

A novel approach for investigation of specific and cross-reactive IgE epitopes on Bet v 1 and homologous food allergens in individual patients

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Abstract

Background: A clinically relevant allergic reaction requires recognition of at least two different epitopes on the surface of the allergen by IgE. These epitopes may be specific or cross-reactive. Moreover, patterns of IgE reactivity may be patient-specific. The aim of our study was to compare specific and cross-reactive IgE epitopes and epitope patterns between individual patients. We used Bet v 1-related food allergy as a model.

Methods: Five patients were investigated by cross-competitive ELISA for specific and cross-reacting IgE to Bet v 1, and its homologues Gly m 4 (soybean), Ara h 8 (peanut), and Pru av 1 (cherry). Allergen-specific as well as cross-reactive IgE epitopes were assessed by competitive immunoscreening of a phage-displayed random 7-mer peptide library using polyclonal purified IgE from individual sera. The resulting peptide mimics were mapped on the surface of the 3D-structure of the allergens using a computer-based algorithm.

Results: Competitive immunoscreening and epitope mapping identified patient-specific IgE epitope patterns. However, one IgE-binding surface area that was recognized by all patients and two recognized by three patients were identified on all four proteins. These results are consistent with the determination of IgE cross-reactivity of the individual patients' sera against the four recombinant allergens by cross-competitive ELISA.

Conclusions: Selection of phage-displayed peptide mimics with serum IgE from allergic patients in combination with computer-based mapping of the peptide mimics onto the surface of the three-dimensional allergen structure is a promising novel tool to investigate IgE epitope specificity in individual patients. Such basic information on epitope structure may be used for prediction of cross-reactivity and potential allergenicity of novel foods.

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1. Introduction

In type-1 allergy, the allergic reaction is mediated by allergen-specific IgE. Cross-linking of the allergen by allergen-specific IgE bound to high affinity receptors on

mast cells and basophils leads to effector cell degranulation and release of a variety of mediators of the allergic reaction, including histamine (Holowka and Baird, 1996). Cross-linking requires at least two binding sites on the surface of the allergen and antibodies specific for each of these epitopes. Specific IgE may cross-react with proteins sharing sufficient sequential and structural homology with the original immunogen (Vieths et al., 2002). If at least one binding site is conserved on the homologous allergen, serological cross-reactivity will be observed.

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IgE antibodies may bind to continuous or discontinuous epitopes of an allergen. The first resemble a part of the primary sequence, whilst the latter consist of amino acids being in proximity to each other as a consequence of the three-dimensional folding of the protein. Continuous epitopes can be determined by analysis of IgE-binding to overlapping peptides derived from the primary sequence (Reese et al., 2001). Obviously, this is not possible for discontinuous epitopes. These epitopes have been investigated by resolution of the structure of antigen–antibody complexes (Mirza et al., 2000), by site-directed mutagenesis (Ferreira et al., 1998; Neudecker et al., 2003a; Wiche et al., 2004; Holm et al., 2004), and by selection of peptide mimics (Jensen-Jarolim et al., 1998; Suphioglu et al., 2001). Mimics are small molecules such as peptides that mimic discontinuous sites of the protein and compete with the native protein for antibody binding. If they are able to induce antibodies with the same biological activity as the antibody used as template they are called mimotopes (Meloan et al., 2000).

Birch pollen-related food allergy is an excellent model to study IgE cross-reactivity. There are a high number of birch pollen-allergic patients with individual clinical patterns of serological and clinical cross-reactivity to various fruits, vegetables, nuts, and legumes. Since about 90% of patients with birch pollen allergy are sensitized to the major birch pollen allergen, Bet v 1, in the majority of the patients, IgE cross-reactivity is mediated by Bet v 1-homologous food allergens (Vieths et al., 2002). Recent publications described patients with birch pollen-associated allergy to legumes. The responsible allergens were identified as Gly m 4 in soybean and Ara h 8 in peanut (Kleine-Tebbe et al., 2002; Mittag et al., 2004a,b). Legume allergens have a lower degree of sequence identity with Bet v 1 than fruit allergens such as Pru av 1 from cherry (Vieths et al., 2002). Thus, they are likely to exhibit less cross-reactivity with IgE originally raised against Bet v 1. Bet v 1-homologous proteins have been shown to bind IgE mainly via discontinuous epitopes (Vrtala et al., 2001; Neudecker et al., 2003a). For Pru av 1 and Bet v 1 some epitope data are already available (Mirza et al., 2000; Spangfort et al., 2003; Ganglberger et al., 2000; Ferreira et al., 1998; Neudecker et al., 2003a; Wiche et al., 2004).

Our aim was to compare at single patient level IgE-binding sites on Bet v 1 and the homologous food proteins Gly m 4 (soy), Ara h 8 (peanut), and Pru av 1 (cherry) in patients with birch pollen-related food allergy. The three-dimensional structures of Bet v 1, Pru av 1, and Gly m 4 (Gajhede et al., 1996; Neudecker et al., 2001, 2003b) were used in the present study to identify areas on the surface of the allergens that represent potential discontinuous IgE epitopes. Conclusions about cross-reactivity in the individual patients were drawn from surface areas on the four proteins mimicked by IgE-binding peptides and from the frequency of selection of peptide mimics matching with surface areas on the different proteins. Results were compared with *in vitro* analysis of IgE cross reactivity in cross-competitive ELISA which was performed by mutual inhibition of recom-

binant Bet v 1, rGly m 4, rAra h 8, and rPru av 1 for each patient.

2. Methods

2.1. Patients

Five birch pollen-allergic patients (four female, one male) with a mean age of 22 ± 5.0 years were selected for the current study. They had food allergy to soy, peanut, and four of them to cherry, respectively. Food allergy to peanut was confirmed in four patients and to soy in all patients by DBPCFC (Table 1) (Mittag et al., 2004b,a; Ballmer-Weber et al., 2002). All of them had a convincing history of other allergies to foods that are known to cross-react with birch pollen and to cause an oral allergy syndrome (OAS). Levels of specific IgE to rBet v 1, rPru av 1, rGly m 4, and rAra h 8, were determined by CAP-FEIA using self-prepared biotinylated CAPs for the recombinant legume allergens. Total IgE was above 100 IU/ml in all patients and especially high in patient #2 (1915 IU/ml).

2.2. Direct ELISA for determination of allergen-specific IgE

The recombinant food allergens were produced and purified as described previously (Scheurer et al., 1997; Kleine-Tebbe et al., 2002; Mittag et al., 2004a). Recombinant Bet v 1 was obtained from Biomay (Vienna, Austria). Microtiter plates (Maxisorp, Nalge Nunc International, Rochester, NY) were coated overnight at 4 °C using 100 µl per well of 2.5 µg protein/ml in phosphate buffered saline (PBS), pH 6.6. All further steps were performed at room temperature. After blocking for 1 h with 200 µl of 2% (w/v) skim milk in PBS, the plate was incubated for 30 min with 16-fold diluted serum. The plate was washed, and incubated for 1 h with rabbit-anti-human-IgE conjugated with horseradish peroxidase (HRP) (DAKO, Glostrup, Denmark). Following a final wash step, tetramethylbenzidine (TMB) was added as substrate. Color development was stopped after 10 min by adding 100 µl 0.2 mol/l sulphuric acid. Absorption was measured at 450 nm.

