

Vaccines for birch pollen allergy based on genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v 1

V. Mahler^{*†}, S. Vrtala[†], O. Kuss[‡], T. L. Diepgen[‡], R. Suck[§], O. Cromwell[§], H. Fiebig[§], A. Hartl[¶], J. Thalhamer[¶], G. Schuler^{*}, D. Kraft[†] and R. Valenta[†]

^{*}Department of Dermatology, University of Erlangen, Germany, [†]Department of Pathophysiology, University of Vienna, Austria, [‡]Department of Clinical Social Medicine, University of Heidelberg, [§]Allergopharma Joachim Ganzler KG, Reinbek, Germany and [¶]Institute of Chemistry and Biochemistry, University of Salzburg, Austria

Summary

Background We have recently engineered recombinant derivatives of the major birch pollen allergen Bet v 1 (rBet v 1 fragments and trimer) with strongly reduced allergenic activity.

Objective The aim of this study was the *in vivo* characterization of potential allergy vaccines based on Al(OH)₃-adsorbed genetically modified rBet v 1 derivatives in mice.

Methods BALB/c mice were immunized either with courses of nine injections of increasing doses of Al(OH)₃-adsorbed rBet v 1 wild-type, rBet v 1 fragments, rBet v 1 trimer or Al(OH)₃ alone in weekly intervals or with three high-dose injections applied in intervals of 3 weeks. Humoral immune responses to rBet v 1 wild-type and homologous plant allergens were measured by ELISA and Western blotting, and the ability of mouse antibodies to inhibit the binding of allergic patients' IgE to Bet v 1 was studied by ELISA competition experiments.

Results In both schemes, hypoallergenic rBet v 1 derivatives induced low IgE but high IgG1 responses against rBet v 1 wild-type. The IgG1 antibodies induced by genetically modified rBet v 1 derivatives cross-reacted with natural Bet v 1 and its homologues from alder (Aln g 1) as well as hazel (Cor a 1) and strongly inhibited the binding of birch pollen allergic patients' IgE to Bet v 1 wild-type.

Conclusion Genetically modified hypoallergenic rBet v 1 derivatives induce blocking antibodies *in vivo*. Their safety and efficacy for the treatment of birch pollen and associated plant allergies can now be evaluated in clinical immunotherapy studies.

Keywords genetic engineering, hypoallergenic allergen derivatives, immunotherapy, rBet v 1, type I allergy

Submitted 12 July 2002; revised 1 August 2003; accepted 30 September 2003

Introduction

Type I allergy, a genetically determined IgE-mediated hypersensitivity, affects almost 25% of the population [1]. Specific immunotherapy, the only curative approach towards allergy treatment, is based on the administration of increasing doses of the disease-eliciting allergens [2]. Although the clinical efficacy of specific immunotherapy has been documented in numerous controlled clinical studies, the underlying mechanisms are not completely understood [3, 4]. This may be attributed in part to the fact that both treatment of allergic diseases and analysis of clinical and immunological effects have been performed with natural allergen extracts consisting of mixtures of allergenic and non-allergenic components.

Another disadvantage of specific immunotherapy is the risk of inducing life-threatening anaphylactic side-effects due to

the administration of active allergens. Therefore, reduction of anaphylactic side-effects and simultaneous preservation of immunogenic properties of therapeutic allergen preparations have been long-sought goals. One way to increase the immunogenicity of a therapeutic allergen preparation and to delay the systemic release of allergens from the site of application is adsorption of allergen extracts to adjuvants [5]. Alternatively and/or in addition, chemical modification of allergen extracts was found to reduce allergenic activity [6–9].

The application of molecular biological techniques for the characterization of allergens has not only provided us with an increasing number of recombinant allergens suitable for diagnostic purposes but also opens new avenues for the generation of hypoallergenic allergen derivatives [10–16].

We have recently produced recombinant derivatives of the major birch pollen allergen, Bet v 1, which exhibited a more than 100-fold reduced allergenic activity *in vitro* and *in vivo* when compared with rBet v 1 wild-type [17–22]. Bet v 1 was chosen as a model allergen since it represents one of the most important environmental allergens. It is recognized by more than 25% of allergic individuals and 95% of birch pollen allergic patients [23, 24], and contains most of the IgE

Correspondence: Rudolf Valenta, Department of Pathophysiology, University of Vienna, Vienna General Hospital, AKH, Währinger Gürtel 18–20, A-1090 Vienna, Austria.

E-mail: rudolf.valenta@akh-wien.ac.at

epitopes present in pollen extracts from birch and related trees of the *Fagales* order and in plant-derived food [25, 26].

However, before hypoallergenic Bet v 1 derivatives can be considered for immunotherapy of allergic patients, their immunogenic properties must be evaluated *in vivo*. In this study we describe the characterization of potential allergy vaccines, which are based on $\text{Al}(\text{OH})_3$ -adsorbed genetically modified rBet v 1 derivatives (rBet v 1 fragments and rBet v 1 trimer), and compare them with rBet v 1 wild-type in BALB/c mice. We studied the induction of IgE and IgG subclass responses and compared different immunization schemes. Furthermore, we investigated whether the antibodies induced by vaccination with the recombinant allergen derivatives cross-react with the major allergens of alder and hazel pollen and whether they inhibit the binding of IgE from birch pollen allergic patients to the rBet v 1 wild-type allergen. Our finding that the genetically engineered rBet v 1 derivatives induced IgG1 and IgG2a/b antibodies in mice which cross-reacted with natural Bet v 1, as well as Bet v 1-related allergens from alder and hazel and blocked allergic patients' IgE binding to Bet v 1 is discussed regarding their use for the treatment of birch pollen and related allergies.

Materials and methods

Production of adsorbates containing recombinant antigens

Recombinant Bet v 1a was expressed in *Escherichia coli* and purified as described [27]. Recombinant Bet v 1 fragments, F1 and F2, comprising aa 1–73 (without methionine; MW 8100 Da) and aa 74–159 (MW 9461 Da) of Bet v 1, respectively, were expressed in *E. coli* and purified [17]. A stable recombinant trimer consisting of three covalently linked copies of Bet v 1a was produced by ligation of three copies of the Bet v 1a-encoding cDNA into plasmid pET-17b and expression of the recombinant trimer in *E. coli* BL21 (DE3) [19].

