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Determination of the allergenic activity of birch pollen and apple prick test solutions by measurement of β -hexosaminidase release from RBL-2H₃ cells. Comparison with classical methods in allergen standardization

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Background: A murine *in vitro* model of the allergic type I reaction was set up to determine the biologic activity of extracts without involvement of human beings. It is based on β -hexosaminidase release from passively sensitized RBL cells after allergen challenge. The intended application of this RBL cell assay in the field of quality control of allergenic extracts requires its comparison with established methods.

Methods: The activity of five standardized birch-pollen prick test solutions was determined in parallel by RBL assay, direct IgE binding, IgE-binding inhibition, major allergen content, histamine-release assay, and skin testing.

Results: The RBL cell-release assay corresponded well to other methods if a reagent raised against natural birch-pollen extract was used for passive sensitization. However, in the case of a reagent against recombinant Bet v 1, only a decreased activity was observed, presumably because a reduced number of epitopes were recognized by the monospecific reagent. In contrast to standardized birch-pollen extracts, nonstandardized apple extracts showed poor activity in all assays.

Conclusions: This murine model might be a useful tool in the quality control of allergenic extracts. It combines properties of assays based on standardized antisera and of assays that consider IgE cross-linking properties.

Recently, we described a murine model of the allergic type I reaction that has been developed to determine the biologic activity of allergenic extracts without the direct involvement of human beings (1, 2). This model is based on two steps:

- 1) generation of specific reagenic antibodies in mice
- 2) the performance of an allergen dose-dependent mediator-release assay from passively sensitized rat basophil leukemia (RBL-2H3) cells.

Due to its sensitivity, this assay is appropriate to investigate final products, including highly diluted allergenic extracts. The procedure of passive sensitization of RBL cells by monospecific reagenic sera allows us to analyze almost any kind of allergenic extract, including rarely occurring allergens. The intended application of this RBL cell assay in the field of quality control of allergenic extracts requires its comparison with established methods of allergen standardization. The major concern is that its specificity depends on a murine reagenic serum which results from an artificial sensitization procedure. In this assay, the murine IgE cannot be replaced by human IgE due to the species restriction of the Fc_ϵ -receptor of RBL-2H3 cells. Thus, the goal of this study was to determine whether and how far results of the murine RBL assay correspond to allergenic activities determined by established methods. We favored a comparative investigation of well-characterized extracts such as those of birch pollen. These are dominated by only one major allergen. Thus, significant interferences caused by the presence of two or more major allergens in the biologic assay were not expected. We selected five standardized prick test solutions (PTS) from birch pollen which were provided by different manufacturers. The allergenic activity was determined in parallel by RBL cell assay and established methods such as IgE-binding inhibition, major allergen content, and histamine release of peripheral blood leukocytes. This was supplemented by evaluation of skin test reactivity. Furthermore, reagenic sera raised against birch-pollen extracts and against recombinant (r) Bet v 1 were compared by the RBL assay. Considering the well-documented cross-reactivity between birch-pollen and apple allergens (3, 4), we extended this study to apple PTS by use of reagenic sera raised against birch-pollen extracts. The results yielded evidence that the mediator-release assay indicates allergen activities similar to those of classical methods of allergen standardization.

Material and methods

Allergens

PTS from birch pollen with a standardized activity of 10 HEP, 50 000 SBE/ml, 10 000 AU/ml, 100 BU/ml, or 2.5 %;

and apple PTS provided by five manufacturers were comparatively investigated. rBet v 1 (obtained from Biomay, Linz, Austria) and Mal d 1 were used as reference allergens. Mal d 1 and crude apple extract were prepared as described elsewhere (4, 5). Birch-pollen extract from MAST Diagnostica, Reinfeld, Germany, was used for sensitization of mice and as reference extract.

Human sera and patients

The serum pool used in EAST and EAST inhibition was prepared from sera of five patients allergic to birch pollen with CAP classes of at least 4 to birch pollen and at least 3 to apple. The skin prick test was performed on 30 patients with clinical symptoms of birch pollinosis. Patients participating in histamine release reported oral symptoms after ingestion of fresh apples and also suffered from birch pollinosis.

Murine reagenic antisera

Reagenic antisera against birch-pollen extract were generated in BALB/c mice by five intraperitoneal injections in biweekly intervals of birch-pollen extract containing 0.1 μ g Bet v 1, or against the recombinant allergen by six injections of 0.1 μ g rBet v 1. Allergens were adsorbed on 1 mg aluminum hydroxide (Merck, Darmstadt, Germany) suspended in 0.5 ml phosphate-buffered saline (PBS), pH 7.4. Ten days after the last immunization, mice were bled by cardiac puncture. Sera were pretested to exclude non-responders. At least eight sera were pooled, aliquoted, and stored at -20°C until use.

