

Mechanisms of allergy

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

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Background: Nearly 60% of birch pollen-allergic patients react exclusively to Bet v 1. With use of the skin blister model, previously only established for installation of crude allergens, we have for the first time characterized the inflammatory response in vivo to recombinant birch pollen allergen, rBet v 1, molecules (rBet v 1 wild type, fragments and trimer).

Objective: Our purpose was to examine whether challenge with rBet v 1 derivatives (fragments and trimer) compared with rBet v 1 wild type differs with respect to influx of activated eosinophils and detectable levels of cytokines/chemokines related to allergic inflammation in skin chambers applied to birch pollen-allergic patients.

Methods: The skin blister chambers were filled for 2 hours with rBet v 1, the derivatives or PBS and heparin (negative control). The fluids were analyzed after 2 and 8 hours. The number of eosinophils was determined and EG2 and CD69 expression measured by flow cytometry. Cytokines and mediators were analyzed by ELISA and RIA techniques.

Results: Comparable numbers of eosinophils were recruited to the chambers challenged with rBet v 1 molecules, but the eosinophils from the rBet v 1 wild-type challenged chambers showed a significantly higher expression of CD69. The levels of eotaxin were similar in all 4 chambers, whereas rBet v 1 wild type induced significantly higher levels of histamine, eosinophil cationic protein, and GM-CSF than the derivatives did. Recombinant Bet v 1 trimer elicited significantly lower levels of IL-4 compared with rBet v1 wild type.

Conclusion: Genetically engineered hypoallergenic rBet v 1 derivatives recruited eosinophils analogously with rBet v 1 wild type. However, the derivatives exhibited a lower capacity to activate eosinophils and to release proinflammatory mediators and T helper type 2-derived cytokines. The derivatives may therefore be candidate molecules for specific immunotherapy of birch pollen allergy with reduced risk of inducing allergenic or inflammatory side effects. (*J Allergy Clin Immunol* 2000;106:101-9.)

Key words: Recombinant birch pollen allergens, rBet v 1, hypoallergenic derivatives, skin blister chambers, eosinophils, CD69, eosinophil cationic protein

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Tree pollen allergens and, above all, allergens from birch pollen (*Betula verrucosa*) are very common and potent elicitors of allergic symptoms.¹ More than 90% of birch pollen-allergic patients in middle and northern Europe display IgE reactivity with the major birch pollen allergen Bet v 1² and nearly 60% of them react solely to Bet v 1.¹ One approach to reduce the anaphylactogenic potential of the major birch pollen allergen Bet v 1³ has been to disrupt the conformation of the molecule by cutting it into two halves. Two fragments representing amino acids 1-74 and 75-160 were produced by recombinant DNA technology and expressed in *Escherichia coli*.⁴ These fragments have almost completely lost their IgE-binding capacity and allergenic activity as a result of loss of their native-like fold. By another approach, three Bet v 1 molecules have been linked together by recombina-

Abbreviations used

BAL:	Bronchoalveolar lavage
ECP:	Eosinophil cationic protein
EG2:	Epitope on ECP, mAb to ECP
MFI:	Mean fluorescence intensity
PBE:	Peripheral blood eosinophil
r:	Recombinant
RAST:	Radioallergosorbent test

nant technique to form a trimer.⁵ One possible explanation for the finding that the recombinant (r) Bet v 1 trimer has low IgE reactivity could be that the oligomerization process has caused a reorientation of IgE epitopes, which although being recognized by IgE antibodies may be less effective in cross-linking effector cell-bound IgE.

We recently demonstrated by the aid of end point skin test titration a reduced IgE reactivity produced by the recombinant derivatives compared with rBet v 1 wild type.^{6,7} According to the established reactivity profiles, patient-tailored forms of immunotherapy (component-resolved immunotherapy) now appear to be possible. However, before hypoallergenic derivatives can be used for specific immunotherapy, extensive studies need to be performed *in vivo* to elucidate their role as triggers of the allergic inflammatory response in comparison with that of wild-type rBet v 1. In contrast to peptide immunotherapy, we aim to produce adsorbates with our hypoallergenic molecules. The proteins will then be subcutaneously deposited and it is therefore important to minimize possible inflammatory activity (eg, eosinophil activation, chemokine and cytokine release).

