

Monoclonal Antibodies Raised against the Major Apple Allergen, Mal d 1, are Useful Tools for Epitope Studies

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Mal d 1, the major apple allergen shows strong cross-reactivity with IgE specific for the major birch pollen allergen, Bet v 1, and is responsible for birch pollen related food allergy to apple. In contrast to other food and pollen allergens, only a few data on the B-cell epitopes of Mal d 1 are available. To obtain data on the antibody binding epitopes of Mal d 1 and to gain insights to the structures responsible for its B-cell cross-reactivity to Bet v 1, the binding characteristics were studied with 3 monoclonal antibodies (MAbs) raised against Mal d 1 and with patients' IgE directed against Mal d 1 and Bet v 1 by immunoblotting and ELISA. The different binding characteristics of these three MAbs indicated that the MAbs 1D6, 2B2 and 3F8 recognized different epitopes on the major apple allergen. MAb 2B2 cross-reacted with Bet v 1 on immunoblots, whereas 1D6 and 3F8 did not. Since 1D6 and 2B2 (but not 3F8) always competed with IgE from the same apple-allergic patients, 1D6 may be a useful tool for Mal d 1 epitope studies and 2B2 for an epitope that cross-reacts with IgE specific for Bet v 1.

Keywords: Apple allergen, epitopes, IgE

INTRODUCTION

Tree pollens are important airborne spring allergens in many parts of the world, particularly in central and northern Europe and in the northern and eastern part of North America. Birch pollen is responsible for the majority of tree pollen-induced allergic diseases, at least in

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Europe. Birch pollen allergens share allergenic determinants with other tree pollens such as alder, hazel or with fruits and vegetables (Halmepuro & Lowenstein, 1985; Ipsen *et al.*, 1985; Calkhoven *et al.*, 1987; Elsayed *et al.*, 1989).

Birch pollen related food allergies to fresh fruits and vegetables are a well-known clinical phenomenon in industrialized countries. Approximately 50–70% of all patients with birch pollinosis exhibit oral symptoms after ingestion of apples, pears, cherries, apricots, hazelnuts or celery tuber, carrots and other vegetable (Vieths, 1997). The most frequent, and therefore best characterized, pollen-fruit syndrome is apple allergy in patients with tree pollen-induced pollinosis (Anderson & Loewenstein, 1978; Dreborg & Foucard, 1983; Ebner *et al.*, 1991).

Both Bet v 1 and Mal d 1, the major allergens of birch and apple, are proteins with a molecular mass of about 17.5kDa. The molecular relationships of Mal d 1 and Bet v 1 have been confirmed by complete sequencing at the nucleic acid level, showing that the two allergens share 56% amino acid sequence identity (Breitender *et al.*, 1989; Vanek-Krebitz *et al.*, 1995; Schoening *et al.*, 1996). The presence of common epitopes on the major allergen of birch pollen (Bet v 1) and related major food allergens, for example Mal d 1 from apple and Pru a 1 from cherry, was clearly demonstrated (Ebner *et al.*, 1991; Vieths *et al.*, 1994a; Scheurer *et al.*, 1997). Moreover, different isoforms of Bet v 1 showed differences in their IgE-binding capacity. In contrast to Bet v 1 isoforms divergent allergenicity of apple strains mainly depends on different expression levels of the major allergen Mal d 1 (Son *et al.*, 1999). Recently, the determination of the three-dimensional structure of Bet v 1 allowed the prediction of three surface areas of the molecule as potential cross-reactive antibody epitopes (Gajhede *et al.*, 1996; Spangfort *et al.*, 1997). To verify the immunodominant IgE epitopes, Bet v 1 was converted into two non-anaphylactic fragments (Vrtala *et al.*, 1997) and modulated by site directed mutagenesis (Ferreira *et al.*, 1998; Scheurer *et al.*, 1999). In comparison with the Bet v 1 studies, only limited information is available about epitopes of Mal d 1. A point mutation of serine in position 111 of Mal d 1 into proline led to a dramatic decrease of the IgE binding capacity (Son *et al.*, 1999). Thus, either the conformation of Mal d 1 was destroyed by this mutation, or S 111 is a part of an IgE-epitope.

