

Development of a Biological Assay to Determine the Allergenic Potential of Foods

L. Vogel, T. Holzhauser and S. Vieths

Paul-Ehrlich-Institut, Division of Allergology, Langen

Correspondence to: Dr. Lothar Vogel, Paul-Ehrlich-Institut, Division of Allergology, Paul-Ehrlich-Str. 51–59, 63225 Langen, Germany, Tel. +49(0)6103/77-5311, Fax +49(0)6103/77-1258, E-mail: voglo@pei.de

Received: June 9, 2006; Revised: August 10, 2006; Accepted: September 12, 2006.

Keywords: Food allergy, allergen detection, biological assay, serum IgE, risk management.

Abbreviations: ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Fc ϵ RI = high affinity receptor for IgE; RBL = rat basophilic leukemia; PBS = phosphate-buffered saline; TBS = tris-buffered saline.

Abstract: Food allergies represent a risk for many people in industrialized countries. Unrecognizable allergenic proteins of foodstuffs may be present as ingredients that are not labeled or as unknown cross-contamination. Such hidden allergens can cause severe reactions in allergics, even at minute quantities, sometimes with fatal outcome. For the verification of the presence of allergenic food constituents, analytical methods such as ELISA and PCR have been developed. However, these tests cannot measure allergenic potential. For this reason, a test system that measures the biological activity of allergens has been developed. It is based on the cellular mechanisms of the type I allergy. Rat basophilic leukemia cells (RBL-2H3) were transfected with the genes of the human high affinity receptor for IgE. The resulting cell line expressed the human receptor α -chain and could bind allergen-specific IgE from allergic subjects, in contrast to the parent cell line. After cross-linking of receptor-bound, allergen-specific human IgE by allergens, the cells released measurable inflammatory mediators. These cells were used for the analysis of a variety of allergen extracts, including extracts prepared from foods containing allergenic hazelnut and peanut. The comparative validation with existing ELISA and PCR for hazelnut and peanut demonstrated similar sensitivity and specificity. The established cell line will be a novel tool in the detection of allergens in complex mixtures, especially to address the issue of their allergenic potential, which cannot be accomplished by classical analytical methods. This will add valuable information about the allergenic potential of food constituents to the risk assessment of foods.

Zusammenfassung: Allergene Lebensmittelbestandteile stellen für viele Nahrungsmittelallergiker in industrialisierten Ländern ein reales Gesundheitsrisiko dar, wenn sie als nicht deklarierte Zutaten oder unerkannte Kontaminationen in Lebensmitteln enthalten sind. Diese so genannten versteckten Allergene können, teilweise in geringsten Mengen, schwere Reaktion bei sensibilisierten Personen auszulösen, manchmal auch mit tödlichem Ausgang. Zum Nachweis allergener Lebensmittelbestandteile wurden bereits verschiedene analytische Methoden, wie z.B. ELISA- und PCR-Verfahren, entwickelt. Allerdings sind diese Methoden nicht in der Lage, das allergene Potenzial solcher Bestandteile zu erfassen. Um jedoch die biologische Aktivität von Allergenen messen zu können wurde ein Testsystem entwickelt, das auf dem Mechanismus der Typ-I Allergie basiert. Dazu wurden basophile Leukämiezellen der Ratte mit den Genen des humanen hochaffinen Rezeptors für IgE transfiziert. Die daraus resultierende Zelllinie exprimiert einen chimären Rezeptor (mit der IgE-bindenden humanen α -Kette) stabil auf ihrer Oberfläche und ist somit in der Lage, auch allergenspezifisches IgE aus Allergikerseren zu binden, was mit der Ausgangszelllinie nicht möglich ist. Die Vernetzung des rezeptorgebundenen IgEs durch das Allergen führt in der Folge zur Freisetzung entzündungsauslösender Mediatoren, die im Zellkulturüberstand gemessen werden können. Diese transfizierte Zelllinie wurde zur Analyse einer Vielzahl von Allergenextrakten eingesetzt, darunter auch Extrakte von Lebensmitteln, die Haselnuss- oder Erdnussallergene enthielten. Der Vergleich des biologischen Testsystems mit den ELISA- und PCR-Verfahren für Hasel- und Erdnuss ergab eine ähnliche Sensitivität und Spezifität. Die etablierte Zelllinie ist ein neues, wertvolles Hilfsmittel für den Nachweis von Allergenen in komplexen, zusammengesetzten Lebensmitteln und im Besonderen zur Erfassung des allergenen Potenzials solcher Nahrungsmittelbestandteile. Somit kann dieses neue Nachweisverfahren helfen, zusätzliche wichtige Informationen für die Risikobewertung von Lebensmitteln zu gewinnen.

1. Introduction

Published data on the prevalence of food allergies vary considerably, but it seems that approximately 1–2 % of adults and up to 8 % of children are affected (Boden et al., 2005; Zuberbier et al., 2004). Minute amounts of the consumed foods can already lead to severe reactions in highly sensitized individuals and life-threatening and even fatal food-related allergic reactions have been reported, as summarized elsewhere (Burks et al., 1999; Wüthrich and Ballmer-Weber, 2001). Because of the risk of severe side effects, immunotherapy as a causative treatment for food allergies cannot be undertaken to date (Wild and Lehrer, 2001). Hence, strict avoidance of the offending food remains the only effective measure to prevent allergic reactions. Although most people concerned are aware of their allergy and try to avoid the consumption of the relevant foods, there is still the risk of unintentional ingestion due to mislabeling of pre-packaged foods, allergen cross-contact during manufacture, or consuming foods in restaurants and catering. For improved transparency of pre-packaged food products in the EU, the eleven most frequent foods that potentially elicit severe allergic reactions have to be labeled when used as ingredients (The European Parliament and Council, 2003). However, the underlying Directives 2000/13/EC and 2003/89/EC do not address the problem of unintentional cross-contact during manufacture.

