Glycosylation enhances allergenic activity of major bee venom allergen Api m 1 by adding IgE epitopes

To the Editor:

Phospholipase A_2 (*Apis mellifera* 1 [Api m 1]), the major bee venom allergen, is a glycoprotein that can bind IgE antibodies of patients with allergy via protein and/or carbohydrate epitopes.[1] It has been suggested that the protein epitopes of Api m 1 are conformation dependant[2]; recently, however, IgE-reactive sequential epitopes have also been described.[3] Carbohydrates present on allergens from plants, insects, mites, molds, and parasites have been considered to be highly cross-reactive epitopes (ie, cross-reactive carbohydrate determinants [CCDs]) with low capacity to induce basophil or mast cell degranulation and hence with low allergenic activity.[4] Accordingly, the presence of CCDs on allergens may cause misleading IgE test results. One important example is that patients with grass pollen allergy who have IgE reactivity against *Phleum pratense* 4 (Phl p 4)-derived CCDs showed IgE reactivity to natural CCD-containing Api m 1 even though they were not sensitized against bee venom.[1] To avoid such false IgE test results, nonglycosylated Api m 1 has been introduced for serologic diagnosis of bee venom sensitivity.[5] Conversely, IgE antibodies to the carbohydrate galactose-α-1,3-galactose (α-GAL) can trigger allergic reactions.[6]

The influence of glycosylation and enzymatic activity on allergenic activity of the major bee venom allergen Api m 1 is a controversial issue. Certain studies suggest similar allergenic activity of natural glycosylated and recombinant nonglycosylated Api m 1,[2] whereas enzymatic activity seems to be important for allergenic activity.[7] To investigate the role of bee-derived glycosylation on structural fold, enzymatic activity, and allergenic activity of Api m 1, we compared purified natural Api m 1 (nApi m 1) with nonglycosylated recombinant Api m 1 (rApi m 1) expressed in insect cells. Recombinant nonglycosylated Api m 1 with a mutated N-glycosylation site was expressed in *Spodoptera frugiperda* (Sf9) insect cells (see the Methods in this article's Online Repository at www.jacionline.org); natural glycosylated Api m 1 was obtained from Sigma-Aldrich (Vienna, Austria). The Coomassie blue–stained SDS-PAGE and corresponding Western blot showed that rApi m 1 was pure and migrated as a 15 kDa band, whereas nApi m 1 appeared in the form of 2 bands, 1 prominent at 16 to 17 kDa and 1 at 14 kDa. According to staining with α-(1,3)-fucose–specific *Aleuria aurantia* lectin, the 16 to 17 kDa nApi m 1 band was glycosylated, whereas rApi m 1 lacked glycosylation (see Fig E1, *A* and *B* and the Methods section in this article's Online Repository at www.jacionline.org). Thus, Fig E1 shows that nApi m 1 consists of a glycosylated fraction and a nonglycosylated fraction. The thin band at 14 kDa corresponds to the nonglycosylated version and the prominent band at 16 to 17 kDa represents the glycosylated version. rApi m 1, which is not glycosylated, gives a single band migrating at 15 kDa.

Both nApi m 1 and rApi m 1 showed specific IgE reactivity by IgE immunoblotting (Fig E1, *C*); however, the levels of IgE specific for nApi m 1 were 1.2- to 2.71-fold higher in 8 of 12 patients with bee venom allergy (see Table E1 and the Methods section in this article's Online Repository at www.jacionline.org). By contrast, there was a good correlation between the rApi m 1-specific IgE levels determined by ImmunoCAP and ELISA, although differences were noted for certain sera (Fig E2 and Table E1 in this article's Online Repository at www.jacionline.org). For both assays (ImmunoCAP and ELISA), rApi m 1 is expressed in the insect cell line Sf9 and lacks the N-linked glycosylation site.[5] One explanation for the observed discrepancies may be that the ImmunoCAP contains large amounts of antigen immobilized to its surface, whereas much lower amounts of antigen are coated to the ELISA plates, which may allow IgG antibodies to compete with IgE for binding in the ELISA, resulting in lower IgE signals. Finally, ImmunoCAPs may bind CCD-specific IgE in a nonspecific manner, which may result in higher IgE levels in the ImmunoCAP measurements for sera containing CCD-specific IgE.[8]

