

Engineering, Purification and Applications of His-Tagged Recombinant Antibody Fragments with Specificity for the Major Birch Pollen Allergen, Bet v 1

Sabine Flicker¹, Sylvia Laffer¹,
Peter Steinberger¹, Bashar Alhani¹, Ying Zhu^{2,a},
Marja-Leena Laukkanen², Kari Keinänen^{2,b},
Dietrich Kraft¹ and Rudolf Valenta^{1,*}

¹ Institute of General and Experimental Pathology,
AKH, University of Vienna, Währinger Gürtel 18–20,
A-1090 Vienna, Austria

² VTT Biotechnology and Food Research,
FIN-02044 Espoo, Finland

* Corresponding author

Type I allergy, an immunodisorder affecting almost 20% of the population worldwide, is based on the production of IgE antibodies against *per se* harmless allergens. We report the expression of hexahistidine-tagged antibody fragments (Fabs) with specificity for Bet v 1, the major birch pollen allergen, in *Escherichia coli*. The cDNA coding for the heavy chain fragment of a mouse monoclonal anti-Bet v 1 antibody, Bip 1, was engineered by PCR to contain a hexahistidine-encoding 3' end. The modified Bip 1 heavy chain cDNA was co-expressed in *E. coli* XL-1 Blue with the Bip 1 light chain cDNA using the combinatorial plasmid pComb3H. His-tagged recombinant (r) Bip 1 Fabs were isolated by nickel affinity chromatography and rBip 1 Fabs without His-tag were purified via affinity to rBet v 1. rBip 1 Fabs with and without His-tag bound specifically to rBet v 1 and, like Bet v 1-specific human serum IgE and rabbit-anti rBet v 1 antibodies, cross-reacted with Bet v 1-related allergens in other plant-species (alder, oak, hazelnut). We demonstrate the usefulness of His-tagged rBip 1 Fabs (1) for the identification of pollen samples containing Bet v 1 by particle blotting, (2) for the detection of Bet v 1-specific IgE antibodies in human serum samples by sandwich ELISA and (3) for the quantification of Bet v 1 in solution. Based on these examples we suggest to use rBip 1 Fabs for the detection of Bet v 1 and Bet v 1-related allergens in natural allergen sources for allergy prevention, as well as for the standardization of natural allergen extracts produced for diagnosis and immunotherapy of birch pollen allergy.

Key words: Allergen / Recombinant antibody fragment / Standardization / Type I allergy.

Introduction

Type I allergy is a genetically determined immunodisorder which is based on the formation of IgE antibodies to innocuous substances (allergens) (Kay, 1996). In sensitized patients, allergen contact induces the crosslinking of effector cell-bound specific IgE antibodies and the release of biological mediators (e.g., histamine, leukotrienes), which leads to the immediate symptoms of Type I allergy (allergic rhinitis, conjunctivitis, asthma) (Segal *et al.*, 1977).

Current forms of allergy diagnosis are based on the detection of allergen-specific IgE antibodies in the patient serum and on *in vivo* provocation (e.g. skin prick testing; Lockey and Bukantz, 1998). While the symptoms of Type I allergy can be controlled by pharmacological treatment, allergen-specific immunotherapy represents the only causative therapeutic approach (Durham and Till, 1998). Immunotherapy is based on the administration of increasing allergen doses in order to induce a state of allergen-specific non-responsiveness. Although several defined recombinant allergens have been produced for diagnostic and therapeutic applications (Valenta and Kraft, 1995; Valenta *et al.*, 1998), current forms of allergy diagnosis and immunotherapy are still performed with natural allergen extracts. These extracts are prepared from allergen sources (e.g. plant pollen, mites, moulds) and thus contain a mixture of allergenic and non-allergenic components. The allergen contents of such extracts therefore need to be standardized with allergen-specific antibody probes (Chapman, 1994; van Ree, 1997). Previously, serum IgE from allergic patients or allergen-specific polyclonal rabbit antisera were used for the standardization of allergen extracts whereas in the last years monoclonal antibodies of defined epitope specificities were introduced for this purpose (Chapman, 1998). Allergen-specific monoclonal antibodies represent valuable tools for the detection of individual allergens in extracts and in the environment (Chapman, 1998). The measurement of allergens in air samples, house dust and food represents the basis for allergen avoidance and thus may reduce the frequency of allergic symptoms in sensitized patients and, perhaps, *de novo* sensitization.

Here we report the expression in *E. coli* and purification of a hexahistidine-tagged Fab with specificity for one of the most frequent environmental allergens, Bet v 1 (Breiteneder *et al.*, 1989). Bet v 1 represents the major allergen

Present addresses:

^a The Haartman Institute, Department of Medical Genetics

^b Department of Biosciences, Division of Biochemistry,
University of Helsinki, FIN-00014 Helsinki, Finland.

from birch pollen (Jarolim *et al.*, 1989). It shares epitopes with the major pollen allergens of trees belonging to the order *Fagales* (Rohac *et al.*, 1991) and with homologous allergens in plant-derived food (fruits, vegetables and spices; Ebner *et al.*, 1995; Jarolim *et al.*, 1998). More than 95% of patients allergic to tree pollen, fruits and vegetables display IgE cross-reactivity to Bet v 1, and approximately 60% of them react exclusively with this protein (Jarolim *et al.*, 1989).

His-tagged Bet v 1-specific Fabs were constructed using a mouse monoclonal anti-Bet v 1 antibody, Bip 1 as a template (Laffer *et al.*, 1996). We purified rBip 1 Fabs by affinity chromatography to recombinant Bet v 1 or to Ni^{2+} and compared their ability to react with Bet v 1 and Bet v 1-homologous allergens. The usefulness of rBip 1 Fabs for the identification of Bet v 1-containing pollen was studied by particle blotting. We also investigated whether rBip 1 Fabs can be used to detect Bet v 1-specific IgE antibodies in human serum samples and to determine Bet v 1 concentrations by sandwich ELISA in solution. Possible advantages of using recombinant His-tagged Bip 1 Fabs for the standardization of allergen extracts and for environmental allergen measurements are discussed.

Results

Analysis of the Bacterial Expression of rBip 1 Fabs

Correct assembly of the heavy chain fragment and light chain in bacteria requires transport into the oxidizing environment of the periplasmic space (Better *et al.*, 1988; Skerra and Plückthun, 1988). When we analyzed the presence of rBip 1 Fabs in culture medium, periplasmic extract, soluble *E. coli* extract and the insoluble pellet fraction of bacteria that were induced and grown for 5 hours or overnight, we found that overnight growth leads to a release of rBip 1 Fabs into the culture medium most likely due to rupture and death of bacteria (Figure 1A). The goat anti-mouse Fab antiserum detected rBip 1 Fabs in the culture medium after overnight and much weaker after 5 hours induction with IPTG (Figure 1A: lanes A). rBip 1 Fabs were abundantly detected in the periplasmic, soluble cellular extract and the insoluble pellet fraction after 5 hours and overnight induction (Figure 1A: lanes B, C, P). ELISA analysis of the different fractions revealed that aliquots of the 50 ml culture medium obtained after overnight induction contained more rBet v 1-reactive rBip 1 Fabs than aliquots of the 1 ml periplasmic and soluble *E. coli* fraction (Figure 1B). The 1 ml periplasmic fraction and soluble cellular extract obtained after 5 hours and overnight induction contained comparable amounts of rBet v 1-reactive rBip 1 Fabs (Figure 1B). The insoluble bacterial pellet fraction contained goat anti-Fab-reactive moieties of the correct molecular weight (Figure 1A: arrow indicates the position of the comigrating heavy chain fragment and light chain at 25 kDa). Mechanical homogenization of induced bacteria yielded soluble cellular extracts rich in functional Fabs (data not shown).

