

# Identification of Multiple T Cell Epitopes on *Bet v I*, the Major Birch Pollen Allergen, Using Specific T Cell Clones and Overlapping Peptides<sup>1</sup>

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**ABSTRACT.** Eleven T cell clones (TCC) with specificity for *Bet v I* were established from the peripheral blood of six birch pollen allergic donors. *Bet v I* is the major allergen of birch (*Betula verrucosa*) pollen and shows high homology to the major allergens of pollens of other trees within the order *fagales* (hazel, alder, hornbeam, oak, etc.), which represent important inhalant allergens in the northern hemisphere. The TCC were shown to react with purified natural, as well as with purified recombinant *Bet v I*. All clones showed the helper cell phenotype (CD3<sup>+</sup>CD4<sup>+</sup>) and expressed the TCR- $\alpha/\beta$ . The cytokine production pattern in response to stimulation with allergen resulted in enhanced production of IL-4 in 9 of 11 clones. The clones were used for T cell epitope mapping on the *Bet v I* molecule. For this purpose, peptides with a length of 12 amino acids each and overlapping for 10 residues were synthesized following the amino acid sequence of *Bet v I*. These 75 peptides were used to stimulate *Bet v I*-specific T cell clones. Our experiments revealed 7 distinct T cell epitopes on the *Bet v I* molecule. The epitopes were scattered over the whole molecule, 2 sequences were in agreement with an algorithm previously described for the prediction of T cell epitopes. In 3 cases, we could identify distinct TCC specificities within single individuals. Furthermore, for each donor, none of the peptides representing epitopes for TCC inhibited the binding of IgE antibodies to *Bet v I*. These results suggest that T cells and IgE antibodies from the same individual recognize different structures on the *Bet v I* allergen. *Journal of Immunology*, 1993, 150: 1047.

**A**ntibody (thus also IgE) production by B lymphocytes requires the help of Ag (allergen)-specific T lymphocytes. These T cells are activated by highly immunogenic peptides presented to them by MHC class II expressing APC after intracellular processing (1, 2). In the last few years, a pathway of IgE regulation, essentially based on the reciprocal activities of IL-4 and

IFN- $\gamma$  was described in mice (3–5). Further studies provided information on similar mechanisms regulating human IgE synthesis (6–11).

For further understanding of the pathogenesis of Type I allergic diseases, the knowledge of a possible influence of the molecular structure of allergens and the epitopes on these proteins seems to be essential. Recently research in this field has been concentrating on the identification and characterization of allergenic proteins using immunochemical and molecular biologic techniques. cDNA cloning has provided an as yet limited (but increasing) number of complete deduced amino acid sequences of atopic allergens (12–26). *Bet v I*, a 17-kDa protein representing the major allergen of birch pollen, was recently cloned, sequenced, and expressed in our laboratory (12, 27) and can therefore conveniently be used as a model

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allergen for studying cellular mechanisms leading to the production of specific IgE. More than 96% of all birch tree pollen-allergic patients exhibit IgE to this allergen and 60% react exclusively to this protein component of birch pollen (28). Because the detection of T cell epitopes on allergens could provide us with new aspects for therapy of Type I allergic diseases, we have established *Bet v I* specific TCC<sup>3</sup> from the peripheral blood of birch pollen-allergic patients. In this study we investigate the phenotype and cytokine-secretion pattern of these clones and describe the specificity of TCC for certain sequence segments (i.e., epitopes) on *Bet v I*.

## Materials and Methods

### Patients and patients' sera

PBMC were obtained from six birch pollen-allergic patients. Type I allergy to birch pollen was proved by typical case history, positive skin tests, and positive radioallergen sorbent test. Furthermore, each patients' plasma was tested for its IgE-binding properties by immunoblot analysis (28) and ELISA using purified natural and recombinant *Bet v I*.