Monoclonal antibodies (MAbs) are useful tools for the characterization of allergen extracts and for the purification of allergenic molecules (Marc-Series *et al.*, 1990). In addition, they could be used for an epitopic definition (mapping) of the major allergen, which could lead to a better understanding of the molecular basis of allergenicity. Recently, MAbs have been raised against Mal d 1 (Fahlbusch *et al.*, 1995).

The present study was undertaken to gain additional information on epitopes of Mal d 1 by further characterizing three of these MAbs and their pattern of competition with IgE from apple-allergic patients.

MATERIALS AND METHODS

Patients' Sera

All sera were collected from birch pollen allergic patients who reported oral allergy-like symptoms after the ingestion of fresh apples at the Hospital Borkum Riff, Department of Dermatology and Allergology (H. Aulepp, Borkum, Germany). Diagnosis was based on a positive skin prick test to both birch pollen extract and apple extract and serological data (EAST class). Patients with positive test results in these assays, a clear anamnesis of spring pollinosis and oropharyngeal symptoms after ingestion of apples, were classified as being allergic to both birch pollen and fruit. Those who were included in the study had an EAST class of at least 2.0 to both purified Bet v 1 and Mal d 1. Sera from a subject with no history of pollen and food allergy were used as a negative control. All sera were stored at -20°C until used.

Monoclonal Antibodies

The MAb 1D6, 2B2 and 3F8 were obtained after immunization of BALB/c mice with purified Mal d 1 as described (Fahlbusch *et al.*, 1995). All antibodies belonged to the IgG1 subclass. Mab B3, raised against a peptide derived from a non-related HIV protein sequence was used as a negative control (Denner *et al.*, 1994).

Allergenic Extracts and Allergens

Apple extract was prepared by a low temperature 'acetone powder' method as described previously (Vieths *et al.*, 1994b; Vieths *et al.*, 1995). Briefly, mature fresh Golden Delicious apples were shock-frosted in liquid nitrogen, homogenized in acetone at -20°C , and the mixture was kept at -20°C overnight. Resulting precipitates were washed twice with acetone and finally with acetone/diethylether (1:1, v:v) at -20°C . The sediment was filtered, freeze-dried and stored at -20°C . To obtain protein extracts, 2 g of acetone powder were extracted with 30 ml of phosphate-buffered saline (0.01 mol l^{-1} , 0.15 mol l^{-1} NaCl, pH 7.4) (PBS) and centrifuged at $20\,000 \times g$ for 45 min (5°C). The supernatant was then passed through a glass fibre pre-filter, lyophilized, and stored at -20°C until use. Freeze-dried birch pollen extract was purchased from Allergopharma (Reinbek, Germany). Mal d 1 and Bet v 1 were isolated by preparative SDS-PAGE followed by electroelution as described (Vieths *et al.*, 1995) and stored freeze-dried at -20°C .

Determination of Specific IgE

Bet v 1 and Mal d 1 were coupled to cyanogen bromide activated filter paper disks at a protein concentration of $1.5 \mu\text{g ml}^{-1}$. Specific IgE was measured by a commercially available enzyme allergosorbent test (EAST) according to the instructions of the manufacturer (Allergopharma, Reinbek, Germany). The results were expressed as EAST classes (corresponding to the classes of PhadebasTM RAST (Pharmacia, Uppsala, Sweden)).

Electrophoresis

Allergen extracts were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE); gel size $180 \times 170 \times 1 \text{ mm}$ according to the method of Laemmli (1970). This system was also used for monitoring the purity of isolated allergens. After separation, proteins were either subjected to Coomassie brilliant blue staining or for immunoblotting as described below.