Therefore, sensitive and specific analytical tests are required for the identification of critical control points during food production and for the verification of obligatory food allergen labeling according to the above-mentioned Directives. In the past decade, numerous applications of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) methods have been developed to fulfill this task. In our laboratory, e.g., several PCR and ELISA tests for important allergens, such as peanut, celery (Holzhauser and Vieths, 1999a; Stephan and Vieths, 2004; Stephan et al., 2004) and hazelnut (Holzhauser and Vieths, 1999b; Holzhauser et al., 2000; Holzhauser et al., 2002) have been developed and validated. Although these methods are very sensitive and specific, they do not address the issue of the allergenic potential of the detectable food constituents. Moreover, PCR-based techniques can only detect the presence of deoxyribonucleic acid (DNA) of the allergenic food from plant and animal origin. Based on the assumption that detectable DNA was not separated from food proteins, a positive PCR test is indicative of the presence of the allergenic food. Similarly, the ELISA detects proteins, but is still not able to make any prediction of their allergenic potential.

Apart from a limited number of clinical trials, such as the threshold-based double-blind placebo-controlled food challenge (DBPCFC), little is known about the allergenic potential of trace amounts of proteins that are present in complex food matrices. In addition, only few data are available on the impact of food processing on the allergenic structures of proteins. It would therefore be desirable to have an assay at hand that also detected the allergenic potential of the relevant proteins. For this, we have developed a biological test, based on the mechanism of type I allergies, that measures the IgE cross-linking properties of allergens. Allergen-specific IgE antibodies bind to high affinity receptors (FcεRI) on mast cells and basophils.

Upon the following encounter with allergen, inflammatory mediators that are responsible for the observed allergic symptoms are released from the cells. Such biological assays, which measure the functional properties of allergenic proteins, may serve as valuable tools to investigate the biological potential of food allergens depending on their concentration and upon structural changes due to various conditions of food processing, such as heat and acidic environment.

Since no appropriate human cell lines were available, RBL-2H3 cells were transfected with the genes of the human FcεRI. Stable transfectants express chimeric receptors and bind human IgE and can be triggered to release mediators (Vogel et al., 2005). For our model investigations, hazelnut and peanut were selected as important allergenic foods because of their high allergenic potential and frequent use in food manufacture. The comparison of the biological assay with established immunochemical and clinical methods demonstrated a good correlation of the results. We show here that allergens can be detected, and their allergenic potential measured, even in complex mixtures such as composed foods. Thus, the biological assay serves as a valuable novel tool in the risk assessment of foodstuffs and may contribute to an increased food safety for consumers, in particular those with food allergies.

2. Materials and Methods

2.1 Cell culture and reagents

The conditions for cell culture were as described previously (Vogel et al., 2005). Briefly, cell lines were grown in a 4:1 mixture of MEM and RPMI (v/v) supplemented with 5 % fetal calf serum and glutamine. Medium for the transfected cell lines contained 1 mg/ml Geneticin. Cells were harvested for use when they had reached at least 95 % confluence. The supernatant of the hybridoma IgEL.b4 (ATCC code TIB-141) was used as a source of murine IgE that can substitute for rat IgE. Purified polyclonal human IgE was obtained from Biogenesis Ltd (Poole, UK). Goat anti-human and anti-mouse IgE antibodies were from Nordic Immunology Laboratories (Tilburg, NL). Mice were immunized with various allergen extracts according to published procedures (Hoffmann et al., 1997; Hoffmann et al., 1999). Sera from subjects having allergen-specific IgE and showing a positive skin test and/or a clear record of allergic symptoms were taken from the serum collection of the Paul-Ehrlich-Institut. Written informed consent was obtained from all blood donors. Chemicals were of highest available purity and purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (München, Germany).

2.2 Mediator release from RBL cells

RBL cells were sensitized either with murine IgE, polyclonal human IgE or sera containing allergen-specific human IgE at pre-determined optimal concentrations as described earlier (Hoffmann et al., 1997; Hoffmann et al., 2003). Cross-linking was induced either with allergen extracts for analysis of allergenic potential or with goat anti-mouse IgE/ anti-human IgE as controls. Allergen extracts and controls were dissolved in Tyrode's buffer containing 50 % D₂O, which had been added to increase and stabilize the secretion of β-hexosaminidase from

RBL cells carrying the humanized Fc ϵ RI (Maeyama et al., 1986). The amount of β -hexosaminidase was measured photometrically and release was determined as the ratio of allergen-specific release and total mediator content (cell lysis with Triton X-100) after correction for spontaneous release of unsensitized cells.

2.3 Determination of allergen-specific serum IgE

Specific IgE antibodies to allergens were measured by the CAP-FEIA system (Phadia AB, Uppsala, Sweden). The determination of IgE antibodies is based on WHO standards and the results are expressed in kU/l. Depending on the amount of specific serum IgE, the read-out is expressed in so-called CAP-classes: class 0 (no allergen-specific IgE) <0.35 kU/l, class 1 <0.7 kU/l, class 2 <3.5 kU/l, class 3 <17.5 kU/l, class 4 <50 kU/l, class 5 <100 kU/l, class 6 >100 kU/l. CAP-classes of 4 and above are considered as highly sensitized.