Next, we investigated whether there are differences regarding the fold and functional properties of the natural and rApi m 1 protein. However, the analysis of nApi m 1 and rApi m 1 by circular dichroism analysis showed that both proteins were folded and no relevant difference regarding secondary structure contents and molar ellipticities could be detected (Fig 1, *A* and the Methods section in this article's Online Repository). Furthermore, nApi m 1 and rApi m 1 occurred as monomeric proteins in physiologic buffers, as demonstrated by size exclusion chromatography (see Fig E3 and the Methods section in this article's Online Repository at www.jacionline.org). Therefore, the higher IgE reactivity of nApi m 1 cannot be explained by differences in the secondary structure; rather, it seems to be due to the attached CCDs, because most of the tested patients were positive for CCD-specific IgE (see Table E1).

The proteins nApi m 1 and rApi m1, but not a control allergen (ie, the birch pollen allergen *Betula verrucosa* 1), showed comparable phospholipase activity during assessment of enzymatic activity in the first 5 minutes of the 15-minute recording period (Fig 1, *B* and see the Methods section in this article's Online Repository at www.jacionline.org). During the last 10 minutes of measurement, a 20% higher activity of nApi m 1 was found, which may be due to the fact that nApi m 1 and rApi m 1 required different pH conditions (nApi m 1 pH = 7.5; rApi m 1 pH = 6) for storage that could affect phospholipase activity (Fig 1, *B*). Next, we investigated the allergenic activity of nApi m 1 and nonglycosylated rApi m 1 in titrated basophil activation experiments (Fig 2 and see the Methods section in this article's Online Repository). Four of the 5 tested patients (patients 6, 3, 9, and 12) (see Table E1) showed stronger basophil activation with nApi m 1 than with rApi m 1, whereas basophil activation was similar for patient 4, who mounted similar levels of IgE specific to nApi m 1 and rApi m 1 (see Table E1). The stronger basophil activation in these 4 patients was associated with higher IgE levels to nApi m 1 than to rApi m 1 (see Table E1). Interestingly, the difference in allergenic activity for nApi m 1 and rApi m 1 was different from that observed for allergens or allergen derivatives, which differ regarding IgE reactivity on account of differences in structural fold, such as (for example) for folded, insect cell– versus unfolded *Escherichia coli*–expressed Par j 2.[9] In fact, for allergen molecules, which show different IgE reactivity on account of

