

Non-anaphylactic surface-exposed peptides of the major birch pollen allergen, Bet v 1, for preventive vaccination

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Summary

Background Almost 100 million allergic patients are sensitized to the major birch pollen allergen, Bet v 1, a 17 kDa protein containing most of the IgE epitopes present in pollens of trees belonging to the *Fagales* order and plant-derived food.

Objective Our aim was to develop an approach for the rational design of B cell epitope-derived, non-allergenic peptide allergy vaccines.

Methods According to the three-dimensional (3-D) structure of birch pollen allergen, Bet v 1, six peptides comprising 25–32 preferably solvent-exposed amino acids were synthesized.

Results Because of lack of secondary structure, the peptides showed no allergenic activity in allergic patients. In a mouse model of birch pollen allergy, peptide vaccination induced Bet v 1-specific IgG and prevented IgE-mediated allergic sensitization to Bet v 1. The protective role of peptide-induced blocking antibodies is demonstrated by inhibition of allergic patients IgE binding to the allergen and by blocking of allergen-induced basophil degranulation.

Conclusion Our results indicate the mechanistic importance of blocking antibodies for allergy vaccination and present a B cell epitope-based approach for the rational design of safe peptide allergy vaccines whenever the structure of the disease-eliciting allergen is known.

Keywords hypoallergenic vaccine, immunotherapy, protective IgG-antibodies, synthetic peptides, three-dimensional structure, type I allergy

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Introduction

The only causative approach towards allergy treatment, allergen-specific immunotherapy, is based on the continuous administration of the disease-eliciting allergens to patients in order to induce allergen-specific non-responsiveness [1, 2]. Numerous studies document the clinical efficacy of specific immunotherapy, but two major disadvantages must be overcome: first, systemic administration of allergens can induce life-threatening anaphylactic side-effects. Second, immunotherapy is performed with allergen extracts consisting of mixtures of allergenic and non-allergenic components that cannot be tailored to the individual patients' sensitization profile. Furthermore, the immunological mechanisms underlying immunotherapy represent a controversial issue [1].

Here, we present a strategy for the rational design of safe peptide allergy vaccines for patient-tailored treatment and use this model to investigate mechanisms for the protective effect of allergen-specific blocking antibodies. The presented approach is based on recent advances made regarding the

structural and immunological characterization of allergens by molecular cloning techniques (reviewed in [3, 4]). It starts with the selection of important allergen sources against which a high percentage of allergic patients is sensitized and requires that the major allergens be identified by molecular cloning. As model allergen source, we have chosen birch pollen because it is widely distributed in Europe, North America, Russia and Australia [5]. Furthermore, the major birch pollen allergen, Bet v 1, contains most of the IgE epitopes present in pollens of trees belonging to the *Fagales* order (birch, alder, hazel, oak, hornbeam) and in plant-derived food (e.g. apple, carrot, nuts) [6, 7]. Moreover, clinical immunotherapy studies performed with crude birch pollen extract suggest that immunotherapy with birch pollen allergens alone may be effective for the treatment of allergies to *Fagales* pollens and plant food [8, 9]. The cDNA and amino acid sequence as well as the three-dimensional (3-D) structure of Bet v 1 have been determined and recombinant Bet v 1, which equals the natural Bet v 1 wild-type, has been produced by recombinant DNA technology [5, 10, 11].

The recognition of Bet v 1 as well as of most other allergens by IgE antibodies of allergic patients depends on conformational epitopes and hence requires a folded molecule [3, 12]. Following this principle, we identified according to sequence and 3-D structure of Bet v 1, peptides, rich in solvent-exposed

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amino acids, that upon active vaccination should induce blocking antibodies. Further important criteria for the selection of the peptides were lack of fold to avoid allergenic activity and a size (25–32 amino acids) sufficient to obtain antibody responses *in vivo*. Reduction of IgE binding and allergenic activity of the peptides was studied by IgE binding assays, basophil histamine release and skin prick tests (SPTs) performed in allergic patients. Using a mouse model for birch pollen allergy, we studied the effects of prophylactic peptide vaccination on Bet v 1-specific allergic sensitization. Finally, we demonstrate that peptide-induced IgG inhibits allergic patients IgE binding to Bet v 1 and Bet v 1-induced basophil degranulation.

Methods

Allergen extracts, allergens

Pollen from trees of the order *Fagales* (birch: *Betula verrucosa*; alder: *Alnus glutinosa*; hazel: *Corylus avellana*; hornbeam: *Carpinus betulus*) were purchased from Allergon (Välinge, Sweden). Aqueous pollen extracts were prepared and checked for quantity and quality of proteins by SDS-PAGE and Coomassie blue staining [13]. Purified recombinant Bet v 1 was purchased from BIOMAY (Vienna, Austria).

Characterization of allergic patients and patients' sera

Birch-pollen allergic patients suffering from allergic rhinoconjunctivitis and/or asthma were selected according to case history indicative for seasonal birch pollinosis and characterized by skin prick testing (SPT) with birch pollen extract and serological CAP-FEIA (Pharmacia Diagnostics, Uppsala, Sweden) testing. Total IgE levels in the sera were determined by CAP measurements (Pharmacia). IgE antibodies specific for rBet v 1 were determined by ELISA [6]. Sera from 60 birch pollen allergic patients and a non-atopic individual were used for IgE competition studies. The birch pollen-allergic patients group consisted of 25 males and 35 females with a mean age of 37 years (ranging from 19 to 60 years).