### Allergen preparations

Natural *Bet v I* was purified from an aqueous extract of birch (*Betula verrucosa*) pollen (Allergen AB, Engelhom, Sweden) by affinity chromatography using immobilized mAb BIP 1 as described (29). The eluted material was dialyzed against PBS, precipitated by the addition of a fourfold volume acetone, and then centrifuged (Minifuge RF, Hereus, Hanau, Germany; 5000 rpm). The pellet was dried in a desiccator, dissolved in 5 M urea, extensively dialyzed against PBS and, after sterile filtration, used for cell culture. Aliquots were analyzed by SDS-PAGE and immunoblot.

Expression and purification of recombinant nonfusion *Bet v I*: the recombinant nonfusion protein was expressed by using the pKK 223-3 expression vector (30) (Pharmacia, Uppsala, Sweden) the promoter of which is regulated by the *lac* repressor and can be induced by adding of isopropyl- $\beta$ -D-thiogalactoside to the medium. Competent *Escherichia coli* JM105 cells transformed with pKK 223-3 containing the complete *Bet v I* cDNA (12) were streaked on LB/ampicillin plates. Individual colonies were picked and grown overnight. One milliliter of an overnight culture was added to 500 ml of LB medium containing 100  $\mu$ g/ml ampicillin. Cultures were grown until the OD<sub>600</sub> reached 0.4. To induce the synthesis of the recombinant nonfusion protein, isopropyl- $\beta$ -D-thiogalactoside was then added to a final concentration of 2.5 mM and the cultures were grown

for 3.5 h at 37°C. The cells of a 500-ml culture of *E. coli* were collected by centrifugation and resuspended in 15 to 20 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 220 mM NaCl. The cells were broken by freezing in liquid nitrogen and thawing at 37°C. This procedure was repeated twice. After centrifugation at 30,000  $\times g$  for 25 min, the SN was dialyzed with four changes of 25-mM imidazole-HCl buffer, pH 7.4. The crude cell extract was introduced into a PBE-94 exchanger column (Pharmacia) at RT. The column was washed with 25 mM imidazole-HCl buffer (pH 7.4) and the protein eluted with 12.5% (v/v) polybuffer 74 titrated to pH 4.0 with HCl. The pooled *Bet v I* fractions from the chromatofocusing step were subjected to HPLC employing a linear gradient of 2-propanol created within 50 min at RT (C8, reverse phase column; solvent A: 0.1% trifluoroacetic acid in water; solvent B; 90% 2-propanol/0.1% trifluoroacetic acid; gradient of 0 to 60% solvent B; flow rate 1.0 ml/min). Subsequently, the protein concentration of the purified sample was determined by the micro-Kjeldahl method using glycine as standard (31). The allergen concentrations applied in proliferation assays were 5  $\mu$ g/ml (1  $\mu$ g/well) for natural and recombinant *Bet v I*. IgE-immunoblots were performed as described previously (28).

### ELISA

An ELISA system was used to investigate *Bet v I*-specific IgE titers of our group of patients. Microtiter plates (M129A, Dynatech MT, Zug, Switzerland) were coated with the anti-*Bet v I* mAb BIP 1 (29) as capture antibody (20  $\mu$ g/ml in PBS) overnight at 4°C. After washing three times with PBS/0.05% Tween 20, the plates were saturated with PBS/0.05% Tween 20/1% human serum albumin at RT for 3 h. Thereafter, 1  $\mu$ g/ml of purified native *Bet v I* in PBS was filled into the coated wells and incubated for 3 h at RT and 1 h at 4°C. After five washings with PBS/Tween 0.05%, patients' plasmas were incubated in five dilutions ranging from 1/4 to 1/64 in PBS/0.05% Tween 20/0.5% human serum albumin overnight at 4°C. The washing procedure was repeated and 100  $\mu$ l of a horse anti-human IgE conjugated with alkaline phosphatase (Allercoat EAST; Kallestad, Chaska, MN) diluted 1/2 in the above mentioned buffer was filled into the wells as detection antibody and remained overnight at RT. After washing, the substrate *para*-nitrophenylphosphate was applied in a concentration of 1 mg/ml in diethanolamine buffer, pH 9.8. The reaction was stopped after a 2-h incubation period at 37°C with 1 M tribasic potassium phosphate. Subsequently, the OD was measured at 405 nm, using the wavelength of 550 nm as reference value. The experiment was also performed using uncoated plates, using coated plates without addition of *Bet v I* and with BIP1-coated plates plus *Bet v I* and a normal human serum pool or

<sup>3</sup> Abbreviations used in this paper: TCC, T cell clone; SN, supernatant; RT, room temperature; IT, immunotherapy.

buffer. Furthermore, a possible reactivity of the anti-IgE antibody with the mAb BIP1 or with any of the peptides was excluded.