2.4 Preparation of allergen and food extracts

Food samples were frozen in liquid nitrogen and subsequently ground with an analytical blender (IKA M20, Staufen, Germany). Sample powders were extracted with phosphate-buffered saline (PBS, 10 mM NaH₂PO₄ x 2H₂O, 70 mM Na₂HPO₄ x 2H₂O, 150 mM NaCl, pH 7.4), tris-buffered saline (TBS, 100 mM Tris(hydroxymethyl)-aminomethan, 100 mM NaCl, 2.5 mM MgCl₂ x 6H₂O, pH 7.4) or 100 mM ammonium carbonate buffer (pH 8.0). The crude extracts were centrifuged at 20,000 x g for 20 min and 4 °C and filtered through 0.45- μ m pore size cellulose acetate filters (Millipore, Schwalbach, Germany), protein concentrations were determined according to a modified Bradford method (Zor and Selinger, 1996) using the Coomassie Plus Protein Assay (Pierce, Rockford, IL; USA) and the extracts were freeze-dried for storage until use.

2.5 Allergen detection and quantification by PCR and ELISA methods

The analysis of peanut by a competitive ELISA and of hazelnut by PCR and sandwich ELISA was performed as previously described (Holzhauser and Vieths, 1999a; Holzhauser and Vieths, 1999b; Holzhauser et al., 2000).

3. Results

3.1 Optimization of the RBL mediator release assay in the human system

3.1.1 Use of heavy water

During the first mediator release assays with transfected RBL cells, hardly any β -hexosaminidase release could be detected when the assay was performed according to the protocol established for RBL-2H3 cells. A first improvement was to incubate the cells with the human IgE during the overnight attachment phase to the microtiter plates. The second important change in the protocol was the preparation of Tyrode's buffer with deuterium oxide (D₂O). Figure 1 shows that the sensitization with murine IgE and cross-linking with anti-mouse IgE was more or less independent of the presence of D₂O, rising from 58 % in Tyrode's buffer without D₂O to 65 % with 75 % D₂O. The sensitiza-

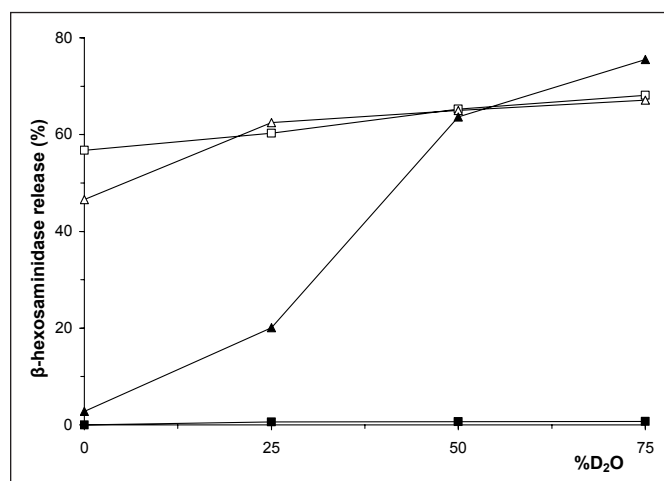


Fig. 1 Influence of deuterium oxide on mediator release from RBL cells. Transfected RBL30/25 (triangles) or RBL-2H3 (squares) cells were sensitized with murine (open symbols) or human (filled symbols) IgE and cross-linked with the appropriate goat anti-IgE antibodies. The graph is representative of three independent experiments.

tion of RBL-2H3 with human IgE did not induce any mediator release after cross-linking with anti-human IgE, confirming the specificity of the IgE receptors. While the sensitization of the transfected cell line RBL-30/25 with murine IgE and cross-linking with anti-mouse IgE resulted in an almost identical curve as was the case with RBL-2H3 cells, a noteworthy mediator release from the transfectants in the human system could only be observed when D₂O was added to the Tyrode's buffer used to prepare the allergen solutions. The release of β -hexosaminidase increased from almost undetectable without D₂O to greater than 70 % with 75 % of D₂O from the transfected RBL-30/25 cell line, showing the steepest rise at D₂O concentrations of 25 % to 50 %. For reasons of economy, Tyrode's buffer for all subsequent studies with the transfected cell line was prepared with 50 % D₂O. In addition, a couple of controls showed that Tyrode's buffer with D₂O, but without the addition of IgE and anti-IgE, did not induce any mediator release by itself. Likewise, no mediator release could be observed when cells were sensitized with IgE from one species and cross-linking was performed with anti-IgE specific for the other (data not shown).

3.1.2 Titration and specificity of patients' sera

Since the amount of allergen-specific IgE in the sera varies considerably between the patients, each serum used in mediator release tests had to be titrated in order to find the optimal dilution for the biological assay. This is shown in Figure 2 for a number of sera from patients that were sensitized to hazelnut proteins. As previously observed with other allergens, the reactivity of each individual serum was quite variable. Some of them even led to less mediator release at higher serum concentration. The optimal dilution range for most sera was 1:20 to 1:40 and a pool of the three sera which lead to the highest mediator release was prepared.

The biological assay is specific for the allergens tested, provided the sera contain the allergen-specific IgE antibodies. In the

case of multi-sensitization, the mediator release will be positive for the appropriate allergens. This is shown in Table 1 for a couple of allergens and selected human sera. For the hazelnut studies, sera from birch pollen-allergic people were selected, since the major allergens in hazelnut and birch pollen, Cor a 1 and Bet v 1, are cross-reactive. Hence, these sera (CG, SH and WB) are positive in the birch pollen and hazelnut CAPs and are also able to induce mediator release from RBL cells. The amount of mediators correlates with the concentration of allergen-specific serum IgE. These sera also contain IgE against grass pollen, which is often observed with birch pollen allergies, and therefore they also react with grass pollen extract in the mediator release assay.