Peptide synthesis

Six Bet v 1-derived peptides of a length between 25 and 32 amino acids, rich in solvent-exposed amino acids were selected according to the 3-D structure of Bet v 1 [10]. Peptides were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc)-strategy with 2-(1H-Benzotriazol-1-yl) 1,1,3,3-tetramethyluronium hexafluorophosphat (HBTU)-activation (0.1 mmol small-scale cycles) on the Applied Biosystems peptide synthesizer Model 433A (Foster City, CA, USA). Preloaded polyethyleneglycol polystyrene (PEG-PS) resins (0.15–0.2 mmol/g loading) (Per Septive Biosystems, Warrington, UK) were used as solid phase to build up the peptides. Chemicals were purchased from Applied Biosystems. Coupling of amino acids was confirmed by conductivity monitoring in a feedback control system. Each of the synthetic peptides contained one cysteine residue in addition to the original sequence, which was attached either to the N- or C-terminus to facilitate coupling to carriers (Table 1). Peptides were

Table 1. Characteristics of non-allergenic Bet v 1-derived synthetic peptides

	Position aa	Sequence	Number of aa	Molecular weight	Isoelectric point (pI)	Fold (NMR)	T cell epitope		AGADIR %
							Human	BALB/c mice	
Peptide 1	1–24	MGVFNYETETTSVIPAAARLFKAFIC	25	2809.3	6.28	–	–	–	0.86
Peptide 2	30–59	LFPKVAPQAISSEVENIEGNGGPGTIKKISFC	31	3202.7	8.63	–	–	–	0.56
Peptide 3	50–79	CGPGTIKKISFPEGFPFKYVKDRVDEVDTN	31	3525	7.00	–	–	–	0.5
Peptide 4	110–139	DGGSILKISNKYHTKGDHEVKAEQVKASKEC	31	3400.8	8.44	–	–	–	0.85
Peptide 5	130–160	CKAEQVKASKEMGETLLRAVESYLLAHSDAYN	32	3556.1	5.49	+ / –	+	+	12.09
Peptide 6	75–104	CVDHTNFKYNSVIEGGPIGDTLEKISNEIK	31	3484.9	4.70	–	+	–	1

Position, sequence, length, molecular weight, isoelectric point, residual fold, presence of T cell epitopes and AGADIR prediction of the Bet v 1-derived peptides. NMR, nuclear magnetic resonance.

cleaved from the resins with a mixture of 250 µL dest. water, 250 µL triisopropylsilan (Fluka, Buchs, Switzerland), 9.5 mL trifluoroacetic acid for 2 h and precipitated in tert-Butyl-methylether (Fluka, Buchs). The identity of the peptides was checked by mass-spectrometry and they were purified to >90% purity by preparative high-pressure liquid chromatography (HPLC) (PiChem, Graz, Austria).

One- and two-dimensional nuclear magnetic resonance

Synthetic peptides were examined for tertiary and/or secondary structure by one- and two-dimensional nuclear magnetic resonance (NMR) on a UNITY VARIAN 600 MHz spectrometer (Varian Inc., Palo Alto, CA, USA) equipped with z-shielded gradient coils using 0.8–1.0 mm samples in 90% H₂O/10% D₂O, 20 mM potassium phosphate buffer pH 7.0, at 20 °C. Water suppression was achieved with WATERGATE. Mixing times used were 70 ms and 150 or 200 ms for TOCSY and NOESY experiments, respectively.

Basophil histamine-release assay

Granulocytes were isolated from heparinized blood samples of three birch pollen-allergic individuals by dextran sedimentation [14]. Cells were incubated with increasing concentrations (10^{-13} –10 µg/mL) of each peptide, an equimolar mix of the six peptides and, for control purposes, with complete rBet v 1. Histamine released in the cell-free culture supernatant was measured by radioimmunoassay (Immunotech, Marseille, France). Total histamine was determined after freezing and thawing of cells. Results are displayed as mean values of triplicate determinations.

Skin prick testing of allergic patients

The *in vivo* allergenic activity of the peptides was studied by SPT in five birch pollen-allergic patients and a grass pollen-allergic patient without birch pollen allergy. SPTs were performed on the individual's forearms as described [12]. Twenty microlitre aliquots containing six concentrations of complete rBet v 1 (0.78, 1.56, 3.12, 6.25, 12.5, 25 µg/mL), 100 µg/mL of each peptide and an equimolar peptide mix were applied. In addition, standardized skin prick solutions (birch pollen extract and histamine) (Allergopharma, Reinbek, Germany) were tested. The mean weal diameter (D_m) was calculated by measuring the maximal longitudinal and transversal diameter and dividing their sum by 2.

Immunization of mice and rabbits

HPLC-purified peptides were coupled to keyhole limpet haemocyanin (KLH: MW 4.5×10^3 – 1.3×10^7 ; Pierce, Rockford, IL, USA) according to the manufacturer's advice and purified using a Conjugation Kit (Sigma, St Louis, MO, USA).

The peptide-based allergy vaccine was evaluated in a mouse model for birch pollen allergy [15]. Four groups each consisting of six mice were studied. Prophylactic vaccination (P+) was performed with a mixture of the six KLH-coupled peptides (5 µg each) adsorbed to 150 µL of CFA (Sigma). Allergic sensitization (S+) was induced by injection of 5 µg rBet v 1 adsorbed to 150 µL Al(OH)₃ (Serva, Heidelberg, Germany). Mice in the P–/S– group received a subcuta-

neous injection of CFA at day 1 and one with Al(OH)₃ at day 32. The P–/S+ group received CFA at day 1 and was sensitized with rBet v 1 adsorbed to Al(OH)₃ at day 32. Mice of the P+/S– group were treated with CFA-adsorbed peptides at day 1 and received Al(OH)₃ at day 32. The P+/S+ group was treated with CFA-adsorbed peptides and sensitized with Al(OH)₃-adsorbed rBet v 1 at day 32. Intradermal skin tests were performed in all mice at day 102 by injecting 100 µL 0.5% Evans blue (Sigma) into the tail vein and subsequent intradermal injection of rBet v 1, birch pollen extract, compound 48/80 and phosphate-buffered saline (PBS) into the abdominal skin as described [15]. Bet v 1-specific mouse IgE and IgG1 were determined by ELISA [16].

To obtain sufficient amounts of peptide-specific IgG antibodies, rabbits were immunized with each of the KLH conjugated-peptides (200 µg/injection) and, for control purposes, with complete rBet v 1 using Freund's complete and incomplete adjuvans (Charles River). Serum samples were obtained in 4-week intervals.

Enzyme-linked immunosorbent assay detection of peptide and rBet v 1-specific human immunoglobulin E antibodies; inhibition of allergic patients immunoglobulin E binding to rBet v 1 by peptide-specific immunoglobulin G

ELISA plates (Nunc Maxisorp, Roskilde, Denmark) were coated with Bet v 1-derived peptides (5 µg/mL) or rBet v 1 as control (5 µg/mL), washed and blocked as described [6]. Subsequently, plates were incubated with 1:3 diluted sera from 60 birch pollen-allergic patients and from a non-atopic individual overnight at 4 °C. Bound IgE antibodies were detected with a 1:1000 diluted alkaline-phosphatase-coupled mouse monoclonal anti-human IgE antibody (Pharmingen, San Diego, CA, USA) as described.

