

Induction of specific histamine release from basophils with purified natural and recombinant birch pollen allergens

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As much as 15% of the population in industrialized countries suffers from type I allergic symptoms (rhinitis, conjunctivitis, and bronchial asthma). One approach toward this disease involves the production of recombinant allergens in *Escherichia coli* and their purification for diagnostic and therapeutic purposes. In this study we compared the IgE-binding capacity of natural and recombinant birch allergens with their functional ability to release histamine from allergic patients' basophils via cross-linking of high-affinity Fc_ϵ -receptors. The recombinant as well as pollen-derived Bet v I and birch profilin (Bet v II) were purified and tested in parallel on IgE immunoblots and in *in vitro* histamine release tests ($n = 21$). We observed an excellent correlation between allergen-induced histamine release and birch pollen RAST ($r = 0.881$, $p < 0.002$). Nonspecific histamine release as a result of a cell toxic effect of the allergen preparations was never observed from basophils of donors without birch pollen allergy. The specificity of the presented effector model is also documented by the specific desensitization of patients' basophils with the recombinant allergens. These data may provide a basis for the use of purified recombinant allergens in sensitive and specific *in vitro* allergy tests monitoring the effector situation in allergic patients, which therefore may represent a possible alternative for *in vivo* diagnostic methods. (*J ALLERGY CLIN IMMUNOL* 1993;91:88-97.)

Key words: Recombinant allergens, Bet v I, profilin, IgE, histamine release, Fc_ϵ -receptor, desensitization of basophils

Type I allergy caused by plant allergens (pollen and plant-derived foodstuff) and indoor allergens (mold, mite, cat, and dog allergens) represents a major health problem in industrialized countries. As much as 15% of the population suffers from type I allergic symptoms (rhinitis, conjunctivitis and bronchial asthma) caused by allergens that are capable of cross-linking

Abbreviations used

cDNA:	Complementary DNA
Bet v I, Bet v II:	Birch pollen allergens
rBet v I, rBet v II:	Recombinant birch pollen allergens
RAST:	Radioallergosorbent assay
RIST:	Radioimmunosorbent assay
PAG buffer:	PIPES (piperazin-1,4-bis(2-ethane sulfonic acid))albumine-glucose buffer
kD:	Kilo Dalton
SDS:	Sodium dodecyl sulfate

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high-affinity Fc_ϵ -receptors of mast cells and basophils via specific IgE, thus leading to release of histamine and other mediators of the allergic effector reaction.¹ One approach to studying the pathogenetic mecha-

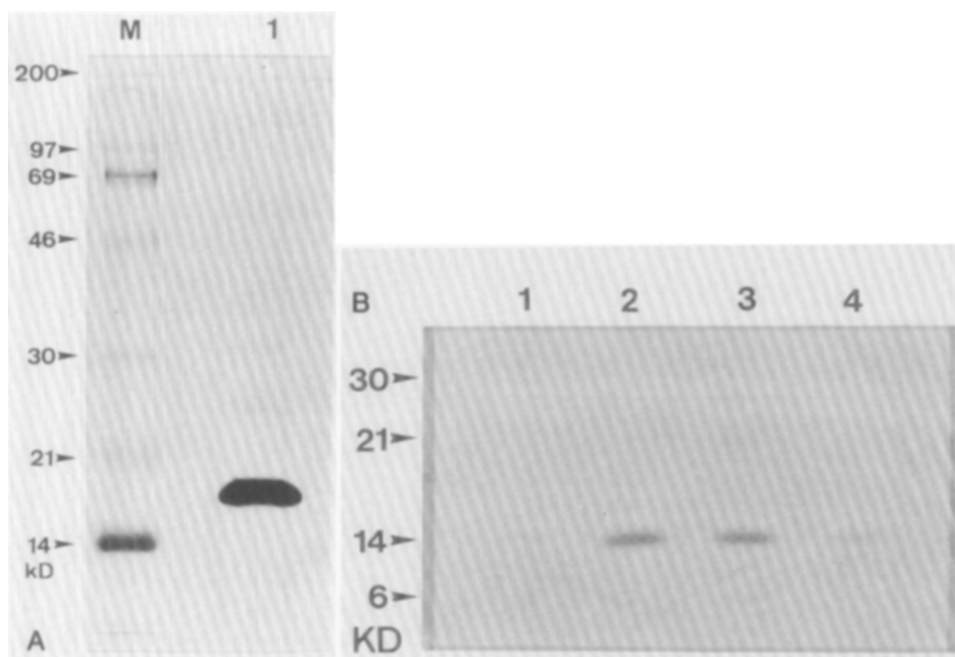


FIG. 1. A, Silver stained polyacrylamide gel with purified recombinant *Bet v I*: Lane M, molecular weight marker; lane 1, purified *rBet v I* at 17 kD. **B,** Coomassie brilliant blue stained polyacrylamide gel containing eluted fractions of *rBet v II*; Lanes 1-4 represent material eluted from poly-(L-proline) sepharose with urea (lane 1, 2 mol/L urea; lanes 2-4, subsequent elutions with 6 mol/L urea).

