displayed very high levels of Bet v 1–specific IgG_4 antibodies at month 6 and 3.5-fold lower levels at month 36 (Table I). At this time point, Bet v 1–specific IgG_1 antibodies were almost reduced to the level before AIT (see Fig E1). In parallel, the IgE-blocking activity decreased only around 2-fold (Table I). The limited reduction in IgE-blocking activity together with the high overlap of predicted IgG_4 epitopes at both time points (Fig 1) suggested a comparable IgG_4 repertoire in the early and late phases of BP-AIT also in this patient. In contrast, patient 5 showed a parallel increase in IgE-blocking activity and IgG_4 levels but no IgG_1 induction during the course of BP-AIT (see Table I and Fig E1). In this individual, the increased IgE-blocking activity may result not only from the enhanced IgG_4 concentration but also from a broader IgG_4 diversity. The latter is strongly suggested by the occurrence of prominent IgG_4-binding areas predicted at month 36 but not at month 6 (Fig 1). We conclude that the Bet v 1–specific IgG_4 repertoire of patient 5 expanded in the course of BP-AIT.

In summary, this study provides first evidence that Bet v 1–specific IgG_4 repertoires induced by BP-AIT do not broadly expand in the course of treatment in most of the patients. Still, 1 of 5 individuals (20%) showed an increase in the IgG_4 diversity with prolonged therapy. We are aware that these conclusions are based on a limited number of patients, in particular in view of the heterogeneous and individual immune response to allergens. However, competitive immunoscreening is laborious and cannot be performed in a high-throughput set-up. Therefore, we sought an additional approach to support our conclusions, namely, the use of 4 chimeric proteins that had been created by grafting surface areas of Bet v 1 onto the scaffold of Api g 1, the Bet v 1–homologue in celery.[9] The IgG_4 reactivity to these chimeras

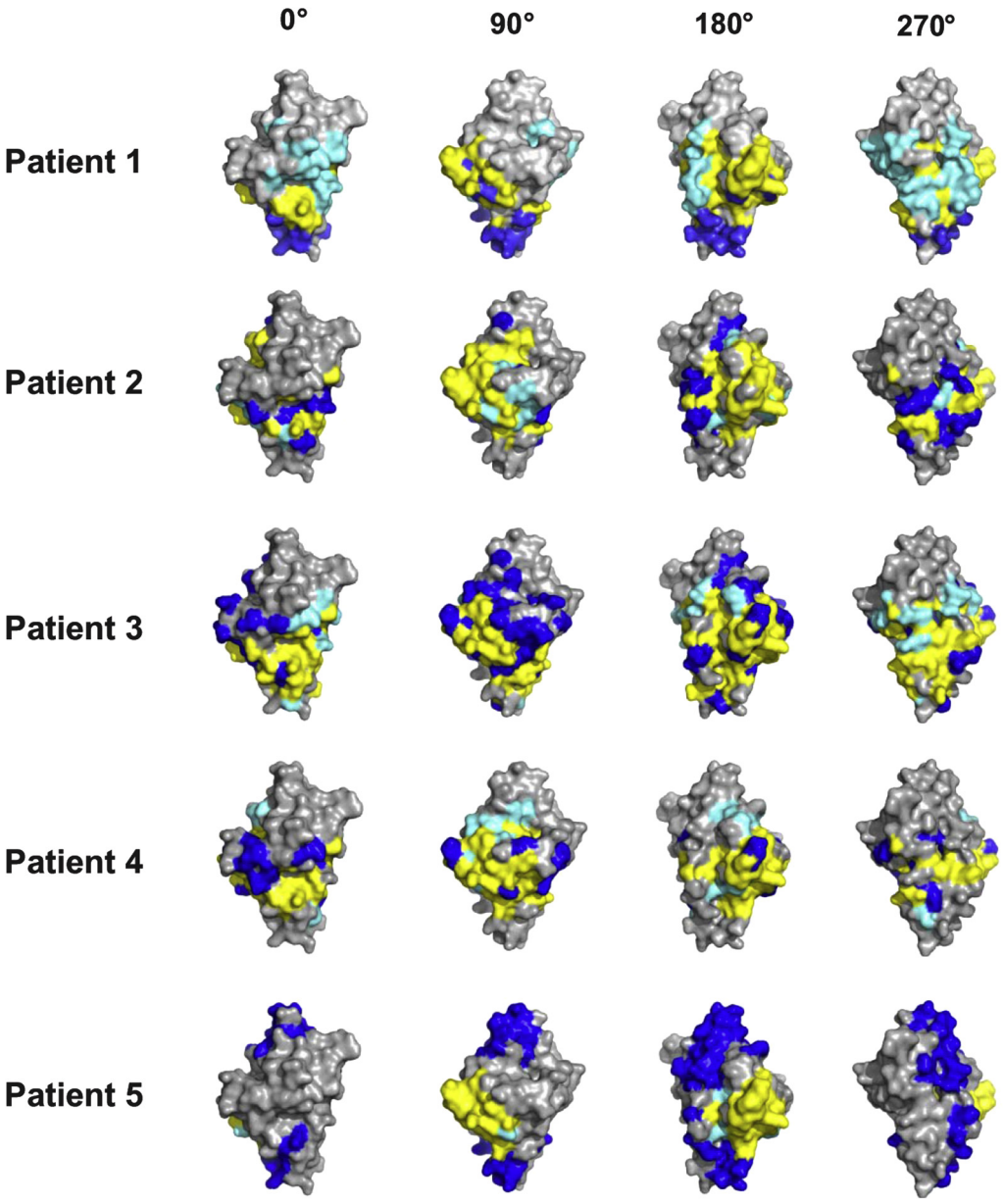

FIG 1. Bet v 1–specific IgG_4-binding areas at 6 and 36 months of BP-AIT largely overlap. The sum of predicted IgG_4 epitopes on Bet v 1 (pdb 1BV1) after 6 *(turquois)* and 36 *(blue)* months of BP-AIT and their overlap *(yellow)* are shown.

TABLE I. Bet v 1–specific IgG_4 levels and IgE-blocking activity at month 6 and 36 of BP-AIT

	IgE-blocking activity (%)*			IgG_4 levels (μg/L)†		
Patient	**Before**	**Month 6**	**Month 36**	**Before**	**Month 6**	**Month 36**
1	1	98	98	0.00	0.72	2.49
2	0	89	92	0.03	0.58	1.12
3	0	96	99	0.28	6.34	26.8
4	0	86	45	0.55	18.5	5.29
5	0	36	62	1.22	1.67	11.8

*The IgE-blocking activity of sera was assessed by facilitated antibody-binding assays.
†Bet v 1–specific IgG_4 levels were determined by ImmunoCAP.

was tested by ELISA. So far, 8 of 9 patients (89%) displayed IgG_4 reactivity to the same chimera after 6 and 36 months of BP-AIT, whereas 1 of 9 individuals (11%) showed a diverse response at both time points (unpublished observations). Identical reactivity patterns at early and late time points of the therapy indicate that the IgG_4 repertoire remained constant for most of the patients during BP-AIT. These preliminary data obtained by a second, entirely different experimental approach to analyze the IgG_4 diversity in the course of BP-AIT strongly underline our conclusions drawn from competitive immunoscreening of phage-displayed peptides and facilitated antibody-binding assays.

Brinda Subbarayal, PhD[a,b]*
Dirk Schiller, PhD[c]*
Christian Möbs, PhD[d]
Wolfgang Pfützner, MD[d]
Beatrice Jahn-Schmid, PhD[a]
Barbara Gepp, MSc[a]
Heimo Breiteneder, PhD[a]
Stefan Vieths, PhD[c]
Barbara Bohle, PhD[a,b]

From [a]the Department of Pathophysiology and Allergy Research and [b]Christian Doppler Laboratory for Immunomodulation, Medical University of Vienna, Vienna, Austria; [c]the Division of Allergology, Paul-Ehrlich-Institut, Langen, Germany; and [d]the Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany. E-mail: barbara.bohle@meduniwien.ac.at.

*These authors contributed equally to this work.

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Host, disease, and antiretroviral factors are associated with normalization of the CD4:CD8 ratio after initiating antiretroviral therapy

To the Editor:

By effectively suppressing plasma HIV-viremia, modern combination antiretroviral therapy (cART) prevents ongoing damage to the immune system from uncontrolled HIV replication and allows immune recovery. Although AIDS-defining conditions are now rare in effectively treated people living with HIV, non–AIDS-defining illnesses such as cardiovascular disease, renal disease, cognitive impairment, and cancer are reported to occur at higher rates and at a younger age than in matched HIV-uninfected control populations.[1] It has been postulated that immunosenescence and persistent immune activation, even in the context of effective control of HIV-viral replication, may be the underlying pathogenic factors that drive such non–AIDS-defining comorbidities[2] and the monitoring of markers of immunosenescence may play a valuable role in the clinical management of people living with HIV. The CD4:CD8 ratio is one such readily available marker.

Few reports have described factors associated with normalization of the CD4:CD8 ratio after the initiation of cART. We aimed to assess such factors within the UK Collaborative HIV Cohort (UK CHIC) study.

Individuals initiating cART (defined as at least 3 antiretroviral agents) from 2000 onwards at the 11 UK CHIC centers that provided $CD8^+$ count and laboratory marker data were eligible. Exclusion criteria included less than 6 months of follow-up after initiating cART, no baseline CD4:CD8 ratio, and pregnancy at any point during or immediately preceding the initiation of cART. Subjects were followed from the initiation of cART to the earliest of discontinuation of cART, death, last recorded date seen, or January 1, 2013.

The CD4:CD8 ratio was calculated for any pair of CD4 and CD8 counts measured on the same day. Where there were repeated CD4 or CD8 counts on any given day, the average of all CD4 or CD8 counts on that day was used to calculate the ratio. Time to normalization of the CD4:CD8 ratio (defined as a ratio of $\geq$1.0 on a minimum of 2 occasions at least 30 days apart) was assessed using Kaplan-Meier and Cox Proportional Hazards models, allowing a maximum follow-up time of 5 years.

In regression analyses, factors considered as potentially associated with normalization of the CD4:CD8 ratio were baseline $CD4^+$ and $CD8^+$ count, plasma HIV RNA, previous AIDS-defining illnesses, type of cART regimen (nucleoside reverse transcriptase inhibitor [NRTI] backbone and third antiretroviral agents), calendar year of starting cART, recent HIV seroconversion (patients known to belong to the UK Register of Seroconverters[3] who initiated cART within 1 year of HIV diagnosis), viral hepatitis coinfection (hepatitis B and C), age, sex, ethnicity, mode of HIV acquisition, and laboratory parameters (hepatic transaminases, albumin, alkaline phosphatase, creatinine, and hemoglobin).