Immunoblotting

Birch-pollen extract was separated by SDS-PAGE using 5 % (w/v) stacking gels (1.0 mm, T=5.0%, C=2.6%) and 13 % (w/v) resolving gels (T=12.8%, C=2.7%). The protein load on the gels was 18 μ g cm^{-1} on 5.5 cm preparative slots (5). Subsequently, the extracts were transferred onto nitrocellulose (NC) membranes by semidry blotting. After blocking with 0.01 M PBS/0.3 % (v/v) Tween 20, cut NC strips (0.3 mm) were either stained with India ink or used for immunodetections. We compared the murine birch-pollen reagenic serum pool, the Bet v 1-specific monoclonal antibody (mAb) BIP1 (from C. Ebner, Vienna, Austria), and the human serum pool from birch-pollen-allergic patients ($n=5$). Primary incubations of NC strips with serum or antibodies were carried out overnight. The human serum

was diluted 1:6.7 in 1 ml PBS containing 0.05 % (v/v) Tween 20 and 0.1 % bovine serum albumin (BSA). Bound IgE was detected by subsequently incubating the NC strips with rabbit polyclonal anti-human IgE, goat polyclonal affinity-purified biotinylated anti-rabbit IgG (both from Dako, Copenhagen, Denmark), and streptavidin horseradish peroxidase (HRP) conjugate (Medac, Hamburg, Germany) at dilutions as previously described (5). The mAb BIP1 was used as culture supernatant 1:100. Bound murine IgG was detected by an affinity-purified rabbit anti-mouse IgG biotin conjugate (Dianova, Hamburg, Germany) followed by streptavidine-HRP incubation, as described above. For detection of specific IgE from murine sera, these were diluted 1:15. Thereafter, strips were further incubated overnight with affinity-purified monoclonal rat anti-mouse IgE biotin conjugate (Pharmingen, Hamburg, Germany), and streptavidine HRP. Bound enzymatic activity was visualized with a commercial enhanced chemiluminescence reaction kit (ECL) (Amersham-Buchler, Braunschweig, Germany) by exposure of NC strips to a radiograph film.

Rat basophil leukemia cell mediator-release assay

Rat basophil leukemia cell subline RBL-2H3 was maintained in Eagle's MEM with 10% FCS. The assay was performed by measurement of β -hexosaminidase release. Briefly, RBL cells were harvested in the stationary phase and plated in 96-well Nunc tissue-culture plates (10^5 cells per well). Cells were attached to the surface during incubation for 18 h at 37°C in 5% CO₂. Passive sensitization was performed by preincubation with reagin pool serum, at a final dilution of 1:50 or 1:100, for 1 h. Subsequently, the cell layer was washed four times in Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA, pH 7.4). Triggering of RBL cells was induced by adding 100 μ l per well of a serial dilution of the allergen in Tyrode's buffer for 1 h in a humidified atmosphere at 37°C. Controls were run without allergen to measure spontaneous release. Total release was obtained by adding 1% Triton X-100 (Sigma, Deisenhofen, Germany) to the buffer. To determine the enzymatic activity of the released β -hexosaminidase, 30 μ l of the supernatant and 50 μ l of *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (1.3 mg/ml in 0.1 M citric acid buffer, pH 4.5) were mixed in a separate 96-well plate. After 1 h at 37°C, the reaction was terminated by adding 100 μ l of 0.2 M glycine solution, pH 10.7, and measured at 405 nm. Results were expressed as percentage of the total release minus the spontaneous release, and plotted on an x, y graph with log x

scale. The allergen concentration in a sample was calculated by the parallel-line method, referring to the standard calibrated in mass or arbitrary units.

Bet v 1 content by mAb-binding inhibition

The Bet v 1 content in allergen extracts was determined by binding inhibition of a Bet v 1-specific mAb. For this, we used the Bet v 1-specific mAb BV09, obtained from ALK Laboratories, Hørsholm, Denmark. The BV09 dilution required for 70% of maximal optical density in ELISA was determined by titration. A volume of 50 μ l from serial dilutions of PTS or from the reference extract was preincubated with 50 μ l of BV09 at 70% maximum dilution.

Subsequently, the free BV09 was detected by ELISA as follows. NUNC Maxisorb 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C by 50 μ l birch-pollen extract diluted in PBS containing 200 ng Bet v 1. Blocking with 0.1% BSA in PBS for 10 min and all further steps were performed at room temperature. The preincubated BV09 dilutions were added for 1 h. Solid-phase fixed BV09 was detected by HRP-labeled IgG from Boehringer, Mannheim, Germany. The volume concentration required for 50% BV09 inhibition was calculated by lin/log interpolation for each extract including the rBet v 1 reference. The ratio of sample to reference indicates the major allergen content in equivalents of rBet v 1.