The ability of peptide-induced rabbit Ig to inhibit the binding of allergic patients' IgE to complete rBet v 1 was investigated by ELISA competition assay as described [17]. ELISA plates were coated with rBet v 1 (1 µg/mL) and pre-incubated either with a 1:250 dilution of each of the anti-peptide antisera (anti-P1–anti-P6), a mixture containing equal volumes of the anti-peptide antisera and, for control purposes, with the corresponding pre-immune sera or a mixture of the pre-immune sera. After washing, plates were incubated with 1:3 diluted sera from 60 birch pollen-allergic patients and bound IgE antibodies were detected with the alkaline-phosphatase-coupled mouse monoclonal anti-human IgE antibody (Pharmingen). The percentage inhibition of IgE binding achieved by pre-incubation with the anti-peptide antisera was calculated as follows: Percentage inhibition of IgE binding = $100 - OD^I/OD^P \times 100$. OD^I and OD^P represent the extinctions after pre-incubation with the rabbits immune and pre-immune serum, respectively.

Rat basophil leukaemia cell degranulation experiments

RBL-2H3 cells were plated in 96-well tissue culture plates (4×10^4 cells/well). To determine allergenic serum activity, passive sensitization of RBL cells was carried out with sera obtained from the four groups of mice after allergic sensitization at a final dilution of 1:30 for 2 h. Unbound antibodies were removed by washing the cell layer two times

in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 1.8 mM CaCl₂, 0.4 mM NaH₂PO₄, 5.6 mM D-glucose, 12 mM NaHCO₃, 10 mM HEPES and 0.1% w/v bovine serum albumin, pH 7.2). Degranulation of RBL cells was induced by adding 100 µL rBet v 1 diluted in Tyrode's buffer ($c = 0.3 \mu\text{g/mL}$) for 30 min at 37 °C. Supernatants were analysed for β -hexosaminidase activity by incubation with 80 mM 4-methylumbelliferyl-N-acetyl- β -D-glucosamide (Sigma) in citrate buffer (0.1 M, pH 4.5) for 1 h at 37 °C [18]. The reaction was stopped by addition of 100 µL glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and the fluorescence was measured at $\lambda_{\text{ex}}: 360/\lambda_{\text{em}}: 465 \text{ nm}$ using a fluorescence microplate reader (Spectrafluor, Tecan, Austria). Results are reported as percentage of total β -hexosaminidase released after addition of 1% Triton X-100. Statistical analysis of antibody levels and RBL results between the groups was performed using the Student's *t*-test.

The anti-allergic activity of peptide-specific IgG was determined by exposing RBL cells that had been loaded with Bet v 1-specific mouse IgE with IgG-containing immune complexes. Immune complexes were formed using 0%, 2%, 5% or 10% of a serum mix obtained from rabbits before and after immunization with the six peptides, serum from an rBet v 1-immunized rabbit or the corresponding pre-immune serum together with 0.01 µg/mL rBet v 1 in Tyrode's buffer for 2 h at 37 °C. Preformed immune complexes (100 µL) were added to Bet v 1-sensitized RBL cells (4×10^4 cells/well) and degranulation was assessed as described for passive sensitization. Results are displayed as mean values of quadruplicate determinations.

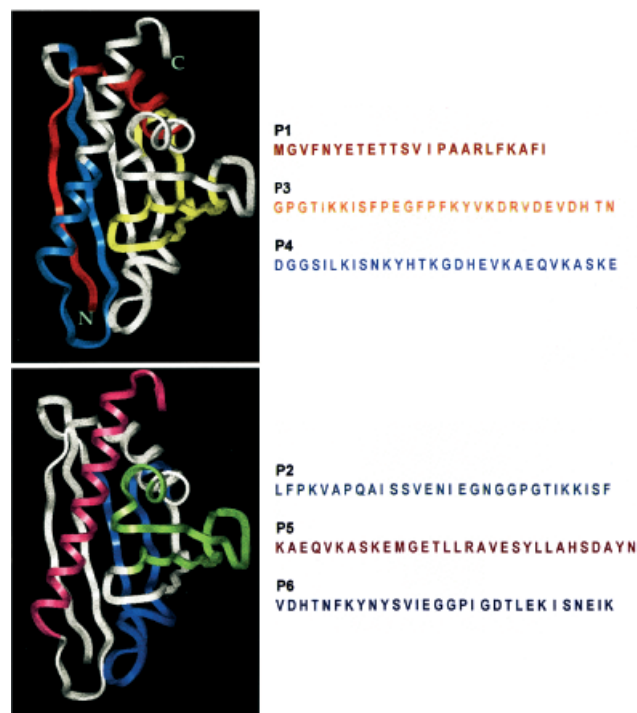


Fig. 1. Ribbon representation of the Bet v 1 three-dimensional structure with the peptides highlighted in different colours. Upper panel: Peptides P1, P3 and P4 are shown in red, yellow and cyan, respectively. Lower panel: Peptides P2, P5 and P6 are displayed in green, magenta and blue, respectively. The N- and C-termini of the Bet v 1 molecule have been indicated.

Results

Characteristics of six synthetic peptides covering the surface of the Bet v 1 allergen

According to the sequence and 3-D structure of the Bet v 1 allergen [5, 10], we have selected six Bet v 1-derived peptides ranging from 25 (P1: 2809.3 Da) to 32 (P5: 3556.1 Da) amino acids in length, which are rich in solvent-exposed amino acids (Fig. 1, Table 1). The isoelectric points calculated for the six peptides ranged from 4.70 (P6) to 8.63 (P2) (Table 1). Together, the six Bet v 1-derived peptides resemble almost all primary structure elements of the Bet v 1 allergen but according to AGADIR prediction [19] lacked secondary structure. NMR confirmed the lack of residual secondary or tertiary structure. By 1D experiments, we found the absence of tertiary structure in all of the six peptides: all the resonances had poor spectral dispersion typical for a mixture of amino acids or peptides without tertiary fold (Fig. 2).