Immunoblotting, Protein Staining and Immunologic Detection

Proteins were transferred to nitrocellulose (NC)-membranes (Schleicher und Schuell, Dassel, Germany) by semi-dry blotting (Khyse-Andersen, 1984) as described (Vieths *et al.*, 1992). Transfer was controlled by staining single NC-strips of 4 mm with 0.1% (w/v) India ink (Pelikan, Hannover, Germany) in PBS containing 0.05% (v/v) Tween 20 and 1% (v/v) acetic acid. Immunodetection was performed with 0.1 ml of patient serum diluted to 1 ml as described (Vieths *et al.*, 1995). Briefly, specific IgE was detected by a peroxidase-based tertiary antibody-amplified system with 3,3', 5,5'-tetramethylbenzidine as the substrate. When used as primary probes, mabs were applied at $1 \mu\text{g ml}^{-1}$, as hybridoma supernatants (1:100), or as indicated. Bound IgG MAb were immunostained by peroxidase/tetramethylbenzidine as described (Vieths *et al.*, 1995). All dilutions were made in 0.01 mol l^{-1} PBS containing 0.1% bovine serum albumin (BSA) and 0.05% (v/v) Tween 20. Blocking buffer was 0.3% Tween 20 in PBS (Vieths *et al.*, 1994a).

In Gel Fragmentation of the Allergen and Separation of the Generated Peptides

After preparative SDS-PAGE and Coomassie staining of apple extract, the major allergen band at 18 kDa was excised from the gel. Gel slices with a length of approximately 0.5 cm

were treated with 50% acetonitrile in 0.2 mol l⁻¹ ammonium carbonate to remove the bulk of SDS, dye, and most of the water (Rosenfeld *et al.*, 1992). Rehydration was performed with 15–50 µl of endoproteinase solution in PBS at a pH of 7.4. All enzymes were obtained from Promega, Madison, WI, USA and were applied in reaction buffer according to the instructions of the manufacturer. The gel was either treated with Glu-C, Lys-C and Clostripain, respectively. Limited proteolysis was optimized in a way that undigested major allergen remained visible after protein staining and multiple fragments of approximately the same intensity were generated in the mass range of 5–17.5 kDa. The following conditions were applied Glu-C: 1:20 (µg per µg of allergen), 2 h 37°C; Lys-C: 1:100 (µg per µg of allergen), 0.5 h 37°C; Clostripain: 1:100 (µg per µg of allergen), 3 h, 37°C. Approximately 5.5 µg of allergen were subjected to one digestion experiment. Digestion was stopped by heating the gel slices in SDS-PAGE sample buffer at 100°C for 3 min under nonreducing conditions. After transfer to 5.5 cm preparative slots, the products were separated by Tricine SDS-PAGE as described (Schägger & von Jagow, 1987). The products were blotted to NC and used for immunologic detection or transferred to PVDF and subsequently subjected to amino acid microsequencing (Vieths *et al.*, 1994a; Petersen *et al.*, 1992).

Microsequencing Analysis

N-terminal microsequencing by Edman degradation was performed with a 473A pulsed liquid protein sequencer with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Weiterstadt, Germany).

Competition Experiments Between MAbs and Patient IgE

Epitope recognition of selected murine MAbs and patient IgE were compared by ELISA inhibition experiments. Microtiter plates were coated with Golden Delicious apple extract using 0.2 µg of protein in 0.5 mol l⁻¹ sodium carbonate, pH 9.6 per well at 4°C for 16 h. After washing, the wells were simultaneously incubated with a fixed amount of patient serum (50 µl, diluted 1:2.5) and increasing amounts of Mal d 1-specific MAbs (0.2–20 µg in 50 µl per well) overnight at ambient temperature. All dilutions were made with incubation buffer. Bound IgE antibodies were detected by subsequent incubations with rabbit anti human-IgE (1:4000, 1 h), goat anti rabbit-IgG biotin conjugate (1:6000, 1 h) (both from Dako, Copenhagen, Denmark) and streptavidine alkaline phosphatase (1:3000, 1 h) (Medac, Hamburg, Germany), all at 37°C. Enzymatic activity was stained with *p*-nitrophenylphosphate using a staining kit (Bio-Rad, Germany). Absorbance was measured at 405 nm. Competition experiments were also performed by immunoblotting inhibition. Immunoblots with separated apple extract were simultaneously incubated with 20 µl of patients' serum and either 50 µg of purified MAb (3F8) or 0.7 ml hybridoma supernatant (1D6, 2B2) diluted to 1 ml and subjected to immunodetection of bound IgE as described (Vieths *et al.*, 1995). Bound peroxidase activity was visualized with the ECL immunoblotting detection reagents (Amersham, Little Chalfont, UK).

