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A New *In Vitro* Model for Testing of Food Allergens: Allergen-specific Mediator Release of Passively Sensitized Rat Basophil Leukaemia Cells

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An assay based on allergen-specific mediator release of rat basophil leukaemia cells (RBL cells) was investigated as a possible new tool for the in vitro testing of food allergens. The RBL-2H3 cells were sensitized passively with diluted murine pool sera containing IgE specific for food and pollen allergens. These sera were obtained from BALB/c mice after low-dose intraperitoneal injection of birch pollen, celery and peanut extracts. Comparative immunoblotting with murine and human IgE demonstrated that the murine IgE response was directed predominantly against known major allergens. Subsequent to sensitization of the RBL cells with antiserum against birch pollen allergens, apple allergy was used as an immunological model of birch pollen related food hypersensitivities. The known cross-reaction of the major allergens of birch pollen and apple was reproduced completely by the mediator release measured in the murine in vitro assay. The ranking of allergenic potency obtained for these allergens agreed closely with the results of histamine release analyses performed with blood samples of allergic patients. In addition, murine sera against celery tuber were used to study the influence of thermal and non-thermal food processing on the allergenic potency of the food. Again, the results corresponded to previous data obtained in allergic humans and indicated a reduction of potency due to the application of heat, but a high residual biological activity in many mildly processed products. Finally, spurious contaminations of peanut protein in commercial foods could be detected specifically at a concentration of at least 0.01%. This assay is suitable for many applications in food allergen research.

Keywords: Food allergens, peanut, celery, apple, IgE, in vitro mediator release, RBL-2H3 cells

INTRODUCTION

Food allergies are an increasing health problem in developed countries. Although the Correspondence to: S. Vieths.

literature lacks exact prevalence data, it has been estimated that approximately 2% of all infants and between 2 and 4% of young adults and adolescents are affected by immune-mediated food hypersensitivities (Wüthrich, 1995; Pastorello *et al.*, 1996). In the light of these facts, one may suspect an influence of industrial food production on allergenicity. The protein structure and composition of foods may be modified by the development of 'novel foods', by new technological processes, by breeding and, in particular, by genetic engineering. Thus, there is an increasing need for developing *in vitro* assays to test the potential allergenicity of food proteins and to determine trace amounts of hidden allergens in processed foods.

Sera from food-sensitized patients containing allergen-specific IgE are commonly applied for the identification and characterization of food allergens. Measuring the IgE-dependent histamine release of human blood basophils after *in vitro* stimulation with allergens represents an assay which mimics one basic mechanism of the type I allergic reaction (Lichtenstein & Osler, 1964; Siraganian, 1974). Therefore, the results of histamine release experiments reflect more closely the biological activity of allergens. However, using materials from humans in food analysis causes ethical and practical problems such as the risk of infection. Moreover, the immune responses to a specific food can be highly variable between allergic individuals and hence assays based on human antibodies are difficult to standardize. Histamine release, in particular, requires the investigation of a panel of patients whose blood samples have to be withdrawn immediately prior to testing.

To overcome some of these problems, a biological allergen assay for commercial allergen products which utilizes an *in vitro* model of the murine type I allergic reaction has been developed (Hoffmann *et al.*, 1997). The method is based on a permanent culture of rat basophil leukaemia cells (RBL cells, subline RBL-2H3) which represent a functional equivalent of mucosal mast cells (Segal *et al.*, 1981). After generation of an IgE response in mice, these cells are sensitized by pooled murine sera containing allergen-specific IgE. Subsequently, the mediator release resulting from allergen challenge is monitored by a simple enzymatic colour reaction.

The present study was undertaken to evaluate possible applications of this assay in food allergen research. Apple, celery and peanut were selected as clinically relevant allergenic plant foods. The specific features of the clinical syndromes and of the known allergens of these foods are briefly summarized in the following.

Apple

Apple allergy is widely used as an immunological model to study the 'oral allergy syndrome' (OAS) which affects a high percentage of birch pollen-allergic patients (Ebner *et al.*, 1991; Fahlbusch *et al.*, 1995; Vieths *et al.*, 1995a,b). In this context, the term OAS is used for allergic symptoms occurring mainly at the mucosa of the lips, tongue and throat after ingestion of several kinds of fresh fruits, vegetables and nuts (see review by Vieths (1997)). Most of these allergic reactions are due to a family of small food proteins that share high sequence identities and common epitopes with Bet v 1, the major birch pollen allergen. After induction of the Bet v 1-specific IgE response, the birch pollen-sensitive patients develop food hypersensitivity when the IgE titre exceeds an individual threshold level and the antibodies cross-react with the related food proteins. However, allergies to these kinds of plant food rarely occur independent of pollen allergy (Dreborg, 1988; Thiel, 1988; Jankiewicz *et al.*, 1996; Schwedler *et al.*, 1996). Generally, the cross-reactive IgE antibodies bind much more strongly to Bet v 1 than to Mal d 1, the major apple allergen (Fahlbusch *et al.*, 1995; Vieths *et al.*, 1995a). OAS patients frequently name apples as the offending food.

Owing to the inherent enzyme activities of the fruit, biologically active apple allergens can not be obtained by simple buffer extractions. Special procedures utilizing, for example, enzyme inhibitors or low temperatures are required to produce suitable extracts. Phenol as

a common preservative of commercial prick test solutions has a disturbing effect on apple allergens (Rudeschko *et al.*, 1995). As a consequence, commercial apple prick test solutions have been repeatedly found to be inactive and unsuitable for the diagnosis of apple allergy (Vieths *et al.*, 1995a,b).