Sensitivity analyses were performed to assess the effects of including time-updated changes in $CD4^+$ cell count from baseline

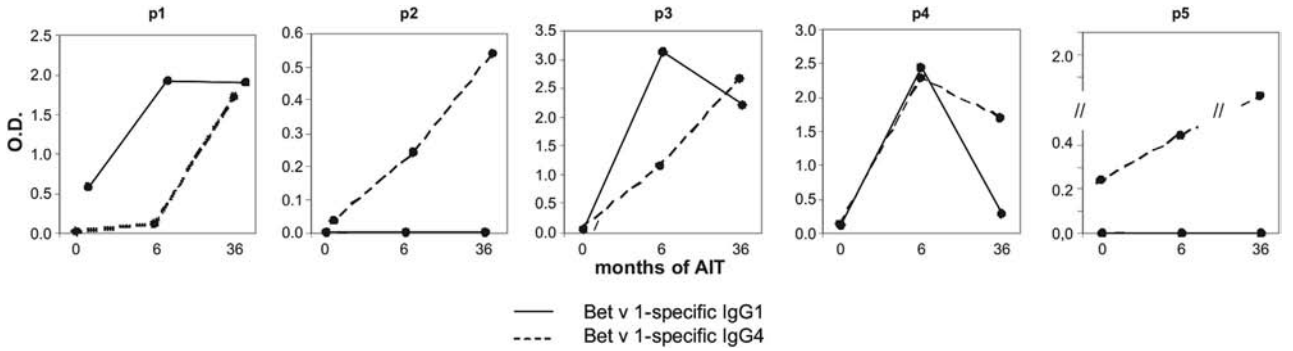

FIG E1. Bet v 1–specific IgG_1 and IgG_4 levels before (0) and after 6 and 36 months of BP-AIT. Antibody levels were determined with ELISA as described previously.[2] *OD*, Optical density.

TABLE E1. Phage-displayed peptides and predicted IgG_4-binding regions on Bet v 1

Patient	BP-SIT (mo)	Peptide sequence	aa residues of predicted IgG_4-binding regions on Bet v 1	% Of identical aa at 6 and 36 mo
		QSVRIMTGADSN	1. S39, I56, S57, G61, V67, V85, G88, G89, I91, G92, Q132, A135, S136, M139	
		KCSLPECVVARE	2. S39, K55, S57, P59, E60, L62, P63, K65, V67, K68, V85, E87, P90, A135, S136	
1	6	SPGLPLQLGSMK	3. S39, K55, S57, P59, G61, L62, P63, K65, K68, G88, G89, P90, G92, Q132, S136, M139	44.7
		SKMAHMERSWEV	4. V2, E6, V85, K97, S99, E101, K115, S117, K119, H121, V128, V133, K134, A135, S136, K137, M139	
		SKMAHMERSWEV	5. E6, V67, V85, S117, K119, V128, K129, A130, E131, V133, K134, A135, S136, K137, E138, M139, E141	
		VQHNTKYSVVIR	6. N4, Y5, T7, T9, I23, K80, Y81, N82, Y83, S99, I102, K103, V105, I113, K115, I116, S117, N118, K119, V133, S136, K137	
		VQHNTKYSVVIR	7. V2, N4, Y5, T7, N82, Y83, V85, K97, S99, I102, K115, I116, S117, N118, K119, H121, V128, Q132, V133, K134, S136, K137	
		YLDLPTNLSHRI	8. T7, T9, I23, L24, D72, D75, N78, Y81, N82, Y83, S99, I102, I113, I116, S117, N118, S136, L143	
		TLRVPPNPNMNV	9. T7, T9, L62, P63, V67, V85, P90, N118, V133, M139, T142, L143, R145	
		GHVTESMTKRSD	10. G1, V2, V67, V85, E87, G88, G89, G92, D93, T94, E96, K97, S99, K119, H121, T122, K123, G124, H126, E127, V128, K129, V133	
	36	GLTKPSLYTMEK	11. S39, K55, S57, P59, E60, G61, L62, P63, K65, Y66, K68, E87, G88, G89, P90, G92, S136, M139	
		VKLNPSMNYLSG	12. S39, K55, S57, P59, G61, L62, P63, K65, Y66, V67, K68, V85, G88, G89, P90, G92, S136, M139	
		SVTESPNRMGLV	13. S39, S57, P59, E60, G61, L62, P63, V67, V85, E87, G88, G89, P90, G92, S136, M139	
		GMSLAQVTAKQN	14. S39, K55, S57, G61, L62, K65, V67, K68, V85, G88, G89, G92, Q132, A135, S136, M139	
		SLTNPWPVSFSP	15. T7, T9, F30, F58, L62, P63, F64, V67, V85, P90, N118, V133, S136, T142, L143	
		YIPPLPQMLTAI	16. I56, P59, L62, P63, Y66, P90, I91, Q132, A135, M139	
		KTASDWMTAQYP	17. S39, K55, S57, P59, P63, K65, Y66, K68, P90, Q132, A135, S136, M139	
		LMTKYHIPEISD	18. S39, K55, I56, S57, P59, E60, L62, P63, K65, Y66, K68, E87, P90, I91, S136, M139	
2	6	SNPLNMLRITSL	19. S39, S40, I44, I53, I56, S57, L62, P63, R70, I86, P90, I91, L95, S136, M139	61.5
		YSFTPHVDFISP	20. D27, F30, P31, S39, S40, V41, I44, I53, I56, S57, F58, P59, F64, Y66, V67, D69, Y83, V85, I86	
		AHNLLYNLKYHP	21. N4, Y5, N82, Y83, P90, L95, K97, K115, N118, K119, H121, K134, A135, K137	
		TTDLVLNMLPLS	22. D25, D27, N28, P31, V33, P35, S39, S40, V41, S57, P59, D69, M139, T142, L143	
		HPSAENPLTMPP	23. E6, T7, P63, P90, S117, N118, A130, E131, A135, S136, E138, M139, E141, T142, L143	
		HPSAENPLTMPP	24. S39, S57, P59, E60, L62, P63, E87, P90, A135, S136, M139	
	36	VSTPANTKYNNR	25. V2, N4, Y5, T7, N82, Y83, V85, P90, K97, S99, K115, S117, N118, K119, V128, V133, K134, A135, S136, K137	
		TSSAPDNWTYWL	26. D25, D27, N28, P31, P35, S39, S40, S57, P59, D69, T142, L143, A146, Y150	
		IAAPNSLRFSPL	27. S39, I56, S57, F58, P59, L62, P63, F64, P90, I91, A135, S136	
		TLTSAPLKWTPR	28. S39, S40, K54, K55, S57, L62, P63, K65, K68, R70, P90, L95, K97, A135, S136	
3	6	HLISSNKPQHTQ	61. N4, T7, N82, P90, L95, K97, S99, I102, K115, I116, S117, N118, K119, H121, Q132, K134, S136, K137	62.5
		WNISSAKPMLPY	62. S39, S40, I44, I53, K54, K55, I56, S57, L62, P63, K65, Y66, K68, Y83, I86, P90, I91, L95, K97, A135, S136, M139	
		WNISSAKPMLPY	63. S39, K55, I56, S57, P59, L62, P63, K65, Y66, K68, P90, I91, A135, S136, M139	
		GMLSSLKPPIAH	64. S39, K55, I56, S57, P59, G61, L62, P63, K65, K68, G88, G89, P90, I91, G92, A135, S136, M139	
		SINAWVPWLIDS	65. I23, D25, D27, N28, P31, V33, P35, S39, S40, V41, I56, S57, P59, D69, L143, A146	
		SWMTSFKHSANS	66. F3, N4, T7, N82, K97, S99, K115, S117, N118, K119, H121, K134, A135, S136, K137, M139	
		AFMSSAKLPDSR	67. S39, K55, S57, F58, P59, L62, P63, F64, K65, K68, P90, A135, S136, M139	
		SSAAMLHSTKSL	68. T7, L95, K97, S99, K115, S117, K119, H121, K134, A135, S136, K137, M139	
	36	ESIHASGKMGRH	69. S39, K55, I56, S57, E60, G61, K65, K68, E87, G88, G89, I91, G92, A135, S136, M139	
		ANHLSGNNYGIS	70. S39, S40, I44, I53, I56, S57, L62, Y66, Y83, I86, G88, G89, I91, G92, L95, A135, S136	
		ANHLSGNNYGIS	71. N43, I44, G48, G49, G51, I53, I56, S57, Y66, Y81, N82, Y83, I86, G88, G89, I91, S99	
		GTTTLNHNYSAK	72. N4, Y5, T7, N82, Y83, L95, K97, S99, K115, S117, N118, K119, H121, K134, A135, S136, K137, G140	
		GTTTLNHNYSAK	73. N4, Y5, T7, T9, K80, Y81, N82, Y83, S99, K103, K115, S117, N118, K119, S136, K137, G140, L143	
		YHPNGMNPYTKA	74. K55, P59, G61, P63, K65, Y66, K68, G88, G89, P90, G92, A135, M139	
		NDNYPATIKHSN	75. I23, D27, P50, I53, K54, I56, K68, D69, D72, K80, Y81, N82, Y83, I86, K97, S99, I102, I116, S117, N118, K119	
		SVDMPHPYGQRP	76. S39, S40, V41, S57, P63, Y66, V67, D69, R70, Y83, V85, G88, G89, P90, G92, S136, M139	

(Continued)

