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The Significance of Isoallergenic Variations in Present and Future Specific Immunotherapy

Key Words

Isoallergens
 Tree pollen
 T cell stimulation
 Bet v 1
 Car b 1

Abstract

The isoallergenic variation of the tree pollen major allergens has been studied by 2D gel electrophoresis, and by analysis of several recombinant clones. The studies have included both antibody-based and T cell stimulation assays. Bet v 1, the major allergen of birch, forms at least 24 spots when conventional extracts are analyzed by 2D gel electrophoresis. Comparison of Bet v 1-encoding DNA sequences reveals a considerable number of amino acid substitutions. This sequence variation can theoretically account for the number of spots observed in 2D gels. Whereas pools of serum from allergic individuals and monospecific antibodies raised in rabbits bind to most if not all spots in 2D gels, analyses of individual serum and/or murine monoclonal antibodies show individual patterns of reactivity with various subsets of spots. These observations point to a model in which amino acid substitutions induce local perturbations of the allergen surface, causing differences in epitope structure. Furthermore, analysis of pollen from individual trees shows that each tree produces individual subsets of Bet v 1 spots. When analyzed in stimulation assays, T cell clones also display differences in reactivity to different isoallergens. In conclusion, we have shown that Bet v 1 is heterogenous, and that individual trees produce various subsets of isoallergens which display differences in reactivity both towards antibodies and T cells. A careful selection of isoform may therefore be of major importance if recombinant allergens or synthetic peptides are to be used for conventional immunotherapy.

Introduction

Most, if not all, pollen allergens form numerous spots when conventional extracts are analysed by 2D gel electrophoresis [1–5]. Variation in both apparent molecular weight and pI is generally noted. Each spot may represent more than one isoallergen, since individual amino acid substitutions may or may not induce changes in pI. The frequency of amino acid substitutions may be as high as 25%, as seen for Amb a 1 [6]. Bet v 1 forms over 20 spots, even though the

sequence variation does not seem to exceed 13% [7]. However, the sequence variation observed is sufficient to theoretically account for the number of spots. Isoallergenic variation has also been described for the mite allergens [8, 9], indicating that the phenomenon may be more general. Since even single amino acid substitutions in some cases have been shown to induce significant effects in epitope structure, we have taken an interest in investigating the epitope structure of isoallergens.

Materials and Methods

Pollen Extracts. Conventional extracts of *Betula verrucosa* and *Carpinus betulus* pollens were prepared as described elsewhere [10]. Pollens from individual birch trees were obtained from catkins collected during the pollination season April–June 1993. The catkins were shaken in sealed containers and extracted [10].

Antibodies. Human serum samples originated from birch-pollen-allergic patients [11]. Rabbit monospecific antibodies against Bet v 1 were raised as described previously [12]. Monoclonal antibodies (mAbs) were raised in BALB/c mice against purified Bet v 1 or Car b 1, and clones were propagated according to their reactivity with the major allergens of selected Fagales species.

2D SDS-PAGE. Isoelectric focusing was performed in immobililine (Pharmacia) pH 4–7 gels and SDS-PAGE [13]. Electroblots of the 2D gels were incubated with various antibodies (human serum, monospecific and monoclonal) and visualized with appropriate secondary antibodies (^{125} I-anti-IgE, alkaline-phosphatase-conjugated antirabbit or antimouse antibodies [10, 13].

Inhibition Assay. Inhibition of the binding of ^{125} I-Bet v 1 to solid-phase- (paramagnetic particles, Biomag US) immobilized mAbs was performed using pollen extracts or soluble bacterial lysates in concentrations corresponding to approximately $5 \times K_d$.

Recombinant Allergens. Genes encoding Bet v 1 or Car b 1 were amplified by PCR using degenerate primers and cDNA made from RNA isolated from pollen [14, 15]. The recombinant allergens were expressed as soluble nonfusion proteins.

T cell Clones. Bet v 1-reactive T cell clones were isolated from a donor clinically allergic to birch pollen. The clones were generated from freshly isolated peripheral blood mononuclear cells (PBMCs) and from a long-term Bet v 1-reactive T cell line using standard limiting dilution techniques in the presence of irradiated autologous Epstein-Barr virus (EBV)-transformed B cells, 10^3 /well, and irradiated allogeneic PBMCs, 10^4 /well, as antigen-presenting cells (APCs), 0.5 μ g/ml of PHA (Sigma), 10% Lymphocult T (Biotest Folex, Germany) 10 μ g/ml Bet v 1 [16]. Growing clones were split when necessary and restimulated every 12 days.

Proliferation Assays. Antigen-specific proliferation was assessed by stimulation of the T cells, 10^4 /well, by serial dilutions of antigen using irradiated autologous EBV-transformed B cells as APCs. The cells were cultured for 4 days, the last 12–18 h in the presence of tritiated methylthymidine, 0.5 μ Ci/well. Finally, the cultures were harvested and specific incorporation expressed as mean cpm of triplicate cultures.

