EPITOPE GRAFTING: RE-CREATING A CONFORMATIONAL BET V 1 ANTIBODY EPITOPE ON THE SURFACE OF THE HOMOLOGOUS APPLE ALLERGEN MAL D 1

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Birch allergic patients often experience oral allergy syndrome upon ingestion of vegetables and fruits most prominently apple, that is caused by antibody cross-reactivity of patients IgE antibodies to proteins sharing molecular surface structures with the major birch pollen allergen Bet v 1. Still, to what extent two molecular surfaces need to be similar for clinically relevant antibody cross-reactivity to occur is unknown. Here we describe the grafting of a defined conformational antibody epitope from Bet v 1 onto the surface of the homologous apple allergen Mal d 1. Engineering of the epitope was accomplished by genetic engineering substituting amino acid residues in Mal d 1 differing between Bet v 1 and Mal d 1 within the epitope defined by the mAb BV16. The kinetic parameters characterizing the antibody binding interaction to Bet v 1 and to the mutated Mal d 1 variant, respectively, were assessed by biacore experiments demonstrating indistinguishable binding kinetics. This demonstrates that a conformational epitope defined by a high affinity antibody-allergen interaction can successfully be grafted onto a homologous scaffold molecule without loss of epitope functionality. Furthermore, we show that increasing surface similarity to Bet v 1 of Mal d 1 variants by substitution of 6-8 residues increased the ability to trigger basophil histamine release with birch allergic patients’ blood not responding to natural Mal d 1. Conversely, reducing surface similarity to Bet v 1 of a Mal d 1 variant by substitution of 3 residues abolished histamine release in one patient reacting to Mal d 1.

Type 1 hypersensitivity, i.e. IgE-mediated allergy, is a substantial health problem in countries having adapted to Western lifestyle (1;2). Grass pollen and house dust mite are the most important allergen sources world-wide (3), but on a regional basis local pollen may be the cause of even higher prevalences of sensitization. In Scandinavia, inhalation allergy to birch pollen is among the most prevalent (4). Patients allergic to birch pollen most often also react to pollens of the related trees alder and hazel (5) prolonging the season with symptom load. Hazel pollinates in February-March, alder typically in March, and birch in April-May. Birch is the quantitatively dominating species reaching average pollen counts in the peak season of about 500 grains per m³, which is about 10 times the level of hazel and alder, and consequently most patients are sensitized to birch pollen. Birch pollen allergic patients have an increased risk of symptoms upon ingestion of foods, such as nuts and certain vegetables and fruits, for example apple (6;7). Symptoms induced by the foods are typically mild and restricted to the region around the mouth, for example itching and swelling of lip or tongue and throat irritation. This phenomenon is referred to as the Oral Allergy Syndrome, (OAS) (8;9).
Analysis of allergic patients serum IgE by crossed immuno-electrophoresis have revealed that all birch pollen allergic patients have IgE directed to the major allergen Bet v 1 (4). Although patients may occasionally react to other allergens, IgE to Bet v 1 accounts for more than 90% of the IgE directed towards birch pollen allergens (4). Molecular studies have shown the presence of major allergens homologous to Bet v 1 in extracts of hazel and alder pollen. The current model for cross-reactivity is that the major allergens with ≥ 75% sequence identity have common molecular structures (i.e. epitopes) on their surfaces, that are recognized by the same patient IgE antibodies (10). Also, more distantly related species, such as apple, contain molecules homologous to Bet v 1 in extracts of hazel and alder pollen. The molecular mechanism underlying OAS upon ingestion of apple is thought to be the same as those responsible for antibody cross-reactivity in general. Sequence similarity between Mal d 1 and Bet v 1 is obviously reduced compared to the more closely related species (hazel, alder), but studies comparing the molecular surfaces of Bet v 1 and Mal d 1 suggest that the OAS is indeed caused by IgE binding to epitopes which are shared between Bet v 1 and Mal d 1 (13). Still, the conserved surface areas between Bet v 1 and Mal d 1 are smaller compared to conserved surface areas between Bet v 1 and Aln g 1, the major allergen of alder pollen, and consequently, only a fraction of birch allergic patients have IgE directed to the conserved surface areas in Mal d 1 and therefore display OAS upon ingestion of apple.

The dynamics of the processes taking place at the surface of mast cells and basophils have been described in some detail. IgE molecules are anchored to the cell surface through high affinity FcεRI receptors with the ability to float freely over the cell surface. Effector cell activation occurs after simultaneous binding of the same allergen molecule by two or more IgE antibodies (14). As shown in a recent article (15) using a panel of recombinant allergen specific IgE antibodies activation of signal transduction requires that the first interaction is of medium to high affinity to withhold the allergen near the cell surface. The receptor-bound IgE molecules then float to the vicinity of the complex facilitating the second interaction. A consequence of this cooperativity is that if the first interaction is of high affinity the affinity of the second interaction is not critical and even low affinity interactions may play a significant role in cross-linking and therefore also effector cell activation. This suggests that an IgE-allergen interaction through a single high affinity antibody may manifest into clinically significant cross-reactivity in the presence of an additional IgE-allergen interaction of any affinity. Still, the degree of similarity of molecular surface structures on related but non-identical allergens needed to support relevant cross-reactivity in relation to allergy and OAS is still uncertain. Here a well characterized conformational B-cell epitope from the major birch pollen allergen Bet v 1 was re-created onto the surface of Mal d 1, and the kinetic parameters characterizing antibody-epitope interactions were measured using Biacore technology. Furthermore, the allergenicity of several mutated Mal d 1 variants was investigated in IgE binding assays with birch patient serum IgE and in basophil histamine release experiments with blood from birch allergic patients to investigate the effects on cross-reactivity of increasing or reducing surface similarity to Bet v 1 of Mal d 1 variants.

