

Cosensitization to the 3 Nonhomologous Major Cashew Allergens Ana o 1, Ana o 2, and Ana o 3 Is Caused by IgE Cross-reactivity

Kabasser S¹, Radauer C¹, Eber E², Haber ME², Hieden K², Zieglmayer P^{3,4}, Kost LE⁵, Sindher SB⁵, Chinthrajah S⁵, Geiselhart S¹, Hoffmann-Sommergruber K¹, Nadeau KC^{5,6}, Breiteneder H¹, Bublin M¹

¹Institute of Pathophysiology and Allergy Research, Center of Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

²Division of Pediatric Pulmonology and Allergology, Department of Pediatrics and Adolescent Medicine, Medical University of Graz, Graz, Austria

³Vienna Challenge Chamber, Allergy Center Vienna West, Vienna, Austria

⁴Competence Center for Allergology and Immunology, Karl Landsteiner University, Krems, Austria

⁵Sean N. Parker Center for Allergy and Asthma Research at Stanford University, Stanford, USA

⁶Division of Pulmonary and Critical Care Medicine, Department of Medicine, Stanford University, Stanford, USA

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■ Abstract

Background: Cashew nuts often cause strong allergic reactions, which are even more severe than those of peanuts. Ana o 1 (vicilin), Ana o 2 (legumin), and Ana o 3 (2S albumin) are major cashew allergens. Cosensitization to all 3 nonhomologous cashew nut allergens has been observed. We hypothesize that this might be due to IgE cross-reactivity.

Methods: IgE cross-inhibitions were performed with Ana o 1-3 using serum samples from cashew nut-allergic patients. The related hazelnut allergens Cor a 11, 9, and 14 were used as controls. For comparison, IgE cross-reactivity between the hazelnut allergens was investigated using serum samples from hazelnut-allergic patients.

Results: The median percentages of cross-inhibition between Ana o 1, 2, and 3 were 84%-99%. In comparison, the median cross-inhibition values between hazelnut allergens were 33%-62%. The IC₅₀ values revealed the highest IgE affinity to be to Ana o 3 and Cor a 14. Hazelnut legumin Cor a 9 inhibited IgE binding to Ana o 1, 2, and 3, with median percentages of 75%, 56%, and 48%, respectively. No cross-reactivity was observed between allergenic vicilins or between 2S albumins from cashew and hazelnut. Potentially cross-reactive peptides of Ana o 3 identified *in silico* overlapped with previously reported IgE epitopes of all 3 allergens.

Conclusion: IgE with high affinity to Ana o 3 that cross-reacts with the other 2 major nonhomologous cashew nut allergens might be responsible for the high allergenic potency of cashew nut. These cross-reactive IgE types comprise the major fraction of specific IgE in cashew-allergic patients and might be responsible for cross-reactivity between unrelated tree nuts.

Key words: Cashew nut allergy. IgE cross-reactivity. Food allergens. Food allergy. Hazelnut allergy. Hazelnut allergens. Tree nut allergy.

■ Resumen

Antecedentes: Los anacardos suelen causar fuertes reacciones alérgicas, incluso mayores a las del cacahuete. Los principales alérgenos del anacardo son: Ana o 1 (vicilina), Ana o 2 (legumina) y Ana o 3 (albúmina 2S). Se ha observado cosensibilización a los tres alérgenos no homólogos de anacardos. Nuestra hipótesis es que esto podría deberse a la reactividad cruzada de IgE.

Métodos: Se realizaron inhibiciones cruzadas de IgE con Ana o 1-3 utilizando sueros de pacientes alérgicos al anacardo. Los alérgenos de avellana relacionados Cor a 11, 9 y 14 se usaron como controles. A modo de comparación, se investigó la reactividad cruzada de IgE entre los alérgenos de las avellanas utilizando sueros de pacientes alérgicos a las avellanas.

Resultados: Las medianas del porcentaje de inhibición cruzada entre Ana o 1-3 fueron del 84-99%. En comparación, las medianas de las inhibiciones cruzadas entre alérgenos de avellana fueron del 33 al 62 %. Los valores de IC₅₀ revelaron mayor afinidad de la IgE por Ana o 3 y Cor a 14. La legumina de avellana (Cor a 9) inhibió la unión de IgE a Ana o 1, 2 y 3 con medianas de 75 %, 56 % y 48%, respectivamente. No se observó reactividad cruzada entre las vicilinas o entre las albúminas 2S de anacardo y avellana. *In silico* se identificaron péptidos que potencialmente eran responsables de la reactividad cruzada de Ana o 3 superpuestos con epítomos IgE previamente identificados de los tres alérgenos.

Conclusión: La IgE con alta afinidad por Ana o 3, que reacciona de forma cruzada con los otros dos alérgenos principales no homólogos del anacardo, podría ser responsable de la alta potencia alérgica del anacardo. Estas IgE de reactividad cruzada comprenden la fracción principal de IgE específica en pacientes alérgicos al anacardo y podrían ser responsables de la reactividad cruzada entre frutos secos no relacionados.

Palabras clave: Alergia al anacardo. Reactividad cruzada de IgE. Alérgenos alimentarios. Alergia a alimentos. Alergia a las avellanas. Alérgenos de las avellanas. Alergia a los frutos secos.

Summary box

• What do we know about this topic?

Reactions induced by cashew nuts are recognized as a major cause of food allergy-induced anaphylaxis. Sensitization to the cashew nut allergens Ana o 1 (vicilin), Ana o 2 (legumin), and Ana o 3 (2S albumin) was each individually predictive of a clinical reaction.

