

Purification and Characterization of Recombinant *Bet v* I, the Major Birch Pollen Allergen

IMMUNOLOGICAL EQUIVALENCE TO NATURAL *Bet v* I*

(Received for publication, February 1, 1993, and in revised form, May 14, 1993)

Fátima D. Ferreira‡§, Karin Hoffmann-Sommergruber‡, Heimo Breiteneder‡, Karin Pettenburger‡, Christof Ebner‡, Wolfgang Sommergruber¶, Renate Steiner‡, Barbara Bohle‡, Wolfgang R. Sperr||, Peter Valent||, Andreas J. Kungl**‡‡, Michael Breitenbach¶¶, Dietrich Kraft‡, and Otto Scheiner‡§§

From the ‡Institut für Allgemeine und Experimentelle Pathologie, Universität Wien, Währinger Gürtel 18–20, A-1090 Vienna, Austria, the ¶Ernst Böhringer Institut für Arzneimittelforschung, A-1120 Vienna, Austria, the ||AKH, Medizinische Abteilung, Hämatologie und Hämostaseologie, Universität Wien, A-1090 Vienna, Austria, the ¶¶Institut für Genetik und Allgemeine Biologie, Universität Salzburg, A-5020 Salzburg, Austria, and the ‡‡Max-Planck-Institut für Biochemie am Klopferspitz, DW-8033 Martinsried, Germany

Pollen from trees of the order *Fagales* (e.g. birch, alder, hazel, oak, and hornbeam) are a major cause of Type I allergies observed in early spring. Previously, we reported the cloning and sequencing of *Bet v* I, the major birch pollen allergen, which showed high sequence similarities to a family of plant pathogen-activated genes (Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O., and Breitenbach, M. (1989) *EMBO J.* 8, 1935–1938). Here, we present the results on the expression, purification, and characterization of recombinant *Bet v* I produced in *Escherichia coli* as fusion and non-fusion protein, respectively. The purified recombinant proteins were analyzed to verify purity and structural integrity, and their immunological properties were compared to those of *Bet v* I isolated from birch pollen (natural *Bet v* I). Immunoblot analyses showed that the recombinant proteins are specifically recognized by monoclonal antibodies raised against natural *Bet v* I as well as by IgE from birch pollen-allergic patients. However, enzyme-linked immunosorbent assays revealed a decreased IgE-binding activity of the recombinant fusion *Bet v* I compared to the non-fusion and natural *Bet v* I proteins, which probably results from conformational changes due to the fusion tail. Recombinant non-fusion *Bet v* I was equivalent to natural *Bet v* I with respect to IgE-binding properties, the ability to induce *in vitro* proliferation of allergen-specific T-cell clones, and the ability to release histamine from basophils derived from birch pollen-allergic patients.

individuals are sensitized by allergenic proteins, which originate from various sources including pollens of grasses, weeds, and trees, spores of molds, feces from mites, and dander from pets and other domestic animals (Kaplan, 1985). These proteins induce IgE synthesis by B-cells, an event clearly shown to be T-cell dependent (Vercelli and Geha, 1989). Cross-linking of allergen-specific IgE bound to high affinity Fc_ε receptors (Fc_εRI) on the surface of effector cells (mast cells and basophils) leads to immediate release of inflammatory mediators, e.g. histamine, arachidonic acid, and its metabolites.

For a number of years, Type I (IgE-mediated) allergic diseases have shown a stupendous increase. According to some authors, the incidence of Type I allergies in developed countries may be as high as 15–20% (Ishizaki *et al.*, 1987; Wüthrich, 1991). For this reason, diagnosis and therapy of these diseases represent a major point of interest. As of yet, allergen extracts used for *in vitro* as well as for *in vivo* diagnostic procedures (radioimmunoassays, skin prick tests) are in general biochemically poorly characterized. Thus, it can be expected that an exact characterization of IgE-binding allergens will significantly improve accuracy and reliability of diagnosis of Type I allergies (Scheiner *et al.*, 1992; Valenta *et al.*, 1992). Furthermore, therapy of these diseases (rhinitis and/or conjunctivitis, allergic asthma bronchiale) is frequently accomplished by injection of the allergen assumed to be responsible for the allergic response (hyposensitization treatment). To be successful, this procedure demands not only accurate diagnosis, but also pure and well characterized allergen preparations. Today, allergen extracts in use are prepared from natural sources and contain, besides the allergen(s), additional components, mostly proteins, to which the patient is not allergic. Taking all this into account, it is easy to see that recombinant techniques offer new aspects in providing pure and well characterized tools for diagnostic and therapeutic purposes. However, before use, any highly purified recombinant allergen has to be shown to be equivalent to its natural counterpart with respect to physicochemical and immunological parameters.

In most parts of the temperate climate zone of the world, including northern parts of the United States, Canada, and even the southernmost areas of Australia, pollen from early flowering trees represents a major source of Type I allergens. Among these tree pollens, birch (*Betula verrucosa*) pollen seems to contain the most potent allergenic molecules. Birch pollen contains a single major allergen with a molecular mass

The mechanisms of induction and regulation of allergic response in humans are not yet well understood. Predisposed

* This work was supported by Grants 3/8603 from the Austrian Forschungsförderungsfonds der Gewerblichen Wirtschaft and S6002-Bio from the Austrian Fonds zur Förderung der Wissenschaftlichen Forschung (F. W. F.) (to O. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Inst. f. Genetik u. Allg. Biologie, Universität Salzburg, Hellbrunner Straße 34, A-5020 Salzburg, Austria.

** Recipient of an "Erwin Schrödinger Auslandsstipendium" (J0724/CHE), from the F. W. F.

§§ To whom correspondence and reprint requests should be addressed.

of 17 kDa (Ipsen and Loewenstein, 1983), designated *Bet v I*.¹ More than 96% of all tree pollen-allergic patients exhibit IgE to *Bet v I*, and 60% react exclusively to this allergen (Jarolim *et al.*, 1989a). Previously, a cDNA clone coding for *Bet v I* was isolated and sequenced in our laboratory (Breiteneder *et al.*, 1989). It shows high sequence similarities to the single major pollen allergens from alder, *Aln g I* (Breiteneder *et al.*, 1992), from hornbeam, *Car b I* (Larsen *et al.*, 1992), and from hazel, *Cor a I* (Breiteneder *et al.*, 1993). This observation is in good agreement with data demonstrating strong cross-reactivities between these allergens (Rohac *et al.*, 1991), thus providing the molecular basis for the observed cross-sensitivity of tree pollen-allergic patients (Eriksson *et al.*, 1987).