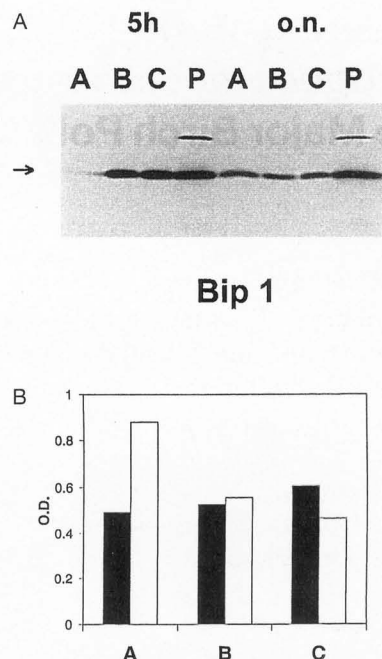


Fig. 1 Detection of rBip 1 Fabs (A) and Detection of rBet v 1-Reactive Recombinant Fabs (B) in Bacterial Extracts.

(A) Nitrocellulose-blotted extracts ('A': culture medium obtained after induction; 'B': periplasmic *E. coli* fraction; 'C': soluble *E. coli* extract obtained after freeze/thawing; 'P': insoluble pellet fraction) obtained after five hours (5 h) or overnight (o.n.) induction were detected with an alkaline phosphatase-coupled goat anti-Fab antiserum. The arrow indicates the position of light and heavy chain fragments (25 kDa) which comigrate under reducing conditions. (B) Aliquots of culture medium ('A'), periplasmic extract ('B') and soluble *E. coli* extract ('C') obtained after 5 hours (black bars) or overnight induction (white bars) were exposed to ELISA plate-coated rBet v 1. Bound Fabs were detected with an alkaline phosphatase-coupled goat anti-mouse Fab antiserum and visualized by color reaction. The amount of bound Fabs corresponds to the OD (optical density) values displayed on the y-axis.

Purification of Soluble Bip 1 Fabs by Single Step Affinity Chromatography

Recombinant Bip 1 Fabs without His-tag were purified by single step affinity chromatography using Sepharose-bound rBet v 1 (Laffer *et al.*, 1996). His-tagged rBip 1 Fabs were purified by Ni^{2+} chelation affinity chromatography. Figure 2A shows the detection of goat anti-mouse Fab-reactive moieties in nitrocellulose-blotted aliquots of total extracts before exposure to the nickel matrix (lane T: 1.25%), unbound material (lane F: 1.25%), wash fractions (lanes W1-W3: 1.25% of each) and eluted fractions (lanes E1, E2: 10% of each). The comigrating heavy chain fragment and the light chain were detected by the goat anti-mouse Fab antiserum at 25 kDa in the total bacterial extract and in the elution fractions when separated under reducing conditions (Figure 2A: lanes T, E1, E2). The assembly of recombinant heavy chain fragments and light chains to hetero- or homodimers is evident from the analysis of the fractions separated by SDS-PAGE under non-reducing conditions (Figure 2B): the goat anti-mouse Fab react-

ed with bands of approximately 44 and 50 kDa in the material eluted from the nickel matrix (Figure 2B: lanes E1, E2). While the 50 kDa band corresponds to the molecular

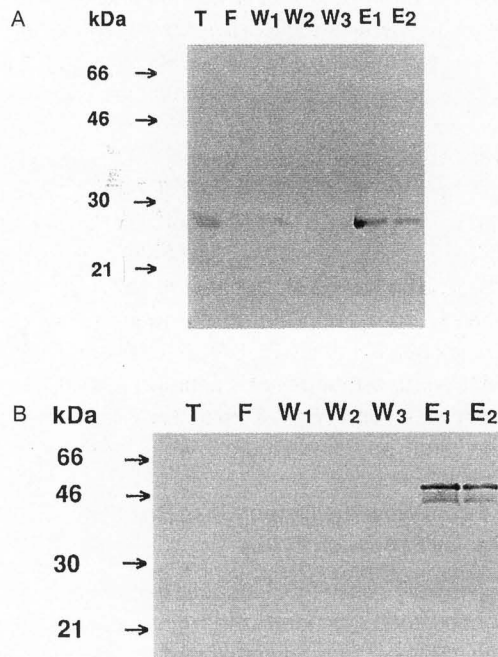


Fig. 2 Purification of Recombinant Bip 1-His Fab by Binding to a Ni^{2+} -Sephrose Matrix.

Total extract containing His-tagged rBip 1 (T) was incubated overnight with Ni^{2+} -Sephrose matrix. Aliquots of the total extract (T), unbound material (F), the wash fractions (W1–W3) and the eluted fractions (E1+E2) were separated by SDS-PAGE under reducing (A) or non-reducing (B) conditions and blotted onto nitrocellulose. rFabs were detected with an alkaline phosphatase-coupled goat anti-mouse Fab antiserum. The molecular masses are indicated in kDa on the left margin.

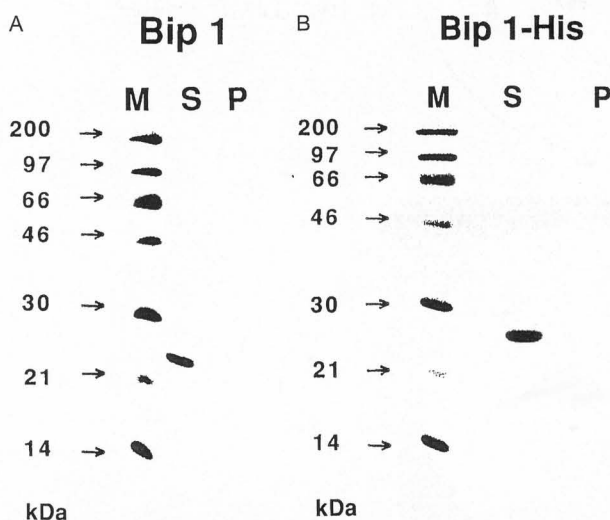


Fig. 3 Purification of rBip 1 Fabs and His-Tagged rBip 1.