Immobilization of allergen from prick test solutions on paper disks

As previously described (3), allergenic extracts were coupled to cyanogen bromide-activated paper disks (Hycor, Kassel, Germany) with 40 μ l PTS, diluted with 100 μ l 0.1 mM NaHCO₃ per disk. The reference extracts from birch pollen and apple were coupled with a solution of 12 μ g/ml of protein. IgE binding was performed by overnight incubation with 50 μ l per disk of human serum pool from birch/apple allergics. IgE specifically bound to the solid phase was semiquantitatively determined by a commercial enzyme allergosorbent test (EAST) (Allergopharma, Reinbeck, Germany) by the calibration system of the manufacturer. The results were expressed as EAST classes (4).

IgE-binding inhibition

The IgE-inhibiting potency of the PTS was evaluated by EAST as described above. The inhibitor serum pool was obtained from five patients who were highly allergic to birch

pollen or apple, and diluted to an optical density of approximately 70% of the EAST signal (usually 1:5 in 0.05 M Tris buffer, pH 7.4, 0.15 M NaCl, 0.03% BSA, and 0.1% Tween 20. Paper disks, coupled with extracts from birch pollen (MAST), or self-prepared crude apple extracts coupled to paper disks served as the solid phase (4). Prick test solutions or reference extract were serially diluted (threefold in nine steps) and used as inhibitors. The allergen disks were incubated with a premix of 50 μ l of diluted serum and 50 μ l of the inhibitor overnight at room temperature. Subsequently, the EAST was continued by the procedure described above. Relative extract potencies were calculated by comparison of 50% inhibition concentration from inhibiting PTS and the reference extract (6).

Basophil histamine release

Five patients allergic to birch pollen and with reported oral symptoms after ingestion of fresh apples participated in histamine-release assays. The control group consisted of three volunteers without known allergies to pollen and apples and without specific IgE against these allergens. Written, informed consent was obtained from all patients and serum donors. None of the donors took any medication for allergic diseases during the week before testing. No participant had been hyposensitized before. The assay was carried out as previously described (7, 8). Briefly, serial dilutions of the allergenic extract, each in a final volume of 400 μ l, were prepared and mixed with 200 μ l of freshly drawn heparinized whole blood. After 1-h incubation at 37°C, the released histamine was extracted by 1-butanol and condensed by *o*-phthaldialdehyde in an autoanalyzer (Bran and Luebbe, Hamburg, Germany). Measurement was done in a fluorescence spectrometer (Perkin Elmer, Überlingen, Germany) at 300/450 nm (excitation/emission). The total release was determined by incubating blood samples with 3% perchloric acid. The allergen-induced histamine release was calculated as the percentage of the total individual amount of histamine.

Skin prick test

Skin testing was done by the prick method on the forearms with five commercial prick test solutions 1:10 diluted in NaCl (0.15 M). Histamine (0.1%) and NaCl (0.15 M) served as positive and negative controls, respectively. All tests were carried out according to the recommendations of the European Academy of Allergology and Clinical Immunology (9). The skin reactivity was determined by the wheal

diameter. The positive patient group (birch pollinosis) comprised 30 subjects; the negative group, five subjects. Informed consent was obtained from all participants. The skin test was performed outside the pollen season at the Borkum Riff Hospital (Borkum, Germany). For statistical analysis, the reactivity of the five commercial extracts was compared by the Wilcoxon ranking test.

Results

Murine reaginic sera

The development of serum IgE in BALB/c mice was directly monitored by the RBL cell mediator-release assay. Four or five applications of birch-pollen extracts containing 0.1 μ g Bet v 1 were sufficient to induce specific IgE in more than 80% of the mice. However, in the case of rBet v 1, at least six applications were required to achieve at least 70% responders. Isotype control experiments to confirm the IgE nature of the induced reaginic activity were performed as previously described (1). SDS-PAGE immunoblotting of birch-pollen extracts revealed similar binding patterns for both the human and murine IgE serum pools used in this study. These were dominated by the major allergen Bet v 1 at 17 kDa and a 34/35-kDa protein, which probably represents dimeric Bet v 1 (10), as shown in Fig. 1. Additionally, the serum pool raised against rBet v 1 recognized exclusively but weakly the 17-kDa Bet v 1 (data not shown).

Degranulation of RBL-2H3 cells

To study the allergenic activity of five standardized PTS from birch pollen and apples, we investigated their degranulating efficiency by assaying β -hexosaminidase release from passively sensitized RBL-2H3 cells. To evaluate the influence of the specificity of the sera used for passive sensitization, we compared passive sensitization by murine reaginic sera raised against birch-pollen extract with those raised against rBet v 1. After sensitization, RBL cells were challenged by serial dilutions of the birch-pollen PTS in the range from 10^{-4} to 10^{-7} , or by rBet v 1 concentrations between 10 ng and 10 pg per ml. Apple PTS were tested in the range from 1:30 to 1:300. As shown in Fig. 2, all birch-pollen PTS, as well as the Bet v 1 reference, caused a characteristic dose-response curve. The allergenic activity of each tested extract was calculated by parallel-line assay (11) and expressed as equivalents of the rBet v 1 reference in mass