Recombinant antigens were dissolved in 144 mM sodium chloride, 10 mM sodium hydrogen carbonate, 0.25% w/v phenol, pH 8.0, and mixed with sterile aluminiumhydroxide suspension (Superfos Biosector, Copenhagen, Denmark) to achieve final concentrations of 1 mg Al^{3+} /mL and 100 µg protein/mL. An adsorbate containing an equimolar mixture of the two fragments was produced by mixing adsorbates prepared with each fragment in the ratio 0.46 F1:0.54 F2 to achieve equimolar concentrations of the two fragments. The resulting adsorbates contained a final total protein concentration of 100 µg/mL. Adsorbates were assessed for stability at three different temperatures (25 °C, 4 °C, –20 °C) for extended periods (30, 60, 90 days). During the storage periods, the proteins were analysed for integrity by SDS-PAGE and protein contents were determined in the supernatants of centrifuged samples of the aluminium hydroxide suspensions using the Lowry method [28].

Immunization of mice

One hundred and forty-four (72 males, 72 females) eight-week-old BALB/c mice were purchased from Charles River (Kislegg, Germany). The animals were maintained in the

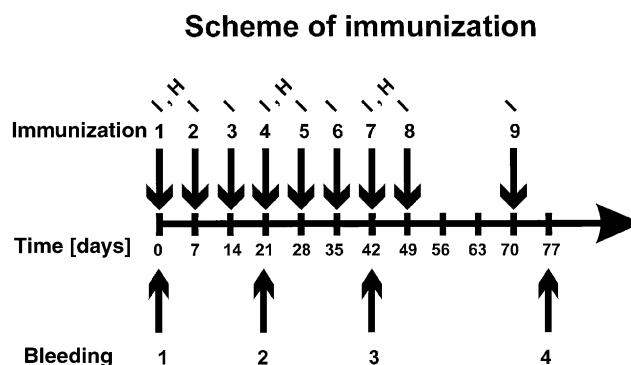


Fig. 1. Scheme of immunization. Groups of mice received either a series of 8 weekly injections containing increasing antigen doses (I) followed by one final injection on day 70 or three high-dose immunizations (H) on days 0, 21 and 42. Serum samples were collected from all mice on days 0, 21, 42 and 77.

animal care unit of the Department of Pathophysiology of the University of Vienna according to the local guidelines for animal welfare. Groups of 16 mice each (eight males, eight females) received subcutaneous immunizations in the neck either in an increasing-dose (I) scheme or as a high-dose (H) scheme (Fig. 1). Mice in the I scheme received increasing doses (0.1, 0.2, 0.4, 0.8, 1.25, 2.5, 5, 10 µg) of either $\text{Al}(\text{OH})_3$ -adsorbed rBet v 1 fragments 1 and 2, rBet v 1 trimer or rBet v 1 in weekly intervals (days 0, 7, 14, 21, 28, 35, 42, 49) until the maintenance dose of 10 µg (equivalent to 1/10 of the conceivable human dose) was reached (day 49). A control group received corresponding injections of an $\text{Al}(\text{OH})_3$ suspension. After a 1-month interval, a final injection of the maintenance dose was given at day 70 (Fig. 1). Mice in the H scheme were injected three times in 3-week intervals (days 0, 21, 42) with the maintenance dose (10 µg) (Fig. 1).

Clinical, laboratory and histological investigations of mice

The well-being of all animals was regularly assessed. Prior to each injection, the body weight of the animals was determined and the injection sites were examined for local responses. Blood samples were collected at days 0, 21, 42, and on completing the study at day 77, in order to assess humoral antibody responses. At day 77, mice were killed after bleeding. Liver, heart, kidneys, spleen and lungs from all animals were weighed and histopathology was obtained for liver, spleen, lungs, lymph nodes, thymus, skin from the injection sites and remote from the injection sites. Blood samples from day 77 were analysed for haematocrit, haemoglobin, erythrocyte counts, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), leucocyte, neutrophil and platelet counts.

ELISA measurement of specific IgE, IgG1 and IgG2a/b antibodies

Mouse antibodies with specificity for rBet v 1 and rBet v 1 fragments F1 and F2 were measured by ELISA as previously described [29]. Optical density (OD) values for the individual plates were determined after the same incubation periods. To exclude plate-to-plate variabilities, reference sera were included on each of the different plates. Results are displayed as

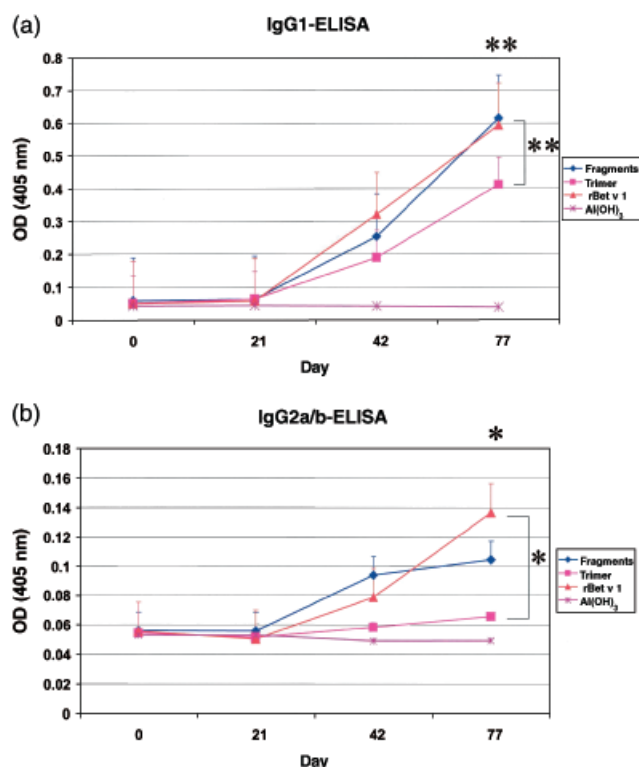


Fig. 2. (a, b) IgG1, IgG2a/b responses to rBet v 1. Groups of 16 mice each were immunized with rBet v 1 fragments, trimer, rBet v 1 wild-type or $\text{Al}(\text{OH})_3$ (inset) according to an increasing-dose scheme. Mean optical density (OD) values and standard errors (y-axis) of 16 immunized mice corresponding to the antibody levels are displayed for the different bleedings (x-axis). *P*-values are as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Asterisks above the graph report results from global tests for differences between treatments at the respective bleeding points.

mean values of OD measurements from sera of all 16 animals of each group for each bleeding.