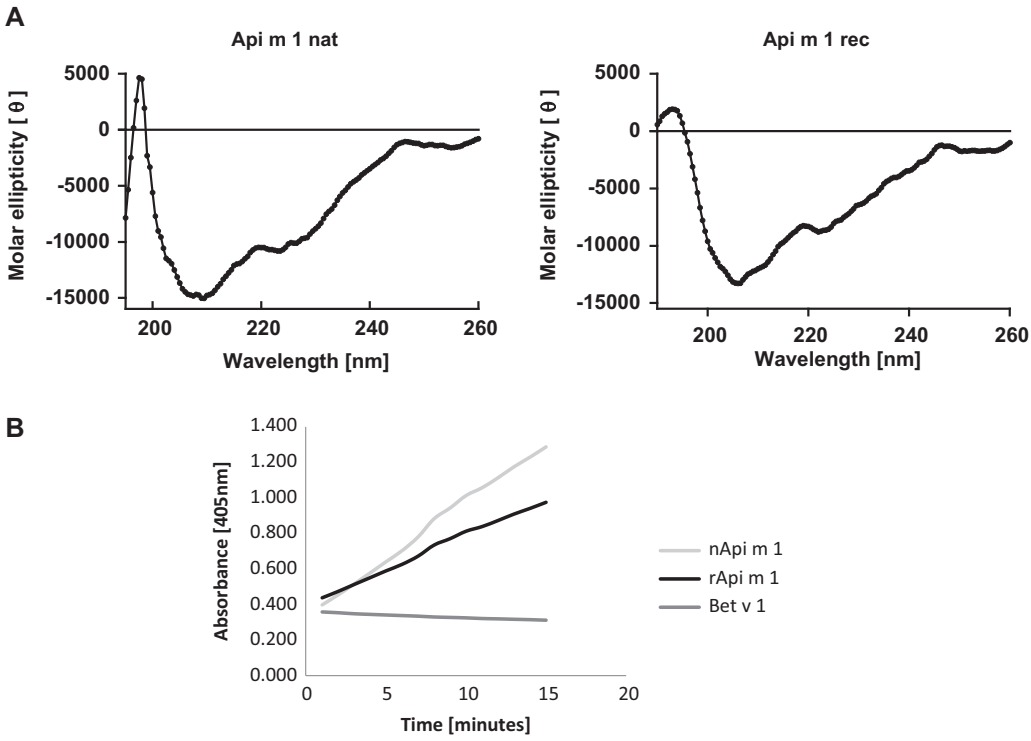

FIG 1. Circular dichroism analysis of nApi m 1 and rApi m 1. **A,** Scans showing molar ellipticity (y-axes) at given wavelengths (x-axes) are presented for nApi m 1 (Api m 1 nat [*left*]) and rApi m 1 (Api m 1 rec [*right*]). **(B)** Phospholipase A_2 activity of nApi m 1 and rApi m 1. Results are expressed as time-dependent increases in absorbance (mOD405nm/min). Mean values of duplicate measurements are shown.