**EXPERIMENTAL PROCEDURES**

**Cloning of constructs** Generation of rMal d 1 variants was initiated on a previously cloned DNA construct (13) coding for Mal d 1 isoform (Genbank accession no.: Q8L6K9) inserted into the *Escherichia coli* expression vector pMAL-c (New England Biolabs, Hertfordshire, UK). First, nucleotides targeted for mutation that were located closely together in the DNA
sequence were mutated by PCR-based overlap extension using sense and anti-sense mutation-specific oligonucleotide primers accommodating each mutation along with sense and anti-sense oligonucleotide primers accommodating either upstream or downstream neighbor mutations or the N-terminus/C-terminus of Mal d 1, respectively. Secondly, the PCR products were purified, mixed and used as templates in a PCR assembling reaction with oligonucleotide primers accommodating the N-terminus and C-terminus of rMal d 1. The N-terminus primer included nucleotides coding for a Factor Xa cleavage site for generation of rMal d 1 with authentic N-terminus. Both oligonucleotide primers contained nucleotide overhang for in frame directional cloning into the multiple cloning site of pMAL-c. After transformation into *Escherichia coli* K-12, strain DH5α correct nucleotide sequence of inserts were verified on both DNA strands by DNA sequencing.

**Protein expression and purification**

Expression vector pMAL-c containing inserts coding for rBet v 1 (Z80104), rMal d 1 (Q8L6K9) or rMal d 1 variants, respectively, were over-expressed in *Escherichia coli* K-12, strain DH5α yielding fusion proteins with amino-terminal maltose binding protein. Purified molecules with authentic N-terminus were obtained by amylose-affinity purification followed by Factor Xa cleavage (Protein Engineering Technology APS, Denmark) and size exchange chromatography as described previously in yields of 3-5 mg/L (16). Protein concentrations were determined by UV absorption at 280 nm in a Shimadzu UV-1601PC (Columbia, MD) spectrophotometer. All purified protein preparations appeared as single bands with an apparent molecular weight of 17.5 kDa after silver-stained SDS-polyacrylamide electrophoresis (data not shown).

**Circular dichroism spectroscopy**

Circular dichroism spectra were obtained using an OLIS DSM 10 CD spectrophotometer (On Line Instrument Systems, Bogart, GA), equipped with cylindrical (31-Q-1/CD) or square (21-Q-1/CD) 0.1 cm light path quartz cuvettes (Starna, Hainault, UK). The spectra were recorded from 260 nm to 184 nm collecting data at every second nm, 38 data points per spectrum. The temperature of the cuvette was maintained constant using a Julabo Model F30-C bath/circulator temperature control module (Julabo Labortechnik, Seelbach, Germany). Spectra were obtained in 0.01M sodium phosphate buffer, pH 7.2, at a concentration of 0.2 mg protein per mL. Each spectrum represents the arithmetic mean of four determinations corrected for buffer absorption and normalized to \( \Delta \varepsilon = 0 \) at 260 nm. The mDeg was transformed to \( \Delta \varepsilon \) using:

\[
\Delta \varepsilon = \frac{m_{\text{Deg}}}{32980 \cdot c \cdot l} \ 	ext{Abs units} / (M \cdot \text{cm})
\]

Where mDeg is the circular dichroism signal, c is the concentration in mol/L as measured by amino acid analysis and l is the length of the light path in cm.

**Biacore experiments**

Biacore experiments were set up according to the manufactures recommendations (Biacore® 2000 Instrument Handbook, January 2001; Getting started Biacore® 3000, October, 1998), in brief the monoclonal antibody BV16 (1 to 10 \( \mu \)g/ml in HBS-EP biacore buffer) was injected into the four flow-channels on the Biacore Sensor Chip CM5 for 300 sec at a flow rate of 30 \( \mu \)L/min (charging phase), followed by a 150 sec flow (30 \( \mu \)L/min) of HBS-EP buffer (equilibrium phase). The antigen i.e. rBet v 1 (Z80104), rMal d 1 (Q8L6K9) or one of the rMal d 1 variants was injected for 180 sec (association phase), followed by a HBS-EP buffer flow (30 \( \mu \)L/min) for up to 1000 sec (dissociation phase), and the flow-channels were regenerated by a pulse of 10 mM glycine solution, pH 1.8 for 30 sec at a flow rate of 30 \( \mu \)L/min. The data used to estimate \( k_1 \) (M\(^{-1}\)·s\(^{-1}\)), \( k_1 \) (s\(^{-1}\)) and \( K_d \) (M) values were obtained from runs performed with 5 different antigen concentrations (1 to 150 \( \mu \)g/mL).

