

Occupational sensitization to ribosome-inactivating proteins in researchers

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Summary

Background Ribosome-inactivating proteins (RIPs) are expressed in many plants. Because of their anti-infectious and anti-proliferative effects, intensive research is going on for applying these toxins in therapy against viral infections or malignancies. Recently, we demonstrated that type I allergy against RIPs from elderberry can occur.

Objective Stimulated by our study, a group of RIP researchers reported that some of the employees had suspected allergy to RIPs.

Methods and Results We tested their sera in ELISA on natural RIPs. Specific IgE in four subjects were found against dianthin30, gelonin, momordin, PAP-S, saporin, ricin and volkensin. In contrast, asparin and lychnin did not show any IgE binding. When separating extracts of plants containing the toxins in SDS-PAGE, RIPs appeared to be the predominant constituents. Interestingly, among the other plant proteins, they were exclusively recognized by IgE in immunoblot. RIPs derived from close botanical families share high sequence homologies. Nevertheless, in IgE inhibition experiments with human sera, cross-reactivity between RIPs also derived from non-related plants could be demonstrated.

Conclusion We conclude that sensitization and IgE induction to RIPs may occur upon exposure. This has to be considered when applying them in therapy against malignancies or viral infections.

Keywords cross-reactivity, IgE, occupational, RIPs, type I allergy

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Introduction

Ribosome-inactivating proteins (RIPs) are enzymes that trigger the catalytic inactivation of ribosomes. They are present in a large number of plants and have also been found in fungi, algae and bacteria [1–3]. An overview on RIPs is given in Table 1. A high number of RIPs have been detected in plants belonging to various families, particularly *Caryophyllaceae*, *Sambucaceae*, *Cucurbitaceae*, *Euphorbiaceae*, *Phytolaccaceae* and *Poaceae* [3].

RIPs are divided into three broad groups: type I RIPs consist of a single peptide chain having enzymatic activity, with a molecular weight in the range of 21–38 kDa. They inhibit cell-free protein synthesis, but are relatively non-toxic to cells and animals. Type II RIPs (56–69 kDa) consist of an enzymatic A chain similar to type I RIPs, connected by a disulphide linkage to a slightly larger B chain. A type III of RIPs has been proposed, including RIPs consisting of a single chain with additional peptide segments [2, 4, 5]. Most B chains of Type II RIPs are lectins, many of them with specificity for sugars, e.g. for sialic acid or *N*-acetylgalacto-

samine, which facilitate the entry of the toxins into cells, where the A chains can exert their enzymatic activity. One exception is a RIP from *Sambucus* which does not bind sugars [6]. Thus, some type II RIPs are potent toxins, the best known being ricin, which alter ribosomes and possibly other structures with consequent cell damage and death [1, 7, 8]. However, other type II RIPs having the same enzymatic and lectin properties as ricin, are much less toxic to cells and animals. In some plants RIPs are present in many or even in all tissues (roots, leaves, stems, bark, flowers, fruits, seeds), while in other plants they are confined to a single tissue. Often, different isoforms are found, sometimes even in the same tissue [9, 10]. In some species the occurrence of both type I and type II RIPs has been described [10–12]. Because of their anti-viral and anti-proliferative effects [13], RIPs are used in popular medicine and one of them, trichosanthin, in Chinese traditional medicine as an abortifacient. Intensive research is going on to apply them traditional linked either by chemical linkage or as recombinant fusion proteins to appropriate carriers, mostly monoclonal antibodies (immunotoxins), for experimental therapy of cancer or viral infections, such as HIV (Table 2).