nisms leading to allergy involves the molecular characterization of allergens and their use to establish in vitro allergy models. For this purpose complementary DNA (cDNA) cloning techniques have proved to be potent tools in the recent time.² With use of serum IgE from individuals allergic to birch pollen we had isolated the cDNA encoding the major birch pollen allergen, *Bet v I*,³ and birch profilin (*Bet v II*)⁴ from birch pollen expression cDNA libraries. We then expressed both allergens in *Escherichia coli* as recombinant nonfusion proteins and showed comparable IgE-binding capacity of pollen-derived as well as recombinant allergens on IgE immunoblots.⁵ The terminus allergen, however, does not only relate to the IgE-binding capacity of a protein but also requires functional properties that lead to allergic effector mechanisms, in particular the release of mediators from mast cells and basophils via cross-linking of high-affinity Fc ϵ -receptors. To perform specific in vitro cellular assays, we purified two natural pollen allergens, *Bet v I* and *Bet v II* and their recombinant counterparts, *rBet v I* and *rBet v II*. The natural as well as recombinant birch pollen allergens were then tested in histamine release experiments with basophils from patients allergic to tree pollen. Thus a specific test system was established that allowed us to monitor the effector cell situation of allergic patients in vitro.

MATERIAL AND METHODS

Purification of pollen-derived *Bet v I* and *Bet v II*

Ninety-five percent pure birch (*Betula verrucosa*) pollen was obtained from Allergon AB (Engelholm, Sweden). Pollen-derived *Bet v I* was isolated via affinity chromatography to anti-*Bet v I* MA b BIP 1, as described.⁶ For the preparation of birch pollen profilin, birch pollen was ground under liquid nitrogen by use of mortar and pestle and extracted as described.⁷ Birch pollen profilin was then isolated via affinity chromatography to poly-(L-proline) sepharose.⁸ Amount and purity of the proteins was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis⁹ and IgE immunoblots^{10, 11} before use in histamine release assays.

Expression and purification of *rBet v I* and *rBet v II*

Escherichia coli JM105 cells containing the plasmid pKK223-3 with the inserted cDNAs encoding *Bet v I* and *Bet v II* were grown and induced with 1 mmol/L final concentration of isopropyl- β -thiogalactoside to produce recombinant allergens as described.⁵ Four hours after induction of protein synthesis *E. coli* were harvested by centrifugation and washed twice in 0.9% sodium chloride. The recombinant allergens were then purified to homogeneity and tested for their IgE-binding properties as described.⁷ Fig. 1, A and B show stained SDS-polyacrylamide gels of the purified recombinant proteins.