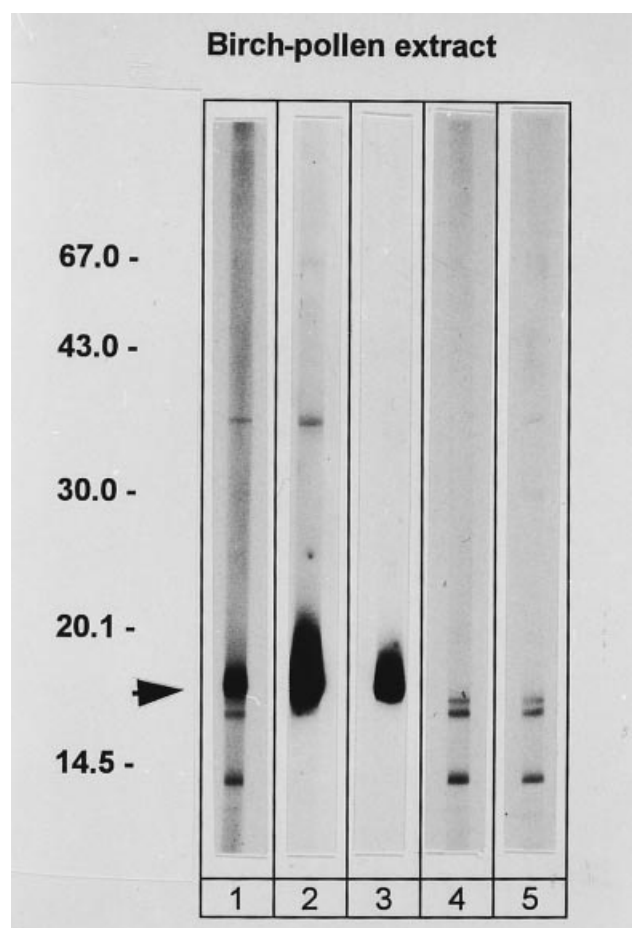


Figure 1. SDS-PAGE and immunoblot of birch-pollen extract. Immunodetection: lane 1) murine IgE serum pool raised against birch-pollen extract; lane 2) IgE pool from birch-pollen allergic patients; lane 3) murine IgG mAb BV09 specific for Bet v 1; lane 4) murine IgE serum pool specific for PLA₂; lane 5) buffer control (arrowhead indicates Bet v 1 position).

units. Obviously, the determined Bet v 1 content was influenced by the origin of the reagent used for passive sensitization (Fig. 3). Sensitization with reagent raised against birch-pollen extract resulted in an apparent allergen content of 10–220 μ g Bet v 1 per ml. Otherwise, passive sensitization with reagent raised against rBet v 1 resulted in a lower allergen content of 7–74 μ g Bet v 1 per ml. The ranking between lower active extracts was similar for both reagents:

$$10 \text{ HEP} < 10\,000 \text{ AU} < 50\,000 \text{ SBE}$$

For extracts of higher activity, a ranking was obtained only with the antiextract reagent:

$$50\,000 \text{ SBE} < 2.5\% < 100 \text{ BU}$$

The cross-reactivity between the major allergens of birch pollen and apple allows us to investigate apple extracts by RBL cells sensitized to reagent raised against birch-pollen

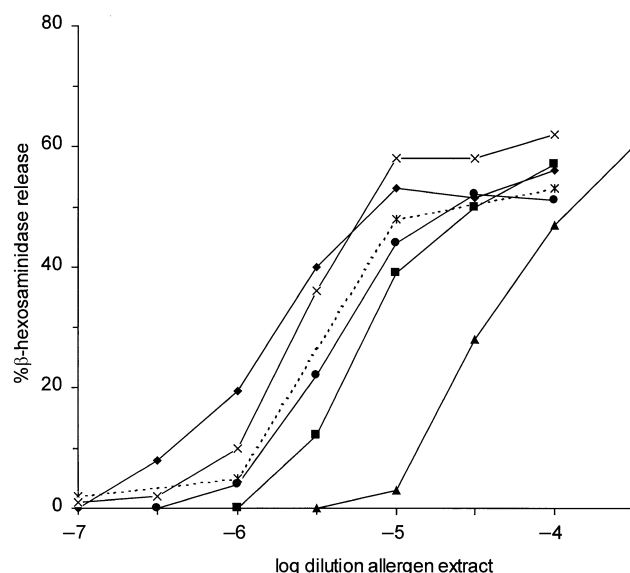


Figure 2. Determination of biologic activity in PTS from birch-pollen extracts by degranulation of RBL-2H₃ cells. Degranulation was measured by β -hexosaminidase release in supernatant. RBL-2H₃ cells were sensitized to reagent raised against birch-pollen extract and triggered by serial dilutions of recombinant birch-pollen allergen rBet v 1 adjusted at 100 μ g/ml (dotted line) (★) or PTS (solid lines) with labeled activities of 10 HEP (▲); 10 000 AU/ml (■); 50 000 SBE/ml (●); 2.5% (X), and 100 BU/ml (◆).

extracts. At a concentration of 1 μ g/ml, the purified apple allergen Mal d 1 caused a maximal β -hexosaminidase release of 50–70% in sensitized RBL cells. This is equivalent to the activity of 1 ng Bet v 1 per ml. In contrast, four of five PTS from apples caused only a maximal release below 10% and were thus considered inactive. One apple PTS induced a maximal release of 20% in the dilution at 1:30.