Celery

In spite of its participation in the OAS of birch pollen- and mugwort pollen-sensitive patients, the capacity of extracts from celery tuber to bind specific IgE is known to partially resist the processing and heating of the food (Wüthrich *et al.*, 1990). In contrast to fresh fruits such as apples, celery elicits more severe allergic reactions in a relatively high number of cases (Pauli *et al.*, 1985; Wüthrich, 1993; Andre *et al.*, 1994). Three important allergenic structures have been identified in this vegetable: the major allergen Api g 1, a Bet v 1 related celery protein, the 'plant pan allergen' profilin and IgE-binding carbohydrate side chains of a variety of celery glycoproteins (Breiteneder *et al.*, 1995; Schöning *et al.*, 1995; Vieths *et al.*, 1995c; Jankiewicz *et al.*, 1996). Owing to immunological studies and partial amino acid sequencing, the major allergens of celery and carrot share a high degree of sequence identity (Schöning *et al.*, 1995). A high degree of thermolability has been demonstrated for Api g 1, whereas profilin and carbohydrate epitopes were found to be more resistant to the application of heat (Jankiewicz *et al.*, 1996, 1997a). As in the case of apple, the current opinion is that native pollen allergens represent the immunogenic stimulus for the induction of celery-reactive IgE that causes food allergy as an epiphenomenon of pollen allergy. This might also be reflected by the fact that it was not possible to identify patients who, at the serological level, showed stronger reactions to heat-denatured celery than to the native vegetable (Jankiewicz *et al.*, 1996).

Peanut

Peanuts are among the most allergenic foods known, affecting children as well as adults. The allergenic activity of this legume resists many kinds of technological processes including roasting (Loza & Brostoff, 1995). Several cases of anaphylactic deaths have been caused by the unwanted ingestion of peanut-containing foods (Yunginger *et al.*, 1991; Sampson *et al.*, 1992; Sampson, 1996). In addition to its importance in the US, the problems of peanut allergy have attracted increasing attention in Europe over recent years (Uhlemann *et al.*, 1993; Loza & Brostoff, 1995; Ewan, 1996; Sampson, 1996). Up to 17 peanut proteins in the molecular mass between 10 kDa and approximately 200 kDa have been shown to bind IgE from peanut-allergic patients (Uhlemann *et al.*, 1993). One heat-stable major allergen, Ara h 1, has a mass of 63.5–66.0 kDa and is recognized by the IgE of at least 90% of peanut-sensitive patients (Burks *et al.*, 1991, 1995; Uhlemann *et al.*, 1993). Since peanuts are widely used in processed foods, peanut proteins as food ingredients and unwanted contamination of foods by peanuts are important causes of allergic reactions to so-called 'hidden allergens'. Thus, specific and sensitive techniques are required to detect hidden peanut proteins and peanut allergens in processed foods.

MATERIALS AND METHODS

Patients and Human Sera

Five patients allergic to birch pollen who reported oral symptoms after ingestion of fresh apples participated in the histamine release assays. The control group consisted of three volunteers without known allergies to pollen and food who presented negative IgE antibody assays for birch pollen and apple. The serum of a patient who was allergic to birch pollen and apple and a human serum containing specific IgE against celery were taken from the serum collection of the Paul-Ehrlich-Institute. Written informed consent was obtained

from all participants and serum donors. A pooled serum of three patients allergic to peanuts was kindly supplied by Dr W.-M. Becker, Borstel Research Center (Borstel, Germany).

Antibodies

Specific IgE was raised against extract from birch pollen, the major birch pollen allergen Bet v 1, native celery, microwave-heated celery (100°C, 30 min) and roasted peanut in BALB/c mice by repeated low-dose intraperitoneal injections. BALB/c mice were injected four to five times with allergen extract containing 1 µg of protein or 0.1 µg of purified allergen adsorbed to 5 mg of Al(OH)₃ in 0.5 ml of phosphate-buffered saline (PBS) at intervals of 2 weeks. Final bleeding was performed 10 days after the last immunization. Sera of at least four responding mice (criterion: at least 30% mediator release at an allergen concentration of 0.1 mg ml⁻¹ and a serum dilution of 1:50) were pooled and stored at -20°C until used (Hoffmann *et al.*, 1997; Jankiewicz *et al.*, 1997b). The occurrence of specific IgE was monitored directly by the mediator release assay as described (Hoffmann *et al.*, 1997). Hybridoma supernatant containing the Bet v 1-specific monoclonal antibody (MAb) BIP 1 was donated by Dr C. Ebner (AKH, Vienna, Austria). MAb PN-t, specific for the major peanut allergen (Ara h 1), was produced at the Borstel Research Center and purified by ammonium sulphate precipitation (Dr W.-M. Becker).

Allergen Extracts and Allergens

Prick test solutions of birch pollen and apple were obtained from a commercial manufacturer. Recombinant (r) Bet v 1 and rAra h 1 were purchased from Biomay (Linz, Austria). The major apple allergen, Mal d 1, was purified by SDS-PAGE/electroelution as described previously (Vieths *et al.*, 1995a). Extract of roasted peanuts (Uhlemann *et al.*, 1993) and purified Ara h 1 were kindly supplied by Dr W.-M. Becker. Celery tubers of the 'Brillant' variety were extracted in the native state, after microwave heating for 30 min at 100°C and after cooking (20 min, 100°C) with PBS as described (Jankiewicz *et al.*, 1996). In addition, cooking water, γ-irradiated celery, celery powder and a heat-sterilized preserve of pickled celery were used (for processing and extraction, see also Jankiewicz *et al.* (1997a)). Native carrots were extracted by the same procedure. The prick test solutions were stored at 4°C. All other preparations were freeze dried and kept at -20°C until used.

Additional Food Extracts

The extracts from commercial food products used in the evaluation of the RBL cell assay for peanut allergens (Tables 1 and 2) were prepared as follows. After freezing with liquid nitrogen the foods were ground to a homogeneous powder using an analytical mill (IKA M 20, IKA Labortechnik, Staufen, Germany). Powdered sample (1 g) was extracted with 20 ml of 0.025 M-Tris-tricine buffer, pH 8.6, for 3 h at 50°C in a water-bath. The samples were vortexed in intervals of 30 min. For defatting, the samples were stored overnight at 4°C which resulted in the fat congealing on the top of the suspension. Afterwards, aliquots of the extracts were centrifuged at 20 000 × g, 15°C, for 45 min, subsequently passed through 1.2 and 0.45-µm nitrocellulose (NC) membrane filters, and stored at -20°C until used. This procedure was selected since all samples were analyzed independently for peanut protein by an immuno-amplified rocket immunoelectrophoresis (Holzhauser *et al.*, 1997). Chocolate samples with known amounts of ground and roasted peanuts (0.0, 0.1 and 0.5% respectively) were supplied by Dr R. Matissek, Institute of Food Chemistry of the German Confectionary Industry (Cologne, Germany). Samples with lower amounts of peanuts were obtained by spiking an extract from peanut-free chocolate with various amounts of peanut-protein.