Soluble (lanes S) and pellet fractions (lanes P) obtained after high-speed centrifugation of 5 μg purified rBip 1 Fabs without (A: Bip 1) and with His-tag (B: Bip 1-His) were analyzed by SDS-PAGE under reducing conditions and Coomassie Blue staining. Molecular weights are indicated on the left margin.

mass expected for the disulfide bond-linked Fab hetero- or homodimers, the 44 kDa band may result from degradation

The analysis of the solubility of rBip 1 and His-tagged rBip 1 Fabs was performed by centrifugation of the purified material and subsequent SDS-PAGE and Coomassie Blue staining. When purified rBip 1 Fabs and His-tagged rBip 1 Fabs were separated under reducing conditions, the heavy chain fragments and light chains comigrated at 25 kDa. All recombinant Fabs were found in the soluble supernatant fractions (Figure 3A, B: lanes S). The yields of His-tagged rBip 1 Fab were typically in the range of 1–3 mg/liter culture (data not shown).

rBip 1 Fabs React with Natural Bet v 1 and Bet v 1-Homologous Allergens from Other Plant Species

Bet v 1-related allergens occur in pollen of trees belonging to the order *Fagales* and in plant-derived food (fruits, vegetables and spices) (Valenta and Kraft, 1996). We show that rBip 1 Fabs as well as His-tagged rBip 1 Fabs react with nitrocellulose-blotted natural Bet v 1, with the major alder pollen allergen, *Aln g 1* (Breiteneder *et al.*, 1992), and to a lesser extent with the major allergen of oak pollen, *Que*

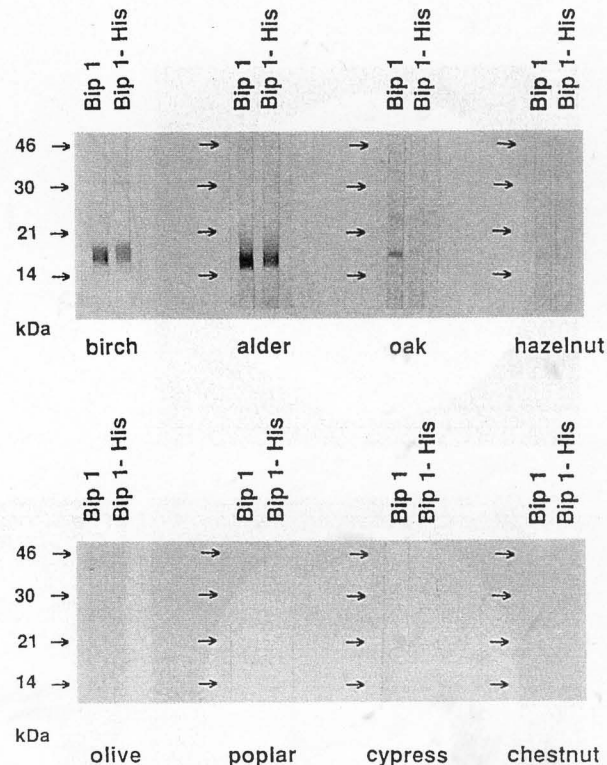


Fig. 4 Crossreactivity of rBip 1 Fabs and His-Tagged rBip 1 Fabs with Natural Bet v 1 and Bet v 1-Homologous Allergens in Different Plant Species.

Bip 1 Fabs without (Bip 1) and with His-tag (Bip 1-His) were tested for their reactivity with nitrocellulose-blotted tree pollen (birch, alder, oak, olive, poplar, cypress, chestnut) and hazelnut extract. Bound Fabs were detected with an alkaline phosphatase-coupled goat anti-mouse Fab antiserum.

a 1 (Ipsen *et al.*, 1990), and with a Bet v 1-related allergen in hazelnut (Hirschwehr *et al.*, 1992) at approximately 17 kDa (Figure 4). No reactivity was observed to nitrocellulose-blotted pollen extracts from olive, poplar, cypress and chestnut, which reportedly do not contain Bet v 1-related allergens (Figure 4). The reactivity profile of the recombinant Bip 1 Fabs thus closely resembles that of IgE antibodies of Bet v 1 allergic patients (Niederberger *et al.*, 1998) and rabbit-anti-Bet v 1 antisera.

rBip 1 Fabs Can Be Used to Detect Bet v 1-Containing Pollen Grains by Particle Blotting

Environmental measurements of pollen allergen loads are currently performed by air sampling and analysis of pollen morphology. We demonstrate that rBip 1 Fabs can be used for the immunological identification of pollen grains containing Bet v 1 or Bet v 1-related allergens by particle blotting (Figure 5). Filter-bound pollen grains from birch, timothy grass and mugwort released proteins after hydration which could be stained with Ponceau S (Figure 5A). rBip 1 Fabs specifically react with Bet v 1 which is expressed in birch-, but not in timothy grass- or mugwort pollen (Figure 5B). A rabbit anti-Phl p 5 antiserum detects grass- but not birch- or mugwort pollen grains (Figure 5C).

rBip 1 Fabs Allow the Detection of Bet v 1-Specific IgE Antibodies in Sera by Sandwich ELISA

Allergen extracts used for current forms of *in vitro* allergy diagnosis consist of difficult to define mixtures of various unrelated allergens and non-allergenic moieties and thus do not allow to measure IgE antibodies with specificity for single allergen molecules. We demonstrate that rBip 1 Fabs can be used to specifically immobilize Bet v 1 but not an unrelated allergen (timothy grass pollen allergen, Phl p 5) to solid phases (e. g. ELISA plates) (Figure 6). Serum IgE from three birch pollen allergic patients reacted specifically with Bet v 1 immobilized via rBip 1 Fabs (Figure 6, black bars: 1–3), whereas no significant IgE binding was found when serum from a non-atopic individual was used (Figure 6, black bar: N). When ELISA-plate bound rBip 1 Fabs were incubated with a Bet v 1-unrelated allergen (Phl p 5) no significant levels of Bet v 1-specific IgE could be bound from the serum samples (Figure 6, white bars: 1–3, N).

rBip 1 Fabs Allow the Detection of Bet v 1 in Solution by Sandwich ELISA

Allergen extracts used for diagnostic and therapeutic purposes need to be standardized regarding their allergen contents. Figure 7 demonstrates that rBip 1 Fabs can be