In this paper we describe the expression and purification of two recombinant forms (fusion and non-fusion proteins) of *Bet v I*, the major birch pollen allergen. Using various analytical criteria, the purity and structural integrity of the proteins were evaluated. In addition, the immunological properties of the purified recombinant proteins produced in *Escherichia coli* were compared to those of *Bet v I* isolated from birch pollen.

EXPERIMENTAL PROCEDURES

Cloning of Recombinant *Bet v I* as a Non-fusion Protein in pKK233-2—Restriction enzyme digests, gel purification of DNA fragments, fill in reactions, DNA ligation reactions, and transformation of *E. coli* were done according to standard techniques (Sambrook *et al.*, 1989). The *Bet v I* cDNA fragment (Breiteneder *et al.*, 1989) was subcloned into the *NcoI* site of the expression plasmid pKK233-2 (Pharmacia, Uppsala, Sweden). Competent *E. coli* strain JM105 was transformed with the resulting plasmid pKK233-2/*Bet v I* and grown to an OD₆₀₀ of 0.4. Isopropyl- β -D-thiogalactopyranoside was then added to a final concentration of 2.5 mM and incubation continued for 3 h at 37 °C.

Construction of Recombinant *Bet v I* as a Fusion Protein in pEX34B—The expression of recombinant *Bet v I* as a fusion protein was achieved by using the plasmid pEX34B (one of the three possible reading frames). This vector directs the synthesis of a hybrid protein under the control of the λ P_L promoter, in which foreign proteins are produced fused to the first 98 amino acids of the MS2 polymerase (Strebel *et al.*, 1986). A linker region of 42 nucleotides was synthesized encoding a cleavage site for the protease factor Xa using an Applied Biosystems DNA-Synthesizer (model 380 A). This oligonucleotide sequence encoded the peptide EFPSTMNQTIEGR (the underlined amino acid residues correspond to the Factor Xa recognition sequence). The linker was ligated to the *Dde*-*EcoRI* fragment of the *Bet v I* cDNA (Breiteneder *et al.*, 1989) and inserted into the *EcoRI* site of pEX34B, resulting in the plasmid pEX60. For expression of the pEX60 plasmid, competent *E. coli* strain AR58 was transformed and grown to an OD₆₀₀ of 0.2. Induction was done by a temperature shift from 28 to 42 °C, and cells were incubated for an additional 2 h.

Purification of Recombinant Non-fusion *Bet v I* (rnfBet v I)—After expression of the recombinant non-fusion protein, cells were harvested by centrifugation and the pellet resuspended in 50 mM Tris-HCl, pH 7.5, containing 220 mM NaCl. The cells were then disrupted by freezing in liquid nitrogen followed by thawing at 37 °C. This step was repeated twice. Insoluble material was removed by centrifugation at 30,000 $\times$ g/25 min/4 °C. In the next purification step, 200 parts/million of Biocryl BPA-1000 (TosoHaas, Stuttgart, Germany) was added to the crude bacterial cell extract followed by centrifugation at 30,000 $\times$ g/25 min/4 °C. The clear supernatant was dialyzed against 25 mM imidazole-HCl buffer, pH 7.4, and applied to a PBE-94 exchanger column (Pharmacia) at room temperature. The column was washed with 25 mM imidazole buffer and bound proteins eluted with 12.5% (v/v) Polybuffer 74-HCl (Pharmacia), pH 4.0. Fractions

containing *Bet v I*, as monitored by dot-blot immunoassays, were pooled and subjected to HPLC employing a linear gradient of 2-propanol created within 60 min at room temperature (C₈ Hypersil-WP 300, 10 μ m, 8 $\times$ 250 mm; solvent A: 0.1% trifluoroacetic acid in water; solvent B: 90% 2-propanol, 0.1% trifluoroacetic acid; gradient of 0–60% solvent B; flow rate 1.0 ml/min). UV absorbance was monitored at 280 nm. Protein concentration of the purified samples was determined by the micro-Kjeldahl method using glycine as standard (Jacobs, 1959).

Purification of the Recombinant Fusion *Bet v I* (rfBet v I)—After expression of the MS2 polymerase/*Bet v I* fusion protein, the cells were harvested by centrifugation and disrupted as described above. The 30,000 $\times$ g pellet containing the fusion protein was solubilized in 8 M urea and sequentially dialyzed against 50 mM Tris-HCl, 220 mM NaCl buffer, pH 7.5, and 25 mM imidazole-HCl buffer, pH 7.4. Subsequently, the purification procedure was carried out as described for the rnfBet v I.

Birch Pollen Extract and Purification of Natural *Bet v I* (nBet v I)—Protein extract from birch (*B. verrucosa*) pollen (Allergon AB, Engelhom, Sweden) was prepared as previously described (Hirschwehr *et al.*, 1992). Pollen-derived *Bet v I* (nBet v I) was purified by affinity chromatography with an immobilized anti-*Bet v I* monoclonal antibody (Jarolim *et al.*, 1989b) followed by reversed-phase HPLC.

SDS-PAGE, Immunoblots, and Isoelectric Focusing—Intermediate fractions of partially purified natural and recombinant *Bet v I* and the final purified proteins were analyzed by SDS-PAGE according to the method of Laemmli (1970), using 15% acrylamide gels. Proteins in the gels were visualized by staining with Coomassie Brilliant Blue R-250.

For immunoblot analysis, proteins were separated by 15% SDS-PAGE and electroblotted (Towbin *et al.*, 1979) onto nitrocellulose membranes (Schleicher and Schuell). IgE immunoblots were performed as previously described (Jarolim *et al.*, 1989a). Bound IgE was detected using ¹²⁵I-rabbit anti-human IgE (Pharmacia). A serum pool from 20 non-allergic healthy donors (NHS) was used as control. Immunoblots using monoclonal anti-*Bet v I* antibodies, BIP 1 and BIP 4 (Jarolim *et al.*, 1989b), were performed as described by Rohac *et al.* (1991) using rabbit anti-mouse IgG, Fc-specific (Jackson ImmunoResearch, MD) and ¹²⁵I-donkey anti-rabbit IgG (Amersham, United Kingdom) antibodies. Autoradiography was performed at –70 °C for 12–48 h with intensifying screens.

Isoelectric focusing was done using an Immobiline system (Pharmacia), pH range 4.0–7.0, according to the instructions of the manufacturer. Protein bands were stained with Coomassie Brilliant Blue G-250, according to Neuhoff *et al.* (1988).

Capillary Electrophoresis—High performance capillary electrophoresis was performed on the P/ACE System 2000 (Beckman, Palo Alto, CA) using uncoated fused silica capillaries. Ten μ g of rnfBet v I and nBet v I, respectively, were diluted 1:10 in 100 mM borate buffer, pH 8.35, and injected electrokinetically at 29 kV for 5 s. UV absorbance was monitored at 214 nm.