Statistical analysis

Results are presented as mean values with standard errors in the figures and as least-squares means (adjusted means). Statistical significance of differences in antibody induction (IgG1, IgG2, IgE) was assessed within repeated measurement ANOVA models unless otherwise stated. Included factors were time of bleeding, type of vaccine, allergen dose used for immunization and gender. For evaluation of immunization schemes (I vs. H) over vaccines, an additional interaction term between vaccine and dose scheme was included. All pairwise comparisons were Bonferroni adjusted to avoid inflation of α -error. To enhance the readability of figures, we report asterisks for *P*-values as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

SDS-PAGE and immunoblotting

Protein extracts from birch (*Betula verrucosa*), alder (*Alnus glutinosa*) and hazel (*Corylus avellana*) pollen (Allergon AB, Valinge, Sweden) were prepared as described [25]. Protein contents and quality of the extracts were analysed by analytical 12.5% SDS-PAGE [30] and Coomassie blue staining [31]. Comparable amounts (100 $\mu\text{g}/\text{cm}$ preparative gel) of each protein extract were blotted onto nitrocellulose

membranes (Schleicher & Schuell, Dassel, Germany) [32]. Nitrocellulose-blotted allergen extracts were probed with two representative immune sera (fourth bleeding, day 77) and the corresponding pre-immune sera from each treatment group and bound IgG1 antibodies were detected as described [29].

Competitive ELISA for assessment of inhibition of patients' IgE binding to rBet v 1 by murine sera

Patients with birch pollen allergy (*n* = 10) were characterized by a case history indicative of birch pollen allergy, positive skin prick test results, RAST and IgE immunoblotting as described [25, 26].

ELISA plates (Nunc-Maxisorb[®], Nunc, Roskilde, Denmark) were coated with 100 $\mu\text{L}/\text{well}$ of 0.5 μg rBet v 1/mL overnight at 4 $^{\circ}\text{C}$. After five Tris-buffered saline containing Tween (TBST) washes, plates were blocked with TBST, 1% w/v bovine serum albumin (BSA) for 2 h at 37 $^{\circ}\text{C}$. Then, plates were incubated with mouse sera from each treatment group (sera from the fourth bleeding, day 77 and corresponding pre-immune sera, day 0), each diluted 1:100 in TBST, 0.5% w/v BSA. After five TBST washes, plates were incubated with human sera from birch pollen allergic patients diluted 1:5 in TBST, 0.5% w/v BSA overnight at 4 $^{\circ}\text{C}$. After five TBST washes, bound human IgE was detected with alkaline phosphatase (AP)-coupled mouse monoclonal anti-human-IgE antibodies (PharMingen, San Diego, CA, USA) diluted 1:1000 in TBST, 0.5% w/v BSA and was incubated at 37 $^{\circ}\text{C}$ and then at 4 $^{\circ}\text{C}$ for 1 h. After a further five washes with TBST, the ELISA substrate (Sigma, St Louis, MO, USA) was added and the OD of the colour reaction was read at 405 nm after 15 min. All measurements were performed as duplicates.

The percentage of inhibition of IgE after pre-incubation with immune sera was calculated as follows: % inhibition = $100 - (\text{OD}_{\text{immune}} \times 100) / \text{OD}_{\text{pre-immune}}$.

$\text{OD}_{\text{pre-immune}}$ and $\text{OD}_{\text{immune}}$ represent the OD after pre-incubation with murine pre-immune and immune sera, respectively.

Rat basophil leukaemia (RBL) cell mediator release

RBL-2H3 cells were plated in 96-well tissue culture plates ($4 \times 10^4/\text{well}$) and incubated for 24 h at 37 $^{\circ}\text{C}$ using 7% CO_2 . First, experiments were performed to study the anaphylactic activity of sera from mice (eight females, eight males) that had been immunized with rBet v 1, rBet v 1 trimer and rBet v 1 fragments 1 and 2, respectively, using either the I or the H scheme. RBL cells were loaded with the respective sera, diluted 1:20 in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl_2 , 1.8 mM CaCl_2 , 0.4 mM NaH_2PO_4 , 5.6 mM D-glucose, 12 mM NaHCO_3 , 10 mM HEPES and 0.1% BSA, pH 7.2) and cross-linked with rBet v 1 (*c* = 0.3 $\mu\text{g}/\text{mL}$). In a second type of experiments, the blocking activity of rabbit anti-rBet v 1 derivative antisera on Bet v 1-induced basophil degranulation was studied. Rabbit α Bet v 1 trimer or rabbit α Bet v 1 fragment immune complexes were formed by incubating 0%, 2%, 5% or 10% rabbit sera or normal rabbit serum with 0.1 $\mu\text{g}/\text{mL}$ rBet v 1 in Tyrode's buffer for 2 h at 37 $^{\circ}\text{C}$. These immune complexes were exposed to RBL cells, which had been passively sensitized (1:30 diluted sera, 2 h, 37 $^{\circ}\text{C}$) with anaphylactic murine sera raised against rBet v 1

wild-type [29]. RBL supernatants were analysed for β -hexosaminidase activity by incubation with 80 μ M 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide (Sigma, Vienna, Austria) in citrate buffer (0.1 M, pH 4.5) for 1 h at 37 °C [33]. The reaction was stopped by the addition of 100 μ L glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7) and fluorescence was measured at λ_{ex} 360/ λ_{em} 465 nm using a fluorescence microplate reader (Spectrafluor, Tecan, Austria). Results are reported as percentage of total β -hexosaminidase released after the addition of 1% Triton X-100.

Results

Stability and toxicity assessment of recombinant allergen adsorbates

The analysis of the individual proteins and adsorbates revealed no evidence of degradation during the course of the stability study (30, 60 and 90 days storage). The pH of the fragment adsorbates was in the range between 7.9 and 8.2, and that of the trimer adsorbates was between 8.0 and 8.3. Repeated dose toxicity studies in mice revealed no evidence for histopathological alterations induced by the allergen adsorbates at the injection site or systemically other than those induced by the adjuvant alone. Comparing the haematological parameters in the different mouse groups, no significant differences were found between the groups receiving active preparations or adjuvant alone (data not shown).