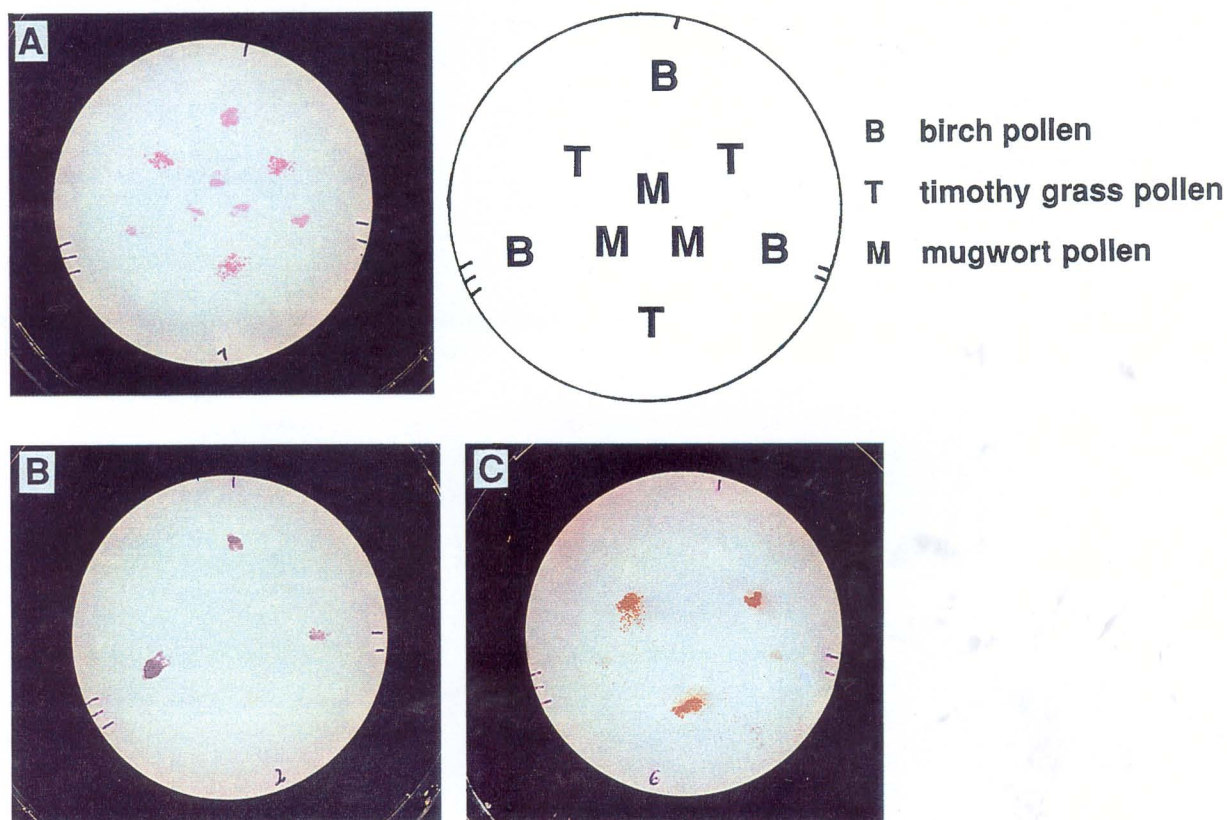


Fig. 5 Detection of Pollen Grains Releasing Bet v 1 by Particle Blotting.

Pollen grains from birch ('B'), timothy grass ('T') and mugwort ('M') were dotted with a toothpick on nitrocellulose filters in the order indicated. Released proteins were stained with Ponceau S (A: red spots). Released Bet v 1 was detected with His-tagged rBip 1 Fabs (B: purple spots), and Phl p 5 with a rabbit anti-Phl p 5 antiserum (C: brown spots).

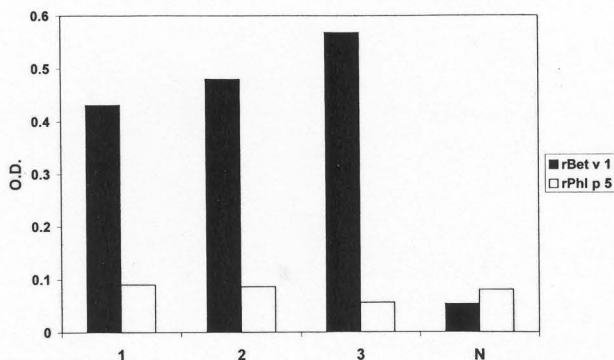


Fig. 6 Sandwich ELISA for the Measurement of Bet v 1-Specific IgE Antibodies in Sera.

ELISA plate-coated His-tagged rBip 1 Fabs were incubated with rBet v 1 (black bars) or rPhl p 5 (white bars) and then exposed to sera from three birch pollen allergic patients containing Bet v 1-specific IgE antibodies (x-axis: 1, 2, 3) or serum from a non-atopic individual (N). OD (optical density) values displayed on the y-axis correspond to the amount of bound IgE antibodies.

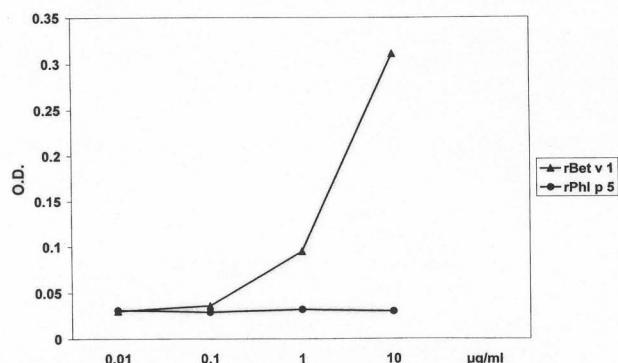


Fig. 7 Sandwich ELISA for the Detection of Bet v 1 in Solution.

ELISA plate-coated His-tagged rBip 1 Fabs were incubated with increasing concentrations (x-axis: 0.01–10 µg/ml) of rBet v 1 or rPhl p 5. Bound rBet v 1 was detected with a biotinylated rabbit anti-Bet v 1 antiserum. OD (optical density) values (y-axis) correspond to the amount of bound Bet v 1.

used to immobilize specifically Bet v 1 out of solutions containing different allergen concentrations. The amount of biotinylated Bet v 1-specific rabbit antibodies which reacted with rBip 1 Fab-bound Bet v 1 increased depending on the concentration of Bet v 1 in solution (Figure 7). Dissolved Bet v 1 could be best detected in a concentration range between 1–10 µg/ml whereas Phl p 5, an allergen unrelated to Bet v 1, was not detected (Figure 7).

Discussion

We report the expression of a mouse monoclonal IgG₁ antibody, Bip 1, with specificity for the major birch pollen allergen, Bet v 1 (Breiteneder *et al.*, 1989), as His-tagged rFab in *E. coli*. Bet v 1, a 17 kDa protein, is recognized by IgE antibodies of more than 95% of birch pollen allergic patients, and almost 60% of those react exclusively with

this allergen (Jarolim *et al.*, 1989; Niederberger *et al.*, 1998). Cross-reactivity of patients IgE with Bet v 1-related allergens present in pollen of trees belonging to the order *Fagales* (alder, hazel, hornbeam, oak) (Niederberger *et al.*, 1998) and in plant-derived food (fruits, vegetables and spices) (Ebner *et al.*, 1995; Valenta and Kraft, 1996; Jarolim *et al.*, 1997) is the reason why most of the Bet v 1 allergic patients exhibit also clinical symptoms after contact with various other plant species and their products.