Two of the peptides (P5, P6) comprised sequence motifs that were described to stimulate Bet v 1-specific human T cells, whereas the other four peptides (P1, P2, P3, P4) did not contain relevant Bet v 1-specific human T cell epitopes (Table 1) [20]. Accordingly, no significant induction of lymphoproliferative responses was observed when we exposed peripheral blood mononuclear cells from birch pollen-allergic patients to the peptides (data not shown). Extensive T cell

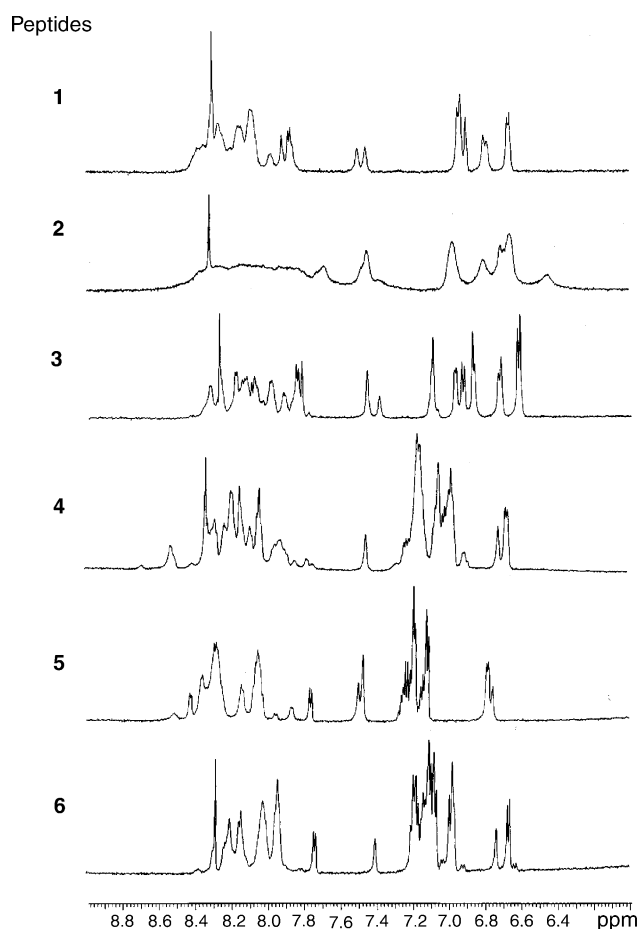


Fig. 2. ¹H 1D spectra of the amide regions of the six peptides. The spectra were recorded at 20 °C on a 600 MHz spectrometer.

epitope mapping studies performed in mice have demonstrated that only peptide 5 contains a major T cell epitope (BV 139: MGETLLRAVESY), whereas the other five peptides had failed to induce significant T cell proliferation in BALB/c mice (Table 1) [21].

Surface-exposed Bet v 1-derived peptides lack immunoglobulin E binding capacity and allergenic activity

The IgE binding capacity of the six Bet v 1-derived, unfolded peptides was compared with that of the complete rBet v 1 wild-type allergen using sera from 60 birch pollen-allergic patients (total IgE levels: 24–2000 kU/L (mean: 300 kU/L); birch pollen extract-specific IgE: 3–100 kUA/L (mean: 47 kUA/L)). All patients had rBet v 1-specific IgE antibodies ranging between 0.081 and 2.530 OD units (mean: 1.416 OD units) but showed no IgE reactivity to any of the peptides (P1–P6) or to an equimolar mix of the peptides (OD < 0.005 units).

Next, we compared the Bet v 1-derived peptides with complete rBet v 1 for their capacity to induce histamine release from basophils of three birch pollen-allergic individuals. As exemplified for one patient in Fig. 3 we found that

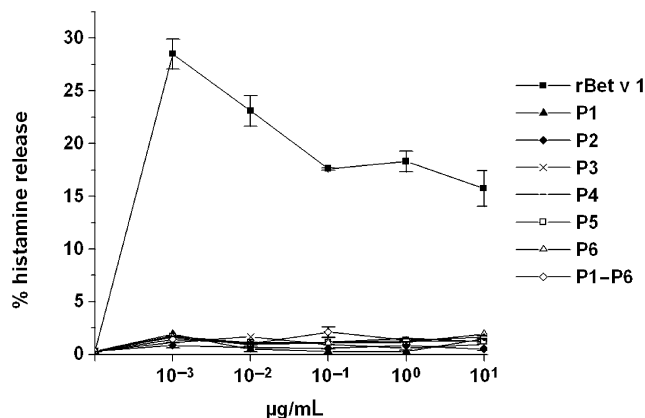


Fig. 3. Induction of histamine release from basophils of a birch-pollen allergic patient. Granulocytes of a birch pollen-allergic patient were incubated with various concentrations (x-axis) of rBet v 1 wild-type, Bet v 1-derived peptides (P1, P2, P3, P4, P5, P6) and an equimolar mix of the six peptides (P1–P6). The percentage of histamine released into the cell-free culture supernatant is displayed on the y-axis. Results are displayed as mean values of triplicate determinations $\pm$ SD.

neither the individual peptides nor an equimolar mix of the peptides induced release of histamine up to concentrations of 10 μ g/mL whereas complete rBet v 1 wild-type induced a dose-dependent release of histamine with a maximum release already at a concentration of 1 ng/mL.

In vivo testing in birch pollen-allergic patients also demonstrated lack of allergenic activity of the Bet v 1-derived peptides. As exemplified in Fig. 4, we performed SPTs in five birch pollen-allergic patients and an allergic individual without birch pollen allergy (Table 2). None of the Bet v 1-derived peptides induced any immediate skin reaction when applied at a concentration of 100 μ g/mL or as peptide mix containing 100 μ g/mL of each peptide (Fig. 4, Table 2). Complete rBet v 1 wild-type already induced immediate-type skin reactions in three patients (Table 2: #1–3) at a concentration of < 0.78 μ g/mL. A concentration of 3.12 μ g/mL was sufficient to induce weal reactions in all five birch pollen-allergic patients (Table 2). All birch pollen-allergic patients displayed immediate skin reactions to birch pollen extract. The grass pollen-allergic patient without birch pollen allergy (Table 2: #6) showed no reactions to birch pollen extract, rBet v 1 and Bet v 1-derived peptides (Table 2) but exhibited a weal reaction after prick testing with timothy grass pollen extract (5.5 mm mean diameter). All individuals reacted with histamine (positive control) (Table 2). Since local and even systemic late-phase reactions to allergens and, in particular to T cell epitope-containing peptides have been reported [22], we checked the application sites 24–48 h after SPT but found no evidence for peptide-induced late-phase reactions in any of the tested individuals.