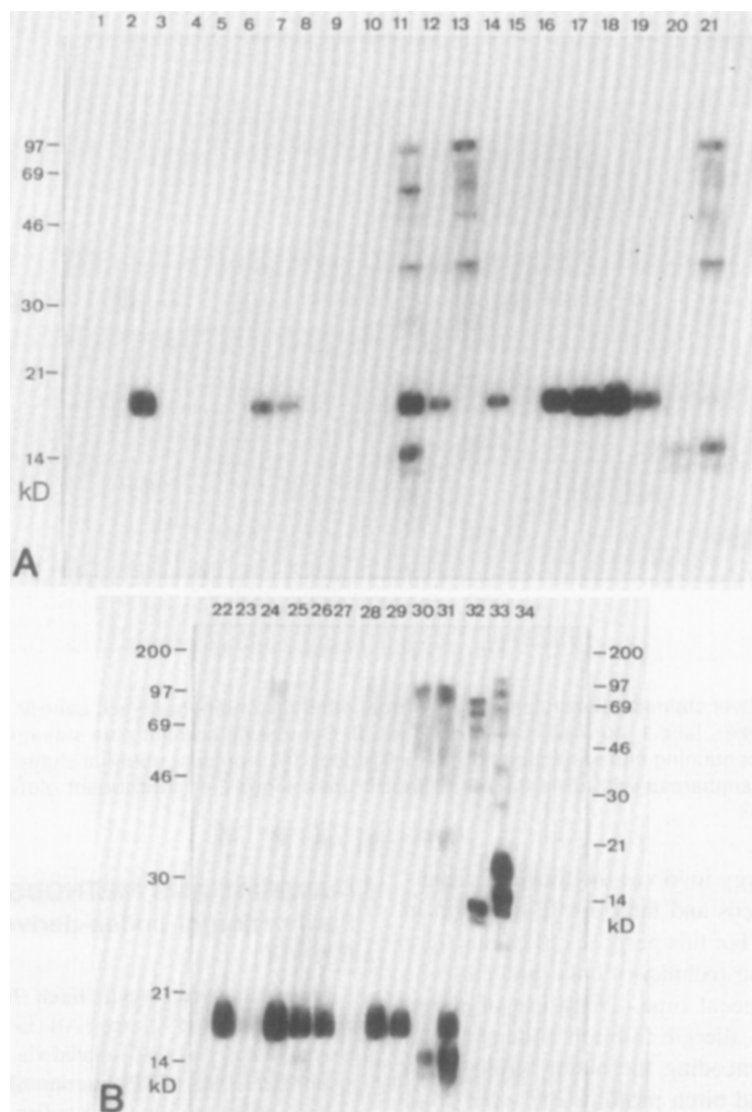


FIG. 2. A and B, IgE immunoblot with total birch pollen proteins: **A**, 20 individuals (2 to 21) and in **B** additional 12 individuals (22 to 33) were tested for IgE reactivity with total birch pollen proteins. Lanes 1 and 34 represent buffer control without addition of patients' serum. Molecular weights for lanes 32 to 34 indicated on *right*. IgE binding to *Bet v I* seen at 17 kD and IgE reactivity to *Bet v II* found at 14 kD.

Characterization of patients allergic to tree pollen, patients allergic to different allergens, and nonallergic patients

All individuals tested in this study were characterized by case history, skin prick test, and radioallergosorbent test (RAST; Pharmacia, Uppsala, Sweden) as performed by Jarolim et al.¹¹ Normal nonallergic individuals (donors 4, 5, 8, 9, 10, and 15) were included as a control.

IgE immunoblots with birch pollen extracts and purified recombinant birch pollen allergens

To compare the amount of allergen-specific serum IgE with the *in vitro* histamine release tests, supernatants from the basophil preparation were directly used for the immunoblots. The dextran supernatants, which contained 1:2 prediluted plasma, were diluted 1:5 in buffer G (50

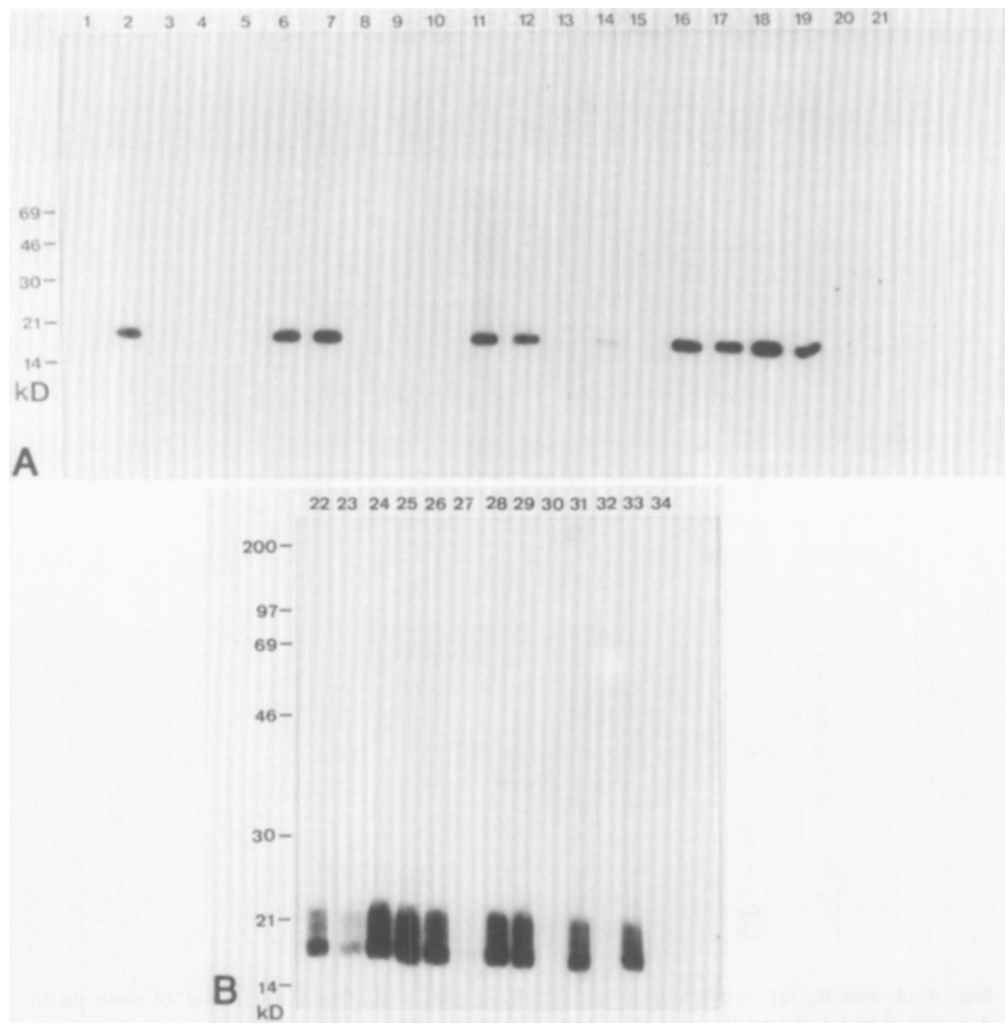


FIG. 3. A and B, IgE immunoblot with purified *rBet v I*: The same panel of sera as in Fig. 2, A and B was tested for IgE binding with purified *rBet v I*.