The Bet v 1-specific monoclonal antibody, Bip 1, recognizes a discontinuous epitope on Bet v 1 which is distinct from the IgE epitopes but crossreacts with most of the Bet v 1-related plant allergens (Laffer *et al.*, 1996). We therefore propose to use His-tagged rBip 1 Fabs for the detection of Bet v 1 and Bet v 1-related allergens in air samples to predict and estimate environmental allergen loads (Schappi *et al.*, 1997; Acevedo *et al.*, 1998; Ormstad *et al.*, 1998). rBip 1 Fabs may likewise be used to monitor the contents of Bet v 1-related food allergens to select fruits and vegetables with low allergen contents and to reveal the presence of Bet v 1-related allergens in complex food preparations. The detection of Bet v 1 and Bet v 1-related allergens with a well-described reagent (rBip 1 Fabs) may thus represent a basis for preventive measures that reduce the allergen exposure in sensitized individuals. Perhaps more important, rBip 1 Fabs may be used for the standardization of natural allergen extracts produced for diagnosis and treatment of birch pollen allergy (Ramirez *et al.*, 1997; Weber *et al.*, 1998). In this context it is worth noting that the contents of Bet v 1 in pollen may greatly vary depending on the tree species (Ahlholm *et al.*, 1998) and environmental factors affecting plant growth and pollen maturation (Helander *et al.*, 1997). The contents of Bet v 1-related allergens in plant food may likewise vary in different parts of fruits and depending on the horticultural species (Jensen-Jarolim *et al.*, 1998) or treatment of the food (Jankiewicz *et al.*, 1996).

The suggestion to use His-tagged rBip 1 Fabs for the detection of Bet v 1 and Bet v 1-related allergens is supported by our experiments which demonstrate reactivity of rBip 1 Fabs with Bet v 1 and Bet v 1-homologous allergens. Furthermore we exemplify the usefulness of rBip 1 Fabs for three practical applications.

First, we show that Bet v 1-releasing pollen grains can be specifically detected by particle blotting. This principle can be applied to detect Bet v 1-containing pollen grains on air sample filters and thus allows to monitor environmental allergen loads. The measurement of environmental allergen loads may be useful for preventive measures but should also allow to correlate clinical symptoms in populations with local allergen loads in the course of epidemiologic studies.

Second, we demonstrate that Bet v 1 can be specifically immobilized by rBip 1 Fabs to solid phases (e. g. ELISA plates) thus allowing the measurement of IgE antibodies to Bet v 1 and Bet v 1-related allergens that are present in complex allergen extracts. This principle is based on the fact that rBip 1 Fabs recognize Bet v 1 epitopes which are

different from those detected by human IgE antibodies.

Finally, we demonstrate that rBip 1 Fabs can be used to establish a sandwich ELISA for the detection and quantification of Bet v 1 in solutions. This sandwich ELISA may be used for the quantification of Bet v 1 in extracts prepared from various allergen sources as well as for the standardization of natural allergen extracts used for diagnostic and therapeutic purposes.

Recombinant allergen-specific Fabs can be produced in a well-controlled manner avoiding expensive and laborious tissue culture work and represent reagents with a well-defined epitope specificity. Moreover, rFabs can be modified by genetic engineering to facilitate their detection and, perhaps, to increase their affinity (Gram *et al.*, 1992; Laukkanen *et al.*, 1995).

However, several disadvantages need to be considered as well: 1. Monoclonal rFabs react with only one epitope and thus may display weaker or no reactivity with less homologous proteins (e. g. weak reactivity of rBip 1 Fabs with the Bet v 1-related hazelnut allergen). 2. Another disadvantage of rFabs expressed in bacteria is that not all of the expressed heavy chain fragments and light chains may form functional Fabs. In this context, we noted that a considerable fraction of rFabs was produced as insoluble proteins and that the amount of goat anti-mouse Fab-reactive material did not always agree with the amount of Bet v 1-reactive material (Figure 1A, B). It should also be pointed out that the expression system used delivered rather low amounts of rFabs (approximately 1–3 mg/l culture) which required the construction of a His-tagged version of Bip 1 Fab that could be purified by single step Ni²⁺ chelation affinity chromatography.

In conclusion, we believe that well-defined allergen-specific Fabs may represent valuable reagents for environmental allergen measurements, the standardization of allergen extracts used for diagnosis and therapy (Valenta *et al.*, 1997). Allergen-specific Fabs which compete with human IgE antibodies may perhaps be used for therapeutic purposes in order to catch and immobilize allergens on the surface of mucous membranes.

Materials and Methods

Materials

Pollen from birch, alder, oak, olive, poplar, cypress, chestnut, mugwort and timothy grass were purchased from Allergon (Välinge, Sweden). *E. coli* strain XL1-Blue was obtained from Stratagene (La Jolla, CA, USA), and plasmid pComb3H was kindly provided by Carlos F. Barbas, Scripps (La Jolla, CA, USA; Barbas *et al.*, 1991). pComb3H contains a human consensus sequence to the amino-terminus of the heavy chain (EVQLLE), and instead of the pelB leader peptide, the ompA sequence was placed in front of the light chain. The vector is provided as pComb3HSS form, containing a heavy chain stuffer of 300 bp and a light chain stuffer of 1200 bp. Alkaline phosphatase-coupled goat anti-mouse Fab antiserum was purchased from Pierce (Rockford, IL, USA). An alkaline phosphatase-coupled mouse

monoclonal anti-human IgE antibody was obtained from Pharmingen (San Diego, CA, USA).

Rabbit anti-Bet v 1 and rabbit anti-Phl p 5 antisera were obtained by immunizing rabbits with purified recombinant Bet v 1 or Phl p 5 using complete and incomplete Freund's adjuvant (Charles River, Kisslegg, Germany). A [¹²⁵I]-labeled donkey anti-rabbit Ig antiserum was purchased from Amersham (Buckinghamshire, UK).

Purified recombinant allergens (rBet v 1, rBet v 2, rPhl p 5) that were expressed in *E. coli* and purified as described (Valenta *et al.*, 1991; Vrtala *et al.*, 1996; Hoffmann-Sommergruber *et al.*, 1997), were obtained from BIOMAY (Linz, Austria).

Methods

Expression of Soluble Recombinant Bip 1 Fabs *E. coli* XL1-Blue cells containing the Bip 1 Fab-expressing pComb3H plasmid (Laffer *et al.*, 1996) were grown in SB medium containing 50 µg/ml ampicillin at 37°C up to an O.D._{600nm} of 0.6–1. After induction with 3 mM IPTG, cells were either incubated for five hours or overnight at 32°C. Induced *E. coli* cells were harvested by centrifugation at 1700 g for 15 min at 4°C and culture medium was stored at –20°C for analysis. Cells were resuspended in phosphate buffered saline containing 100 µg/ml polymyxin B sulfate (Sigma, Steinheim, Germany), incubated for 2 min at room temperature and centrifuged at 1700 g for 15 min at 4°C to obtain the periplasmic fraction in the supernatant. Soluble *E. coli* extracts were obtained by freezing and thawing of *E. coli* cells for three times and recovering the supernatant after centrifugation (1700 g for 15 min at 4°C). The insoluble pellet fraction was also kept for analysis. Aliquots of each fraction (culture medium, periplasmic extract, soluble *E. coli* extract and insoluble pellet fraction) were analyzed by 12.5% SDS-PAGE (Fling and Gregerson, 1986) and blotted onto nitrocellulose (Schleicher and Schuell, Dassel, Germany) (Towbin *et al.*, 1979). In addition to the described fractionation, total cellular extracts were obtained by homogenizing bacterial cell pellets in PBS containing 5 mM PMSF with an ultraturax (Ika, Stauffen, Germany). Nitrocellulose sheets were blocked twice for 5 min and once for 30 min with TBST (50 mM Tris-HCl, 150 mM NaCl, pH 7.5, containing 0.05% v/v Tween 20) containing 0.5% (w/v) BSA at room temperature and were then incubated for 2 hours with an alkaline phosphatase-coupled goat anti-mouse Fab antiserum (Pierce) diluted 1:2500 in TBST/0.5% (w/v) BSA. The nitrocellulose sheets were washed in TBST and AP-buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM MgCl₂). Bound Fabs were visualized by addition of NBT and BCIP diluted in AP-buffer as described (Laffer *et al.*, 1996).