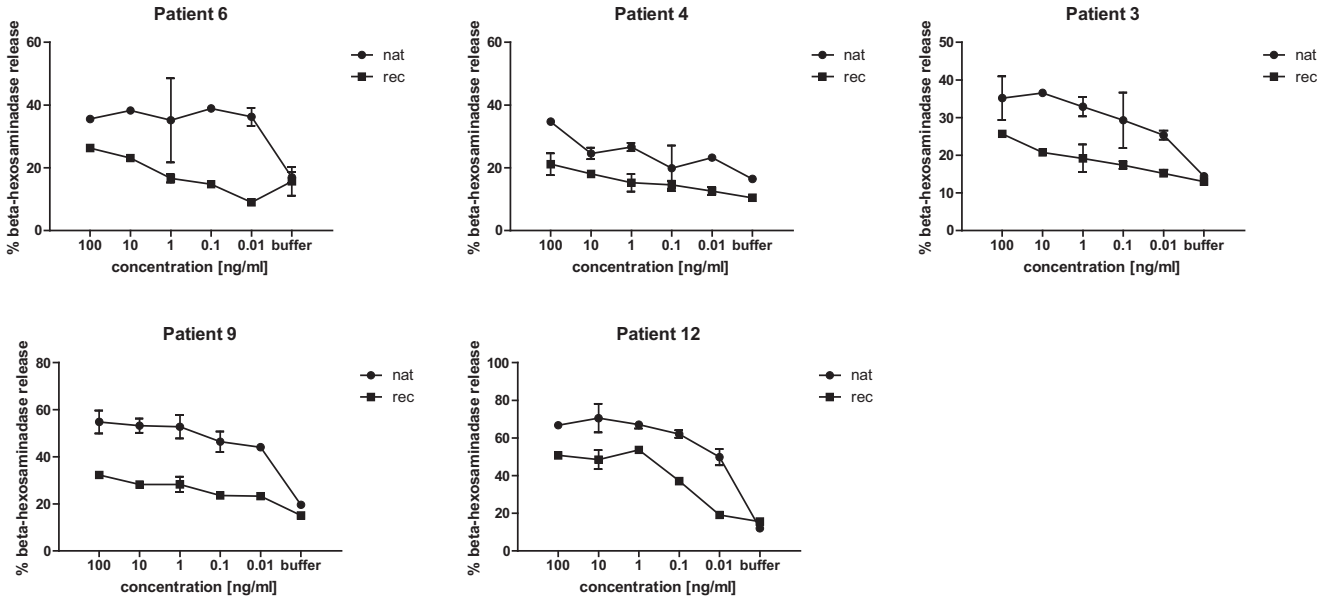

FIG 2. Allergenic activity of nApi m 1 and rApi m 1. Rat basophil leukemia cells were loaded with serum IgE from patients with bee venom allergy (Table E1 [patients 3, 4, 6, 9, and 12]) and incubated with different concentrations of nApi m 1 or rApi m 1 (100, 10, 1, 0.1, and 0.01 ng/mL) (x-axes) or buffer alone. β-hexosaminidase releases are displayed as mean percentages of duplicate determinations displaying the ranges of variance of total β-hexosaminidase release minus buffer on the y-axes.

alterations of their structural fold, a shift of the dose-response curve can be observed in basophil activation; in contrast, nApi m 1 and rApi m 1 showed a comparable dose dependency of basophil activation, but nApi m 1 induced stronger release of ß-hexosaminidase at each of the concentrations (Fig 2). Such a qualitative difference in basophil activation is usually observed when basophils contain more allergen-specific IgE.[E4]

Our study thus demonstrates that structural fold, enzymatic activity, IgE binding capacity, and allergenic activity of Api m 1 do not depend on glycosylation, although glycosylation may increase

IgE reactivity through the additional presence of IgE-reactive CCDs, which in the case of Api m 1 enhances the allergenic activity of the allergen. Our findings are different from what has been described for other glycosylated allergens. For example, natural Lyc e 2 from tomato bound IgE only in the glycosylated form and only the glycosylated Lyc e 2 was able to trigger histamine release.[E5] The nonglycosylated house dust mite allergen Der p 1 had enzymatic activity, was folded correctly, and induced histamine release in the basophil activation test, although no evidence was provided that glycosylation enhances allergenic activity, as we found for Api m 1.[E6] By contrast, Bla g 2, a German cockroach allergen, seems to be an example similar to Api m 1 because it has been shown that removal of sugars resulted in a reduction of IgE binding and allergenic activity, as demonstrated in histamine release experiments with human basophils.[E7-E9]

According to the available data, one can envisage different possibilities as to how glycosylation may affect the allergenic activity of an allergen. Carbohydrate epitopes may mask protein-based IgE epitopes, as has been shown for the grass pollen allergen Phl p 4.[E10] Carbohydrates may improve the fold of an allergen and hence stabilize conformational IgE epitopes, which would enhance allergenic activity.[E11] Finally, carbohydrates may add carbohydrate IgE epitopes in addition to protein-based IgE epitopes. In fact Api m 1 seems to be a first example for the latter case, because glycosylation adds carbohydrate-specific IgE epitopes to the peptide IgE epitopes on an otherwise folded, IgE-reactive, allergenic and enzymatically active, nonglycosylated protein to increase its allergenic activity. This mechanism may be also operative for Bla g 2, where the carbohydrates seem to contribute to IgE antibody binding to the protein within the same epitope.[E7,E9]

This result has important implications for the use of Api m 1 for diagnostic purposes and presumably for therapeutic purposes as well. nApi m 1 seems to be more effective in inducing basophil activation and hence may be more potent in effector cell–based assay and in *in vivo* provocation tests, whereas folded, nonglycosylated insect cell–expressed rApi m 1 may increase the specificity of *in vitro* IgE testing because it avoids misleading test results due to the presence of CCDs.

