

Biologic allergen assay for in vivo test allergens with an in vitro model of the murine type I reaction

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Background: The determination of the biologic activity of allergenic extracts in human beings is limited for ethical and practical reasons. The establishment of a simplified in vitro model, which mimics a main event of the type I reaction, should provide true benefit for manufacturers, researchers, and clinicians.

Objective: This study was designed to develop and evaluate a mediator release assay based on rat basophil leukemia cells for the purpose of investigating allergenic extracts.

Methods: Rat basophil leukemia cells were passively sensitized with murine IgE raised against allergens and stimulated by serial dilutions of allergenic extracts in a dose-related manner. The allergen-specific degranulation was monitored by measuring the release of β -hexosaminidase.

Results: The investigation of standardized commercial allergen products for in vivo diagnostics (birch pollen, cat dander, and bee venom) allowed a quantitative description of differences in biologic activity in accordance with the declared activity units. The Fel d 1 content was determined in 17 cat dander extracts and correlated well with the results of a two-site binding ELISA ($r = 0.93$, log/log). Extremely low allergen amounts in the range of 10 to 100 pg/ml could be easily detected. Moreover, the cross-reactivity pattern of patients allergic to birch pollen could be reproduced with extracts of hazel, alder, apple, and celery.

Conclusion: The assay is suitable for supplementing quality control of allergenic extracts and for the determination of biologic activity of final allergen products. As a research tool, it allows the study of IgE cross-linking properties of modified and recombinant allergens at an early stage before they are tested in human beings. (*J Allergy Clin Immunol* 1997;99:227-32.)

Key words: Biologic allergen assay, animal model, final allergen products, rat basophil leukemia cell, murine IgE

In the last decade, allergen standardization by biologic and immunochemical methods has improved the efficiency and safety of allergenic extracts for in vivo application in human beings. Allergenic extracts are today biologically standardized by using skin test techniques to assign allergy units corresponding to reference preparations.¹⁻³ However, the determination of biologic activity of allergen products in human beings is limited for ethical and practical reasons.

The aim of this study was to investigate whether a mediator release assay, based on the permanently growing rat basophil leukemia (RBL)-2H3 cell line⁴ (RBL assay), might serve as a tool with which to determine the biologic activity of final allergen products. For this, we raised specific murine IgE against cat dander, birch and grass pollen, and phospholipase A₂ (PLA₂) from bee venom. These murine sera were used to passively sensi-

Abbreviations used

HEP:	Histamine equivalent potency units
PLA ₂ :	Phospholipase A ₂
RBL:	Rat basophil leukemia
SQ-U:	Standardized quality units

tize RBL cells. We investigated basic properties of the RBL assay such as detection threshold, selectivity, and specificity of the murine IgE; cross-reactivity, and reproducibility. The biologic activities of standardized final products for prick, provocation, and intradermal testing were investigated with cat dander and birch pollen extracts or bee venom as clinically important examples.

METHODS

Allergens

Standardized prick test solutions from cat dander, birch pollen, and timothy grass pollen (each 10 histamine equivalent potency units [HEP]) or PLA₂ from bee venom (Sigma, Deisenhofen, Germany) were applied for immunization and stimulation of sensitized cells. For stimulation experiments, prick test solutions from alder pollen, hazel pollen (both 10 HEP), bee venom (300, 100, and 10 μ g), and birch pollen- or cat

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dander-specific provocation and intradermal test solutions (450,000 standardized quality units [SQ-U] or 4500 SQ-U) were tested separately. All these final products were obtained from ALK Laboratories (Horsholm, Denmark). RBL assay and two-site binding ELISA were compared by investigating cat dander extracts from different suppliers or crude extracts prepared in our laboratory from cat hair.⁵ Purified Fel d 1 (kindly provided by C. Schou, ALK), recombinant Bet v 1 (a gift from O. Scheiner, Vienna, Austria), and PLA₂ were used as reference allergens. Natural Bet v 1 and the major apple allergen Mal d 1 were purified as previously described.⁶ Crude apple and celery extracts were prepared as described by Schönig et al.⁷