NH₂-terminal Sequence Analysis—Thirty μ g of purified rnfBet v I was subjected to electrophoresis and transferred onto polyvinylidene difluoride membrane (Millipore). The protein band was visualized by Coomassie Blue R-250 staining, excised, and applied to a 6*6 mm Blott Cartridge of an Applied Biosystems 477A pulsed liquid-phase sequencer. Amino acid derivatives were analyzed on-line by reversed-phase HPLC (Applied Biosystems model 120A).

Quasielastic Laser Light Scattering—A solution containing rnfBet v I at 0.5 mg/ml was investigated with a laser light scattering system which has been set up by Prof. Glatter and his co-workers. Before measurements, the sample was filtered through a 0.2- μ m filter. Scatter was detected at 90° and the acquired data were analyzed as described previously (Schnablegger and Glatter, 1991; Glatter *et al.*, 1991).

Patients and Sera—Birch pollen-allergic patients were selected according to typical case history, positive skin prick test, and RAST classes > 3.5. Twenty non-allergic individuals (case history, skin prick test, RAST) were selected as control. Sera were stored at –20 °C.

ELISA and Cross-inhibition Experiments—Microtiter plates (Dynatech M129 A) were coated overnight at 4 °C with 20 μ g/ml of the monoclonal antibody BIP 1 (Jarolim *et al.*, 1989b) as “catching antibody.” Remaining binding sites were saturated by incubation with 110 μ l of phosphate-buffered saline, 1% human serum albumine, 0.05% Tween 20 (buffer A) for 3 h at room temperature. The plates were then incubated with increasing concentrations of *Bet v I* (0.125 μ g, 0.25 μ g, 0.5 μ g, 1.0 μ g, and 2.0 μ g/ml of purified nBet v I or rnfBet

¹ The abbreviations used are: *Bet v*, *Betula verrucosa*; *Aln g*, *Alnus glutinosa*; *Cor a*, *Corylus avellana*; *Car b*, *Carpinus betulus*; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; nBet v I, natural *Bet v I*; NHS, normal human serum; RAST, radioallergo sorbent assay; rfBet v I, recombinant fusion *Bet v I*; rnfBet v I, recombinant non-fusion *Bet v I*; PAGE, polyacrylamide gel electrophoresis.

v I, and with 0.25, 0.5, 1.0, 2.0, and 4.0 $\mu\text{g/ml}$ of the r*Bet v I*). For optimal binding conditions, the plates were first incubated for 3 h at room temperature and then for 1 h at 4 °C. After washing five times with phosphate-buffered saline, 0.05% Tween, a serum pool (diluted 1:2, 4, 8, and 16 in buffer A) from 12 birch pollen-allergic patients containing IgE antibodies reacting exclusively to *Bet v I* was applied onto the BIP 1/*Bet v I*-coated plates and incubated overnight at 4 °C. After washing, the plates were incubated (4 h at room temperature and then overnight at 4 °C) with alkaline phosphatase-conjugated horse anti-human IgE antibodies (Kallestad, Chaska) and developed with the substrate *p*-nitrophenyl phosphate. The optical density was measured at 405 nm (with 550 nm as reference wavelength), using an ELISA reader (Dynatech MR 600). Adequate controls were performed for each incubation step of the ELISA assay and a serum pool from 20 non-allergic individuals (NHS) assured the specificity of the IgE-detection system.

For cross-inhibition experiments, the above mentioned *Bet v I*-specific serum pool was diluted 1:6 in buffer A and preincubated (5 h at room temperature followed by 2 h at 4 °C) with 20.0, 10.0, 5.0, 2.5, 1.25, 0.6, and 0 $\mu\text{g/ml}$ of n*Bet v I* or r*Bet v I* preparations. Twice that amount was used when testing r*Bet v I* because the calculated (and experimentally estimated) molecular mass of r*Bet v I* was twice as large as that of n*Bet v I* or r*Bet v I*. ELISA plates were coated with 20 $\mu\text{g/ml}$ BIP 1 followed by 2 $\mu\text{g/ml}$ of purified n*Bet v I*. Afterward, the ELISA assay was performed as described above. The specificity of the inhibition assay was assured by using a non-related allergen (cat dander) under identical conditions.

RAST—Coupling of purified n*Bet v I* or r*Bet v I* to activated paper discs was carried out essentially as described by Ceska *et al.* (1972), using 1 μg of *Bet v I*/disc. RAST was performed using commercially available birch pollen extract-coated discs (Pharmacia) and r*Bet v I* or n*Bet v I*-coated discs (described above), according to the manufacturer's instructions.

T-cell Proliferation Assays—Isolation of *Bet v I*-specific T-cell clones from the peripheral blood of seven birch pollen-allergic patients (as indicated by typical case history, positive skin tests, and positive RAST) and proliferation assays were done as previously described (Parronchi *et al.*, 1991; Ebner *et al.*, 1993).

Histamine Release Assay—Basophils from non-allergic individuals and from *Bet v I*-allergic patients were isolated from peripheral blood as previously described (Lichtenstein and Osler, 1964) with some modifications (Valent *et al.*, 1989). After incubation with various concentrations of purified allergens or with mAb E-124-2-8 anti-IgE (Immunotech, Marseille, France), histamine was measured in the cell-free supernatant using a commercial radioimmunoassay (Immunotech, Marseille, France). Total histamine of cell suspensions was determined after cell lysis in distilled water.

RESULTS

Purification of Recombinant *Bet v I*—Fusion and non-fusion forms of the full-length *Bet v I* cDNA were expressed in *E. coli* as intracellular proteins. Initially, to optimize the extraction conditions, *E. coli* cells were harvested and lysed by different techniques (sonication, lysozyme treatment) in the presence or absence of detergents and protease inhibitors (data not shown). Finally, SDS-PAGE and immunoblot analyses showed that undegraded r*Bet v I* was quantitatively extracted in Tris-HCl buffer by repeated freeze-thaw cycles, in the absence of detergents and protease inhibitors. High levels of expression of the r*Bet v I* with the expected M_r value of 30,000 were obtained, but the fusion protein was only extractable under strong denaturing conditions (6–8 M urea). After renaturation by dialysis against 50 mM Tris-HCl, 220 mM NaCl, pH 7.5, the fusion *Bet v I* reacted with BIP 1, a monoclonal antibody raised against natural *Bet v I* (Jarolim *et al.*, 1989b), as determined by immunoblot analysis. After centrifugation, the high speed supernatant was fractionated using a chromatofocusing column. Pretreatment of the supernatant with positively charged polymeric particles (Biocryl BPA-1000) drastically reduced its viscosity by removing nucleic acids and other contaminants and consequently improved the loading capacity and the overall performance of the chromatofocusing step. Fractions eluting at pH 5.8–5.5 or

at pH 5.5–5.2 contained r*Bet v I* or r*Bet v I*, respectively, and few contaminating *E. coli* proteins. After further fractionation on a reversed-phase HPLC column, the recombinant *Bet v I* proteins appeared homogeneous as determined by SDS-PAGE (Fig. 1A). The purified r*Bet v I* produced in *E. coli* showed the same electrophoretic mobility as *Bet v I* isolated from birch pollen. These results are shown in Fig. 1, A–C, using both Coomassie staining and immunoblotting techniques.