TABLE E1. *(Continued)*

Patient	BP-SIT (mo)	Peptide sequence	aa residues of predicted IgG_4-binding regions on Bet v 1	% Of identical aa at 6 and 36 mo
4	6	WNQGSPNLRYTS	40. T7, T9, Q36, L62, P63, G89, P90, N118, Q132, S136, G140, T142, L143, R145	53.8
		WNQGSPNLRYTS	41. S39, S40, S57, L62, P63, Y66, R70, Y83, G88, G89, P90, G92, L95, S136	
		VSTAKYETLNEQ	42. N4, Y5, E6, T7, E8, T9, K80, Y81, N82, Y83, S99, E101, K103, V105, K115, S117, N118, K119, V133, S136, K137, E141, L143	
		VSTAKYETLNEQ	43. V2, N4, Y5, E6, T7, N82, Y83, V85, L95, K97, S99, E101, K115, S117, N118, K119, V128, Q132, V133, K134, A135, S136, K137	
		VPTECSGRTSCT	44. G1, V2, V67, V85, E87, G88, G89, P90, G92, T94, E96, S99, T122, G124, E127, V128, V133	
		DPLDQGKMLRTP	45. K55, P59, G61, L62, P63, K65, K68, G88, G89, P90, G92, Q132, M139	
		SIPRYYHTENSP	46. S39, S40, E42, I44, I53, I56, S57, P63, Y66, R70, Y83, I86, E87, P90, I91, S136	
	36	SIPRYYHTENSP	47. I23, P50, I53, I56, R70, E73, Y81, N82, Y83, I86, S99, E101, I102, I116, S117, N118	
		SIPRYYHTENSP	48. N28, P31, S40, E42, N43, I44, E45, N47, I53, I56, S57, Y66, R70, Y83, I86, E87	
		SSPSAAQWLNIG	49. S39, I56, S57, P59, G61, L62, P63, G88, G89, P90, I91, G92, Q132, A135, S136	
		LPGRAHDPWKVP	50. V41, K54, K55, L62, P63, K65, V67, K68, D69, R70, V85, G88, G89, P90, G92, L95, K97, A135	
		HPATSNTKYWIN	51. S39, S40, I44, I53, K54, K55, I56, S57, P63, K65, Y66, K68, Y83, I86, P90, I91, K97, A135, S136	
		HPATSNTKYWIN	52. N4, Y5, T7, N82, Y83, P90, K97, S99, I102, K115, I116, S117, N118, K119, H121, K134, A135, S136, K137	
		FTTESGGLNPHA	53. S39, S57, F58, P59, E60, G61, L62, P63, F64, E87, G88, G89, P90, G92, A135, S136	
		VEAHNQWTGLVA	54. G1, V2, N4, G92, T94, L95, E96, N118, H121, T122, H126, E127, V128, A130, E131, Q132, V133, A135	
		YHPNGMNPYTKA	55. K55, P59, G61, P63, K65, Y66, K68, G88, G89, P90, G92, A135, M139	
		APTIAYNTFMPM	56. I44, I53, I56, F58, P63, F64, Y66, Y83, I86, P90, I91, A135, M139	
		APTIAYNTFMPM	57. T9, F22, I23, F30, P31, P35, I56, I102, I116, M139, T142, A146, Y150	
		APTIAYNTFMPM	58. F22, I23, N28, F30, P31, P35, I56, F58, P59, M139, T142, A146, Y150	
		APTIAYNTFMPM	59. I56, F58, P59, P63, F64, Y66, P90, I91, A135, M139	
		APTIAYNTFMPM	60. F30, P31, N43, I44, I53, I56, F58, P59, F64, Y66, Y83, I86, M139	
		IYGQVLSTVQYR	29. S39, I56, S57, G61, L62, Y66, V67, V85, G88, G89, I91, G92, Q132, S136	
		DLTFTVNPLSKA	30. S39, K55, S57, F58, P59, L62, P63, F64, K65, V67, K68, V85, P90, A135, S136	
5	6	STIAERSIHPFN	31. S39, I56, S57, F58, P59, E60, P63, F64, E87, P90, I91, A135, S136	36.8
		NLSTGEKPEVTK	32. S39, K55, S57, P59, E60, G61, L62, P63, K65, V67, K68, V85, E87, G88, G89, P90, G92, S136	
		SLSSMNTIRVPG	33. S39, I56, S57, P59, G61, L62, P63, V67, V85, G88, G89, P90, I91, G92, S136, M139	
		HGVMTVSTTEKF	34. S39, K55, S57, F58, E60, G61, F64, K65, V67, K68, V85, E87, G88, G89, G92, S136, M139	
		HMGMTKINYSAL	35. S39, K55, I56, S57, G61, L62, K65, Y66, K68, G88, G89, I91, G92, A135, S136, M139	
		LSSHKATSTRAD	36. D93, T94, L95, K119, H121, T122, K123, H126, K129, A130, K134, A135, S136	
	36	LSSHKATSTRAD	37. R17, D25, K32, L143, R145, A146, S149, L152, A153, H154, S155, D156, A157	
		SLPFGPNTTIRP	38. S39, I56, S57, F58, P59, G61, L62, P63, F64, G88, G89, P90, I91, G92, S136	
		LESHYTQASYTQ	39. T9, T10, E141, T142, L143, A146, E148, S149, Y150, L152, A153, H154, A157, Y158	

Identical peptides in the same individual (boldface) or in different patients (italics) are indicated.

CHAPTER IV:

Manuscript 3

Letter to the editor

Monitoring the epitope recognition profiles of IgE, IgG1, and IgG4 during birch pollen immunotherapy

Barbara Gepp[1], Nina Lengger[1], Christian Möbs[2], Wolfgang Pfützner[2], Christian Radauer[1], Barbara Bohle[1,3], Heimo Breiteneder[1]

[1]Department of Pathophysiologie and Allergy Research, Medical University of Vienna, Austria

[2]Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany

[3]Christian Doppler Laboratory for Immunomodulation, Medical University of Vienna, Austria

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Letter to the Editor

Monitoring the epitope recognition profiles of IgE, IgG_1, and IgG_4 during birch pollen immunotherapy

To the Editor:

It is well established that allergen-specific IgG antibodies induced during allergen-specific immunotherapy (AIT) can block the IgE-mediated cascade of allergic inflammation.[1] Although this role of IgG has been intensively studied, little is known about the epitope diversities of immunoglobulin subclasses induced during AIT. Thus far, analyses of IgG_4-binding patterns to major cow's milk[2] and peanut[3] allergens during oral AIT revealed discrepant results. The overall diversity of IgG_4 specific for cow's milk allergens changed little,[2] whereas the IgG_4 repertoire specific for peanut allergens was broadly expanded in a polyclonal fashion, including *de novo*–generated specificities different from IgE.[3] Binding of IgE to Bet v 1, the major birch pollen allergen, is dependent on the protein's native conformation.[4] We have previously assessed the diversity of IgG_4 antibodies specific for Bet v 1 during subcutaneous AIT with birch pollen using competitive immunoscreening of phage-displayed peptides and found that the Bet v 1–specific IgG_4 repertoire did not broadly expand in most patients.[5] However, competitive immunoscreening is laborious and can be performed only with a limited number of serum samples. Therefore our previous work was restricted to the analysis of IgG_4 diversity at 2 time points (before and after 3 years) of AIT by using sera of 5 patients.

Here, we applied a chimera-based approach to monitor development of the Bet v 1–specific IgE, IgG_1, and IgG_4 repertoires in narrow time intervals during 3 years of AIT in more subjects.[6] Four chimeras were generated by grafting 4 different nonoverlapping, contiguous, Bet v 1–derived surface areas onto the Bet v 1 homolog from celeriac, Api g 1, by replacing Api g 1–specific surface residues by corresponding residues from Bet v 1 (see Fig E1 in this article's Online Repository at www.jacionline.org).[7] Api-Bet-1 contains the P-loop, Api-Bet-2 contains the region opposite the P-loop, Api-Bet-3 contains the C-terminus and surrounding residues, and Api-Bet-4 contains the C-terminal α-helix of Bet v 1. Sera from 11 patients with birch pollen allergy with improved symptom and medication scores were collected before (time point 0) and after 1, 3, 6, 12, 18, 24, 30, and 36 months of AIT.[6] Patients received weekly doses of birch pollen extract (ALK-depot SQ 108; ALK-Abelló, Hørsholm, Denmark) subcutaneously until a maintenance dose of 100,000 standard quality units per injection was reached, followed by monthly maintenance injections for 3 years. Antibody binding to Bet v 1, the 4 Bet v 1–specific areas on the chimeras, and Api g 1 (scaffold protein control) was assessed by means of ELISA. Sera were not available from all time points from 3 subjects (p1, p5, p8). Proteins (2 μg/mL) were coated onto 96-well microtiter plates (MaxiSorp; Nalge Nunc International, Roskilde, Denmark) in carbonate buffer (50 mmol/L, pH 9.6). Nonspecific binding sites were saturated for 2 hours at room temperature with 1% human serum albumin in Tris-buffered saline containing 0.5% Tween-20 (TBST). Sera were diluted 1:20 (IgE), 1:50 (IgG_1), or 1:200 (IgG_4) in TBST with 0.5% human serum albumin and applied in duplicates overnight at 4°C. Bound antibodies were detected with alkaline phosphatase–conjugated mouse anti-human IgE (BD PharMingen, Heidelberg, Germany), IgG_1 (Acris Antibodies, Herford, Germany), or IgG_4 (BD PharMingen) mAbs. OD was measured after color development with Sigma FAST p-nitrophenyl phosphate tablets (Sigma-Aldrich, St Louis, Mo) at 405 nm. Sera from 8 nonallergic subjects served as negative controls. OD values for these controls were not significantly different from the buffer controls when IgE, IgG_1, or IgG_4 binding to the proteins was assessed. For all 11 immunotherapy sera, OD values exceeding the mean value of the 8 nonallergic subjects by more than 5 SDs were considered positive. To assess specific antibody binding to the grafted region on each chimera, the OD value measured for the scaffold protein Api g 1 was subtracted from the chimera-specific OD value and considered positive when the difference exceeded 5 times the SD of the negative control values.

In the majority of patients, Bet v 1–specific IgE levels increased during the early phase of treatment, followed by a gradual decrease (Fig 1). Seven (64%) of 11 patients displayed lower IgE levels after 36 months of AIT compared with those before therapy (Fig 1). All patients had Bet v 1–specific IgG_4, and 7 (64%) of 11 patients had Bet v 1–specific IgG_1 (Fig 1). In these subjects Bet v 1–specific IgG_1 antibody levels increased earlier than IgG_4 antibody levels (median duration required to reach half-maximum concentrations of Bet v 1–specific IgG_1 and IgG_4: 4.5 and 19.5 months; $P = .016$, Wilcoxon signed-rank test). Accordingly, chimera-specific IgG_1 appeared earlier than IgG_4 (Fig 2).

The patterns of IgE recognition of the 4 Bet v 1–specific areas on the chimeras and Api g 1 did not change over time in most patients, indicating that the overall IgE epitope diversity during AIT remained constant (Fig 2). Similarly, once induced, chimera recognition was constant in 4 (57%) of 7 patients (p3, p5, p7, and p10) for IgG_1 and 8 (73%) of 11 patients (p1-p5, p7, p8, and p11) for IgG_4, albeit with quantities varying over time (Fig 2). These findings match our previous results achieved by means of competitive immunoscreening of phage-displayed peptides, showing that the early established IgG_4 repertoire did not change with prolonged AIT.[5]

IgE binding to all 4 chimeras was observed in 7 (64%) of 11 patients (Fig 2). In contrast, only 2 (p3 and p10) and 3 (p2, p4, and p7) patients showed IgG_1 or IgG_4 binding to all chimeras, respectively. We conclude that among the immunoglobulin classes investigated, IgE showed the highest epitope diversity. Therefore, most likely, not all IgE-binding epitopes could be blocked by AIT-induced IgG_1 or IgG_4 because of direct epitope competition. It was shown that IgA, IgG_2, and IgG_3 also possess blocking activity,[8] which could contribute to the reported amelioration of allergic symptoms. However, IgG_2 and IgG_3 were not induced during AIT,[5] and IgA induction was low.[6] Steric inhibition by binding of IgG to sites adjacent to IgE epitopes on the grafted regions could also contribute to IgE blocking.[1] In addition, mechanisms not depending on epitope-matched blocking IgG antibodies might contribute to the success of immunotherapy by mixed immune complexes containing IgE and IgG, such as inhibition of IgE-facilitated antigen presentation, as shown in our previous study,[5] and inhibition of mast cell degranulation.