Murine serum antibodies

Cat dander-, birch pollen-, and timothy grass pollen-specific antisera were generated in BALB/c mice by four or five injections of 5 µl of prick test solutions. To induce PLA₂ antiserum, CBA/J mice (both mouse strains from Charles River, Sulzfeld, Germany) were injected five times with 0.1 µg. Allergens were adsorbed on 5 µg aluminum hydroxide (Merck, Darmstadt, Germany) suspended in 0.5 ml of phosphate-buffered saline, pH 7.4 (0.15 mol/L), and administered intraperitoneally. Ten days after the last immunization, the mice were anesthetized and bled by cardiac puncture. Sera were pretested to exclude nonresponders. At least five sera were pooled and stored in 50 µl aliquots at -20°C until use.

RBL cell mediator release assay

RBL cell subline RBL-2H3, a generous gift from Dr. I. Pecht of the Weizman Institute (Rehovot, Israel), was maintained in Eagle's minimal essential medium with 10% fetal calf serum. The assay was performed by measurement of β-hexosaminidase. Briefly, the cells were harvested in the stationary phase and plated in 96-well Nunc tissue culture plates (10⁵ cells per well). Cells were attached to the surface during incubation for 18 hours at 37°C in 5% CO₂. Passive sensitization of the cells was performed by preincubation with serum in a final dilution of 1:50 in Eagle's medium or as indicated. Subsequently, cells were washed four times in Tyrode's buffer (130 mmol/L NaCl, 5 mmol/L KCl, 1.4 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.6 mmol/L glucose, 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, and 0.1% bovine serum albumin [pH 7.4]). For cross-linking, RBL cells were incubated with 100 µl of serial dilutions of the allergen in Tyrode's buffer for 1 hour in a humidified atmosphere at 37°C. Controls were run without allergen to measure spontaneous release and with the buffer containing 1% Triton X-100 (Sigma) to quantify the total release. For measurement of the released β-hexosaminidase, we transferred 20 µl of the supernatant into a second 96-well plate. The enzymatic activity was detected by addition of 50 µl of p-nitrophenyl N-acetyl β-d-glucosaminide as substrate (1.3 mg/ml in 0.1 mol/L citric buffer, pH 4.5). For termination of the reaction, 100 µl of 0.2 mol/L glycine solution, pH 10.7, was added. Absorbance was measured at 405 nm. Results are expressed as percent of the total release after subtraction of spontaneous release and are plotted on an x-y graph by 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.⁸

Isotype control experiments

Murine antiserum was divided into three aliquots, which were heated for 30 minutes or 60 minutes at 56°C. One control was

run without treatment. Subsequently, cells were preincubated by inactivated serum. Cross-linking was tested with allergen, anti-IgE (diluted 1:1000; Nordic, Tilburg, Netherlands), anti-IgG₁, or IgG₂ (1 µg/ml, both from Sigma).

Fel d 1 determination by two-site binding ELISA

The assay was performed according to a method previously described.⁹ Briefly, Maxisorb 96-well plates were coated with the monoclonal antibody Mc a Fel d 1 105802, followed by blocking, washing, and addition of the diluted sample. Solid-phase-bound Fel d 1 was detected with the horseradish peroxidase-labeled rabbit antibody HRP-a Fel d 1 170993. Both antibodies were kindly provided by C. Schou (ALK). The Fel d 1 content from each sample was calculated from a calibration curve of purified Fel d 1 or a reference extract with known Fel d 1 content.

RESULTS

Induction of specific IgE in mice

The immunization experiments of this study revealed that four or five injections of 5 µl of prick test solution or 0.1 µg of purified allergen adsorbed to Al(OH)₃ were sufficient to induce specific IgE in more than 80% of BALB/c mice with cat dander, birch, or timothy grass pollen extract and in CBA/J mice with PLA₂. The development of serum IgE in mice was directly monitored by RBL assay because of its IgE-isotype specificity. Nonresponders and "weak" responders, detected by a serum dilution of 1:50 and an allergen concentration of 100 ng/ml (or 1:1000 diluted prick test solutions), were excluded from pooling.