TABLE 1. β-Hexosaminidase release of RBL-2H3 cells passively sensitized with anti-peanut reaginic antiserum (1:100) after stimulation with various native and heated food extracts

Food extract	Protein concentration (µg ml ⁻¹)	% β-Hexosaminidase release after pretreatment	
		37°C/15 min	100°C/15 min
Pistachio	2.5	1	0
	25	8	1
	250	60	1
White bean	2.5	6	0
	25	24	0
	250	34	17
Soya bean	2.5	3	0
	25	5	0
	250	9	0
Hazelnut	2.5	3	1
	25	3	1
	250	35	1
Sunflower seed	2.5	1	1
	25	14	1
	250	21	6
Sesame seed	2.5	30	0
	25	44	0
	250	62	10
Almond	2.5	0	0
	25	13	0
	250	81	7
Peanut	0.2	39	37
	2.0	34	40
	20	38	38

Electrophoresis and Immunoblotting

Electrophoresis and immunoblotting were carried out as previously described (Vieths *et al.*, 1992a, 1995a). Briefly, the extracts were separated by discontinuous SDS-PAGE according to the method of Lämmli (1970), 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 µg cm⁻¹ on 5.5-cm preparative slots. After separation, the extracts were transferred on to NC membranes by semi-dry blotting. After blocking with 0.01 M-PBS/0.3% (v/v) Tween 20, cut NC strips (0.4 mm) were either stained with indian ink or used for immunodetections. The latter were achieved by incubating one NC strip in 1.0 ml of serum, diluted in PBS containing 0.05% (v/v) Tween 20 and 0.1% bovine serum albumin (BSA). Primary incubations with antibodies were carried out overnight. The human sera were diluted 1:6.7. Bound human IgE antibodies were detected by subsequently incubating the NC strips with: (1) a rabbit polyclonal anti-human IgE (1:4000, 1 h), (2) a goat polyclonal affinity-purified biotinylated anti-rabbit IgG (1:6000, 1 h) (both from Dako, Copenhagen, Denmark) and (3) with streptavidin-horseradish peroxidase (HRP) conjugate (1:20 000, 20 min) (Medac, Hamburg, Germany). MAbs were either used at 1 µg ml⁻¹ (PN-t) or as culture supernatants, diluted 1:100. Bound murine IgG was detected by an affinity-purified rabbit anti-mouse IgG biotin conjugate (1:3000, 1 h) (Dianova, Hamburg, Germany) followed by streptavidin-HRP incubation as above. For detection of specific IgE

TABLE 2. Analysis of a range of commercial food products for peanut protein by the RBL cell mediator release assay

Sample	Peanut declared on label	Maximal release (%)	Extract dilution at maximal release
Model chocolates			
0.0% PN	no	0.9	1:4
0.1% PN	yes	32.1	1:100
0.5% PN	yes	33.1	1:100
Commercial chocolates			
a	no	3.2	1:10
b	no	1.0	
c	no	0.6	
d	no	12.1	
e	yes	18.0	
f	yes	21.0	
Peanut flips	yes	16.4	1:100
Icecream	yes	18.7	1:1000
Rice snack 'nuts'	yes	23.9	1:1000
Rice snack 'wakkanai'	no	0.2	1:10
Peanut toffee	yes	18.2	1:100
Cream toffee	no	0.3	1:10
Nougat crisp	no	0.9	1:10
Coconut sweet	no	1.0	1:10
Refined peanut oil	yes	0.0	1:10
Peanut extract	—	32.1	0.1 µg ml ⁻¹

in murine sera, these were diluted 1:15. The overnight incubation was followed by using purified rat anti-mouse IgE biotin conjugate (1:750, 2 h) (Pharmingen, obtained from Dianova, Hamburg, Germany), and again streptavidin-HRP. Bound enzymatic activity was visualized with a commercial enhanced chemoluminescence reaction kit (ECL) (Amersham-Buchler, Braunschweig, Germany) by exposing the blots to an X-ray film for 5–30 s immediately after incubation with the ECL reagent.

Rat Basophil Leukaemia Cell Mediator Release Assay

The RBL-2H3 cells applied in this study represent functional analogues of mucosa mast cells which have been selected for maximal mediator release. The cells can be sensitized passively with murine and rat IgE, but not with human IgE. The mediator release assay (RBL assay) was performed as described previously (Hoffmann *et al.*, 1997). Briefly, permanently growing RBL-2H3 cells were harvested in the stationary phase, transferred at 10⁵/well to 96-well cell culture plates (Nunc, Wiesbaden, Germany) and cultivated for 18 h at 37°C in Eagle's minimal essential medium (MEM) containing 5% of fetal calf serum (FCS). The attached cells were sensitized passively for 1 h with pooled murine reagenic antiserum, diluted in Eagles MEM, 5% FCS. The sera were diluted as follows: anti-birch pollen: 1:100; anti-native celery: 1:65; anti-heated celery: 1:30; anti-peanut: 1:100. After sensitization, the cells were washed three times with Tyrode's buffer. Subsequently, the RBL cells were incubated with 100 µl of serial allergen extract dilutions

in Tyrode's buffer in a humidified atmosphere at 37°C for 1 h. The specific release was assayed by measurement of β -hexosaminidase activity which is present in the same granula as histamine (Schwartz *et al.*, 1981). After transfer of 20 µl of the supernatant to a second microtitre plate, the released enzymatic activity was detected by hydrolysis of *p*-nitro-phenyl-*N*-acetyl β -D-glucosaminide for 1 h. Absorbance was measured at 405 nm. The total release was quantified by treating the cells with the buffer containing 1% Triton X-100. Controls were run with Tyrode's buffer without allergen to measure spontaneous release, and by stimulation of the sensitized cells with non-related extracts (e.g. anti-celery with peanut extract). Results were expressed as per cent of the total release after subtraction of the spontaneous release (Hoffmann *et al.*, 1997; see also Jankiewicz *et al.*, 1997b).