2.3. Cross-competitive ELISA

IgE cross-reactivity of the individual patients was assessed for each combination of 2 of the 4 homologous allergens, resulting in 16 inhibition assays per patient. Coating and blocking were performed as described above. Sixty µl of nine-fold diluted serum were pre-incubated for 2 h with 60 µl of 0.001, 0.1, and 10 µg/ml of rBet v 1, rGly m 4, rAra h 8, or rPru av 1. Subsequently, 100 µl of the mixture were added to the coated plate and incubated for 10 min. After washing, bound IgE was detected as described for the ELISA above. Only experiments in which the maximum IgE-binding (B_0) was at least two-fold the level of non-specific binding were used to calculate the inhibition levels. As a result, the inhibition of

Table 1

Clinical history of food allergy and other allergies, and IgE-reactivity of the investigated patients

Patient	Food allergy	Non-food allergy	Specific IgE CAP (kU/l)					Total IgE (kU/l)
			Bet v 1	Bet v 2	Pru av 1	Gly m 4	Ara h 8	
1	P, S, c, f, n, v	Grass pollen	81.7	<0.35	15.9	7.74	21.5	608
2	P, S, c, f, n, v	Grass pollen, cat	>100	14.8	37.0	18.41	22.8	1915
3	P, S, c, f, n, v	Grass pollen	36.1	<0.35	7.27	10.4	14.08	119
4	P, S, C, f, n, v	Grass pollen, cat	34.9	7.76	6.05	9.34	12.5	219
5	p, S, f, n, v	House dust mite, AD	20	<0.35	9.95	3.98	6.74	131

Upper case letters: food allergy confirmed by DBPCFC; p, peanut; s, soy; c, cherry; f, fruits; n, nuts; v, vegetables; AD, atopic dermatitis.

IgE-binding to Ara h 8 and Gly m 4 could not be evaluated properly in patients #1 and #5.

2.4. Selection of phage-displayed IgE epitope mimicking peptides

Two millilitre aliquot of a tosyl-activated M280 Dynabeads suspension (Dynal Biotech, Oslo, Norway) were loaded with 125 µg rabbit-anti-human IgE (DAKO, Glostrup, Denmark), and blocked with 2% (w/v) skim milk in PBS. Patient serum (600 µl) was diluted in 9.4 ml PBS with 0.05% Tween 20 and 0.1% skim milk, and incubated overnight at 4 °C with 200 µl coated beads suspension. After extensive washing, 100 µl were used immediately for the first round of selection, while the remaining 100 µl were restored for the second selection round.

First selection round: The IgE coated beads were incubated for 3 h with 2¹¹ phages from a library of phages displaying linear random 7-mer peptides (Ph.D.-7 Phage Display Peptide Library Kit, New England BioLabs, Beverly, MA, USA), and washed with dilution buffer to remove unbound phages. The phages still bound to the beads were amplified by direct infection of ER2738 *E. coli* cells (provided with the phage library). Phage amplification was allowed overnight at 30 °C. Phages were purified by PEG-precipitation and titered.

Second selection round: The beads with immobilized IgE were incubated with 2¹¹ amplified phages selected in the first round. Now, the beads were washed and aliquoted for specific elution with 500 µl of 25 µg protein/ml of recombinant allergen in PBS with 0.1% (w/v) skim milk. One aliquot was eluted with one allergen. Titres of eluted phages were determined. Single colonies were picked and isolated phage preparations were made and processed as described by the library manufacturer. Amino-acid sequences of the displayed peptides were deduced from the DNA sequences obtained for each phage clone using M13 phage specific primers.

2.5. Phage ELISA for determination of IgE-reactivity to phage-displayed peptides

Plates were coated with rabbit-anti-human IgE (DAKO, Glostrup, Denmark) overnight, blocked with 200 µl of 2% (w/v) skim milk in PBS, and incubated with 100 µl of 50-fold diluted serum. After washing, the wells were incubated for 1 h with supernatants from single phage infected ER2738 *E. coli*

cultures containing isolated phages. A mouse-anti-M13 antibody conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for detection of bound phages. Following a final wash step, tetramethylbenzidine (TMB) was added as substrate. Color development was stopped after 10 min by adding 100 µl of 0.2 mol/l sulphuric acid. Absorption was measured at 450 nm.

Wild-type phage without an insert coding for a peptide was used as negative control. The results were corrected for the background and a corrected optical density (OD) of more than 0.2 was regarded as a positive result.

2.6. Mapping of peptides on the allergen surfaces using 3D-structures

Amino acids corresponding to the sequence of the selected peptides were localised on the respective allergens using published three-dimensional structures for Bet v 1 and Pru av 1, and a preliminary structure for Gly m 4 (Gajhede et al., 1996; Neudecker et al., 2001, 2003b). A homology model of the three-dimensional structure of Ara h 8 was obtained by applying Modeler, version 5.0 (copyright 1989–1998 Andrej Sali) using the structures known for Bet v 1, Pru av 1, and Gly m 4 as models.

The peptides obtained by immunoscreening of the phage-displayed peptide library were localised on the protein surface using a computer-based algorithm (Epitope mapping tool (EMT), Novozymes A/S). In short, amino acids were searched consecutively in the order given by the peptide sequence. Restrictions were made on distance from neighbouring residues (10 Å), accessible surface area for each residue (minimum 5 Å²), and maximum distance between any two residues in the epitope (<25 Å). The existence of a deep, hydrophobic cavity in Bet v 1 and its homologues (Gajhede et al., 1996; Markovic-Housley et al., 2003) allowed non-sense connectivity through the protein core rather than on its surface. Thus, the distribution of the EMT-identified amino acids on the protein surface was further analysed using PyMOL (DeLano Scientific LLC, San Carlos, CA, USA).

Further, specific adjustments were applied to avoid a systematic bias. Indeed, a peptide might not match with a specific surface area because an included amino acid was selected in a position for only one but not all its properties. A single proline has an entropic advantage to occur in the peptide due to rigidification of the peptide's conformation. Therefore,

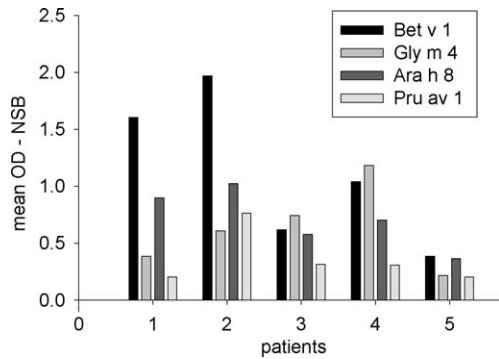


Fig. 1. ELISA screening results for five patients' sera that were chosen for further investigation by cross-competitive ELISA and for selection of phage-displayed peptide mimics.

it may mimic any other residue constraint in the allergen. The entropic advantage is by far largest for a first constraint, thus multiple prolines should not receive the same treatment. Glycine has few possibilities to contribute enthalpically to binding. However, it may allow the formation of a peptide turn in the bound state, that is necessary to achieve binding, and thus no matching glycine would be found within the mimicked surface. Furthermore, it is known that arginine is unfavourable to be displayed on phage due to systematic bias in the assembly of phage particles for the peptide library (Peters et al., 1994). As their most prominent feature is their positive charge, lysine residues may be selected in peptides instead of arginines.

3. Results

3.1. Direct ELISA for determination of IgE-reactivity

The IgE-reactivity to rBet v 1, rGly m 4, rAra h 8, and rPru av 1 was assessed for the individual sera by means of ELISA (Fig. 1). Overall, IgE-reactivity as determined by ELISA was consistent, but not identical, with the CAP data (Table 1). Patients #1 and #2 were most reactive to rBet v 1 and rAra h 8. Patient #2 was more reactive to rGly m 4 and rPru av 1, as compared to patient #1. The reactivity profile of patient #5 was similar to the reaction profiles observed for patients #1 and #2, but the specific IgE levels were 2–4-fold lower. Serum IgE from patients #3 and #4 reacted predominantly with rGly m 4 and rBet v 1, followed by rAra h 8 and rPru av 1.