Isotype control experiments

To confirm that the mediator release of RBL-2H3 cells was only IgE-mediated, cat dander-, birch pollen-, and timothy grass pollen-specific pooled sera were heated for 30 minutes and 60 minutes at 56°C.¹⁰ This heating procedure destroyed any reaginic activity. Furthermore, cross-linking by anti-IgG₁ and anti-IgG₂ failed to induce any mediator release in contrast to the strong release induced by stimulation with anti-IgE or allergen.

Determination of the detection threshold

The lowest detectable amount of allergen that evokes a significant mediator release was determined by a checkerboard titration: serum for preincubation of the cells was diluted from 1:25 to 1:800, and the allergen concentration for cross-linking ranged from 1 µg/ml to 1 pg/ml purified reference allergen. As expected, the lowest detectable amount was inversely correlated to the serum dilution used for pretreatment of RBL cells. With pooled sera, diluted 1:50 to 1:200, purified allergens with concentrations between 10 and 100 pg/ml could be specifically detected. This sensitivity is sufficient for the investigation of highly diluted final allergen products, such as solutions for intradermal testing.

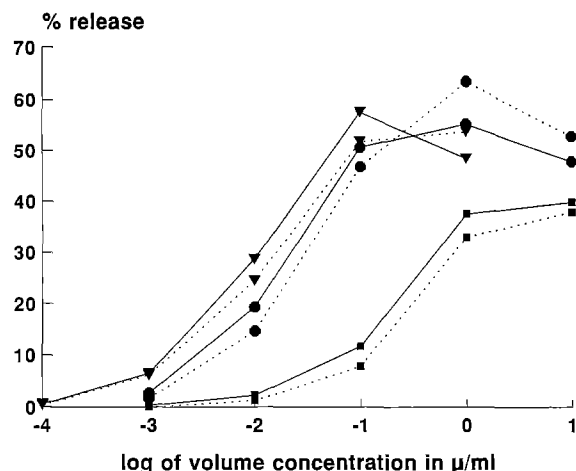


FIG. 1. Cross-reactivity between prick test solutions from birch, alder, and hazel pollen. RBL cells were sensitized with IgE from antisera raised against birch pollen. β -Hexosaminidase release was detected after stimulation with tree pollen prick test solution of birch (filled triangles), alder (filled circles), and hazel pollen (filled squares). From each species two batches (dotted or continuous line) were compared. The declared activity of all solutions was 10 HEP.

Specificity testing of the murine sera

The specificity of the antisera was studied by sensitizing the RBL cells with antisera specific for cat dander, birch pollen, timothy grass pollen, or PLA₂. Stimulation was performed with the previously mentioned prick test solutions (diluted 1:1000) or PLA₂ (100 ng/ml). With the exception of the allergens used for immunization, we did not find any mediator release above the spontaneous release resulting from the stimulation of the cells with the nonrelated allergens.

Cross-reactivities

As a clinically important example, birch pollen-related cross-reactivities were investigated. RBL cells were sensitized with pooled birch pollen-specific antisera. Cross-linking was performed with prick test solutions from related tree pollens such as hazel and alder, each standardized and adjusted to 10 HEP by the supplier. A 10 HEP birch pollen solution from the same manufacturer served as the reference. Titration of two batches from each allergen revealed no differences between alder pollen and birch pollen. In the case of hazel pollen, however, a 10-fold higher concentration was required to evoke the same β -hexosaminidase release obtained with alder and birch (Fig. 1). Birch pollen-sensitized RBL cells were also sensitive to plant food allergens such as apple or celery. Recombinant Bet v 1 was used as the reference. In addition, natural Bet v 1 was compared (with the recombinant Bet v 1) in the same experiment. The resulting dose-related mediator release plots were nearly identical for recombinant and natural Bet v 1 preparations (Fig. 2). In the case of the cross-reactive major apple allergen, Mal d 1, a 1000-fold higher