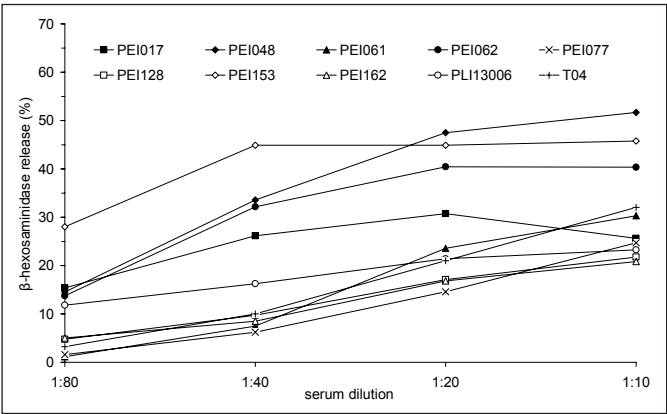


Fig. 2 Titration of sera from hazelnut-allergic patients. Transfected RBL-30/25 cells were sensitized with sera in different dilutions, containing hazelnut-specific IgE. Cross-linking was performed with hazelnut extract at predetermined optimal concentration (2 µg/ml).

Except for serum SH, no peanut-specific IgE was detected in CAP analysis, and consequently sensitization of RBL cells with these sera did not induce release of mediators in response to peanut extracts. For comparison, the results for a serum of a mite-allergic person (JS) without allergies to birch, grass or peanut are given, showing positive CAP values and measurable mediator release only in response to extracts from house dust mites. The correlation between allergen-specific IgE measured by CAP and the capability of the sera to passively sensitize RBL cells was excellent. The only exemption was serum SH that contained a moderate level of peanut-specific IgE, but did not mediate peanut-specific release in the mediator release assay.

3.2 Analysis of food samples with the mediator release assay

The applicability of the mediator release assay has been tested for a couple of often consumed foods. In Fig. 3, the analyses of hazelnut extracts are shown. Transfected RBL-30/25 cells were sensitized with pooled sera from hazelnut allergics and cross-linked with extracts from different hazelnut varieties (Fig. 3A). Virtually no difference could be seen in the allergenic potential of the hazelnut varieties since all curves run in parallel and show almost identical release values for each concentration. The analysis of four different batches of PBS extracts prepared from the variety Piemonte at different times during one year (Fig. 3B) demonstrated a very consistent quality of the extracts. One critical issue in the preparation of food extracts can be the buffer used for the extraction. Figure 3C, however, shows that in the case of hazelnuts there was no marked difference in the dose-dependent mediator release, regardless which extraction buffer (ammonium carbonate and phosphate- or tris-buffered

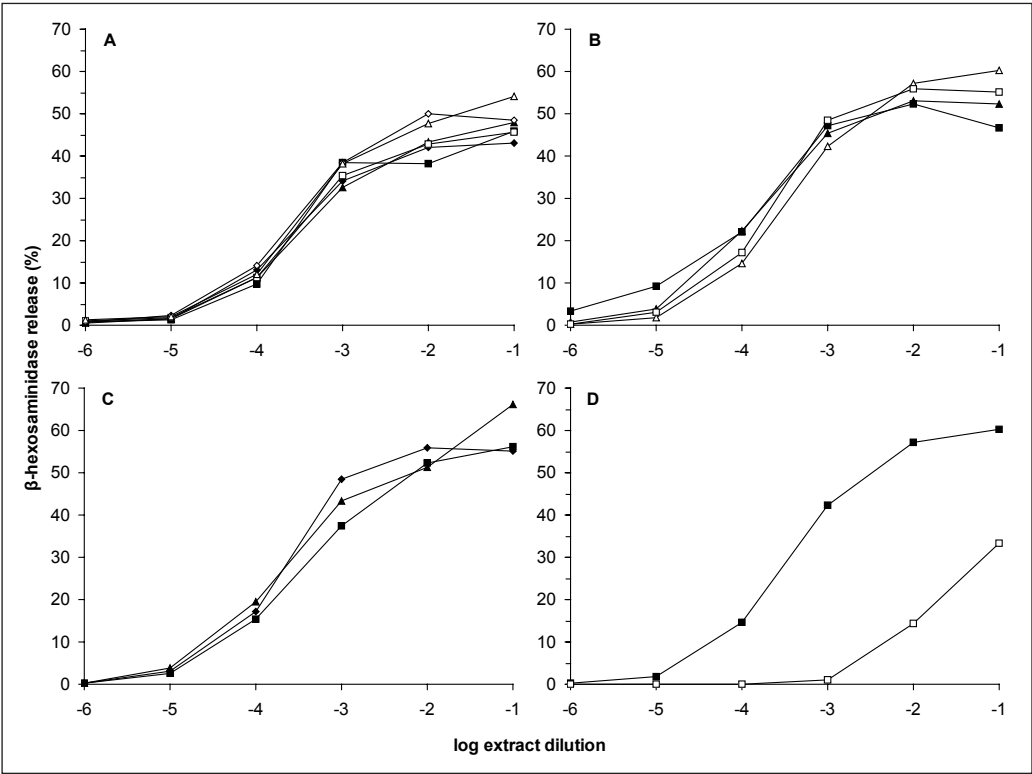


Fig. 3 Analysis of hazelnut extracts. Transfected RBL-30/25 cells were sensitized with a serum pool (from three allergics) and crosslinked with extracts from different hazelnut varieties (A): Mortarella (■), Nocciolo Ordu (◆), Piemonte (▲), San Giovanni (□), Tonda Giffioni (◇), or Tonda Romana (Δ). Panels B-D show the analysis of extracts of the hazelnut variety Piemonte, namely (B) different batches of PBS extracts (Jan.: ■; June: ▲; July: □; Dec.: Δ), (C) different extraction procedures (ammonium carbonate ■; PBS ◆; and TBS ▲) and (D) extracts from raw (■) or roasted (□) hazelnuts.