**Specific human serum IgE inhibition assay**

Individual patient sera or serum pools of equals volume of 10 sera from birch pollen allergic patients was used for specific serum IgE
inhibition assays. All patients had a clinical history of birch pollinosis and were RAST class 2 or more against birch extract. Information with respect to clinical history of apple allergy was not available. rBet v 1 was biotinylated at a molar ratio of 1:5 (rBet v 1:biotin). The inhibition assay was performed on ADVIA Centaur System (Bayer, Kgs. Lyngby, Denmark) as follows: A serum sample (25 µl) was incubated with paramagnetic beads (solid phase) coated with a monoclonal mouse anti human IgE antibody (ALK-Abelló, Hørsholm, Denmark), washed, resuspended and incubated with a mixture of biotinylated rBet v 1 and inhibitor (non-biotinylated molecules i.e. rBet v 1, rMal d 1 or derivates molecules) in dilution series. The amount of biotinylated rBet v 1 bound to the serum IgE on the solid phase was estimated from the measured relative light units (RLU) after incubation with acridinium-ester labeled streptavidin. The degree of binding was calculated as the ratio between the RLU's obtained using buffer and rBetv 1, rMal d 1 or derivates as inhibitor, respectively.

**Histamine release in human basophils**

Heparinized blood (20 ml) was drawn from birch pollen allergic patients with a history of seasonal hay fever, stored at room temperature, and used within 4 hours. 100 µl of pipes buffer pH 7.4 or 100 µl of allergen dilutions in Pipes buffer pH 7.4, 1 ng, 3 ng, 10 ng, 30 ng, 100 ng, 1000 ng of rBet v 1 (Z80104), rMal d 1 (Q8L6K9) or mutated rMal d 1 variants were added to 96 well plates in triplicates. Plates were preheated to 37°C before 100 µl of preheated (37°C) blood, diluted 1:5 with Pipes buffer pH 7.4, were added to wells with different allergen dilutions or Pipes buffer. Plates were incubated 30 minutes at 37°C before centrifuged 10 minutes at 800g in a centrifuge with a plate-rotor. Supernatants from each of the wells were transferred to wells in new micro-titer plates that were lid and sealed with sealing tape before incubated at -20°C. All histamine release experiments were performed in triplicates.

**Histamine ELISA assay**

Histamine release in each sample was measured using the ELISA based Enzyme Immunoassay Kit ref. 2015 (Immunotech, France) following the recommendations of the supplier. 100 µl of sample or standard solutions were acylated with 25 µl of acylation buffer and 25 µl of acylation reagent to permit later binding to anti-histamine-antibodies. 50 µl of acylated histamine samples, standards or negative controls were then added to anti-histamine-antibody coated wells along with 200 µl of alkaline phosphatase conjugated histamine and incubated 2 hours at 4°C with shaking, 250 rpm. Plates were rinsed 3 times with diluted wash solution before 200 µl of Para-nitrophenylphosphate substrate solution was added to the plates that were incubated 30 minutes at room temperature with shaking, 250 rpm, before 50 µl of stop solution were added. The ability of the histamine samples and histamine standards (1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 1000 nM) to inhibit binding of alkaline phosphatase conjugated histamine to anti-histamine antibody coated wells were measured as relative absorbance at 405 nm.

**Sequence analysis and molecular surface illustrations**

BLAST searches with the amino acid sequence of Mal d 1 (Q8L6K9) were performed at the Internet server of the National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov/BLAST/ and at the Swiss Institute of Bioinformatics (http://www.expasy.org/tools/blast/). Three-dimensional structure models of Mal d 1 and mutated variants were obtained using the program Deepview/Swiss-pdb-viewer v4.0.1 and the SWISS-MODEL Comparative Protein Modelling Server (http://swissmodel.expasy.org/) (17) using the pdb coordinates (http://www.rcsb.org/pdb) for Bet v 1 (1BV1) as template and the amino acid sequences of respective Mal d 1 molecules or Mal d 1 variants. The program DS ViewerPro version 5.0 (http://www.accelrys.com) was used for depiction of the water accessible molecular surfaces. Unless stated otherwise amino acid residues conserved between Bet v 1 and Mal d 1
were colored red and conservative substitutions were colored blue in all shown models. Substitution of amino acid residues within the following groups were considered conservative: Val-Leu-Ile-Met, Ser-Thr-Cys, Phe-Tyr-Trp, Asp-Glu, Asn-Gln, and Lys-Arg-His.

RESULTS

Serum IgE antibody cross-reactivity

To visualize the basis for birch allergic patients’ serum IgE antibody cross-reactivity towards the apple allergen Mal d 1 the amino acid sequences of 77 published different Mal d 1 isoforms were analyzed with respect to sequence identity to Bet v 1. Structural similarities between Bet v 1 and Mal d 1 enabled modeling of the 3D-structure of Mal d 1 isoforms upon combining amino acid sequence information of the different Mal d 1 isoforms with the pdb coordinates from the known structure of Bet v 1 (18). In figures 1A, 1B and 1C the modeled water accessible molecular surfaces of Mal d 1 isoforms have been color coded with respect to amino acid identity to Bet v 1. In figure 1A red color indicates amino acid residues in Mal d 1 that in ≥90% of available sequences of Mal d 1 isoforms are identical to the corresponding residues found in Bet v 1, while blue indicates naturally occurring conservative substitutions. Figures 1B and 1C show the actual conservation of molecular surface structures to Bet v 1 of Mal d 1 isoforms (Genbank accession no.: Q43550) and (P43211) having highest (65%) and lowest (55%) amino acid identity, respectively, to Bet v 1 among available sequences. Figure 1A points out three surface patches that are well conserved between Bet v 1 and most Mal d 1 isoforms. These surface patches vary in shape and size in the molecules in figures 1B and 1C. Interestingly, figure 1B shows an additional patch (Patch 4), that only exists in a subgroup of published Mal d 1 isoforms represented by Mal d 1 (Q43550). Patch 4 is not present in the subgroup represented by Mal d 1 (P43221) shown in figure 1C.