Immunoglobulin responses to rBet v 1 derivatives administered in increasing concentrations

Sera from the differently immunized groups of mice were analysed for the presence of rBet v 1-specific IgG1 and IgG2a/b antibodies (Figs 2a and b). Figures 2a and b display the antibody responses determined for each group ($n = 16$) that had received a course of nine injections of increasing antigen concentrations (i.e. I scheme). Serum samples obtained at the fourth bleeding (day 77) contained the highest levels of Bet v 1-specific IgG1 antibodies (Fig. 2a). The strongest IgG1 response was induced by repeated vaccination with the rBet v 1 fragment mixture and the unmodified rBet v 1 followed by the trimer (Fig. 2a). The mean rBet v 1-specific IgG1 antibody responses (least-squares means) at day 77 were 0.622 (fragments), 0.593 (rBet v 1), 0.411 (trimer), 0.03 ($\text{Al}(\text{OH})_3$). All responses were significantly greater than those with $\text{Al}(\text{OH})_3$ alone ($P < 0.001$). Sera from mice immunized with the equimolar fragment mixture mounted significantly higher IgG1 responses to fragment 2 than to fragment 1 ($P < 0.001$, data not shown). At day 77, IgG1 antibody levels induced by fragments ($P = 0.004$) and rBet v 1 wild-type ($P = 0.009$) are significantly higher than those induced by trimer (Fig. 2a). Recombinant vaccine formulations also induced IgG2a/b antibodies (Fig. 2b). rBet v 1 fragment mixture and rBet v 1 wild-type induced higher levels of rBet v 1-specific IgG2a/b than the trimer formulation (Fig. 2b). The mean IgG2a/b antibody responses (least-squares means) obtained at the fourth bleeding (day 77) were 0.136 (rBet v 1), 0.103

(fragments), 0.065 (trimer) and 0.049 ($\text{Al}(\text{OH})_3$). IgG2a/b antibodies are induced the strongest by the wild-type with significantly higher levels ($P = 0.021$) than by the trimer.

Of the three recombinant preparations, the trimer induced significantly more IgE than fragments ($P = 0.036$) and rBet v 1 wild-type ($P = 0.041$) at day 77 (data not shown).

Immunoglobulin responses to rBet v 1 derivatives administered as three high-dose injections

Comparing Bet v 1-specific IgG1 and IgG2a/b antibody levels of mice of the I scheme (comprising a course of nine injections with antigen amounts gradually increasing to 10 μ g) with mice of the H scheme (consisting of three injections of the maintenance dose of 10 μ g antigen) no significant differences were found at the third bleeding (Figs 2a and 3). At the time of third and fourth bleeding, immunization according to the H scheme with rBet v 1 fragments (Fig. 4a: $P = 0.008$ at day 42), rBet v 1 wild-type (Fig. 4c: $P = 0.015$ at day 42; $P = 0.037$ at day 77) and trimer (Fig. 4b) induced lower IgE levels than the I scheme.

Hypoallergenic rBet v 1 derivatives induce IgG1 antibodies that cross-react with natural Bet v 1 and Bet v 1-related allergens

Sera from mice treated with rBet v 1 fragments, trimer and rBet v 1 wild-type exhibited IgG1 reactivity to nitrocellulose-blotted natural Bet v 1 in a birch pollen extract at 17 kDa (Fig. 5a, lanes 1i–4i). Pre-immune sera obtained from the same mice, as well as sera from mice treated with the adjuvant alone, did not contain Bet v 1-specific IgG1 (Fig. 5a, lanes 1p–4p; $\text{Al}(\text{OH})_3$). The immune sera also recognized the major alder pollen allergen Aln g 1 (Fig. 5b) and the major hazel pollen allergen Cor a 1 (Fig. 5c) at 17 kDa.

Hypoallergenic rBet v 1 derivatives induce antibodies that inhibit binding of IgE from allergic patients to rBet v 1 wild-type

Representative serum samples (one male, one female) from each mouse group obtained at the fourth bleeding, together with the corresponding pre-immune sera as controls, were

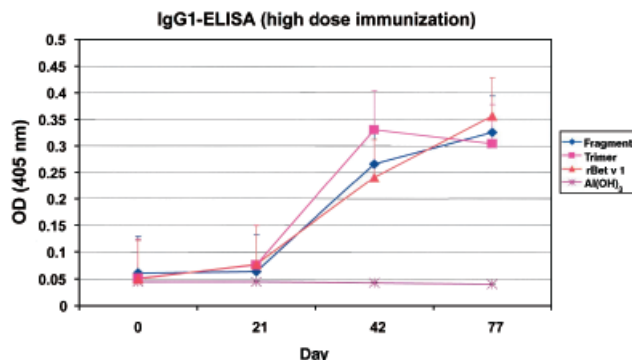


Fig. 3. IgG1 response to rBet v 1 after immunization according to the high-dose scheme. Groups of 16 mice each were immunized with rBet v 1 fragments, trimer, rBet v 1 wild-type or $\text{Al}(\text{OH})_3$ (inset) according to the high-dose scheme. Mean optical density (OD) values (y-axis) of 16 immunized mice corresponding to the antibody levels are displayed for the different bleedings (x-axis).