Basophil Histamine Release

The assay was carried out as described by Siraganian (1974, 1975) and Siraganian and Brodsky (1976) respectively. Whole blood samples of the donors were heparinized and used within 1 h after drawing. None of the donors took any medication against allergic diseases for over 1 week prior to testing. No participant had been hyposensitized before. In short, the blood samples were incubated with serial allergen extract dilutions and the samples were treated according to the procedures described in the literature. After extraction with 1-butanol and condensation with *o*-phthalaldehyde in an autoanalyzer (Bran & L  bbe, Hamburg, Germany, formerly produced by Technicon), the reaction product was measured in a fluorescence spectrometer (Perkin Elmer,   berlingen, Germany). Excitation was performed at 300 nm which is not the optimum but which causes a signal exclusively originating from histamine (Lorenz *et al.*, 1972; Fischer & Schmutzler, 1980). Emission was measured at 450 nm. The spontaneous release was tested by incubating blood samples in buffer without allergen. The total histamine content was assayed in blood samples treated with 3% perchloric acid. The allergen-induced histamine release was calculated as per cent of the total individual amount of histamine. Histamine standards were run at concentrations of 1.0–15 ng ml⁻¹. The correlation coefficients of the standard curves ranged between 0.9986 and 0.9999.

RESULTS

Apple Allergy as an Immunological Model of Pollen-related Food Hypersensitivities

The experiments described in the following were conducted to investigate whether the murine model was suitable to simulate the phenomenon of pollen-related allergies to food. In total, 25 BALB/c mice were immunized by five injections of 5 µl of a birch pollen prick test solution adsorbed to Al(OH)₃. The IgE of 90% of these mice caused more than 30% mediator release at a serum dilution of 1:50 and a stimulator concentration of 100 ng ml⁻¹ of rBet v 1. These were classified as high responders and the sera of at least four animals were pooled and used for passive sensitization. In addition, 0.1 µg of Mal d 1 and rBet v 1 were injected into 10 and 6 mice respectively, following the same procedure, and six animals were immunized with 1 µg ml⁻¹ protein from Golden Delicious apple extract. As demonstrated by ELISA (not shown), all sera contained IgG specific for the immunogen. In contrast, the RBL assay indicated an IgE response exclusively with birch pollen extract and rBet v 1, but not with the cross-reactive apple protein and apple extract. The specificity of the murine IgE against birch pollen was tested by immunoblotting of birch pollen extract. As shown in Figure 1, the IgE of the serum pool Bet4 (*n* = 10) (lane 1) bound to Bet v 1 and to one additional band of higher molecular mass. The recognition of Bet v 1 by a specific MAb of the IgG isotype and by IgE from an allergic individual is shown in lanes 2 and 3 respectively. Bet v 1 was not detected by a reagenic murine antiserum raised against phospholipase A₂ (PLA 2) from bee venom (lane 4). Owing to their limited amount the murine sera had to be used at high dilutions, and relatively high concentrations of the

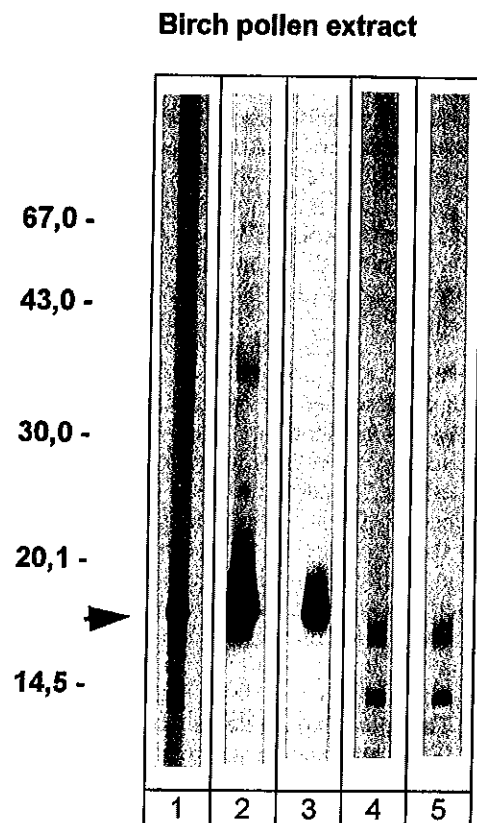


FIG. 1. SDS-PAGE immunoblot analysis of birch pollen extract. Immunodetection: lane 1: IgE from the murine anti-birch pollen pool Bet4; lane 2: IgE of a birch pollen-allergic patient; lane 3: murine IgG-MAb BIP 1, specific for the major allergen Bet v 1; lane 4: murine IgE specific for PLA 2 from bee venom; lane 5: buffer control. The position of Bet v 1 is indicated by an arrow.

detection reagents had to be applied. As indicated by the buffer control (lane 5) and by anti-PLA 2 (lane 4), this caused non-specific staining of some bands below 17 kDa. However, these could be distinguished from the specific IgE response.