mmol/L sodium phosphate, pH 7.5, 0.5% Tween 20, 0.5% bovine serum albumin, 0.05% NaN_3). These dilutions were then used to detect IgE-binding proteins on nitrocellulose strips containing total birch pollen proteins (Fig. 2, A and B), purified *rBet v I* (Fig. 3, A and B) or purified *rBet v II* (Fig. 4, A and C). Bound IgE was detected with rabbit antihuman IgE antibody labeled with iodine 125 (Pharmacia) as described.¹¹

In vitro histamine release with blood basophils and purified natural and recombinant allergens

After informed consent was obtained from the donors, heparinized blood samples were taken, and peripheral blood cells were fractionated by incubation in 1.1% dextran 70

and 8 $\mu\text{mol/L}$ ethylenediamine tetraacetic acid for 90 minutes at room temperature.¹² Granulocytes were washed and incubated with various concentrations of purified natural and recombinant allergens (dose range, 10^{-4} to $10 \mu\text{g/ml}$) and anti-IgE MAb E-124-2-8 (as a positive control) for 30 minutes at 37°C . Histamine was measured in the cell-free supernatants by radioimmunoassay (Immunotech, Marseille, France).

Allergen-specific desensitization of patient's basophils was done essentially as described by Sobotka et al.,¹³ Dembo and Goldstein,¹⁴ MacGlashan and Lichtenstein,¹⁵ and Kazimierzak et al.¹⁶ In brief, blood basophils that had been prepared as described above were washed and preincubated in PIPES-albumine-glucose (PAG) buffer with *rBet v I* or *rBet v II*. The cells were then washed and incubated in PAG

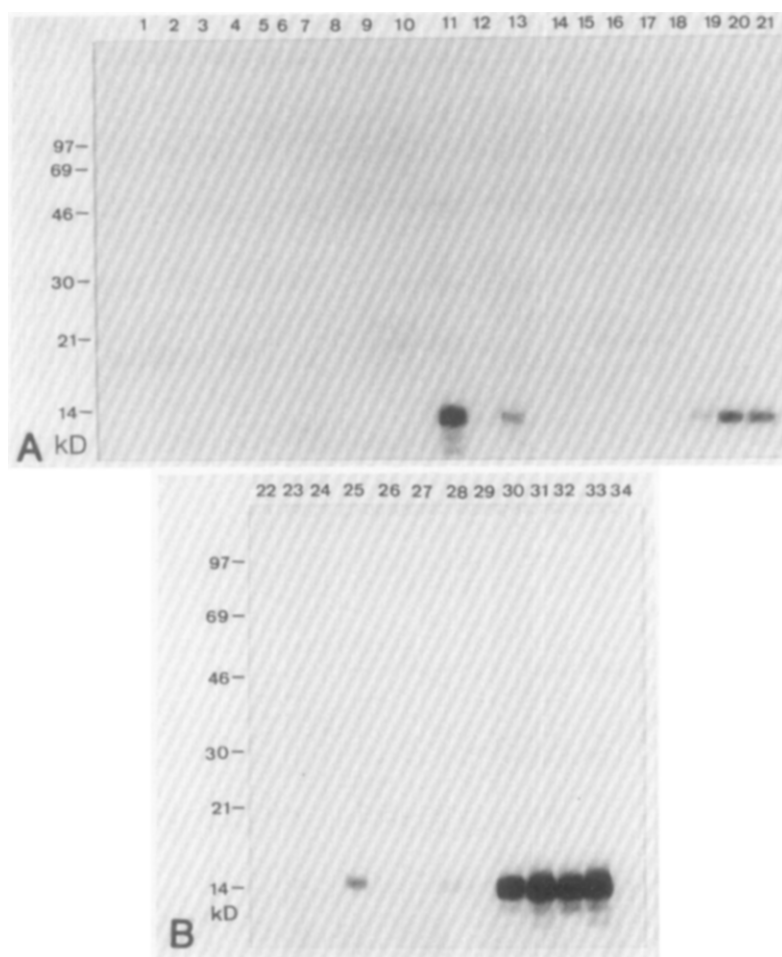


FIG. 4. A and B, IgE immunoblot with purified *rBet v II*: the same panel of sera as in 2, A and B and 3, A and B was tested for IgE reactivity with purified *rBet v II*.

buffer containing 1 mmol/L CaCl_2 and either recombinant allergens or antihuman IgE MAb E-124-2-8.