Tab. 1 Test of specificity of selected sera towards different extracts of inhalant allergens.

Serum	JS		CG		SH		WB	
diagnosed allergy/ investigated extract	mites		birch, grass		birch, grass		birch, grass, mites	
	release ¹	CAP ²	release	CAP	release	CAP	release	CAP
birch pollen	0.2	<0.35	39.5	63.0	27.1	49.7	30.0	54.3
grass pollen	0.3	<0.35	23.8	75.0	30.9	97.7	10.8	10.0
hazelnut	0.2	<0.35	41.0	25.2	22.7	14.2	3.5	4.6
peanut	0.2	<0.35	1.8	1.2	0.7	10.6	2.6	<0.35
<i>Dermatophagoides farinae</i>	26.8	66.0	2.6	4.5	0.3	<0.35	n.d. ³	>100
<i>Dermatophagoides pteronissinus</i>	23.1	61.0	0.5	1.0	0.4	<0.35	n.d.	84.4

¹ release: maximal mediator release at optimal concentrations of serum and allergen, given in % of total cellular β -hexosaminidase content

² allergen-specific serum IgE in kU/l

³ n.d.: not determined

saline) was used. The influence of food processing on allergenicity is shown in Figure 3D. Roasting of hazelnuts did significantly reduce hazelnut allergenicity, most likely due to denaturation of heat-sensitive hazelnut allergens. With roasted hazelnut extract, a more than 100-fold higher protein concentration was necessary for a comparable release of mediators that was elicited by raw hazelnut protein extract.

Next, self-prepared model chocolates without hazelnut and peanut were spiked with increasing amounts of hazelnut and peanut, respectively. The hazelnut spikes ranged from 1 % down to 0.001 % in a 10-fold dilution series, the peanut spikes ranged from 1 % down to 0.01 %. Sera from mice immunized with either hazelnut or peanut extract were pooled and RBL-2H3 cells passively sensitized with the pooled sera. Subsequently, RBL cells were incubated with the corresponding extracts, prepared from the hazelnut- or peanut-spiked whole-milk chocolates. The unspiked chocolates were analyzed simultaneously. The curves of the spiked samples were ideally parallel and the calculations of the relative potencies at the half-maximal mediator release levels represented the tenfold differences of the allergen concentrations in an excellent manner (Fig. 4). The unspiked control chocolates did not induce any mediator release, even at the highest applied concentration. In addition, Figure 4A shows that two samples of chocolate contained considerable amounts of hazelnut proteins. Sample A, which originated from the cleanup of a chocolate production line, had hazelnut between 0.01 and 0.1 %. Sample B, a whole milk chocolate without labeled hazelnut and bought at a local retailer, had hazelnut between 0.001 and 0.01 %. In both chocolate samples, low levels of hazelnut clearly had cross-linking properties for the release of physiologically active mediators. Thus, the allergenic potential of spurious amounts of hazelnut in chocolate was demonstrated in vitro. Furthermore, the allergenic potency was quantified in comparison to spiked samples. The presence of hazelnut was additionally verified with ELISA (Holzhauser and Vieths, 1999b) and PCR (Holzhauser et al., 2000). In both samples hazelnut was detectable by PCR and ELISA. Moreover, hazelnut protein was quantified by ELISA at 35 μ g/g (sample A) and 4 μ g/g (sample B), corresponding to approximately 0.04 % and 0.004 % hazelnut, respectively. The calculation of hazelnut from quantified hazelnut protein was based on the finding that

hazelnut protein and whole hazelnut from spiked samples correlated at a ratio of approximately 1:10. Quantification of hazelnut in chocolate by ELISA and relative potency of hazelnut measured in RBL cell assay were in complete agreement. The mediator release of peanut-sensitized RBL 2H3 cells after cross-

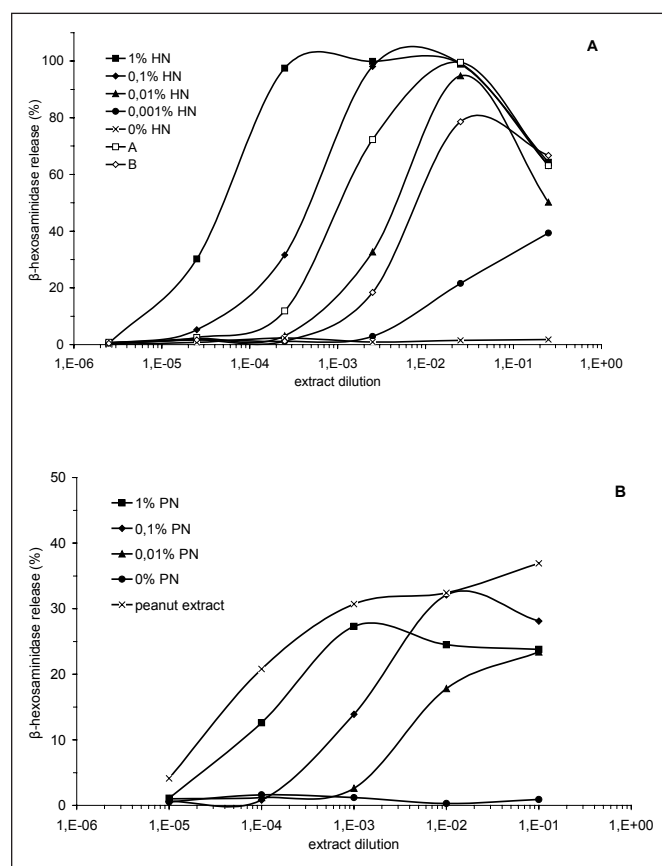


Fig. 4 Detection of hazelnut and peanut in chocolate preparations in the murine system. RBL-2H3 cells were sensitized with murine hazelnut- (A) or peanut-specific (B) serum pools and cross-linked with extracts from model chocolates spiked with the specified amounts of hazelnut or peanut, respectively. In panel A, curve A and curve B represent whole milk chocolate samples with unknown amounts of hazelnut that were also detected and quantified by PCR and ELISA, respectively.

linking by extracts from chocolate with increasing amounts of peanut (Fig. 4B) had similar characteristics as that of the hazelnut-sensitized cells; the semi-quantitative determination of the allergenic potency of peanut in chocolate was also comparable.