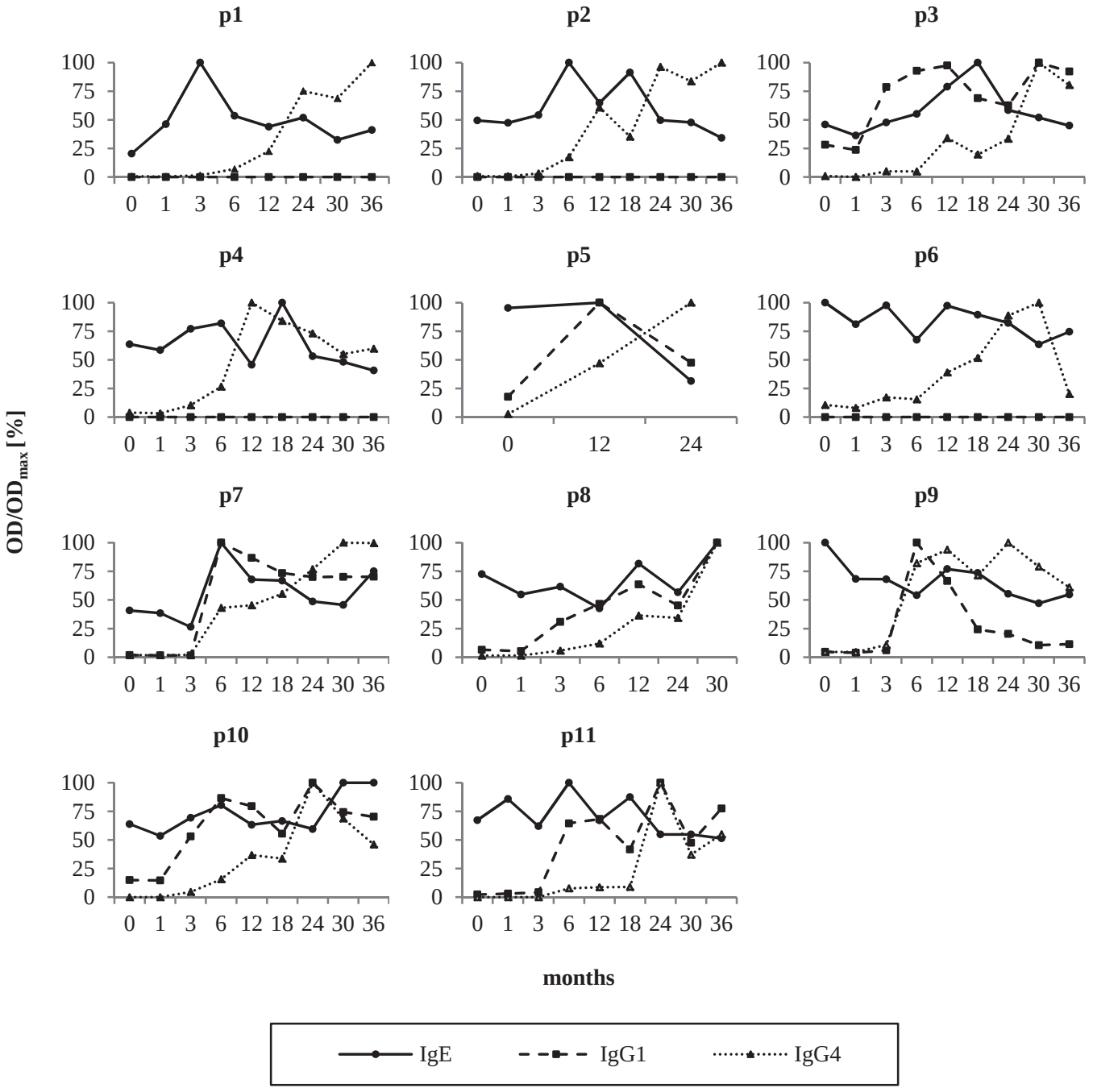

FIG 1. Bet v 1–specific IgE, IgG_1, and IgG_4 responses of 11 patients during AIT with birch pollen. Relative absorbance values are based on the highest value (= 100%) within each isotype for each patient.

Furthermore, our data revealed that IgE, IgG_1, and IgG_4 recognized different epitope profiles (Fig 2). For example, patient 1 showed IgE binding exclusively to Api-Bet-2 and Api-Bet-3 but had high amounts of IgG_4 directed against Api-Bet-1. Patient 3 had IgE and IgG_1 binding mainly to Api-Bet-1, Api-Bet-2, and Api-Bet-3 but developed solely Api-Bet-3–specific IgG_4. For patient 7, IgE and IgG_4 binding to all 4 chimeras was observed, whereas Api-Bet-2–specific IgG_1 was not detected.

In summary, this is the first study that longitudinally monitored IgE, IgG_1, and IgG_4 repertoires in narrow intervals during 3 years of subcutaneous AIT. The grafted Bet v 1–specific areas on our chimeras are approximately 2000 $Å^2$ offering potential space for 2 or 3 epitopes[7] and allow an approximation of the epitope diversities of different allergen-specific immunoglobulin subclasses. Our data provide evidence that the allergen-specific IgE repertoire and the AIT-induced IgG_1 and IgG_4 profiles did not expand over time but differed among each other. Our finding that AIT-induced IgG_1 antibodies developed earlier than IgG_4 antibodies is in agreement with previous reports that IgG_1 dominates the early and IgG4 dominates the late IgG response in

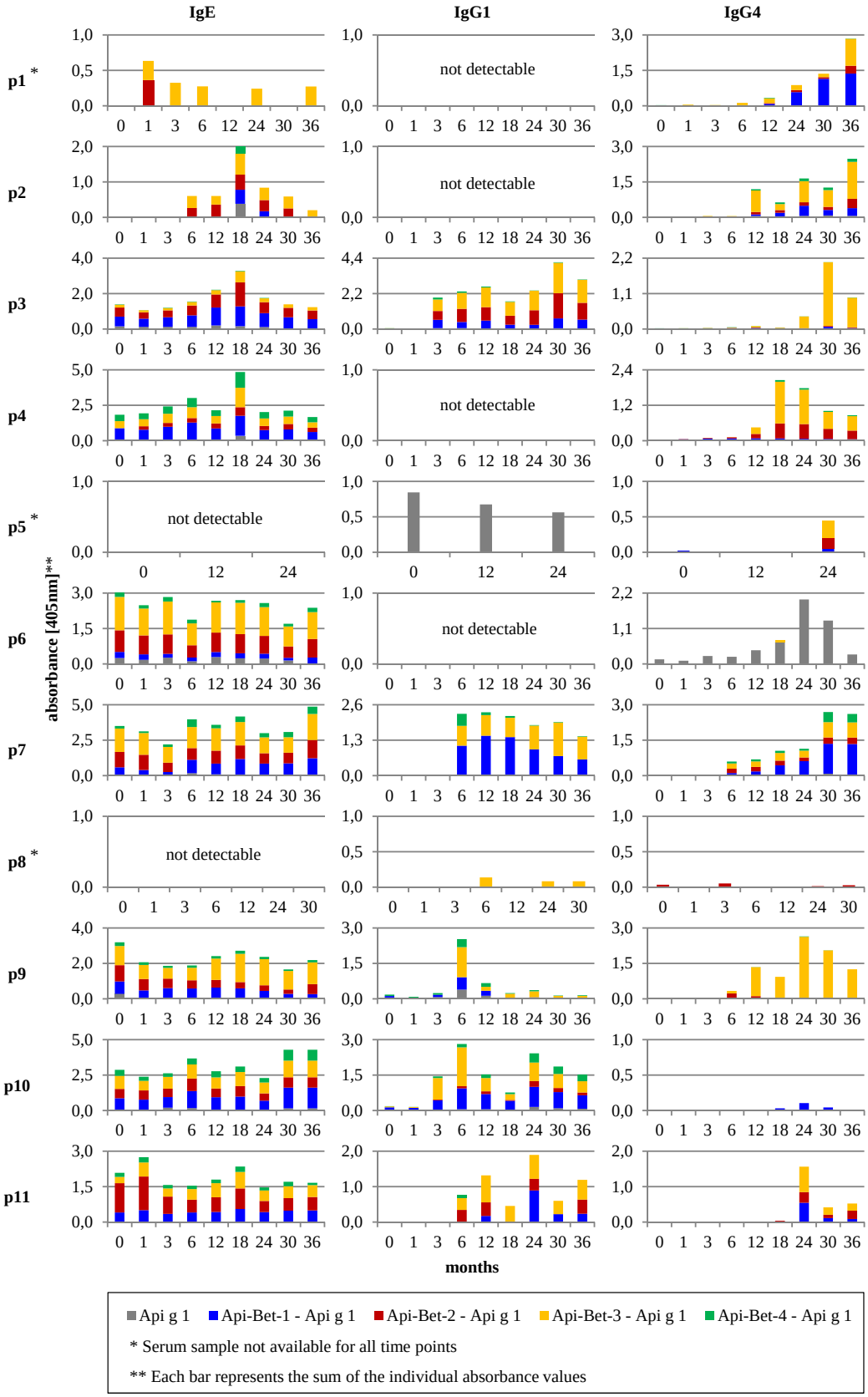

FIG 2. IgE, IgG_1, and IgG_4 binding to Api g 1 and the 4 Bet v 1–specific areas on chimeras of 11 patients during AIT with birch pollen. OD values for Api g 1 (scaffold protein) were subtracted from chimera-specific OD values.

patients with AIT.[9] Both IgG_1 and IgG_4 displayed more restricted epitope diversities than IgE antibodies. Together, our findings contribute to elucidating the complex immune mechanisms that occur during AIT.

Barbara Gepp, MSc[a]
Nina Lengger, BMA[a]
Christian Möbs, PhD[b]
Wolfgang Pfützner, MD[b]
Christian Radauer, PhD[a]
Barbara Bohle, PhD[a,c]*
Heimo Breiteneder, PhD[a]*

From [a]the Department of Pathophysiology and Allergy Research and [c]the Christian Doppler Laboratory for Immunomodulation, Medical University of Vienna, Vienna, Austria, and [b]the Department of Dermatology and Allergology, Philipps University Marburg, Marburg, Germany. E-mail: barbara.bohle@meduniwien.ac.at. Or: heimo.breiteneder@meduniwien.ac.at.

*These authors contributed equally to this work.