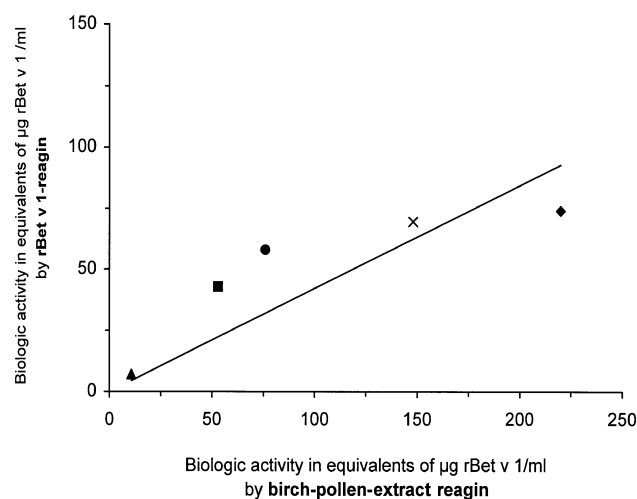


Figure 3. Comparison of biologic activity of five birch-pollen PTS determined by RBL cell degranulation when reagent against either birch-pollen extract or against recombinant major allergen (rBet v 1) was used. ($r=0.70$; $y=0.42x$). Each symbol represents standardized extract: 10 HEP (▲); 10 000 AU/ml (■); 50 000 SBE/ml (●); 2.5% (X), and 100 BU/ml (◆).

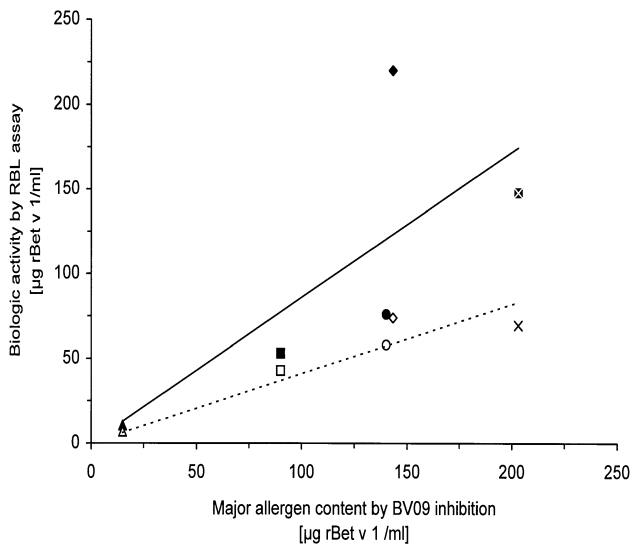


Figure 4. Quantification of birch-pollen major allergen content in five PTS by ELISA binding inhibition. Comparison with biologic activity determined by β -hexosaminidase release from RBL cells. Solid line, filled symbols: RBL cells passively sensitized with reagin raised against birch-pollen extract ($r=0.73$; $y=0.86x$). Dotted line, open symbols: passive sensitization with reagin against rBet v 1 ($r=0.92$; $y=0.41x$). Each symbol stands for standardized extract: 10 HEP ($\blacktriangle$); 10 000 AU/ml ($\blacksquare$); 50 000 SBE/ml ($\bullet$); 2.5% ($\times$), and 100 BU/ml ($\blacklozenge$).

Major allergen content by mAb-binding inhibition

The ELISA binding inhibition of the Bet v 1-specific mAb BV09 by serial dilutions of birch-pollen PTS or rBet v 1 was applied to determine the Bet v 1 content of the PTS. The dilution of a PTS which inhibited the BV09 binding to a degree of 50% was between 1:4 and 1:50. In comparison, about 5–7 μ g rBet v 1 per ml was required for 50% inhibition of the binding. By examination of the 50% inhibition concentrations, we obtained allergen contents of 15–203 μ g Bet v 1 per ml. Fig. 4 illustrates the correlation between the inhibition of the mAb BV09 and the RBL cell-degranulation assay. Interestingly, in RBL assay studies with anti-birch-pollen-extract reagin, the resulting amount of Bet v 1 was generally equivalent to that derived by BV09 inhibition ($y=0.86x$), but, not as well correlated ($r=0.73$). In contrast, sensitization with anti-rBet v 1 reagin led to a closer correlation of results ($r=0.93$). With the latter reagin, the detected amount of Bet v 1 was clearly lower than in results from BV09 inhibition ($y=0.41x$).