These studies were finally extended to the human system, where the transfected cell line RBL-30/25 was sensitized with a serum pool from five patients with an allergy against hazelnuts. Degranulation was induced by a variety of commercial samples containing or not containing hazelnut proteins. The results, shown in Figure 5, demonstrate that neither the negative controls (chocolate matrix or extraction buffer) nor samples without hazelnut proteins (cereals or peanut flakes) could induce a measurable release of β -hexosaminidase. The analysis of extracts of nougat or hazelnut chocolates, however, resulted in an increased mediator release in a dose-dependent fashion. From the known concentration of protein in the hazelnut extract, it was estimated that the biological assay was able to detect hazelnut allergens down to approximately 0.001%. In addition, most samples were analyzed by hazelnut-specific PCR and ELISA and the comparison of the results is shown in Table 2. In samples where hazelnut was detected by ELISA and PCR, specific release of mediators was also measurable. Moreover, there was a quantitative correlation between the amount of hazelnut present in the sample, as measured by ELISA, in comparison to the maximal dilution of sample extracts that would still result in 5% specific mediator release compared to total release. It was demonstrated that the biological assay can be used as a tool in the analysis of the allergenic potential of foods in a semi-quantitative manner.

4. Discussion

A biological test system, based on the mechanism of type I allergies, has been developed that allows to determine the allergenic potential of extracts from a variety of sources (Vogel et al., 2005). Here it is shown that its use can be extended to the analysis of allergens in complex matrices such as composed foods. The transfected cell line binds human IgE and can be

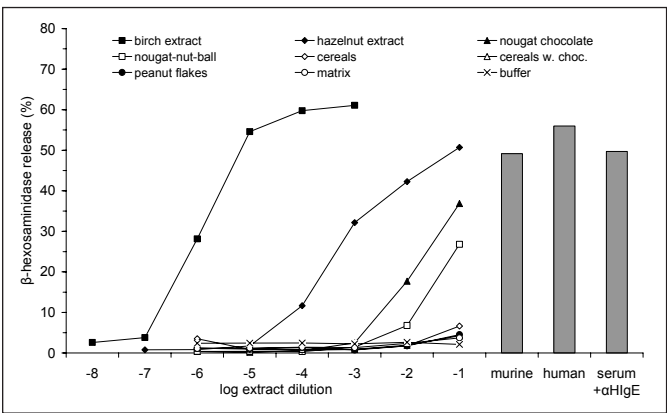


Fig. 5 Detection of hazelnut in food samples with transfected RBL cells. RBL-30/25 cells were sensitized with a hazelnut-specific pool of sera of five allergics and cross-linked with extracts from the specified extracts from commercial food samples as well as negative controls (chocolate matrix and extraction buffer). Columns represent the positive controls for assay performance, where cells were sensitized with murine IgE, polyclonal human IgE or the serum pool and cross-linked with the appropriate anti-IgE antibodies.

induced to release mediators after the cross-linking of receptor-bound IgE molecules by allergens. In contrast to the rat cell line, a noteworthy release is only observed in the presence of deuterium oxide. There is evidence that D₂O has a variety of effects in mammalian cells although the exact mechanisms are unknown (Lowe et al., 1995; MacDonald et al., 1991). With respect to mediator release from mast cells, the stabilizing activity towards microtubules (Gillespie and Lichtenstein, 1972) and reactive oxygen species formed during signal transduction (Thannickal and Fanburg, 2000) are discussed. As stated by Maeyama et al. (1986), D₂O could also have an effect during the initial phases of signal transduction by influencing the interaction of membrane components or the activity of phospholipase C. After several rounds of recloning and selection of high-releaser clones, it has become obvious that in the case of

Sample	ELISA (ppm hazelnut protein) ¹	PCR ¹	% Mediator release human	
			max. release	> 5% at dilution
Peanut flakes	–	–	4.5%	./.
Mixed cereals	701	+	6.6%	1:20
Cereals with chocolate	n.d. ²	n.d.	4.2%	./.
Nougat-chocolate bar	16,120	+++	32.5%	1:240
Hazelnut chocolate	17,123	+++	23.1%	1:325
Hazelnut extract	n.d.	n.d.	40.0%	1:50,000
Chocolate matrix	–	–	3.7%	./.
Buffer	–	–	2.1%	./.

Tab. 2 Comparison of assays for hazelnut detection in foods.

¹ – below limit of detection (ELISA: ~1 µg hazelnut per gram chocolate; PCR: ~2–4 µg hazelnut per gram chocolate, 1 µg/g equals 1 ppm)

² n.d. not done

./.. not applicable

polyclonal human IgE and anti-human IgE cross-linking, D₂O could be omitted from the Tyrode's buffer (unpublished observations). However, when using human sera, especially those with lower concentrations of allergen-specific IgE, it is still necessary to have D₂O present in the buffer used in the steps after sensitization.

