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Studies on the Allergenicity of the Amino-Terminal Epitope (Bet v I 23-38) from Birch Pollen Allergen

Key Words

Allergens
Birch pollen
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Abstract

An N-terminal peptide of the major allergen of birch (Bet v I 23-38) was selected for studying the activity of this segment on the basis of optimal hydrophilicity as it was tentatively suggested to be a surface exposed epitope. In addition two control peptides in the region 1-38 were similarly used for comparative assignment of the allergenicity. Peptide analogues from the amino acid terminal region, amino acid residues No. 23-38 of Bet v I, were synthesized by semiautomatic solid-phase peptide synthesis. In vitro and in vivo biological activity studies were performed on these analogous peptides. The IgE-binding capacity of the synthetic peptide 23-38 was examined using the following tests: specific IgE inhibition, skin prick test, nasal provocation and Prausnitz-Küstner inhibition. The results of these investigations suggested that the region 23-38 from the birch and hazel major allergen encompassed a single haptenic epitope.

Introduction

Allergy to birch and other tree pollen is common in North Europe, and represents considerable clinical problems during early spring. Depending on the dose, mode of exposure and other environmental factors, tree pollen allergens induce allergic diseases such as rhinitis, conjunctivitis or bronchial asthma in genetically disposed individuals [1]. Knowledge about tree pollen has increased during the last two decades. The major allergens of birch and hazel pollen have been isolated and purified to homogeneity and amino acid sequenced. Several isoallergens in birch,

hazel and alder pollen have been studied [2-12, and others].

The helical assignment of the N-terminal region of the major birch and hazel pollen allergens suggested that amino acid residue 23-38 is surface located and could encompass an antibody-binding site. This entire region was predicted to be representative of the amino-terminal segment of the Bet v I molecule and demonstrated to encompass an allergenic epitope [13-15].

The purpose of this communication is to study in detail the biological activity of peptide 23-38 using in vivo as well as in vitro specific IgE test methods.

Table 1. IgE-binding capacity of the crude and GFC-purified peptide material Bet v I 23-38 in sera from 2 tree-pollen-allergic individuals and one serum pool from 10 individual birch-allergic patients (RAST inhibition percent)

Peptide concentration mmol/l	Serum No. 7		Serum No. 23		Pool	
	C	G	C	G	C	G
1,110	36	19	49	12	60	24
555	25	5	32	10	39	4
277	21	0.9	21	7	20	0
137	19	15	17	5	9	2
68	16	13	16	10	12	0
Bet v I ¹	100		100		100	
Cor a I ¹	80		90		78	
OA ¹	15		2		9	

C = Crude preparation; G = gel filtration chromatography preparation.

¹ Crude hazel and ovalbumin extracts were used as controls at concentrations of 50 nmol/l.

Materials and Methods

Solid-Phase Peptide Synthesis, Purification and Conjugation of the Peptide Bound to Bovine Serum Albumin

The region 23–38 was obtained by solid-phase peptide synthesis (SPPS), purified and conjugated to BSA as previously described [14]. Gel filtration chromatography (GFC) and reversed-phase (RP) high-performance liquid chromatography (HPLC) were used for the purification of the peptides [13, 14]. The coupling to BSA was performed by activation with 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (EDC) in 6.9 mmol sodium N-hydroxysulpho-succinimide. The reaction was terminated after 16 h and the solvent dialyzed (membrane cut-off 3,500 daltons) against 50 mmol/l ammonium bicarbonate pH 8.5 [13, 14]. The amino acid analysis was performed after randomly selected coupling stages to check the complete insertion of Boc-amino acids, and the amino acid sequence was performed to ascertain the linearity of the sequence as described in detail elsewhere [13].

Specific IgE Inhibition Tests

To investigate the IgE binding of the synthetic peptides, a modified procedure of that described by Yman was performed [5, 13, 15]. Individual sera from 2 patients with clinical histories of tree pollen (Bet v I and Aln i I), total IgE of 471 and 56 × 10³ IU/l and RAST class 3 to birch pollen, respectively, were prepared. In addition, a serum pool (RAST class 3) prepared from equal aliquots of sera from 10 birch pollen allergic patients with positive skin prick test (SPT) towards tree pollen. SPT was performed with birch pollen extracts, and was evaluated 3+ and 2+ as compared to histamine chloride solution (10 mg/ml) that gave a 2+ reaction [16]. The sera were onefold diluted and 100-μl aliquots were pre-incubated with an equal volume of the different peptide dilutions as shown in table 1. Negative controls using an unrelated peptide were also included in the test battery to ascertain the reaction's specificity.