Vaccination with Bet v 1-derived peptides prevents the development of allergic sensitization to Bet v 1 and induces Bet v 1-specific immunoglobulin G responses

Prophylactic vaccination with a mix of the six peptides led to a reduction of Bet v 1-specific IgE production in mice (Fig. 5). The prophylactic treatment was accompanied by a strong and highly significant induction of Bet v 1-specific IgG responses, whereas almost no induction of Bet v 1-specific IgE levels and skin sensitivity were noted (Fig. 5). Loading of RBL with sera from peptide-vaccinated and sensitized mice (group 4) containing high levels of allergen-specific IgG induced significantly less RBL degranulation compared with those formed



Fig. 4. Immediate-type skin reactions to rBet v 1 and to rBet v 1-derived synthetic peptides. Two birch pollen-allergic patients (1, 2) were skin prick tested with Bet v 1-derived peptides, complete rBet v 1, birch pollen extract and histamine as shown in the scheme. Weal reactions were surrounded after 20 min.

Table 2. Summary of immediate-type skin reactions to complete rBet v 1- and Bet v 1-derived peptides

Individual	Mean weal diameter																
	rBet v 1 (µg/mL)								Birch	Histamine	100 µg/mL						
	0.19	0.39	0.78	1.56	3.12	6.25	12.5	25			P1	P2	P3	P4	P5	P6	P1–P6 mix
1	0	2.5	4	4.5	5	6	13	ND	5.5	5	0	0	0	0	0	0	0
2	0	2	3.5	5	5.5	7	7.5	8	7.5	6	0	0	0	0	0	0	0
3	ND	ND	2	3	4	6	6.5	8	8	7	0	0	0	0	0	0	0
4	ND	ND	0	0	2	3	4	4.5	8	5	0	0	0	0	0	0	0
5	ND	ND	0	2	2.5	4	11	5	9	5.5	0	0	0	0	0	0	0
6	ND	ND	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0

Five birch pollen-allergic patients (#1–5) and an allergic individual without birch pollen allergy (#6) were tested. The mean weal diameters (mm) are displayed for rBet v 1, for birch pollen extract, histamine, the individual peptides and a mixture of the six peptides.

ND, not determined.

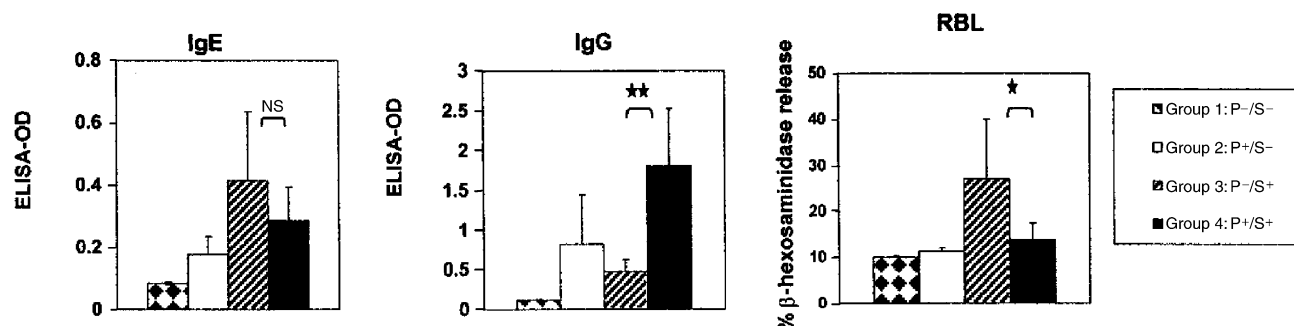


Fig. 5. Prophylactic vaccination with Bet v 1-derived peptides reduces Bet v 1-specific IgE production and sensitivity to Bet v 1 in mice. The development of Bet v 1-specific IgE and IgG1-antibody responses (after prophylactic vaccination and sensitization) is displayed for four groups of mice (Group 1: P –/S – = no prophylaxis/no sensitization; Group 2: P +/S – = prophylaxis/no sensitization; Group 3: P –/S + = no prophylaxis/sensitization; Group 4: P +/S + = prophylaxis/sensitization). The mean OD levels $\pm$ SD of each group corresponding to the amount of bound Bet v 1-specific IgE and IgG1 levels are shown on the y-axes (IgE, IgG1). The percentage of β -hexosaminidase release induced from rat basophil leukemia (RBL) cells with Bet v 1-immune complexes is displayed for each group (RBL). NS, not significant; * P < 0.05; ** P < 0.001.

with sera from non-vaccinated and sensitized mice (group 3) (Fig. 5). Vaccination did not induce antibodies with allergenic activity because immune complexes formed with sera from vaccinated, non-allergic mice (group 2) failed to induce RBL degranulation (Fig. 5).

Immunization with Bet v 1-derived peptides induces immunoglobulin G antibodies that inhibit the binding of birch pollen allergic patients immunoglobulin E to Bet v 1

Immunization with peptides induced Bet v 1-specific IgG in rabbits similar to that observed for non-allergenic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1 [23].

The capacity of Bet v 1 peptide-induced antibodies to inhibit the binding of allergic patients IgE to complete rBet v 1 was studied by ELISA competition using sera from 60 birch pollen-allergic patients (Table 3). For all but three allergic patients sera, preincubation with peptide-induced rabbit IgG inhibited the binding of patients IgE to Bet v 1. The strongest inhibition of IgE binding was observed after pre-incubation with anti-P2 (68% average inhibition), anti-P6 (60% average inhibition), anti-P3 (53.6% average inhibition) and anti-P5 (41.6% average inhibition). Anti-P4 antibodies showed a lower capacity to inhibit serum IgE binding to Bet v 1 (average inhibition: 28.5%) and anti-P1 antibodies raised against the N-terminus of Bet v 1 weakly inhibited IgE

binding to Bet v 1 (average inhibition: 11.4%). Interestingly, a mixture of anti-P1 to anti-P6 antibodies did not exhibit a stronger inhibition of IgE binding (average inhibition: 56.9%) than antibodies induced against single peptides (e.g. anti-P2, anti-P6 antibodies) (Table 3).