RBL cells were sensitized with the murine anti-birch pollen serum pool Bet4 ($n = 10$) and subsequently incubated with serial dilutions of rBet v 1 and Mal d 1 and with commercial prick test solutions of birch pollen and apple. The resulting dose-related mediator releases are depicted in Figures 2(a) and (b) respectively. The same allergens and extracts were used to trigger blood basophils of five patients who were allergic to birch pollen and apples. As shown in Figures 2(c) and (d) the course of the allergen-specific histamine release was similar to the results of the RBL cell mediator release assay. In both the murine and the human system, rBet v 1 was clearly more active than Mal d 1, approximately 1000-fold in the RBL cell assay and approximately 100-fold with human basophils respectively. A high allergenic potency was also detected in the birch pollen prick test solution, whereas a commercial product from apples showed very low allergenic activity in both assays (Figures 2(b) and (e)). No histamine release ($< 15\%$) was measured with blood samples of the three non-allergic controls.

Mediator Release due to Allergens from Native and Processed Celery Tuber

Food products prepared from celery tubers were used to study the influence of food

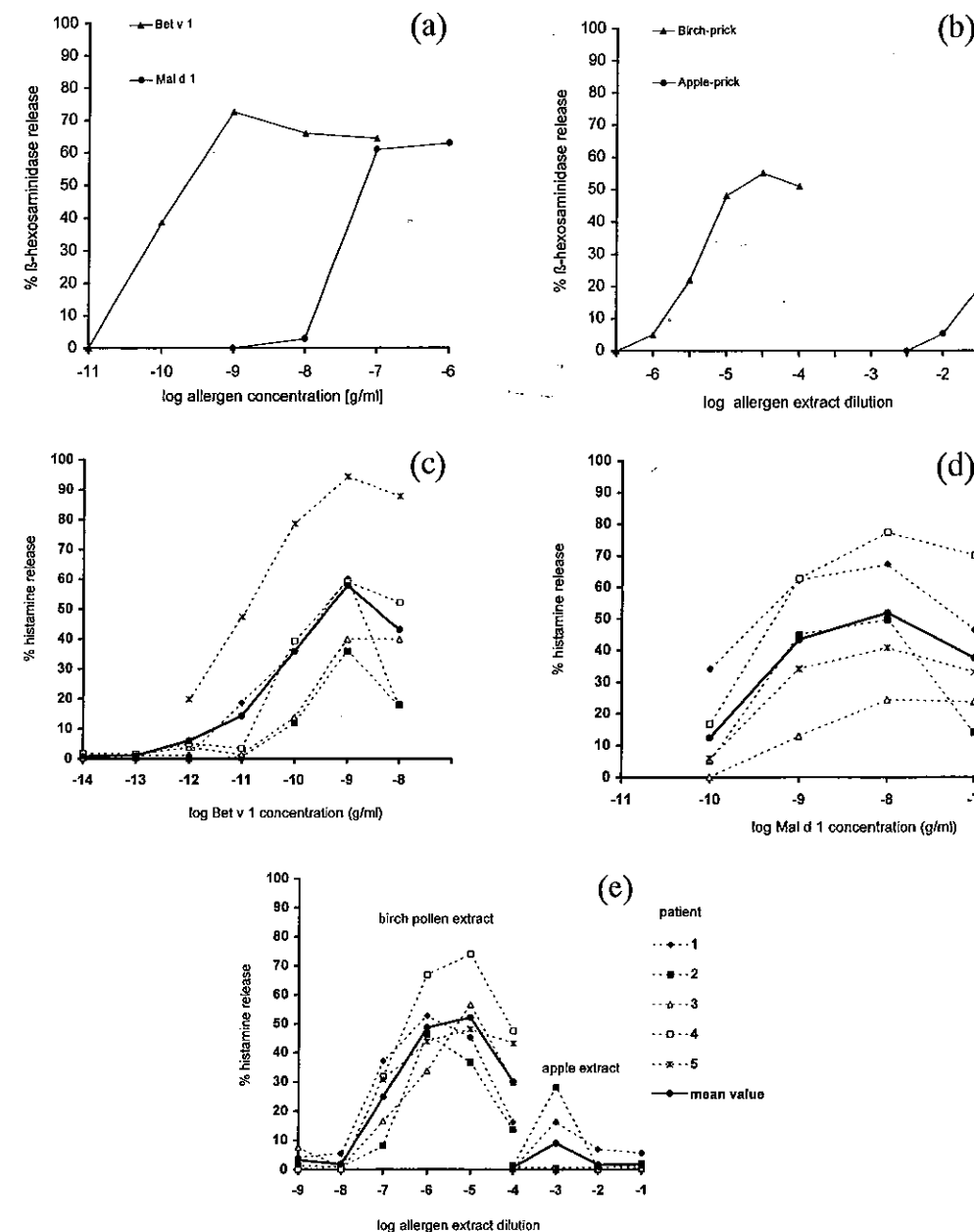


FIG. 2. Comparative mediator release experiments in the murine and human system. RBL cells sensitized with the anti-birch pollen murine serum pool Bet4, stimulation by Bet v 1 and Mal d 1 (a) and by skin prick test solutions from birch pollen and apples (b) as indicated. Basophil histamine release of five patients allergic to birch pollen and apples, stimulation by Bet v 1 (c), Mal d 1 (d) and by skin prick test solutions from birch pollen and apples (e) as indicated.

processing on allergenicity and to test the possibility of creating neoallergens by heat processing. A strong allergenic response to a heated food concomited by a weak reaction to native proteins from the same source has been reported for molluscs (Carrillo *et al.*, 1991, 1992), pecan nuts (Malanin *et al.*, 1995) and for fish in the classical Prausnitz-

Celery extract

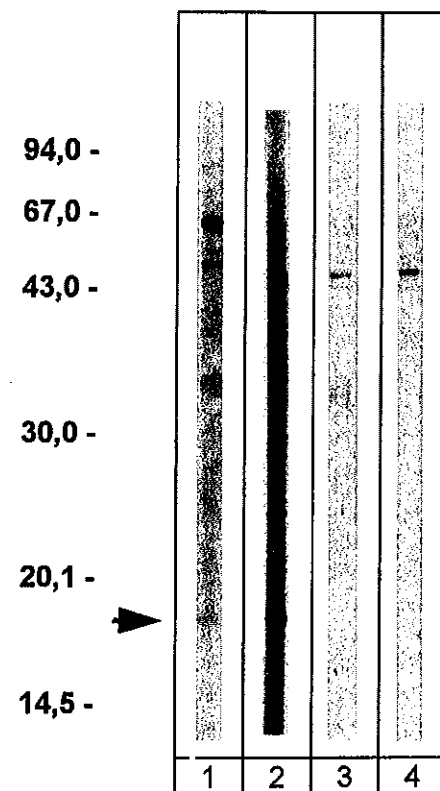


FIG. 3. SDS-PAGE immunoblot analysis of extract from native celery. Immunodetection: lane 1: murine IgE against native celery; lane 2: IgE of a celery-sensitized patient; lane 3: murine IgE specific for PLA 2 from bee venom; lane 4: buffer control. The position of the major allergen Api g 1 is indicated by an arrow.