The *Bet v I* fusion gene was designed such that it successively coded for 98 NH₂-terminal amino acids of the MS2-polymerase, 14 residues of a linker region containing the blood coagulation factor Xa recognition sequence (Ile-Glu-Gly-Arg), and the whole sequence of *Bet v I*. It was expected that digestion of the fused protein would release a protein having the same NH₂-terminal sequence as the n*Bet v I*. However, attempts to cleave the purified *Bet v I* fusion protein failed.

Analytical Characterization—The purified r*Bet v I* was characterized by different analytical methods to evaluate its purity and examine its primary structure. Analytical reversed-phase HPLC and capillary electrophoresis showed that the r*Bet v I* preparation was 99% pure (Fig. 2A). Although *Bet v I* isolated from birch pollen migrated as a single band on SDS-PAGE (Fig. 1A, lane 1), capillary electrophoresis showed that the preparation was quite heterogeneous. At least three different components (3.19, 3.31, and 3.43 min migration times) could be identified (Fig. 2B). However, the migration time of the r*Bet v I* corresponded to that of the major component seen in the natural *Bet v I* preparation (3.17 and 3.19 min, respectively).

Automated NH₂-terminal sequence analysis of two different r*Bet v I* preparations showed that the initiating methionine was removed, as is the case with the natural protein from birch pollen. A single sequence was detected, which corresponded exactly to the first 20 amino acids at the NH₂ terminus of *Bet v I*, as predicted from the cDNA sequence (Breiteneder *et al.*, 1989).

Analysis of the purified *Bet v I* proteins by isoelectric focusing on an immobilized pH gradient ranging from 4.0 to 7.0 showed an intense double band in the pH range of 5.1–5.2 (Fig. 3). This value is in good agreement with the calculated isoelectric point of 5.22, based on the amino acid sequence. Two additional protein bands with more basic pI values (5.55 and 5.60) were also observed in the r*Bet v I* preparation, which closely coincided with protein bands seen in the n*Bet v I*. Four or five additional protein bands in n*Bet v I* had no correspondents in the r*Bet v I* preparation.

Laser light scattering measurements of r*Bet v I* (0.5 mg/ml) revealed that the main fraction in solution represents monomers with a hydrodynamic radius of 2.2 nm (data not shown). Larger particles were also detected in the volume distribution, but, as judged by their size, they seem to be due to the presence of non-protein contaminants rather than to larger aggregates of *Bet v I*. From this analysis, we concluded that after purification *Bet v I* exists mainly in a monomeric form and/or in small aggregates.

Immunological Properties—It remained to be established whether the r*Bet v I* and r*Bet v I* purified from *E. coli* share immunological properties with *Bet v I* isolated from birch pollen. Two monoclonal antibodies BIP 1 and BIP 4 (Jarolim *et al.*, 1989b) raised against n*Bet v I* showed strong reactivity to both r*Bet v I* and r*Bet v I*, as illustrated in Fig. 1C.

Immunoblot experiments using a serum pool derived from birch pollen allergic patients revealed strong IgE binding to n*Bet v I* as well as to r*Bet v I* and r*Bet v I* (Fig. 1B, lanes 1–3, respectively). No signals were obtained when the primary

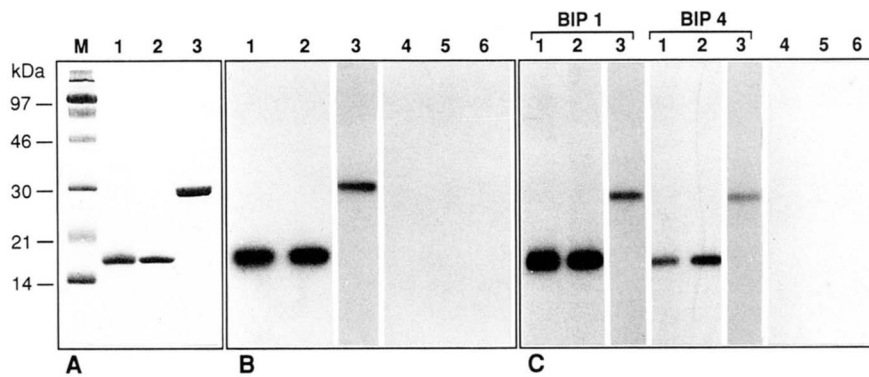


FIG. 1. Analysis of purified natural and recombinant *Bet v I* proteins by SDS-PAGE and immunoblotting. A, Coomassie-stained gel: lane M, molecular mass markers; lane 1, *nBet v I* purified by immunoaffinity chromatography and reversed-phase HPLC; lanes 2 and lane 3, *rnfBet v I* and *rfBet v I*, respectively, purified by chromatofocusing and reversed-phase HPLC. For immunoblot analysis, purified proteins were electrophoresed and blotted onto nitrocellulose: lanes 1 and 4, *nBet v I*; lanes 2 and 5, *rnfBet v I*; lanes 3 and 6, *rfBet v I*. B, blotted proteins were probed with a serum pool from birch pollen-allergic patients (lanes 1–3) or with NHS (lanes 4–6). Bound IgE was detected with ^{125} I-rabbit anti-human IgE. (C), blotted proteins were probed with monoclonal anti-*Bet v I* antibodies, BIP 1 and BIP 4 (lanes 1–3). Bound antibodies were detected with rabbit anti-mouse IgG followed by ^{125} I-donkey anti-rabbit IgG. In lanes 4–6, the primary antibody was omitted from the reaction procedure.

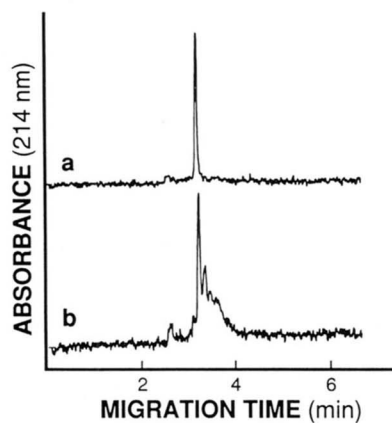


FIG. 2. Analysis of purified *rnfBet v I* (a) and *nBet v I* (b) by high performance capillary electrophoresis. Experimental details are described under "Experimental Procedures."