IgE-binding activity of immobilized PTS

Coupling of allergen extracts from PTS to activated disks is a convenient method to screen their general allergenic

Table 1. IgE-binding activity of birch-pollen PTS detected by direct EAST and EAST inhibition. Direct EAST: birch-pollen PTS bound to solid phase and IgE binding with serum pool from birch/apple-allergic patients. EAST inhibition: preincubation of serum pool with PTS and determination of specific IgE by EAST

PTS with	10 HEP	10 000 AU/ml	50 000 SBE/ml	2.5%	100 BU/ml	Reference
EAST class	4	3	3	4	4	3
EAST inhibition (relative potency)	0.27	1.02	1.49	1.86	2.13	1.00

activity. This requires the detection of their capacity to bind allergen-specific IgE. Here we used the serum pool from birch-pollen- and apple-sensitized patients. The IgE-binding activity of the tested birch-pollen extracts resulted in EAST classes between 3 and 4, including the reference (Table 1). However, in the case of apple PTS, only the extract which was active in the RBL cell degranulation showed detectable IgE-binding activity. However, its EAST class was only 1, whereas the self-prepared apple extract used as reference resulted in an EAST class of 4 (Table 2).

IgE-binding inhibition

The EAST IgE-binding inhibition of the pool serum from allergic, birch-pollen sensitized patients was used to quantify the total allergenic activity of birch-pollen PTS by a procedure comparable to RAST inhibition. Relative potencies according to the reference were calculated on the basis of interpolated 50% inhibition concentrations. From all birch-pollen PTS, a dilution between 1:100 and 1:10 was required for 50% binding inhibition. Table 1 shows relative potencies by EAST inhibition. Fig. 5 illustrates their correlation with BV09 inhibition ($r=0.91$) and RBL degranulation by pollen-extract reagin ($r=0.90$). In the case of apple PTS, their expected lower amount of allergen required inhibition at extract dilutions from 1:5 to undiluted. Due to nonspecific interference of the glycerol contained in the PTS at dilutions below 1:10, these concentrations could not be investigated. Thus, no activity was detectable in the commercial apple extracts at dilutions above this level.

Table 2. Allergenic activity of apple PTS detected by IgE-binding capacity in EAST. EAST results obtained with serum pool from birch/apple-allergic patients and apple PTS from five different suppliers (A–E) bound to solid phase

Apple PTS	A	B	C	D	E	Reference
EAST class	1	0	0	0	0	4

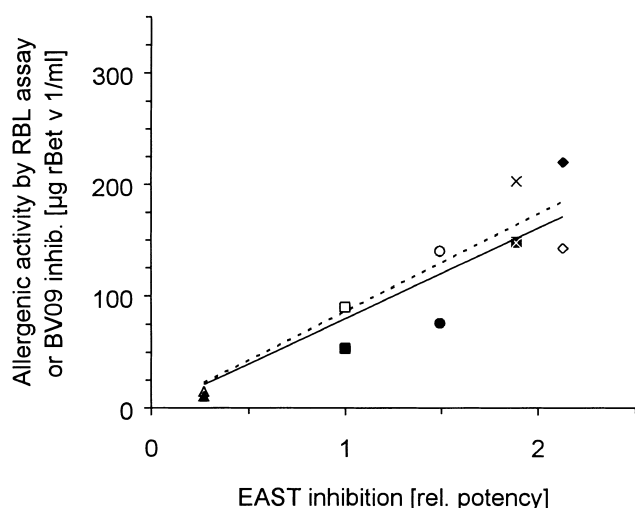


Figure 5. Measurement of allergenic potency of five PTS by EAST inhibition. Comparison with major allergen content determined by BV09 inhibition (open symbols, dotted line; $r=0.91$) and allergenic activity by RBL cell degranulation with birch-pollen-specific reagent (filled symbols, solid line; $r=0.90$). Each symbol marks standardized extract: 10 HEP ($\blacktriangle$); 10 000 AU/ml ($\blacksquare$); 50 000 SBE/ml ($\bullet$); 2.5% ($\times$), and 100 BU/ml ($\blacklozenge$).

Basophil histamine release

To compare the biologic activity of different PTS, we investigated their ability to induce histamine liberation from peripheral blood leukocytes of subjects with birch-pollen allergy combined with apple hypersensitivity, and from nonallergic control subjects. Freshly drawn whole blood was incubated by serial dilutions of birch-pollen PTS in the range from $1:10^4$ to 10^9 . Apple PTS were diluted from $1:10^1$ to 10^4 . The rBet v 1 was tested between 10 ng/ml and 1 pg/ml, and the purified apple major allergen Mal d 1 was tested in the range from 10 μ g to 10 ng/ml. Plotting the histamine release from the allergic patient group ($n=5$) resulted in a nearly bell-shaped dose-response curve for birch-pollen PTS, rBet v 1, and Mal d 1 (Fig. 6). All three negative donors were without detectable release ($<5\%$).