Epitope grafting

The mAb BV16 binds an epitope on Bet v 1. Previously, the precise epitope was identified from the crystal structure of the Bet v 1-BV16 Fab complex (19) and a clear inhibition of birch allergic patients’ serum IgE by mAb BV16 was shown (19) illustrating the relevance of the BV16 defined epitope on Bet v 1 for IgE-binding. Here, the requirements for Bet v 1-specific antibody cross-reactivity between Bet v 1 and Mal d 1 were investigated in further detail in a model system including anti-Bet v 1 mAb BV16 together with rBet v 1, rMal d 1 and variants of Mal d 1 (Table I). The x-ray structure of the complex revealed 16 amino acid residues (Figure 2A) in rBet v 1 that constitute a 931 Å² conformational B-cell epitope on the Bet v 1 surface. Figure 2B (right model, Bet v 1) depicts the footprint of the BV16 epitope on rBet v 1. Five residues within the antibody footprint are different between rBet v 1 and rMal d 1 (Q8L6K9) seen as blue or white colored residues in the left model, depicting Mal d 1. Center model illustrates the grafting of the “BV16 epitope” into the Mal d 1 variant (Mut-2782) through substitution of these five residues to residues found in corresponding positions in Bet v 1. Figure 2C shows a Biacore diagram illustrating the interaction between the mAb BV16 and each of the three molecules. The BV16 antibody did not bind Mal d 1 with any measurable strength. Kinetic parameters for the interaction with Bet v 1 and the Mal d 1 variant with the grafted BV16 epitope are displayed below the respective models. The very similar kinetic parameters characterizing antibody binding to Bet v 1 and Mut-2782, respectively, as both being of high affinity shows full recognition of the grafted epitope on the engineered Mal d 1 variant Mut-2782 by BV16.

With the aim to further investigate the structural basis for birch allergic patients’ antibody cross-reactivity with Mal d 1 additional Mal d 1 variants were engineered (table I). An amino acid sequence alignment of Bet v 1, Mal d 1 and all mutated variants is shown in figure 3A. Mut-2782 (figure 2B), Mut-2781 (figure 3B) and Mut-2762 (figure
3C) all illustrates Mal d 1 variants with increased surface similarity to the Bet v 1 allergen. Mut-2762 was designed with the intension to increase surface similarity to Bet v 1 in a surface area on Mal d 1 clearly separated from the epitope defined by mAb BV16. As seen from figure 3C Mut-2762 contains a surface area shared with Bet v 1 that is very similar to “Patch 4”, shown in figure 1B, that is shared between a subgroup of naturally occurring isoforms of Mal d 1 and Bet v 1. In Mut-2781, Mut-2782 and Mut-2762 mutations are located in surface areas that on naturally occurring Mal d 1 isoforms are specific to Mal d 1, and all targeted residues were substituted to residues that are present in the corresponding positions in Bet v 1. In contrast, figure 3D displays the modeled molecular surface of Mut-2760 that contains mutations intended to reduce surface similarity to Bet v 1. Here, three residues N28, K32, E45 shared between Mal d 1 and Bet v 1 were substituted with residues differing from the ones found in the corresponding positions of known Bet v 1 isoforms. These residues were chosen for substitution based on previous data showing that they are critical residues in Bet v 1 for binding birch allergic patients serum IgE antibodies (20).

**Maintained structural integrity of rBet v 1, rMal d 1 and mutated rMal d 1 variants** In order to confirm the structural integrity of wild type and mutated molecules the presence of secondary structure elements were analyzed by circular dichroism (CD) spectroscopy. Figure 3, panel E shows an overlay of CD-spectra obtained at 15°C. A CD-spectrum of heat-denatured rBet v 1 obtained at 90°C is included for comparison. The spectra of rBet v 1, rMal d 1 and mutated variants all have negative and positive amplitudes at 212-216 nm and 192-194 nm, respectively, characteristic for nBet v 1. All five spectra are different from the CD-spectrum of heat denatured rBet v 1, which is characterized by negative and positive amplitudes at 200 nm and 186 nm, respectively. This suggests that the containment of secondary structure elements is unaffected by the introduced point mutations and that all of the molecules have maintained structural integrity. CD spectroscopy was not applied to Mut-2782. However, BV16 binds a conformational epitope on Mut-2781 and Mut-2782 with identical kinetics strongly indicating identical folding patterns. Therefore, in this study the reported effects of introducing mutations was in all cases caused by local changes in surface topography and charge distribution and was not caused by loss of overall structure integrity.