Statistical analysis-correlation of birch pollen RAST with histamine release tests

For RAST measurements the following scores were used: RAST 0 = 0, RAST 0.1 to 1 = 1, 1.1 to 2 = 2, 2.1 to 3 = 3, 3.1 to 4 = 4, greater than 4 = 5. Maximal percentages of histamine release obtained with the optimal allergen in optimal concentration received the following scores: Percentage release 0% to 5% = 0, 6% to 10% = 1, 11% to 20% = 2, 21% to 30% = 3, 31% to 40% = 4, greater than 40% = 5. Patients 22 and 26 who did not release histamine even on stimulation with antihuman IgE had to be excluded from the statistical analysis because of lack of basophil sensitivity. In all other cases a correlation between the RAST and histamine release scores was obtained according to the linear regression and correlation test.

RESULTS

Purification of *rBet v I* and *rBet v II*

Recombinant birch pollen allergens, *rBet v I*, and *rBet v II*, were purified from *E. coli* lysates by two different methods. In the case of *rBet v I* a combination of chromatofocusing and high-pressure liquid chromatography (HPLC) purification delivered a pure protein (Fig. 1, A). In the case of *rBet v II*, the purification was based on the affinity of profilins to poly-(L-proline).⁸ Since to our knowledge profilins do not occur in prokaryotic organisms, highly pure *rBet v II* could be obtained in a single-step purification procedure from *E. coli* (Fig. 1, B). Both recombinant proteins showed comparable molecular weights as the pollen-derived allergens in SDS-polyacrylamide gels, suggesting that little or no posttranslational modification

TABLE 1. Summary of histamine releases with recombinant and pollen-derived tree pollen allergens, correlation with birch pollen RAST and IgE immunoblot data

Donors	Histamine release				Immunoblot			RAST
	<i>nBet v I</i>	<i>rBet v I</i>	<i>nBet v II</i>	<i>rBet v II</i>	<i>a-IgE</i>	<i>Bet v I</i>	<i>Bet v II</i>	
2	+	+	+	—	+	+	—	4.9
4	—	—	—	—	+	—	—	0
5	—	—	—	—	+	—	—	0
6	+	+	+	—	+	+	—	3.5
7	+	+	+	—	+	+	—	4
9	—	—	—	—	+	—	—	0
10	—	—	—	—	+	—	—	0
12	+	+	nt	—	nt	+	—	2.2
14	—	±	—	—	nt	+	—	2.5
15	—	—	—	—	+	—	—	0
16	+	nt	—	—	+	+	—	3.2
21	nt	nt	+	+	+	+	+	3.4
22	—	—	—	—	—	+	—	4.6
23	+	+	+	+	±	+	+	3.4
24	+	+	+	—	±	+	—	4
25	+	+	+	+	+	+	+	5
26	—	—	±	±	±	+	—	4.3
27	+	—	+	+	+	—	+	2.7
28	+	+	+	+	+	+	+	4.5
29	nt	nt	+	+	+	—	+	3
30	+	+	+	+	+	+	+	4

n: Natural; *r*: recombinant; *a-IgE*, antihuman IgE; *nt*, not tested; ±, specific release of histamine below 200% of buffer control.

*Release observed with high concentrations of allergen only.

of these allergens occurs. Recombinant *Bet v I* ranges at 17 kD (Fig. 1, A) and recombinant profilin at 14 kD (Fig. 1, B). Both molecular weights correlate with the molecular weight that can be calculated from the cDNA sequences and hence deduced amino acid sequences of both allergens.^{3,4} In the case of *Bet v II* a smaller protein with molecular weight of approximately 12 to 13 kD could be found in pollen extracts (Fig. 2, A and B), which shared identical IgE-binding properties as the 14kD component and similarly was purified by affinity to poly-(L-proline)-sepharose.¹⁷

IgE immunoblots with total birch pollen proteins and purified *rBet v I* and *Bet v II*

As in a previous study performed with unpurified recombinant allergens we obtained comparable reactivity of patients' IgE with natural as well as recombinant allergens. The use of purified recombinant allergens, however, allowed a much more specific detection of specific IgE. Patients 23 and 28 gave only weak IgE binding to *Bet v II* on immunoblots containing total birch pollen extracts (Fig. 2, B), whereas

with purified recombinant allergens the signals were more pronounced (Figs. 3, B, 4, B). The basophils of these patients showed specific dose-dependent histamine release with natural as well as recombinant allergens. Both *rBet v I* and *rBet v II*, when used together were sufficient to detect all individuals allergic to tree pollen, and none of the control persons reacted with birch pollen-derived or purified recombinant allergens on IgE-immunoblots. Each of the allergen preparations retained their ability to bind patient's IgE on the immunoblots (total birch pollen proteins-Fig. 2, A and B, purified *rBet v I* Fig. 3, A and B, and purified *rBet v II* Fig. 4, A and B).