The correlation of mediator release with data from immunochemical analysis and clinical observation is very good, provided that the sera used in the biological test system are evaluated and carefully chosen (Hoffmann et al., 2003; Vogel et al., 2005; Kaul et al., 2006). This is no problem for more prevalent allergies, since enough patients can be recruited for blood donation. In the case of peanut, however, the availability of adequate sera has been limited. Although allergies to peanut are often severe, even sera from highly allergic patients did not lead to a measurable mediator release (similar to serum SH in Table 1). The reason for this observation is presently unclear and under investigation. Despite this drawback, the mediator release assay gives consistent results with coefficients of variation between 10–15% and a sensitivity that can detect low amounts of allergenic proteins (10^{-9} – 10^{-11} g) and in some cases even in the femtogram-range (Hoffmann et al., 1995). The comparison of three cell-based assays, namely mediator release from RBL-2H3 or transfected RBL-30/25 cells and stripped human basophils, respectively, resulted in almost identical data (Hoffmann et al., 2003; Kaul et al., 2006). Since the method using stripped basophils requires freshly prepared human cells, its application in routine analysis is restricted for practical and ethical reasons. However, the assays based on the RBL cell lines, grown in permanent culture, can be used alternatively. In cases where sera from allergic persons are not available, RBL-2H3 cells can be sensitized with sera from immunized mice. Sometimes, it may be difficult to immunize mice with certain allergens. In these cases, the transfected cell line can be applied for the analysis with appropriate human sera.

The applicability of the biological test system has been shown for a couple of questions such as the analysis of allergen extracts used for diagnosis and therapy (Hoffmann et al., 2003), characterization of clinically relevant allergens (Mittag et al., 2004; Mittag et al., 2005), or the evaluation of mutated protein molecules that should have a reduced allergenicity (Gadermaier et al., 2005; Wallner et al., 2005). As demonstrated in the present publication, the method has been extended to address the biological activity of allergens in complex mixtures such as foods. The preparation of the samples follows established procedures and so far all extractions performed yielded reproducible results that were in agreement with ELISA and PCR methods. The limit of detection of the cell-based assays (RBL-2H3, RBL-30/25) was comparable to that of ELISA and PCR, namely in the range of 0.01 to 0.001% for hazelnut and peanut in complex food matrices such as chocolate, which corresponds to 10–100 ppm (mg/kg). In a threshold-based double-blind placebo-controlled food challenge (DBPCFC) with peanut, 2 mg of peanut flour induced objective symptoms and 5 mg peanut lead to severe systemic reactions in one study subject (Hourihane et al., 1997). In another threshold-based DBPCFC study with hazelnut-allergic subjects, 6.4 mg hazelnut elicited objective symptoms in one study subject (Wensing et al., 2002). Based on the

assumption that 100 g of a foodstuff that contains traces of peanut and hazelnut would be eaten at once, objective symptoms for peanut and hazelnut would be expected at a concentration of 20 ppm and 64 ppm respectively. These quantities of allergenic peanut and hazelnut were reliably detectable with the biological test systems described in this study.

Taken together, we have shown that the cell-based mediator release assays are suitable for the determination of the allergenic potential of proteins in a variety of settings. Because of the difficulties encountered in clinical threshold studies, investigation of the allergenic potential of traces of allergenic foods in a clinical setting is impossible on a routine basis. Since immunochemical methods only quantify potentially allergenic proteins, and PCR can only verify the presence of the allergenic food, a biological assay that measures potential allergenicity and determines the allergenic potency semi-quantitatively can serve as a valuable supplement in the risk assessment of foods that contain molecules with unknown allergenic potential. Thus, the functional assay contributes to an increased level of safety for the allergic consumers.

5. Acknowledgments

Murine IgE-containing sera were the kind gift of Dr. A. Hoffmann (Langen, Germany). The expert technical assistance of Lina Hatahet is gratefully acknowledged. This work was in part funded by grants from the German Research Foundation (DFG, Vi165/3), Research Center Karlsruhe (PUG P96002) and the German Federal Ministry of Health (1720/I-11A).

6. References

- Boden, M., Dadswall, R. and Hattersley, S. (2005) Review of statutory and voluntary labelling of food allergens. *Proc Nutr Soc* 64:475–480.
- Burks, A. W., Jones, M. S., Wheeler, J. G. and Sampson, H. A. (1999) Anaphylaxis and food hypersensitivity. *Immunol Allergy Clin North America* 19:533–552.
- Gadermaier, G., Dedic, A., Jahn-Schmid, B., Ebner, C. and Ferreira, F. (2005) Hypoallergenic variants of Art v 1, the major mugwort pollen allergen. *J Allergy Clin Immunol* 115:S89.
- Gillespie, E. and Lichtenstein, L. M. (1972) Histamine from human leukocytes: studies with deuterium oxide, colchicine and cytochalasin B. *J Clin Invest* 51:2941–2947.
- Hoffmann, A., Jamin, A. and Haustein, D. (1995) Generation of cat dander specific IgE in mice permits detection of femtograms of Fel d 1 by RBL cell mediator release assay. *Allergo J* 4:42.
- Hoffmann, A., Vieths, S. and Haustein, D. (1997) Biologic allergen assay for in vivo test allergens with an in vitro model of the murine type I reaction. *J Allergy Clin Immunol* 99:227–232.
- Hoffmann, A., Jamin, A., Fötisch, K., May, S., Aulepp, H. and Haustein, D. (1999) 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. *Allergy* 54:446–454.
- Hoffmann, A., Kaul, S., Fötisch, K., Danz, N., Lüttkopf, D., Hatahet, L., Kastner, B., Vogel, L., Hölz, G. and Vieths, S. (2003) Mediator release assays based on human and murine IgE: potential and limitations in allergen standardisation. In: Löwer, J. et al. (eds.) *Arbeiten aus dem Paul-Ehrlich-Institut*. Frankfurt a.M., Sperlich Verlag, pp. 76–85.