Eosinophil recruitment is suggested to be triggered by specific stimuli such as IL-4, IL-5, and the chemokine eotaxin as well as GM-CSF.⁸ Previous studies have shown that eosinophils recruited to the lungs in patients with symptomatic asthma are in an activated state. For example, increased levels of released eosinophil cationic protein (ECP) have been detected in bronchoalveolar lavage (BAL) fluid⁹ and sputum¹⁰ in allergic patients. Furthermore, an increased expression of the EG2-epitope on intracellular-expressed ECP in lung tissue has also been noted.¹¹ Freshly isolated blood eosinophils and neutrophils do not express CD69, but eosinophils can after *in vitro* stimulation with GM-CSF, IL-5, IL-3,^{12,13} and IFN- γ ,¹² mobilize CD69 to the cell surface. Moreover, it has been reported that BAL eosinophils express surface CD69.¹³

In previous studies we demonstrated an increased recruitment of eosinophils to skin chambers incubated with anti-IgE in atopic subjects¹⁴ as well as with relevant crude allergen¹⁵ compared with control chambers. Given that different birch pollen allergen may elicit various local inflammatory responses, we compared the induction of eosinophil accumulation and mediator release by rBet v 1 fragments and rBet v 1 trimer with that of rBet v 1 wild type in blister fluid from skin chambers applied on patients with birch pollen allergy. Expression of the

eosinophil activity markers CD69 and the EG2 epitope on intracellular ECP, as well as levels of released ECP, IL-4, GM-CSF, IFN- γ , and eotaxin, were measured.

MATERIAL AND METHODS

Subjects

The study group consisted of 9 allergic patients, 4 men and 5 women, aged 18 to 57 years (mean age 32 years) with a history of birch pollen-related allergic rhinitis and/or mild bronchial asthma (Table I). The patients were positive to rBet v 1 in skin prick test $\geq 3+$, had end point concentrations from intradermal titration tests in 10-fold dilution steps up to 1 mg/mL rBet v 1 (7.5–15 mm wheal at 10 min), and were RAST rBet v 1 positive ≥ 2 kU_A/L (Pharmacia CAP RAST System, Pharmacia & Upjohn, Uppsala, Sweden) (Table I).

All subjects were asymptomatic and drug free except for inhaled β_2 -agonists, which were allowed on an as-needed basis. There was no evidence of current airway infection. The study was performed out of the pollen season. The subjects gave their informed consent to participate in the study, which was approved by the Ethics Committee of the Karolinska Hospital and by the Swedish Medical Product Agency.

Skin chambers

Two skin blisters were induced, as previously described,¹⁵ on the volar aspect of each forearm with a gentle suction. The blisters were covered overnight with a plastic eye chamber and the next morning the epidermal blister roofs were gently removed. A transparent plastic skin chamber with an inner volume of 1 mL was placed over each unroofed blister and secured with tape. The chambers were washed four times with sterile PBS, pH 7.4, containing 20 IU/mL heparin. Three chambers were filled in random order with one of the three recombinant products, generally in 10 times end point concentrations (ie, instilled concentration of 10 μ g/mL) for rBet v 1 wild type as determined by intradermal titration, and one chamber served as negative control (PBS + heparin 20 IU/mL), in a total volume of 0.5 mL to visualize the net influx of mediators and inflammatory cells. Allergen specificity for all involved recombinant birch pollen allergens was ascertained from two previous trials in non-birch atopic control subjects.^{6,7} Subjects exhibiting RAST rBet v 1 levels above 50 kU_A/L (patients No. 7, 8, and 9, Table I) were for safety reasons challenged with 10 times less concentration (ie, 1 μ g/mL rBet v 1 wild type).

After 2 hours of incubation the blister fluids were harvested and the skin chambers were washed twice with PBS-heparin to avoid a carryover effect of any recombinant product. The chambers were then refilled with PBS-heparin (1 mL) and left for an additional 6 hours (a total of 8 hours after the start of challenge) before final removal.