#### Allergen-specific TCC

Allergen-specific TCC were obtained by a technique described (32). Briefly,  $0.5 \times 10^6$  PBMC were stimulated with individual optimal *Bet v I* concentrations (5 to 20  $\mu\text{g/ml}$ , determined by titration in an proliferation assay using PBMC) in Aim V medium (GIBCO, Grand Island, NY) supplemented with 2 mM/liter glutamine and  $2 \times 10^{-5}$  M 2-ME in 24-well, flat bottomed culture plates (3524 Costar, Cambridge, MA) for 5 days. Subsequently, human rIL-2 (20 U/ml, Genzyme, Cambridge, MA) was added and the culture continued for an additional 7 days. Viable T cell blasts were then separated by Ficoll/Hypaque (Pharmacia), and *Bet v I* specificity was assessed by a [ $^3\text{H}$ ]TdR (Amersham, Amersham, UK) incorporation assay. Thereafter, T cell blasts were seeded in limiting dilution (0.3 cells/well) in 96-well, round bottom plates (Nunc, Nunc, Denmark) in the presence of  $10^5$  irradiated (5000-rad) allogeneic PBMC as "feeder cells," 1% PHA v/v (GIBCO) and rIL-2 (4 U/well) in the above described medium. Growing microcultures were then expanded at weekly intervals with fresh feeder cells and rIL-2. The specificity of TCC was assessed as previously described (32). Briefly,  $2 \times 10^4$  T cell blasts were incubated in triplicate cultures in 96-well plates in the presence of  $10^5$  autologous PBMC, purified *Bet v I* and whole birch pollen extract. Microtiter plates were then incubated for 48 h at 37°C, 5% CO<sub>2</sub>, humidified atmosphere. After pulsing for 16 h with 0.5  $\mu\text{Ci/well}$  of [ $^3\text{H}$ ]TdR, cells were harvested and the incorporated radioactivity was measured by scintillation counting. When the stimulation index (ratio between cpm obtained in cultures with TCC plus autologous irradiated PBMC plus Ag and cpm obtained in cultures containing TCC and PBMC alone) was greater than 10, responses were considered as positive.

#### Analysis of the phenotype of TCC

The FITC-labeled mAb anti-Leu-4/CD3, anti-Leu-3a/CD4, anti-Leu-2a/CD8, anti-T cell Ag Receptor TCR-1  $\alpha/\beta$  WT31 and anti-TCR- $\gamma/\delta$  I (Becton Dickinson, Mountain View, CA) were used to analyze the phenotype of the TCC. TCC and irradiated feeder cells alone were harvested and washed and  $10^5$  cells were incubated for 30 min on ice with the appropriate dilution of mAb or isotype-matched control antibodies. After washing three times with PBS containing 0.3% BSA and 0.3% NaN<sub>3</sub>, the cells were stained with propidium iodide to allow exclusion of dead cells and analyzed on a FACScan (Becton Dickinson).

#### Stimulation of specific TCC for cytokine production

A total of  $10^6$  clone cells were incubated with  $10^6$  irradiated autologous APC in the presence of *Bet v I* (5  $\mu\text{g/ml}$ ). SN from the allergen-stimulated cultures were collected after 72 h of incubation. Cytokine assays: IL-4 and IFN- $\gamma$  levels in the SN were measured using sandwich ELISA techniques provided by Genzyme, Boston, MA (IFN- $\gamma$ ) and Quantikine R&D Systems, Minneapolis, MN (IL-4). IL-2 was determined in a solid-phase ELISA obtained from Medgenix Diagnostix, Fleurus, The Netherlands. Recombinant and natural cytokine standards were used as references. SN collected from cultures containing allergen and irradiated feeder cells alone were used as controls.