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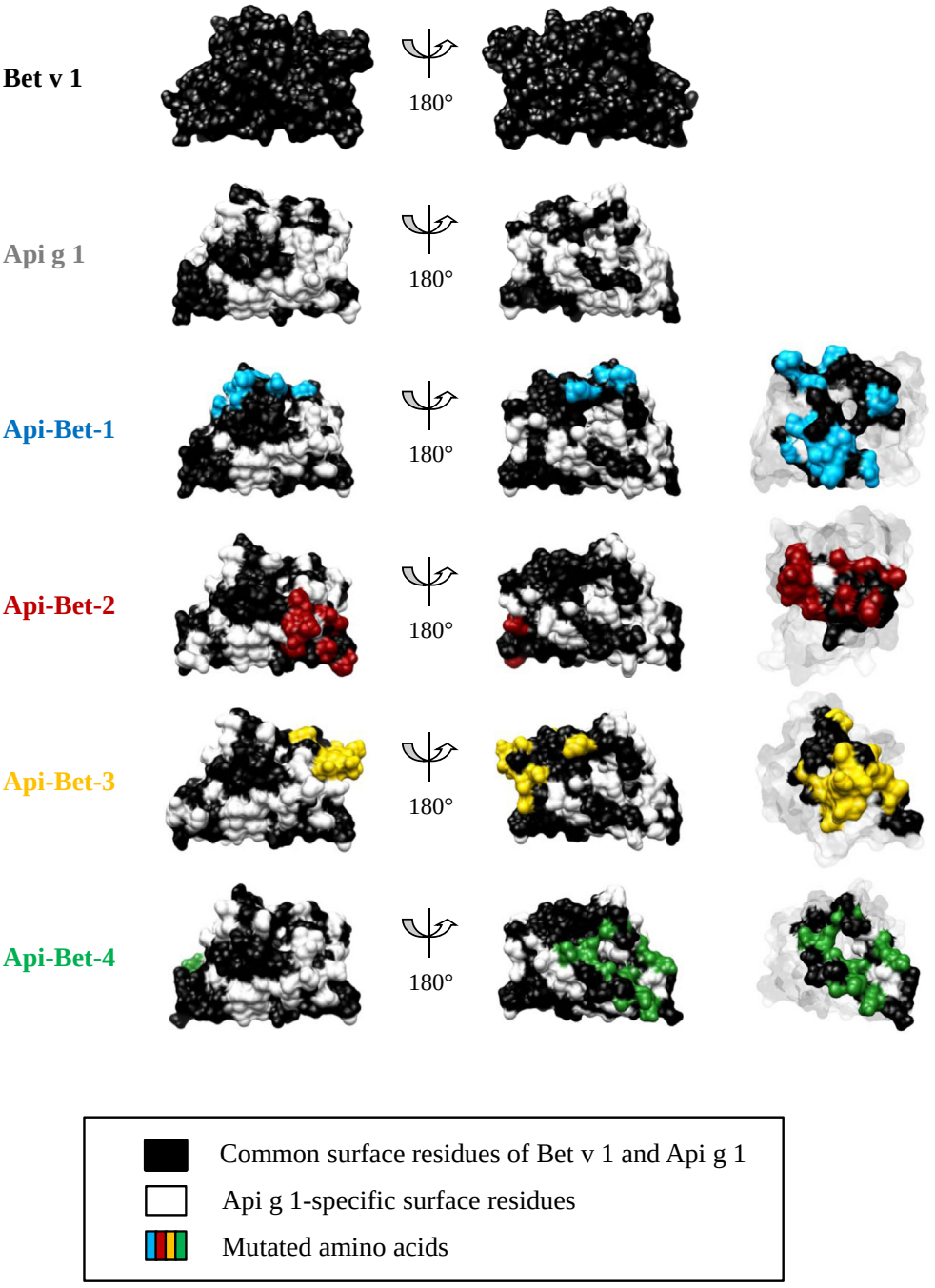


FIG E1. Structural comparison of Bet v 1, Api g 1, and the 4 chimeras. Front and back views (rotated by 180° around a vertical axis) of the parent molecules and chimeras are depicted. At right, views centered at the Bet v 1–specific areas on the chimeras are shown.

CHAPTER V:

Discussion

DISCUSSION

The location of IgE-binding epitopes of allergens is crucial for a better understanding of the IgE immune response, for the prediction of cross-reactivity and for the design of safer hypoallergenic variants of allergens for AIT. Due to the fact that Bet v 1-specific IgE binds exclusively to conformational epitopes, the determination of antibody binding areas is not trivial. A simple and fast method to perform epitope mapping by using synthetic overlapping peptides spanning the complete sequence of Bet v 1 cannot be applied. Therefore, more sophisticated and indirect approaches have to be used.

Three types of analytical tools have been used to identify conformational epitopes: (1) structural variants which include natural variants, mutants obtained by site-directed mutagenesis or by grafting of larger areas, (2) antibodies of various formats that bind to (or close to) the IgE epitope (e.g. mouse monoclonal antibodies, Fab fragments, scFv antibodies) and thus compete with the IgE antibody, and (3) molecules that mimic the allergen in its interaction with the antibody (mimotopes) and the *in silico* prediction of IgE-binding epitopes [117]. However, all these methods have advantages and disadvantages.

For site-directed mutagenesis, point mutations can have a major impact on IgE binding by either directly destroying the IgE binding epitope or by altering the overall structure of the allergen. One example for this was described by Scheurer et al. [128]. The authors reported that the exchange of S112 to proline in Bet v 1 and Pru av 1 altered their tertiary structure to such an extent that it lead to a complete loss of allergenicity. Hence, the correct 3D-structure of the expressed mutant has to be confirmed for the correct interpretation of the data. Furthermore, the analysis of only a single amino acid instead of around 15 residues that are involved in a typical epitope [138] and, in many cases, the expression of a large number of mutants are major limits of this approach.

X-ray crystallography of an antibody-antigen complex is the gold standard for epitope determination [195]. However, the generation of human monoclonal IgE antibodies derived from the repertoires of allergic individuals and the crystallization together with the allergen in complex. Only in a few cases it was possible to study the human IgE-allergen interactions by using X-ray crystallography. For example, the three-dimensional structure of the complex between the major respiratory grass pollen allergen Phl p 2 and its specific human IgE-derived Fab and the crystal structure of an IgE Fab fragment in complex with beta-lactoglobulin, one of the major allergens of bovine milk, was published [196, 197]. Moreover, Levin et al. [137] tried to crystallize Bet v 1 in complex with a Bet v 1-specific human monoclonal IgE antibody, however, it was not possible to obtain crystals. Surprisingly, the authors successfully mapped the

epitopes of their Bet v 1-specific recombinant human IgE single chain antibodies by using synthetic overlapping 14-mer Bet v 1-derived peptides. The fact that IgE from patients' sera only recognizes conformational epitopes of Bet v 1 [120], indicates, that the binding specificity of the Bet v 1-specific human monoclonal IgE might have been changed by random pairing of the VH and VL regions during the cloning process. A further concern of recombinant IgE mAbs is the lack of control whether the isolated clone is representative for the patients' allergen-specific IgE repertoire.

An indirect approach using murine monoclonal antibodies was also used for epitope studies. For example, Bet v 1 was co-crystallized with an Fab fragment of the monoclonal murine IgG1 BV16 [138]. The fact that BV16 was able to inhibit IgE binding to Bet v 1 indicates that the epitope defined by BV16 overlaps with an IgE binding epitope. However, different binding characteristics of murine IgG and human IgE to Bet v 1 are a crucial limitation of this method and may lead to wrong conclusions. For instance, IgGs raised by immunization of mice with two Bet v 1-derived peptides (aa 29-58 and aa 73-103) were able to bind to those peptides and to the correctly folded Bet v 1 whereas patients' IgE, as mentioned above, was not able to bind to the to the randomly coiled N- and C-terminal halves of Bet v 1 [120, 135].

Mimotopes are peptides which mimic the structure of an epitope. They are obtained from a phage display library by biopanning with allergen-specific patients' IgE. This method is quite simple and fast. However, the pool of obtained peptide sequences when panning against polyclonal sera can be large and diverse. In addition, the sequences of the mimotopes often bear no resemblance to the surface residues of the allergen. Hence, mapping the epitope onto the allergen surface requires algorithms which in many cases yield inconsistent results. For example, Bet mim 1, a mimotope of the murine anti-Bet v 1 antibody BIP 1 was predicted to be located at position 58-67 (Figure 20; page 29) [143]. However, we showed that BIP 1 binds to the Bet v 1-specific area of the chimera Api-Bet-1 with high specificity. In Figure 25, it is clearly visible that the Bet v 1-specific region of Api-Bet-1 shows little overlap with Bet mim 1.

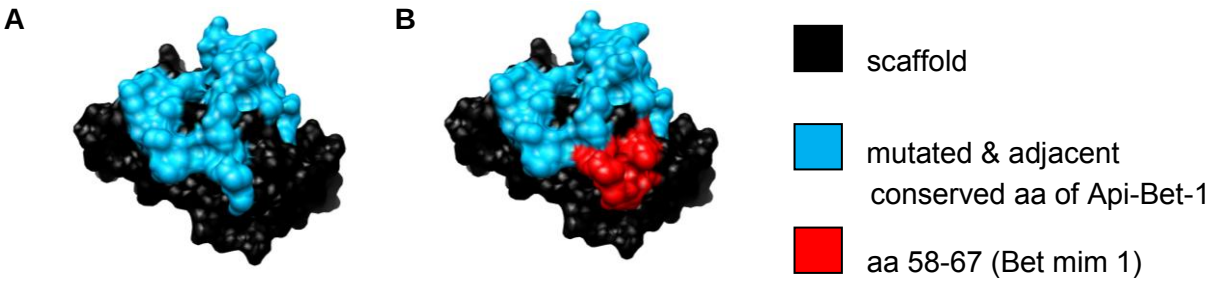


Figure 25: Predicted epitope area of the monoclonal antibody BIP 1. A) Api-Bet-1 showing the Bet v 1-specific surface area (mutated and adjacent conserved amino acids of Bet v 1 and Api g 1) in blue. **B)** The predicted mapping of the mimotope Bet mim 1 is shown in red.

For our studies [198-200], we grafted four different non-overlapping Bet v 1-specific surface areas onto the scaffold molecule Api g 1, the Bet v 1-homologue from celeriac. In this way, four chimeras, termed Api-Bet-1 to Api-Bet-4, were generated (Figure 26). In each chimera, 10-13 Api g 1-specific amino acid residues were exchanged by the Bet v 1-specific ones at the corresponding positions.

A very important benefit of the chimera approach is the possibility to evaluate the binding capacity of a high number of polyclonal sera to a defined conformational area. In our first study [198], 64 patients' sera were tested for their IgE recognition patterns of the four chimeras. In the third study [200], we tested 11 patients' sera at 9 different time points during AIT for their IgE, IgG1 and IgG4 binding ability to the chimeras.

The surface area of the mutated residues for each chimeric protein ranges from 701 Å² to 1093 Å² (Figure 26). Together with the adjacent conserved amino acids, the surface area of the contiguous Bet v 1-specific area per chimera ranges from 1528 Å² to 2125 Å², which corresponds to 20-27% of the overall Bet v 1 surface area. The size of an epitope is around 600-900 Å² [201], hence the Bet v 1-specific area on each chimera harbors the potential space for 2 to 3 non-overlapping epitopes. The simultaneous binding of IgE antibodies to at least two different epitopes on allergens is a prerequisite for the cross-linking of mast cell and basophil-bound IgE antibodies by allergens. Therefore, the investigation of the biologic relevance of IgE specific for the grafted area by performing basophil activation tests with polyclonal patients' sera is possible.