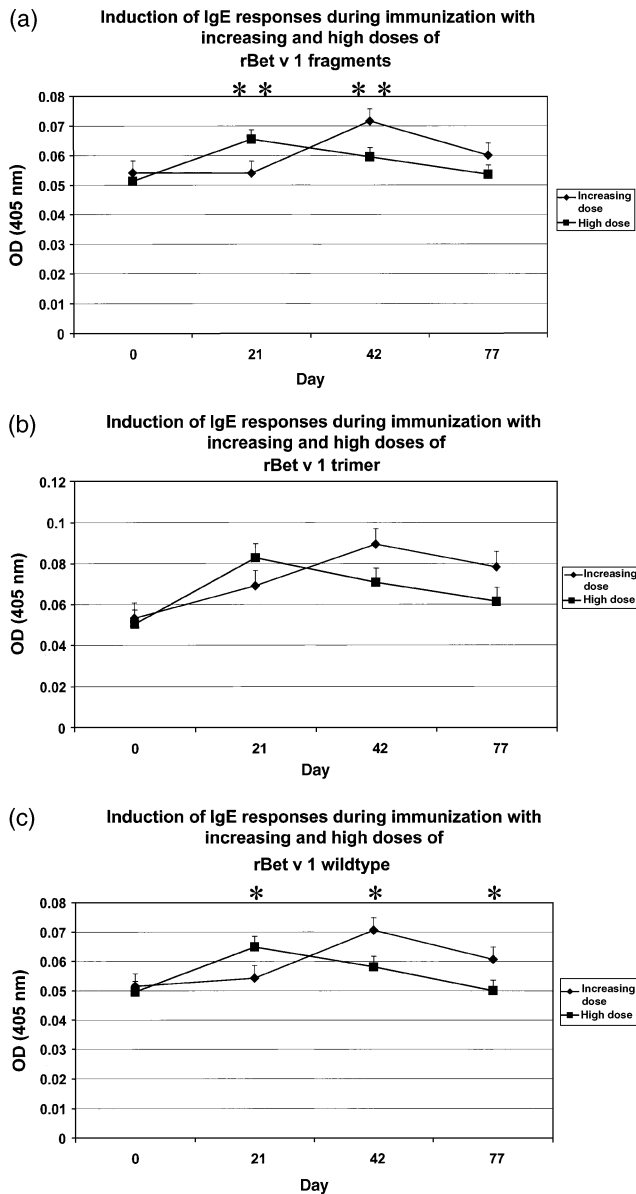


Fig. 4. (a–c) IgE responses to rBet v 1 in mice having received high- or increasing-dose immunizations. Mean optical density (OD) values with standard errors corresponding to Bet v 1-specific IgE levels (y-axis) are displayed for the mouse groups (a: fragments; b: trimer; c: wild-type) treated by the increasing- and high-dose scheme for the different bleedings (x-axis). *P*-values are as follows: **P* < 0.05, ***P* < 0.01, ****P* < 0.001. Asterisks above the graph report results from global tests for differences between dose schemes at the respective bleeding points.

analysed for their ability to block human IgE binding to rBet v 1 (Table 1). Despite the fact that mouse sera were diluted 1:100, they potentially blocked IgE binding to Bet v 1. Sera from rBet v 1 fragment-immunized mice (increasing scheme) inhibited patients' IgE binding between 58.3% and 89.3% (mean 74.6%) (Table 1). Pre-incubation with sera from trimer-immunized mice (increasing scheme) yielded an inhibition of IgE binding between 14.6% and 85.2% (mean 50.2%) and sera from mice immunized with rBet v 1 wild-type achieved an inhibition of patients' IgE binding from 32.8% to 84.0% (mean 62.8%). A substantial inhibition of IgE binding to rBet v 1 was also obtained with sera from

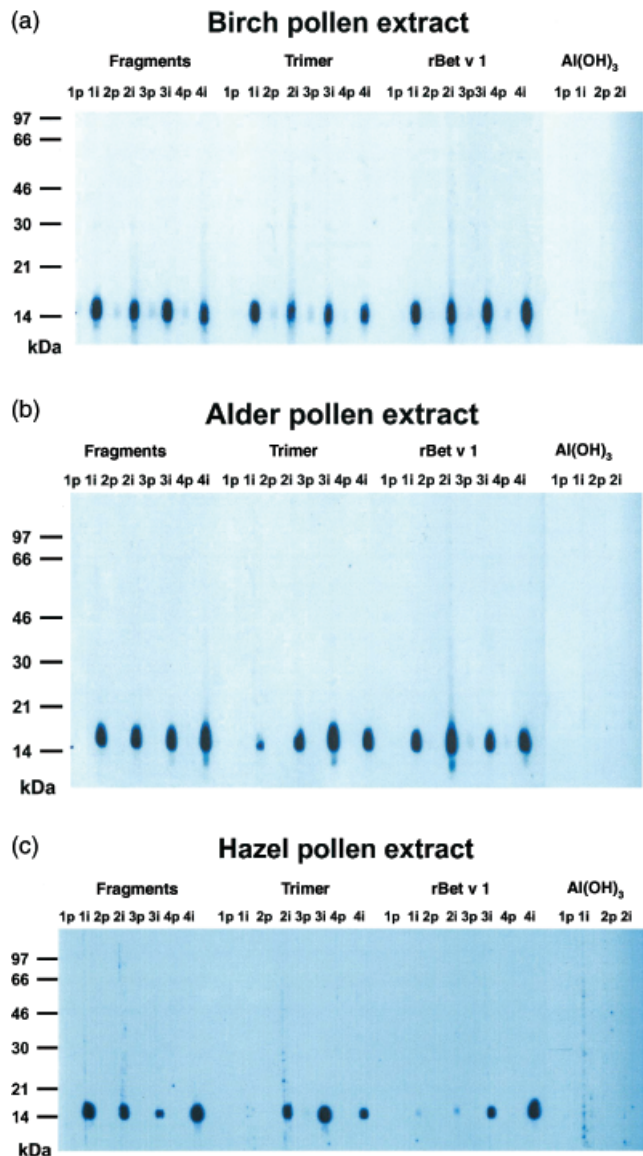


Fig. 5. (a–c) IgG1 reactivity of mice to nitrocellulose-blotted pollen extracts. Sera (p, pre-immune sera; i, immune sera) from mice immunized with rBet v 1 fragments, trimer, rBet v 1 wild-type (#1 and 2: increasing-dose protocol; #3 and 4: high-dose scheme) or Al(OH)₃ alone were exposed to nitrocellulose-blotted birch pollen (a), alder pollen (b) and hazel pollen (c) extract. Molecular masses are displayed in kDa at the left side.

mice immunized according to the H scheme (fragments: 18.1–59.5%, mean 45.2%; trimer: 11.6–46.6%, mean 27.4%; wild-type: 0–52.9%, mean 24.9%) (Table 1).

Sera from mice immunized according to the high-dose protocol induce less RBL mediator release than sera from mice immunized with increasing doses

To confirm that high-dose immunization induces less IgE production than immunization according to the increasing-dose protocol, RBL cells were incubated with sera from the various groups and challenged with rBet v 1.