Ethical Aspects

Informed consents were obtained from the volunteers before the blood samples were delivered and SPT, Prausnitz-Küster (PK) inhibition tests or nasal provocation tests were performed. The emergency measures to treat anaphylactic reactions are regularly secured at the hospital. The volunteers were informed about the disadvantages and the risks of the test, and were allowed to leave the investigation at any time during the procedure. They accepted to deliver blood samples and to participate in SPT and PK tests. None of the patients were treated by systemic corticosteroids or had undergone specific immunotherapy.

Skin Prick Tests

The 16 amino acid residues Bet v 23–38 was used for SPT at concentrations of 6.0, 3.0 and 0.24 mmol/ml, respectively. The crude and a GFC-purified peptide were tested in 10 birch-allergic and 9 non-allergic controls. The allergic individuals were selected for the investigations from the outpatient referred from the Allergy Section, Department of Thoracic Medicine. They had clinical history of tree pollen allergy, RAST class 2–4 towards birch pollen allergens, and SPT wheal reaction equivalent to at least that of 10 mg/ml histamine chloride. Control individuals had no clinical histories of allergy, serum IgE concentrations below 20 × 10³ U/l, and negative SPT reactions towards birch pollen extracts.

The SPT were performed as double-blind tests [17, 18], and the coding of the reagents and practical part were independently performed by two different investigators. The wheal areas were measured in mm².

Nasal Provocation

Nasal provocation tests with tree-pollen extracts and the synthetic peptides, respectively, were done on 4 volunteers, including two tree-pollen allergic and 2 nonallergic controls. Prior to the provocations the volunteers were SPT-examined to confirm that they did not react towards birch pollen extracts. The 2 allergic individuals showed SPT reactions equivalent to or more than the area produced by histamine chloride solution, and RAST classes 2–4 to birch pollen allergen.

A 4% xylocaine spray was used for a local anesthetic. The preparations were applied on the inferior and medial parts of the inferior turbinates by a cotton sponge. First, 0.9% NaCl was applied on the left inferior turbinate of all individuals tested. Biopsies were taken after 1 and 5 min by a scalpel. Then, the unconjugated peptide at the concentrations of 1.2 mmol/l was applied on the right inferior turbinate, and biopsies were similarly taken after 1 and 5 min. Three weeks later the BSA-conjugated peptide at identical concentrations was applied on the left inferior turbinate.

Whenever clinical reactions such as itching, sneezing, rhinorrhea or nasal stenosis were experienced, biopsies were taken and no further provocation done that day. If no symptoms of allergic reactions occurred, Bet v I at a concentration of 50 nmol/l was applied on the right inferior turbinate and biopsies taken. Those who reacted to the BSA-conjugated peptide were provoked again 3 weeks later by application of Bet v I.

Transmission Electron Microscopy (TEM)

The nasal biopsies were fixed for 24 h in 2% glutaraldehyde solution containing 0.1 M cacodylate buffer and 0.1 M sucrose. They were postfixed for 1 h in 1% osmium tetroxide in cacodylate buffer and dehydrated in upgraded ethanol concentrations to 100%. After 2 × 10 min in propyleneoxide and 12 h in a 1:1 mixture of propyleneoxide

Fig. 1. The N-terminal amino acid sequence of the major birch pollen allergen Bet v Ia, and Bet v Ib (23–38) as compared to other homologous regions.

Residue No.	-	-	25	-	-	-	-	30	-	-	-	-	35	36	37	38
SPPS peptide	I	L	D	G	D	N	L	F	P	K	V	A	P	Q	A	I
Cor a I	V	-	-	-	-	K	-	L	-	-	-	-	-	-	-	L
Bet v I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bet v I (cDNA)	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bet v Ia	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	I
Bet v Ib	-	-	-	-	-	K	-	-	-	-	-	-	-	-	X	Q

and Epon 812, the fragments were embedded in pure Epon 812. The final polymerization was carried out at 60 °C for 3 days. Sections were cut on an ultramicrotome, Reichert Om U3. The sections were mounted on copper grids and stained with uranyl acetate for 30 min and lead citrate for 8 min. They were studied under a Phillips EM 300 transmission electron microscope. The evaluation of the nasal mast cells was done according to Drake-Lee [19].

PK Inhibition Tests

Those were performed as originally described [18, 20, 21]. Serum from a birch-allergic patient (J.H.J.), with RAST class 3, positive SPT toward birch pollen allergens and clinical symptoms with rhinorrhea and conjunctivitis when exposed to birch allergens, was used in the investigations. Serum was collected May 1986 and May 1989. The last was tested for HIV, cytomegalovirus and hepatitis B surface antigen antibodies, and found negative.

Two healthy volunteers (H.V. and J.A.), both with total IgE less than 20×10^3 U/l, RAST class 0 and negative SPT towards birch pollen allergens, were tested using the first serum on the volar forearms as described previously.