Histamine release tests with purified natural as well as recombinant tree pollen allergens

Sixteen patients allergic to birch pollen and five nonallergic individuals were tested for histamine release with natural and *rBet v I* and *Bet v II*. In all patients allergic to birch pollen whose basophils were sensitive to antihuman IgE stimulation, natural as well as recombinant birch allergens released histamine

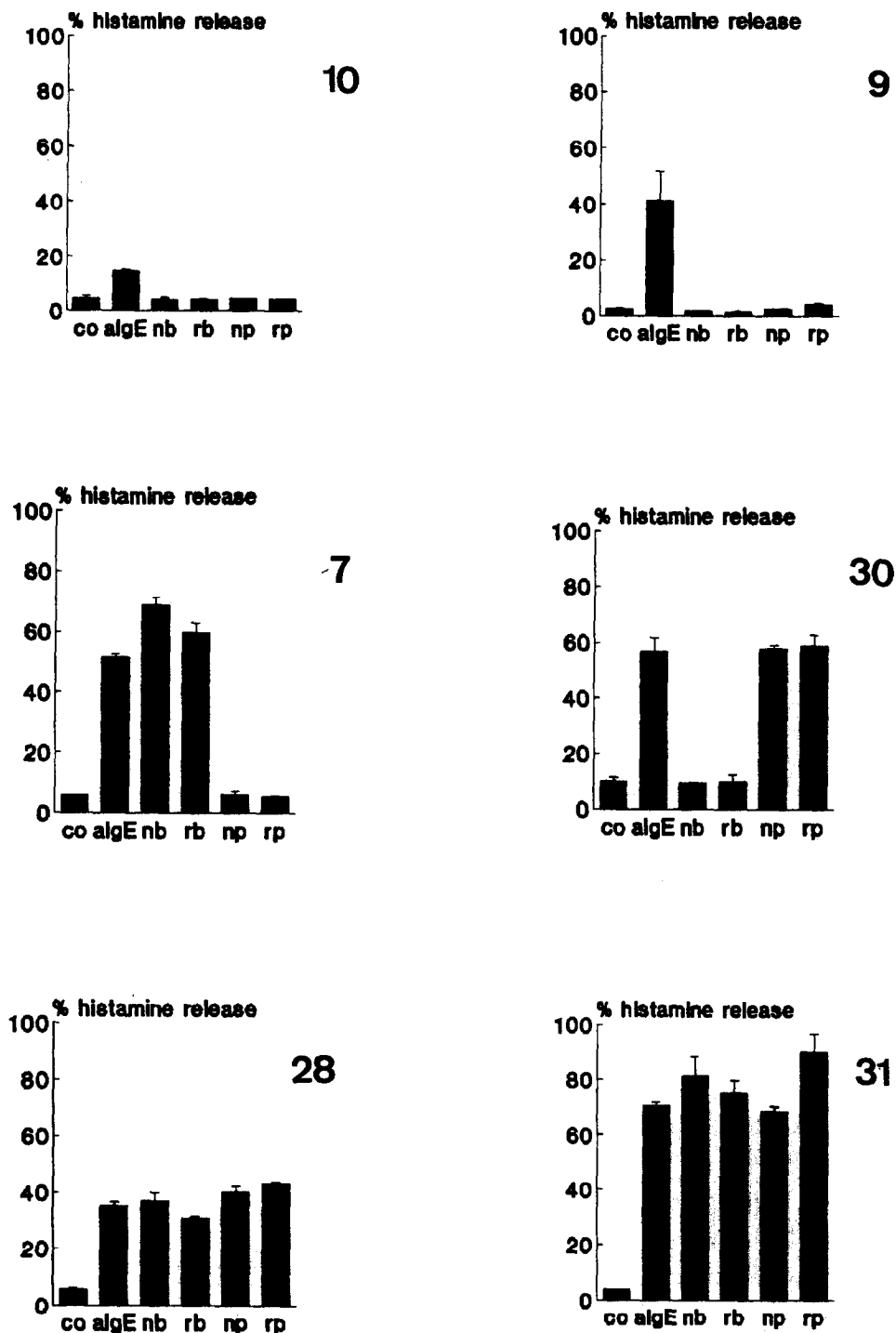


FIG. 5. Comparison of histamine release tests: histamine releases from patient's basophils (patients 7, 9, 10, 28, 30, and 31 indicated as in Table I) with natural birch pollen allergens (*nb*, natural birch pollen allergen; *np*, natural *Bet v II*), with recombinant birch pollen allergens (*rb*, recombinant birch pollen allergen; *rp*, recombinant *Bet v II*), with the buffer control (*co*) and with monoclonal anti-IgE antibody MAb E-124-2-8 are displayed as bar diagrams. Results reflect mean values of triplicate determinations after stimulation with an optimal concentration of antigen resulting in maximum percentage of histamine release.