Figure 6a shows that rBet v 1 always induced much less mediator release from RBL cells that had been incubated with mouse sera obtained after immunization with the H scheme

Table 1. Inhibition of patients' IgE binding to rBet v 1 by murine antisera

	Percentage inhibition of patients' (P1–P10) IgE binding to rBet v 1										
Murine antiserum raised against	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	Mean inhibition
Increasing-dose scheme											
Fragments (m)	75.05	89.38	79.97	81.32	76.31	86.66	65.66	65.27	60.52	80.60	76.07
Fragments (f)	72.33	87.00	79.89	78.04	67.74	73.57	63.30	76.32	58.36	76.65	73.32
Trimer (m)	39.54	35.83	36.80	35.25	28.93	33.04	31.62	35.95	14.65	21.59	31.32
Trimer (f)	67.58	85.26	72.47	72.30	65.59	80.79	56.77	70.00	50.00	71.66	69.24
rBet v 1 (m)	63.24	75.10	59.30	44.66	61.45	69.31	42.56	58.01	42.65	62.41	57.87
rBet v 1 (f)	75.18	84.01	76.88	73.16	73.20	79.64	32.80	61.38	47.03	75.88	67.92
High-dose scheme											
Fragments (m)	57.14	59.53	53.56	51.87	45.81	55.65	39.56	52.60	24.24	18.18	45.81
Fragments (f)	49.93	56.81	49.70	51.89	40.34	51.99	31.01	43.41	29.90	42.58	44.76
Trimer (m)	21.08	33.46	20.93	27.65	20.64	28.05	11.63	26.50	13.23	17.99	22.12
Trimer (f)	38.44	46.66	35.79	41.86	24.85	42.22	14.63	31.84	20.53	31.02	32.78
rBet v 1 (m)	15.37	21.16	13.59	7.91	6.12	25.40	−10.28	15.24	23.30	38.07	15.59
rBet v 1 (f)	41.77	52.93	46.70	26.55	22.91	28.72	17.76	24.46	33.71	47.87	34.34

ELISA-plate bound rBet v 1 was pre-incubated with representative sera from male (m) or female (f) mice (increasing-, high-dose scheme) which were immunized with fragments, trimer, rBet v 1, or Al(OH)₃, respectively. The percentage inhibition of IgE binding is displayed for sera from 10 birch pollen allergic patients (P1–P10). The right column shows the mean percentage inhibition of IgE binding for the patients.

than with mouse sera obtained after immunization with the I scheme. This is in agreement with the results of the ELISA experiments (Fig. 4), which showed that immunization with the H scheme induced significantly lower IgE levels than immunization with the I scheme.

Antibodies raised against Bet v 1 derivatives inhibit Bet v 1-induced release of mediators from RBL cells

To demonstrate the protective activity of antibodies induced with rBet v 1 derivatives, RBL cell mediator release inhibition experiments were performed. rBet v 1 was pre-incubated with rabbit anti-rBet v 1 trimer, anti-rBet v 1 fragment antisera and a normal rabbit serum, respectively, and then exposed to RBL cells that had been loaded with Bet v 1-specific IgE. Figure 6b shows that rabbit anti-rBet v 1 trimer as well as anti-rBet v 1 fragments antisera cause a dose-dependent inhibition of degranulation and β -hexosaminidase release from RBL cells with an almost complete suppression when 10% serum was added. No relevant inhibition of mediator release was noted when a normal rabbit serum was pre-incubated with Bet v 1.

Discussion

Recently, several research groups have started to produce genetically modified allergen derivatives, which preserve many of the primary structure elements of the respective wild-type allergens, but exhibit strongly reduced allergenic activity (reviewed in [10, 11]). The reduction of allergenic activity of a given allergen derivative can be evaluated by *in vitro* assays (e.g., IgE binding assays, basophil histamine release tests) and by *in vivo* provocation testing (e.g., skin testing), but it is equally important to determine whether the allergen derivative can induce an immune response that may protect against exposure to the wild-type allergen.

In this study we have evaluated the immunogenic activities of vaccine formulations, which are based on genetically modified hypoallergenic derivatives of the major birch pollen allergen Bet v 1. In order to obtain vaccine formulations that may receive permission from regulatory authorities for clinical evaluation in allergic patients, Al(OH)₃ adsorbates of two hypoallergenic rBet v 1 fragments and of a hypoallergenic rBet v 1 trimer were prepared following a protocol that is currently used for allergen extract-based immunotherapy. The immunogenicity of both vaccines was compared with that of the active wild-type allergen by immunizing mice. The potential protective effect of the vaccine was evaluated by testing whether antibodies induced in mice can inhibit the binding of allergic patients IgE to the wild-type allergen.

Both vaccine formulations containing genetically modified rBet v 1 derivatives induced strong IgG1 responses to rBet v 1 wild-type when administered in an I scheme but induced only weak *de novo* IgE production. Notably, we found that a high-dose regimen consisting of three injections of the maintenance dose also induced strong IgG1 responses to rBet v 1 wild-type, but even lower *de novo* IgE responses than the I scheme. Perhaps even more important was the finding that sera obtained from mice having received the high-dose immunization induced much less degranulation of rat basophils than sera from mice treated with increasing doses.

Our finding that genetically modified rBet v 1 derivatives induced IgG1 antibodies in mice which cross-reacted with the major allergens of alder and hazel pollen indicates that they may not only be useful for the treatment of birch pollen but also for the treatment of birch-associated allergies (e.g., allergy to alder and hazel pollen). In this context, it is noteworthy that previous clinical immunotherapy studies with natural birch pollen extract indicated that treatment with birch pollen extract reduced allergic symptoms to pollen of trees of the *Fagales* order and even allergy to apple [34–36].