The skin chamber fluid was collected in plastic tubes after 2 and 8 hours of incubation for further analysis. The code for randomization of the test products was broken after completion of all blister fluid analysis.

Recombinant allergens

Recombinant Bet v 1 wild type and rBet v 1 fragments comprising amino acid 1–74 and 75–160 were expressed in *E coli* and purified as described earlier^{3,4,16} and rBet v 1 trimer was engineered by expressing three copies of the Bet v 1 complementary DNA³ in *E coli* BL21 (DE3) as a stable trimer.⁵

Preparation of recombinant allergens

Lyophilized recombinant Bet v 1, Bet v 1 fragments (amino acid 1–74 and 75–150 mixed in equal amounts), and rBet v 1 trimer were dis-

TABLE I. Characterization of study population

Patient No.	Sex	Age (y)	Symptoms	Duration of symptoms (y)	Total IgE (kU/L)	RAST rBet v 1 (kU _A /L)	End point titration intradermal test rBet v 1 (μg/mL)	rBet v 1, trimer, and fragment mix instilled in skin chamber (μg/mL)
1	F	48	R	28	55	2.2	1	10
2	F	28	RC	4	120	3.2	1	10
3	M	25	R	6	39	7.8	1	10
4	M	57	A	5	105	13	1	10
5	M	40	A + RC	22	336	26	1	10
6	F	27	RC	8	379	37	1	10
7	F	27	A + RC	25	174	73	1	1
8	M	18	RC	5	733	90	1	1
9	F	22	A + RC	13	809	190	0.1	1

RAST, Radioallergosorbent test; F, female; R, rhinitis; RC, rhinoconjunctivitis; M, male; A, asthma.

solved in PBS to 1 mg/mL. Protein preparations and dilutions of different recombinant proteins were adjusted to the same protein concentrations. Aliquots were stored at -20°C until the test day when they were diluted to respective working concentration (1–10 μg/mL) with PBS and heparin (final concentration 20 IU/mL) to avoid clotting.

Histamine measurements

The histamine levels were measured in the skin blister fluid collected after 2 hours. Analyses were performed with use of the commercially available histamine RIA kit (Immunotech, Marseilles, France). The limit of detection was set to 0.2 nmol/L histamine according to the manufacturer.

ECP in skin chamber fluid

ECP levels in the blister fluids, collected after 2 and 8 hours, were measured with the Pharmacia CAP System ECP FEIA (Pharmacia & Upjohn). The detection limit of ECP was 2.3 μg/L.

GM-CSF, eotaxin, IFN- γ , and IL-4 in skin chamber fluid

Levels of GM-CSF, eotaxin, and IFN- γ were measured with the Quantikine immunoassay (R&D Systems, Minneapolis, Minn) in skin chamber liquid obtained after 2 and 8 hours. The minimal detectable levels were according to the manufacturer as follows: GM-CSF 2.8 pg/mL, eotaxin 5.0 pg/mL, and IFN- γ 3.0 pg/mL.

Levels of IL-4 were measured with the Cytoscreen Immunoassay kit (Biosource International). The minimal detectable concentration of IL-4 was 0.27 pg/mL according to the manufacturer.

All assays mentioned above were performed according to the instructions of the manufacturer. We have in earlier studies performed control experiments that showed no evidence that the antigen or the diluent (containing heparin) placed initially in the skin chambers interfered with the eotaxin measurements.¹⁵

Preparation of peripheral blood leukocytes

From each person 5 mL of peripheral blood was collected in citrate tubes (Vacutainer, 5 mL, with 0.5 mL of 0.129 mol/L 9NC sodium citrate, Becton Dickinson Immunocytometry Systems, San Jose, Calif) before the allergen and PBS solution were added to the skin chambers. The blood was hemolyzed in 150-μL portions by dilution in 3 mL of $+4^{\circ}\text{C}$ ammonium chloride–EDTA lysing solution (154 mmol/L ammonium chloride, 1.5 mmol/L potassium carbonate, 0.1 mmol/L EDTA, pH 7.2), washed in 2 mL of $+4^{\circ}\text{C}$ 0.15 mmol/L PBS, pH 7.4, supplemented with 0.1 mmol/L EDTA and 0.02% sodium azide (PBS-EDTA), and kept on ice until further use.