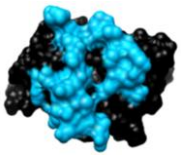
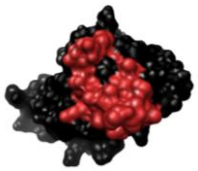
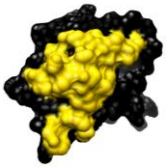
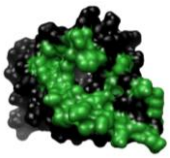
	Api-Bet-1	Api-Bet-2	Api-Bet-3	Api-Bet-4
mutated & adjacent conserved residues scaffold				
mutated residues:	11 amino acids	10 amino acids	13 amino acids	11 amino acids
surface area of Bet v 1:	**7764 Å²**			
surface area of mutated residues:	1093 Å²	904 Å²	1086 Å²	701 Å²
area of mutated & adjacent conserved residues:	**2125 Å²**	**1528 Å²**	**2040 Å²**	**1939 Å²**
% of the Bet v 1 surface area:	**27%**	**20%**	**26%**	**25%**

Figure 26: Comparison of the Bet v 1-specific surface areas of the four chimeras. Homology models were prepared with MODELLER 9.14 [202]. As templates, Bet v 1.0101 (4A88), Bet v 1.0112 (1BV1) and Api g 1.0101 (2BK0) were used. The overall quality of the models was assessed by molpdf, DOPE (Discrete Optimized Protein Energy), ProSa (Protein Structure Analysis) [203] and Procheck [204]. The mutated and adjacent conserved surface residues of the chimeras are depicted in blue (Api-Bet-1), red (Api-Bet-2), yellow (Api-Bet-3) and green (Api-Bet-4). The surface residues of the scaffold are shown in black. The surface area calculations were performed with GETAREA [205].

The Bet v 1-specific areas of all four chimeras represent almost the whole surface area of Bet v 1 (Figure 26). We determined the diversity of the Bet v 1-specific IgE response for 64 patients by using our chimeras and tested also for high IgE-binding areas [198]. We found that the Bet v 1-specific areas of Api-Bet-1, -2, and -3 were recognized by 70-81% of the patients' sera, whereas the grafted area on Api-Bet-4, which represents the C-terminal α-helix was only recognized by 45% of the patients' sera. Api-Bet-3 with the C-terminus of Bet v 1 exhibited the highest IgE binding capacity. These data were confirmed in our third study [200], where IgE binding to all four chimeras was observed in 7 of 11 patients (64%).

The most striking result of our first study [198] was that a total of 12 different IgE recognition profiles with numbers of recognized chimeras between 0 and 4 were observed. Consistent with our observations, many studies reported a high diversity of the patients' IgE antibodies when binding to Bet v 1 homologues [144] or mutants [124, 129, 139]. Thus, using a serum pool of

birch pollen allergic patients to investigate IgE epitopes on Bet v 1, which was done in some studies [133, 138, 139], does not reveal patient-specific information and may lead to non-representative conclusions. Similar to Bet v 1-specific IgE, we further found that the Bet v 1-specific IgG1 and IgG4 responses are highly patient-specific [200].

The concept of dominating IgE epitopes on Bet v 1 is also supported by the study of Mirza et al. [138]. The authors showed that the P-loop binding antibody BV16 inhibited ~40% of the binding of birch pollen-allergic patients' serum IgE to Bet v 1. The importance of the P-loop region as an important IgE epitope was furthermore confirmed by several studies [126, 128, 129, 133, 139, 140, 144]. Dominating conformational IgE epitopes were moreover described to be present in grass pollen allergens. Using well-defined monoclonal antibodies, Flicker et al. [206] demonstrated that the majority of the IgE-binding sites on the major timothy grass pollen allergen Phl p 1 cluster in the C-terminal portion of the allergen. The authors further showed that a Phl p 2-specific human IgG1-antibody strongly inhibited the binding of allergic patients' IgE to the allergen [207]. The inhibition by a full-size antibody was clearly higher than by an Fab fragment, which indicates that the antibody inhibits spatially clustered epitopes. Moreover, a polyclonal antibody specific for the 25-mer C-terminal peptide of the ryegrass allergen Lol p 1 was shown to inhibit basophil histamine release by more than 90% [208].

If IgE epitopes are spatially clustered on the allergen surfaces, passive immunization, which means the administration of allergen-specific monoclonal blocking IgG antibodies, would be a good concept to diminish allergic symptoms. Flicker at al. showed in a mouse model that passive immunization with monoclonal IgG antibodies specific for Bet v 1, Phl p 1 and Phl p 5 is effective for treatment and prevention of allergy [209]. We observed that the majority of the Bet v 1-specific patients' IgE in our studies [198, 200] recognized three or all four Bet v 1-specific surface areas, indicating that IgE binding epitopes are located across the entire surface of Bet v 1. These data are confirmed by the fact that when combining all IgE epitope mapping studies of Bet v 1, almost the whole surface area of Bet v 1 has been shown or predicted to be involved in IgE binding (Figure 27 & Figure 28). Thus, the administration of only a single Bet v 1-specific monoclonal blocking antibody will not yield satisfying results [198].

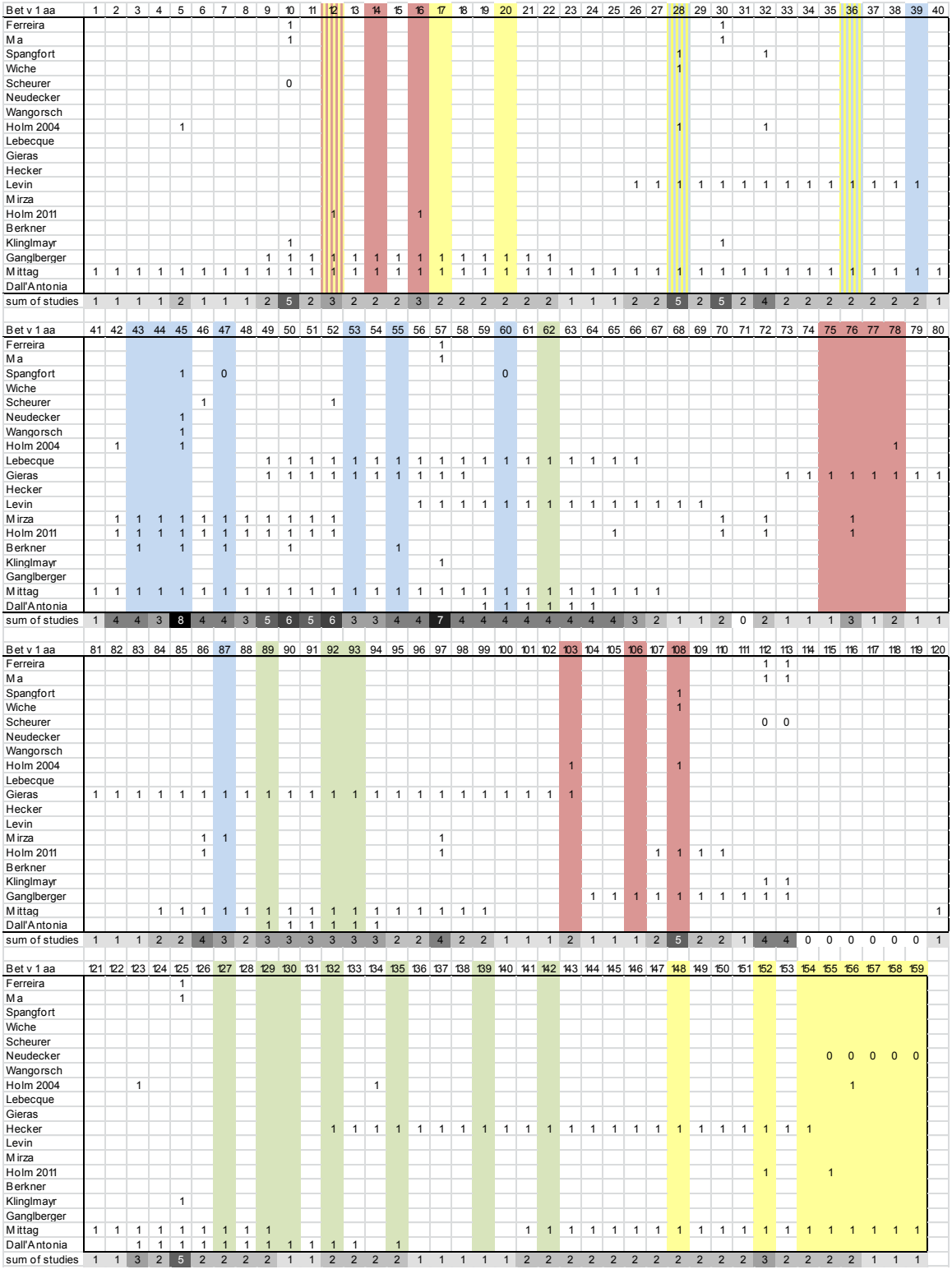


Figure 27: Amino acids involved in IgE-binding to Bet v 1 according to the literature. On the left side, the first authors of the studies showing involvement of certain amino acids for IgE binding to Bet v 1 (marked with 1 in the table) are listed. Amino acids marked with 0 were shown not to be involved in IgE binding to Bet v 1. The sequence of Bet v 1 is shown at the top of each panel. At the bottom of each panel, the sum of the studies which showed involvement is depicted. Amino acids mutated in the chimeras are labeled in blue (Api-Bet-1), red (Api-Bet-2), yellow (Api-Bet-3) and green (Api-Bet-4). Those residues which were mutated in two chimeras are depicted with hatched bars in the respective colors.

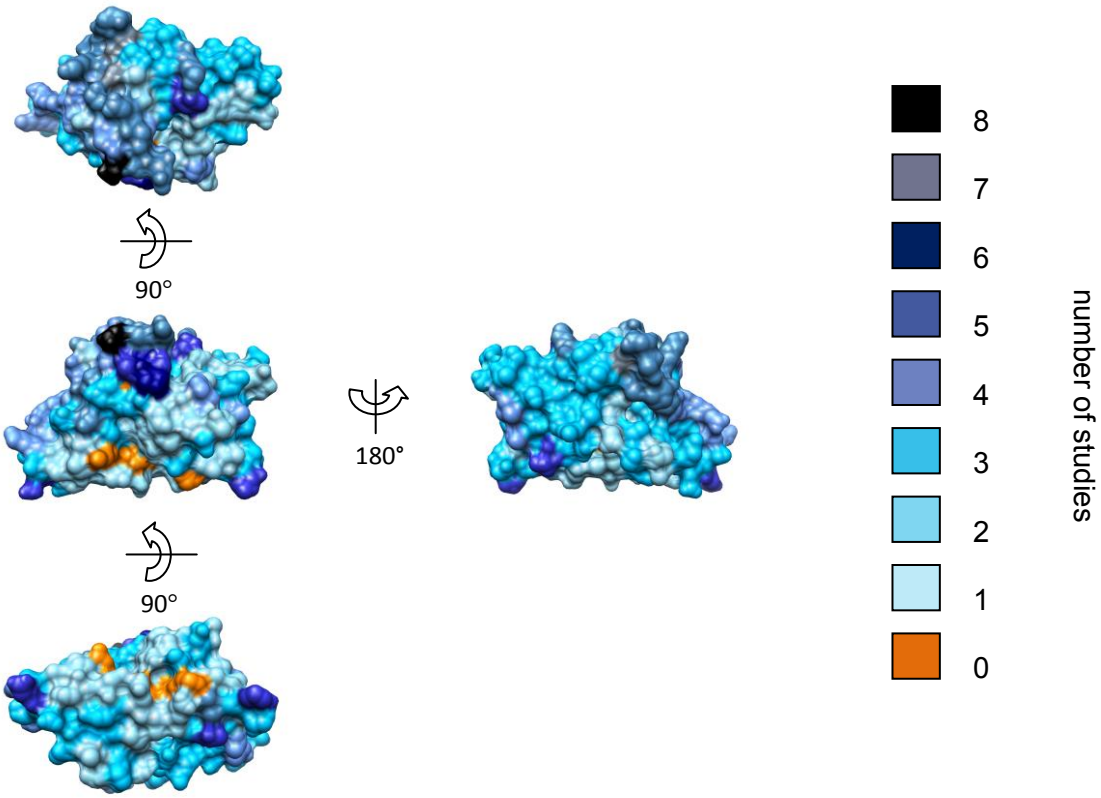


Figure 28: Bet v 1-specific surface residues and their involvement in IgE-binding. Surface residues of Bet v 1.0112 (1BV1) are colored according to the number of studies where they were shown to be involved in IgE binding. The models were prepared with UCSF Chimera [84].