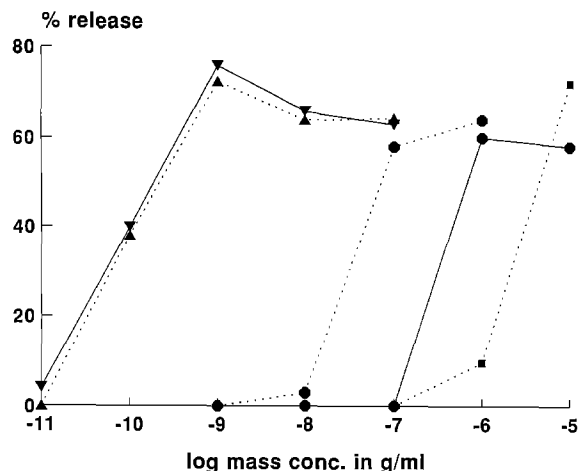


FIG. 2. Cross-reactivity between isolated allergens or extracts from birch pollen, apple, or celery. RBL cells were sensitized with IgE from antisera raised against birch pollen. Degranulation was measured by β -hexosaminidase release after stimulation with extract from apple (filled circles, continuous line), the apple major allergen Mal d 1 (filled circles, dotted line), and celery (filled squares, dotted line). For comparison the closely related release triggered by natural Bet v 1 (filled triangles, continuous line) and recombinant Bet v 1 (filled triangles, dotted line) is shown.

concentration was necessary to induce the same biologic effect as with Bet v 1. From crude apple extract and from celery, 10-fold and 100-fold higher concentrations, respectively, were required when compared with Mal d 1 on the basis of the total protein amount.

Calculation of the allergen concentration and reproducibility

Because of variations of the cell culture system, the β -hexosaminidase activity released by a single allergen concentration lacks sufficient accuracy for quantitative measurements. In contrast, the distances between the titration curves show much less variation. Therefore allergenic activity was calculated by the parallel line method, comparing the distances of the linear segments of sample and reference graphs.⁸ Reproducibility of the method was determined by analyzing four cat dander extracts and a Fel d 1 curve as the reference. Over 5 days, the day-to-day variation was 15.2%. Variation between different plates within 1 day ranged from 11.7% to 12.6%. Variation within one plate ranged from 9.7% to 11.8%.

Comparison of RBL assay and two-site binding ELISA to determine the major allergen content of cat dander extracts

The feasibility of obtaining quantitative results in terms of mass units (or arbitrary units, depending on the reference) by the RBL assay was tested by comparing it with a two-site binding ELISA. Seven cat dander extracts from different suppliers and 10 crude cat hair extracts prepared in our laboratory from cat hair samples were simultaneously analyzed with both methods,

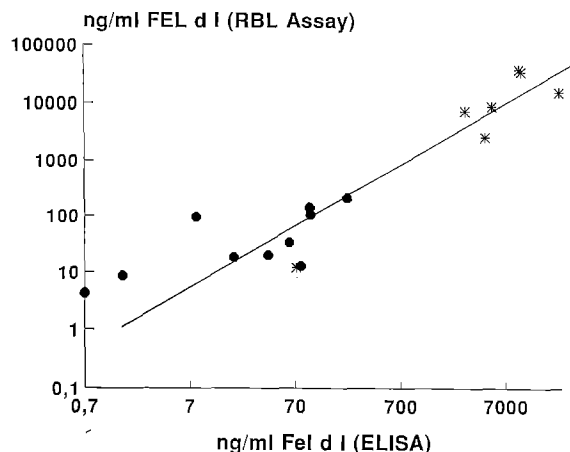


FIG. 3. Comparison of Fel d 1 concentration of extracts determined by ELISA or RBL assay. The Fel d 1 content from seven cat dander test solutions (filled circles) and 10 cat hair extracts (asterisk) was measured by two-site binding ELISA. The biologic activity detected by RBL assay is also expressed in mass units of Fel d 1 with the same reference. A log/log correlation ($r = 0.93$) was calculated.

and the Fel d 1 content was calculated. Comparison of the two assays resulted in a log/log correlation of $r = 0.93$ (Fig. 3).