• How does this study impact our current understanding and/or clinical management of this topic?

Patients allergic to cashew nut are primarily sensitized to 2S albumin Ana o 3, which induces highly cross-reactive IgEs that also recognize Ana o 1 and Ana o 3 and contribute to cross-reactivity between cashew and hazelnut allergens.

Introduction

Peanut and tree nuts such as cashew nut, hazelnut, and walnut are sources of potent allergenic proteins. The reactions induced by cashew nut and peanut can be particularly severe [1-4] and are recognized as leading causes of food allergy-induced anaphylaxis, mainly among children and young adults [5-7]. Furthermore, the incidence of severe clinical reactions to cashew nut has been reported to be higher than to peanut [3,4,8,9]. It seems that the increased reports of cashew nut allergy have paralleled the increasing consumption of this nut over the last 3 decades [5]. Studies on threshold dose distributions have shown that ingestion of an amount as small as 0.9 mg of cashew nut protein [3,10,11], or even only skin and mucosal contact [12,13], may cause severe clinical reactions. The potency of cashew nut allergens, therefore, is high, and equivalent to or even higher than that of peanut [14]. Notably, the allergic reactions were triggered in more than three-quarters of cases at the first known exposure [10,12,15,16]. Similar clinical observations in peanut allergy led to the hypothesis that early environmental exposure to peanut (through impaired skin or the airway) accounts for early sensitization [17].

Cashew nut contains about 44% lipids and 19% proteins [18], and most of the protein content is made up by seed storage proteins comprising the major cashew allergens Ana o 1, 2, and 3 [19]. Ana o 1 (vicilin) [20] and Ana o 2 (legumin) [21] belong to the cupin superfamily, and Ana o 3 belongs to the 2S albumin protein family [22]. Studies in populations from various geographical regions performed with the only commercially available cashew nut allergen, Ana o 3, showed that sensitization to this allergen is highly predictive of clinical reactivity in cashew nut-sensitized patients [23-25]. However, the outcomes of a large study including 173 patients with suspected cashew nut allergy showed that all 3 components (Ana o 1, Ana o 2, and Ana o 3) were each individually predictive of failure in oral food challenge [26]. Interestingly, higher median values of specific IgE to the cashew nut components Ana o 2 and 3 than to whole cashew nut extract have been recorded; the same is also true of peanut [27].

In our previous study, we found unexpected IgE cross-reactivity between the 3 nonhomologous peanut allergens Ara h 1-3 [28]. A later study analyzing antibodies produced by B cells from peanut-allergic patients confirmed the presence of IgEs with high affinity and cross-reactivity to the unrelated peanut allergens [29]. Meanwhile, it became evident that highly similar Ara h 2-specific IgE sequences are shared

between peanut-allergic patients [30,31], indicating that common immunoglobulin rearrangements may contribute to pathogenesis and that peanut allergens are recognized in a similar manner.

Given the high clinical relevance of cashew nut allergy and the above-described similarities to peanut allergy, we hypothesized that the high allergenic potency of cashew nut is due to IgE cross-reactivity between the 3 individual cashew nut allergens. To test our hypothesis, we performed IgE cross-inhibitions with cashew nut allergens Ana o 1-3 using well characterized serum samples from cashew-allergic patients. For comparison, IgE cross-reactivity between the 3 hazelnut allergens was assessed using serum samples from hazelnut-allergic patients.

Methods

Patient Serum Samples

In this study, serum samples from 10 cashew nut-allergic and 10 hazelnut-allergic patients were used (Table 1). The samples were collected from patients with a convincing history of cashew nut or hazelnut allergy or a positive result in a double-blind, placebo-controlled food challenge (DBPCFC) with cashew nut or hazelnut plus sIgE ≥ 0.35 kU/L. DBPCFCs were performed at the Sean N. Parker Center for Allergy and Asthma Research at Stanford University using a standardized methodology according to validated guidelines [32,33] and as previously described [34]. Four samples from atopic patients with no history of food allergy and negative sIgE were used as a negative control group. Total IgE levels were measured using ALEX (Macro Array Diagnostics GmbH) and allergen-specific IgE levels obtained by quantitative IgE ELISA [35]. The use of clinical data and serum samples for this study was approved by the local ethics committees (Stanford Institutional Review Board, the Ethics Committee of the Medical University of Vienna, and the Ethics Committee of the Medical University of Graz), and signed informed consent was obtained from all patients.

Purification and Identification of Cashew Nut and Hazelnut Allergens

Purification and identification of cashew nut (Ana o 1-3) and hazelnut allergens (Cor a 9, 11, and 14), as well as of recombinant Ana o 3 and Cor a 14, is described in Methods and Figure S1A in the Supplement.

Table 1. Cashew Nut Extract and Allergens (Ana o 1, 2, 3) of 10 Cashew Nut–Allergic Patients Used for the Study: Clinical Characteristics and sIgE Levels.