3.2. Cross-competitive ELISA

Cross-inhibition of IgE-binding to rBet v 1, rGly m 4, rAra h 8, and rPru av 1 was tested in competitive ELISA with sera from all patients. The experimental output of these experiments is exemplified for patient #2 (Fig. 2). The inhibition data for the individual sera and allergens are summarised in Table 2.

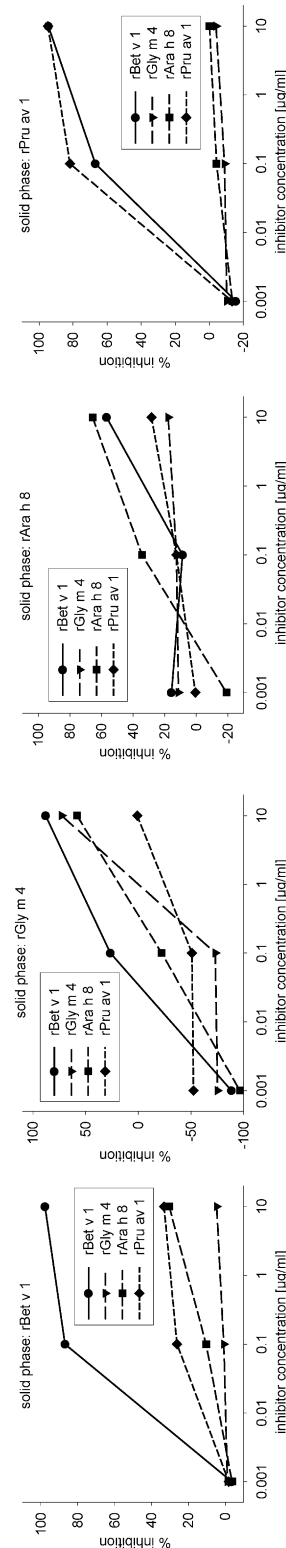


Fig. 2. Cross-competitive ELISA in patient #2 with rBet v 1, rGly m 4, rAra h 8, and rPru av 1 as inhibitors of IgE-binding to each of these allergens.

Table 2

Maximum inhibition and inhibitor concentration for 50% inhibition in cross-competitive ELISA

Coated allergen	Inhibitor	Patients									
		#1		#2		#3		#4		#5	
		Max	IC50	Max	IC50	Max	IC50	Max	IC50	Max	IC50
Bet v 1	Bet v 1	98.4	0.01	97.7	0.01	96.7	0.01	96.6	0.01	85.7	0.01
	Gly m 4	25.9	np	4.7	np	31.2	np	39.5	np	17.3	np
	Ara h 8	50.8	10	30.4	np	59.3	1	37.5	np	38.8	np
	Pru av 1	26.8	np	33.2	np	16.6	np	16.9	np	29.5	np
Gly m 4	Bet v 1	$B_0 < 2^* \text{NSB}$		88	1	89.4	0.01	52.2	0.1	$B_0 < 2^* \text{NSB}$	
	Gly m 4			72.7	5	80.9	0.1	100.5	0.01		
	Ara h 8			58.2	5	105.6	0.05	84.5	1		
	Pru av 1			0.9		34.6	np	12.7	np		
Ara h 8	Bet v 1	60.5	0.1	56.7	10	27.3	np	38.5	np	$B_0 < 2^* \text{NSB}$	
	Gly m 4	29.7	np	17.8	np	0	np	0	np		
	Ara h 8	40.7	np	65.6	1	60.2	5	72.8	1		
	Pru av 1	9.6	np	28.3	np	13.3	np	0	np		
Pru av 1	Bet v 1	78	0.05	94.5	0.05	96.3	0.01	96.2	0.05	63.5	1
	Gly m 4	18.2	np	0	np	41.9	np	49.3	np	13	np
	Ara h 8	6.1	np	0	np	35.5	np	39.7	np	0	np
	Pru av 1	76.6	0.01	94.7	0.05	102.2	0.01	101.4	0.05	76.4	1

Max: maximum inhibition (%); IC50: inhibitor concentration at 50% inhibition; np: estimation of IC50 was not possible because of a weak maximum inhibition.

Self-inhibition with Bet v 1 was high (>85%) at high inhibitor level, and 50% inhibition of IgE-binding was obtained with 0.01 $\mu\text{g/ml}$ (IC50). Comparable results were obtained for rPru av 1 with IC50 values ranging between 0.01 and 1.00 $\mu\text{g/ml}$. Among the legume allergens, self-inhibition with Gly m 4 resulted in IC50 values (0.01–5.00 $\mu\text{g/ml}$) that varied significantly from patient to patient. Among the allergens tested in this study, Ara h 8 revealed to be the weakest self-inhibitor of IgE-binding with IC50 values >1 $\mu\text{g/ml}$.

In the five patients, a high preference of IgE for binding to rBet v 1 was observed. Only Ara h 8 was capable of inhibiting IgE-binding to Bet v 1 for >50%, and only, when sera for patient #1 (IC50 = 10 $\mu\text{g/ml}$) and patient #3 (IC50 = 1 $\mu\text{g/ml}$) were used. Inhibition of IgE-binding to Pru av 1 by rBet v 1 and rPru av 1 was equally effective, with IC50 values ranging between 0.01 and 1 $\mu\text{g/ml}$. None of the legume allergens could inhibit IgE-binding to Pru av 1 for >50%. In spite of this strong cross-inhibition, rBet v 1 and rPru av 1 behaved differently when used in a competitive ELISA on the legume

allergens. IgE-binding to Gly m 4 was significantly inhibited by rBet v 1 (IC50 = 0.1–1.0 $\mu\text{g/ml}$) but not by rPru av 1. Similarly, rBet v 1, but not rPru av 1, could compete with Ara h 8 for binding IgE from patient #1 (IC50 = 0.1 $\mu\text{g/ml}$) and patient #2 (IC50 = 10 $\mu\text{g/ml}$).

The legume allergens rGly m 4 (IC50 = 0.01–5.00 $\mu\text{g/ml}$) and rAra h 8 (IC50 = 0.05–5.00 $\mu\text{g/ml}$) were equally potent in inhibiting binding of IgE to rGly m 4. In contrast, rGly m 4 could not inhibit IgE-binding to Ara h 8. Interestingly, rAra h 8 inhibited only moderately (41%) binding to rAra h 8 of IgE from patient #1.

3.3. Selection of peptides mimicking IgE epitopes

Competitive elution was performed with rBet v 1 and rGly m 4 for patients #1–#5, and additionally with rAra h 8 and rPru av 1 for patients #1 and #2.