Preparation of leukocytes from skin chamber fluids

After 8 hours of incubation, leukocytes were obtained from the skin chamber fluid. The fluid was centrifuged for 5 minutes at 300g in $+4^{\circ}\text{C}$. The cell free supernatant was divided in a number of portions and stored at -70°C until it was further analyzed for the presence of IL-4, IFN- γ , GM-CSF, eotaxin, ECP, and histamine. The cell pellets were resuspended in PBS-EDTA and divided into four different tubes for flow cytometric analysis of total cell number, percentage of eosinophils, and expression of EG2 and CD69 (see below).

Cell membrane fixation and permeabilization (the FOG method)

To enable detection of eosinophils by flow cytometric methods and to analyze intracellular antigens, the leukocyte preparations from blood and skin exudates were fixed and permeabilized according to the FOG method.¹⁷ The fixation of the cell membrane was performed with 200 mL of 4% (wt/vol) paraformaldehyde (Sigma Chemical, St Louis, Mo) for 10 minutes at $+20^{\circ}\text{C}$. The cells were then washed once in 3 mL of PBS-EDTA and centrifuged at 400g for 7 minutes at $+4^{\circ}\text{C}$.

Cell membrane permeabilization was achieved by exposing the cells to 200 mL of 0.74% (wt/vol) *n*-octyl- β -D-glucopyranoside (Sigma Chemical) dissolved in PBS for 6 minutes at $+20^{\circ}\text{C}$. The cells were then washed once in 3 mL of PBS-EDTA and centrifuged at 400g for 7 minutes at $+4^{\circ}\text{C}$.

Immunofluorescence staining of cell surface and intracellular antigens

The cell surface antigen was detected by use of FITC-conjugated mAb against CD69 (50 mg/mL) (clone L78, Becton Dickinson). To determine the nonspecific binding, isotype-matched IgG1 (100 mg/mL) (clone DAK-GO1, Dako A/S, Denmark) control antibody was used.

The FITC-conjugated mAb EG2 (40 mg/mL) (Pharmacia & Upjohn) was used to detect the intracellular expression of the EG2 epitope on ECP after the cells had been subjected to FOG treatment.

All cell suspensions with antibodies were incubated on ice for 30 minutes at $+4^{\circ}\text{C}$ and then washed twice in PBS-EDTA.

Analysis with flow cytometry

The different leukocyte preparations were analyzed and counted in an EPICS XL flow cytometer (Beckman Coulter, Hialeah, Fla). In the flow cytometer the cells are distinguished by their different light-scattering properties; forward scatter reflects the cell size and

side scatter reflects the complexity/granularity. In the current study three different leukocyte clusters were distinguished in untreated blood samples and blister fluids: lymphocytes, monocytes, and granulocytes. In the FOG-treated preparations, lymphocytes, neutrophils + monocytes, and eosinophils were detected as well-separated populations as previously described.¹⁷ The peripheral blood eosinophils (PBE) were identified by their high scatter signals, surface expression of CD9, and expression of the EG2 epitope on intracellular ECP as previously described.^{17,18}

The instrument was calibrated daily with standardized 10- μ m fluorospheres (Flow-Check, Beckman Coulter). Flow-set (Beckman Coulter), another fluorosphere with controlled fluorescence intensity, was used to standardize the mean fluorescence intensity (MFI) before each experiment. The percentage and absolute number of positively immunostained leukocytes was determined and a quantification of antigen was obtained by measuring the MFI units of the positive cell population.

Statistical analysis

Data are presented as medians (interquartile range). Differences between the variables were first analyzed by Friedman ANOVA. Paired comparisons between respective recombinant products and negative control within patients were performed with use of the nonparametric-method Wilcoxon paired test. Correlations between the different parameters were sought by calculating the Spearman correlation coefficient. Differences were considered statistically significant at $P < .05$.