More important than the fact that genetically modified rBet v 1 derivatives induced IgG antibodies to rBet v 1 wild-type

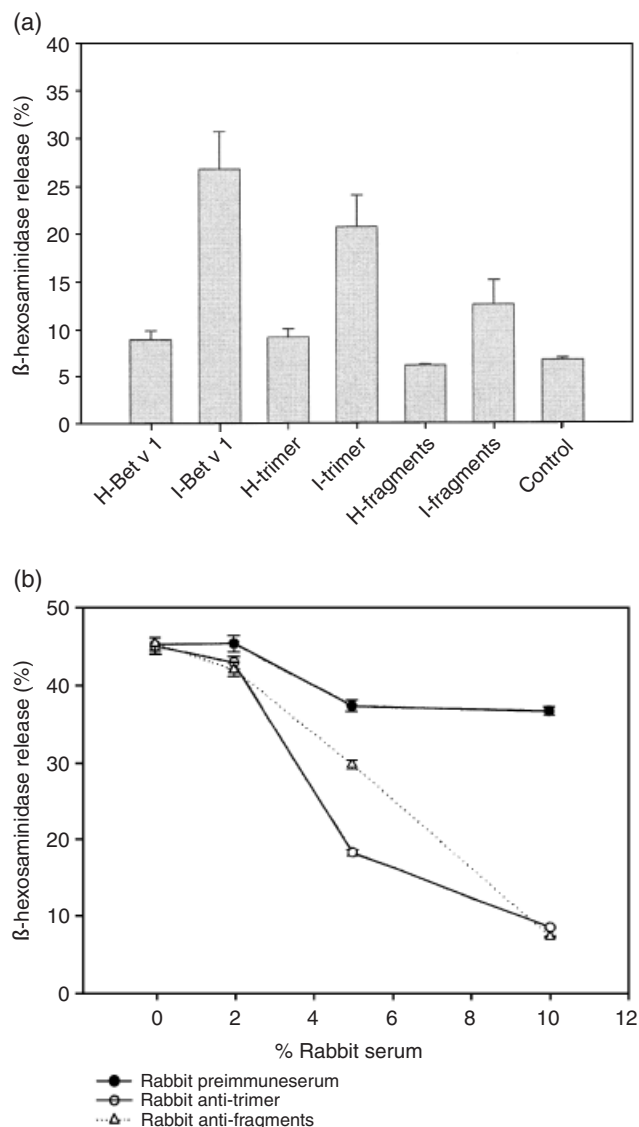


Fig. 6. (a) Induction of mediator release from rat basophil leukaemia (RBL) cells with sera from immunized mice. RBL cells were loaded with mouse sera obtained at day 77 after immunization with rBet v 1, rBet v 1 trimer and rBet v 1 fragments, respectively, using the high-dose (H) or the increasing-dose (I) scheme. Release of β -hexosaminidase (y-axis: % release) was induced with rBet v 1. Results are displayed as mean values $\pm$ SD. (b) Rabbit anti-rBet v 1 derivative antibodies inhibit Bet v 1-induced RBL cell mediator release. rBet v 1 was pre-incubated with different dilutions (x-axis: 0%, 2%, 5% and 10%) of rabbit anti-rBet v 1 trimer (white circles), rabbit anti-rBet v 1 fragments (triangles) and rabbit pre-immunesera (black circles), respectively, and then exposed to RBL cells loaded with mouse anti-rBet v 1 IgE. The percentage of β -hexosaminidase release into the supernatants is displayed as mean $\pm$ SD on the y-axis.

and to Bet v 1-homologous allergens is the finding that mouse-derived antibodies strongly inhibited the binding of birch pollen allergic patients' IgE to Bet v 1. Despite the fact that mouse sera were diluted 1:100 for the blocking experiments, we found that fragment- and trimer-specific antibodies induced by the increasing-dose protocol yielded a mean inhibition of human IgE binding of 75% and 50%, respectively. The mean inhibition obtained with sera from mice immunized with the high-dose protocol ranged between 45% in the case of the fragments and 27% for the trimer. These results indicate that the genetically modified rBet v 1

derivatives contain sufficient structural elements to induce protective IgG antibodies *in vivo*, which either bind to or close to epitopes defined by human IgE antibodies and thus prevent IgE binding to the wild-type allergen. This finding is in agreement with recent reports re-confirming the importance of allergen-specific blocking antibodies of the IgG isotype for the successful outcome of specific immunotherapy [37, 38]. In this context, it has been shown that blocking antibodies not only suppress allergen-induced effector cell activation but inhibit IgE-mediated allergen presentation to T cells, and thus can reduce proliferation and cytokine release from allergen-specific T cells [37, 39]. That IgG antibodies induced by the Bet v 1 derivatives have indeed protective activity is demonstrated by the inhibition of Bet v 1-induced RBL degranulation with rabbit sera containing such antibodies whereas normal rabbit serum had no relevant effect (Fig. 6b).

Skin test studies have shown that allergic patients tolerated approximately the 100-fold dose of the rBet v 1 derivatives [20–22]. We thus hope that it may be possible to develop immunotherapy protocols that are based on a few high-dose injections, an assumption that is supported by the notion that the high-dose immunization scheme also induced blocking antibody responses. Another advantage of using recombinant hypoallergenic allergen derivatives for specific immunotherapy may be that treatment can be conducted with pharmaceutically defined allergen preparations according to the patients' sensitization profile [40, 41]. As a next step, clinical immunotherapy trials will have to evaluate the efficacy and safety of the hypoallergenic rBet v 1 derivative vaccine formulations in birch pollen allergic patients.

Acknowledgements

This work was supported by grant Y078GEN of the Austrian Science Foundation, CeMM (Center of Molecular Medicine, a basic research institute within the companies of the Austrian Academy of Sciences), Pharmacia AB Diagnostics (Uppsala, Sweden), Allergopharma Joachim Ganzer KG (Reinbek, Germany) and BIOMAY (Vienna, Austria). V. M. was supported by grant Ma 1997/1–1 of the German research society (DFG).

References

- Kay AB. Allergy and allergic diseases. Oxford: Blackwell Science, 1997.
- Bousquet J, Lockey R, Mallon HJ and the WHO panel members. Allergen immunotherapy: therapeutic vaccines for allergic diseases. A WHO position paper. *J Allergy Clin Immunol* 1998; 102:558–62.
- Durham SR, Till SJ. Immunological changes associated with allergen immunotherapy. *J Allergy Clin Immunol* 1998; 102:157–64.
- Valenta R. The future of antigen-specific immunotherapy of allergy. *Nat Rev Immunol* 2002; 2:446–53.
- Møllerup MT, Hahn GW, Poulsen LK, Mallon HJ. Safety of allergen-specific immunotherapy Relation between dosage regimen, allergen extract, disease and systemic side-effects during induction treatment. *Clin Exp Allergy* 2000; 30:1423–9.