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REFERENCES

1. Mittermann I, Zidarn M, Silar M, Markovic-Housley Z, Aberer W, Korosec P, et al. Recombinant allergen-based IgE testing to distinguish bee and wasp allergy. J Allergy Clin Immunol 2010;125:1300-7.
2. Müller UR, Dudler T, Schneider T, Crameri R, Fischer H, Skrbic D, et al. Type I skin reactivity to native and recombinant phospholipase A2 from honeybee venom is similar. J Allergy Clin Immunol 1995;96:395-402.
3. Zahirovic A, Koren A, Kopac P, Strukelj B, Korosec P, Lunder M. Identification of bee venom Api m 1 IgE epitopes and characterization of corresponding mimotopes. J Allergy and Clin Immunol 2019;143:791-4.
4. Mari A. IgE to cross-reactive carbohydrate determinants: analysis of the distribution and appraisal of the in vivo and in vitro reactivity. Int Arch Allergy Immunol 2002; 129:286-95.
5. Jakob T, Köhler J, Blank S, Magnusson U, Huss-Marp J, Spillner E, et al. Comparable IgE reactivity to natural and recombinant Api m 1 in cross-reactive carbohydrate determinant-negative patients with bee venom allergy. J Allergy Clin Immunol 2012;130:276-8.
6. Platts-Mills TA, Schuyler AJ, Tripathi A, Commins SP. Anaphylaxis to the carbohydrate side chain alpha-gal. Immunol Allergy Clin North Am 2015;35:247-60.
7. Blank S, Michel Y, Seismann H, Plum M, Greunke K, Grunwald T, et al. Evaluation of different glycoforms of honeybee venom major allergen phospholipase A2 (Api m 1) produced in insect cells. Protein Pept Lett 2011;18:415-22.
8. Hemmer W, Altmann F, Holzweber F, Gruber C, Wantke F, Wöhrl S. ImmunoCAP cellulose displays cross-reactive carbohydrate determinant (CCD) epitopes and can cause false-positive test results in patients with high anti-CCD IgE antibody levels. J Allergy Clin Immunol 2018;141:372-81.
9. Dorofeeva Y, Colombo P, Blanca M, Mari A, Khanferyan R, Valenta R, et al. Expression and characterization of recombinant Par j 1 and Par j 2 resembling the allergenic epitopes of Parietaria judaica pollen. Sci Rep 2019;9:15043.

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CXXC5 variant in an immunodeficient patient with a progressive loss of hematopoietic cells

To the Editor:

Primary immunodeficiency disorders (PIDs) are a group of heterogenous genetic defects that affect the development and/or function of the immune cells. An increasing number of novel gene mutations have been identified in patients with PIDs thanks to the advent and improvement of next-generation sequencing technologies. In the present study, we performed whole exome sequencing on a patient to identify the cause for progressive decline in antibody and blood cell production. The patient had an immunodeficiency starting in early childhood and suffered recurrent otitis media, sinusitis, and an occasional pneumonia starting at approximately 4 years of age. His IgG level was 150 mg/dL (reference range for age 444-1187 mg/dL), and his IgA and IgM levels were also reported to be low. He was diagnosed with hypogammaglobulinemia and began receiving immunoglobulin replacement therapy, but he continued to have recurrent severe sinopulmonary and gastrointestinal infections. At around 13 years of age, when he was first seen by 1 of the authors of this letter (H.R.H.). He had a normal number of B cells, but in the next 20 years, he experienced a progressive loss of all major types of blood cells (Fig 1, *A*). The loss was most severe in the lymphoid compartment. In particular, in the B-cell compartment he had hardly any detectable B cells in the peripheral blood by his early 30s.