Results and Discussion

When analyzed by 2D gel electrophoresis, a birch pollen extract forms numerous spots. 24 of these represent the Bet v 1 major allergen (fig. 1). When stained with a pool of patients' serum or a monospecific rabbit antibody, most if not all spots may be shown to bind antibody. However, if single serum or murine mAbs are used in immunoblotting, various subsets of spots are stained, indicating that the Bet v 1 isoallergens differ in epitope structure, fig. 2. Analysis by 2D immunoblotting of extracts made from pollen from ten indi-

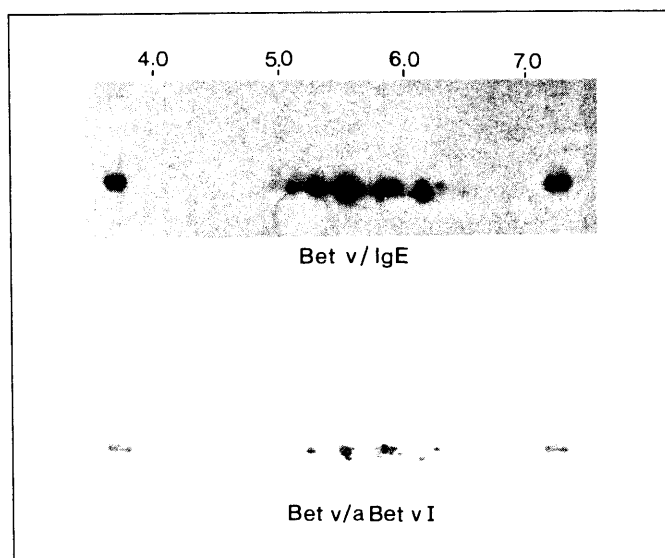


Fig. 1. Sections of 2D immunoblots of birch pollen extract using allergic patients' serum pool ($n = 38$; upper panel) or rabbit monospecific anti-Bet v 1 (lower panel).

vidual birch trees indicates that neither the full spectrum of isoallergens nor just a single isoallergen is present in each tree. From 3 to 6 of the 24 spots have been found to stain with monospecific rabbit antibodies (fig. 2).

Differences in epitope structure between isoallergens are more precisely determined in carefully controlled quantitative assays using homogeneous preparations of recombinant allergens. We have produced recombinant isoallergens of Bet v 1 and Car b 1 and they have been analyzed in a quantitative radioinhibition immunoassay using a panel of mAbs. Differences in epitope structure between subsets of isoallergens are demonstrated (table 1). When using IgE in these assays results are not as clear-cut due to the polyclonal nature of serum IgE. However, the inhibitory capacity of different isoallergens for the binding of IgE from individual serum to solid-phase birch extract appears to differ (data not shown).

As shown in table 1, Car b 1 isoallergens may be divided into two groups on the basis of sequence similarity. None of the recombinant Car b 1 isoallergens react with the mAb BV10, which is specific for Bet v 1, whereas they all react with mAb CB11, originally raised against Car b 1. On the other hand, mAb BV16 defines an epitope which is present on group 2 Car b 1 isoallergens but not on group 1 isoallergens. A closer examination of the amino acid substitutions between groups 1 and 2 reveals that in most of these positions, the group 2 sequences are identical to the sequence of Bet v 1, meaning that group 2 Car b 1 isoallergens