Patient no.	Age, y	Sex	Allergy confirmed by	Cashew nut–related symptoms	Other nut allergies	Total IgE, kU/L	qELISA sIgE, kU/L							
							Cashew nut	Ana o 1	Ana o 2	Ana o 3	Hazel-nut	Cor a 11	Cor a 9	Cor a 14
CA1	4	M	Positive history	Anaphylaxis	None	360	>100	>100	>100	>100	2.34	<0.35	1.54	6.41
CA2	6	F	Positive history	Urticaria, vomiting	Hazelnut	1324	>100	>100	>100	>100	>100	13.91	93.54	>100
CA3	11	F	DBPCFC	Wheezing	Peanut, almond, hazelnut, walnut	956	>100	>100	>100	>100	83.42	18.83	66.14	2.83
CA4	6	M	DBPCFC	Wheezing	Peanut, almond, hazelnut, walnut	652	>100	>100	>100	>100	24.68	<0.35	16.73	15.77
CA5	8	M	DBPCFC	Cough	Peanut, almond, hazelnut, walnut	1355	>100	>100	>100	99.23	4.40	<0.35	2.87	<0.35
CA6	5	M	Positive history	Urticaria	Peanut, hazelnut	317	49.15	56.30	65.74	51.74	2.05	<0.35	<0.35	1.66
CA7	4	M	Positive history	Anaphylaxis	Peanut	87	36.63	29.43	48.77	27.04	1.61	<0.35	1.45	<0.35
CA8	5	F	Positive history	Vomiting, angioedema, eczema	Hazelnut, walnut, pistachio, macadamia	374	>100	>100	>100	>100	>100	17.32	27.17	>100
CA9	4	M	Positive history	Rash, vomiting, angioedema	None	227	>100	52.07	>100	37.42	2.93	11.92	0.83	3.92
CA10	3	M	Positive history	Rash, urticaria, cough	None	ND	62.93	>100	91.73	>100	<0.35	<0.35	<0.35	1.83
Median						374	128.54	150.94	170.38	154.82	4.40	15.62	9.80	5.17
Range						87-1355	37-850	29-837	49-1199	27-624	2-155	12-19	1-94	2-141

Abbreviations: DBPCFC, double-blind, placebo-controlled food challenge; qELISA, quantitative enzyme-linked immunosorbent assay.

Dose-dependent IgE Inhibition ELISA Assay

Dose-dependent IgE inhibition ELISA assays with the 3 cashew nut allergens and hazelnut allergens were performed as described in the Supplement. To evaluate the nature of the epitopes involved in cross-reactivity, Ana o 2, Ana o 3, and Cor a 9 were reduced and alkylated as described in the Supplement.

IgE Immunoblot and Inhibition

IgE binding and cross-reactivity were analyzed using immunoblotting and inhibition with a human-derived monoclonal anti–Ana o 3 IgE antibody (Indoor Biotechnologies Ltd), as well as a pool of sera from cashew-allergic patients as described in the Supplement.

Identification of Potentially Cross-reactive Peptides

Potentially cross-reactive peptides were identified by comparing the mature protein sequence of Ana o 3 with the

mature sequences of Ana o 1 and 2 and Cor a 9. The mature sequence of Cor a 14 was compared with that of Cor a 9 and 11 and Ana o 3. The sequence comparisons were performed as previously described for the peanut allergens Ara h 1-3 [28] with modifications (see Supplement).

Results

IgE Coreactivity to Ana o 1-3 is High in Cashew Nut–Allergic Patients

Analysis of 10 serum samples from cashew nut–allergic patients (Table 1) showed that IgE coreacted to Ana o 1, 2, and 3, with statistically significant Pearson correlation coefficients of between 0.87 and 0.98 ($P<.001$ and $P<.0001$) (Figure 1A). As a control group, 10 samples from hazelnut-allergic patients were tested for IgE reactivity to hazelnut extract and the 3 hazelnut allergens (Table 2 and Figure 1B). All patients were sensitized to Cor a 9, and 9 had sIgE to Cor a 14. Five patients were

Table 2. Hazelnut Extract and Allergens (Cor a 9, 11, 14) of 10 Hazelnut-Allergic Patients Used for the Study: Clinical Characteristics and sIgE Levels.

Patient no.	Age, y	Sex	Allergy confirmed by	Hazelnut-related symptoms	Other nut allergies	Total IgE, kU/L	qELISA sIgE, kU/L				
							Hazelnut	Cor a 11	Cor a 9	Cor a 14	Ana o 3
HA1	33	F	Positive history	Anaphylaxis	All tree nuts (not almond)	1982	38.62	7.53	20.59	24.9	16.63
HA2	27	F	DBPCFC	Anaphylaxis	Peanut, walnut	1470	24.00	<0.35	7.90	9.56	24.10
HA3	7	M	Positive history	Dyspnea	Peanut, walnut	2119	75.37	6.63	59.42	14.18	59.92
HA4	2	M	Positive history	Angioedema	None	68	28.85	<0.35	3.00	32.11	<0.35
HA5	34	F	Positive history	Diarrhea, vomiting, angioedema, rash	Peanut	864	20.96	2.95	4.88	15.18	<0.35
HA6	1	M	Positive history	Angioedema, dyspnea, stridor	Peanut, walnut, almond	ND	56.58	<0.35	3.50	92.91	ND
HA7	27	F	Positive history	Angioedema, dyspnea	Almond, walnut	ND	8.93	<0.35	2.7	4.93	<0.35
HA8	10	F	DBPCFC	Vomiting	Almond, cashew nut, walnut	1249	87.67	13.05	23.83	84.26	31.42
HA9	11	F	DBPCFC	Vomiting	Peanut, almond, cashew nut, walnut	956	>100	32.29	>100	38.58	29.26
HA10	26	F	Positive history	Angioedema	Peanut, walnut, almond, pecan	729	32.85	<0.35	5.41	<0.35	<0.35
Median						1103	35.74	7.53	5.41	24.90	29.26
Range						68-2119	9-173	3-32	3-130	5-93	17-60

Abbreviations: DBPCFC, double-blind, placebo-controlled food challenge; qELISA, quantitative enzyme-linked immunosorbent assay; ND, not determined.