RESULTS

Monitoring of patients during the study

All patients completed the study. The patients generally had transient mild local pruritus beneath applied skin chambers on the volar side of forearms. No late reactions, either local or systemic, were reported by any subject. One patient (patient No. 7, Table I) had strong itching and continuous erythema on both arms during the second hour of challenge. These symptoms gradually disappeared after the chambers were flushed at 2 hours.

Recovery of skin exudates

Recovered fluid at 2 and 8 hours was more than 95% in all chambers. However, the recovery in one control chamber at 8 hours (patient No. 5, Table I) was due to leakage only 15% (only analyzed as a negative control for histamine).

rBet v 1 derivatives and rBet v 1 have the same capacity to recruit leukocytes and eosinophils to skin chamber fluid

Peripheral blood leukocytes (lymphocytes, monocytes, and granulocytes) were counted before allergen was applied to the chamber. The number of leukocytes in blood from the patient group ($n = 9$) was $5.1 (4.1-7.2) \times 10^9/L$, which is within the normal range of our reference material at the Karolinska Hospital ($4-11 \times 10^9$ leukocytes/L). When the chamber fluid was analyzed after 8 hours ($n = 9$), we found that the number of recruited leukocytes (lymphocytes, monocytes, and granulocytes) did not differ among the chambers instilled with rBet v 1 $5.7 (4.9-10.2) \times 10^9/L$, rBet v 1 fragment mix $4.1 (2.0-$

$6.1) \times 10^9/L$, or rBet v 1 trimer $4.4 (3.9-12.2) \times 10^9/L$. However, there was a significantly lower number of leukocytes ($P < .05$), $1.7 (0.8-6.6) \times 10^9/L$, in the control chamber compared with the three challenged chambers.

The number of eosinophils in blood was $0.23 (0.13-0.45) \times 10^9/L$, which is within the normal range for healthy subjects at the Karolinska Hospital, $(0.05-0.40) \times 10^9$ eosinophils per liter. The number of recruited eosinophils at 8 hours did not differ among the chambers filled with rBet v 1 $0.22 (0.17-0.27) \times 10^9/L$, the fragment mix $0.12 (0.08-0.15) \times 10^9/L$, or the trimer $0.21 (0.08-0.44) \times 10^9/L$. However, there was a lower number of eosinophils ($P < .05$) in the control chamber compared with the three challenged chambers.

rBet v 1 derivatives and rBet v 1 have the same ability to up-regulate the intracellular expression of the EG2 epitope on ECP in skin blister fluid

The expression of the EG2 epitope on intracellular ECP was detected in eosinophils obtained from skin chamber fluid after 8 hours ($n = 8$) (not shown). The expression of EG2 was increased on the eosinophils from all four chambers: rBet v 1 $60.0 (46.6-66.3)$ MFI, fragment mix $50.5 (43.0-56.1)$ MFI, trimer $50.9 (40.6-63.0)$ MFI, and control $45.7 (40.6-58.4)$ MFI compared with EG2 expression on PBE.¹⁵ We did not note any significant differences in the EG2 expression between the different chambers.

rBet v 1 derivatives induce lower surface expression of CD69 on eosinophils from skin chamber fluid compared with rBet v 1

The surface expression of CD69 was measured both on eosinophils from peripheral blood ($n = 9$) and on eosinophils from the skin chamber fluid at 8 hours ($n = 8$). There was a low expression of CD69 on PBE $1.3\% (0.7\%-2.8\%)$. When measuring CD69 expression on eosinophils from the four skin chamber fluids, we found a marked increase compared with PBE. There was a consistent and significantly higher expression ($P < .05$) of CD69 on eosinophils from the rBet v 1 chamber $92.8\% (87.8\%-95.0\%)$ compared with the chambers challenged with the fragment mix $84.7\% (65.8\%-87.9\%)$, trimer $70.5\% (54.3\%-88.8\%)$, or control $80.4\% (44.2\%-87.8\%)$ (Fig 1).

rBet v 1 derivatives induce lower levels of ECP and GM-CSF but equivalent amounts of eotaxin in skin chamber fluid compared with rBet v 1