In comparison of the dose-response relation in five PTS with five subjects, it was obvious that from the PTS with 10 HEP a 10-fold lower dilution was required to reach maximal histamine release (Table 3). A calculation of relative potencies for each PTS was not possible due to variations between tested individuals, as shown in Fig. 6. The ranking between less and more active birch-pollen extracts was comparable to other methods. Investigating apple extracts, we found that 1 μ g/ml of the major apple allergen Mal d 1 was as effective in triggering basophils from the tested birch/apple-pollen-sensitized subjects as 1 ng/ml from rBet v 1. Although the susceptibility of the donor leukocytes was

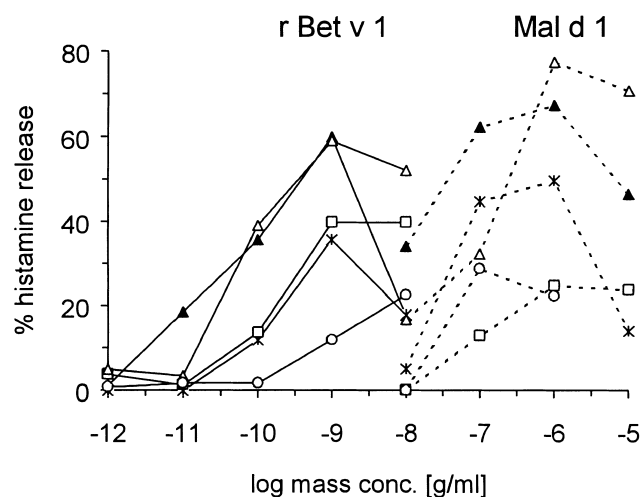


Figure 6. Dose-dependent histamine release from peripheral blood leukocytes from five birch-pollen allergics with apple hypersensitivity. Comparison of rBet v 1 (solid lines) with Mal d 1 (dotted lines). Each symbol represents one subject.

clearly proven by reactivity to Mal d 1, we were not able to demonstrate histamine-releasing activity by apple PTS.

Skin test reactivity of birch-pollen PTS

To determine whether results obtained by *in vitro* methods correspond to skin test reactivity, all five PTS were compared in birch-pollen-sensitized patients. The results obtained from 30 tested patients were recorded in wheal diameter reactivity and plotted as shown in Fig. 7. The results illustrate a considerable deviation of the reactivity between individuals. In comparison of the results by Wilcoxon signed rank test ($P<0.05$) (12), it was evident that the activity of extracts with 10 HEP and 10 000 AU was significantly weaker than the activity of extracts with 2.5%, 50 000 SBE, or 100 BU.

Table 3. Histamine release from peripheral blood leukocytes by birch pollen PTS. Log of dilution of extract required to induce maximal release

Allergen extract	Patient no.				
	1	2	3	4	5
100 BU	6	6	5	5	5
2.5%	6	6	5	n.i.	n.i.
10 HEP	5	4	4	4	4
50 000 SBE	6	6	5	5	5
10 000 AU	6	6	5	5	5
n.i.: not investigated.					

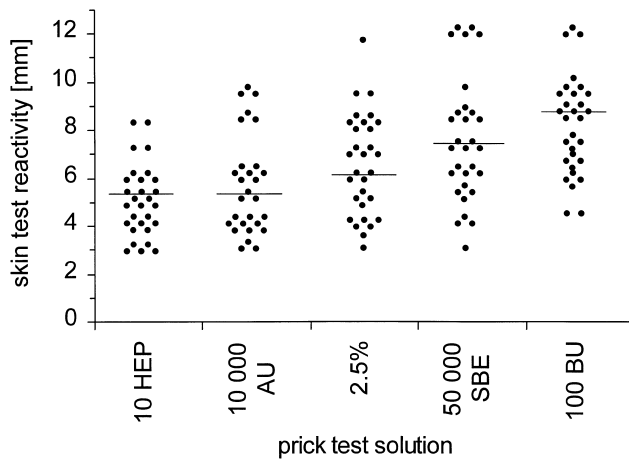


Figure 7. Skin test reactivity: birch-pollen PTS (1:10 diluted) from five manufacturers were pricked in parallel in birch-pollen-sensitized allergic patients ($n=30$). Diameter of wheal was recorded after 20 min. Each dot represents reactivity of one subject. Median is indicated by bars.

Discussion

Recently, we set up a murine *in vitro* model of the type I reaction which allowed us to determine the biologic activity of highly diluted allergenic extracts without the involvement of allergic patients. It is based on allergen-dependent degranulation of RBL-2H3 cells, passively sensitized to murine IgE. Comparative investigation of a selection of standardized birch-pollen extracts should help to ascertain the reliability of this assay. Doubtless, the most crucial point of this method is the murine reagent used for passive sensitization of RBL cells. The immunization schedule based on repeated applications of low doses of antigen (13, 14) seems to be generally suitable to induce Bet v 1-specific IgE in BALB/c, DBA/2, and BP2 mice (15).