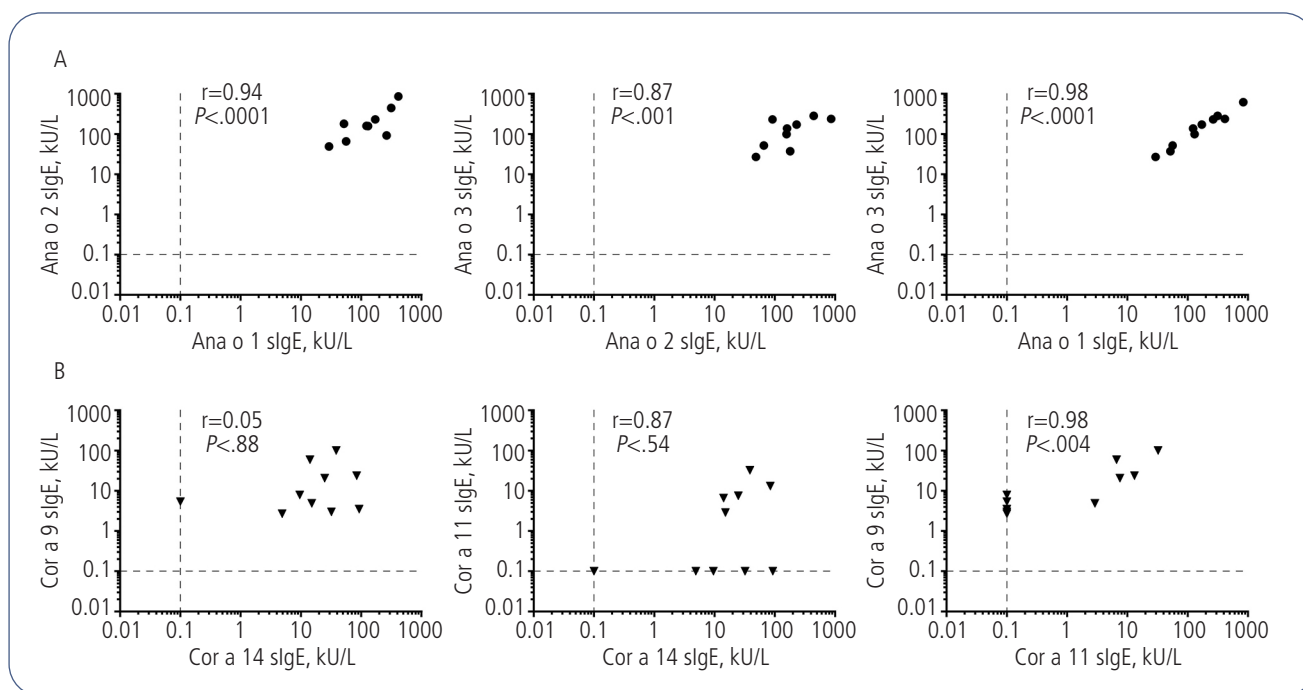


Figure 1. Correlation between specific IgE to cashew nut and hazelnut allergens using sera from (A) cashew nut-allergic and (B) hazelnut-allergic patients. Negative values were set to 0.01. Pearson correlation coefficients (r values), and P values were calculated using GraphPad Prism.

cosensitized to Cor a 9, 11, and 14. A statistically significant Pearson correlation coefficient of 0.91 ($P=.004$) was found only for the correlation between sIgE to Cor a 9 and sIgE to Cor a 11. The coefficient of the correlation between IgE to Cor a 11 and IgE to Cor a 14 was 0.24; the coefficient of the correlation between Cor a 14 and Cor a 9 was 0.05.

The 3 Nonhomologous Cashew Nut Allergens Are Highly Cross-reactive Between Each Other

To test the cross-reactivity between the 3 cashew nut allergens, dose-dependent IgE inhibition was performed

with 10 individual serum samples from cashew nut-allergic patients (Figures 2 and 3 and Figure S2 in the Supplement). Preincubation of the samples with Ana o 3 at a concentration of 10 $\mu\text{g/mL}$ reduced IgE binding to Ana o 1 by a median of 99% (range, 87%-100%) and to Ana o 2 by a median of 90% (range, 51%-100%). Similarly, Ana o 2 inhibited IgE binding to Ana o 1 and 3 by 96%-100% (median, 99%) and 82%-100% (median, 94%) (Figure 2A). Ana o 1 inhibited IgE binding to Ana o 2 by 54%-96% (median, 84%) and to Ana o 3 by 64%-100% (median, 86%).