Overall, the number of phages eluted by competition with the respective allergens did not reflect the IgE-reactivity in

Table 3

Number and IgE-binding properties of peptides mimicking IgE epitopes that were selected by the individual sera

Patient	Number of eluted phages counted in 10^{-1} dilution				Phage ELISA results							
	Bet v 1	Gly m 4	Ara h 8	Pru av 1	Bet v 1		Gly m 4		Gly m 4		Pru av 1	
					(%) Positive	(%) OD > 2	(%) Positive	(%) OD > 2	(%) OD > 2	(%) OD > 2	(%) Positive	(%) OD > 2
1	1040	390	600	220	78	58	57	52	75	47	52	33
2	70/80	200	180/100	210	16	3	10	0	19	8	24	5
3	100	70			43	43	81	48				
4	100	120			10	10	33	14				
5	170/160	220/160			31	0	49	7				

In patients #3–#5 no elution with Ara h 8 and Pru av 1 was performed. (%) Positive, percentage of eluted and tested phages that were positive for IgE-binding in phage ELISA; (%) OD > 2, percentage of phages with strong IgE-binding in phage ELISA defined as corrected OD of more than two.

CAP-FEIA nor ELISA. The number of phages was rather similar ($N = 70$ – 220) for all patients but patient #1 (Table 3). Serum IgE of patient #1 resulted in considerable higher phage counts when eluted with rBet v 1 ($N = 1040$), rAra h 8 ($N = 600$) and rGly m 4 ($N = 390$), but not with Pru av 1 ($N = 220$). Surprisingly, competitive immunoscreening using IgE from patient #2 for phage-binding and rBet v 1 for elution produced the lowest number of phages ($N = 70$ – 80), while the serum revealed the highest IgE reactivity to rBet v 1 in both CAP-FEIA and ELISA. In agreement with the ELISA data, IgE from patients #3, #4, and #5 produced comparable numbers of phages for rBet v 1 and rGly m 4.

The percentage of IgE-binding phages confirmed by phage ELISA was both patients and allergen dependent. Sera from patient #1 (78% (rBet v 1) – 50% (rPru av 1)) and patient #3

(43% (rBet v 1) – 81% (rGly m 4)) produced phage clones that were most likely to be confirmed by phage ELISA. For the individual allergens, the rate of confirmation by phage ELISA varied among the patients. Thus, 10% (patient #4) to 78% (patient #1) of the phages selected by elution with rBet v 1 could be confirmed. Only 16% of the phages isolated by using the serum from the in ELISA high responding patient #2, were positive in phage ELISA. For Gly m 4, 10% (patient #2) to 81% (patient #3) of the eluted phages were confirmed by phage ELISA.

Differences were also observed in the intensity of the signals in phage ELISA. For example, 58% of the isolated phages obtained with serum IgE of patient #1 and confirmed to bind IgE in phage ELISA gave a strong signal in phage ELISA ($OD_{450\text{ nm}} > 2$). In contrast, none of the phages

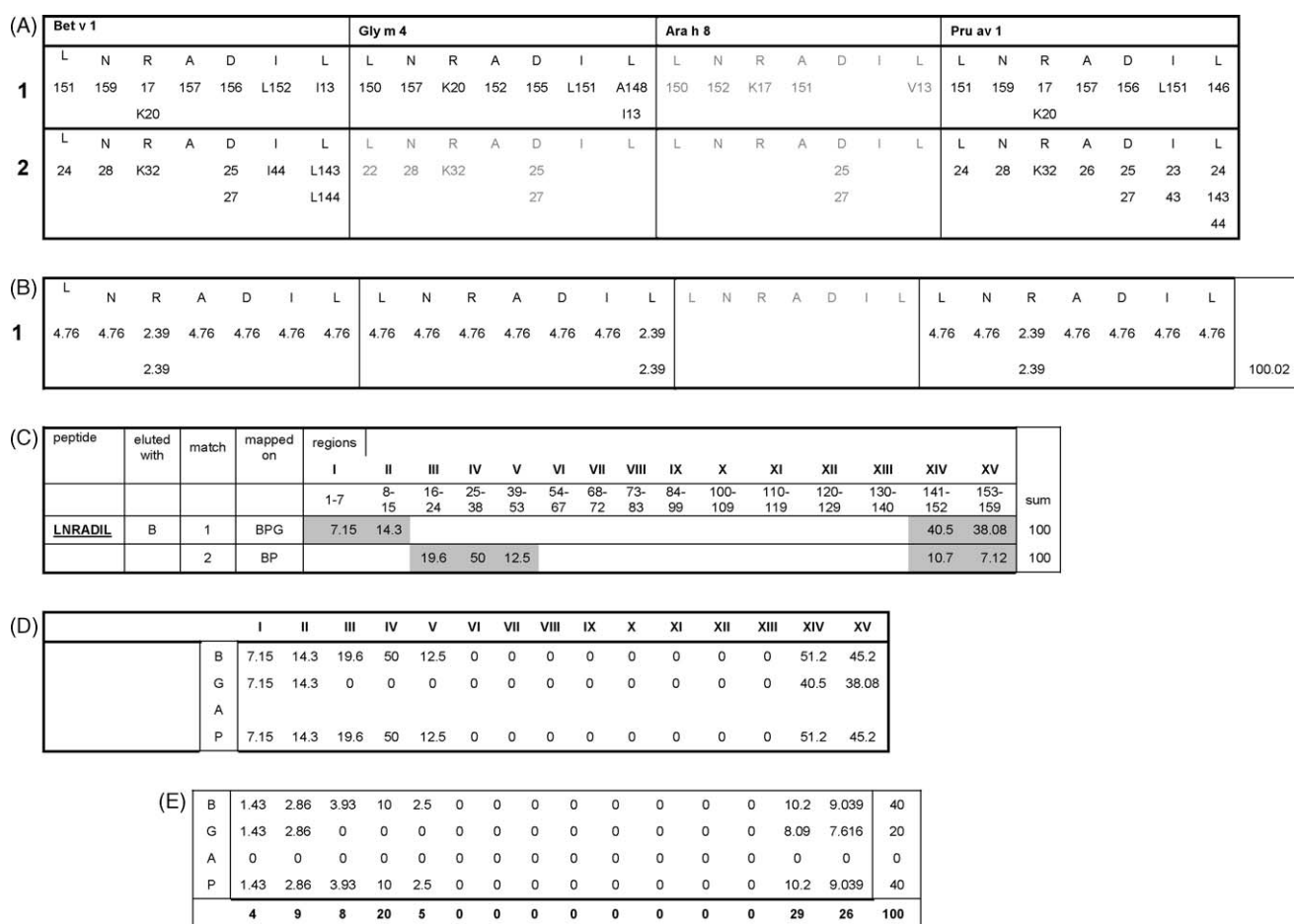


Fig. 3. Example for analysis of the mimicked surface areas and calculation of the results given in Table 3. The peptide LNRADIL was selected with serum of patient #3 and eluted with Bet v 1 two times. (A), Two possible surface areas (number 1 and 2) were found on Bet v 1 that could be mimicked by the peptide LNRADIL and similarly on Pru av 1. Only the first match is also conserved on Gly m 4 and neither 1 or 2 on Ara h 8. This is due to differences in the amino acid sequences and the three-dimensional structure. The residue numbers of corresponding amino acids differ slightly because of gaps in Gly m 4 and Ara h 8 sequences compared to Bet v 1 and Pru av 1 sequence. (B), For each mimicked amino acid a value representing its importance is calculated. In example of match 1, seven amino acids have to be mapped on three proteins, which results in a total of 21 amino acids. Therefore, in this case, 21 were set 100%. This is necessary to avoid a bias due to the number of proteins on that a surface area was mimicked by this peptide. In case that an amino acid in the peptide could mimick more than one surface amino acid the value was split into equal parts per each mimicked amino acid. (C), This table shows the calculation of the shares of amino acids per regions of the protein surface. Those regions were defined as sequential subunits of the proteins with considering amino acid groups as mimicked by the peptides. The share of amino acids involved in each region was summed up for each match. (D), Results from C were split up per each protein. (E), Results from D were transferred by setting the sum of all value 100%. E gives the percentage of involvement of a region on each protein.

selected with serum of patient #2 and confirmed by ELISA had an OD_{450 nm} > 2 (Table 3).