One of the weaknesses when using chimeras is that artificial proteins are generated which might not display exactly the same three-dimensional structures of the grafted areas as the wildtype proteins. Circular dichroism spectra of our chimeric proteins were measured and revealed secondary structures comparable with the wildtype proteins. However, the sensitivity of CD measurements might be too low to detect minor structural changes. Hence, crystallization or NMR-spectroscopy of the chimeras would provide more detailed information, but involves a great deal of time and effort, especially if the structures of all chimeras should be evaluated [210, 211].

The OD values of Bet v 1 when testing with patients' sera were in most cases much higher than the OD values of the chimeras, although, in theory, the sum of all chimera-specific OD values should approximate the Bet v 1-specific OD value. Small structural changes in the grafted areas could be an explanation for this observation. Another explanation could be that antibodies specific for the edge region of the grafted areas might be washed away due to low affinity, which results in a lower OD value of the chimera. An illustrative example of different affinities of antibodies recognizing overlapping epitopes on Bet v 1 was shown by testing two Bet v 1-

specific monoclonal antibodies, BIP 1 and BIP 4 [198]. Both antibodies bound to Bet v 1 and Api-Bet-1, but not to Api g 1 and the other chimeras. Thus, both antibodies recognize an epitope at or near the P-loop, which is not present in Api g 1. Interestingly, BIP 4 showed considerably less binding to Api-Bet-1 than to Bet v 1, while BIP 1 bound to both proteins with equal intensity. Similarly, some clones from the patients' IgE may bind to epitopes represented by one of the chimeras, albeit with reduced affinity. The fact that BIP 1 and BIP 4 exclusively bound to Bet v 1 and Api-Bet-1 indicates however, that a Bet v 1-like area is present on the chimera. The same was shown for Api-Bet-3 with a specific single-chain variable antibody fragment.

Another point to consider when working with chimeras is that their expression and purification can be a laborious and time-consuming task. In our experimental setup, the *E. coli* strain BL21(DE3) was used as the host for protein expression. All four chimeras were inserted into the pET28a(+) vector. The expression levels of Api-Bet-1 and -4 were high and the chimeric proteins were soluble. The yield of purified proteins from 1L bacterial culture was 40 mg for Api-Bet-1 and 50 mg for Api-Bet-4. In contrast, Api-Bet-2 and -3 were not soluble. Therefore, those chimeras were cloned into the pET32a(+) vector and expressed as fusion proteins with thioredoxin. However, after lysis of the cells, the major part of the fusion proteins remained in the pellet. Therefore, the insoluble fraction was denatured in 8 M urea and refolded to obtain small amounts of soluble fusion-proteins. After the cleavage of the fusion proteins with protease, approximately 2 mg from 1L bacterial culture of Api-Bet-2 and -3 were obtained. Similarly, difficulties with the expression of chimeric proteins were also reported by Alcocer et al. [212]. The authors succeeded in expressing only six out of nine chimeras which were derived from two 2S albumins, Ber e 1 from Brazil nut and SFA8 from sunflower seed.

The design of the chimeras is of high importance. The surface residues to be mutated in our chimeras were selected in order to reproduce four separate Bet v 1-specific surface areas. However, the sizes of the grafted areas are rather large (Figure 26) and thus will have to be reduced for the next generation of chimeras in order to allow a more detailed epitope-specific investigation. The challenge will be to find a good compromise between minimized patches and a manageable number of chimeric proteins that can be expressed and purified. Another issue to decide will be whether the grafted regions on several chimeras should overlap or be located next to each other.

We used the Bet v 1-homologue from celeriac, Api g 1.0101, as scaffold molecule because it binds only small amounts of IgE of birch pollen allergic patients and its 3D-structure is available [192]. Api g 1.0101 has 41% sequence identity with Bet v 1.0101 and is recognized by 44% of the patients' sera [198]. Despite this high recognition rate, in almost all cases, notably higher amounts of IgE were bound by the chimeras than by Api g 1.0101. To assess specific antibody binding to the grafted region on each chimera, the OD value measured for Api g 1.0101 was

subtracted from the chimera-specific OD value. In order to avoid this step, a non-IgE binding scaffold should be chosen to improve the chimera design. Following our publication, such an approach was chosen by Berkner and colleagues [140]. However, in this study the authors grafted only one individual area, the P-loop region of Bet v 1, onto a non-IgE-binding protein with a Bet v 1-like fold, the enzyme (S)-norcoclaurine synthase, and subsequently observed enhanced IgE binding to this chimera. Moreover, in our hands the P-loop region of Bet v 1 did not turn out to be the most important IgE-binding area of this allergen [198]. Notably, many options of most probably non-IgE binding structural Bet v 1-homologues exist, which can be found in all three domains of life [88] and might be an even better choice as a template.

Detailed knowledge of the location of IgE-binding epitopes would be valuable for future attempts to increase the resolution of allergy diagnosis from component-resolved to epitope-resolved diagnosis. Seventy percent of all birch pollen allergic individuals react with foods which contain proteins that share high degrees of sequence similarity with Bet v 1 and therefore cause pollen-related food allergies [82]. Until now, it is not clear if the lack or the low affinity of cross-reactive IgE antibodies or the presence of blocking antibodies results in oral tolerance of birch pollen allergic patients. Geroldinger-Simic et al. [213] found elevated food allergen-specific IgG4/IgE rations in hazelnut- or apple-tolerant birch pollen allergic patients. In contrast, Guhsl et al. [214] reported that no significant correlation between plant food allergy and increased or reduced levels of IgE, IgG1, IgG4 or IgA specific for most Bet v 1-related allergens was observed. When we investigated the relationship between reported food allergies and the IgE recognition to a certain chimera we found no significant correlation. A reason for this could be that the grafted areas are too large to assess such correlations. Furthermore, it would be of high interest to analyze chimera binding patterns of immunoglobulin classes other than IgE from birch pollen allergic individuals and correlate this information with their reported food allergies.

The presence of blocking IgA antibodies in saliva and the oral mucosa could be a possible explanation why some birch pollen allergic individuals tolerate Bet v 1-related foods [215]. Therefore, in future projects, not only the antibody repertoires present in patients' sera but also antibodies in the saliva should be investigated for their epitope specificities. Once chimeras with defined individual epitopes of Bet v 1 will be available, it might be possible to define clinically relevant cross-reactive epitopes. Those chimeras could be included on an allergen chip and hence would enable a patient-tailored diagnosis and therapy.

The clinical benefit of birch pollen immunotherapy for birch pollen-related food allergy is still under debate. Improvements as well as unchanged clinical symptoms to birch pollen-related foods were reported [216-219]. Some patients even developed allergic reactions to foods during the course of therapy [220]. A future AIT concept for birch pollen allergic patients with no

reported food allergies could be treatment with a hypoallergenic variant of Bet v 1 which lacks cross-reactive epitopes to prevent them from developing food allergies.

Strong evidence for the efficacy of immunotherapy in birch pollen allergy-induced allergic rhinoconjunctivitis has been well-documented by numerous trials [221]. Nevertheless, only 73% of 746 patients allergic to Bet v 1 or the major allergens of timothy grass (Phl p 1/Phl p 5) reported good or very good improvements of allergic symptoms after AIT [193]. In order to improve birch pollen immunotherapy, it is crucial to understand the development of the different immunoglobulin classes during AIT. Numerous studies exist which revealed that the induction of allergen-specific IgGs, which act as blocking antibodies, is essential for successful AIT [222]. However, limited data exist about the diversities and specificities of the IgG subclasses inducted during AIT. Recently, Vickery and colleagues published that during oral peanut immunotherapy, the allergen-specific IgG4 repertoire broadly expanded in a polyclonal fashion including *de novo* specificities [183]. Similarly, Hoh et al. [223] used high-throughput DNA sequencing of antibody heavy chain repertoires and reported that due to peanut OIT, IgG4 was found to be more mutated at 5 longitudinal time points compared to IgE. This indicated that more mutated and potentially higher-affinity IgG4 antibodies were generated during immunotherapy.

In contrast, we revealed that the individual recognition profiles of the early established Bet v 1-specific IgG4 repertoire remained mainly constant during AIT [199]. These results were obtained by two entirely different methods, phage display and the chimera-based approach [199]. Competitive immunoscreening of phage-displayed peptides revealed that the predicted IgG4 epitopes at 6 and 36 months of birch pollen immunotherapy overlapped by 37%-63%. These results were confirmed by IgG4-ELISA with the chimeric proteins, where 8 of 9 patients (89%) displayed IgG4 reactivity to the same chimera after 6 and 36 months of immunotherapy.

In a second study, we used sera from the same patient cohort to monitor the development of Bet v 1-specific IgE, IgG1, and IgG4 repertoires before and after 1, 3, 6, 12, 18, 24, 30, and 36 months of effective subcutaneous birch pollen immunotherapy [200]. In total, sera from 11 patients were investigated by ELISA. Interestingly, IgE binding to all 4 chimeras was observed in 7/11 patients (64%). In contrast, only 2 and 3 patients' sera showed IgG1 or IgG4 binding to all chimeras, respectively. The fact that Bet v 1-specific IgE showed a higher epitope diversity than IgG1 or IgG4, indicating that most likely not all IgE epitopes were blocked by AIT-induced IgG by means of direct epitope competition.

Furthermore, we tested five AIT-treated patients' sera for their activity to reduce the binding of Bet v 1-IgE complexes to $CD23^+$ B cells. Notably, four of five sera showed 86-98% IgE-inhibition already after 6 months of immunotherapy [199]. These results indicate that not only direct epitope competition but rather the induction of high levels of allergen-specific IgG is crucial for

successful AIT. Nevertheless, many studies indicate that the induction of high levels of allergen-specific IgG does not always correlate with a clinical improvement [149]. Most likely, several mechanisms including inhibition of the IgE-facilitated allergen presentation and the induction of IgE epitope-matched IgG antibodies are essential for successful AIT and synergistically lead to the amelioration of allergic symptoms.

The importance of direct epitope competition of IgE and IgG antibodies was confirmed by a study of Savilahti and colleagues [184]. The authors reported that a failure to complete oral immunotherapy with cow's milk due to adverse reactions was associated with a lower overlap of the induced IgG4 antibodies with IgE epitopes.