Peptide-specific immunoglobulin G antibodies inhibit Bet v 1-induced allergic reactions

Next we investigated the anti-allergic activity of peptide-specific IgG using RBL cells. RBL cells were preloaded with IgE antibodies from birch pollen-allergic mice. Exposure of Bet v 1 in the presence of increasing amounts of sera containing peptide-specific rabbit IgG inhibited degranulation of RBL cells as determined by β -hexoaminidase release (Fig. 6). RBL degranulation was also blocked when Bet v 1 was added in the presence of rabbit IgG obtained by immunization with the complete Bet v 1 allergen. Addition of preimmune IgG from peptide- or rBet v 1-immunized rabbits did not reduce Bet v 1-induced RBL degranulation (Fig. 6).

Discussion

In this study, we demonstrate that safe, hypoallergenic allergy vaccines, which induce protective antibodies, can be developed

Table 3. Rabbit anti-Bet v 1 peptide antisera inhibit serum IgE binding of birch pollen-allergic patients to rBet v 1

Patient	% inhibition						
	Anti-P1	Anti-P2	Anti-P3	Anti-P4	Anti-P5	Anti-P6	Anti-P1-P6
1	16	82	76	59	72	80	86
2	0	88	78	49	70	76	85
3	16	92	86	50	76	85	92
4	39	66	47	28	38	60	67
5	0	75	44	60	31	62	48
6	0	88	75	38	59	76	90
7	20	92	80	40	68	86	93
8	17	68	69	44	66	65	67
9	13	0	0	0	0	0	0
10	0	0	0	0	0	0	0
11	34	70	55	0	13	56	62
12	10	44	41	24	27	28	0
13	18	54	62	31	33	53	0
14	12	72	51	0	52	61	48
15	0	51	6	18	14	51	6
16	13	81	56	27	52	72	69
17	13	87	75	48	46	80	86
18	15	89	73	44	65	85	90
19	46	82	35	0	51	61	92
20	0	85	61	34	59	77	84
21	14	69	54	13	44	56	53
22	0	72	62	35	52	65	71
23	17	86	74	51	69	81	87
24	11	68	39	19	43	51	37
25	15	83	73	48	67	77	78
26	8	51	37	31	29	51	0
27	0	79	65	33	60	78	84
28	5	72	59	17	45	70	75
29	0	66	44	21	38	41	33
30	0	40	18	0	0	31	13
31	6	79	80	25	66	46	82
32	7	79	67	38	51	68	78
33	0	22	21	4	20	21	0
34	0	62	44	7	47	51	65
35	0	72	57	16	45	64	61
36	13	69	52	12	46	57	54
37	2	22	23	7	12	14	0
38	0	0	0	0	0	0	0
39	0	89	66	0	50	80	87
40	9	78	63	32	61	79	66
41	16	90	80	64	49	87	91
42	11	89	76	49	45	79	86
43	25	80	69	28	3	81	74
44	0	67	28	17	8	50	62
45	23	82	73	37	76	76	80
46	23	84	85	47	76	82	86
47	5	73	64	18	32	60	42
48	7	82	65	38	20	71	83
49	2	74	68	38	56	63	48
50	13	64	42	35	29	62	58
51	20	47	42	15	0	28	27
52	14	55	38	35	15	48	13
53	7	87	69	52	34	80	85
54	37	82	81	71	79	78	74
55	16	82	52	29	39	72	85
56	21	87	74	51	61	81	88
57	30	75	60	45	55	66	65
58	4	23	0	0	0	28	9
59	7	62	43	17	41	59	13
60	11	68	39	19	43	51	37
Mean	11.4	68	53.6	28.5	41.6	60	56.9

The percentage inhibitions of IgE binding to complete rBet v 1 obtained with the individual anti-peptide antisera (anti-P1 to anti-P6) and a mixture of the anti-peptide antisera (anti-P1 to anti-P6) are displayed for sera from 60 birch pollen-allergic patients. The mean percentage inhibitions are shown at the bottom of the table.

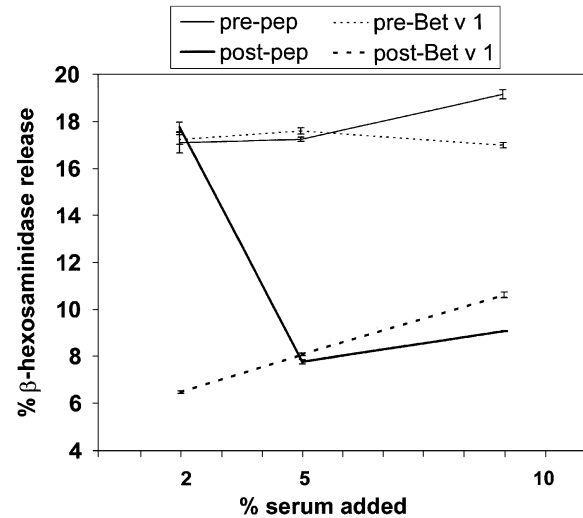


Fig. 6. Rabbit anti-Bet v 1 peptide antisera inhibit Bet v 1-induced β -hexosaminidase release from sensitized rat basophil leukaemia (RBL) cells. RBL cells were loaded with serum IgE from Bet v 1-allergic mice and were subsequently exposed to rBet v 1 in the presence of increasing amounts of sera (x-axis: v/v percentage of added serum) containing anti-Bet v 1 (post-Bet v 1), anti-Bet v 1 peptide-specific IgG (post-pep) or the corresponding preimmune IgG (pre-Bet v 1, pre-pep). The percentage of released β -hexosaminidase is displayed on the y-axis. Results are displayed as mean values of quadruplicate determinations $\pm$ SD.

for allergens with known sequence and 3-D structure. According to the sequence and 3-D structure of the major birch pollen allergen, Bet v 1 [5, 10], a major allergen for 25% of allergic patients, we have prepared six non-allergenic peptides, rich in solvent-exposed amino acids covering the surface of the Bet v 1 molecule. While complete rBet v 1 represents a folded molecule consisting of mixed α helical and β -sheet structure [10, 12], none of the Bet v 1-derived peptides assumed secondary structure. The B cell epitope-derived Bet v 1-peptides were synthesized with an additional cysteine residue, which was added either at the N- or C-terminus of the peptides to facilitate coupling to carriers for vaccination purposes. Despite the presence of the additional cysteine per peptide, we have no evidence from HPLC and mass spectroscopy for dimer formation via disulphide bonds. Even if such an event would have occurred, it had no effect on IgE reactivity and allergenic activity because none of the Bet v 1 allergic patients showed IgE reactivity to the peptides and the peptides did not induce histamine release or skin reactions in patients. The lack of allergenic activity of the Bet v 1-derived peptides can be explained by the absence of secondary structure of the peptides because birch pollen-allergic patients IgE antibodies primarily recognize conformational epitopes [12].