3.4. Mapping of peptides displayed by eluted phages on the allergen surface

Each peptide could be mapped on the surface of the eluting protein and in many cases also on homologous proteins' surfaces (see example in Fig. 3A). This is consistent with the confirmed existence of cross-reacting IgE antibodies. An individual set of peptides was selected with each patient's serum and peptides were selected multiple times. No identical peptides were selected with sera of two different patients. Overall, 69 different peptide mimics were eluted with the sera of the five patients. The 37 peptides with the strongest binding to IgE in phage ELISA (OD_{450 nm} > 2) were selected for mapping on the allergen surfaces (Table 4).

Peptides selected with IgE from different patients mimicked at times the same surface areas, but nevertheless, each patient was found to have an individual pattern of surface sites mimicked by the selected peptides. Especially peptides with strong IgE-binding activity in phage ELISA (OD_{450 nm} > 2) or peptides eluted multiple times and with different allergens were often found to mimic more than one surface area on a protein. This was also the case for all three selected pairs of peptides showing minimal amino acid variations and for some peptides with lower IgE-binding capacity.

3.5. Assessment of IgE-binding surface areas that were mimicked by the selected peptides

Regions were defined as subunits of the primary sequence considering amino acids that were simultaneously mimicked by the same peptide (Fig. 3C). Amino acids mimicked by the peptides were assigned to the regions. The percentage of amino acids of a peptide mimicking each region was calculated (see Fig. 3). The frequency of recognised peptides for each region on each protein per patient is shown in Table 5.

IgE-binding surface areas representing possible epitopes were defined by grouping regions that were mimicked simultaneously by different peptides. Most peptides were found to mimic discontinuous areas, as the surface areas consisted of more than one sequential region that were not neighbouring in the primary sequence. Individual patterns of regions were found for the individual sera. Patients showed most divergent results for the regions, VIII, IX, XII, and XIII, which were mimicked by most of the peptides in some patients and by none in the others. In all patients, the regions IV, V, VI, XIV, and XV were found to be mimicked by peptides and thus are likely to be IgE-binding regions. Regions VII, X, and XI were least important in any of the patients.

Three antigenic areas that are likely to be IgE-binding epitopes could be identified (Fig. 4). They consisted of regions mimicked by combinations of different peptides recognised by the same and also by different patients. The first antigenic

Table 4

Eluting allergens and number of elution of the 37 peptide mimics that were mapped on the allergen surfaces

Patient	Eluted peptide mimics	Eluting allergens	Times eluted
#1	LEKGPW	BGAP	11
	AAKGTPW	BGAP	8
	EPDKMTT	B	1
	EPDKSII	BA	3
	EAGKHSG	A	1
	QVELPKG	G	1
	KLPLTKG	G	2
	QKTAASY	BGP	5
	EAAKQGE	G	1
	GKQPASD	B	1
	AVTFSKG	B	1
#2	GKIGMYP	BAP	4
	GKDRSIQ	B	1
	IKLPALF	BAG	4
	FKLKAIE	P	1
	HLVEKGY	B	1
	ITADMYT	P	1
	MPNYDRL	P	1
#3	KNLEEPK	BG	11
	KYLEEPV	BG	2
	LNRAIDL	B	2
	WPKDITD	B	1
	TPIRHRS	G	1
	QYPWRLA	G	1
	FDMATNP	G	1
	SLYELTH	G	1
	TAIHAVW	G	1
	DHLPSPW	G	1
#4	YKIGDNW	B	1
	YKSDNIR	G	1
	KSDNNVT	B	1
	SKTDNLT	G	1
	AFNRASD	G	1
#5	YLMEVQK	BG	7
	YEMDAPK	G	4
	ERHLTFT	G	1
	TLKQAPA	B	1

Row 2: amino acids, one letter code; row 3: B, Bet v 1; G, Gly m 4; A, Ara h 8; P, Pru av 1.

area is formed by regions I, IX and XII that were mimicked by a total of 14 peptides selected with sera of patients #1, #3, and #5. The second area was recognized by all patients and consists of regions XIV and XV and at least one out of regions II, III, and IV. Peptides mimicking these antigenic areas could be equally mapped on each of the four proteins. The third potential IgE-binding site formed by regions IV, V, and VI was mimicked by peptides selected with IgE from patients #3 to #5. However, peptides mimicking this combination were less frequently selected. This potential epitope could be mapped on all four allergens, but less frequently on Pru av 1.

With serum of patient #1 mainly peptides mimicking the first and the second antigenic surface area were selected, whereas in the other patients, additional surface areas were mimicked.

Table 5

Frequency of each of the 15 regions of being involved in surface areas on that peptide mimics selected with the five patients' sera were mapped; in the five patients, individual patterns of frequently recognized regions and regions with strongest differences in recognition frequency between the four proteins were found.

antigenic areas	I 1-7	II 8-15	III 16-24	IV 25-38	V 39-53	VI 54-67	VII 68-72	VIII 73-83	IX 84-99	X 100-109	XI 110-119	XII 120-129	XIII 130-140	XIV 141-152	XV 153-159	total
patient #1																
Bet v 1	1.8	0.2	0.1	3.9	0.6	0.4	0.4	0.0	7.5	0.0	0.1	5.3	4.8	2.5	0.2	28
Gly m 4	0.7	0.0	0.1	3.5	0.6	0.2	0.4	0.0	7.1	0.0	0.0	4.9	3.4	1.0	0.2	22
Ara h 8	1.0	0.0	0.1	5.7	1.4	2.4	0.4	0.0	6.2	0.0	0.0	3.2	0.6	1.0	0.2	22
Pru av 1	1.8	0.2	0.1	3.9	0.6	0.4	0.4	0.0	7.5	0.0	0.1	5.3	4.8	2.5	0.2	28
total	5	0	0	17	3	3	2	0	28	0	0	19	14	7	1	100
patient #2																
Bet v 1	0.0	2.3	3.8	2.8	2.0	5.1	1.2	2.1	4.4	1.1	0.7	0.0	0.0	4.5	2.2	32
Gly m 4	0.0	0.5	1.4	1.8	1.3	3.9	0.1	1.2	1.8	0.2	0.0	0.0	0.0	2.1	1.5	16
Ara h 8	0.0	0.5	2.3	2.4	2.7	4.7	0.8	1.8	2.9	0.2	0.0	0.0	0.0	3.6	2.2	24
Pru av 1	0.0	2.3	3.8	2.2	3.7	0.4	2.0	2.8	2.3	1.1	0.7	0.0	0.0	4.5	2.2	28
total	0	6	11	9	10	14	4	8	11	2	1	0	0	15	8	100
patient #3																
Bet v 1	1.4	1.5	1.5	1.5	2.1	0.5	1.7	0.4	3.8	1.0	0.8	2.7	0.1	4.9	2.4	26
Gly m 4	1.7	1.9	2.6	1.8	1.9	3.2	0.2	1.5	3.5	1.0	1.4	3.0	0.1	2.8	2.8	29
Ara h 8	1.2	1.5	0.3	0.1	1.7	3.1	0.2	0.7	2.9	0.6	0.8	1.9	0.1	1.7	1.8	19
Pru av 1	1.4	2.7	0.9	1.5	2.1	0.5	0.2	0.7	3.8	0.8	0.8	2.7	0.1	4.5	3.2	26
total	6	8	5	5	8	7	2	3	14	3	4	10	0	14	10	100
patient #4																
Bet v 1	0.0	0.1	2.6	3.9	7.1	3.3	1.1	4.6	0.0	2.4	1.0	0.0	0.0	2.9	3.2	32
Gly m 4	0.0	0.1	1.4	6.0	6.1	2.6	0.4	3.5	0.0	2.1	1.0	0.0	0.0	1.2	1.7	26
Ara h 8	0.0	0.2	2.2	2.2	1.9	0.4	0.6	4.4	0.0	2.1	1.0	0.0	0.0	0.3	1.1	16
Pru av 1	0.0	0.1	2.5	2.4	3.1	2.6	1.0	4.4	0.0	2.4	1.0	0.0	0.0	2.9	3.2	25
total	0	0	9	14	18	9	3	17	0	9	4	0	0	7	9	100
patient #5																
Bet v 1	3.1	0.9	1.2	0.0	2.2	1.0	0.0	0.4	1.4	1.3	1.2	3.8	1.4	4.4	0.9	23
Gly m 4	3.1	0.9	1.3	2.3	3.2	3.1	0.4	0.4	1.4	1.3	1.2	3.8	1.4	5.0	1.1	30
Ara h 8	3.1	0.6	1.1	2.3	3.2	3.1	0.8	2.5	1.4	1.5	1.4	3.8	1.4	2.6	1.1	30
Pru av 1	2.1	1.3	0.6	0.0	0.0	0.0	0.0	0.4	0.6	1.3	1.2	2.3	1.4	4.1	1.4	17
total	11	4	4	5	9	7	1	4	5	5	5	14	5	16	4	100