Unfortunately, we did not have access to sera from patients whose birch pollen immunotherapy was unsuccessful. However, our chimeras would be an excellent tool to compare the epitope specificities of induced antibodies in sera from patients who underwent successful or non-effective birch pollen AIT. Such a comparison might lead to new insights into why immunotherapy is not efficient in some allergic individuals.

It was furthermore reported that the cross-linking of FcεRI and FcγRIIB on the surface of mast cells and basophils due to the presence of allergen-specific IgG and IgE might lead to the inhibition of mediator release [175]. In theory, the induction of allergen-specific IgG1 antibodies might be beneficial for successful AIT. IgG1 binds with a significantly higher affinity to FcγRII than IgG4 [40]. However, although the immunotherapy had been effective in all patients included in our study [200], Bet v 1-specific IgG1 had only been induced in 7 of 11 birch pollen allergic individuals.

The birch pollen allergic patients from our studies [199, 200] had been treated with birch pollen extracts over a period of 3 years. Extract-based immunotherapies, however, have several disadvantages, which include the variability in composition and allergen content of the extract [224]. These problems can be overcome by using purified natural or recombinant allergens. However, in order to be able to administer high doses of allergens and to avoid side effects, different kinds of hypoallergenic variants with reduced IgE reactivity have been designed. According to our results the entire surface area of Bet v 1 is allergenic [198-200]. Hence, the design of hypoallergenic variants of Bet v 1 which are based on the mutation of selected high IgE binding areas seems not to be possible.

Presently, concepts that focus on destroying IgE epitopes by fragmentation or oligomerization of Bet v 1 hold more promise for improving birch pollen immunotherapy. Birch pollen-allergic patients were treated with a mixture of two recombinant Bet v 1 halves with preserved T-cell epitopes (aa 1-74; aa 75-160) [120] or with a recombinant Bet v 1 trimer [225]. Patients from

both studies developed protective IgG antibodies and had a reduced IgE memory boost during the birch pollen season [226-229].

Moreover, due to the fact that IgE binding epitopes of Bet v 1 are conformation-dependent [120], folding variants of Bet v 1 which do not have a correct 3D-structure are promising candidates for specific immunotherapy. IgE binding to those variants is reduced but T cell reactivity is retained. Treatment of Bet v 1 with NaOH modifies the structure and thus prevents IgE binding to the allergen [230]. Patients treated with a Bet v 1 folding variant revealed lower symptom medication scores than the birch pollen extract-treated group [231]. Another fold variant of Bet v 1 was produced by minimal changes of the sequence which led to a loss of the Bet v 1–like fold [232]. This construct was shown to be beneficial in a therapeutic Bet v 1 mouse model.

In summary, we are of the opinion that a second generation of improved chimeras will lead to an even better understanding of the location and importance of Bet v 1-specific IgE epitopes. This knowledge would pave the way for new diagnostic tools and hypoallergenic variants of Bet v 1. Improved diagnostic methods might help to provide hints to why immunotherapy is not successful in approximately 30% of treated patients [193]. In addition, such diagnostic methods might enable an early evaluation of the therapeutic outcome and thus would help to avoid 3 years of injections, if the immunotherapy were predicted to be unsuccessful.

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Reshaping the Bet v 1 fold modulates T(H) polarization. J Allergy Clin Immunol, 2011. 127(6): p. 1571-8.

LIST OF FIGURES

LIST OF ABBREVIATIONS

aa	Amino acid
AIT	Allergen-specific immunotherapy
B_R1	IL-10-secreting regulatory B cells
Breg	B regulatory cells
CD	Cluster of differentiation
CDRs	Complementarity determining regions
C	Constant
CSBP	Cytokinin-specific binding protein
CTLA-4	Cytotoxic T lymphocyte antigen 4
D	Diversity
DC	Dendritic cell
DOPE	Discrete Optimized Protein Energy
EAACI	European Academy of Allergy and Clinical Immunology
EBV	Epstein-Barr Virus
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen-binding
FcεRI	High affinity IgE receptor 1
FcεRII	Low affinity IgE receptor 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H	Heavy
HR	Histamine receptor
Ig	Immunoglobulin
IL	Interleukin
ILC2	Group 2 innate lymphoid cells
J	Joining
L	Light

mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
NCS	(S)-Norcoclaurine synthases
NMR	Nuclear magnetic resonance
NOD	Nucleotide oligomerization domain
OAS	Oral allergy syndrome
OD	Optical density
OIT	Oral immunotherapy
PD-1	Programmed death 1
PDB	Protein Data Bank
PR	Pathogenesis-related proteins
Q3OS	Quercetin-3-O-sophoroside
RDD	Rapid drug desensitization
scFv	Single-chain variable fragment
SCIT	Subcutaneous immunotherapy
SLIT	Sublingual immunotherapy
SPADE	Surface comparison-based Predication of Allergenic Discontinuous Epitopes
StAR	Steroidogenic acute regulatory protein
TCR	T-cell receptor
Th	T helper cell
T_R1	IL-10-secreting regulatory T cells
Treg	T regulatory cells
TSLP	Thymic stromal lymphopoietin
V	Variable
WHO/IUIS	World Health Organization and International Union of Immunological Societies

APPENDIX

CURRICULUM VITAE

PERSONAL DETAILS

Name	DI Barbara Gepp
Date of birth	17/11/1985
Place of birth	Vienna
Nationality	Austria

EDUCATION

since 04/2010	PhD thesis at the Department of Pathophysiology and Allergy Research, Medical University of Vienna
04/2009 – 03/2010	Master thesis at the University of Natural Resources and Applied Life Sciences, Vienna; Department of Biotechnology: "Effects of the secretome from senescent endothelial cells on adipose derived stem cells"
10/2007 – 03/2010	Master course Biotechnology at the University of Natural Resources and Applied Life Sciences (BOKU), Vienna
01/2008 – 06/2008	ERASMUS Exchange Program at the National University of Ireland, Galway
09/2004 – 09/2007	Bachelor course Food- and Biotechnology, University of Natural Resources and Life Sciences (BOKU), Vienna
09/2000 - 06/2004	Grammar school (BORG), Mistelbach

WORK EXPERIENCE

since 04/2010	Medical University of Vienna, Department of Pathophysiology and Allergy Research (PhD project)
04/2009 – 09/2009	University of Natural Resources and Applied Life Sciences, Vienna, Department for Biotechnology (Master thesis project)
09/2008	Octapharma AG, Vienna
07/2008 – 08/2008	Akras Flavours GmbH, Biedermannsdorf
09/2007	Brennerei Starrein GmbH, Starrein
08/2007	Brau Union Österreich AG, Schwechat
09/2006	OMV Refining & Marketing, Schwechat
07/2006	MAPAG GmbH, Gumpoldskirchen

AWARDS AND GRANTS

2016 **Travel Grant** for the 14th Immunology Winter School on Basic Immunology Research in Allergy and Clinical Immunology, February 4-7, 2016, Cortina d'Ampezzo, Italy

2015 **Poster Prize** at the ÖGAI Symposium 50-Years of B-Lymphocytes, December 11, 2015, Vienna, Austria

Travel Grant for the 6th International Symposium on Molecular Allergology, November 19-21, 2015, Lisbon, Portugal

Best Oral Presentation Award at Annual Congress of the European Academy of Allergy and Clinical Immunology 2015, June 6-10, Barcelona, Spain

2014 **Poster Prize** at the Annual Congress of the European Academy of Allergy and Clinical Immunology 2014, June 7-11, Copenhagen, Denmark

2012 **Travel Grant** for the Annual Congress of the European Academy of Allergy and Clinical Immunology 2012, June 16-20, Geneva, Switzerland

Best Oral Presentation Award and **Poster Prize** at the Annual Congress of the European Academy of Allergy and Clinical Immunology 2012, June 16-20, Geneva, Switzerland

2011 **Travel Grant** for the XXII World Allergy Congress, December 4-8, 2011, Cancun, Mexico

Poster Prize at the Annual meeting of the Austrian Society for Allergology and Immunology, September 15-17, 2011, Graz, Austria

Poster Prize at the 30th Congress of the European Academy of Allergy and Clinical Immunology, June 11-15, 2011, Istanbul, Turkey

INVITED TALK

2013 **Pirquet-Club:** "Epitope grafting: Eine neue Methode zur Charakterisierung des Bet v 1-spezifischen IgE Repertoires von Birkenpollenallergikern" organized by the Austrian Society of Allergology and Immunology. Medical University of Vienna, December 2, 2013, Austria

INVITED CHAIR

2011 Chairperson at the XXII World Allergy Congress, December 4-8, 2011, Cancun, Mexico

PUBLICATIONS

Gepp B, Lengger N, Bublin M, Hemmer W, Breiteneder H, Radauer C. (2014): *Chimeras of Bet v 1 and Api g 1 reveal heterogeneous IgE responses in patients with birch pollen allergy.* J Allergy Clin Immunol. 134: 188-194

Subbarayal B, Schiller D, Möbs C, Pfützner W, Jahn-Schmid B, Gepp B, Breiteneder H, Vieths S, Bohle B. (2015): *The diversity of Bet v 1-specific IgG4 antibodies remains mostly constant during the course of birch pollen immunotherapy.* J Allergy Clin Immunol. 136: 1680-1682

Gepp B, Lengger N, Möbs C, Pfützner W, Radauer C, Bohle B, Breiteneder H. (2015): *Monitoring the epitope recognition profiles of IgE, IgG, and IgG during birch pollen immunotherapy.* J Allergy Clin Immunol. doi: 10.1016/j.jaci.2015.10.022.

PRESENTATIONS AT INTERNATIONAL CONGRESSES

Gepp B, Balazs N, Hemmer W, Radauer C, Breiteneder H. (2010): *Analysis of the importance of selected epitopes for IgE binding to Bet v 1 using epitope grafting.*
Poster presentation at the EAACI-ISMA 2010 – 4th International Symposium on Molecular Allergology, October 29-31, 2010, Munich, Germany

Gepp B, Balazs N, Hemmer W, Radauer C, Breiteneder H. (2010): *Analysis of IgE binding to Bet v 1 and homologous food allergens by epitope grafting.*
Poster Presentation at the Annual Meeting of the Austrian Society for Allergology and Immunology in co-operation with the national societies from Croatia, Czech Republic, Hungary, Slovakia and Slovenia. December 3-5, 2010, Vienna, Austria

Gepp B, Balazs N, Hemmer W, Radauer C, Breiteneder H. (2011): *Epitope grafting reveals patient specific IgE binding profiles to the major birch pollen allergen Bet v 1.* Allergy 66 Suppl. 94: 99.
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PRESENTATIONS AT NATIONAL CONGRESSES AND SYMPOSIA

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Gepp B, Lengger N, Möbs C, Pfützner W, Radauer C, Bohle B, Breiteneder H. (2015): *Longitudial analysis of Bet v 1-specific epitope repertoires during birch pollen immunotherapy.*
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Gepp B, Lengger N, Kitzmüller C, Radauer C, Bohle B, Breiteneder H. (2015): *A chimeric protein containing the C-terminus of Bet v 1 is a potent inducer of basophil degranulation.*
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