#### Overlapping peptides

A panel of 75 peptides was synthesized according to the *Bet v I* sequence by Cambridge Research Biochemicals Limited, Cambridge, UK, using the pin synthesis method (33). The dodecapeptides were overlapping for 10 amino acids. For our proliferation assays, a concentration of 1  $\mu\text{g/well}$  was used.

#### Inhibition experiments

Plasma samples diluted 1/8 in PBS were incubated overnight with 40  $\mu\text{g/ml}$  of the peptide which induced proliferation in the clones derived from the respective patient. Thereafter, *Bet v I*-specific IgE was determined by ELISA as described above. The titer was compared with a control sample in the same dilution, which was not preincubated with peptide.

## Results

#### IgE-immunoblots

All patients exhibited IgE-binding to *Bet v I* (Fig. 1). Specific IgE bound with comparable strength to both natural and recombinant *Bet v I*. ELISA assays revealed anti-*Bet v I* IgE-titers in the range of 4 to 32.

#### Flow cytometry analyses

FACS analysis revealed the helper cell phenotype (CD3<sup>+</sup> CD4<sup>+</sup> CD8<sup>-</sup>) for all clones. The TCR could be identified in all cases as TCR- $\alpha/\beta$ .

#### Specificity of TCC

Eleven TCC could be identified as specific for *Bet v I*; stimulation with natural as well as with recombinant *Bet v I* led to strong proliferation. The clones and the respective counts produced by [ $^3\text{H}$ ]TdR incorporation in response to stimulation with natural and recombinant *Bet v I* are shown in Table I.

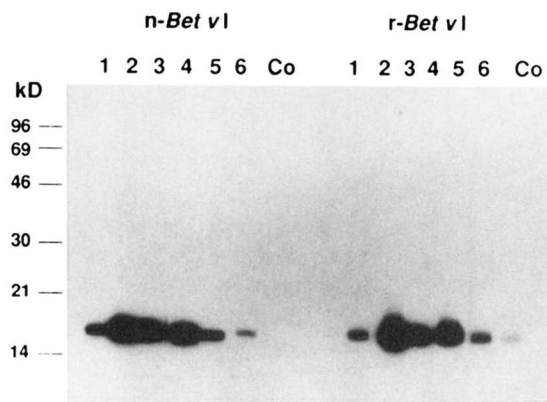


**FIGURE 1.** Western blot experiment: The reactivity of patients' IgE with natural and recombinant *Bet v I* is compared. Numbers 1–6: patients plasma: AS, HC, FS, SS, BE, DF. n-*Bet v I*: purified natural *Bet v I*, r-*Bet v I*: purified recombinant *Bet v I*. Control: A serum pool of 13 non-allergic donors.

#### Cytokine pattern

In response to stimulation of the TCC with allergen 9/11 clones secreted detectable amounts of IL-4 into the culture SN, some of them high amounts (BE15, DF17, HC5, HC26, SS6). IFN- $\gamma$  levels were low in all cases, in 4/11 TCC SN not detectable (sensitivity threshold: 1 U/ml). Seven of 11 clones produced IL-2, 5 of 11 TCC produced levels of IL-2 lower than 1 U/ml. Clones BE15 and SS10 produced huge amounts of IL-4 and high levels of IL-2 in response to allergen stimulation. According to their cytokine production pattern, two clones (FS3 and FS5) could be classified as Th-1-like TCC. The results of the TCC stimulation are summarized in Table II.