Küstner passive transfer experiment (Prausnitz & Küstner, 1921), but not for celery. However, Wüthrich *et al.* (1990) were the first to show that residual allergenic activity can be present in cooked celery and even in the cooking water.

For the experimental model, IgE was raised successfully in BALB/c mice against extract from native celery and celery that had been microwave heated for 30 min at 100°C. Comparative immunoblotting analyses of celery extract are presented in Figure 3. As observed with birch pollen, one band was stained non-specifically (lanes 3 and 4). However, in native celery the major allergen Api g 1 was specifically recognized at 16 kDa by anti-native murine IgE and by IgE from a celery-sensitized patient (lanes 1 and 2 respectively; position of Api g 1 indicated by an arrow). In addition, a strong band was recognized by murine IgE at 67 kDa. Several additional minor allergens bound IgE from the patients' serum. No bands were stained with the murine serum against heated celery, most probably due to the limited detection threshold of the method (not shown).

In a first series of release experiments the RBL cells were sensitized with the reagenic serum against native celery and incubated with serial dilutions of the native immunogen, microwaved celery, cooked celery and the cooking water. As demonstrated by Figure 4(a), native celery was highly active in this system, whereas heat processing led to a strong decrease of the allergenicity. The extended release experiments illustrated by Figure 4(b)

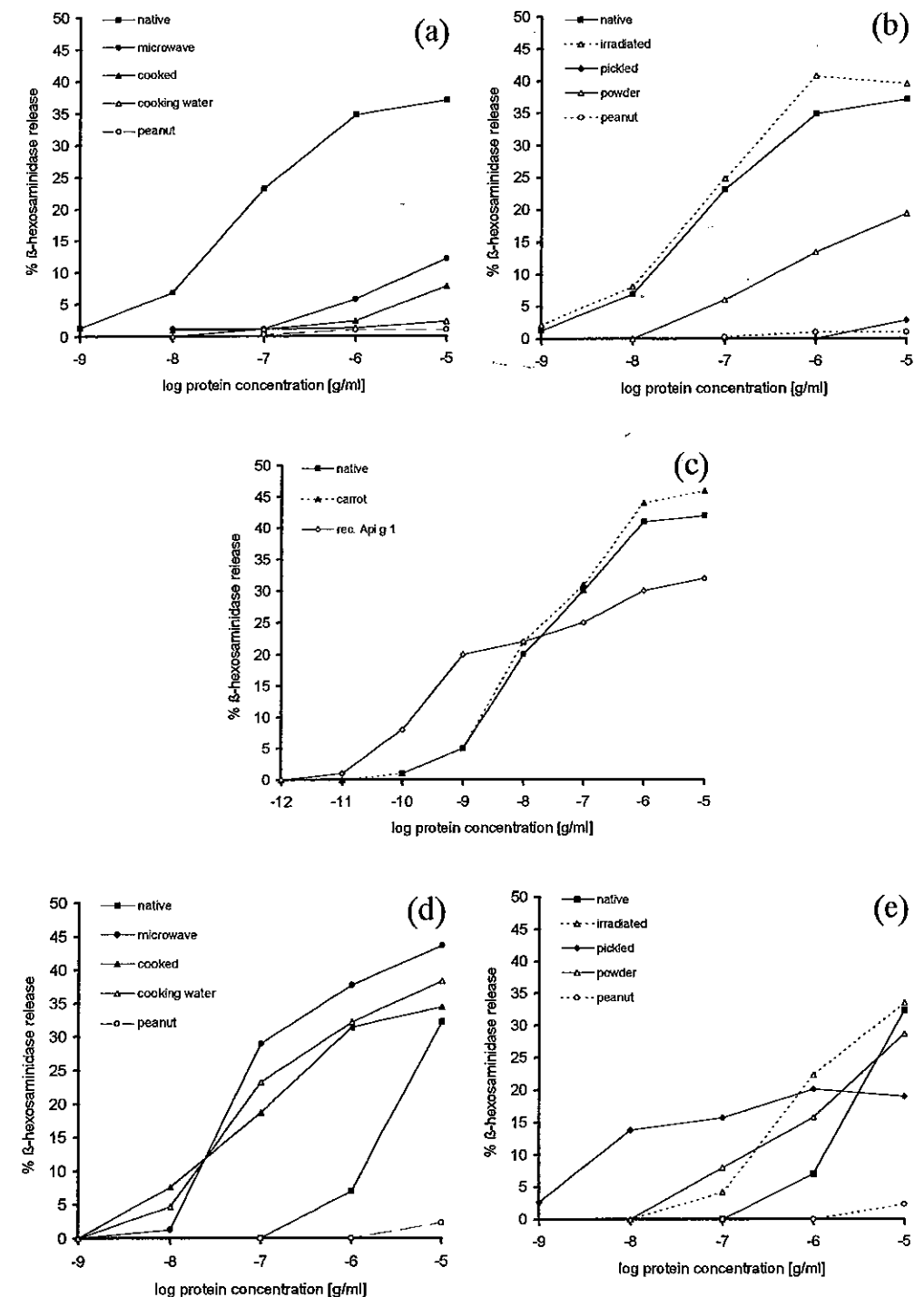


FIG. 4. Results of the mediator release assay with RBL cells sensitized against celery roots. (a, b) Passive sensitization with murine IgE against native celery, release by extracts from native and processed celery tuba as indicated. (c) Sensitization as in (a), release by the major allergen rApi g 1, native celery and carrot extract as indicated. (d, e) Passive sensitization with murine IgE against heated celery, release by extracts from native and processed celery tuba as indicated.

indicated a high allergenicity for γ -irradiated celery and celery powder, but the release was negligible with pickled celery and with peanut as the negative control. Figure 4(c) shows that rApi g 1 caused a considerable mediator release with this serum, but contrary to Bet v 1, it did not mimic the allergenicity of the crude allergen extract completely. As a cross-reactive allergenic food, carrot caused a dose-dependent mediator release similar to extract from native celery.