RESULTS

Fragmentation of the Allergen

Multiple allergen fragments of Mal d 1 were successfully generated by the optimized procedure with all three enzymes. After separation of the digests by Tricine SDS-PAGE the most intensive bands occurred at the following molecular masses (kDa): Glu-C: 18.0 (Mal d 1), 17.5, 15.6, 11.8, 10.7, 7.8, 6.7; Lys C: 18.0 (Mal d 1), 14.2, 12.9, 12.5, 10.4, 8.8, 7.8; Clostripain: 18.0 (Mal d 1), 15.0, 13.0, 10.0, 7.0 (results not shown).

TABLE 1. Antigenicity of Mal d 1 fragments after partial proteolysis with specific endoproteases

MAb	Mass of Mal d 1 antigenic fragment (kDa) generated by		
	Glu-C	Lys-C	Clostripain
1D6	17.5	12.5	15.0
	15.6	10.4	13.0
	11.8		10.0
	10.7		
	6.7		
2B2	17.5	12.5	15.0
	15.6	10.4	13.0
			10.0
3F8	11,8	—	15.0
		10.0	13.0

Immunologic Reactivity of the Fragments

The antigenic properties of the fragments were studied with the three MABs raised against Mal d 1. Multiple fragment bands were immunostained by all three antibodies, but not by the control MAb B3. The antigenic patterns obtained by digestion with Glu-C, Lys-C and Clostripain are summarized in Table 1. As an example, Figure 1 shows the recognition of Mal d 1 fragments which occurred after Clostripain treatment by the MABs 1D6, 2B2, 3F8 and B3 which was directed against a partial sequence of a HIV protein as the negative control.

For studying potential allergenicity, sera of 24 patients who suffered from spring pollinosis and oral symptoms after apple ingestion and who showed IgE antibodies against Mal d 1 and a pooled serum from four additional patients, were used for probing Tricine SDS-PAGE blots of the digests. Besides the clear recognition of Mal d 1 by all sera, only nine of these 25 sera (36%) including the serum pool showed IgE binding to fragments: all nine sera bound to a

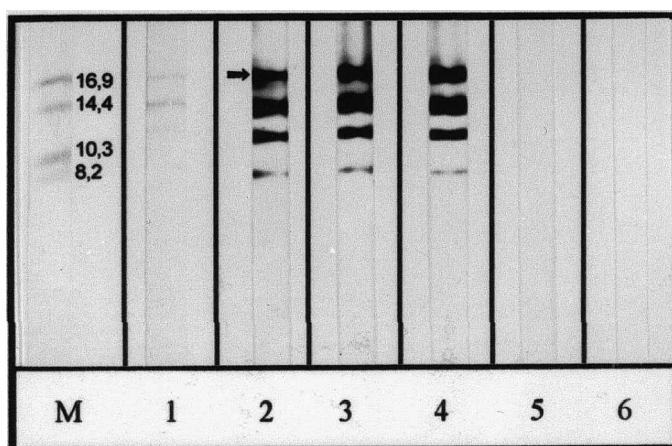


FIG. 1. Binding of the Mal d 1-specific MABs 1D6, 2B2 and 3F8 to fragments of the allergen after treatment with Clostripain, separated by SDS-PAGE and semidry blotting onto NC membranes. M: molecular mass marker proteins; lane 1: protein stain with india ink; lane 2: 1D6; lane 3: 2B2; lane 4: 3F8; The mab B3 (lane 5) was specific for a HIV peptide and served as a negative control. Lane 6: buffer control. The position of undigested Mal d 1 is indicated by an arrow.