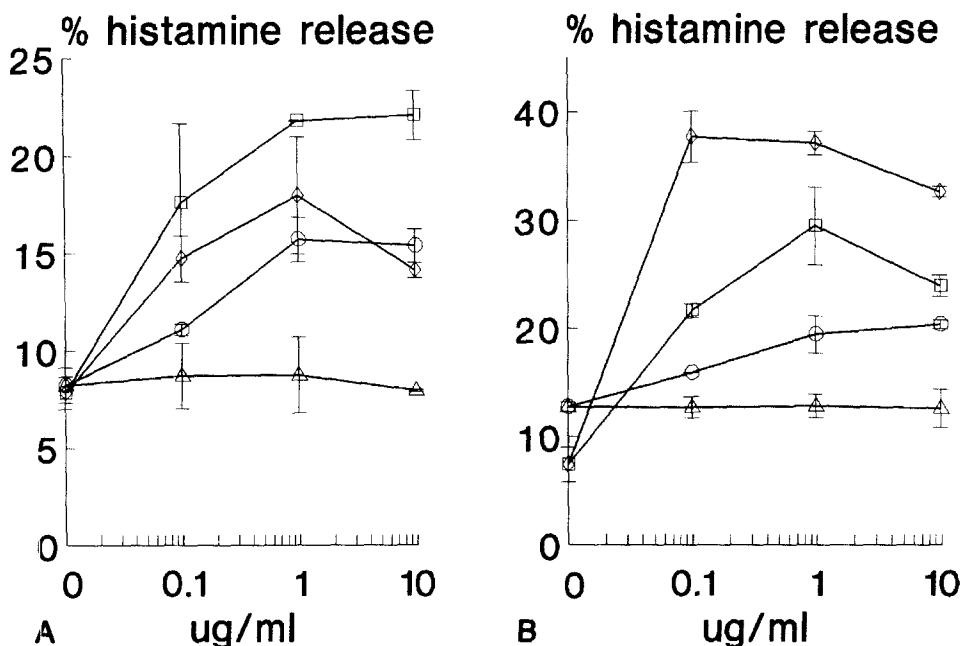


FIG. 6. Allergen-specific desensitization of patient's basophils-dose dependency of histamine release: **A**, basophils from a patient allergic to *Bet v I* were desensitized with *rBet v I*; **B**, basophils from an individual allergic to profilin were desensitized with *rBet v II*. In both cases the preincubation of basophils with recombinant allergens led to complete desensitization (**A**, rhombus indicates release with recombinant allergens from untreated and triangle from desensitized basophils). Histamine release from basophils with monoclonal anti-IgE E-124-2-8 was less affected by allergen-specific desensitization (*square* represents not desensitized and *circle* desensitized basophils). Histamine release expressed as a percentage of total histamine. Values are means of triplicate determinations, and *error bars* indicate standard deviation.

from the peripheral blood basophils in a comparable and dose-dependent way (Table I and Fig. 5). The capability to release histamine depended on the presence of allergen-specific IgE as determined by comparing histamine release data with IgE-immunoblots (Table I). In some cases (donors 2, 6, 7, and 24) high concentrations ($>10 \mu\text{g/ml}$) of natural allergens yielded histamine release, although no IgE binding was seen on the immunoblots. This is most likely due to contaminating trace amounts of other allergens in the protein preparation. These effects were never observed with purified recombinant pollen allergens. Two patients (donors 22 and 26) did not show histamine release on allergen stimulation, although specific IgE was found in the immunoblots. The basophils of both patients were found not sensitive even on stimulation with antihuman IgE. Specific and dose-dependent histamine release was observed even in patients with low amounts of allergen-specific IgE (patients 23 and 28) indicating the high sensitivity of the recombinant test system. As determined by the linear regression and correlation test, excellent cor-

relation ($r = 0.881$, $p < 0.002$) was obtained between RAST measurements and percentages of histamine releases.

Allergen-specific desensitization of patient's basophils

To demonstrate the specificity of the presented effector model, *rBet v I* and *rBet v II* were used to desensitize patient's basophils in an allergen-specific manner as described by Sobotka et al.,¹³ Dembo and Goldstein,¹⁴ MacGlashan and Lichtenstein,¹⁵ and Kazimierzak et al.¹⁶ Patient's basophils were preincubated with *rBet v I* or *Bet v II* in Ca^{2+} free buffer. Subsequent incubation of the cells with the respective allergens showed specific desensitization (Fig. 6, *A* and *B*). In no case was histamine release observed as a result of nonspecific lysis of the cells.