The first documented allergic reaction to RIPs was elicited by castor bean (*Ricinus communis*) in an occupational setting in industrial workers [14]. Formation of IgE has also been

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Table 1. List of type I and type II RIPs with corresponding plants examined in this study

	MW* (kDa)	Family	Species	Source	Characteristics and utilization	References
<i>Type I RIPs</i>						
Asparin	30.5–29.8	Asparagaceae	<i>Asparagus officinalis</i>	Seeds	Popular vegetable, most commonly eaten in May	[23, 26]
Dianthin30†	29.5	Caryophyllaceae	<i>Dianthus caryophyllus</i>	Leaves	87% homology with saporin-S6 Anti-viral property including HIV-1 infection	[27–30]
Gelonin†	30.0	Euphorbiaceae	<i>Gelonium multiflorum</i>	Seeds	Anti-viral effect Anti-melanoma immunotoxin Anti-tumour activity	[31–35]
Lychnin†	26.6	Caryophyllaceae	<i>Lychnis chalcidonica</i>	Seeds		[26, 28]
Momordin†	29.1	Cucurbitaceae	<i>Momordica charantia</i>	Seeds	Source of vitamin C, A, phosphorus and iron Has been used by natives for cancer, diabetes, infectious diseases treatment	[31, 36–38]
PAP-S† (pokeweed anti-viral protein)	29.0	Phytolaccaceae	<i>Phytolacca americana</i>	Seeds	Juice from berries has been used for pimples, boils, joint pain Anti-viral activity	[36, 39–43]
Saporin†	29.5–31.6	Caryophyllaceae	<i>Saponaria officinalis</i>	Seeds, leaves, roots	DNA-nuclease activity Immunotoxin Anti-tumour activity	[31, 32, 36, 40, 44–46]
<i>Type II RIPs</i>						
Ricin†	63–67	Euphorbiaceae	<i>Ricinus communis</i>	Seeds	Anti-HIV and anti-tumour effect	[31, 47, 48]
Volkensin	62	Passifloraceae	<i>Adenia volkensii</i>	Roots		[49]

*Molecular weights from reference [3].

†The structure of these RIPs has been determined by crystallization or by X-ray diffraction analysis.

RIPs, ribosome-inactivating proteins.

Table 2. References on applications of immunotoxins or conjugates containing RIPs

	HIV	Tryp., Leish.	CD80/CD86 tumors	Hodgkin disease	Leukaemia	Melanoma	Breast cancer	Human bladder tumour	HSP 65 ⁺ tumours	Pain
Asparin										
Dianthin30	[29]	[45]		[27]	[27]					
Gelonin			[32]	[32]	[50]	[33]	[34, 51]			
Lychnin										
Momordin				[52]	[53]			[40]		
PAP-S	[54]	[45]		[52]	[55]			[40]		
Ricin	[56]			[57]	[58]		[59]			[60]
Saporin	[61]	[45]	[32]	[52]			[62]	[40]	[63]	[60]
Volkensin										[60]

HIV, human immunodeficiency virus; Tryp., *Trypanosoma brucei rhodesiense*; Leish., *Leishmania infantum*; HSP 65 kDa, heat shock protein 65; RIPs, ribosome-inactivating proteins.

observed in HIV-infected patients treated with trichosantin (type I RIP) and in patients treated with immunotoxins containing RIPs [15].

This study examines the sensitization profile of people who have been working with RIPs for several years in a research laboratory. Some of them reported having developed allergic symptoms upon contact with RIPs during this period. We collected observational data of these subjects and compared them with the results obtained from serum analysis in ELISA and immunoblot. Furthermore, we characterized the purified RIPs and the corresponding plant extracts

with regard to their IgE-binding capacity and possible cross-reactivity.