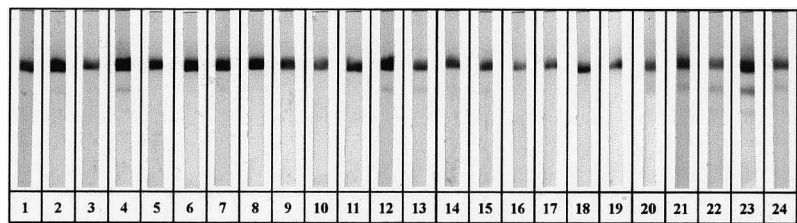


FIG. 2. Immunodetection of Mal d 1 digests by IgE from 23 apple allergic patients and a pooled serum from four apple allergic patients after treatment of the allergen with Clostripain. Nine sera (serum 2, 4, 12, 13, 20, 21, 22, 23, 24) of 24 investigated apple-allergic patients' sera bind the 15 kDa fragment of Mal d 1.

15kDa peptide produced by Clostripain, and three sera to a 17.5kDa band after Glu-C treatment. No IgE-reactive fragments occurred after partial proteolysis with Lys-C. In general, no bands were stained with serum of a nonatopic individual nor with serum from a pollinosis patient with IgE exclusively directed against the minor allergen profilin (not shown). In contrast to the MAbs, patient IgE caused more intensive staining of complete Mal d 1 when compared to fragments in all cases.

As an example, Figure 2 shows such blots with Mal d 1 cleaved by Clostripain tested with 23 sera from apple-allergic patients and a pooled serum from four apple-allergic patients, nine of which presented IgE against the 15 kDa fragment (serum 2, 4, 12, 13, 20, 21, 22, 23, 24). After blotting onto PVDF, partial N-terminal sequences were obtained from five fragments including the two which were recognized by specific human IgE: Glu-C: 17.5 kDa: GVYTFE (Mal d 1 1–6), 11.8 kDa: GDGGPGT (Mal d 1, 46–57), 10.7 kDa: GVYTVE (Mal d 1, 1–6), 7.8 kDa: TKVL (Mal d 1, 102–105), Clostripain: 15 kDa: LFKFVLD (Mal d 1, 18–24).

Binding Characteristics of Three Mal d 1-Specific MAbs with Apple and Birch Pollen Extracts

Binding characteristics of three MAbs to Mal d 1 and Bet v 1 were investigated by immunoblotting and ELISA. All three MAbs 1D6, 2B2 and 3F8 bound strongly to the major apple allergen, Mal d 1. Only 2B2 showed a weak cross-reactivity with Bet v 1, the major birch pollen allergen, by immunoblotting (Figure 3). When tested by ELISA with Bet v 1 and Mal d 1, 1D6 showed no cross-reactivity with Bet v 1, whereas 2B2 and 3F8 bound to the major birch pollen allergen under these conditions, indicating that 3F8 may recognize a conformational epitope on the major birch pollen allergen (data not shown).

Competitive Binding of MAbs and Patient's IgE to Apple Extract

Competition assays were performed in order to test whether the MAbs and IgE from apple-allergic patients recognize identical or related epitopes on Mal d 1. Microtiter plates were coated with Golden Delicious apple extract and incubated with human sera containing IgE against Mal d 1 at a serum dilution of 1:10. Without competition, the absorption after detection of bound IgE ranged from 0.6 to 2.5, depending on the specific patient. Buffer controls and nonallergic sera produced optical densities between 0.05 and 0.1. Increasing amounts of 1.0µg to 20µg of the MAbs 1D6, 2B2 or 3F8 were added to the human serum dilution and the resulting mixtures were subjected to the ELISA. These experiments were conducted with seven patients' sera. Dose related inhibitions that exceeded 20% at MAb concentrations of 10µg ml⁻¹ were obtained with the MAbs 1D6 and 2B2 for five of the sera (Table 2). In contrast, no inhibition resulted with MAb 3F8.