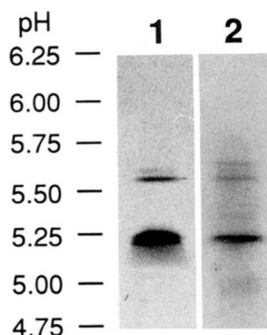


FIG. 3. Analytical isoelectric focusing of *Bet v I* proteins. Purified proteins were analyzed on immobilized pH gradient between 4.0–7.0. Lane 1, *rnfBet v I*; lane 2, *nBet v I*. β -Lactoglobulin A (pI 5.2), and bovine carbonic anhydrase B (pI 5.85) were used as isoelectric point markers (not shown).

antiserum was replaced by NHS (lanes 4–6), demonstrating the specificity of the antibody reaction, or when the primary antiserum was omitted from the reaction procedure (data not shown). Furthermore, IgE binding to purified *rnfBet v I* and to *nBet v I* was quantitatively evaluated in ELISA-titration experiments. At any evaluated protein concentration or serum dilution, *nBet v I* and *rnfBet v I* showed identical IgE-binding

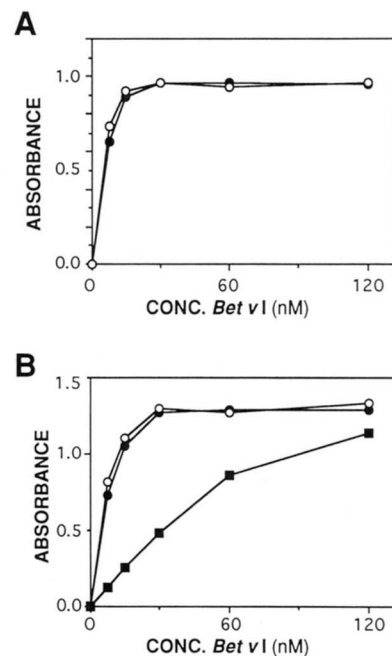


FIG. 4. Quantitative analysis of human IgE binding to recombinant *Bet v I* proteins. A, serial concentrations of purified *rnfBet v I* (○) and *nBet v I* (●) were tested in an ELISA assay for reactivity with birch pollen-allergic patients' IgE. To test whether denaturing and renaturing had any influence on the behavior of the protein, *rnfBet v I* was denatured in 8 M urea and renatured by purification on reversed-phase HPLC. B, serial concentrations of purified *rnfBet v I* (○), *rfBet v I* (■), and *nBet v I* (●) were tested in an ELISA assay for reactivity with birch pollen-allergic patients' IgE. All three protein preparations were purified as described under "Experimental Procedures."

capacity (Fig. 4A). This indicates that the conformations of the two proteins are equivalent, even after denaturing and renaturing *rnfBet v I*, as described in the legend. Interestingly, equimolar amounts (doubled protein amounts) of *rfBet v I* showed decreased IgE reactivity in all cases (Fig. 4B). To achieve comparable IgE-binding levels, a 4-fold concentration of the fusion protein had to be used. On the other hand, *nBet v I* and *rnfBet v I* had the same inhibitory effect on IgE binding to *nBet v I* as shown by ELISA assays (Fig. 5). Amounts as low as 1 μ g/ml serum for *nBet v I* and 0.6 μ g/ml

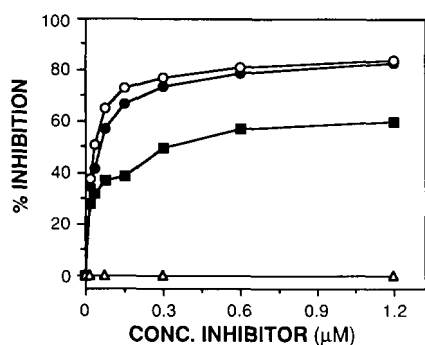


FIG. 5. Inhibition of IgE binding to purified nBet v I. A serum pool from birch pollen-allergic patients was absorbed with serial concentrations of purified rnfBet v I (○), rfBet v I (■), and nBet v I (●) and subsequently tested in an ELISA assay for reactivity with nBet v I. Cat dander allergen (Δ) was used as control. The values of percentage of inhibition were calculated as following: $[1 - (\text{OD in the presence of inhibitor} / \text{OD in the absence of inhibitor})] \times 100$.

TABLE I

Proliferative responses of human Bet v I-specific T-cell clones to natural and recombinant Bet v I presented by autologous peripheral mononuclear cells

Values are given in counts/min $\times 10^{-3}$.

	Clones								
	AS16	AS27	BE15	DF16	DF17	FS3	HC5	SS10	MB20
Control ^a	0.18	0.12	0.14	0.16	0.20	0.05	0.06	0.70	0.09
nBet v I ^b	5.02	5.70	8.51	51.60	36.40	3.10	69.30	133.70	15.30
rnfBet v I ^b	4.99	4.50	10.60	50.70	25.10	1.50	68.10	80.60	0.11
rfBet v I ^b	4.18	7.10	ND ^c	52.50	ND	1.40	61.20	83.80	0.08

^a T-cell clone + irradiated autologous peripheral mononuclear cells.

^b T-cell clone + irradiated autologous peripheral mononuclear cells + antigen.

^c ND, not determined.

for rnfBet v I resulted in 50% inhibition of IgE binding to nBet v I. Equivalent amounts of rfBet v I led only to 30% inhibition. The IgE binding activity of the purified proteins was also evaluated in a standard radioimmunoassay (RAST), which is routinely employed for *in vitro* allergy diagnosis. Comparable IgE binding was obtained for rnfBet v I- and nBet v I-coated paper discs and crude birch pollen extract-coated paper discs (values representing means from triplicate experiments were 6.5 , 5.7 , and 7.3×10^3 counts/min, respectively).