Materials and methods

Study population

Serum samples used in this study were collected from 17 persons who are employees in the research laboratory of the Department of Experimental Pathology, University of

Table 3. Characteristics of the examined employees

Number	Sex	Age (years)	Self-reported symptoms to RIPs	After occupational exposure during	Other allergies
1	M	45	Ocular reactions, dermatitis	2 years	–
2	F	28	–	–	–
3	M	25	–	–	Penicillin
4	M	28	–	–	–
5	M	50	–	–	Conservants
6	F	26	–	–	–
7	F	36	Ocular and respiratory reactions	3 years	Grass pollen
8	F	59	–	–	Mosquito bites
9	M	72	Rhinitis, asthma, conjunctivitis	2 years	–
10	F	25	–	–	–
11	M	63	Asthma	8–9 years	–
12	F	32	–	–	–
13	F	31	–	–	–
14	F	47	–	–	Detergents
15	F	31	–	–	Pollen
16	F	35	–	–	Nickel
17	F	29	–	–	Pollen

M, male; F, female; RIPs, ribosome-inactivating proteins.

Bologna, Italy (see Table 3). The gender ratio was 6:11 (male:female); the mean age was 38.9 years. Some of them suffered from different allergic symptoms, four reported reactions upon RIP exposure. By ELISA measurements, all sera of the employees were tested for RIP-specific IgE. For immunoblotting, we pooled the sera in equal parts of patients 1, 5, 7 and 9, who were chosen based on their high specific IgE levels to RIPs.

Measurements of specific immunoglobulin E antibodies by enzyme-linked immunosorbent assay

Microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4 °C with RIPs (10 µg/mL in 50 mM NaHCO₃, pH 9.6). Thereafter, plates were washed with TBS (0.9% NaCl, 50 mM Tris-base, pH 7.4)/0.5% Tween 20, Herck, Darmstadt, Germany (TBST), and blocked for 1 h at 37 °C and 1 h at 4 °C with TBST/1% bovine serum albumin (BSA). Sera were added at a dilution of 1:5 in TBST/0.1% BSA and incubated overnight at 4 °C. Plates were washed and incubated for 1 h at 37 °C and 1 h at 4 °C using peroxidase-labelled anti-human IgE antibody, diluted 1:1000 (Pharmin-gen, BD Biosciences, San Diego, USA). The reaction was developed by adding 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) in citric acid (pH 4.0) and hydrogen peroxide. The optical density was measured at 405–490 nm in an ELISA-Reader (Spectramax Plus, Molecular Devices, Munich, Germany).

Plant extracts and ribosome-inactivating proteins

RIPs were purified by the methods described previously [16]. The crude plant extracts were prepared by homogenizing

plant materials in phosphate-buffered saline (0.14 M NaCl, 5 mM Na-phosphate buffer, pH 7.4, PBS) (8 mL/g). After overnight stirring at 4 °C, the extracts were centrifuged, and the supernatants were collected. Their protein concentrations (w/v) were: *Adenia volkensii* 3.31 mg/mL, *Asparagus officinalis* 4.18 mg/mL, *Dianthus caryophyllus* 4.61 mg/mL, *Gelonium multiflorum* 22.02 mg/mL, *Lychnis chalcidonica* 19.9 mg/mL, *Momordica charantia* 7.22 mg/mL, *Phytolacca americana* 10.09 mg/mL, *R. communis* 10.9 mg/mL and *Saponaria officinalis* 17.3 mg/mL. The RIPs were adjusted to 1 mg/mL either in H₂O or in PBS.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting

The extracts and RIPs were separated on 12% SDS-PAGE according to the protocol from Laemmli [17]. Briefly, 15 µL of extract or RIP was heated to 95 °C for 5 min in reducing Laemmli buffer before loading them to SDS-PAGE (Biometra, Göttingen, Germany). Plant and RIP protein masses were estimated by Coomassie brilliant blue staining of the gel and comparison with molecular weight markers (RPN 800, Amersham, Buckinghamshire, UK). Electrobolt of proteins was performed onto a nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany).