As a result of extensive work in the field of allergen characterization, the molecular nature, 3-D structures and epitopes of the most common allergens have been determined [4]. It should hence be possible to use the described approach for the general development of allergy vaccines. This assumption is supported by our finding that hypoallergenic peptides could also be synthesized for allergens containing continuous (i.e. sequential, linear) IgE epitopes [23, 24].

In a mouse model for birch pollen allergy, we demonstrate that peptide vaccination reduced Bet v 1-specific IgE production and prevented Bet v 1-induced allergic reactions

(i.e., basophil degranulation). This activity was associated with a strong induction of Bet v 1-specific IgG antibodies by the vaccine with reduced IgE production. Passive transfer of mouse sera to RBL cells and subsequent allergen-induced degranulation confirmed that peptide-vaccinated mice indeed exhibited reduced production of IgE (i.e. anaphylactic) antibodies.

Whether the reduced IgE production in peptide-vaccinated mice was controlled by T cells or was because of capture of the sensitizing allergen by vaccine-induced IgG antibodies cannot be definitively answered. In this context, it should, however, be mentioned that only one of the six peptides (i.e. peptide #5) contained a Bet v 1-specific T cell epitope recognized by the BALB/c mice used in our model [21]. Furthermore, the peptide vaccine was designed to incorporate antigenic structures that should primarily induce blocking antibodies.

Several recent findings have rekindled the interest in the concept that blocking IgG antibodies can protect against type I allergy (reviewed in [25, 26]). First, evidence has been provided that IgG antibodies are induced in the course of allergen extract-based immunotherapy, which inhibit basophil histamine release [27, 28]. Second, it was shown that therapy-induced antibodies may inhibit IgE-mediated T cell activation and subsequent release of pro-inflammatory cytokines [29].

Therefore, the inhibitory characteristics of peptide-induced antibodies were studied in more detail. We found that peptide-induced antibodies strongly inhibited the binding of birch-pollen allergic patients IgE to the complete rBet v 1 allergen. The degree of inhibition with the peptide antisera was almost comparable with that reported by Vrtala et al. [30] for rabbit antibodies raised against complete recombinant Bet v 1. Interestingly, we noted that the inhibition of IgE binding obtained with a mixture of all six peptide antisera was not higher than that obtained with antisera raised against certain single peptides, indicating that a few B cell epitope-derived peptides may be sufficient for vaccination against birch pollen allergy.

The biological significance of the competition results was confirmed by the demonstration that peptide-induced IgG almost completely suppressed allergen-induced degranulation of RBL cells. Whether epitope competitions of IgG with IgE or Fcγ-receptor-mediated effects or both mechanisms are responsible for reduced basophil degranulation cannot be answered at this point [31]. However, the strong inhibition of allergen-induced immediate reactions in the RBL system demonstrates that peptide vaccination induces blocking antibodies, which inhibit immediate allergic reactions.

The peptide vaccine approach described by us is different from previous attempts to use T cell epitope-containing peptides for modulation of allergen-specific T cell responses [32, 33]. While administration of free, soluble T cell epitope-containing peptides either aims at inducing tolerance or at switching towards T helper type 1-responses, our approach uses adjuvant-bound surface-exposed peptides for the induction of blocking IgG antibodies against solvent-exposed allergen domains. Since we intend to use adjuvant-bound peptides for the induction of blocking antibodies, it is unlikely that injection of peptides, even when they contain T cell epitopes, will cause T cell-mediated systemic reactions as have

been reported for T cell epitope-containing peptides of the major cat allergen, Fel d 1 [22, 33].

Hypoallergenic peptides containing surface-exposed amino acids can be produced in a well-controlled manner and represent therapeutic reagents, which can be easily purified and standardized. The latter seems important in view of the fact that vaccines based on crude allergen extracts have numerous disadvantages. For example, it has been demonstrated that allergen extracts may be contaminated with allergens from other sources [34], contain high levels of endotoxins [35] and induce IgE sensitizations to new components [36]. In addition to the advantage that peptide vaccines are well-defined, it should be possible to administer high doses with a low risk of inducing anaphylactic side-effects, particularly when they are bound to adjuvants. Administration of such high peptide doses is expected to induce a vigorous production of IgG antibodies, which may be accompanied by a beneficial modulation of various effector cell responses [26]. Since peptide vaccination of mice did not induce sensitization, our approach may be especially suitable for prophylactic vaccination.

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References

- 1 Durham SR, Till SJ. Immunologic changes associated with allergen immunotherapy. *J Allergy Clin Immunol* 1998; 102:157–64.
- 2 Valenta R. The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol* 2002; 2:446–53.
- 3 Valenta R, Kraft D. Recombinant allergen molecules: tools to study effector cell activation. *Immunol Rev* 2001; 179:119–27.
- 4 Valenta R, Kraft D. From allergen structure to new forms of allergen-specific immunotherapy. *Curr Opin Immunol* 2002; 14:718–27.
- 5 Breiteneder H, Pettenburger K, Bito A et al. The gene coding for the major birch pollen allergen Betv1, is highly homologous to a pea disease resistance response gene. *EMBO J* 1989; 8:1935–8.
- 6 Niederberger V, Pauli G, Gronlund H et al. Recombinant birch pollen allergens (rBet v 1 and rBet v 2) contain most of the IgE epitopes present in birch, alder, hornbeam, hazel, and oak pollen: a quantitative IgE inhibition study with sera from different populations. *J Allergy Clin Immunol* 1998; 102:579–91.
- 7 Kazemi-Shirazi L, Pauli G, Purohit A et al. Quantitative IgE inhibition experiments with purified recombinant allergens indicate pollen-derived allergens as the sensitizing agents responsible for many forms of plant food allergy. *J Allergy Clin Immunol* 2000; 105:116–25.