**Increasing surface similarity to Bet v 1 of Mal d 1 variants increased binding of birch allergic patients’ serum IgE** Compared to wildtype rMal d 1 the mutated variants Mut-2781 and Mut-2762 with point mutations that increase surface similarity to Bet v 1 showed increased binding of birch patients’ pooled serum IgE. Figure 4 (left column) shows the binding of human serum IgE to biotinylated rBet v 1 inhibited by rBet v 1, rMal d 1 or mutated Mal d 1 variants, respectively. Figures 4A and 4B show the degree of inhibition when testing serum pools A and B, respectively. Serum pool A is a pool of sera from 10 birch pollen allergic patients tested positive for IgE against Bet v extract (ALK-Abelló, Magic Lite®, class 3) and Mal d extract (Pharmacia, CAP®, class 2-4). Serum pool B is a pool of 10 sera from patients tested positive for IgE against Bet v extract (Magic Lite®, class 3) but negative against Mal d extract (CAP®, class 0). Figures 4C and 4D show the degree of inhibition when testing individual patient sera. In all four graphics rBet v 1 shows full inhibition at the highest inhibitor concentration whereas wild type rMal d 1 inhibits IgE-binding poorly. Compared to rMal d 1 the two mutated Mal d 1 variants with increased surface similarity to Bet v 1 inhibit IgE-binding to Bet v 1 to a larger extent.

**Increasing surface similarity to Bet v 1 of Mal d 1 variants increased the ability to trigger histamine release in birch allergic patients’ basophils** Recombinant Bet v 1, rMal d 1, Mut-2781 and Mut-2762 were tested in standard
basophil histamine release assays. Figure 4 (right column) shows the result of 3 histamine release experiments (E, F, G) with blood drawn from three individual birch allergic patients not reacting to apple. The increased surface similarity to Bet v 1 of the Mal d 1 variants mut-2781 and mut-2762 enabled both molecules to trigger basophil histamine release with blood from these patients.

Reduction of surface similarity to Bet v 1 of a Mal d 1 variant affected histamine release from individual patients basophils differently

rBet v 1, rMal d 1 and the mutated variant Mut-2760 were tested in IgE inhibition experiments with individual sera from five birch pollen allergic patients and biotinylated Bet v 1, figure 5 (left column). rBet v 1 inhibits binding fully at maximum inhibitor concentration whereas rMal d 1 and Mut-2760 inhibited binding of different patients’ serum IgE to biotinylated rBet v 1 poorly. Blood obtained from the same five individual patients were used in basophil histamine release assays, figure 5 (right column). Here, rBet v 1 triggered strong histamine release responses with basophils from all patients whereas notable patient-to-patient variations were seen with rMal d 1. In basophils from patient (n) no histamine release occurred within the measured antigen concentration range. In basophils from patients (o), (p) and (q) 10 to 100-fold higher concentrations of rMal d 1 were needed to initiate histamine release when compared to rBet v 1. In basophils from patient (r) the difference was considerable less i.e. 0 to 3-fold. Still, rMal d 1 had the ability to reach histamine release levels (maximum release) equal to those obtained with rBet v 1 in basophils from four patients (o, p, q, r). The Mal d 1 variant Mut-2760 with point mutations that reduce surface similarity to Bet v 1 affected histamine release in basophils from individual patients differently. As with wildtype rMal d 1 no histamine release occurred within the measured antigen concentration range when testing basophils from patient (n). With basophils from patient (o) histamine release was abolished in the measured antigen concentration range. In basophils from patient (p) a 10-fold reduced potency of Mut-2760 to trigger histamine release was seen compared to Mal d 1. In basophils from patient (q) and (r) no difference was seen.

DISCUSSION

Bet v 1 in complex with an antibody fab-fragment from the mAb BV16 raised against natural Bet v 1 has previously been crystallized (19). In the present study the molecular surface of Mal d 1 which shares 60% amino acid identity to Bet v 1 and is not recognized by mAb BV16 was altered by introduction of five point mutations. After these alterations all of the 16 amino acid residues that constitute the BV16 epitope on the surface of rBet v 1 were present in the corresponding surface area on the Mal d 1 variant Mut-2782, as if the epitope had been grafted onto the molecular surface. The kinetics of the complex formation between BV16 and Mut-2782 and the complex formation between BV16 and Bet v 1 were nearly identical which strongly indicates a perfect fit between epitope and paratope in both complexes and confirm that full functionality of the assembled conformational epitope on the engineered Mal d 1 variant has been achieved. The identical CD spectra obtained here for Bet v 1 and Mal d 1 indicate equal folding patterns that must be assumed to be very important for the formation of this non-linear epitope and in this case a prerequisite for epitope grafting. The allergen preparations used did not activate basophils passively sensitized with a monoclonal humanized recombinant IgE antibody form of BV16 (see supplemental data section, Fig. E1) excluding possible oligomerization/ aggregation of the allergen.

We have previously illustrated the molecular basis of birch allergic patients’ antibody cross-reactivity to the group 1 allergen from apple by analysis of 17 Mal d 1 isoform sequences (13). Here, a total of 77 different Mal d 1 isoform sequences originating from twelve different apple varieties were analyzed with the purpose
of identifying structurally identical surface patches on Bet v 1 and Mal d 1 large enough to accommodate antibody binding. Available crystal structures of antibody-antigen interactions suggest that a typical epitope covers approximately 600-900 Å$^2$ on the surface of the antigen (21;22). Here, four such patches of sufficient sizes (figure 1) on Mal d 1 were identified where birch allergic patients’ serum IgE may bind. Surprisingly, despite the dominance of conserved residues in the models in figure 1 our IgE binding data show that the large majority of birch allergic patients’ specific IgE antibodies fail to bind Mal d 1 in a competition assay with biotinylated Bet v 1 (figure 4). This indicates that few IgE antibody specificities in patients’ IgE-repertoires bind surface areas with high affinity that are conserved between Bet v 1 and Mal d 1. Still, it is tempting to suggest that the presence of IgE directed against the surface patches conserved among Bet v 1 and Mal d 1 increases the risk of cross-allergic reactions as those seen with OAS.