#### Epitope mapping

Stimulation of the 11 *Bet v I*-specific TCC with the overlapping peptides revealed seven T cell epitopes. TCC FS3, SS6, and SS10 reacted with three neighboring peptides, a sequence near the N terminus (TTSVIPAARLFK, SVIP AARLFKAF, IPAARLFKAFIL). One sequence of peptides included an epitope relevant for two clones derived from different donors (BE15, DF 17; amino acids 141–159: ET/LL/RA/VE/SYLLAHSDAYN), this sequence is located at the C terminus of *Bet v I*. The two TCC established from donor AS (AS16, AS27) reacted both with a sequence within amino acids 111–126, the peptides GSILKISNKYHT and ILKISNKYHTKG induced proliferation. The stimulating epitopes for the remaining three TCC were: GF/PF/KY/VKDRVDEVDHTN (DF16; amino acids 61–78), YS/VIEGGPIGDTLE (FS5; 83–96), DH/TN/FKYNYSVIEGGP (HC5; 75–90), and GV/FN/YETETTSVIPAA (HC26; 1–16). The complete sequence of *Bet v I*, and peptides with the ability to stimulate different TCC to proliferate are shown in Figure 2.

#### Inhibition assays

In ELISA inhibition experiments, high concentrations (40  $\mu$ g/ml) of peptides representing T cell epitopes were in no case able to diminish IgE binding to *Bet v I*, suggesting a difference between T cell epitopes, and epitopes that are recognized by IgE antibodies (data not shown).

#### Discussion

In the present study, we isolated *Bet v I*-specific TCC from the peripheral blood of birch pollen-allergic patients. By using purified natural as well as purified recombinant *Bet v I* in proliferation assays, 11 TCC established from 6 donors could be identified as specific for this allergen. *Bet v I*, the major allergen of birch pollen, is an important allergen causing symptoms in tree pollen-allergic individuals in the flowering season in early spring. *Bet v I* is highly homologous and cross-reacts with other tree pollen allergens of the order *fagales* (34) and can thus be regarded as a representative tree pollen allergen. Flow cytometry analysis of the specific TCC reflected the Th phenotype (CD3<sup>+</sup>CD4<sup>+</sup>), all the clones expressed the TCR- $\alpha/\beta$  S. TCC were expanded and subsequently tested for reactivity with 75 dodecapeptides, which were synthesized according to the 159 amino acid sequence of *Bet v I*, and spanned the whole molecule, overlapping by 10 amino acids.

In previous investigations, the descriptions of peptides coding for T cell epitopes have varied greatly. In one case the typical length of peptides binding to MHC II molecules and eliciting maximal T cell proliferation was described as 13–17 amino acids long (35), in another, reactivity was even observed with three amino acid peptides in high concentrations (36). In this study, the T cell epitopes of 11 out of 13 clones could be defined by using dodecapeptides. Two TCC did not react with any of the peptides or with recombinant *Bet v I* despite strong reactivity with natural *Bet v I* (data not shown), suggesting the lack of crucial amino acids or amino acid sequences (recombinant proteins and peptides synthesized according to this sequence represent only one possible isoform of the allergenic protein). Our experiments revealed a variety of peptides with the ability to stimulate distinct *Bet v I*-specific TCC to proliferate: 7 different T cell epitopes were identified for 11 specific TCC (Fig. 2). The peptides were scattered over the molecule, 2 of the epitopes (amino acids 11–22 and 61–72; Fig. 2) matched with a prediction algorithm using motifs in the primary structure of a protein (37). No correlation of epitopes with loci of a high immunogenicity index, as predicted by a method analyzing the secondary structure (38), could be found.

IT, i.e., inducing tolerance to an allergen in an allergic patient by injecting increasing doses of specific allergen,

Table I

Proliferative responses of human *Bet v I*-specific T cell clones to natural *Bet v I*, recombinant *Bet v I*, and the peptide causing maximal proliferation, presented by autologous PBMC