- 8 Petersen BN, Janniche H, Munch EP et al. Immunotherapy with partially purified and standardized tree pollen extracts. I. Clinical results from a three-year double-blind study of patients treated with pollen extracts either of birch or combinations of alder, birch and hazel. *Allergy* 1988; 43:353–62.
- 9 Asero R. Effects of birch pollen-specific immunotherapy on apple allergy in birch pollen-hypersensitive patients. *Clin Exp Allergy* 1998; 28:1368–73.
- 10 Gajhede M, Osmark P, Poulsen FM et al. X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nat Struct Biol* 1996; 3:1040–5.
- 11 Ferreira FD, Hoffmann-Sommergruber K, Breiteneder H et al. Purification and characterization of recombinant Bet v 1, the major birch pollen allergen. Immunological equivalence to natural Bet v 1. *J Biol Chem* 1993; 268:19574–80.
- 12 Vrtala S, Hirtenlehner K, Vangelista L et al. Conversion of the major birch pollen allergen, Bet v 1, into two nonanaphylactic T cell epitope-containing fragments: candidates for a novel form of specific immunotherapy. *J Clin Invest* 1997; 99:1673–81.
- 13 Vrtala S, Grote M, Duchene M et al. Properties of tree and grass pollen allergens: reinvestigation of the linkage between solubility and allergenicity. *Int Arch Allergy Immunol* 1993; 102:160–9.
- 14 Valent P, Besemer J, Muhm M, Majdic O, Lechner K, Bettelheim P. Interleukin 3 activates human blood basophils via high-affinity binding sites. *Proc Natl Acad Sci USA* 1989; 86:5542–6.
- 15 Wiedermann U, Jahn-Schmid B, Bohle B et al. Suppression of antigen-specific T- and B-cell responses by intranasal or oral administration of recombinant Bet v 1, the major birch pollen allergen, in a murine model of type I allergy. *J Allergy Clin Immunol* 1999; 103:1202–10.
- 16 Vrtala S, Ball T, Spitzauer S et al. Immunization with purified natural and recombinant allergens induces mouse IgG1 antibodies that recognize similar epitopes as human IgE and inhibit the human IgE-allergen interaction and allergen-induced basophil degranulation. *J Immunol* 1998; 160:6137–44.
- 17 Denepoux S, Eibensteiner PB, Steinberger P et al. Molecular characterization of human IgG monoclonal antibodies specific for the major birch pollen allergen Bet v 1. Anti-allergen IgG can enhance the anaphylactic reaction. *FEBS Lett* 2000; 465:39–46.
- 18 Hoffmann A, Vieths S, Haustein D. Biologic allergen assay for in vivo test allergens with an in vitro model of the murine type I reaction. *J Allergy Clin Immunol* 1997; 99:227–32.
- 19 Munoz V, Serrano L. Elucidating the folding problem of helical peptides using empirical parameters. *Nat Struct Biol* 1994; 1: 399–409.
- 20 Ebner C, Szepefalusi Z, Ferreira F et al. Identification of multiple T cell epitopes on Bet v 1, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J Immunol* 1993; 150:1047–54.
- 21 Bauer L, Bohle B, Jahn-Schmid B et al. Modulation of the allergic immune response in BALB/c mice by subcutaneous injection of high doses of the dominant T cell epitope from the major birch pollen allergen Bet v 1. *Clin Exp Immunol* 1997; 107: 536–41.
- 22 Haselden BM, Kay AB, Larche M. Immunoglobulin E-independent major histocompatibility complex-restricted T cell peptide epitope-induced late asthmatic reactions. *J Exp Med* 1999; 189:1885–94.
- 23 Focke M, Mahler V, Ball T et al. Nonanaphylactic synthetic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1, for allergy vaccination. *FASEB J* 2001; 15:2042–4.
- 24 Ball T, Fuchs T, Sperr WR et al. B cell epitopes of the major timothy grass pollen allergen, Phl p 1, revealed by gene fragmentation as candidates for immunotherapy. *FASEB J* 1999; 13:1277–90.
- 25 Flicker S, Valenta R. Renaissance of the blocking antibody concept in type I allergy. *Int Arch Allergy Immunol* 2003; 132:13–24.
- 26 Valenta R, Ball T, Focke M et al. Immunotherapy of allergic disease. *Adv Immunol* 2004; 82:105–53.
- 27 Visco V, Dolecek C, Denepoux S et al. Human IgG monoclonal antibodies that modulate the binding of specific IgE to birch pollen Bet v 1. *J Immunol* 1996; 157:956–62.
- 28 Mothes N, Heinzkill M, Drachenberg KJ et al. Allergen-specific immunotherapy with a monophosphoryl lipid A-adjuvanted vaccine: reduced seasonally boosted immunoglobulin E production and inhibition of basophil histamine release by therapy-induced blocking antibodies. *Clin Exp Allergy* 2003; 33:1198–208.
- 29 van Neerven RJ, Wikborg T, Lund G et al. Blocking antibodies induced by specific allergy vaccination prevent the activation of CD4+ T cells by inhibiting serum-IgE-facilitated allergen presentation. *J Immunol* 1999; 163:2944–52.
- 30 Vrtala S, Akdis C, Budak F et al. T cell epitope-containing hypoallergenic recombinant fragments of the major birch pollen allergen, Bet v 1, induce blocking antibodies. *J Immunol* 2000; 165:6653–9.
- 31 Ravetch JV, Bolland S. IgG Fc receptors. *Annu Rev Immunol* 2001; 19:275–90.
- 32 Müller U, Akdis CA, Fricker M et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J Allergy Clin Immunol* 1998; 101:747–54.
- 33 Oldfield WL, Larche M, Kay AB. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 2002; 360:47–53.
- 34 Van der Veen MJ, Mulder M, Witteman AM et al. False-positive skin test responses to commercially available dog dander extracts caused by contamination with house dust mite (*Dermatophagoides pteronyssinus*). *J Allergy Clin Immunol* 1996; 98:1028–34.
- 35 Trivedi B, Valerio C, Slater JE. Endotoxin content of standardized allergen vaccines. *J Allergy Clin Immunol* 2003; 111:777–83.
- 36 Moverare R, Elfman L, Vesterinen E, Metso T, Haahtela T. Development of new IgE reactivities to allergenic components in pollen extracts during specific immunotherapy studied with immunoblotting and the Pharmacia CAP system. *Allergy* 2002; 57:423–30.