Detection of rBet v 1-Reactive rBip 1 Fabs by ELISA Purified recombinant Bet v 1 (Hoffmann-Sommergruber *et al.*, 1997), or, for control purposes, rBet v 2 (Valenta *et al.*, 1991), were coated to ELISA plates (Greiner, Kremsmünster, Austria) in 0.1 M sodium bicarbonate pH 9.6 (10 µg/ml) overnight at 4°C. Plates were washed twice with TBST containing 0.5% (w/v) BSA and blocked with TBST containing 3% (w/v) BSA at 37°C for three hours. After blocking, 100 µl of culture medium, periplasmic extract and soluble extract, respectively, were added to the wells and incubated overnight at 4°C. Plates were then washed 5 times with TBST/0.5% (w/v) BSA and incubated with the alkaline phosphatase coupled goat anti-mouse Fab antiserum (Pierce) diluted 1:2500 in TBST/0.5% (w/v) BSA at 37°C for two hours. Plates were washed five times and bound goat anti-Fab antibodies were visualized by colour reaction using alkaline phosphatase substrate (Sigma).

Purification of rBip 1 Fabs via Affinity to Recombinant Bet v 1 For the purification of rBip 1 Fabs without His-tag an affinity matrix was prepared by coupling 4 mg of purified recombinant Bet v

1 to an AminolinkTM column (Pierce) according to the manufacturer's advice. Fab containing *E. coli* extract was cleared by centrifugation at 25 000 *g* and filtered (Macherey-Nagel, Düren, Germany) to remove debris prior to application to the affinity column. The extract was applied to the column which was then washed extensively with phosphate-buffered saline until no protein could be detected in the wash fractions (photometry 280 nm). Bound rBip 1 Fabs were eluted with 100 mM Glycine-HCl, pH 2.7 and neutralized in 1 M Tris, pH 9.5. Ten 1 ml eluates were collected. Their protein contents were measured by photometry at 280 nm. Most rBip 1 Fabs were found in fraction 4 which was selected for testing the solubility of the eluted proteins. For this purpose 50 µl of fraction 4 were centrifuged at 13 000 *g* for 5 min. Pellet and supernatant were analyzed by 12.5% SDS-PAGE under reducing conditions and Coomassie Brilliant Blue staining (Bradford, 1976).

Construction and Expression of His-Tagged Bip 1 Fabs A cDNA of the Bip 1 heavy chain fragment containing a hexahistidine-encoding DNA fused to its 3'-end was obtained by PCR amplification of the Bip 1 heavy chain cDNA with the following primer pair: #2377 5' tac aac tag tt ataat ggt gat ggt ggt gat ggc tag tac aat ccc tgg gc 3', #2378 5' tac act cga gtc tgg agg tgg cc 3'. The *SpeI* site is printed in italics and the *XhoI* site is underlined. The amplified PCR product was digested with *XhoI* and *SpeI*, isolated by preparative agarose gel electrophoresis and cloned into the Bip 1 Fab-expressing plasmid (Laffer *et al.*, 1996) where the original Bip 1 heavy chain-encoding cDNA had been removed. For the production of soluble His-tagged Bip 1 Fabs the gene III sequence was deleted by digestion with *SpeI* and *NheI* and religation to yield a plasmid expressing His-tagged Bip 1 Fabs. *E. coli* XL1-Blue cells containing the pComb3H plasmid expressing His-tagged Bip 1 Fabs were grown in SB medium containing 50 µg/ml ampicillin at 37 °C up to an O.D._{600nm} of 0.6–1. After an overnight induction with 3 mM IPTG at 32 °C, cells were harvested by centrifugation at 1700 *g* for 15 min at 4 °C and stored at –20 °C until use. All molecular biological manipulations were performed as described (Sambrook *et al.*, 1989).

Purification of His-Tagged Recombinant Bip 1 Fabs *E. coli* cells (1g) containing recombinant His-tagged Bip 1 Fabs were resuspended in 5 ml buffer A (50 mM NaH₂PO₄, 50 mM Na₂HPO₄, 300 mM NaCl pH. 7.5, 5 mM PMSF, 1 mM Imidazol) containing 1 mg/ml lysozyme and incubated on ice for 35 min. The cells were then homogenized for 1 min with an ultraturrax (IKA, Heidelberg, Germany). The extract was incubated on ice with 10 µg/ml RNaseA (Boehringer-Mannheim, Mannheim, Germany) and 5 µg/ml DNase I (Boehringer-Mannheim) for 15 min and debris was removed by centrifugation (16 000 *g*, 15 min, 4 °C). The resulting extract (Bip-His extract) was then mixed and incubated overnight at 4 °C with the Ni-NTA superflow matrix (Qiagen, Hilden, Germany) that was equilibrated with buffer A. The Ni-NTA matrix containing bound His-tagged Bip 1 Fabs was washed ten times with buffer A containing 20 mM imidazole. His-tagged Bip 1 Fabs were eluted twice with 0.5 ml buffer A containing 250 mM imidazole. Fifty µl aliquots of each washing and elution fraction were analyzed by 12.5% SDS-PAGE under reducing and non-reducing conditions and stained with Coomassie Brilliant Blue (Biorad, Richmond, CA, USA) or blotted onto nitrocellulose and probed with an alkaline phosphatase-coupled goat anti-mouse Fab antiserum.

Reactivity of Nitrocellulose-Blotted Bet v 1 and Bet v 1-Homologous Allergens with Anti-Bet v 1 Antibodies Hundred mg of pollen from birch, alder, oak, olive, poplar, cypress, and chestnut were homogenized in 5 ml SDS-sample buffer (Vrtala *et al.*, 1993) using an ultraturrax (Ika) and extracted by boiling for 5 minutes. Hazelnut extracts were prepared by grinding fresh hazelnuts with a mill. Hundred mg of this powder were further

homogenized in 5 ml SDS-sample buffer with the ultraturrax and boiling. Boiled extracts were centrifuged at 12 000 *g* for 5 min in a bench centrifuge to remove insoluble debris. Fifty µl aliquots of each supernatant were analyzed by SDS-PAGE and Coomassie Blue staining. Comparable amounts of extracted proteins were blotted onto nitrocellulose.