Detection of human immunoglobulin E in immunoblot

Serum samples were selected according to their IgE-binding capacity in ELISA and were used either purely or were pooled at equal parts. Blocking, washing and diluting were performed in 50 mM sodium phosphate buffer, pH 7.5, 0.5% Tween 20 (PBST), containing 0.5% BSA. After blocking, blots were incubated overnight at 4 °C with 1 mL sera (diluted 1:5). After washing, the binding of serum IgE to the blotted plant extract or RIP proteins was detected by I¹²⁵-rabbit-anti-human IgE (Pharmacia-Diagnostics, Vienna, Austria), diluted 1:20 in blocking buffer. Blots were exposed to BIOMAX-MS films (Kodak, Rochester, NY, USA) at –70 °C for 3–5 days.

Immunodot

RIPs (1 µg/dot) were dotted in duplicates onto a nitrocellulose membrane (Schleicher&Schuell). Dot strips were air-dried and blocked with PBST, 0.5% BSA. One millilitre of the pooled sera, diluted 1:5 in blocking buffer, was pre-incubated overnight at 4 °C with 20 µg RIP. After incubation of strips with the pre-incubated sera (overnight at 4 °C), bound IgE was detected as described above.

Deglycosylation experiments

Deglycosylation of nitrocellulose-blotted RIPs was performed with 0.01 mol/L NaIO₄ in acetate buffer (pH 5.0) at room temperature in the dark. The reaction was stopped by extensive washes with ddH₂O.

Results

Characteristics of the study population

Self reports were collected from 17 adults working in a research laboratory and being in daily or in rare contact with RIPs. The characteristics of the researchers are presented in Table 3. Four of the 17 screened persons, namely numbers 1, 7, 9 and 11, reported immediate-type symptoms elicited upon exposure RIPs during their work. Among them, only individual number 7 reported concomitant sensitization to other type I allergens.

The immunoglobulin E-binding capacity of ribosome-inactivating proteins in enzyme-linked immunosorbent assay and on immunoblot

The specific IgE antibody levels to RIPs were tested in ELISA (Fig. 1). As negative control, we used serum from a non-allergic person. First, we examined the IgE binding to type I RIPs: particularly high levels of IgE were observed in the serum of individual number 9, who reacted to dianthin30, gelonin, momordin, PAP-S and saporin; number 1 showed IgE binding to gelonin, mormordin and PAP-S, number 5 to mormordin and PAP-S and number 7 to saporin only. No IgE reactivity to type I RIPs asparin and lychnin could be observed (data not shown). With respect to type II RIPs, only serum number 9 showed IgE reactivity to volkensin. To the type II RIP ricin, IgE binding was detected in sera 5 and 9. The IgE-binding patterns in ELISA were mostly paralleled by the immunoblot results.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblot

Purified RIPs were separated under reducing conditions by SDS-PAGE and were visualized by Coomassie staining (Fig. 2, lanes 3). As expected, the type I RIPs dianthin30, gelonin, momordin, PAP-S and saporin showed molecular weights at or close to 30 kDa. With the type II RIP ricin, two bands at 30 and 32 kDa, and in volkensin a single band at 32 and a double band at 45 kDa were detected. Also plant extracts were separated by SDS-PAGE under the same conditions and stained by Coomassie to study relative their contents of RIPs (Fig. 2, lanes 1). Whereas gelonin, PAP-S, saporin and ricin represented the most abundant protein in the respective plant extracts, momordin and volkensin were found in moderate amounts and dianthin30 only in minor quantities in the corresponding extracts.