high recognition frequency for this region compared to the other proteins
 low recognition frequency for this region compared to the other proteins

4. Discussion

About 70% of birch pollen-allergic patients have birch pollen-associated food allergy which is often mediated by Bet v 1-homologous allergens from foods (Eriksson et al., 1982). Numerous Bet v 1-homologous food allergens from fresh fruits, vegetables, nuts, and legumes have been identified (Vieths et al., 2002). However, divergent patterns of serological cross-reactivity and clinically relevant allergic reactions to foods occur in the individual patients (Ferreira et al., 1998; Holm et al., 2004). Patients included in this study had birch pollen allergy in combination with several food allergies. We used a novel approach to investigate IgE-reactivity to the homologous allergens Bet v 1, Gly m 4, Ara h 8, and

Pru av 1 on the IgE epitope level and analyzed IgE cross-reactivity in each patient by cross-competitive ELISA. As expected, cross-reactivity between food allergens and Bet v 1 was observed but interestingly the results for IgE specificity varied strongly from patient to patient.

Bet v 1-homologous proteins bind IgE mainly via discontinuous epitopes (Vrtala et al., 2001; Neudecker et al., 2003a). Discontinuous Bet v 1 IgE epitopes have been investigated by determination of the X-ray structure of an antigen–antibody complex (Mirza et al., 2000) and by selection of peptide mimotopes (Jensen-Jarolim et al., 1998). Moreover, critical amino-acids have been determined on Bet v 1 and Pru av 1 by site-directed mutagenesis (Neudecker et al., 2003a; Ferreira et al., 1998; Wiche et al., 2004; Holm et al., 2004). In none

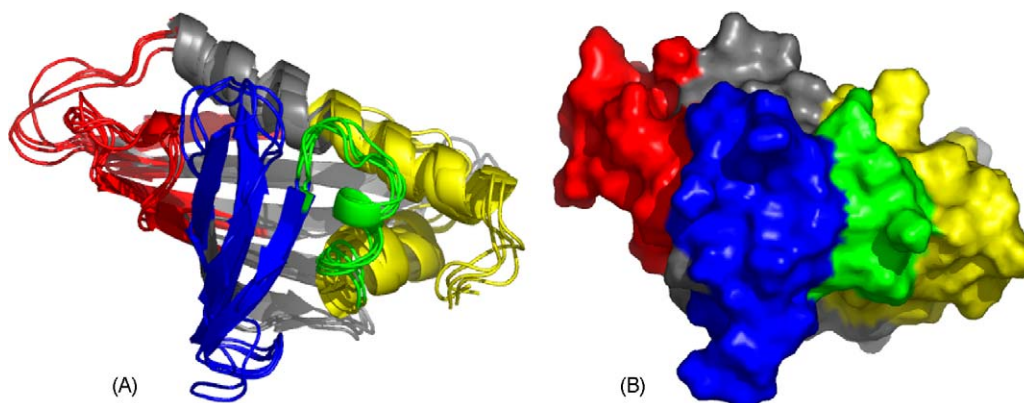


Fig. 4. Location of IgE-binding surface areas on the Bet v 1 surface that were characterized by the polyclonal IgE response of the majority of the studied patients and that were conserved on all four proteins. Antigenic area 1 (red): regions I, IX, XII; antigenic area 2 (yellow): regions XIV, XV, II, III, IV; antigenic area 3 (blue): regions IV, V, VI; Region IV is coloured green because it is involved in areas 2 and 3. (A), Secondary structure view of an overlay of Bet v 1, Gly m 4, Ara h 8, and Pru av 1. (B), Surface view of Bet v 1 in the same orientation.

of these studies the complete spectrum of IgE-binding of an individual patient was evaluated.

Competitive immunoscreening of phage-displayed peptides was performed using the entire polyclonal IgE response of individual patients. Peptides bound by IgE antibodies specific for Bet v 1 or the food allergens were eluted competitively with the respective allergen. Therefore, the resulting peptides are very likely to represent or mimick parts of binding sites of the eluting allergen.

Serum IgE was captured from serum by immobilised anti-IgE. In this approach, a higher diversity of the patient's IgE-response reduces the likelihood of immobilization of Bet v 1- or Bet v 1 homologue-specific IgE to the solid phase. This might be reflected by the lower number of peptide mimics specifically eluted in patients #2, #4, and #5 who had additional allergies to animal dander or house dust mite. Furthermore, patient #5 suffered from atopic dermatitis which involves induction of high amounts of IgE with very different specificities, and patients #2 and #4 were sensitized to Bet v 2. Accordingly, the highest numbers of peptides with strong IgE-binding capacity ($OD_{450\text{ nm}} > 2$ in phage ELISA) were eluted after selection with sera of patients #1 and #3 who had limited spectra of IgE responses and patient #1 was mono sensitised to the Bet v 1 family. Taken together, the results are in full accordance with a specific procedure of the selection and elution of phage-displayed peptide mimics.

For some peptides, more than one good match was found on the protein surface which could be explained by either of two ways. First, IgE-binding sites may exist repeatedly on the protein surface which would mean that single monoclonal IgE antibodies with such specificity could activate mast cells even in the absence of other IgE specificities. It was found for example for the major peanut allergen, Ara h 2, that a dominant linear motif occurs more than one time in the protein (Hales et al., 2004). Second, a particular peptide could be flexible enough to exhibit different conformations that can hypothetically bind to two different IgE antibodies. Each of these antibodies would recognize one of the surface sites on

that the peptide was mapped. This could also explain that they were mainly detected with a high signal ($OD_{450\text{ nm}} > 2$) in phage ELISA or eluted more frequently because such a peptide would be bound by two antibodies of different specificity.