Investigation of final allergen products for in vivo diagnostic by the RBL assay

The potency of test solutions from cat dander, birch pollen, and bee venom to degranulate sensitized RBL cells was investigated in a dose-dependent manner. In the case of cat dander and birch pollen, serial dilutions of two batches of prick, provocation, and intradermal test solutions were tested. Results are shown in Fig. 4, A and B. From the reference graph, mean Fel d 1 concentrations of 26.5 $\mu\text{g/ml}$, 2.1 $\mu\text{g/ml}$, and 9.7 ng/ml were calculated for prick, provocation, and intradermal test solutions, respectively. In the case of birch pollen, we calculated mean Bet v 1 contents of 10.4 $\mu\text{g/ml}$, 0.43 $\mu\text{g/ml}$, and 2.35 ng/ml, respectively, with regard to the mass units of the recombinant Bet v 1. Bee venom is known to induce nonspecific mast cell degranulation.¹¹ Indeed, a 1:10 diluted solution with 300 $\mu\text{g/ml}$ bee venom had strong degranulating effects on RBL cells without IgE pretreatment. However, the effect did not occur in the case of highly diluted samples (10^{-2} to 10^{-4}), which were generally investigated. For specific degranulation experiments, cells were sensitized with mouse antiserum raised against PLA₂. Cross-linking was performed by using bee venom prick test solution with a labeled amount of 300, 100, and 10 $\mu\text{g/ml}$. Purified PLA₂ was used as the reference. Fig. 4, C, shows the ranking between the activity of the bee venom prick test solutions, which was in accordance with the declared venom concentration. Referring to purified PLA₂ from bee venom, we computed a PLA₂ content of 72 $\mu\text{g/ml}$ PLA₂ within 300 $\mu\text{g/ml}$ strength, 32 $\mu\text{g/ml}$ within 100 $\mu\text{g/ml}$

strength, and 1.4 $\mu\text{g/ml}$ within 10 $\mu\text{g/ml}$ strength prick test solutions, respectively.

DISCUSSION

In this study we developed an in vitro model that mimics a main event of the type I reaction, the allergen-dependent degranulation of mast cells sensitized by IgE. This model has been applied as a new approach to determine the biologic activity of allergenic extracts, particularly final products for in vivo testing. The potency estimation of highly diluted extracts, such as those used for intradermal testing, has until now been particularly difficult or even impossible. In comparison with the well-known histamine release assay, there are several advantages. One of them is independence from recruiting and presence of patients with allergy and from individual seasonal immunologic status. Because of the species restriction of the Fc ϵ receptor, the assay requires murine or rat IgE. In the case of selected experimental questions, this provides the opportunity to sensitize cells by IgE from monospecific antisera directed against rare allergenic components or recombinant allergens, which are difficult to obtain from human beings. Thus protocols to induce specific IgE are essential to run this assay. For this assay, the schedule of repeated application of minute doses of allergens adsorbed to AlOH₃^{12, 13} could be successfully transferred to the allergens that were studied in this investigation. The development of specific IgE in the murine serum before pooling was directly monitored by the RBL assay. The isotype control experiments demonstrate its excellent IgE-isotype specificity in accordance with data from the literature.^{14, 15} The nature of this murine IgE is a crucial point for this assay. It is remarkable that immunization by crude allergens induced IgE that seems to be exclusively or predominantly directed to major allergens. This is supported by the high specific release induced by Fel d 1 and Bet v 1 and by a preliminary immunoblot study (latter data not shown). Slight variations in the IgE response occur despite a standardized immunization schedule and the use of inbred strains. They were compensated by pooling of at least five sera. We were not able to detect different properties between different serum pools. Such IgE serum pools were generally used for preincubation of RBL cells. As expected, the detection limit of the assay is inversely correlated to the serum dilution. A 50-fold serum dilution (or higher) was sufficient to determine allergens in the concentration range below 100 pg/ml. This allows the investigation of even final allergen products with very low concentrations, such as intradermal test solutions, with moderate consumption of murine antisera.