Among the 3 allergens, Ana o 3 was the most potent in reducing IgE binding to Ana o 1, 2, and itself, with median

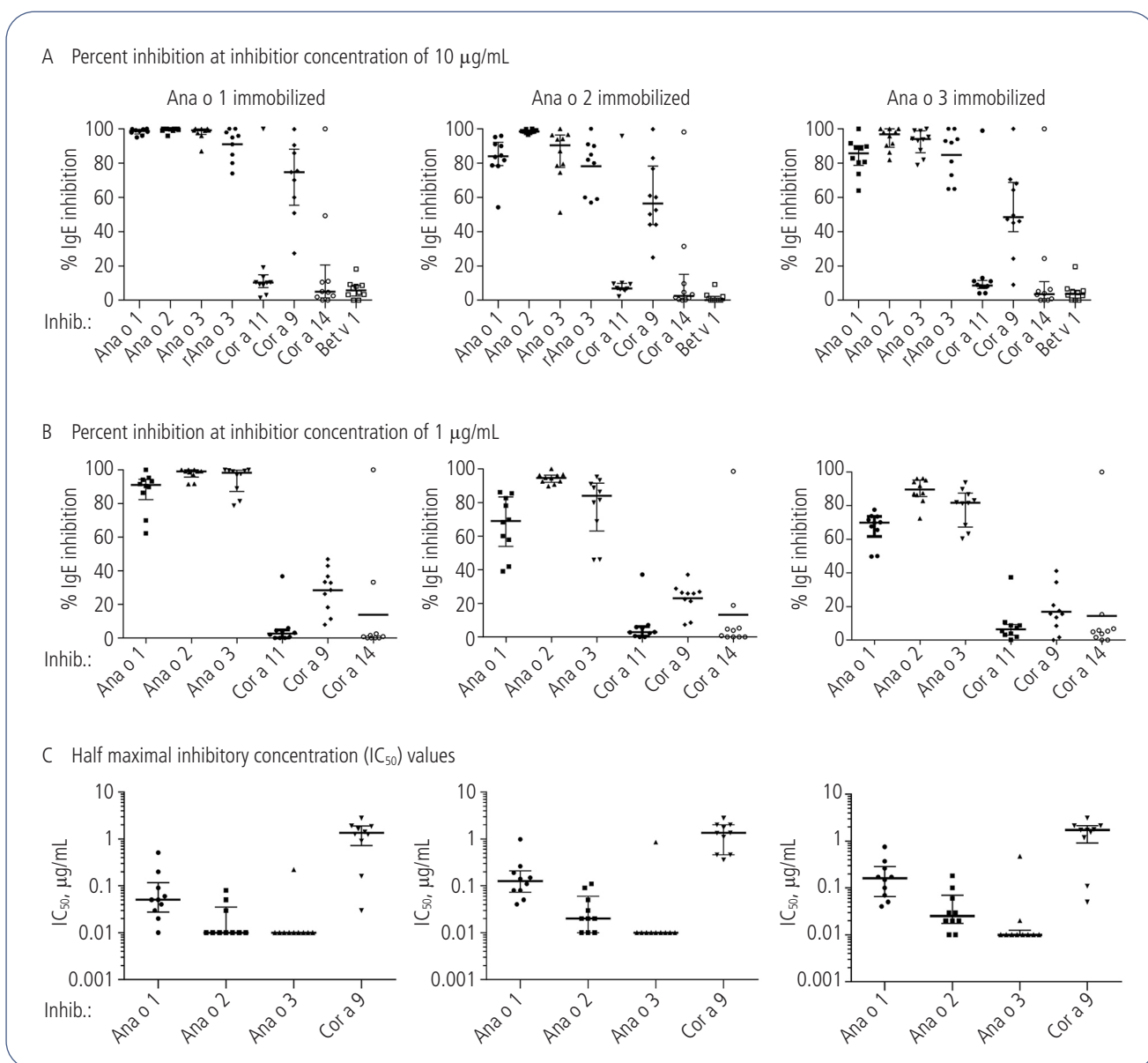
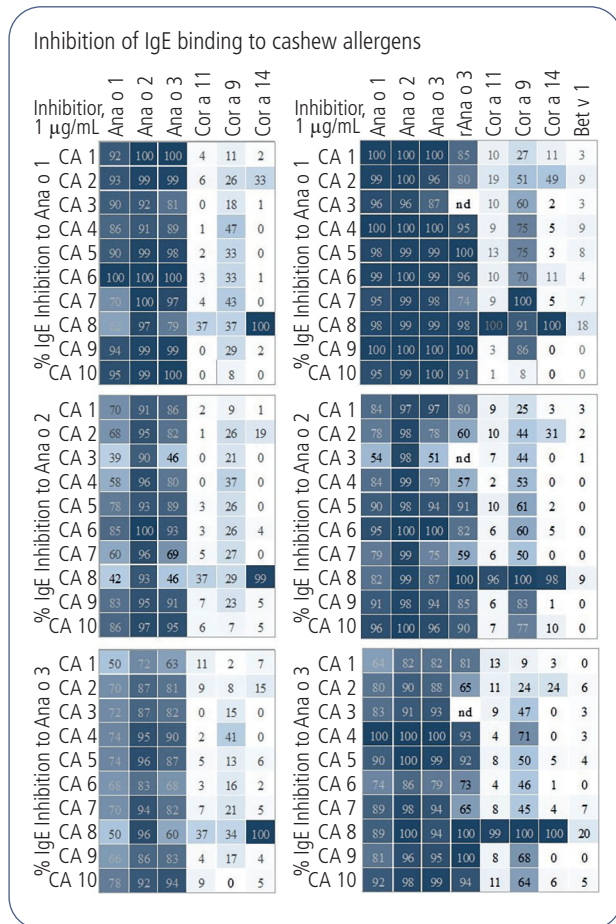


Figure 2. IgE cross-reactivity between cashew nut allergens Ana o 1, Ana o 2, and Ana o 3 tested using an IgE ELISA inhibition assay. Cross-inhibition was tested in a dose-dependent manner with serum samples from patients with cashew nut allergy ($n=10$). A, Values are expressed as maximum inhibition at 10 $\mu\text{g/mL}$. B, Values represent inhibition at 1 $\mu\text{g/mL}$ of inhibitor. Recombinant Ana o 3 and Bet v 1 were used for inhibitions as additional control allergens only at a concentration of 10 $\mu\text{g/mL}$. C, Inhibitor concentrations for half-maximal inhibition (IC_{50}) with interquartile ranges. Inhib. indicates inhibitor. Median values (solid lines) with interquartile ranges are shown.



cross-reacted to Ana o 1 and 2. Strong signals to both Ana o 1 at about 50 kDa and Ana o 2 at about 55 kDa were obtained by analysis under nonreducing conditions and under reducing conditions, and binding to the 30-kDa acidic and 20-kDa basic Ana o 2 subunits was visible.