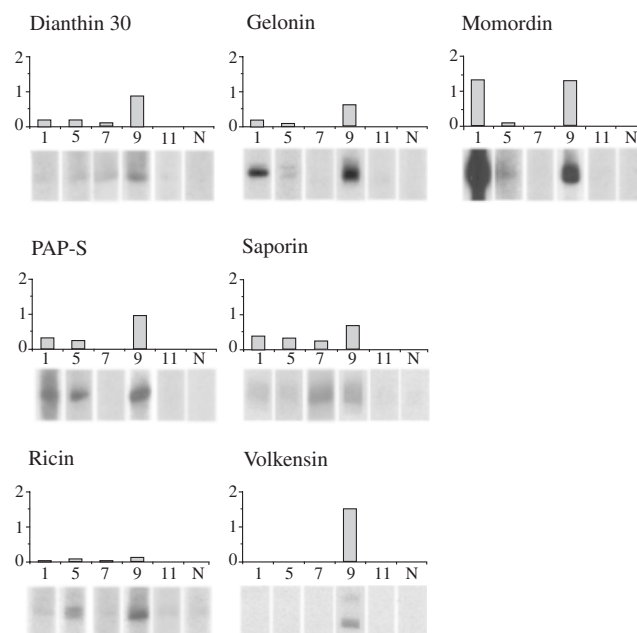


Fig. 1. Detection of specific IgE to purified ribosome-inactivating proteins (RIPs) in ELISA in comparison with immunoblot. Diagrams show specific IgE to RIPs (as indicated). The y-axis shows the optical density (OD_{405–490nm}). Specific IgE to blotted type I and type II RIPs was detected with radiolabelled anti-IgE antibody. The reactivity patterns of subjects 1, 5, 7, 9, 11 compared with a negative (N) control are shown.

The plant extracts were also tested in IgE immunoblotting using a serum pool (Fig. 2, lanes 2). IgE binding was observed at the molecular weights of the RIPs gelonin, momordin, PAP-S and to a minor extent, dianthin30, saporin, ricin and volkensin. Thus, the binding of polyclonal serum IgE was predominantly directed towards the RIPs in most extracts, possibly due to a biased exposure of researchers to RIPs in the lab. Besides, minor bands with IgE-binding capability were observed in the plant extracts containing gelonin, momordin, PAP-S, saporin and ricin.

Cross-reactivity studies of ribosome-inactivating proteins

The cross-reactivity assay is based on the inhibition of IgE binding to RIPs after pre-incubation of pooled sera with other RIPs (Fig. 3). Dianthin30 demonstrates cross-reactivity with gelonin, ricin and saporin; gelonin with all other RIPs; momordin with gelonin, PAP-S and ricin; PAP-S with momordin and ricin; ricin with saporin; saporin with dianthin30, gelonin and ricin; and volkensin does not show

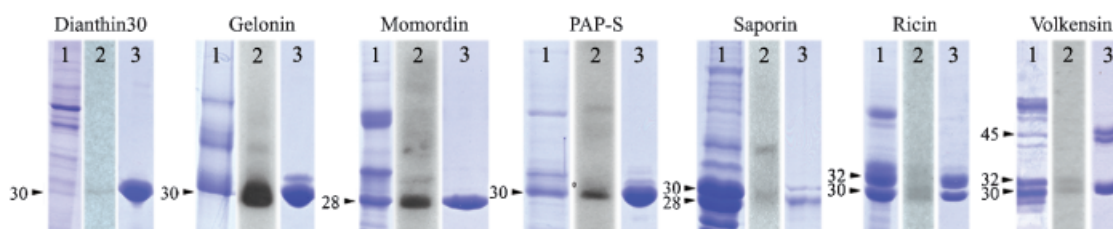


Fig. 2. IgE-binding characteristics of plant extracts containing ribosome-inactivating proteins (RIPs). Lane 1: plant extracts separated by SDS-PAGE under reducing conditions, stained by Coomassie. Lane 2: corresponding immunoblot of plant extracts using pooled sera. IgE binding was visualized by radiolabelled anti-IgE antibody. Lane 3: purified RIPs were separated by SDS-PAGE under reducing conditions and stained with Coomassie.