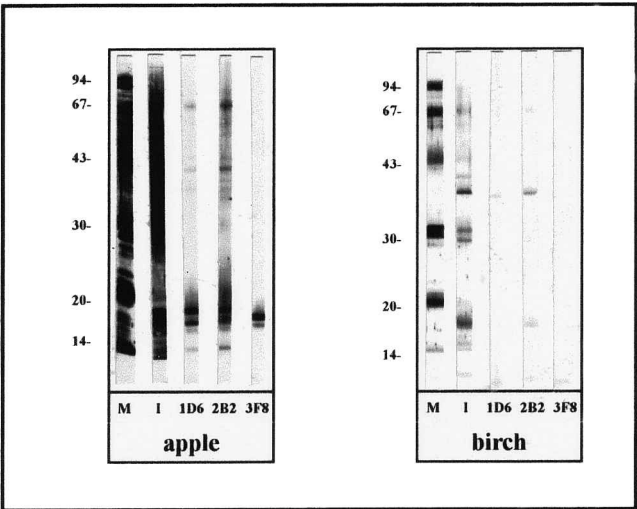


FIG. 3. Control of MAb-binding characteristics to Mal d 1 and Bet v 1. Apple and birch pollen extracts were separated ($10\text{ }\mu\text{g cm}^{-1}$) by SDS-PAGE and semidry blotting onto NC membranes I: protein stain with india ink; M: molecular mass marker proteins.

These results were confirmed by immunoblot inhibition between the MABs and IgE from four patients' sera which also demonstrated the allergen specificity of the competition. All four sera showed identical inhibition patterns. As demonstrated in Figure 4 for three of the sera (Bo 14, Bo 28, Bo 204), 1D6 and 2B2 strongly quenched the Mal d 1-band on apple immunoblots. No inhibition was observed with 3F8.

DISCUSSION

Despite the great progress in analyzing the molecular properties of allergens there remains a lack of knowledge about the allergenic and antigenic B- and T-cell epitopes of many of these

TABLE 2. Competition between IgE from apple allergic patients and mabs for binding to Mal d 1 in ELISA

Patients' serum	% of IgE ELISA inhibition by MAb ($10\text{ }\mu\text{g ml}^{-1}$)			EAST class to	
	1D6	2B2	3F8	Mal d 1	Bet v 1
Bo 4	8	15	4	3	4
Bo 14	43	70	0	4	4
Bo 15	1	3	0	3	4
Bo 16	nd	nd	14	4	4
Bo 21	26	52	nd	3	3
Bo 28	31	40	0	4	4
Bo 200	nd	nd	0	3	4
Bo 204	47	50	0	4	4
BS	59	62	0	2	3

nd: Not done.

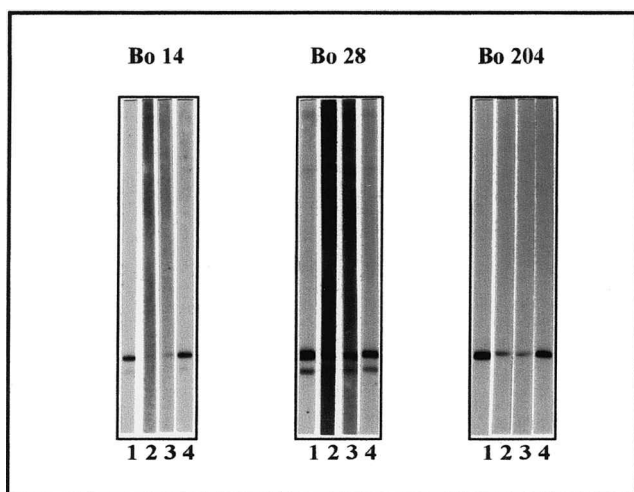


FIG. 4. Immunoblot inhibition using electrophoretically separated apple extract at the solid phase. 20 μ l of patient's sera were diluted to 1 ml and tested for IgE binding to Mal d 1 with and without simultaneous incubation by MAbs specific for Mal d 1. The additional band at 16 kDa recognized by patient Bo28 represents an isoallergen of Mal d 1. Lane 1: serum without inhibitor, lane 2: serum + MAb 1D6, lane 3: serum + MAb 2B2, lane 4: serum + MAb 3F8.

molecules. The present investigation yielded evidence that allergen-specific MAb and patient IgE recognize related structures on Mal d 1, the major apple allergen.