His family history showed that his parents and siblings did not have any symptoms associated with immunodeficiency (see

METHODS

Immunologic characterization of glycosylated and nonglycosylated Api m 1

IgE binding of nApi m 1 and rApi m 1 was studied by using sera from clinically well-characterized patients with bee venom allergy by IgE immunoblotting and ELISA, as described.[E1]

Grading of the severity of clinical reactions according to Mueller was available for 11 of the 12 patients (Table E1). IgE specific to bee venom extract (i1) was quantified by ImmunoCAP (Thermo Scientific, Phadia AB, Uppsala, Sweden). Carbohydrate-specific IgE was determined by ELISA by using recombinant glycosylated horse heart myoglobin and CCD and/or bromelain ImmunoCAPs.[E2]

The anonymized analysis of the sera was performed with approval by the ethical committee of the Medical University of Vienna (EK1641/2014). Lectin blots of natural and rApi m 1 were performed as described previously.[E2]

Biophysical and biochemical analysis of nApi m 1 and rApi m 1

nApi m 1 and rApi m 1 were analyzed by size exclusion chromatography using cytochrome C and carbonic anhydrase as molecular weight standards.[E3] Far-UV circular dichroism spectrometry of Api m 1 proteins was performed as previously described.[E2] The enzymatic activity of nApi m 1 and rApi m 1 was assessed with the sPLA2 Assay Kit (Cayman Chemical) (Sanova, Vienna, Austria) according to the recommendations of the manufacturer. Recombinant *Betula verrucosa* 1 was used as a negative control. Results are expressed as time-dependent absorbance increases (mOD405nm/min). Mean values with deviations less than 5% of duplicate measurements are shown. A 1,2-dithio analog of diheptanoyl phosphatidylcholine was used as the substrate and following hydrolysis of the thioester bond at the sn-2 position by phospholipase A free thiols were detected by using 5,5′-dithio-bis-(2-nitrobenzoic acid).

Determination of allergenic activity by basophil activation

The allergenic activity of glycosylated and nonglycosylated Api m 1 was determined by using rat basophil leukemia cells expressing the human high-affinity IgE receptor FcεRI. The rat basophil leukemia cells were loaded with serum IgE from patients with bee venom allergy. The release of β-hexosaminidase was measured as described.[E3]

REFERENCES

E1. Valenta R, Duchene M, Ebner C, Valent P, Sillaber C, Deviller P, et al. Profilins constitute a novel family of functional plant pan-allergens. J Exp Med 1992;175:377-85.

E2. Gattinger P, Mittermann I, Lupinek C, Hofer G, Keller W, Stojkovic UB, et al. Recombinant glycoproteins resembling carbohydrate-specific IgE epitopes from plants, venoms and mites. EBioMedicine 2019;39:33-43.

E3. Villazala-Merino S, Rodriguez-Dominguez A, Stanek V, Campion NJ, Gattinger P, Hofer G, et al. Allergen-specific IgE levels and the ability of IgE-allergen complexes to cross-link determine the extent of CD23-mediated T-cell activation. J Allergy Clin Immunol 2020;145:958-67.

E4. Gieras A, Focke-Tejkl M, Ball T, Verdino P, Hartl A, Thalhamer J, et al. Molecular determinants of allergen-induced effector cell degranulation. J Allergy Clin Immunol 2007;119:384-90.

E5. Foetisch K, Westphal S, Lauer I, Retzek M, Altmann F, Kolarich D, et al. Biological activity of IgE specific for cross-reactive carbohydrate determinants. J Allergy Clin Immunol 2003;111:889-96.

E6. Burtin D, Chabre H, Olagnier B, Didierlaurent A, Couret MN, Comeau D, et al. Production of native and modified recombinant Der p 1 molecules in tobacco plants. Clin Exp Allergy 2009;39:760-70.

E7. Li M, Gustchina A, Glesner J, Wünschmann S, Vailes LD, Chapman MD, et al. Carbohydrates contribute to the interactions between cockroach allergen Bla g 2 and a monoclonal antibody. J Immunol 2011;186:333-40.

E8. Glesner J, Wünschmann S, Li M, Gustchina A, Wlodawer A, Himly M, et al. Mechanisms of allergen-antibody interaction of cockroach allergen Bla g 2 with monoclonal antibodies that inhibit IgE antibody binding. PLoS One 2011;6:e22223.