The specificity of the assay was investigated by cross-wise testing of RBL cells sensitized with antisera against PLA₂, cat dander, birch, and timothy grass pollen with all four allergens. No cross-sensitivities were observed. This high specificity of the antisera and the low detection limit allow the detection of low dose contaminations of final allergen products by foreign allergens in accor-

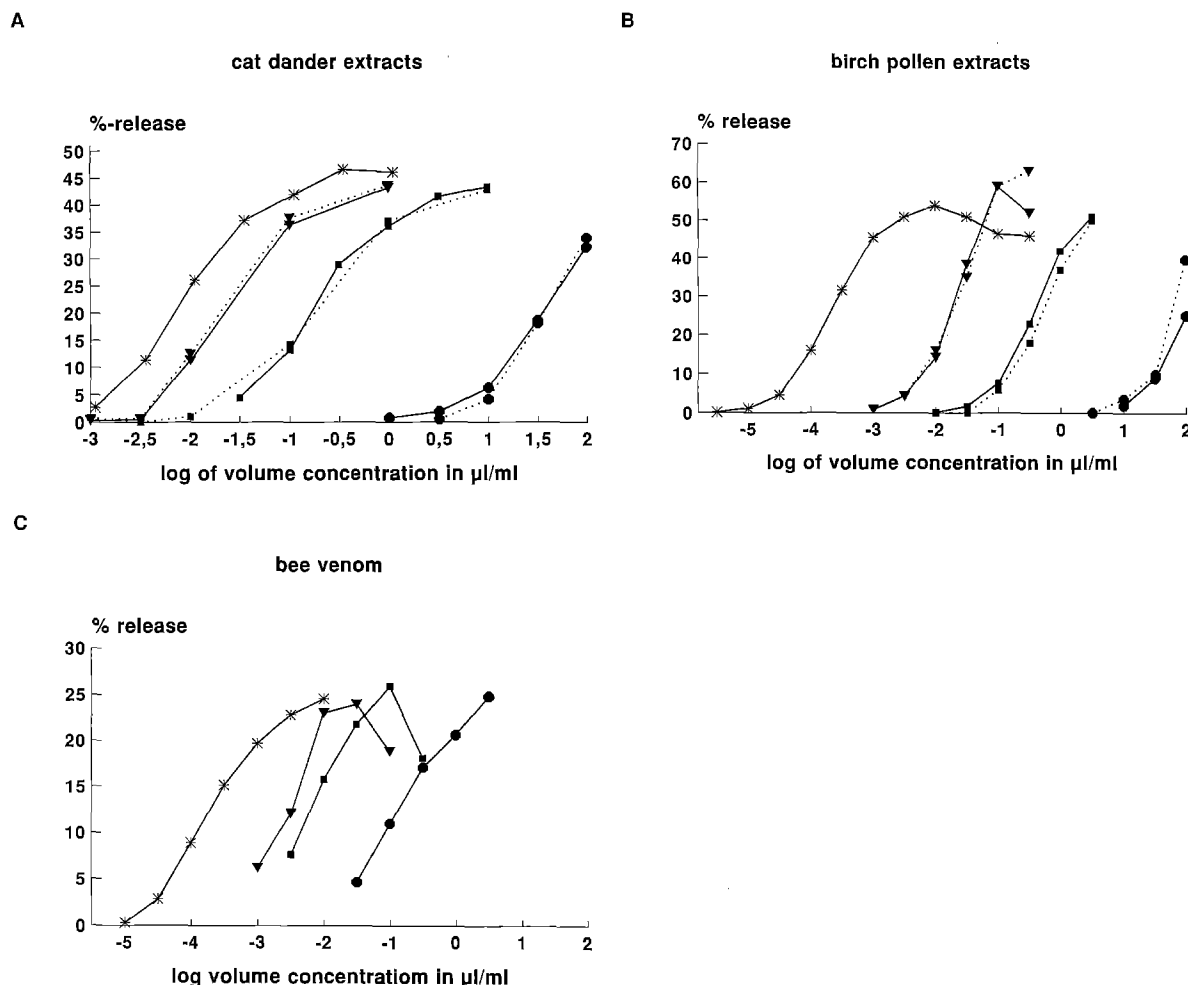


FIG. 4. Comparison of standardized diagnostic allergen solutions of different strengths by RBL assay. **A**, Cat dander extracts. RBL cells were sensitized with IgE from antisera raised against cat dander. β -Hexosaminidase release was determined after stimulation by serial dilutions of cat dander extracts with a labeled amount of 10 HEP (filled triangles), 100,000 SQ-U (filled squares), 1000 SQ-U (filled circles) from each of two batches (dotted or continuous line). Fel d 1 (asterisk) reference (50 μ g/ml). **B**, Birch pollen extracts. RBL cells were sensitized with anti-birch pollen serum. Stimulation by birch pollen test solutions with a labeled amount of 10 HEP (filled triangles), 100,000 SQ-U (filled squares), or 1000 SQ-U (filled circles) from each of two batches (dotted or continuous line). For reference, recombinant Bet v 1 (asterisk), 1 mg/ml, was used. **C**, Bee venom. RBL cells were sensitized with anti-PLA₂ serum. Mediator release was induced by incubation with serial dilutions of bee venom prick test solutions with a declared content of 300 μ g (filled triangles), 100 μ g (filled squares), and 10 μ g (filled circles) per milliliter. As a reference, we used bee venom PLA₂ (asterisk), 1 mg/ml.

dance with the requirements of the European Economic Community notes for guidance.¹⁶

Clinically known cross-reactivities were studied with birch pollen-related plant allergens. Cells sensitized with anti-birch pollen IgE showed a high degree of cross-reactivity with prick test solutions from alder and hazel pollen, as well as with extracts from apple and celery and the purified major apple allergen Mal d 1. Hazel pollen cross-reacted 10 times more weakly than alder pollen extract of the same labeled biologic activity. Because the activity of this test solution was not determined by an independent method, the reason for this unexpectedly low degree of cross-reactivity could not be