For the detection of Bet v 1 and Bet v 1-homologous allergens by rBip 1 Fabs and His-tagged rBip 1 Fabs, nitrocellulose strips were blocked twice for 5 min and once for 30 min with TBST at room temperature and then incubated overnight with 1:1 in TBST/0.5% BSA diluted bacterial extracts containing rBip 1 Fabs (rBip 1 Fab: culture medium; His-tagged rBip 1: Bip-His extract) at 4 °C. Membranes were washed with TBST/0.5% BSA and bound rFabs were detected with an alkaline phosphatase-coupled goat anti-mouse Fab antiserum (Pierce).

Particle Blotting Pollen grains from birch, timothy grass and mugwort were applied to a nitrocellulose filter in a defined order using toothpicks. Filters were placed on water-soaked Whatman paper for one hour to release allergens. Filter-bound proteins were detected with Ponceau S as described (Sambrook *et al.*, 1989). Membrane-bound Bet v 1 was detected with His-tagged rBip 1 and visualized with an alkaline phosphatase-conjugated goat anti-mouse Fab antiserum as described for immunoblotting. The major timothy grass pollen allergen, Phl p 5, was detected with a rabbit anti-rPhl p 5 antiserum diluted 1:2000 in TBST. Bound rabbit antibodies were reacted with a horseradish peroxidase-conjugated donkey anti-rabbit Ig antiserum (Amersham) diluted 1:1000 in TBST and visualized with diaminobenzidine substrate (Sigma) as described (Sambrook *et al.*, 1989).

Sandwich ELISA for the Detection of Bet v 1-Specific IgE Antibodies Purified His-tagged rBip 1 Fabs were diluted in 0.1 M sodium bicarbonate (1 µg/ml) and coated to ELISA plates (Greiner) overnight at 4 °C. Plates were washed three times with PBSTT [PBS containing 0.1% (v/v) Tween, 0.01% (w/v) thimerosal] and blocked with PBSTT 1% (w/v) BSA at room temperature for four hours. After blocking, wells were incubated overnight at 4 °C with 100 µl aliquots containing either rBet v 1 or, for control purposes, rPhl p 5 dissolved in PBS, 0.01% (w/v) thimerosal, 0.1% (w/v) BSA at a concentration of 10 µg/ml. Plates were then washed three times with PBS/0.01% (w/v) thimerosal and incubated with sera from birch pollen allergic patients or a non-atopic person diluted 1:2 in TBST/0.5% (w/v) BSA overnight at 4 °C. Bound human IgE antibodies were detected with an alkaline phosphatase-conjugated mouse monoclonal anti-human IgE antibody (Pharmingen) diluted 1:1000 in TBST. Optical densities corresponding to the amounts of bound IgE antibodies were measured in an ELISA reader (Dynatech) at 405 nm and represent mean values of duplicate determinations with an error of less than 5%.

Sandwich ELISA for the Determination of Bet v 1 in Solution Purified His-tagged rBip 1 Fabs were coated to ELISA plates and plates were washed and blocked as described for the sandwich ELISA. Wells were then incubated with 100 µl aliquots of recombinant allergens (rBet v 1, rPhl p 5) at different concentrations (10 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml) diluted in PBS/0.01% (w/v) thimerosal, 1% BSA at 4 °C overnight. Plates were washed with PBSTT three times and incubated with a biotinylated rabbit anti-rBet v 1 antiserum diluted 1:1000 in PBS/0.01% thimerosal, 0.1% BSA at 4 °C overnight. Bound rabbit antibodies were detected with peroxidase-conjugated streptavidin [0.125 ng/ml in PBS/0.01% (w/v) thimerosal, 0.1% (w/v) BSA] for three hours at room temperature. Plates were washed with PBS/0.01% (w/v) thimerosal and reacted with *ortho*-phenyldiamin substrate (Sigma). Optical densities were determined at 490 nm and

represent results of duplicate determinations with an error of less than 5%.

Acknowledgements

This study was supported by the Austrian Ministry of Research and Transport and by the Technical Research Centre of Finland (MASTO research program).