E9. Do DC, Yang S, Yao X, Hamilton RG, Schroeder JT, Gao P. N-glycan in cockroach allergen regulates human basophil function. Immun Inflamm Dis 2017;5:386-99.

E10. Zafred D, Nandy A, Pump L, Kahlert H, Keller W. Crystal structure and immunologic characterization of the major grass pollen allergen Phl p 4. J Allergy Clin Immunol 2013;132:696-703.

E11. Ball T, Edstrom W, Mauch L, Schmitt J, Leistler B, Fiebig H, et al. Gain of structure and IgE epitopes by eukaryotic expression of the major Timothy grass pollen allergen, Phl p 1. FEBS J 2005;272:217-27.

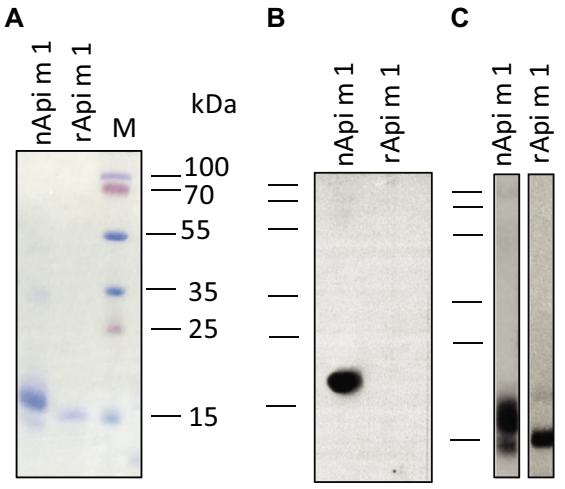

FIG E1. A, Coomassie blue–stained SDS-PAGE showing nApi m 1 and rApi m 1. Nitrocellulose-blotted nApi m 1 and rApi m 1 stained with the biotinylated *Aleuria aurantia* lectin (AAL) (**B**) or with serum IgE from a patient with bee venom allergy **(C)**. Molecular weights are indicated with the molecular weight marker M.

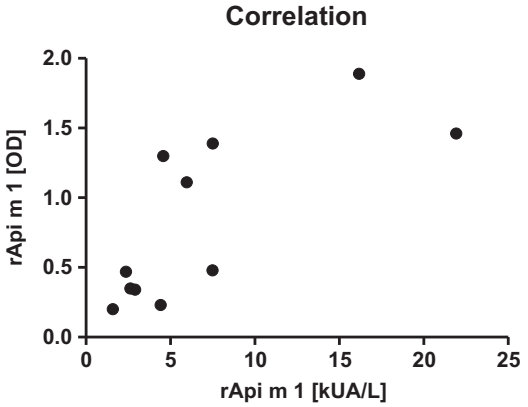

FIG E2. Correlation of levels of sIgE to rApi m 1 as determined by ImmunoCAP (kUA/L) and ELISA (OD) (Pearson correlation coefficient $R = 0.7453$; $P < .0005$).