The serum was sterile filtered through a 0.45 µm Millex-HA filter and diluted in 0.9% NaCl (1 ± 1.5). Thereafter, it was incubated at room temperature for 1 h with equal volumes of BSA-conjugated or purified Bet v I 23–38, both diluted in 0.9% NaCl to peptide concentrations of 1.2 µmol/l. Thereafter, intradermal (i.d.) injections were done on the left forearm of the two volunteers. Each of them was sensitized in two sites, 100 µl each, with the respective samples. After another 48 h, the sites were challenged by Bet v I, and the wheal reactions of these sites compared to the reactions of sites sensitized with the nonabsorbed serum.

Results

Peptide Synthesis

The covalent structure of Bet v I 23–38, as deduced from the AA sequence of the native Bet v I and Cor a I (fig. 1) was synthesized by SSPS. The purification, amino acid analyses and amino acid sequence of its entire chain was performed identically to procedures earlier described [22]. The homology between this region and the previously published ones is depicted in figure 1.

Table 2. SPT wheal relative to histamine chloride (10 mg/ml) in 10 birch-allergic patients and in 9 nonallergic controls

Test solution	Patient (n = 10) wheal area in mm ² mean (range)	Controls (n = 9) wheal area in mm ² mean (range)	p values
Physiological saline	0.10 (0–0.60)	0	0.1
GFC-purified Bet v I 23–38			
240 mmol/l	0.18 (0–0.79)	0	0.05
3,000 mmol/l	0.20 (0–0.95)	0	0.004
6,000 mmol/l	0.46 (0–0.94)	0.13 (0–0.63)	0.009
Crude Bet v I 23–38			
240 mmol/l	0.19 (0–1.0)	0.07 (0–0.52)	0.111
6,000 mmol/l	0.56 (0–1.05)	0.35 (0–0.90)	0.089
24,000 mmol/l	0.81 (0.43–1.58)	0.51 (0.28–0.83)	0.060
Bet v I, 50 mmol/l	1.63 (1.11–2.63)	0.05 (0–0.47)	0.0001
Cor a I, 50 mmol/l	1.59 (0–3.49)	0.04 (0–0.32)	0.0005

The Wilcoxon two-sample test was used to compare the SPT reactions of allergic patients and controls. The level of significance chosen was 5% ($p < 0.05$).

Specific IgE Inhibition (RAST)

The IgE binding of the unconjugated peptide Bet v I 23–38 has been tested using two individual sera (7 and 23) and a serum pool prepared from 10 individual birch-allergic patients [14]. The results are shown in table 1. An inhibition of more than 20% was obtained at peptide concentrations of 277 nM or higher, confirming a specific IgE binding under the experimental conditions.

Skin Prick Tests

The SPT results obtained with the two groups of birch-allergic and nonallergic persons are presented in table 2. The Wilcoxon two-sample test was used to compare the SPT reactions of allergic patients and controls. The level