Finally, the different allergen preparations were analyzed for their ability to (i) induce *in vitro* proliferation of T-cells from birch pollen-allergic patients and (ii) to release histamine from allergic patients' blood basophils, via cross-linking of specific IgE bound to Fc_ε receptors. Nine Bet v I-specific T-cell clones established from the peripheral blood of seven birch pollen-allergic donors (Ebner *et al.*, 1993) were tested. In eight of nine clones, stimulation with rnfBet v I or rfBet v I led to strong proliferation of the cells. In this case, no significant differences could be observed in the ability of the purified natural and recombinant Bet v I to activate allergen-specific T-cell clones. One clone (MB20) did not react with either rnfBet v I or rfBet v I proteins, although strong proliferative response was obtained using nBet v I. These results are summarized in Table I. nBet v I and rnfBet v I induced IgE-mediated histamine release from birch pollen-allergic patients' blood basophils. As shown in Fig. 6A, this effect was dose dependent and comparable for both proteins. Blood basophils isolated from non-allergic individuals did not re-

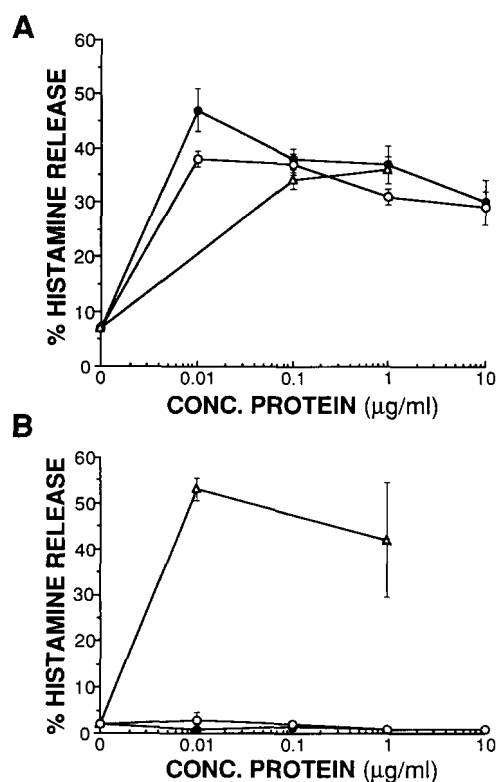


FIG. 6. *In vitro* histamine release assay with purified natural and recombinant Bet v I. A, blood basophils from a birch pollen-allergic patient were incubated with various concentrations of rnfBet v I (○), nBet v I (●), and monoclonal anti-IgE E-124-28 (Δ). B, for control experiments, basophils from a non-allergic individual were treated as in A. Results of histamine release are expressed as percentage of total histamine and each value represents the mean of triplicate determinations.

lease histamine upon stimulation with the purified allergens (Fig. 6B).

DISCUSSION

Recently, several reports included data on heterologous expression of different allergens (Yang *et al.*, 1991; Greene *et al.*, 1991; Bond *et al.*, 1991; Olsen and Mohapatra, 1992). However, in most cases these recombinant allergen products were truncated forms and/or insoluble fusion proteins that were not further characterized. In the present study, we report on the expression, purification, and characterization of two recombinant forms of Bet v I, the major birch pollen allergen: an insoluble fusion form and a soluble non-fusion protein.

The recombinant fusion construct pEX60 produced between 5 and 6 mg of fusion Bet v I protein/liter, but the poor solubility of the fusion product in aqueous buffers made this system less attractive, despite the advantage of high level expression. In addition, site-specific cleavage of the purified MS2-polymerase/Bet v I fusion protein with the protease Factor Xa was not successful, which until now limits the further use of this recombinant product for functional studies or clinical tests. This result is not entirely surprising, since the cleavage efficiency of this enzyme is influenced not only by the amino acid residues adjacent to the recognition sequence but also by the three-dimensional structure in the fusion protein (Uhlén *et al.*, 1992).

The bacterial system used for the expression of the non-fusion form produced between 200 and 300 μg/l of Bet v I. This protein (rnfBet v I) had the same amino terminus as the natural allergen. The efficient removal of methionine from

the *Bet v I* amino terminus in *E. coli* is consistent with the described substrate specificity of prokaryotic methionyl aminopeptidases (Miller *et al.*, 1987; Ben-Bassat *et al.*, 1987; Hirel *et al.*, 1989). These proteases show absolute specificity for the amino-terminal methionine, and their catalytic efficiency is significantly influenced by the structure of the second amino acid residue in the polypeptide chain. Adjacent residues having radii of gyration of 1.29 Å or less (*e.g.* glycine, alanine, threonine, and proline) are preferred. The penultimate amino acid residue in the *Bet v I* molecule is a glycine. According to the *N*-end rule (Bachmair *et al.*, 1986), the amino-terminal processing of *Bet v I* and consequent exposure of a glycine residue at its amino terminus may also prevent rapid degradation of the protein.

Although the *rnfBet v I* preparation appeared to be homogeneous, as judged by SDS-PAGE and zonal capillary electrophoresis, some microheterogeneity was detected by isoelectric focusing. This charge heterogeneity cannot be attributed to glycosylation, as proteins synthesized in *E. coli* are usually not glycosylated. A similar effect was observed with *nBet v I*. Although a single consensus site for *N*-glycosylation is present in the *Bet v I* sequence (Breiteneder *et al.*, 1989), mass spectrometry analysis of *Bet v I* isolated from pollen conclusively showed that the protein is not glycosylated (Jilek *et al.*, 1993); thus, glycosylation does not contribute to the observed microheterogeneity. Deamidation of asparagine or glutamine residues is a commonly observed form of post-translational protein modification and could be one explanation, at least for the component with experimental pI values slightly lower than the theoretical pI value. This is a phenomenon which was previously observed for recombinant hirudin and recombinant variants of human α_1 -antitrypsin (Bischoff *et al.*, 1992). Studies using synthetic peptides proposed that asparagine residues which occur in the sequence Asn-Gly are particularly susceptible to deamidation (Geiger and Clarke, 1987; Lura and Schirch, 1988). In this case, Asn⁴⁷ in the *Bet v I* sequence might be a potential deamidation site. However, mass spectrometry analysis of proteolytic fragments from *rnfBet v I* confirmed the entire sequence and its integrity. In addition, no postsynthetic modifications were found, except the NH₂-terminal cleavage of methionine (Jilek *et al.*, 1993). Modification via deamidation is therefore very unlikely and the structural basis for the reported observations is still unclear. It is possible that the different protein bands seen in the isoelectric focusing represent different native forms in solution attributable to the same chemical species. Although it is generally accepted that globular proteins in their native state assume a single conformation, several reports showed that some proteins may exhibit multiple stable conformations. Using multidimensional NMR methods, Fox *et al.* (1986) showed that two distinct folded conformations of recombinant staphylococcal nuclease coexist in solution. NMR analysis of a nuclease mutant (Pro¹¹⁷ → Gly) obtained by site-directed mutagenesis, showed that *cis/trans* isomerism at a single peptide bond between the proline residue and its preceding residue was the origin of multiplicity of conformational states (Evans *et al.*, 1987). In preparations of *nBet v I*, isoforms (Jilek *et al.*, 1993) may contribute additionally to the heterogeneity detected by capillary electrophoresis and isoelectric focusing.