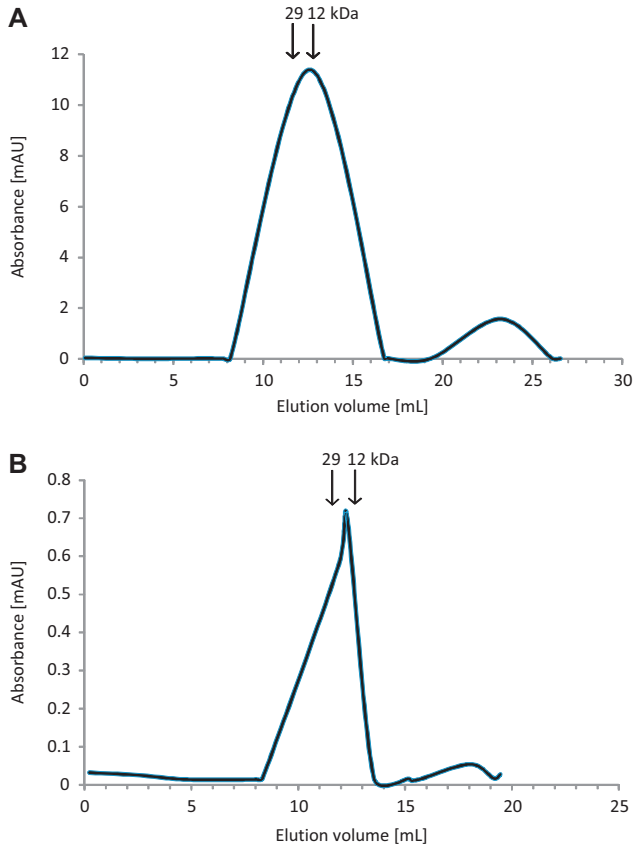

FIG E3. Size exclusion chromatography of nApi m 1 (**A**) and rApi m 1 (**B**). Elution volumes of marker proteins (cytochrome C, 12 kDa; carbonic anhydrase, 29 kDa) are indicated by arrows. Shown are elution volumes (x-axis) and absorbance units (y-axis).

TABLE E1. Clinical and serologic characterization of patients with bee venom allergy

Patient ID	Total serum IgE level (kU/L)*	Bee venom IgE level (kUA/L)†	rApi m 1 (kUA/L)‡	nApi m 1 OD, range of individual measurements from lowest to highest§‖	nApi m 1 OD, mean§‖	rApi m 1 OD, range of individual measurements from lowest to highest¶‖	rApi m 1 OD, mean‖¶	Fold increase nApi m 1 to rApi m 1	Mueller grade#	CCD-specific IgE detected with the tested markers**
1	496	100	7.51	1.70-1.73	1.72	1.37-1.41	1.39	1.2	2	No
2	304	8.81	2.37	0.45-0.46	0.46	0.46-0.47	0.47	0.98	2	No
3	393	100	5.96	1.69-1.73	1.71	1.1-1.12	1.11	1.5	2	Yes
4	nd	44.3	4.57	1.32-1.34	1.33	1.28-1.32	1.30	1.02	4	No
5	474	3.83	16.17	1.89-1.88	1.88	1.89-1.89	1.89	0.99	2	Yes
6	63	1.96	4.43	0.40-0.40	0.4	0.22-0.23	0.23	1.73	2	Yes
7	566	54.1	21.93	1.92-1.95	1.94	1.43-1.49	1.46	1.3	2	Yes
8	385	79.8	2.91	0.48-0.52	0.5	0.33-0.35	0.34	1.62	2	Yes
9	nd	10.9	2.63	0.95-0.96	0.95	0.34-0.35	0.35	2.71	2	Yes
10	212	17	7.49	0.89-0.93	0.91	0.45-0.50	0.48	1.9	3	Yes
11	18	7.82	1.59	0.20-0.22	0.21	0.2-0.2	0.2	1.05	2	No
12	477	nd	nd	1.79-1.83	1.81	1.30-1.33	1.32	1.37	nd	Yes

ID, Identifier; *nd*, not done.

*ImmunoCAP (Phadia AB) total IgE level.

†ImmunoCAP (Phadia AB), bee venom extract, i1, positive (≥0.35 kU/L).

‡IgE reactivity to nonglycosylated rApi m 1, i208 measured by ImmunoCAP.

§IgE reactivity to glycosylated natural Api m 1 measured by ELISA.

‖All ELISA measurements were performed in duplicate with a variation of less than 6%.

¶IgE reactivity to nonglycosylated rApi m 1 measured by ELISA.

#Clinical severity in terms of Mueller grade.

**IgE reactivity to CCDs was determined by using the recombinant CCD marker HHM2,[E8] CCD-ImmunoCAP, and Bromelain-ImmunoCAP.