Furthermore, serum pool A, with IgE reactivity to birch and apple (figure 4A) and serum pool B, with IgE reactivity to only birch (figure 4B) only showed modest differences in IgE binding capacities to rMal d 1 implying that even small differences among birch allergic patients’ IgE repertoires may determine the ability to potentiate allergic reactions to apple and may determine whether or not a particular patient experience OAS.

Here, we show that changing the allergen epitope composition by increasing or reducing the surface similarity to Bet v 1 in confined surface areas on Mal d 1 variants has a huge impact on the histamine release patterns obtained with basophils from birch allergic individuals. Amino acid residues E45, P108, N28 and K32 all present in surface structures on Bet v 1 that are conserved among the group 1 Fagales allergens have previously been demonstrated to be critical residues for binding of birch allergic patients serum IgE to Bet v 1 (23). In later studies with the homologous protein from cherry, Pru av 1, residues E45 (24) and N28 (25) have likewise been identified as critical residues for IgE binding. Here we show (figure 5) notable differences between birch allergic patients’ basophil histamine release profiles upon testing of wild type rMal d 1 and the mutated variant Mut-2760 (N28T, K32Q, E45S) with point mutations that decrease surface similarity to Bet v 1. This result illustrates that allergic patients’ IgE repertoires can be expected to vary from patient to patient and that different IgE antibodies binding different epitopes on Mal d 1 may be responsible for OAS in individual patients. In addition, the unresponsiveness toward Mut-2760 seen with blood from patient o suggests that histamine release in this patient caused by wild type rMal d 1 is due to the presence of few or as little as two different IgE-binding epitopes. This result may explain why some birch allergic patients suffer from OAS to apple despite the result in figure 4 showing effectively no inhibition of serum IgE-binding to Bet v 1 by Mal d 1.

In addition, the classical view states that the minimum requirements for basophil- and mast cell activation involves cross-linking of antigen by two receptor-bound IgE antibodies that binds two non-overlapping epitopes on the same antigen. However, it has been hypothesized (26) that one high affinity IgE antibody on the surface of an effector cell may act as a stable surface anchor hereby facilitating binding to a second IgE antibody even if the second antibody involved binds the antigen with very poor affinity. This theory was recently supported by data from basophil activation experiments with a panel of humanized recombinant IgE antibodies binding the house dust mite allergen rDer p 2 with low, medium or high affinity (15). Here, the combination of a single high affinity IgE specificity combined with a single low affinity IgE specificity was almost as effective as the combination of two high affinity IgE specificities and both combinations resulted in potent histamine release responses. Therefore, whether or not an individual suffers from OAS may be
determined by the existence of a single IgE antibody specificity binding a single “birch-like epitope” present on Mal d 1 with medium to high affinity causing effector cell activation through cross-linking of antigen in complexes with low affinity IgE antibodies. Together, this indicates that the number of “birch-like epitopes” in Mal d 1 bound by high affinity IgE that is responsible for antibody cross-reactivity in a particular birch allergic patient may be few and interestingly that low-affinity IgE antibodies may play an important role in cross-allergies.

Specific immunotherapy (SIT) is a highly effective treatment of allergic disease that reduces both the immediate allergic symptoms as well as late phase responses (27). Current treatment depends on the use of standardized allergen extracts administrated to patients for a period of 3-5 years. SIT based on subcutaneous injection of allergen extract is associated with a small but significant risk of inducing systemic allergic adverse reactions. Several concepts for the improvement of vaccines for subcutaneous SIT have therefore been proposed. Most concepts aim for a reduction in the anaphylactic potential of the vaccine component either through disruption of the tertiary structure of the allergen e.g. allergen derived peptides (28;29) or through disruption of epitopes by substitution of surface exposed single amino acid residues on structurally intact allergens (20;23). A vaccine for SIT based on allergen derived peptides focuses solely on a modulation of the existing T-cell response whereas the latter concept also focuses on maintaining surface structures for the induction of so called blocking IgG antibodies (30) (31) reactive with the native allergen. The approach, however, is hampered by the heterogeneity of allergic patients’ responses and the concept may be dependent on the introduction of mutations in numbers leading to structural destabilization of the resulting molecule.

Conceptually, a novel approach to rational allergy vaccine engineering might be based on epitope grafting i.e. the building of allergen specific antibody binding epitopes into a stable homologous scaffold molecule that with respect to binding allergic patients’ IgE antibodies ideally should be inert. The idea is to generate a vaccine with reduced anaphylactic potential by splitting the allergen surface onto separate vaccine molecule components. Ideally, the size of the surface graft on each scaffold molecule should allow for several overlapping epitopes to exist but restricted in size to allow only one IgE antibody to bind within the surface graft at the same time. Potentially, such a multi component based vaccine will be unable to activate effector cells as this would require specific IgE antibodies to bind two or more non-overlapping epitopes on the same scaffold molecule. The vaccine, however, would be able to generate blocking IgG antibodies directed against all the overlapping epitopes present within the grafted surface areas present on the different vaccine components. In line with the results of other studies (32;33) we have demonstrated reactivity with Mal d 1 of Bet v 1-specific T-cell lines and in addition, the T-cell recognition of rMal d 1 variants was not influenced by the mutations investigated in the present study (see Fig E2 in the supplemental data section). Still, a scaffold molecule that is very distantly related but structurally similar to the allergen in question would probably lack most if not all allergen specific T-cell epitopes. Grafting of both specific B-cell epitopes as well as specific T-cell epitopes might therefore be necessary in order to obtain a suited vaccine candidate. The grafting of the BV16 epitope from Bet v 1 to Mal d 1 performed in this study was a first attempt to follow such a strategy and this study shows that it is possible to shape a conformational B-cell epitope onto the surface of a homologous scaffold molecule having 60% amino acid identity to Bet v 1 and maintain epitope functionality. Further work is needed to show whether a conformational epitope can be grafted onto a homologous scaffold molecule that is exceedingly more distant related to Bet v 1.
Reference List