| TCC                            | [ <sup>3</sup> H]TdR Incorporation (cpm × 10 <sup>-3</sup> ) |      |      |      |      |      |      |      |      |      |       |
|--------------------------------|--------------------------------------------------------------|------|------|------|------|------|------|------|------|------|-------|
|                                | AS16                                                         | AS27 | BE15 | DF16 | DF17 | FS3  | FS5  | HC5  | HC26 | SS6  | SS10  |
| Control <sup>a</sup>           | 0.18                                                         | 0.12 | 0.14 | 0.16 | 0.2  | 0.15 | 0.03 | 0.06 | 0.5  | 0.5  | 0.7   |
| n- <i>Bet v I</i> <sup>b</sup> | 5.02                                                         | 5.7  | 8.51 | 51.6 | 36.4 | 19.5 | 5.24 | 69.3 | 11.9 | 9.03 | 133.7 |
| r- <i>Bet v I</i> <sup>b</sup> | 4.99                                                         | 4.5  | 10.6 | 50.7 | 25.1 | 10.5 | 1.43 | 68.1 | 6.5  | 5.77 | 83.8  |
| Epitope <sup>b</sup>           | 2.14                                                         | 2.46 | 10.2 | 48.8 | 53.6 | 4.46 | 1.73 | 34.4 | 47.2 | 30   | 100.7 |

<sup>a</sup> + Irradiated autologous PBMC, no Ag.

<sup>b</sup> + Irradiated autologous PBMC + Ag.

Table II

Cytokine production by human *Bet v I*-specific TCC in response to Ag-specific stimulation

|                           | AS16 | AS27 | BE15 | DF16 | DF17 | FS3  | FS5 | HC5  | HC26 | SS6  | SS10 |
|---------------------------|------|------|------|------|------|------|-----|------|------|------|------|
| IL-4 (pg/ml) <sup>a</sup> | 92   | 53   | 4635 | 46   | 751  | <10  | <10 | 3901 | 1900 | 2600 | 300  |
| IFN-γ (U/ml) <sup>a</sup> | 2.8  | <1   | <1   | 7.1  | <1   | 10.2 | 5.2 | <1   | 1.7  | 4.4  | 4.6  |
| IL-2 (U/ml) <sup>a</sup>  | >10  | <1   | >10  | <1   | <1   | >10  | >10 | 1.5  | 1.1  | 6    | >10  |

<sup>a</sup> One million T cells were stimulated with 5 μg *Bet v I* in 1 ml medium; cytokines were measured in 72-h culture supernatants by specific ELISA.