References

- Acevedo, F., Vesterberg, O., and Bayard, C. (1998). Visualization and quantification of birch-pollen allergens directly on air-sampling filters. *Allergy* 53, 594–600.
- Ahlholm, J.U., Helander, M.L., and Savolainen, J. (1998). Genetic and environmental factors affecting the allergenicity of birch (*Betula pubescens* ssp. *czerepanovii*) pollen. *Clin. Exp. Allergy* 28, 1384–1388.
- Barbas, C.F. III, Kang, A.S., Lerner, R.A., and Benkovic, S.J. (1991). Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl. Acad. Sci. USA* 88, 7978–7982.
- Better, M., Chang, C.P., Robinson, R.R., and Horwitz, A.H. (1988). *Escherichia coli* secretion of an active chimeric antibody fragment. *Science* 240, 1041–1043.
- Bradford, M.M. (1976). A rapid sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Breitenbach, M., and Scheiner, O. (1989). The gene coding for the major birch pollen allergen, Bet v 1, is highly homologous to a pea disease resistance response gene. *EMBO J.* 8, 1935–1938.
- Breiteneder, H., Ferreira, F., Reikerstorfer, A., Duchene, M., Valenta, R., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Kraft, D., and Scheiner, O. (1992). Complementary DNA cloning and expression in *Escherichia coli* of Aln g 1, the major allergen in pollen of alder (*Alnus glutinosa*). *J. Allergy Clin. Immunol.* 90, 909–917.
- Chapman, M. (1994). Monoclonal antibody immunoassays: quantitative methods for allergen standardization. *Arb. Paul Ehrlich-Inst. Bundesamt Sera Impfstoffe Frankfurt a. M., Band* 87, 120–124.
- Durham, S.R., and Till, S.J. (1998). Immunologic changes associated with allergen immunotherapy. *J. Allergy Clin. Immunol.* 102, 157–164.
- Ebner, C., Hirschwehr, R., Bauer, L., Breiteneder, H., Valenta, R., Ebner, H., Kraft, D., and Scheiner, O. (1995). Identification of allergens in fruits and vegetables: IgE cross-reactivities with important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J. Allergy Clin. Immunol.* 95, 962–969.
- Fling, S.P., and Gregerson, D.S. (1986). Peptide and protein molecular weight determination by electrophoresis using a high molarity Tris buffer system without urea. *Anal. Biochem.* 155, 83–88.
- Gram, H., Marconi, L.A., Barbas, C.F. III, Colet, T.A., Lerner, R.A., and Kang, A.S. (1992). *In vitro* selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. *Proc. Natl. Acad. Sci. USA* 89, 3576–3580.
- Helander, M.L., Savolainen, J., and Ahlholm, J. (1997). Effects of air pollution and other environmental factors on birch pollen allergens. *Allergy* 52, 1207–1214.
- Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W.R., Valent, P., Rohac, M., Rumpold, H., Scheiner, O., and Kraft, D. (1992). Identification of common allergic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J. Allergy Clin. Immunol.* 95, 927–936.
- Hoffmann-Sommergruber, K., Susani, M., Ferreira, F., Jertschin, P., Ahorn, H., Steiner, R., Kraft, D., Scheiner, O., and Breiteneder, H. (1997). High-level expression and purification of the major birch pollen allergen, Bet v 1. *Protein Expr. Purif.* 9, 33–39.
- Ipsen, H., and Hansen, O.C. (1990). Structural similarities among major allergens of tree pollens. In: *Epitopes of atopic allergens*. A.H. Sehon, D. Kraft, G. Kunkel, eds. (Brussels, Belgium: The UCB Institute of Allergy), pp. 3–8.
- Jankiewicz, A., Aulepp, H., Baltes, W., Bogl, K.W., Dehne, L.I., Zuberbier, T., and Vieths, S. (1996). Allergic sensitization to native and heated celery root in pollen-sensitive patients investigated by skin test and IgE binding. *Int. Arch. Allergy Immunol.* 11, 268–278.
- Jarolim, E., Rumpold, H., Endler, A.T., Ebner, H., Breitenbach, M., Scheiner, O., and Kraft, D. (1989). IgE and IgG antibodies of patients with allergy to birch pollen as tools to define the allergen profile of *Betula verrucosa*. *Allergy* 44, 385–395.
- Jensen-Jarolim, E., Leitner, A., Hirschwehr, R., Kraft, D., Wütherich, B., Scheiner, O., Graf, J., and Ebner, C. (1997). Characterization of allergens in Apiaceae spices: anise, fennel, coriander and cumin. *Clin. Exp. Allergy* 27, 1299–1306.
- Jensen-Jarolim, E., Santer, B., Leitner, A., Grimm, R., Scheiner, O., Ebner, C., and Breiteneder, H. (1998). Bell peppers (*Capiscum annuum*) express allergens (profilin, pathogenesis-related protein P23 and Bet v 1) depending on the horticultural strain. *Int. Arch. Allergy Immunol.* 16, 103–109.
- Kay, A.B., ed., *Allergy and allergic diseases*, (Oxford, UK: Blackwell Science), pp. 835–857.
- Keinänen, K., and Laukkanen, M.L. (1994). Biosynthetic lipid-tagging of antibodies. *FEBS-Lett.* 346, 23–126.
- Laffer, S., Vangelista, L., Steinberger, P., Kraft, D., Pastore, A., and Valenta, R. (1996). Molecular characterization of Bip 1, a monoclonal antibody that modulates IgE binding to birch pollen allergen, Bet v 1. *J. Immunol.* 157, 4953–4962.
- Laukkanen, M.L., Orellana, A., and Keinänen, K. (1995). Use of genetically engineered lipid-tagged antibody to generate functional europium-chelate-loaded liposomes: application in fluoroimmunoassay. *J. Immunol. Meth.* 85, 95–102.
- Lockey, R.F., and Bukantz, S.C. (1998). *Allergens and allergen immunotherapy*, (New York, USA: Marcel Dekker, Inc.).
- Niederberger, V., Pauli, G., Grönlund, H., Fröschl, R., Rumpold, H., Kraft, D., and Valenta, R. (1998). Recombinant birch pollen allergens (rBet v 1 and rBet v 2) contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak: A quantitative IgE inhibition study with sera from different populations. *J. Allergy Clin. Immunol.* 102, 579–591.
- Ormstad, H., Johansen, B.V., Gaarder, P.I. (1998). Airborne house dust particles and diesel exhaust particles as allergen carriers. *Clin. Exp. Allergy* 28, 702–708.
- Ramirez, J., Carpizo, J.A., Ipsen, H., Carreira, J., and Lombardero, M. (1997). Quantification in mass units of Bet v 1, the main allergen of *Betula verrucosa* pollen, by monoclonal antibody based ELISA. *Clin. Exp. Allergy* 27, 926–931.
- Rohac, M., Birkner, T., Reimitzer, I., Bohle, B., Steiner, R., Breitenbach, M., Kraft, D., Scheiner, O., Gabl, F., and Rumpold, H. (1991). The immunological relationship of epitopes on major pollen allergens. *Mol. Immunol.* 28, 897–906.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (Cold Spring Harbor, USA: Cold Spring Harbor Laboratory Press).
- Schappi, G.F., Suphioglu, C., Taylor, P.E., and Knox, R.B. (1997). Concentrations of the major birch tree allergen Bet v 1 in pollen

- and respirable fine particles in the atmosphere. *J. Allergy Clin. Immunol.* 100, 656–661.
- Segal, D.M., Taurog, J.D., and Metzger, H. (1977). Dimeric immunoglobulin E serves as a unit signal for mast cell degranulation. *Proc. Natl. Acad. Sci. USA* 74, 2993–2997.
- Skerra, A., and Plückthun, A. (1988). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* 240, 1038–1041.
- Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci.* 76, 4350–4354.
- Valenta, R., Duchene, M., Vrtala, S., Birkner, T., Ebner, C., Hirschwehr, R., Breitenbach, M., Rumpold, H., Scheiner, O., and Kraft, D. (1991a). Recombinant allergens for immunoblot diagnosis of tree-pollen allergy. *J. Allergy Clin. Immunol.* 88, 889–894.
- Valenta, R., Duchene, M., Pettenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1991b). Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 253, 557–560.
- Valenta, R., Sperr, W.R., Ferreira, F., Valent, P., Sillaber, C., Tejkl, M., Duchene, M., Ebner, C., Lechner, K., Kraft, D., and Scheiner, O. (1993). Induction of specific histamine release from basophils with partially purified natural and recombinant birch pollen allergens. *J. Allergy Clin. Immunol.* 91, 88–97.
- Valenta, R., and Kraft, D. (1995). Recombinant allergens for diagnosis and therapy of allergic diseases. *Curr. Opin. Immunol.* 7, 751–756.
- Valenta, R., and Kraft, D. (1996). Type I allergic reactions to plant-derived food: a consequence of primary sensitization to pollen allergens. *J. Allergy Clin. Immunol.* 97, 893–895.
- Valenta, R., Flicker, S., Eibensteiner, P., Steinberger, P., Laffer, S., Dolecek, C., and Kraft, D. (1997). Recombinant allergen-specific antibody fragments: Tools for diagnosis, prevention and therapy of Type I allergy. *Biol. Chem.* 378, 745–748.
- Valenta, R., Vrtala, S., Laffer, S., Spitzauer, S., and Kraft, D. (1998). An update on allergens-Recombinant allergens. *Allergy* 53, 552–561.
- van Ree, R. (1997). Analytic aspects of the standardization of allergenic extracts. *Allergy* 52, 795–805.
- Vrtala, S., Grote, M., Duchene, M., van Ree, R., Kraft, D., Scheiner, O., and Valenta, R. (1993). Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *Int. Arch. Allergy Immunol.* 102, 160–169.
- Vrtala, S., Susani, M., Sperr, W.R., Valent, P., Laffer, S., Dolecek, C., Kraft, D., and Valenta, R. (1996). Immunologic characterization of purified recombinant timothy grass pollen (*Phleum pratense*) allergens (Phl p 1, Phl p 2, Phl p 5). *J. Allergy Clin. Immunol.* 97, 781–787.
- Weber, B., Fiebig, H., and Cromwell, O. (1998). Quantification of major allergens as part of the quality control of commercially produced allergen extracts. *Allergologie* 21, 116–124.

Received April 1, 1999; accepted November 8, 1999