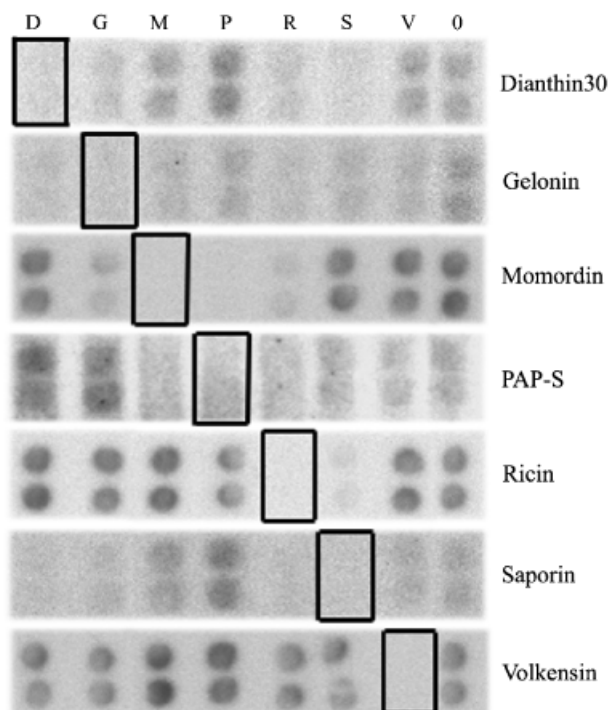


Fig. 3. Cross-reactivity analysis of ribosome-inactivating proteins (RIPs) in an immunodot. The toxins were dotted in duplicate on horizontal lanes; the vertical lanes represent nitrocellulose strips incubated with serum pool containing IgE towards RIPs. Serum pool was pre-incubated with the following toxins: dianthin30 (D), gelonin (G), momordin (M), PAP-S (P), ricin (R), saporin (S) and volkensin (V). Bound IgE was detected with radioactive-labelled anti-IgE. Frames: Complete inhibition of IgE-binding to homologous dotted RIP. Non-pre-incubated serum pool is shown in lane 0.

cross-reactivity. To elucidate whether carbohydrate epitopes were causing the observed cross-reactivities, we performed deglycosylation experiments using the periodate method on blotted RIPs (Fig. 4). Deglycosylation of blotted RIPs had no effect on the IgE-binding capacity in most cases, except for mormordin and PAP-S, where treatment resulted in complete loss of IgE binding. This suggests that the IgE epitopes of dianthin30, gelonin, saporin, ricin and volkensin are constituted by the polypeptide chain rather than by carbohydrate moieties.

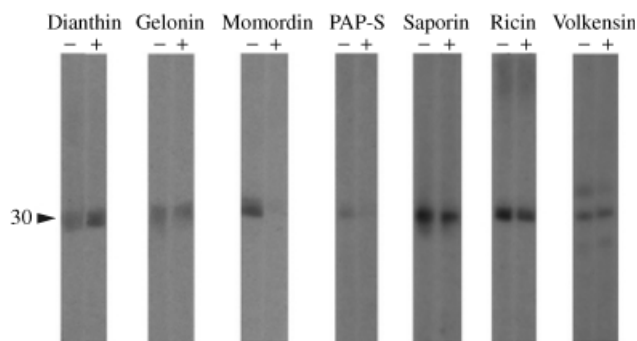


Fig. 4. Effects of deglycosylation on IgE binding to ribosome-inactivating proteins (RIPs). Blotted purified RIPs were treated by periodate (lanes +), or left untreated (-). Thereafter, strips were incubated with allergics sera. Bound IgE was detected by radiolabelled anti-IgE. The autoradiograms are shown. The arrow indicates a molecular mass of 30 kDa.