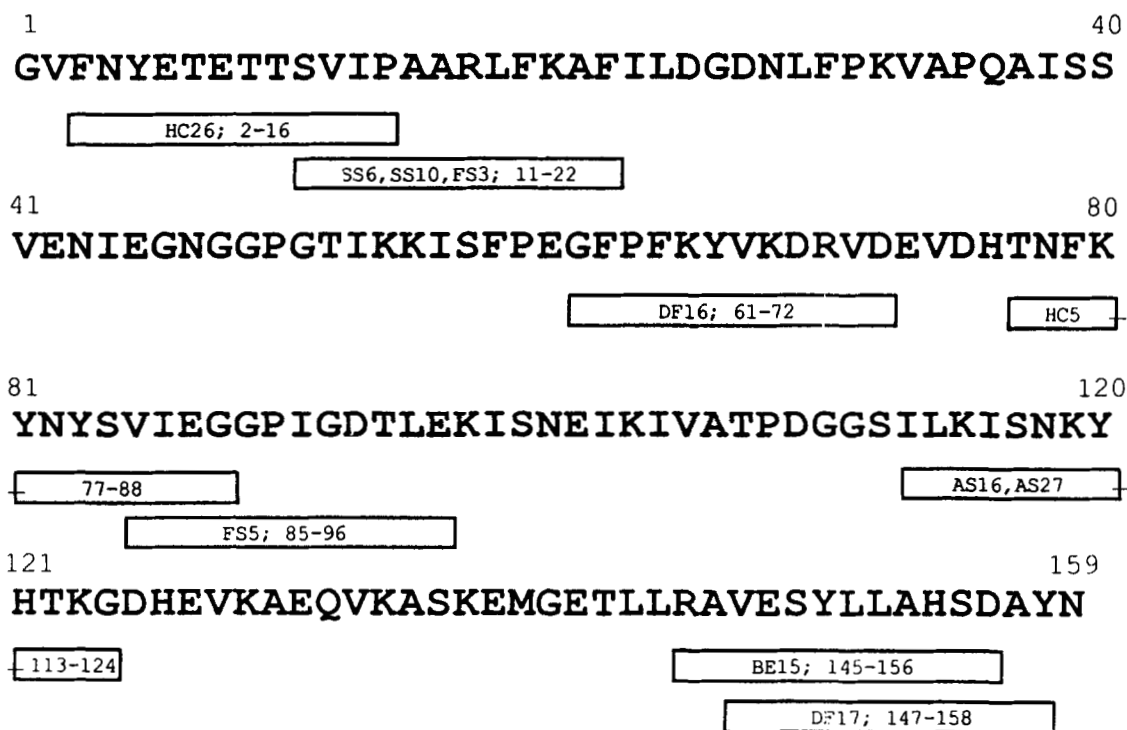


**FIGURE 2.** Schematic diagram of the amino acid sequence of *Bet v I*. The positions of the peptides, causing maximal proliferation in the respective TCC, are indicated by boxes underneath the sequence of *Bet v I*.

was proved to be an effective treatment for patients suffering from Type I allergy resulting from airborne allergens (39, 40). Until now, allergen extracts containing a protein mixture of allergens (pollen, mites, pet danders, molds, etc.) have been used for this treatment in spite of the fact that many patients exhibit IgE only to one single component (28) or to few proteins of this mixture. In these

cases, a therapy with purified—preferably recombinant—proteins or perhaps just fragments of them (peptides representing T cell epitopes) could make this therapy more efficient and improve the prognosis of IT outcome. There is evidence that the epitopes stimulating allergen-specific T cells are distinctly different from epitopes detected by IgE antibodies (41). If such peptides were specific for T

cells, the risk of side effects such as anaphylaxis during IT could be eliminated and higher concentrations of the vaccine could be given. To answer the question of whether this is true for our peptides, inhibition experiments were performed. Patients' plasma derived from the same blood samples from which PBMC for T cell cloning were separated were preincubated with peptides representing T cell epitopes and tested in ELISA for *Bet v* I-specific IgE. In this highly sensitive assay, the peptides representing the T cell epitopes were not able to inhibit IgE binding to *Bet v* I, indicating that they do not represent IgE epitopes.

Different strategies have been proposed to induce tolerance at the T cell level. Approaches using epitopes in high concentrations or modifying the peptides in such a way so that they compete for binding to MHC were described (42–44). Even nonrelated peptides with the ability to bind to particular MHC molecules (45) or TCR gene products (46) have been used to block MHC-Ag-TCR binding and thus to induce anergy (Ag-specific non-responsiveness) in specific T cell clones. However, our finding that the T cell epitopes differ not only between distinct individuals but also within single individuals is striking and diminishes the optimistic hope of treating allergic patients using only a few immunodominant peptides. Other allergens will have to be analyzed for epitopes before definitive statements can be made, but it seems that the most effective individual IT would be the treatment with complete recombinant molecules, which have to be tested for their immunologic properties (27).

Most of the clones investigated produced IL-4, and practically no IFN- $\gamma$  upon stimulation with allergen (Table II) and can thus be made responsible for directing the antibody response toward IgE production (6–11, 47). In our opinion, new insights on the mechanisms of the IgE-mediated diseases could be gained from the comparison of allergic patients with nonatopic individuals. Healthy individuals also show a reactivity to birch pollen Ag (28). This immune response is normally restricted to IgG antibody production. Considering this, more investigations should be done in the future to compare the cytokine production pattern of allergen-specific T cell clones derived from allergic and nonallergic individuals (48) and to examine whether these specific T cells are stimulated by the same epitopes. Such studies could reveal a difference between "immunodominant" and "disease-related" epitope peptides. It is conceivable that particular structures or properties of allergens cause the T cells to evolve into a Th-2-like phenotype. It has already been shown that Th-2-like cells are not defective in producing cytokines characteristic for the Th-1 type, but that other stimuli are required to induce this production (41). The experimental conditions for such investigations should exclude every possible source of error by employing a highly specific system,

using synthesized or recombinant peptides representing epitopes and T—as well as B—cell clones.

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