Very different results were obtained when the RBL cells were sensitized with the antiserum against microwave-heated celery. In this case, the most intensive release occurred with the heat-processed immunogen, cooked celery and the cooking water, whereas native celery triggered a strong response only at the maximal protein concentration of $10 \mu\text{g ml}^{-1}$ (Figure 4(d)). Figure 4(e) shows that high or considerable allergenicity related to anti-heated celery was also present in γ -irradiated celery, pickled celery and celery powder, but not in the control extract from peanut. The maximal release due to stimulation by rApi g 1 in the latter system was below 50% of its activity related to anti-native celery IgE (result not shown).

Determination of Hidden Allergenic Activity of Peanut Proteins in Commercial Food Products

Specific IgE was also raised against extract from roasted peanut. Immunoblotting revealed that multiple allergen bands were detected by IgE of the murine serum pool and by a serum pool from three peanut-allergic patients. When compared to human IgE, the murine reaction pattern was somewhat poorer (Figure 5, lanes 1 and 2). As demonstrated by the IgG-MAb PN-t which was specific for Ara h 1 (lane 3), the major allergen was detected at 66–67 kDa by both murine and patients' IgE. After passive sensitization of RBL-2H3 cells with the murine reaginic antiserum at a serum dilution of 1:100, the cells responded with a strong mediator release to the stimulation with both peanut extract and purified Ara h 1 (Figure 6(a)). Comparable to rApi g 1 from celery, the purified peanut allergen only partly reproduced the allergenicity of the crude protein extract. To perform a series of initial model experiments on the detection of 'hidden allergens', extract from peanut-free chocolate was spiked with peanut protein corresponding to 0.01%–1.0% of whole peanut in chocolate. The resulting dose release plots of the subsequent RBL cell mediator release assay are presented in Figure 6(b). Allergenic activity was clearly detected in all samples except peanut-free chocolate.

Possible interferences with other food proteins were studied with extracts of white bean, soya bean, hazelnut, almond, sunflower seed, sesame seed and pistachio. Many of these extracts caused relatively high non-specific signals in the mediator release assay, but these interferences decreased drastically when the food extracts were heated to 100°C for 15 min (Table 1). In contrast, the mediator-releasing capacity of peanut extract survived the heating procedure. Afterwards, heat-treated extracts of two model chocolates containing 0.1 and 0.5% of ground and roasted peanuts and extracts of selected food products from the German market were analyzed. The results of these experiments are summarized in Table 2. With the exception of one chocolate sample where peanut was not listed as ingredient, a specific release was only obtained in the samples which were declared as containing peanut protein. No release was caused by a sample of refined peanut oil.

DISCUSSION

In vitro mediator release of passively sensitized RBL-2H3 cells was developed as a new tool in food allergen research. Prior to prospective testing of novel foods the assay was applied to simulate known phenomena of food allergy. As a prerequisite, IgE specific for food allergens or cross-reacting pollen allergens had to be raised in mice. As shown for pollen, bee venom and epithelia, this procedure is reproducible and works with a range of

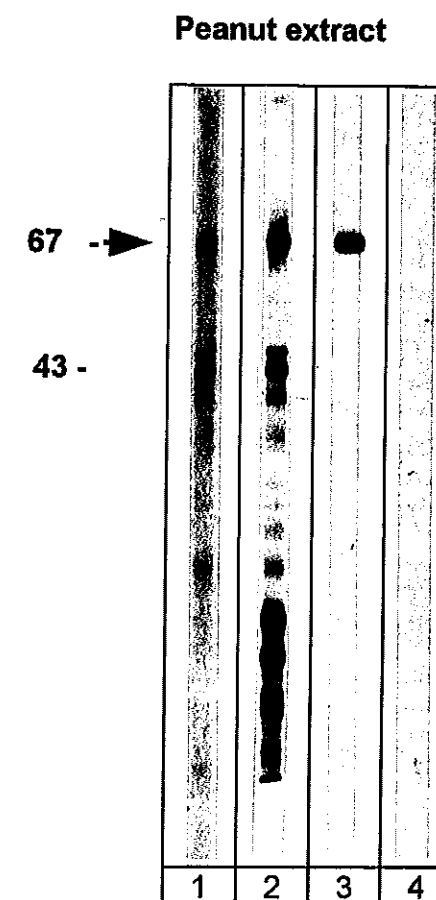


FIG. 5. SDS-PAGE immunoblot analysis of peanut extract. Immunodetection: lane 1: IgE from the murine serum pool raised against peanut protein; lane 2: IgE from 3 peanut-allergic patients; lane 3: murine IgG-MAb PN-t, specific for the major allergen, Ara h 1; lane 4: murine IgE specific for PLA 2 from bee venom. The position of Ara h 1 is indicated by an arrow.

known allergens (Hoffmann *et al.*, 1997). Although the route of immunization applied in the study was injection and not ingestion or inhalation, our immunoblot results indicate that the murine IgE response elicited in this way is predominantly directed against major allergens. In the case of peanut, however, the multiplicity of the human IgE pattern was not completely reproduced by immunoblotting of murine IgE. However, it should be borne in mind that an extract from a single food was used for the laboratory immunizations, whereas allergic humans underlie multiple antigenic stimuli, some of which may cause cross-reactive recognition of peanut proteins by IgE. Owing to the limited amount of murine serum and possibly a poorer quality of the secondary antibody, one reason for this difference might be a lower sensitivity of murine IgE immunoblotting.