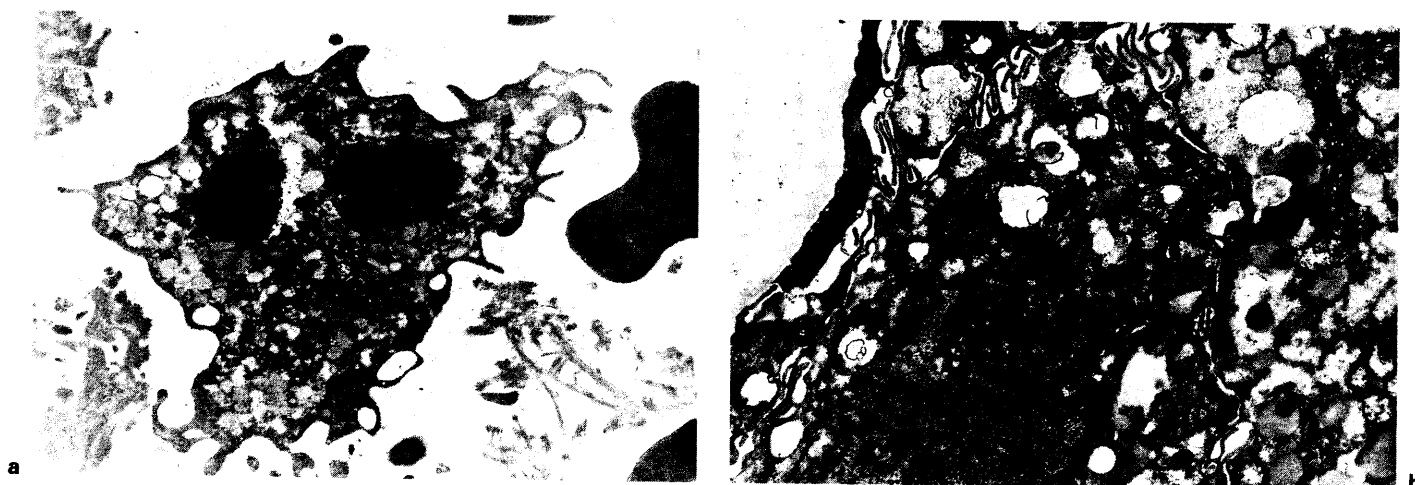


Fig. 2. **a** Tissue from the inferior turbinate of an allergic person (TEM $\times 6,800$) 60 s after application of 0.9% NaCl. No degranulation of mast cells can be observed. **b** Mast cells from an allergic individual 60 s after challenge of the turbinate with BSA-conjugated Bet v I 23-38 (TEM $\times 6,800$). Massive degranulation of mast cells can be seen.

Table 3. PK inhibition tests with Bet v I 23-38 and BSA-conjugated Bet v I 23-38 done on 2 volunteers

Volunteer	PK inhibition, %	
	Bet v I 23-38	BSA-Bet v I 23-38
H.V.	56	50
J.A.	27	45

The inhibition of the skin wheal reactions are given relative in percent of areas obtained by challenging nonabsorbed serum sites on the skin with Bet v I.

tides, and were accompanied by more severe rhinorrhea, nasal stenosis, irritation of the conjunctiva, and ipsilateral flushing of the cheek compared to the peptide.

Transmission Electron Microscopy

The findings by TEM were consistent with the clinical observations. No mast cell degranulation could be observed after application of any of the peptides in the control group. The biopsies taken from the allergic volunteers after application of NaCl and unconjugated Bet v I 23-38 did not show any mast cell degranulation. However, both BSA-conjugated Bet v I 23-38 and Bet v I led to massive degranulations of mast cells in the 2 allergic patients (fig. 2a, b).

PK Inhibition Tests

In the PK inhibition tests both the peptides and the BSA-conjugated peptides seemed to bind specific IgE antibodies visualized by inhibiting the challenge of these sites by Bet v (table 3).

Discussion

The peptide Bet v I 23-38 from the major birch-pollen allergen was selected by conventional secondary structure peptide analysis algorithms [23] and demonstrated to be antigenically and allergenically reactive in the utilized test systems using both rabbit polyclonal antibodies and hu-

of significance chosen was 5%. The differences in reactions to purified peptides in the allergic and nonallergic groups were statistically significant.

Nasal Provocation Tests

Neither the Bet v I peptides nor the entire crude extract could initiate inflammatory reactions in the 2 controls (H.V., T.D.). In the birch-allergic patients (B.R., T.G.) the nonconjugated Bet v I 23-38 initiated a moderate degree of itching in the nose after 15-60 s. The BSA-conjugated Bet v I 23-38 caused sneezing and itching after 15-60 s. After another 15-60 s rhinorrhea, nasal stenosis and ipsilateral conjunctival irritation could be observed.

The native allergen Bet v I gave sneezing and itching at the same time intervals as for the BSA-conjugated peptides. However, the native allergen induced local inflammatory reactions faster than the BSA-conjugated pep-

man specific IgE. The specific IgE-binding properties were confirmed in vitro by inhibition of the RAST [14]. Two control peptides in the region 1–38, peptides Bet v I 1–16 and Bet v I 10–24 were used for control. Bet v I 1–16 was similarly active, while 10–24 was not reactive with either human IgE or rabbit polyclonal Ig [13]. SPT demonstrated different reactions in allergic versus nonallergic individuals to the purified peptide material Bet v I 23–38. The nasal provocation tests and indirect PK further confirmed the in vivo IgE-binding capacities of the BSA-conjugated peptide 23–38. The nasal provocation was used as an alternative technique for direct and visual indications of mast cell degradation. No incidence of anaphylactic reactions were experienced during the in vivo testing performed.

The results suggested that the investigated 16 AA sequence from the major birch pollen allergen did encompass a monovalent (haptenic) IgE-binding epitope. Accordingly, this peptide did not induce an IgE-mediated mediator release from mast cells in the skin as did the BSA-conjugated peptide.

By binding this molecule to a carrier protein like BSA, or by formation of various aggregates, it may act as a poly-

valent allergenic polypeptide, inducing release of mediators. This was also illustrated by the activity of the BSA conjugated in all tests performed. The N-terminal segment of Bet v I therefore seemed to encompass a region with hydrophilic location on the molecular surface available for binding antibodies.

Using the N-terminal birch pollen synthetic peptides for investigating its activity in allergen-specific T cell cultures generated from birch-allergic patients is in progress. T cell epitopes and the intact major allergen from birch can be useful tools for immunotherapy of one of the most common type I seasonal allergies.

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