Interestingly, the Ana o 3-specific antibody cross-reacted to the hazelnut legumin Cor a 9, but not to the hazelnut 2S albumin Cor a 14 or vicilin Cor a 11. The binding of the antibody to all 4 allergens was inhibited by rAna o 3, thus confirming the specificity of the antibody.

Identification of Potentially Cross-reactive Peptides

Sequence alignments using EMBOSS Needle revealed significant identities only between homologous allergens from cashew nut and hazelnut (Table S1).

Using a search for similarly short peptides, a comparison of Ana o 1 and 2 with Ana o 3 yielded 2 regions on the Ana o 3 sequence that matched similar peptides on Ana o 1 or 2 (Figure S5). Ana o 3 amino acid residues 40-49 located on the surface exposed 4 Ana o 1 peptides matched to N-terminal helices 1 and 2, whereas the loop region (residues 101-108)

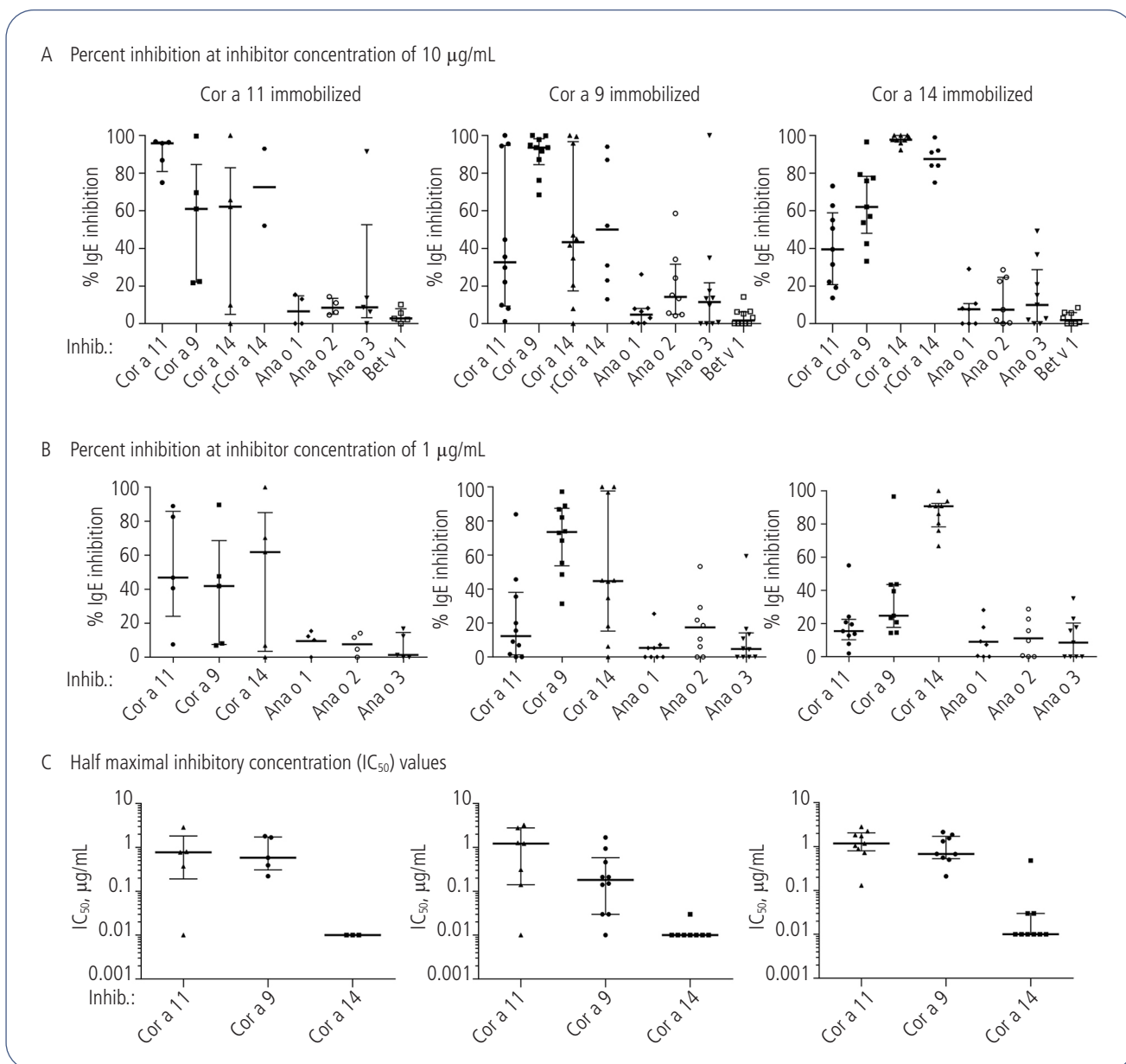


Figure 4. IgE cross-reactivity between hazelnut allergens Cor a 11, Cor a 9, and Cor a 14 tested using an IgE ELISA inhibition assay. Cross-inhibitions were tested in a dose-dependent manner with serum samples from patients with hazelnut allergy ($n=10$). A, Values are expressed as maximum inhibition at 10 $\mu\text{g/mL}$. B, Values represent inhibitions at 1 $\mu\text{g/mL}$ of inhibitor. Recombinant Cor a 14 and Bet v 1 were used for inhibitions as additional control allergens only at a concentration of 10 $\mu\text{g/mL}$. C, IC_{50} with interquartile ranges. Owing to negligible inhibition of hazelnut allergens by Ana o 3, IC_{50} values were excluded from the graph. Inhib. indicates inhibitor. Median values (solid lines) with interquartile ranges are shown.

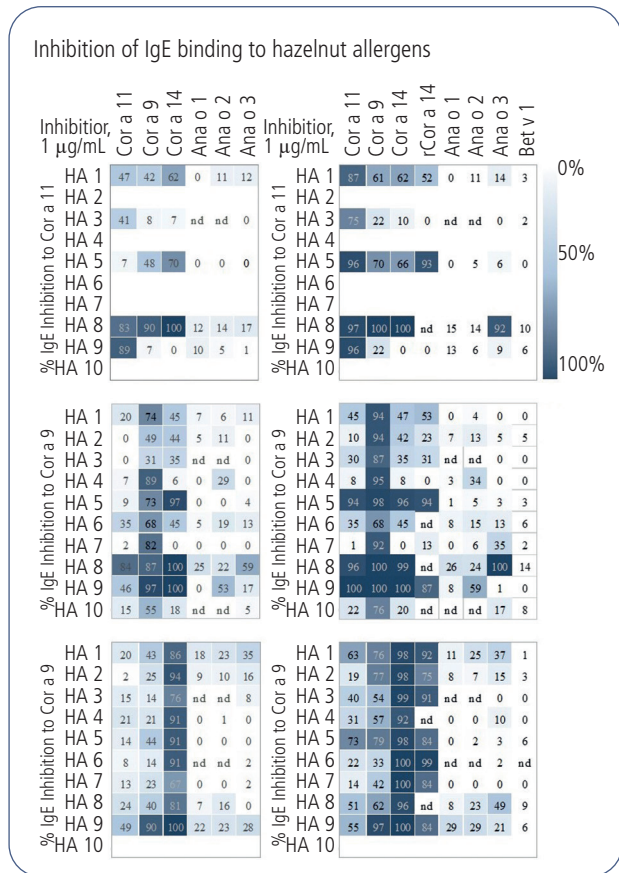


Figure 5. Summary heat map of percent IgE binding inhibition to hazelnut allergens at inhibitor concentrations of 1 µg/mL and 10 µg/mL.

connecting helices 5 and 6 was similar to 1 peptide of Ana o 2. All matching peptides from Ana o 3 were located on the surface and have previously been identified as important IgE-binding regions (Figure S5) [22]. No peptides sharing significant sequence similarity were identified after comparison of Ana o 3 and its hazelnut homolog Cor a 9. Using the same method, only 1 peptide of Cor a 14 matched the sequence of Cor a 11, whereas no peptides matched the sequence of Cor a 9.

Discussion