**FOOTNOTES**

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The abbreviations used are: Bet v 1, Betula verrucosa group 1; Mal d 1, Malus domestica group 1; OAS, Oral Allergy Syndrome; RAST, radioallegrosorbent test; RLU, Relative Light Unit; SIT, Specific Immunotherapy.

**FIGURE LEGENDS**

**Fig. 1.** Modeled molecular surfaces of Bet v 1 (Z80104) and two Mal d 1 isoforms (Q43550) and (P43211) having 65% and 55% amino acid sequence identity to Bet v 1 (Z80104), respectively. Front and back views, (turned 180° vertical axes) are shown. Panel A: Red color indicates residues shared between Bet v 1 and ≥90% of available Mal d 1 sequences. Blue color indicates residues that in ≥90% of Mal d 1 isoforms are either shared with Bet v 1 or conservatively substituted. Three surface patches are framed that are shared between Bet v 1 and 9 out of 10 Mal d 1 sequences. Patch 1 contains 28 residues, E42, I44, E45, G46, G48, G49, G51, T52, T53, K55, Y66, V67, K68, R70, V71, D72, S84, V85, I86, E87, G88, I91, L95, E96, K97, I98, S99, H121. Patch 2 contains 19 residues, L24, D25, D27, N28, L29, P31, K32, V33, A34, P35, Q36, A37, E148, Y150, L151, D156, A157, Y158, N159. Patch 3 contains 11 residues, Y5, E6, E8, E127, V128, K129, E131, V133, K134, K137, E138. Panel B: Red (identity) and blue (homology) show residues shared between Bet v 1 and Mal d 1 isoform Q43550. Four surface patches shared between Bet v 1 and Mal d 1 (Q43550) are framed. Panel C: Red (identity) and blue (homology) show residues shared between Bet v 1 and Mal d 1 isoform P43211. Three surface patches shared between Bet v 1 and Mal d 1 P43211 are framed.

**Fig. 2.** Panel A: Amino acid sequence alignment of residues present within the epitope defined by the binding of the mAb BV16 to Bet v 1 in Bet v 1, Mal d 1 and the mutated Mal d 1 variant Mut-2782. Five residues within the antibody footprint are not shared between Mal d 1 and Bet v 1. All 16 residues within the antibody footprint are shared between Bet v 1 and Mut-2782. Panel B: Modeled molecular surfaces of Mal d 1, Mut-2782 with the BV16 epitope grafted onto it’s surface and Bet v 1. Amino acid residues are color coded according to amino acid identity (red) and homology (blue) to Bet v 1. Right model shows Bet v 1 (Z80104) with 16 amino acid residues colored cyan that make up the BV16 epitope. Left model shows Mal d 1 (Q8L6K9) with the antibody footprint visible in cyan. Center model shows Mut-2782 with the antibody footprint visible in cyan and mutated amino acid residues colored in green. Panel C: Kinetic parameters for the binding of mAb BV16 are shown below each model. No binding to Mal d 1 could be detected whereas very similar kinetic parameters characterize BV16 antibody binding to Bet v 1 and Mut-2782 were found.

**Fig. 3.** Panel A: amino acid sequence alignment of Bet v 1, Mal d 1 and mutated variants. Red and blue colors indicate residues that are identical or homologous to residues in Bet v 1, respectively. Symbols: #, X, @, $ mark positions of introduced point mutations in Mut-2781, Mut-2782, Mut-2762 or Mut-2760, respectively. Amino acid residue positions in Bet v 1 are shown with numbers above aligned sequences. For the C-terminal part of Mal d 1 residue positions are shown below aligned sequences. Panel B: Modeled molecular surfaces of Mal d 1, Mut-2781 and Bet v 1 (front views). Amino acid residues identical or homologous with residues in Bet v 1 are shown in red and blue colors, respectively. Green color shows the positions of 6 introduced point mutations in Mut-2781 that increases surface similarity to Bet v 1 within (five mutations) and around (1 mutation) the
epitope defined by the BV16 antibody. **Panel C**: Modeled molecular surfaces of Mal d 1, Mut-2762 and Bet v 1 (back views). Frame marks Patch 4 previously defined in figure 1B. Green color shows the positions of 8 introduced point mutations in Mut-2762 that increases surface similarity to Bet v 1 within Patch 4. **Panel D**: Modeled molecular surfaces of Mal d 1, Mut-2760 and Bet v 1 (front views). Amino acid residues identical or homologous with residues in Bet v 1 are displayed in red and blue colors, respectively. Frame marks Patch 2 previously defined in figure 1A. Yellow color shows the positions of 3 introduced point mutations in Mut-2760 of which 2 mutations decrease surface similarity to Bet v 1 within Patch 2. **Panel E**: Circular dichroism (CD) spectra. All spectra of mutated molecules Mut-2781 (□), Mut-2762 (△) and Mut 2760 (◇) recorded at 15°C are similar to spectra of wild type molecules rBetv 1 (●) and rMal d 1 (▲) recorded at 15°C that are different from the spectrum of denatured rBet v 1 (x) recorded at 90°C.