There was no detectable level of ECP in the skin chamber fluid after 2 hours (data not shown). However, the level of ECP in blister fluid obtained after 8 hours ($n = 9$) from the chamber containing rBet v 1 was significantly higher ($P < .05$), $54.8 (35.0-63.6)$ mg/L, com-

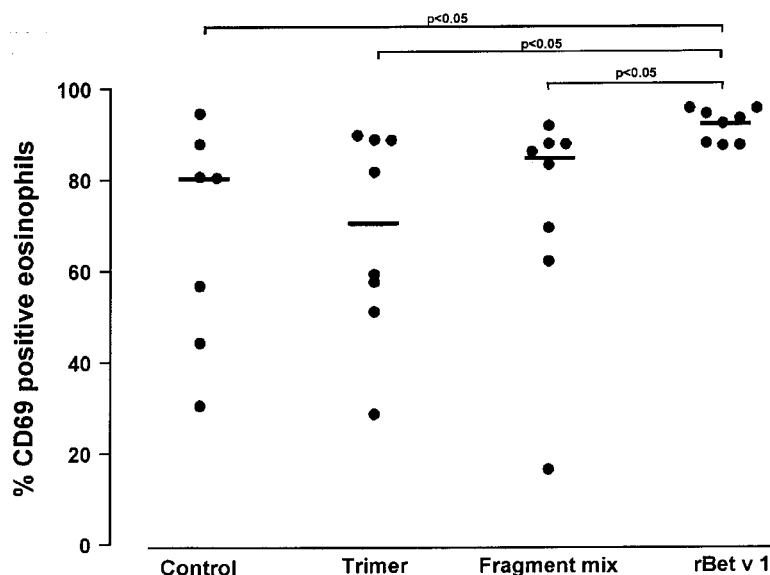


FIG 1. Surface expression of CD69 on eosinophils in the skin blister fluids collected 8 hours after challenge. Each patient is represented by a solid circle, and the median values are marked by lines.

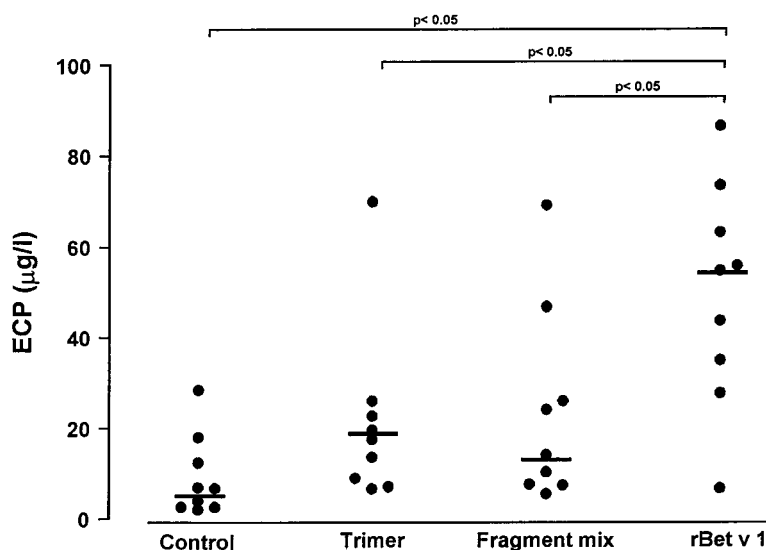


FIG 2. The level of released ECP in the skin blister fluids collected 8 hours after challenge. Each patient is represented by a solid circle, and the median values are marked by lines.

pared with the chambers containing the fragment mix, 14.1 (7.6-26.0) mg/L, trimer 21.3 (9.0-22.7) mg/L, or the control 8.2 (2.6-6.9) mg/L. Moreover, ECP levels in the three challenged chambers were significantly higher ($P < .05$) versus the control (Fig 2).

GM-CSF was measured in fluids after 2 and 8 hours ($n = 8$). There was no detectable level of GM-CSF after 2 hours (data not shown). However, after 8 hours the level of GM-CSF in the chamber challenged with rBet v 1 was significantly higher ($P < .05$), 20.1 (15.4-29.3)

pg/mL, compared with the chamber challenged with the fragment mix, 8.2 (0.0-9.2) pg/mL, trimer 3.2 (0.0-8.8) pg/mL, or control 9.2 (7.0-12.9) pg/mL (Fig 3).