In this study, we used pooled sera from BALB/c mice, which were repeatedly shown to be suitable with respect to their IgE response and their general availability. We observed that a pool size of 8–10 sera yields invariably similar results when comparing PTS in pool switching, indicating that the assay can be used as a standardized tool. Nevertheless, for further distribution of this method, the size of pools can be enlarged as necessary. However, if the composition of the immunogen is changed with respect to the allergen, the reagent sera may differ markedly. This was illustrated by comparing IgE sera raised either against a complete extract from birch pollen or against the recombinant allergen rBet v 1. Interestingly, rBet v 1 seems to be less immunogenic than a complete birch-pollen extract with the same content of Bet v 1. Conversely, the direct comparison of dose-dependent cross-linking properties of recombinant

and natural Bet v 1 revealed no differences, as shown previously (1).

In investigating reagent sera raised against the extract and rBet v 1, a reproducible dose-response curve was obtained with both sera. This allowed us to designate the activity of each birch-pollen extract in equivalents of rBet v 1 in mass units. Remarkably, the apparent Bet v 1 content was approximately two times lower when reagent raised against rBet v 1 was used than with anti-birch-pollen-extract reagent (Fig. 3). This might be partially explained by a restricted specificity of the rBet v 1 reagent to fewer isoforms, in contrast to the polyspecific extract reagent. In addition, birch-pollen minor allergens could also contribute to the release of cells sensitized to the latter reagent serum. By determining Bet v 1 via mAb inhibition with BV09, we obtained a major allergen content which was approximately 85% of that calculated from RBL assay with extract reagent. This general conformity may be explained by the preferred recognition of Bet v 1 by both the mAb and the reagent serum, which, in the latter case, was demonstrated by immunoblotting. Remarkably, the correlation with BV09 inhibition was closer with degranulation by rBet v 1 reagent ($r=0.97$) than degranulation by extract reagent ($r=0.73$) (Fig. 4). Possibly, the latter reagent, also recognizes biologically active structures other than Bet v 1 which were not equally distributed in all extracts. It remains to be elucidated whether this is due to dimeric Bet v 1 structures or other Bet v 1 isoforms (16, 17).

Among the methods that were based on human IgE, the high overall allergenic activity of all birch-pollen extracts and the lack of activity of all apple extracts was initially demonstrated by direct EAST (Tables 1 and 2). By EAST inhibition, we obtained relative potencies which were closely correlated with the biologic activity determined in the RBL cell test or by mAb BV09 inhibition (Fig. 5). This was to be expected since all the probes, i.e., the human IgE serum pool, the mAb BV09, and the extract reagent, preferably recognized the 17-kDa Bet v 1 (Fig. 1). Histamine release from basophils was performed to confirm these results by an assay of the biologic activity of these extracts in man. The histamine-release assay underlies great variation between individuals, as illustrated in Fig. 6 for rBet v 1 and Mal d 1. Therefore, a calculation of relative potencies is not recommended. However, almost all patients showed a ranking of the maximal histamine release which corresponded to the murine RBL cell-degranulation assay. The skin test investigation supported the findings of the ranking of the allergenicity between the extracts. However, the variability between the individuals demonstrated also the natural limitation of *in vivo* testing for standardization

purposes. Thus, support of current methods of biologic allergen standardization by the RBL assay might be advantageous.

An additional part of this study included investigation of apple extracts. However, in contrast to commercially available standardized birch-pollen PTS, the activity of nonstandardized apple extracts was generally poor. Remarkably, only one of five investigated extracts showed weak allergenic activity, as detected by the RBL assay and by direct EAST, whereas a self-prepared apple extract, as well as purified Mal d 1, was highly active. We did not succeed in histamine release with this single apple extract despite the susceptibility of the apple major allergen Mal d 1 to this assay. Because previously performed skin tests revealed no activities (18), the obviously ineffective PTS were excluded from repeated skin testing in this study. Of course, the situation in the case of apple PTS is disappointing. However, we may expect that recently developed methods, particularly for the preparation of active and stable apple extracts for prick testing (4, 19), will improve the quality of such extracts in the near future.

In the present study, we demonstrated that determination of biologic activity by a murine model of the type I reaction yields results in accordance with established methods of allergen standardization. With respect to the controversy on whether allergen standardization should rely on IgE-based techniques or on (major) allergen-specific animal antisera (20), this assay may represent a compromise. It has the advantages of an assay based on defined polyclonal antibodies and considers also the cross-linking properties of allergenic extracts.

Finally, we feel that the present study yields further evidence that murine RBL cell mediator-release assay could serve as a useful tool in the standardization of extracts, at least for selected allergens. Further studies will reveal whether the assay is also applicable to adsorbed and modified extracts and allergens that are difficult to standardize such as those of molds.

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