Fig. 4. IgE inhibition (left column) and histamine release (right column).

**IgE inhibition assay**: Graphs A, B, C, D show the binding of serum IgE (individual sera or serum pools) to biotinylated rBet v 1 upon inhibition with rBet v 1 (●), rMal d 1 (▼) or mutated rMal d 1 variants Mut-2781 (□) and Mut-2762 (△) with increased surface similarity to Bet v 1. Serum pool A, 10 sera sensitive to Bet v and Mal d extracts. Serum pool B, 10 sera sensitive to Bet v extract solely. In all graphs full inhibition is obtained with rBet v 1 as inhibitor. Compared to rMal d 1 that shows very little inhibition the mutated variants Mut-2781 and Mut2762 inhibit binding of serum IgE to biotinylated rBet v 1 to a larger degree. **Histamine release assay**: Graphs D, F, G show the results of histamine release experiments using blood from 3 individual birch allergic patients. No histamine release is observed with rMal d 1 (▼) whereas histamine release is seen with rBet v 1 (●) as well as with the mutated rMal d 1 variants Mut-2781 (□) and Mut-2762 (△) with increased surface similarity to Bet v 1.

Fig. 5. IgE binding and histamine release experiments.

**IgE inhibition assay**: Graphs A, B, C, D, E (left column) show binding of individual (n, o, p, q, r) birch patients’ serum IgE samples to biotinylated rBet v 1 upon inhibition with rBet v 1 (●), rMal d 1 (▼) or a mutated rMal d 1 variant, Mut-2760 (◇), with point mutations that decrease surface similarity to Bet v 1. In all graphs binding of serum IgE is inhibited fully by rBet v 1 whereas little inhibition is caused by rMal d 1 or the variant Mut-2760. **Histamine release assay**: Graphs F, G, H, I, J (right column) show the results of histamine release experiments using blood from the same panel of individual birch allergic patients (n, o, p, q, r). rBet v 1 triggered histamine release in basophils from all five patients whereas notable patient-to-patient variations were seen with rMal d 1 and the variant Mut-2760. Interestingly, three mutations N28T, K32Q and E45S that decrease surface similarity to Bet v 1 of variant Mut-2760 abolished histamine release in patient (o).
Table I. Mutated recombinant Mal d 1 variants

<table>
<thead>
<tr>
<th>Mutated variants</th>
<th>Amino acid substitutions&lt;sup&gt;A&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mut-2782&lt;sup&gt;B, D, G&lt;/sup&gt;</td>
<td>I43N, L44I, D47N, K70R, E76H</td>
</tr>
<tr>
<td>Mut-2781&lt;sup&gt;B, E, G, H&lt;/sup&gt;</td>
<td>I43N, L44I, D47N, G65K, K70R, E76H</td>
</tr>
<tr>
<td>Mut-2760&lt;sup&gt;C, H&lt;/sup&gt;</td>
<td>N28T, K32Q, E45S</td>
</tr>
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<sup>A</sup> Amino acid substitutions were introduced into Mal d 1 (Q8L6K9).
<sup>B</sup> Residues in Mal d 1 was substituted to residues present in corresponding positions in Bet v 1.
<sup>C</sup> Residues in Mal d 1 was substituted to residues not present in corresponding positions in Bet v 1.
<sup>D</sup> All five mutations in Mut-2782 are located within the BV16 antibody footprint illustrated in figure 2.
<sup>E</sup> In addition to the five mutations located within the BV16 antibody footprint Mut-2781 contains an additional mutation G65K located outside the footprint as shown in figure 3B.
<sup>F</sup> (+109D) refers to the insertion of Aspartic acid extending the length of the mutated molecule to 159 amino acid residues.
<sup>G</sup> Mut-2781 and Mut-2782 were tested along with rBet v 1 and rMal d 1 in Biacore experiments with mAb BV16.
<sup>H</sup> Mut-2781, Mut-2762 and Mut-2760 were tested along with rBet v 1 and rMal d 1 in IgE-binding experiments with birch allergic patients’ sera and in histamine release assays with blood from birch allergic patients.
Figure 1
Figure 2

A

Position
BY’16 epitope:

Bet v 1:
Mut-2782:
Mal d 1:

ENIEGNGPGT

B

Mal d 1
Mut-2782
Bet v 1

C

\[ k_1 = 1.2 \pm 0.1 \times 10^5 \, \text{M}^{-1}\text{s}^{-1} \]
\[ k_{-1} = 3.2 \pm 0.3 \times 10^{-5} \, \text{s}^{-1} \]
\[ K_d = 2.7 \pm 0.4 \times 10^{-10} \, \text{M} \]

\[ k_1 = 1.0 \pm 0.1 \times 10^5 \, \text{M}^{-1}\text{s}^{-1} \]
\[ k_{-1} = 2.5 \pm 1.4 \times 10^{-5} \, \text{s}^{-1} \]
\[ K_d = 2.4 \pm 1.4 \times 10^{-10} \, \text{M} \]
Figure 3
Figure 5.
Epitope grafting: re-creating a conformational bet V 1 antibody epitope on the surface of the homologous apple allergen MAL D 1

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