Obviously, the MAbs 1D6, 2B2 and 3F8 bind to different epitope structures on the Mal d 1. This can be deduced from their different binding characteristics to Mal d 1 and Bet v 1 and the results of the competition experiments with patients' IgE: 3F8 cross-reacts with Bet v 1 (Fahlbusch *et al.*, 1995) and showed no competition with IgE. The MAb 1D6 did not cross-react with Bet v 1 and competed with IgE from 5/7 apple allergic patients for the binding to entire Mal d 1. 2B2 showed the same pattern of competition with IgE but cross-reacted with Bet v 1.

Immunoblotting of proteolytic allergen fragments generated from Mal d 1 demonstrated intensive binding to many of these fragments for the three MAbs raised against the apple allergen. Due to Tricine SDS-PAGE the apparent size of these fragments varied between 6.7 and 17.5 kDa. These results indicate that the complete conformational structure is not essential for the antibody reactivity of Mal d 1. The data of the partial N-terminal amino acid sequence of immunoreactive peptides further show that N-terminal and C-terminal fragments seem to carry antigenic epitopes. Due to the entire deduced amino acid sequence determined in our laboratory (Schoening *et al.*, 1996) the two cleavage sites identified by sequencing were in accordance with the specificity of the enzymes and hence were located C-terminal from glutamic acid or arginine residues. Only two of the proteolytic fragments bound IgE (Glu-C: 17.5 kDa, Clostripain: 15 kDa).

Due to the results of immunoblotting the MAbs 2B2 and 1D6 seem to have similar affinities to complete Mal d 1 and to its fragments of larger size (Figure 1). In contrast, patient IgE caused only weak immunostaining of these fragments in the presence of undigested Mal d 1 (Figure 2). Taken together, the results of inhibition experiments and immunoblotting suggest that the MAbs 2B2, 1D6 and IgE bind to related but not to identical structures on the apple allergen.

Our results are in accordance with those obtained with other food allergens such as ovalbumin (Elsayed *et al.*, 1991; Kahlert *et al.*, 1992) and β -lactoglobulin (Ball *et al.*, 1994). For both allergens fragments reacting with IgE have been identified and specific but weak

binding of IgE to synthetic peptides has been demonstrated. Binding to cleavage products has also been shown for pollen allergens such as Phl p 5 from timothy pollen (Petersen *et al.*, 1994). In the case of the rye grass pollen allergen Lol p 11 a peptide of 25 amino acid residues seems to contain approximately 30% of the IgE binding capacity of the entire allergen (De Lalla *et al.*, 1996). Controversial data have been published for Bet v 1 from birch pollen, which has about 60% sequence identity with Mal d 1. Some authors reported a complete loss of the antibody reactivity after enzymatic removal of the 20 N-terminal amino acid residues (Schou, 1995) whereas others described an important continuous epitope (Bet v 1, 23–38) (Vik *et al.*, 1993). The specific but reduced IgE binding capacity of Mal d 1 fragments correlates well with the results of the dramatically reduced allergenicity of Mal d 1 altered by site-directed mutagenesis (Son *et al.*, 1999). On the one hand, our results lead to the conclusion that specific IgE is mainly directed against a conformational epitope on the apple major allergen Mal d 1. On the other hand, the MAbs 1D6 and 2B2 react with Mal d 1 fragments as strong as those with complete Mal d 1 and may thus recognize continuous stretches of the Mal d 1 sequence. However, the results of competitive binding of MAbs and patient's IgE indicate that the conformational IgE epitope is blocked when the MAbs bind to their sequential epitope.

Secondary structure analysis of Bet v 1 and structure prediction of Mal d 1 indicate that these two allergens may share a similar fold. An exposed conformational B-cell epitope could explain a considerable proportion of the regularly observed cross-reactivity between the major allergens from birch pollen and apple. This is more likely to be represented by the reactivity of the MAb 2B2 which, in contrast to 1D6 clearly cross-reacts with Bet v 1 and, similar to 1D6, competes with IgE from apple-allergic patients. Both antibodies may serve as useful tools in extended epitope mapping studies on pollen-related food allergens.

Further work including epitope mapping by using synthetic peptides is in progress to investigate antigenic epitopes of the major apple allergen Mal d 1.

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