Discussion

Type I allergic reactions against different RIPs have already been demonstrated, when people had contact with plants or were treated with medication containing RIPs [18–23]. In a previous study, we could also show that type I allergic reactions against RIPs from elderberry (*Sambucus nigra*) can occur [22]. Referring to this report, a research group working in the field of RIPs research since decades performed a subjective self-report on possible hypersensitivity to RIPs. They found that four out of 17 employees (24%) had suspected different allergic symptoms to RIPs when working with them. The sensitization period of these subjects varied from 2 to 9 years, and also the mode of contact with the toxins was different. A few of them worked the whole day with extracts and toxins, performing extractions, purifications or cytotoxicity experiments. Others were just exposed for a short time (half an hour per day) in the laboratory. None of them had signs of toxicity. Taking castor beans as an example, their average content of ricin is 1 mg/g, whereas its lethal dose is around 1 mg when injected. In contrast, these minute amounts of protein were obviously sufficient to sensitize some researchers. Blinded, we tested all the sera in ELISA for IgE reactivity against RIPs used in their studies (asparin, dianthin30, gelonin, lychnin, momordin, PAP-S, ricin, saporin and volkensin). Comparing these results with the self-reports, the ELISA and immunoblot results mostly supported their observations. Interestingly, one person (number 11) also reported asthma attacks in the laboratory, but the toxin-specific IgE level was inconspicuous, suggesting that the reactions were caused by other environmental factors. In contrast, researcher number 5 did not report complaints upon exposure to RIPs, but exhibited positive IgE reaction to toxins in our immunoassay, indicating a silent sensitization to RIPs. Patient 9, who had been working for decades with RIPs, showed the highest and broadest specific IgE binding to RIPs. We suggest that these sensitizations could explain the rhinitis, asthma and conjunctivitis of this person. Asparin and lychnin did not show specific IgE reactivity in ELISA, which might indicate their lower allergenic potential.

The level of RIPs in plant tissues is highly variable, ranging from traces to hundreds of milligram per 100 g. Notably, in most instances (dianthin30, gelonin, momordin, PAP-S, volkensin), RIPs represented substantial constituents and the predominant IgE-binding entities in the plant extracts, even if other proteins were present in high numbers and quantities. This phenomenon may be due to exclusive exposure to the RIPs and the high specificity of induced IgE to RIPs. In *A. volkensii*, volkensin and a second unidentified IgE-binding component were observed at 45 kDa. Besides, minor bands with IgE-binding capability were observed in the plant extracts containing gelonin, momordin, PAP-S, saporin and ricin. The nature of these bands remains undefined; they may possibly represent RIP precursors or cross-reactive non-related compounds.

RIPs have been described in about 50 plant species covering 17 families. In general, the classification of RIPs is mainly based on structure and function, even if RIPs derived from close botanical families show considerable similarities, as e.g. dianthin with saporin and momordin with trichosanthin [24].

However, these sequence relations are likely to be causative for observed cross-reactivity between RIPs [25]. Our dot inhibition experiments revealed, e.g. cross-reactivity between dianthin30 and saporin, which are derived from the same plant family and have a high amino acid sequence homology. Interestingly, antigenic cross-reactivity was also observed between botanically unrelated toxins, for example dianthin30 with gelonin, PAP-S with ricin, gelonin with dianthin30 or momordin with PAP-S. In the case of momordin and PAP-S, participation of carbohydrate determinants cannot be excluded, whereas in the other instances most likely the polypeptide fold is responsible for the IgE cross-reactivity. Our results should be taken into consideration when planning therapeutic applications of RIPs. Immunotoxins, i.e. monoclonal antibodies chemically coupled to toxins, are selectively toxic for the cells targeted by antibodies. Immunotoxins have been tested for experimental treatment of a variety of malignancies and viral infections over the last 20 years. We found that often cross-reactive RIPs are in therapeutic use for the same disease, like momordin, PAP-S, ricin and saporin against Hodgkin's disease, or dianthin30, PAP-S and ricin against HIV infection (Table 2). Further, volkensin is the only RIP that is not cross-reactive with the other tested RIP molecules.

From our data we conclude that RIPs from different plant origin are potent elicitors of sensitization and IgE induction, and their allergenic as well as cross-reactive potential has to be considered when applying them in therapy.

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