Cashew nut has been reported to cause strong allergic reactions, even exceeding those observed for peanut, suggesting that its allergens are highly potent. However, there are few data to explain the nature of cashew nut allergenicity and subsequently inform studies to identify targets for treatment of cashew nut allergy.

Our findings suggest that the strong IgE coreactivity to the 3 unrelated major cashew nut allergens, Ana o 1-3 observed in our and in a previous study [26] is due to high cross-reactivity between them. As occurs in peanut, the high percentages of IgE cross-inhibition between the 3 allergens indicate that cross-reactive IgE antibodies comprise the major portion of IgE specific to these allergens and might contribute

to the high specific IgE values for each of the 3 allergens. The affinity of these IgE antibodies was highest for the 2S albumin, Ana o 3, potentially supporting the finding that Ana o 3 is a predictive marker of clinical reactivity to cashew nut [23-25]. Similarly, previous studies showing cross-reactivity of the 3 nonhomologous peanut allergens [28] and evidence of cross-reactive IgE antibodies [29] reported the highest affinity of cross-reactive IgE to the 2S albumin Ara h 2, which was confirmed as a predictor of clinical reactivity to peanut [36,37]. Interestingly, in vivo studies [38,39] and in vitro studies [40,41] on peanut allergens showed that treatment with a single allergen or a single allergen-specific antibody induces protection against the peanut extract consisting of multiple allergens. Based on the data recorded for peanut allergens and the present results, we could hypothesize that 1 cashew allergen might be sufficient for successful immunotherapy in cashew-allergic patients.

We also demonstrated IgE cross-reactivity between unrelated allergens from the same nut in hazelnut, albeit to a lesser extent than in cashew nut and peanut. Cross-reactivity was only high in patients cosensitized to all 3 hazelnut allergens and appears not to be associated with sensitization to cashew allergens. Cosensitization and cross-reactivity were strongest between Cor a 9 and 14. However, similar to peanut and cashew nut, the highest IgE affinity was seen for the 2S albumin Cor a 14. For hazelnut, it was shown that sIgE to Cor a 9 had a diagnostic value comparable to that of sIgE to Cor a 14 and that sensitization to both allergens is associated with a more severe hazelnut-allergic phenotype [42,43]. Nevertheless, Cor a 14 was shown to be the most potent hazelnut allergen in basophil activation assays [43].