Seven-mer peptides are too short to mimic complete epitopes as the majority of binding interfaces of protein heterodimers is larger than $600 \text{ \AA}^2$ (Jones and Thornton, 1996). In advantage to longer peptides, 7-mer peptides are very likely to define the essential amino acids of an epitope mimic because unspecific binding to these peptides is too low in affinity for selection of a peptide by an IgE-antibody (Meloan et al., 2000). As the peptides were selected by a polyclonal mixture of IgE and almost the complete protein surface was involved in mimicked binding sites, it was not possible to clearly determine a single epitope. Nevertheless, frequently mimicked regions allowed the definition of antigenic surface areas that are likely to contain IgE-binding sites.

Although we found by our approach different patterns of IgE-specificity in the five patients, the three defined antigenic surface areas were found to bind IgE from all or most of the patients. This is consistent with the extensive cross-reactivity against the four proteins that was found in cross-competitive ELISA. Mimicked surface areas as well as cross-competitive ELISA data obtained for Bet v 1 showed a higher degree of similarity with Pru av 1 than with Gly m 4 and Ara h 8. This was expected as there is a higher degree of sequence identity between Bet v 1 and Pru av 1 than with the legume homologues.

Cross-competitive ELISA with Bet v 1 as inhibitor indicated that no additional epitopes than those cross-reacting with Bet v 1 were present on the food allergens. The overwhelming dominance of Bet v 1 in cross-competitive ELISA in all patients was not completely reflected by the results of peptide mapping. Only slightly more peptides were mimicking surface areas on Bet v 1 than on the other proteins. These findings may be explained by other parameters important for the outcome of the competitive ELISA such as higher affinity of IgE antibodies to Bet v 1.

IgE epitopes or epitope areas described earlier on Bet v 1 and Bet v 1-homologues were found to be recognized by our patients, though with some patient-dependent variation in the surface areas involved. The Bet v 1 epitope described by Mirza et al. (2000); Spangfort et al. (2003) involves mainly amino acids from the regions V, representing the so-called P-loop, and region IX of Bet v 1. It was determined by resolution of the structure of an immune complex of Bet v 1 with the monoclonal mouse IgG Fab BV16. BV16 was shown to inhibit IgE-binding of a serum pool to Bet v 1 for up to 40%. Region V was mimicked by peptides selected with IgE from all and region IX by different peptides from four patients. The combination of both regions was clearly detected only in patient #2, but significant shares of regions VII and VIII were additionally involved in this case. The epitope bound by BV16 is overlapping with two different antigenic areas (no. 1 and 3) determined in our study. Therefore, it is likely that BV16 would be able to inhibit patients' IgE, which is directed against any of these areas.

Neudecker et al. (2003a) investigated the P-loop region of the homologous food allergens Pru av 1 by site-directed mutagenesis. Mutation of glutamic acid 45 in Pru av 1 to tryptophane caused a reduction of IgE-binding to Pru av 1 in a subgroup of cherry-allergic patients. Glutamic acid 45 is conserved in part of region V for which peptide mimics were selected with all patients and Glu45 is conserved in all four investigated proteins. Asparagine 28 was shown to be part of a second epitope as mutation of asparagine 28 to lysine reduced IgE-binding and lowered activity in histamine release assay (Wiche et al., 2004). Asparagine 28 is part of region IV that was found to bind IgE from all of our patients indicating that this residue is part of a frequently recognized IgE epitope. However, in the Ara h 8 sequence it is replaced by serine. Consequently, part of the mimicked surface areas including region IV on the other three proteins could not be found on Ara h 8 due to the lack of asparagine in position 28, especially in patient #3 and #4 (see Table 4 and Fig. 2).

The selection of phage-displayed peptides with monoclonal mouse IgG and human serum IgE has been described earlier (Jensen-Jarolim et al., 1998; Ganglberger et al., 2000; Suphioglu et al., 2001). In contrast to our study, previous approaches were focused on selection of few peptides for which high amounts of IgE existed or which, by performing more rounds of selection, had highest affinity to IgE. Furthermore, selected peptides were eluted unspecifically, increasing the likelihood of unspecific results. Such treatment focussing on a single, highest observed affinity-epitope would be unsuitable to study epitopes involved in cross-reactivity. Ganglberger et al. selected the mimotope Bet mim E from a random circular nonapeptide library using purified Bet v 1-specific IgE. The surface area mimicked by Bet mim E was found to be located between residues 9–22 and 104–113 (Ganglberger et al., 2000). The combination of the corresponding regions as defined in our study (II, III, X, and XI) was mimicked with slight variations by only few peptides selected with IgE from patients #2, #3, and #5. In patient #4 regions X and XI were

combined with VIII. Interestingly, these peptides could be mapped on surface areas of all four proteins indicating that this Bet v 1 epitope is conserved on Bet v 1 homologous allergens from fruits and legumes. In another study, the peptide Bet mim 1 was selected with a murine monoclonal IgG antibody and mapped on residues 58–67 (Jensen-Jarolim et al., 1998). This is corresponding to region VI which was involved in surface areas detected with serum IgE of our patients. However, region VI alone was not mimicked by a peptide.

Multiple-point mutations were introduced into Bet v 1 in order to affect more than one epitope. A six-point mutant of Bet v 1 was shown by Ferreira et al. (Ferreira et al., 1998) to have a strongly reduced IgE-binding capacity compared to unmodified Bet v 1 and single-point mutants. Holm et al. found a reduction of IgE-binding to a Bet v 1 nine-point-mutant of only 27%, and in two of eight patients histamine release was not affected by the mutations at all (Holm et al., 2004). Accordingly, analysis of IgE-binding to sequential epitopes in peanut allergens revealed a high degree of heterogeneity of the IgE-response in individual patients (Shreffler et al., 2004). These studies indicate a high variation of IgE-binding to an allergen between different patients which might be explained with the individual development of antibody specificities in the immune system and its polyclonal nature. However, in our study certain regions seemed to be more likely to bind IgE and, accordingly, investigation of protein interactions revealed the existence of so-called hot spots of binding energy with properties common in the majority of binding sites (Bogan and Thorn, 1998; DeLano, 2002).

Keeping in mind the limited success to generate hypoallergenic point mutants with preserved structure and no or very low IgE-binding capacity in larger patient groups, information on IgE epitopes and IgE epitope specificity of individual patients may support the development of patient tailored reagents for immunotherapy and diagnostic tools (Vrtala et al., 2004; Shreffler et al., 2004). Combined with information about conservation of epitopes on homologous proteins it may also be useful to predict potential cross-reactivity patterns and their clinical relevance. Moreover, the determination of IgE epitopes might allow prediction of potential allergenicity of proteins in novel foods or genetically modified foods.