There were no detectable levels of eotaxin in any chamber fluid after 2 hours ($n = 9$) (data not shown). However, after 8 hours comparable levels of eotaxin were found in all four chambers, rBet v 1 15.1 (15.6-28.8) pg/mL, fragment mix 19.6 (16.1-23.8) pg/mL, trimer 17.0 (12.2-27.3) pg/mL, and control 12.2 (11.0-13.6) pg/mL.

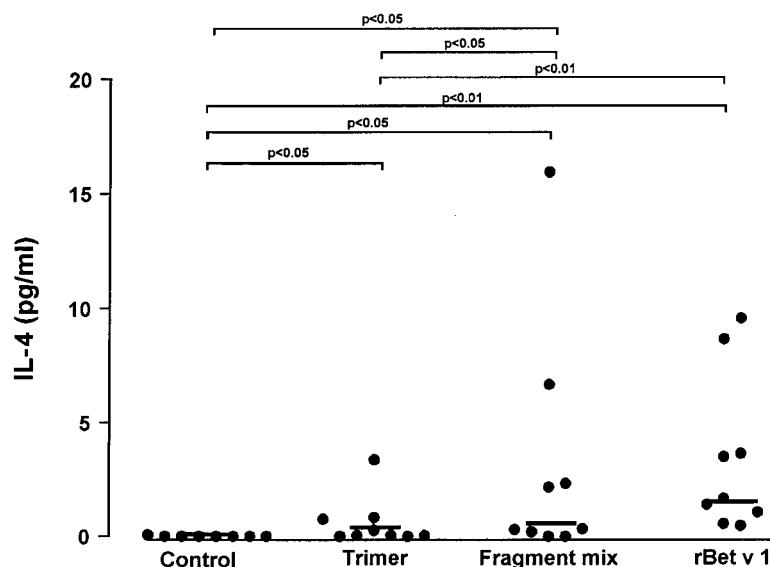


FIG 4. The level of soluble IL-4 in the skin blister fluids collected 8 hours after challenge. Each patient is represented by a *solid circle*, and the median values are marked by *lines*.

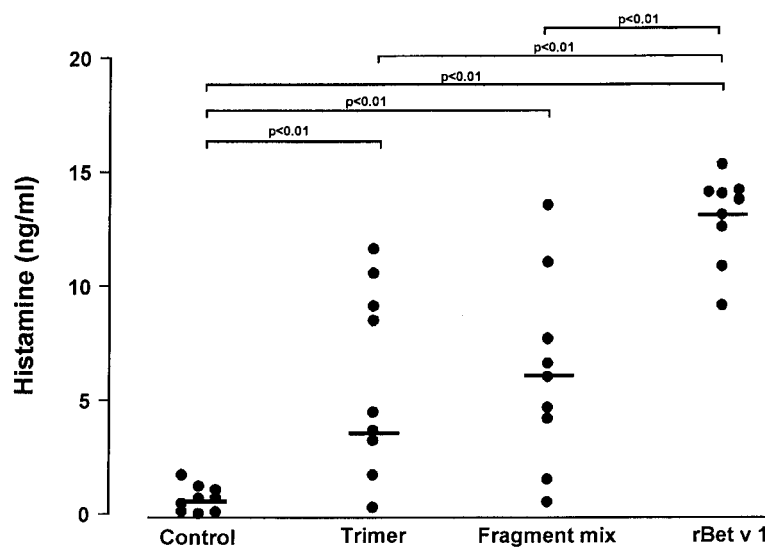


FIG 5. The level of released histamine in the skin blister fluids collected 2 hours after challenge. Each patient is represented by a *solid circle*, and the median values are marked by *lines*.