DISCUSSION

New diagnostic and therapeutic concepts for type I allergy may evolve from the molecular characterization of atopic allergens and the availability of al-

lergens as recombinant proteins.² However, to introduce recombinant proteins in the diagnostic and therapeutic field several prerequisites have to be fulfilled.

One basic requirement is that a reasonable panel of relevant recombinant allergens can be defined and highly purified.^{5, 18} In cases of tree pollen allergy we were able to clone the two most important birch pollen allergens, *Bet v* I and *Bet v* II, from cDNA libraries with use of patient's IgE. We purified both recombinant allergens and compared the IgE-binding capacity of natural and recombinant molecules with their capability to release histamine from allergic patients' basophils via cross-linking of allergen-specific IgE that is bound to the high-affinity Fc ϵ -receptors. We found that natural as well as recombinant allergens gave similar results in IgE immunoblots and released histamine in the in vitro cellular effector model. Carbohydrate moieties or other posttranslational modifications of both tree pollen allergens were not required for their functional properties. The purification protocols that we applied provided recombinant allergens in sufficient purity that did not show any toxic side effects to human basophils.

Moreover, our results indicate that pure allergen preparations can be better obtained from *E. coli* expressing one defined molecule than from pollens or other natural sources that contain other potent allergens. As was described for natural allergens we were able to desensitize patients' basophils allergen specifically with purified r*Bet v* I and r*Bet v* II indicating the specificity of the described effector model. Pure recombinant allergens therefore allow the setup of experimental allergen-specific effector systems and in addition may be useful for several clinical applications. Measurements of specific IgE such as RAST or even IgE immunoblots do not provide information about the allergic patients' ability to respond on allergen contact with release of biologic mediators such as histamine, which are directly linked to the clinical symptoms. This may be attributed to the fact that the functions of allergic effector cells can be modulated by pharmaceutical substances and hormones as well as by cytokines.^{19, 20} The allergen-specific in vitro histamine release test with patients' basophils may therefore provide more information about the effector mechanisms in allergic patients and add substantially to already established clinical allergy tests (RAST, RIST) measuring IgE levels. The application of novel recombinant material in vivo, such as for specific in vivo allergy tests like skin prick tests, and their use for allergen-specific immunotherapy, requires a thorough in vitro testing. The presented effector model

may be regarded as a first promising step toward the in vivo application of recombinant allergens.

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Treatment of allergic rhinitis with intranasal corticosteroids in patients with mild asthma: Effect on lower airway responsiveness

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*The effect of treatment of allergic rhinitis with intranasal corticosteroids on lower airway responsiveness was assessed in a randomized, double-blind, placebo-controlled, crossover study. Twenty-one young patients with perennial allergic rhinitis and asthma, with documented lower airway hyperresponsiveness (PC_{20} methacholine <8 mg/ml), were treated with intranasal aqueous beclomethasone dipropionate and placebo, each given for 4 weeks. Patients recorded rhinitis and asthma symptom scores and monitored peak expiratory flow rates every morning and evening. Patients recorded global assessment of rhinitis and global asthma symptom scores at the beginning and end of each treatment. PC_{20} methacholine was performed at baseline and at the end of each treatment period. Intranasal beclomethasone dipropionate significantly reduced global rhinitis symptom scores ($p = 0.05$) after 4 weeks of treatment. Global asthma scores did not change significantly ($p = 0.2$). Geometric mean PC_{20} methacholine improved significantly after 4 weeks of intranasal beclomethasone, but not after placebo ($p = 0.04$). Daily morning and evening rhinitis symptom scores were lower in patients treated with intranasal corticosteroids over the first 4 weeks of treatment, but carryover effect of steroids precluded comparative analysis of the second 4-week block (morning $p = 0.06$, evening $p = 0.03$). Morning asthma scores tended to decrease ($p = 0.07$). Evening asthma scores were significantly decreased at weeks 2 and 3 ($p = 0.001$, $p = 0.02$, respectively). No change in peak expiratory flow rate was seen. This study confirms that treatment of inflammation in the upper airways indirectly improves asthma symptoms and decreases bronchial hyperreactivity. Ignoring inflammation in the upper airway may lead to suboptimal results in asthma treatment. (*J ALLERGY CLIN IMMUNOL* 1993;91:97-101.)*

Key words: Allergic rhinitis, asthma, intranasal corticosteroids, airway responsiveness

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The relationship between upper and lower airway manifestations of allergy remain incompletely investigated. Many patients with allergic rhinitis, but no history of asthma, have evidence of abnormal pulmonary function, occurring either spontaneously or after bronchoprovocation with methacholine, histamine, or cold air.¹⁻¹³ In some patients with allergic rhinitis and no evidence of asthma, inhalation of pollen causes bronchoconstriction, just as it does in asthmatic