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References

- Ballmer-Weber, B.K., Scheurer, S., Fritsche, P., Enrique, E., Cistero-Bahima, A., Haase, T., Wüthrich, B., 2002. Component-resolved di-

- agnosis with recombinant allergens in patients with cherry allergy. *J. Allergy Clin. Immunol.* 110, 167–173.
- Bogan, A.A., Thorn, K.S., 1998. Anatomy of hot spots in protein interfaces. *J. Mol. Biol.* 280, 1–9.
- DeLano, W.L., 2002. Unraveling hot spots in binding interfaces: progress and challenges. *Curr. Opin. Struct. Biol.* 12, 14–20.
- Eriksson, N.E., Formgren, H., Svenonius, E., 1982. Food hypersensitivity in patients with pollen allergy. *Allergy* 37, 437–443.
- Ferreira, F., Ebner, C., Kramer, B., Casari, G., Briza, P., Kungl, A.J., Grimm, R., Jahn-Schmid, B., Breiteneder, H., Kraft, D., Breitenbach, M., Rheinberger, H.J., Scheiner, O., 1998. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J.* 12, 231–242.
- Gajhede, M., Osmark, P., Poulsen, F.M., Ipsen, H., Larsen, J.N., Joost van Neerven, R.J., Schou, C., Lowenstein, H., Spangfort, M.D., 1996. X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat. Struct. Biol.* 3, 1040–1045.
- Ganglberger, E., Grunberger, K., Sponer, B., Radauer, C., Breiteneder, H., Boltz-Nitulescu, G., Scheiner, O., Jensen-Jarolim, E., 2000. Allergen mimotopes for three-dimensional epitope search and induction of antibodies inhibiting human IgE. *FASEB J.* 14, 2177–2184.
- Hales, B.J., Bosco, A., Mills, K.L., Hazell, L.A., Loh, R., Holt, P.G., Thomas, W.R., 2004. Isoforms of the major peanut allergen Ara h 2: IgE-binding in Children with peanut allergy. *Int. Arch. Allergy Immunol.* 135, 101–107.
- Holm, J., Gajhede, M., Ferreras, M., Henriksen, A., Ipsen, H., Larsen, J.N., Lund, L., Jacobi, H., Millner, A., Wurtzen, P.A., Spangfort, M.D., 2004. Allergy Vaccine Engineering: epitope modulation of recombinant Bet v 1 reduces IgE-binding but retains protein folding pattern for induction of protective blocking-antibody responses. *J. Immunol.* 173, 5258–5267.
- Holowka, D., Baird, B., 1996. Antigen-mediated IgE receptor aggregation and signaling: a window on cell surface structure and dynamics. *Annu. Rev. Biophys. Biomol. Struct.* 25, 79–112.
- Jensen-Jarolim, E., Leitner, A., Kalchauer, H., Zurcher, A., Ganglberger, E., Bohle, B., Scheiner, O., Boltz-Nitulescu, G., Breiteneder, H., 1998. Peptide mimotopes displayed by phage inhibit antibody binding to bet v 1, the major birch pollen allergen, and induce specific IgG response in mice. *FASEB J.* 12, 1635–1642.
- Jones, S., Thornton, J.M., 1996. Principles of protein–protein interactions. *Proc. Natl. Acad. Sci. U.S.A.* 93, 13–20.
- Kleine-Tebbe, J., Vogel, L., Crowell, D.N., Haustein, U.F., Vieths, S., 2002. Severe oral allergy syndrome and anaphylactic reactions caused by a Bet v 1-related PR-10 protein in soybean SAM22. *J. Allergy Clin. Immunol.* 110, 797–804.
- Markovic-Housley, Z., Degano, M., Lamba, D., Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O., Breiteneder, H., 2003. Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. *J. Mol. Biol.* 325, 123–133.
- Meloan, R.H., Puijk, W.C., Sloodstra, J.W., 2000. Mimotopes: realization of an unlikely concept. *J. Mol. Recognit.* 13, 352–359.
- Mirza, O., Henriksen, A., Ipsen, H., Larsen, J.N., Wissenbach, M., Spangfort, M.D., Gajhede, M., 2000. Dominant epitopes and allergic cross-reactivity: complex formation between a fab fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1. *J. Immunol.* 165, 331–338.
- Mittag, D., Akkerdaas, J., Ballmer-Weber, B.K., Vogel, L., Wensing, M., Becker, W.M., Koppelman, S.J., Knulst, A., Helbling, A., Hefle, S.L., van Ree, R., Vieths, S., 2004a. Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. *J. Allergy Clin. Immunol.* 114, 1410–1417.
- Mittag, D., Vieths, S., Vogel, L., Becker, W.M., Rihs, H.P., Helbling, A., Wuthrich, B., Ballmer-Weber, B.K., 2004b. Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens. *J. Allergy Clin. Immunol.* 113, 148–154.
- Neudecker, P., Lehmann, K., Nerkamp, J., Haase, T., Wangorsch, A., Foetisch, K., Hoffmann, S., Roesch, P., Vieths, S., Scheurer, S., 2003a. Mutational epitope analysis of Pru av 1 and Api g 1, the major allergens of cherry and celery: correlating IgE reactivity with three-dimensional structure. *Biochem. J. Pt.*
- Neudecker, P., Lehmann, K., Rosch, P., 2003b. Sequence-specific (1)H, (13)C and (15)N resonance assignments of SAM22, an allergenic stress-induced protein from soy bean. *J. Biomol. NMR* 26, 191–192.
- Neudecker, P., Schweimer, K., Nerkamp, J., Scheurer, S., Vieths, S., Sticht, H., Rosch, P., 2001. Allergic cross-reactivity made visible: solution structure of the major cherry allergen Pru av 1. *J. Biol. Chem.* 276, 22756–22763.
- Peters, E.A., Schatz, P.J., Johnson, S.S., Dower, W.J., 1994. Membrane insertion defects caused by positive charges in the early mature region of protein pIII of filamentous phage fd can be corrected by prfA suppressors. *J. Bacteriol.* 176, 4296–4305.
- Reese, G., Ayuso, R., Leong-Kee, S.M., Plante, M.J., Lehrer, S.B., 2001. Characterization and identification of allergen epitopes: recombinant peptide libraries and synthetic, overlapping peptides. *J. Chromatogr. B: Biomed. Sci. Appl.* 756, 157–163.
- Scheurer, S., Metzner, K., Haustein, D., Vieths, S., 1997. Molecular cloning, expression and characterization of Pru a 1, the major cherry allergen. *Mol. Immunol.* 34, 619–629.
- Shreffler, W.G., Beyer, K., Chu, T.H., Burks, A.W., Sampson, H.A., 2004. Microarray immunoassay: association of clinical history, in vitro IgE function, and heterogeneity of allergenic peanut epitopes. *J. Allergy Clin. Immunol.* 113, 776–782.
- Spangfort, M.D., Mirza, O., Ipsen, H., van Neerven, R.J.J., Gajhede, M., Larsen, J.N., 2003. Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis. *J. Immunol.* 171, 3084–3090.
- Suphioglu, C., Schappi, G., Kenrick, J., Levy, D., Davies, J.M., O’Hehir, R.E., 2001. A novel grass pollen allergen mimotope identified by phage display peptide library inhibits allergen-human IgE antibody interaction. *FEBS Lett.* 502, 46–52.
- Vieths, S., Scheurer, S., Ballmer-Weber, B., 2002. Current understanding of cross-reactivity of food allergens and pollen. *Ann. N.Y. Acad. Sci.* 964, 47–68.
- Vrtala, S., Focke-Tejkl, M., Swoboda, I., Kraft, D., Valenta, R., 2004. Strategies for converting allergens into hypoallergenic vaccine candidates. *Methods* 32, 313–320.
- Vrtala, S., Hirtenlehner, K., Susani, M., Akdis, M., Kussebi, F., Akdis, C.A., Blaser, K., Hufnagl, P., Binder, B.R., Politou, A., Pastore, A., Vangelista, L., Sperr, W.R., Semper, H., Valent, P., Ebner, C., Kraft, D., Valenta, R., 2001. Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1. *FASEB J.* 15, 2045–2047.
- Wiche, R., Gubesch, M., Konig, H., Fotisch, K., Hoffmann, A., Wangorsch, A., Scheurer, S., Vieths, S., 2004. Molecular basis of pollen-related food allergy: identification of a second cross-reactive IgE epitope on Pru av 1, the major cherry allergen (*Prunus avium*). *Biochem. J. Pt.*