The major allergen of apple, Mal d 1, was studied after sensitizing RBL cells with an anti-birch pollen murine serum pool. The clinically important cross-reaction between Mal d 1 and the major birch pollen allergen, Bet v 1, was reproduced perfectly in the model system. With IgE against native celery the RBL assay also demonstrated the cross-allergenicity between celery extract and carrot extract. Both the RBL cell assay and basophil histamine release of allergic patients show the same ranking of the allergenic potency for Bet v 1 and Mal d 1, as well as for commercial skin prick test solutions from

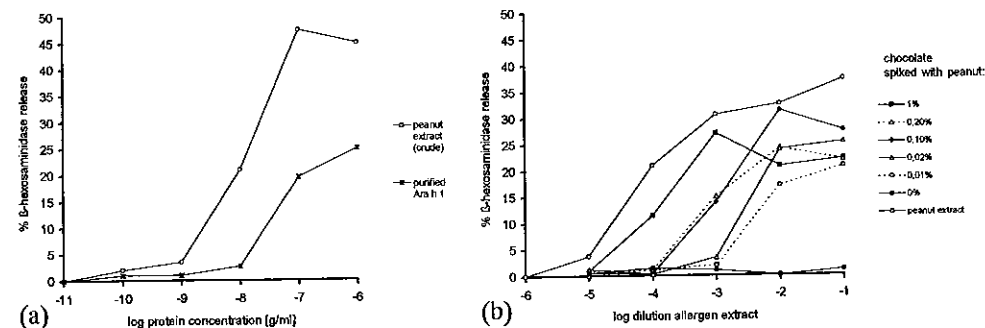


FIG. 6. β -Hexosaminidase release of RBL cells sensitized with a murine serum pool containing peanut-specific IgE (1:100). (a) Release caused by crude peanut extract and by the major peanut allergen, Ara h 1. (b) Stimulation of the sensitized cells with extract from peanut-free chocolate which was spiked with peanut protein in a concentration range corresponding to 0.01–1.0% of peanut as indicated. Reference: peanut extract, containing 2 mg ml^{-1} protein.

birch pollen and apples. In contrast to Bet v 1 and birch pollen, no apple-specific IgE was induced by injecting apple allergens. This is in accordance with the common view that the inhalation of birch pollen raises specific IgE in humans which induces hypersensitivity to apples. A weak capacity of apple allergens to induce a specific IgE response was also indicated by experiments with T-cell lines from apple allergic patients. These cells poorly proliferated when stimulated with apple allergens, but strongly responded to birch pollen (Fahlbusch *et al.*, 1996). However, in the case of the major allergens of celery and peanut, these proteins did not represent the complete allergenicity of the corresponding food extracts. This is in agreement with the more complex immunoblot patterns of these foods obtained with both patients' and murine IgE.

The influence of food processing on allergenic potency was studied with celery. Previous investigations utilizing IgE of celery-sensitized patients indicated that the major celery allergen, Api g 1, is rapidly inactivated by thermal processing of celery tuber, but can remain allergenic after non-thermal treatments, e.g. γ -irradiation or the production of celery powder (Jankiewicz *et al.*, 1996, 1997a). This behaviour is simulated by the present mediator release experiments with RBL cells sensitized to native celery. However, celery-sensitive patients can also present IgE specific for profilin and for carbohydrate side chains of glycoproteins respectively. These structures have been found to be relatively resistant to thermal processing and are candidates to explain the thermostable allergenic component of celery. Neither the current immunoblot results nor the data of the RBL assay indicate that IgE against these structures is present in the murine serum pool against native celery. Thus, the information resulting from the model system is mainly restricted to the major celery allergen. To overcome this incompleteness, one should try to generate IgE against the minor allergens, for example by using purified allergen preparations for immunization. However, remembering the high variability of the allergic immune response, any model system has to be suspected to produce incomplete data.

A murine IgE response was also induced by immunization with an extract of microwave-heated celery. Mediator release experiments with a pooled serum of these mice led to a reversed ranking of the allergenic potency. With this serum, all preparations from heat-processed celery were highly allergenic. Partial denaturation, for example by γ -irradiation, caused a high efficiency of cross-linking with IgE against native as well as against heated celery. The results obtained with murine IgE against heated celery may be interpreted as a model for the creation of 'neoallergens' by heating processes, indicating a high risk to experience allergic reactions after ingestion of processed and spiced foods if patients become sensitized against heated celery tuber.

Peanut belongs to the most important hidden allergens in processed foods. The present results indicate that, when carefully optimized, the RBL cell mediator release assay is suitable to detect spurious amounts of hidden and potentially allergenic peanut protein in many processed foods. Owing to the labelling of peanut, false-negative results have not been obtained in this study. However, the method requires heating of the sample extracts as described to avoid false-positive results. This observation might be due in part to inherent enzymatic activities, cross-linking by lectins or other non-specific mediator release properties present in many of the native food extracts. After heating, matrix interferences causing false-positive results can only be expected from strongly interfering foods such as white bean or sesame seed being present as major constituents of the food. Under practical conditions, this situation will only occur in a very small number of samples. However, in our opinion, careful confirmation of any result by an independent technique is required. Hence, we have developed an immuno-amplified rocket immunoelectrophoresis which completely confirms the results listed in Table 2 (Holzhauser *et al.*, 1997). This is also true for the chocolate sample, where peanut was not declared on the label. Owing to the rocket method the peanut content of this sample was 0.02%. Despite the fact that the RBL cell mediator release assay cannot compete with classical quantitative immunochemical methods such as ELISA, we conclude that the technique is suitable to detect peanut contaminations of processed foods at a concentration level of 0.01%, or even below. Further studies are in progress, in particular on comparative analysis of hidden allergens in processed foods.

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