In the present study we have used different techniques (immunoblotting, RAST, and ELISA) to confirm the immunological integrity of purified recombinant *Bet v I*. In immunoblotting experiments, comparable monoclonal and IgE binding properties were observed for *nBet v I*, *rfBet v I*, and *rnfBet v I* proteins, indicating that the immunogenic/all-

genic epitopes were well preserved during the purification steps. However, the highly sensitive ELISA assay revealed significant decrease in the IgE binding activity of *rfBet v I* compared to *nBet v I* and *rnfBet v I*. In fact, patients' sera preabsorbed with *rfBet v I* retained considerable reactivity to *nBet v I*, suggesting that the IgE-binding sites are not fully accessible on the fusion protein (Fig. 5). From the results shown in Fig. 4A, we conclude that denaturation with 8 M urea and renaturation does not change the IgE binding properties of *rnfBet v I* in quantitative assays. Therefore, the decreased IgE binding ability of *rfBet v I* (Fig. 4B) cannot be attributed to the fact that *rfBet v I* had to be treated with 8 M urea and renatured during its purification. Rather, we think that the difference in IgE binding activity must be caused by the fusion part of the protein.

Although there is no precise information about IgE binding structure(s) on the *Bet v I* molecule, two lines of evidence suggest that functional determinants are present only if they can form from the total sequence. First, when screening a birch pollen cDNA library with patients' IgE, only complete clones of *Bet v I* were reactive (Breiteneder *et al.*, 1989). NH₂ and COOH terminally truncated forms of *Bet v I* expressed in *E. coli* had no IgE binding activity.² Second, fragments obtained by chemical or enzymatic treatment of *nBet v I* were all not able to bind patients' IgE (Ipsen and Hansen, 1989).³ Similar findings have been described for *Der p II* of house dust mite (Lombardero *et al.*, 1990; Chua *et al.*, 1991) and *Amb a I* of ragweed (Olsen and Klapper, 1986). These observations again point to the fact that the decreased IgE binding activity of the *rfBet v I* might result from conformational changes due to the fusion tail.

To demonstrate the presence of T-cell epitopes on the recombinant *Bet v I* proteins, we utilized *Bet v I*-specific T-cell clones. These clones were established from the peripheral blood of birch pollen-allergic patients and were shown to recognize distinct epitopes, which are scattered over the whole *Bet v I* molecule (Ebner *et al.*, 1993). The epitope recognized by one of the clones used in that study (MB20) was not identified, as the clone did not react with any of the 75 dodecapeptides synthesized according to the *Bet v I* sequence, which were used for the T-cell epitope mapping procedure. In the present study, this particular clone also failed to react with both recombinant forms of *Bet v I*, but clearly, the stimulating sequence is present in the mixture of isoforms represented by *nBet v I*. It is possible that a crucial amino acid change within the T-cell epitope (Suhbier *et al.*, 1991) precludes reactivity toward one of the *Bet v I* isoforms, in this case the isoform represented by recombinant *Bet v I*. Interestingly, serum from the same donor (MB) showed strong IgE reactivity with natural as well as with recombinant *Bet v I*. Notwithstanding the decreased IgE binding activity of the *rfBet v I* compared to *nBet v I* and *rnfBet v I*, no significant differences in the ability to activate the other allergen-specific T-cell clones were observed among the three protein preparations. This might be explained by the fact that T-lymphocytes do not recognize intact antigens, but rather fragments of proteins which are generated by antigen-presenting cells after intracellular processing (Schwartz, 1985; Inanue and Allen, 1987). The fusion part of the *rfBet v I* does not seem to influence the processing of the protein in the endocytic pathway, and consequently, binding of the T-cell receptor to the complex formed by the peptide and the major histocompatibility complex class II was not affected.

² R. Valenta, D. Kraft, and O. Scheiner, unpublished observations.

³ M. Tejkl, A. Jilek, F. Ferreira, R. Steiner, D. Kraft, and O. Scheiner, unpublished observations.

Blood basophils and mast cells play a major role in the pathogenesis of IgE-dependent allergic reactions. They store inflammatory mediators (histamine and others) which are released upon activation of the cells by allergens interacting with specific IgE bound to the cell surface via Fc ϵ RI (Kaplan, 1985). Therefore, the process of characterization of allergens should comprise not only assays for their ability to bind patients' IgE but also specific tests for their functional properties leading to allergic effector mechanisms. *In vitro* allergen-specific histamine release tests with patients' basophils constitute a suitable assay system for the latter and, in addition, provides information on the *in vivo* situation of allergic patients. Our results on the *in vitro* histamine-releasing activity of the purified rnfBet v I further demonstrate its functional allergenic properties.

In conclusion, our studies revealed no significant differences between recombinant non-fusion Bet v I and natural Bet v I with respect to IgE binding properties, reactivity with allergen-specific T-cell clones, and histamine release from allergic patients' blood basophils. The results obtained using a recombinant fusion Bet v I molecule indicate that recombinant allergens expressed as fusion proteins might not have the native conformation required for full IgE binding activity. The highly purified recombinant non-fusion allergen could be used for structure determination by x-ray crystallography, which would help in the characterization of epitopes recognized by B-cells.

Acknowledgments—We thank P. Briza for invaluable help and advice concerning HPLC and other protein purification methods as well as for critically reading the manuscript, N. Maurer and O. Glatter (Universität Graz) for the laser light scattering measurements, R. Lunzer and E. Wasserbauer (Sandoz-Forschungsinstitut) for performing capillary electrophoresis analysis, H. Ahorn (Ernst Böhlinger Forschungsinstitut) for performing NH₂-terminal sequence analysis, and L. Koller for critically reading the manuscript.