have been discussions concerning the reliability of EG2 staining and ECP release as eosinophil activity markers. When PBE is analyzed, we believe that it is more a question of the clinical status of the patients and whether activated eosinophils are in the circulation at the time of blood sampling. Furthermore, we suggest that the reliability of EG2 as an activity marker is more associated to the technique and fixative used.^{17,18,21} According to a recent study by Nakajima et al,²² where a Western blot technique was used, the results showed that EG2 reacts with the soluble form of ECP despite the origin. This is

not contradictory to our results because by the use of the FOG technique and flow cytometry all eosinophils are detected as EG2 positive. However, with this method, as opposed to immunohistochemistry techniques, we measure quantitative differences in binding of EG2 to intracellular ECP between unactivated and activated eosinophils. This method has been applied in several studies with reproducible results.^{17,18,23-25}

All recombinant molecules had the same ability to up-regulate the expression of EG2, but the ECP release was significantly lower in the chambers challenged with the

derivatives compared with rBet v 1 wild type. The up-regulation of EG2 is in agreement with our previous in vitro and in vivo results, which show that once eosinophils are recruited to an inflammatory foci the EG2 epitope is up-regulated, probably as a result of the transmigration process.^{15,26}

To further study eosinophil activation we measured the CD69 expression on eosinophils. We were able to detect CD69 expression on eosinophils in all skin blister chambers, with a significantly lower expression in chambers challenged with rBet v 1 derivatives compared with the rBet v 1, after 8 hours of challenge. We suggest that the up-regulation of CD69 and increased release of ECP found in the rBet v 1-challenged chamber is most likely attributed to activation of eosinophils, perhaps by eosinophil-bound Bet v 1-specific IgE. In this study the hypoallergenic derivatives had a reduced ability to activate eosinophils. This hypothesis is in line with earlier reports showing that the hypoallergenic rBet v 1 derivatives exhibited a reduced capacity to activate basophils and mast cells by cross-linking of Bet v 1-specific IgE.³

We hypothesize that the CD69 expression on eosinophils is due to a redistribution of the preformed intracellular pool of CD69 in eosinophils²⁷ and not to eosinophil-platelet interactions. The phenomenon of adherence of platelets to eosinophils has been studied in relation to CD9 expression. Adherence of platelets is known to increase the CD9 expression on eosinophils.²⁸ However, we have in an earlier study shown that CD9 expression was significantly decreased on eosinophils in the skin blister fluids compared with PBE, indicating that platelets were not adhered to eosinophils in skin blister fluids.¹⁵

Besides T cells, eosinophils from blood, nasal mucosa, BAL fluid, and skin are reported to be important sources for GM-CSF and IL-4.²⁹⁻³² Our finding that IL-4 and GM-CSF appeared in the blister fluid collected after 8 hours is in agreement with previous studies reporting messenger RNA expression of IL-4 in 6 hours in late-phase reactions in biopsy specimens³² and GM-CSF³³ in blister fluid after 5 hours challenge with allergen. Our data show that the trimer fragment mix and the control chambers contained significantly lower levels of GM-CSF and IL-4 than did the rBet v 1-challenged chamber. The finding that IL-4 and GM-CSF levels were higher in those chambers, where we found elevated CD69 expression and ECP-release, supports the idea that both cytokines originate from activated eosinophils. Although influx of basophils into skin blister fluids can be registered within 6 hours,³⁴ most studies report a later recruitment (9-24 hours after challenge)^{35,36} and therefore argue against basophils being the major source of IL-4 in our study. Because the levels of IL-4 were just above cutoff detection limit, the interpretation should be done with caution. Furthermore, we found no detectable levels of IFN- γ , which is consistent with the previously reported lack of IFN- γ expression in mite allergen-exposed skin biopsy specimens taken 2 to 48 hours after allergen challenge.³⁷

In conclusion, our results demonstrate that genetically engineered hypoallergenic rBet v 1 derivatives induce

significantly lower activation of eosinophils by means of surface expression of CD69 and ECP release (and levels of GM-CSF and IL-4) compared with rBet v 1 wild type. The previously reported reduced allergenic activity of the rBet v 1 derivatives together with the results obtained in this study encourage to use rBet v 1 derivatives for immunotherapy. Such a study will finally evaluate their clinical and immunologic efficacy.

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