It is a commonly held view that cross-reactivity relies on highly similar primary and tertiary structures between allergens and is hence generally observed between members of the same protein family. Consequently, frequent cosensitization of peanut- and/or tree nut-allergic individuals to multiple nuts and seeds and their extensive in vitro IgE cross-reactivity have been interpreted by cross-reactive epitopes present in homologous allergens from the vicilin, legumin, and 2S albumin protein families [44-46]. Among cashew nut-allergic patients, cosensitization was observed to pistachio, peanut, hazelnut, and almond [3,8,16,47]. However, in most cases, the identity of the cross-reactive allergens was not investigated. Strong clinically relevant cosensitization was found only between cashew nut and the botanically related pistachio [25,48], which could be explained by the high sequence identities ($\geq 70\%$) between their homologous allergens.

As a control, we used the 3 homologous allergens from hazelnut for inhibition of IgE binding to cashew nut allergens in the cashew nut-allergic group and the 3 cashew nut allergens in the hazelnut-allergic group. We did not find cross-reactivity between cashew and hazelnut 2S albumins and vicilins. Interestingly, at the highest inhibitor concentration, the hazelnut legumin, Cor a 9, could moderately inhibit not only IgE binding to the cashew nut legumin, Ana o 2, which is not unexpected (sequence identity, 54%), but also to the nonhomologous cashew nut vicilin, Ana o 1, and the 2S albumin, Ana o 3. Consequently, binding of cross-reactive IgE to Cor a 9 might be responsible for the previously observed cross-reactivity between cashew nut and hazelnut, where

hazelnut extract was a strong inhibitor of cashew nut sIgE, while cashew nut extract was less able to inhibit IgE binding to hazelnut extract [47,49]. The finding is also in line with our results showing that low or no inhibition of IgE binding to hazelnut allergens was achieved by preincubating serum samples from hazelnut-allergic patients with individual cashew nut allergens. The differences between cashew- and hazelnut-allergic patients may be explained by different degrees of cross-reactivity of IgE, depending on the sensitizing allergen. Cashew-allergic patients are primarily sensitized by Ana o 3, which induces highly cross-reactive IgE that also recognizes Cor a 9. In contrast, hazelnut-allergic patients, who are primarily sensitized to Cor a 9, develop IgE with low cross-reactivity. Thus, our study highlights the importance of using purified individual allergens instead of total protein extracts to correctly assess cross-reactivity and commonly observed cosensitization to diverse tree nuts.

Using recombinant Ana o 3 and Cor a 14 allergens and an Ana o 3-specific monoclonal antibody, we showed that the high cross-reactivity observed between the unrelated cashew nut and hazelnut allergens is not due to contamination of the individual allergen preparation by the other allergens. Recombinant Ana o 3 and rCor a 14 are therefore not contaminated with other nut proteins, with no significant differences in inhibition of IgE compared with their natural counterparts. Furthermore, neither sera from patients with cashew allergy nor the Ana o 3-specific monoclonal antibody detected other allergens in the lanes containing each purified allergen. Besides, the Ana o 3-specific antibody isolated from a cashew nut-allergic patient showed the same pattern of cross-reactivity as IgE in the sera of cashew nut-allergic patients in our study.

Our present and previous findings [28] demonstrating cross-reactivity between unrelated seed storage proteins further emphasize that protein sequence identity alone cannot accurately predict cross-reactivity. By searching for short matching peptides, we previously identified cross-reactive IgE epitopes of the unrelated peanut allergens Ara h 1-3 [28]. Using a similar approach, we identified potential epitopes on Ana o 3 with sequences similar to those of peptides from Ana o 1 and 2 that could be recognized by cross-reactive antibodies. This assumption is supported by the fact that the peptides identified overlap with peptides that have previously been reported to bind to IgE from most cashew nut-allergic individuals [20-22]. The adjacent location of the surface-exposed patches presenting the 2 Ana o 3 peptides identified in the present study indicates that they could be part of a conformational epitope. Indeed, our experimental data showed that the native structure of Ana o 3 and, more particularly, that of Ana o 2 and Cor a 9, are substantial for binding of the cross-reactive IgE, since their disruption strongly reduced or eliminated the inhibitory potency of the 3 allergens. Various studies on the 3 cashew nut allergens have shown that their epitopes are conformational and depend on the 3-dimensional structure of the protein [50].

In summary, our results indicate the presence of IgE with high affinity to Ana o 3 that cross-reacts with the other 2 unrelated major cashew nut allergens. Although this needs to be demonstrated, it is highly possible that such cross-reactive antibodies with high affinity are particularly potent at inducing allergen aggregation and IgE-mediated mast cell activation and

might therefore be responsible for the high allergenic potency of cashew nut.

This substantial new information on cross-reactivity between unrelated cashew nut and hazelnut allergens may have important consequences for diagnosis and immunotherapy, not only of cashew nut allergy, but also of other tree nut allergies.

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Conflicts of Interest

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The remaining authors declare that they have no conflicts of interest.

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■ Merima Bublin

Institute of Pathophysiology and Allergy Research
Center of Pathophysiology, Infectiology and Immunology
Medical University of Vienna
Währinger Gürtel 18-20, 1090 Vienna, Austria
E-mail: merima.bublin@meduniwien.ac.at