REFERENCES

- Bachmair, A., Finley, D., and Varshavsky, A. (1986) *Science* **234**, 179–186
- Ben-Bassat, A., Bauer, K., Chang, S. Y., Myambo, K., Boosman, A., and Chang, S. (1987) *J. Bacteriol.* **169**, 751–757
- Bischoff, R., Roecklin, D., and Roitsch, C. (1992) *Electrophoresis* **13**, 214–219
- Bond, J. F., Garman, R. D., Keating, K. M., Briner, T. J., Rafnar, T., Klapper, D. G., and Rogers, B. L. (1991) *J. Immunol.* **146**, 3380–3385
- Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O., and Breitenbach, M. (1989) *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) *J. Allergy Clin. Immunol.* **90**, 909–917
- Breiteneder, H., Ferreira, F., Hoffmann-Sommergruber, K., Ebner, C., Breitenbach, M., Rumpold, H., Kraft, D., and Scheiner, O. (1993) *Eur. J. Biochem.* **212**, 355–362
- Ceska, M., Eriksson, R., and Varga, J. M. (1972) *J. Allergy Clin. Immunol.* **49**, 1–9
- Chua, K. Y., Greene, W. K., Kehal, P., and Thomas, W. R. (1991) *Clin. Exp. Allergy (Cph.)* **21**, 161–166
- Ebner, C., Szépfalusi, Z., Ferreira, F., Jilek, A., Valenta, R., Parronchi, P., Maggi, E., Romagnani, S., Scheiner, O., and Kraft, D. (1993) *J. Immunol.* **150**, 1047–1054
- Eriksson, N. E., Wihl, J. A., Arrandal, H., and Strandhede, S. O. (1987) *Allergy (Cph.)* **42**, 205–214
- Evans, P. A., Dobson, C. M., Kautz, R. A., Hatfull, G., and Fox, R. O. (1987) *Nature* **329**, 266–268
- Fox, R. O., Evans, P. A., and Dobson, C. M. (1986) *Nature* **320**, 192–194
- Geiger, T., and Clarke, S. (1987) *J. Biol. Chem.* **262**, 785–794
- Glatter, O., Sieberer, J., and Schnablegger, H. (1991) *Part. Part. Syst. Charac.* **8**, 274–281
- Greene, W. K., Cyster, J. G., Chua, K. Y., O'Brien, R. M., and Thomas, W. R. (1991) *J. Immunol.* **147**, 3768–3773
- Hirel, P., Schmitter, M., Dessen, P., Fayat, G., and Blanquet, S. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 8247–8251
- Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W. R., Valent, P., Rohac, M., Rumpold, H., Scheiner, O., and Kraft, D. (1992) *J. Allergy Clin. Immunol.* **90**, 927–936
- Inanue, E. R., and Allen, P. M. (1987) *Science* **236**, 551–557
- Ipsen, H., and Hansen, O. C. (1989) in *Epitopes of Atopic Allergens* (Sehon, A. H., Kraft, D., and Kunkel, G., eds) pp. 3–8, Union Chimique Belge Institute of Allergy, Brussels, Belgium
- Ipsen, H., and Loewenstein, H. (1983) *J. Allergy Clin. Immunol.* **72**, 150–159
- Ishizaki, L., Koizumi, R., Ikemori, R. (1987) *Ann. Allergy* **58**, 265–270
- Jacobs, S. (1959) *Nature* **183**, 262
- Jarolim, E., Rumpold, H., Endler, A. T., Ebner, H., Breitenbach, M., Scheiner, O., and Kraft, D. (1989a) *Allergy (Cph.)* **44**, 385–395
- Jarolim, E., Tejkl, M., Rohac, M., Schlerka, G., Scheiner, O., Kraft, D., Breitenbach, M., and Rumpold, H. (1989b) *Int. Arch. Allergy Appl. Immunol.* **90**, 54–60
- Jilek, A., Swoboda, I., Breiteneder, H., Fogy, I., Ferreira, F., Schmid, E., Heberle-Bors, E., Scheiner, O., Rumpold, H., Kraft, D., Koller, H. T., and Breitenbach, M. (1993) in *Molecular Biology and Immunology of Allergens* (Kraft, D., and Sehon, A. H., eds) pp. 39–45, CRC Press Inc., Boca Raton, FL
- Kaplan, A. P. (ed) (1985) *Allergy (Cph.)*
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Larsen, J. N., Ströman, P., and Ipsen, H. (1992) *Mol. Immunol.* **29**, 703–711
- Lichtenstein, L. M., and Osler, A. G. (1964) *J. Exp. Med.* **120**, 507–530
- Lombardero, M., Heymann, P. W., Platts-Mills, T. A. E., Fox, J. W., and Chapman, M. D. (1990) *J. Immunol.* **144**, 1353–1360
- Lura, R., and Schirch, V. (1988) *Biochemistry* **27**, 7671–7677
- Miller, C. G., Strauch, K. L., Kukral, A., Miller, J., Wingfield, P., Mazzei, G., Werlen, R., Graber, P., and Movva, N. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 2718–2722
- Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, W. (1988) *Electrophoresis* **9**, 255–262
- Olsen, J. R., and Klapper, D. G. (1986) *J. Immunol.* **136**, 2109–2115
- Olsen, E., and Mohapatra, S. S. (1992) *Int. Arch. Allergy Appl. Immunol.* **98**, 343–348
- Parronchi, P., Macchia, D., Picchini, M. P., Biswas, P., Simonelli, C., Maggi, E., Ricci, M., Ansari, A. A., and Romagnani, S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 4538–4542
- Rohac, M., Birkner, T., Reimitzer, I., Bohle, B., Steiner, R., Breitenbach, M., Kraft, D., Scheiner, O., Gabl, G., and Rumpold, H. (1991) *Mol. Immunol.* **28**, 897–906
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Scheiner, O., Bohle, B., Breitenbach, M., Breiteneder, H., Duchene, M., Ebner, C., Ferreira, F., Hirschwehr, R., Hoffmann-Sommergruber, K., Pettenburger, K., Rumpold, H., Steiner, R., Tejkl, M., Valenta, R., and Kraft, D. (1992) in *Advances in Allergy and Clinical Immunology* (Godard, P., Bousquet, J., and Michel, F. B., eds) pp. 115–126, Parthenon Publishing Group, Carnforth, United Kingdom
- Schnablegger, H., and Glatter, O. (1991) *Appl. Optics* **30**, 4889–4896
- Schwartz, R. H. (1985) *Annu. Rev. Immunol.* **3**, 237–261
- Strebel, K., Beck, E., Strohmaier, K., and Schaller, H. (1986) *J. Virol.* **57**, 983–991
- Suhrbier, A., Rodda, S. J., Ho, P. C., Csürhes, P., Dunckley, H., Saul, A., Geysen, H. M., Rzepczyk, C. M. (1991) *J. Immunol.* **147**, 2507–2513
- Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
- Uhlén, M., Forsberg, G., Moks, T., Hartmanis, M., and Nilsson, B. (1992) *Curr. Opin. Biotechnol.* **3**, 363–369
- Valent, P., Besemer, J., Muhm, M., Majdic, O., Lechner, K., and Bettelheim, P. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 5542–5546
- Valenta, R., Vrtala, S., Ebner, C., Kraft, D., and Scheiner, O. (1992) *Int. Arch. Allergy Appl. Immunol.* **97**, 287–294
- Vercelli, D., and Geha, R. S. (1989) *J. Clin. Immunol.* **9**, 75–83
- Wüthrich, B. (1991) *Allergy Clin. Immunol. News* **3**, 41–44
- Yang, M., Olsen, E., Dolovich, J., Sehon, A. H., and Mohapatra, S. S. (1991) *J. Allergy Clin. Immunol.* **87**, 1096–1104