highlighted. In the case of apple extracts, the results fit well with serologic and clinical observations.^{6,7} When compared with Bet v 1, the apple allergen Mal d 1 had a 1000-fold lower mediator release activity. These results are in accordance with clinical observations of the higher allergenicity of aeroallergens when compared with reactions elicited by pollen-related plant foods. Interestingly, natural and recombinant Bet v 1 caused identical mediator release plots. This identical allergenic activity has also been determined in patients with allergy by inhibition studies, basophil histamine release, and skin tests.^{17,18}

The suitability of our assay for major allergen quan-

tification was tested with the major cat allergen serving as a clinically relevant example. Investigation of 17 cat dander and hair extracts by two-site binding ELISA and RBL assay revealed a good log/log correlation of $r = 0.93$ (Fig. 3). Discrepancies observed for some samples might be due to the fact that the murine antiserum used in the RBL assay had been raised against whole cat dander extract and not against Fel d 1. Furthermore, divergent epitope specificities and general differences of IgE cross-linking and antibody-binding activities must be taken into consideration.

The investigation of commercial skin test solutions from birch pollen and cat dander revealed excellent agreement between lots of different strengths (Fig. 4, A and B). The differences between the activity of different strength solutions were in the expected range. Slight deviations by a factor of ± 2 are in accordance with findings obtained by comparing major allergen content and biologic activity determined by skin tests.^{19, 20} When bee venom was analyzed, the ranking determined by the RBL assay also followed the ranking of the declared strengths of commercial extracts. However, among the test solutions with a labeled amount of 300 μg and 100 μg of bee venom per milliliter, we calculated at 24% and 32% a higher PLA₂ percentage than expected, in contrast to the 10 μg per ml strength, which contained the expected PLA₂ content of 15%. This overestimation of PLA₂ might be due to additional nonspecific releasing activities at higher bee venom concentrations.¹¹

In summary, the RBL assay permits a convenient determination of the allergenic potency of extracts with regard to their IgE cross-linking properties. This assay should supplement the quality control of allergenic extracts, including highly diluted final products. As a research tool it may be applied to characterize the cross-linking properties of modified extracts and allergenic fragments. Our ongoing studies are focused on investigation of cross-linking properties of aluminum-adsorbed and chemically modified extracts.

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