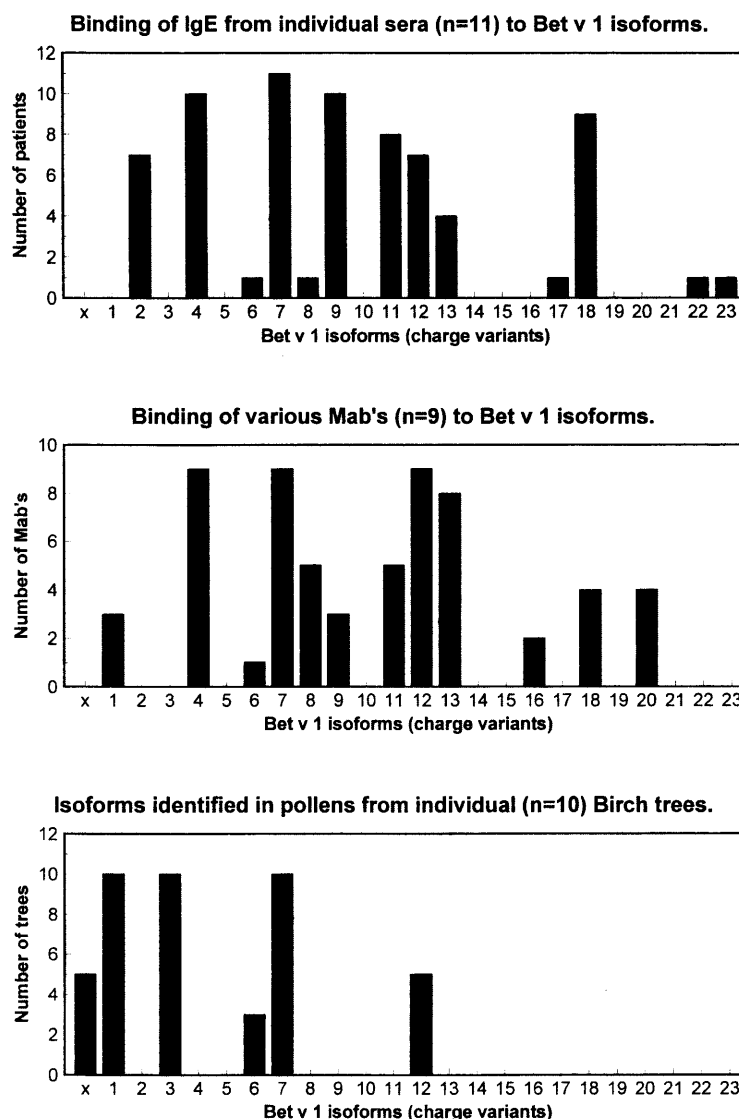


Fig. 2. Summary of 2D immunoblotting experiments. Analysis of individual patients' serum (top panel), individual monoclonal antibodies (middle panel), and extracts from individual trees (lower panel). Spots stained by rabbit monospecific anti-Bet v 1 (fig. 1) were numbered from low to high pI.

exhibit structural similarities with Bet v 1, while group 1 Car b 1 does not. These results support a model in which amino acid substitutions induce local perturbations on the surface of the molecules, leading to differences in epitope structure.

We have analyzed recombinant Bet v 1 isoallergens in the same assay. As shown in table 2, isoallergens with only one amino acid substitution have identical epitope structures when analysed with this panel of mAbs. However, as more amino acid substitutions are introduced, the recombinant isoallergens show differences in their antibody-binding properties.

We generated a series of Bet v 1-reactive T cell clones from a donor clinically allergic to birch pollen. They were

all highly reactive to Bet v 1 purified from birch pollen extract, but only 5 out of 14 clones reacted to a recombinant form of Bet v 1, representing only one isoform. This fraction could be further subdivided, as these clones exhibited different proliferative responses towards major allergens from related species of the Fagales order, Car b 1, Cor a 1, Aln g 1 and Que a 1.

The epitope specificities of the T cell clones that were reactive to the recombinant Bet v 1 isoform were mapped by a combination of deletion mutants fused to maltose-binding protein and overlapping synthetic peptides. Epitopes were situated within residues 1–16, 19–34 and 125–140.

Table 1. Partial deduced amino acid sequences and RIA inhibition data on recombinant isoallergens of Car b 1

		BV10	BV16	CB11
Car b 1, group 1				
GVFN YE AETPSV I PAARLFKSYVLDGDKLIPKVAPQVISSVENVGGNGGPGTIKNITFAEGIPFKFVKERVD EVDNANFK		0	0	+
-----M-----F-----		0	0	+
-----F-----		0	0	+
Car b 1, group 2				
-----T-----AF I --N-----S--V-----E-----K--S---V-Y-----E-I-HT---		0	+	+
-----T-----AF I --NN-----V-----E-----K--S---V-Y-----E--HT---		0	+	+
-----T-----AF I --N-----S--V-----E-----K-----V-Y-----E--HT---		0	+	+
X XXX X X X XX				
Bet v 1				
GVFN YE TETTSV I PAARLFKAF I LDGDNLFPKVAPQA ISSVEN I EGNGGPGTIKK I SFPEGFPFKYVKDRVD EVDHTNFK		+	+	+
1	10 20 30 40 50 60 70 80			

- = Amino acid identities; X = identity between Car b 1 group 2 and Bet v 1, but deviation from Car b 1 group 1. The presence (+) or absence (0) of epitopes defined by selected mAbs is indicated on the right.

Table 2. Recombinant isoallergens of Bet v 1

Amino acid substitutions of Bet v 1	BV10	CB11	BV17	Theoretical pI
None	+	+	+	5.37
L(62)	+	+	+	5.37
V(91), C(112), V(113), N(125)	+	0	0	5.72
V(91)	+	+	+	5.37
G(25), G(69), M(91), N(136)	+	+	0	6.14
R(140)	+	+	+	5.73

Amino acid substitutions (and positions) in relation to the published Bet v 1 sequence [17]. The presence (+) or absence (0) of epitopes defined by selected mAbs is indicated, with the calculated pI on the right.

These results demonstrate that apart from T cell epitopes situated along the Bet v 1 molecule, we have shown the existence of T cell clones with other and distinct isoform specificities. This indicates that isoallergenic variation also plays an important role in the induction of T cell responsiveness to allergens.

Even though differences in epitope structure between isoallergens have been firmly established, the overall picture may be one of general resemblance. Thus, the diversities of epitope structures of isoallergens might have important implications for the use of peptides and recombinant proteins in immunotherapy.

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