- 6 Lee WY, Sehon AH. Abrogation of reaginic antibodies with modified allergens. *Nature* 1977; 267:618–9.
- 7 Marsh DG, Lichtenstein LM, Campell DH. Studies on 'allergoids' prepared from naturally occurring allergens I Assay of allergenicity and antigenicity of formalized rye group I component. *Immunol* 1970; 18:705–22.
- 8 Metzger WJ, Dorminey HC, Richerson HB, Weiler JM, Donnelly A, Moran D. Clinical and immunologic evaluation of glutaraldehyde-modified tyrosine-adsorbed short ragweed extract: a double-blind, placebo-controlled trial. *J Allergy Clin Immunol* 1981; 68:442–8.
- 9 Maasch HJ, Marsh DG. Standardized extracts modified allergens-allergoids. *Clin Rev Allergy* 1987; 5:89–106.
- 10 Valenta R, Kraft D. From allergen structure to new forms of allergen-specific immunotherapy. *Curr Opin Immunol* 2002; 14:718–27.
- 11 Singh MB, de Weerd N, Bhalla P. Genetically engineered plant allergens with reduced anaphylactic activity. *Int Arch Allergy Immunol* 1999; 119:75–5.
- 12 Ferreira F, Rohlf A, Hoffmann-Sommergruber K et al. Modulation of IgE-binding properties of tree pollen allergens by site-directed mutagenesis. In: Sehon A, HayGlass KT, Kraft D, eds. *New horizons in allergy immunotherapy*. Plenum Press, New York and London, 1996; 127–36.
- 13 Smith AM, Chapman MD. Reduction of IgE antibody binding to rDer p 2 variants generated by site-directed mutagenesis. In: Sehon A, HayGlass KT, Kraft D, eds. *New horizons in allergy immunotherapy*. Plenum Press, New York and London, 1996; 391–94.
- 14 Smith AM, Chapman MD. Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. *Mol Immunol* 1996; 33:399–405.
- 15 Takai T, Yokota T, Yasue M et al. Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. *Nat Biotechnol* 1997; 15:754–8.
- 16 Ferreira F, Ebner C, Kramer B et al. Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy. *FASEB J* 1998; 12:231–42.
- 17 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.
- 18 Vrtala S, Akdis CA, 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.
- 19 Vrtala S, Hirtenlehner K, Susani M et al. Genetic engineering of a hypoallergenic trimer of the major birch pollen allergen Bet v 1. *FASEB J* 2001; 15:2045–7.
- 20 van Hage-Hamsten M, Kronqvist M, Zetterstrom O et al. Skin test evaluation of genetically engineered hypoallergenic derivatives of the major birch pollen allergen, Bet v: results obtained with a mix of two recombinant Bet v 1 fragments and recombinant Bet v 1 trimer in a Swedish population before the birch pollen season. *J Allergy Clin Immunol* 1999; 104:969–77.
- 21 Pauli G, Purohit A, Oster J et al. Comparison of genetically engineered hypoallergenic rBet v 1 derivatives with rBet v 1 wild-type by skin prick and intradermal testing: results obtained in a french population. *Clin Exp Allergy* 2000; 30:1076–84.
- 22 Nopp A, Hallden G, Lundahl J et al. Comparison of inflammatory responses to genetically engineered hypoallergenic derivatives of the major birch pollen allergen Bet v 1 and to recombinant Bet v 1 wild type in skin chamber fluids collected from birch pollen-allergic patients. *J Allergy Clin Immunol* 2000; 106:101–9.
- 23 Breiteneder H, Pettenburger K, Bito A et al. The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to a pea disease resistance response gene. *EMBO J* 1989; 8:1935–8.
- 24 Valenta R, Duchêne M, Vrtala S et al. Recombinant allergens for immunoblot diagnosis of tree pollen allergy. *J Allergy Clin Immunol* 1991; 88:889–94.
- 25 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.
- 26 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.
- 27 Hoffmann-Sommergruber K, Susani M, Ferreira F et al. High-level expression and purification of the major birch pollen allergen, Bet v 1. *Protein Expr Purif* 1997; 9:33–9.
- 28 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; 193:265–75.
- 29 Vrtala S, Ball T, Spitzauer S et al. Immunization with purified natural and recombinant allergens induce 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.
- 30 Fling SP, Gregerson DS. Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. *Anal Biochem* 1986; 155:83–8.
- 31 Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein–dye-binding. *Anal Biochem* 1976; 72:248–54.
- 32 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; 76:4350–4.
- 33 Hochreiter R, Stepanoska T, Ferreira F et al. Prevention of allergen-specific IgE production and suppression of an established Th2-type response by immunization with DNA encoding hypoallergenic allergen derivatives of Bet v 1, the major birch pollen allergen. *Eur J Immunol* 2003; 33:1667–76.
- 34 Petersen BN, Janniche H, Munch EP et al. 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.
- 35 Asero R. Effects of birch pollen-specific immunotherapy on apple allergy in birch pollen hypersensitive patients. *Clin Exp Allergy* 1998; 28:1368–73.
- 36 Herrmann D, Henzgen M, Frank E, Rudeschko O, Jäger L. Effect of hyposensitization for tree pollinosis on associated apple allergy. *J Invest Allergol Clin Immunol* 1995; 5:259–67.
- 37 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.
- 38 Ball T, Sperr WR, Valent P et al. Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy. *Eur J Immunol* 1999; 29:2026–36.
- 39 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.
- 40 Valenta R, Lidholm J, Niederberger V, Hayek B, Kraft D, Grönlund H. The recombinant allergen-based concept of component-resolved diagnostics and immunotherapy (CRD and CRIT). *Clin Exp Allergy* 1999; 29:896–904.
- 41 Valenta R, Kraft D. Vaccines for Type I allergy obtained by genetic engineering and synthetic peptide chemistry. In: Hansel TT, Barnes PJ, eds. *New drugs for asthma, allergy and COPD. Progress in Respiratory Research*, Vol. 31. Basel: Karger, 2000; 195–200.