- Holzhauser, T. and Vieths, S. (1999a) Indirect competitive ELISA for determination of traces of peanut (*Arachis hypogaea* L.) protein in complex food matrices. *J Agr Food Chem* 47:603–611.
- Holzhauser, T. and Vieths, S. (1999b) Quantitative sandwich ELISA for determination of traces of hazelnut (*Corylus avellana*) protein in complex food matrices. *J Agr Food Chem* 47:4209–4218.
- Holzhauser, T., Wangorsch, A. and Vieths, S. (2000) Polymerase chain reaction (PCR) for detection of potentially allergenic hazelnut residues in complex food matrices. *Europ Food Res Technol* 211:360–365.
- Holzhauser, T., Stephan, O. and Vieths, S. (2002) Detection of potentially allergenic hazelnut (*Corylus avellana*) residues in food: a comparative study with DNA PCR-ELISA and protein sandwich-ELISA. *J Agr Food Chem* 50:5808–5215.
- Hourihane, J. O., Kilbum, S. A., Nordlee, J. A., Hefle, S. L., Taylor, S. L. and Warner, J. O. (1997) An evaluation of the sensitivity of subjects with peanut allergy to very low doses of peanut protein: a randomized, double-blind, placebo-controlled food challenge study. *J Allergy Clin Immunol* 100:596–600.
- Kaul, S. et al. (2006) Mediator release assays based on human or murine IgE in allergen standardization. *Clin Exp Allergy* (in press).
- Lowe, J., Jardieu, P., van Gorp, K. and Fei D. T. (1995) Allergen-induced histamine release in rat mast cells transfected with the α subunits of Fc ϵ RI. *J Immunol Methods* 184:113–122.
- MacDonald, S. M., White, J. M., Kagey-Sobotka, A., MacGlashan, D. W. and Lichtenstein, L. M. (1991) The heterogeneity of human IgE exemplified by the passive transfer of D2O sensitivity. *Clin Exp Allergy* 21:133–138.
- Maeyama, K., Hohman, R. J., Metzger, H. and Beaven, M. A. (1986) Quantitative relationships between aggregation of IgE receptors, generation of intracellular signals, and histamine secretion in rat basophilic leukemia (2H3) cells. Enhanced responses with heavy water. *J Biol Chem* 261:2583–2592.
- Mittag, D., Akkerdaas, J., Ballmer-Weber, B. K., Vogel, L., Wensing, M., Becker, W. M., Koppelman, S. J., Knulst, A. C., Helbling, A., Hefle, S. L., van Ree, R. and Vieths, S. (2004) Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. *J Allergy Clin Immunol* 114:1410–1417.
- Mittag, D., Vieths, S., Vogel, L., Wagner-Loew, D., Starke, A., Hunziker, P., Becker, W.-M. and Ballmer-Weber, B. K. (2005) Birch pollen-related food allergy to legumes: identification and characterization of the Bet v 1 homologue in mungbean (*Vigna radiata*), Vig r 1. *Clin Exp Allergy* 35:1049–1055.
- Stephan, O. and Vieths, S. (2004) Development of a real-time PCR and a sandwich ELISA for detection of potentially allergenic trace amounts of peanut (*Arachis hypogaea*) in processed foods. *J Agr Food Chem* 52:3754–3760.
- Stephan, O., Weisz, N., Vieths, S., Weiser, T., Rabe, B. and Vatterot, W. (2004) Protein quantification, sandwich ELISA, and real-time PCR used to monitor industrial cleaning procedures for contamination with peanut and celery allergens. *J AOAC Internat* 87:1448–1457.
- Thannickal, V. J. and Fanburg, B. L. (2000) Reactive oxygen species in cell signaling. *Amer J Physiol* 279:L1005–L1028.
- The European Parliament and Council (2003) Directive 2003/89/EC of the European Parliament and of the Council amending Directive 2000/13/EC as regards indication of the ingredients present in foodstuffs. Official J European Union L305:15–18.
- Vogel, L., Lüttkopf, D., Hatahet, L., Haustein, D. and Vieths, S. (2005) Development of a functional in vitro assay as a novel tool for the standardization of allergen extracts in the human system. *Allergy* 60:1021–1028.
- Wallner, M., Stöcklinger, A., Vogel, L., Bohle, B., Hammerl, P., Breiteneder, H., Hoffmann-Sommergruber, K., Vieths, S., Thalhamer, J. and Ferreira, F. (2005) Expression and characterization of Bet v 1 variants created by gene shuffling. *J Allergy Clin Immunol* 115:S89.
- Wensing, M., Penninks, A. H., Hefle, S. L., Akkerdaas, J. H., van Ree, R., Koppelman, S. J., Bruijnzeel-Koomen, C. A. and Knulst, A. C. (2002) The range of minimum provoking doses in hazelnut-allergic patients as determined by double-blind, placebo-controlled food challenges. *Clin Exp Allergy* 32:1752–1762.
- Wild, L. G. and Lehrer, S. B. (2001) Immunotherapy for food allergy. *Curr Allerg Asthma Rep* 1:48–53.
- Wüthrich, B. and Ballmer-Weber, B. K. (2001) Food-induced anaphylaxis. *Allergy* 56 (Suppl. 67):102–104.
- Zor, T. and Selinger, Z. (1996) Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal Biochem* 236:302–308.
- Zuberbier, T., Edenharter, G., Worm, M., Ehlers, I., Reimann, S., Hantke, T., Roehr, C. C., Bergmann, K. E. and Niggemann, B. (2004) Prevalence of adverse reactions to food in Germany – a population study. *Allergy* 59:338–345.



To access this journal online:
<http://www.birkhauser.ch>