

Recombinant allergen-based IgE testing to distinguish bee and wasp allergy

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Background: The identification of the disease-causing insect in venom allergy is often difficult.

Objective: To establish recombinant allergen-based IgE tests to diagnose bee and yellow jacket wasp allergy.

Methods: Sera from patients with bee and/or wasp allergy (n = 43) and patients with pollen allergy with false-positive IgE serology to venom extracts were tested for IgE reactivity in allergen extract-based tests or with purified allergens, including nonglycosylated *Escherichia coli*-expressed recombinant (r) Api m 1, rApi m 2, rVes v 5, and insect cell-expressed, glycosylated rApi m 2 as well as 2 natural plant glycoproteins (Phl p 4, bromelain).

Results: The patients with venom allergy could be diagnosed with a combination of *E coli*-expressed rApi m 1, rApi m 2, and rVes v 5 whereas patients with pollen allergy remained negative. For a group of 29 patients for whom the sensitizing venom could not be identified with natural allergen extracts, testing with nonglycosylated allergens allowed identification of the sensitizing venom. Recombinant nonglycosylated allergens also allowed definition of the sensitizing venom for those 14 patients who had reacted either with bee or wasp venom extracts. By IgE inhibition studies, it is shown that glycosylated Api m 2 contains carbohydrate epitopes that cross-react with natural Api m 1, Ves v 2, natural Phl p 4, and bromelain, thus identifying cross-reactive structures responsible for serologic false-positive test results or double-positivity to bee and wasp extracts.

Conclusion: Nonglycosylated recombinant bee and wasp venom allergens allow the identification of patients with bee and wasp allergy and should facilitate accurate prescription of venom immunotherapy. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Venom allergy, recombinant allergen, in vitro diagnosis, glycosylation

Up to 3% of the general population has a history of systemic anaphylactic reactions to insect stings, which are in large part caused by honey bee (*Apis mellifera*) or yellow jacket (*Vespula vulgaris/Vespula germanica*).¹ Venom immunotherapy (VIT) is highly effective for preventing allergic reactions in patients with allergy but requires a careful diagnosis that aims to identify the culprit insect.¹⁻³ However, the accurate diagnosis of venom allergy and in particular of the culprit insect can be hampered by several factors. For example, it has been reported that more than 30% of patients with systemic reactions to insect stings were negative by skin testing, most likely because of limited sensitivity of the skin test reagents.⁴ Furthermore, serologic diagnosis of insect venom allergy is often difficult because of the presence and recognition of cross-reactive carbohydrate epitopes in insect venom extracts and unrelated allergen sources such as pollen and plant-derived materials (eg, latex) that lack allergenic activity and hence may give rise to false-positive diagnostic results.⁵⁻⁷ Finally, it is difficult and sometimes impossible to define the culprit venom for patients who exhibit IgE double-positivity to different venoms, such as bee and wasp venom.⁸⁻¹⁰

As has been shown for other allergen sources, recombinant allergens may be useful tools to facilitate the diagnosis of bee and wasp allergy.³ The usefulness of recombinant bee and wasp allergens for *in vitro* and *in vivo* diagnosis has been shown in several studies.^{3,11} Several recombinant bee and wasp allergens have been produced in bacterial expression systems as nonglycosylated proteins or in eukaryotic systems as glycosylated allergens and are available for diagnostic purposes.^{3,11-13} In this context, it has been reported that by expression of venom allergens as glycosylated proteins in eukaryotic cells such as baculovirus-infected insect cells, recombinant allergens with increased IgE binding capacity can be obtained.¹⁴ However, several studies provide evidence that IgE reactivity to carbohydrate epitopes can lead to false-positive test results in patients without bee and wasp allergy and may hamper the discrimination of patients with bee and wasp allergy.^{8,15-18}

Here we used a panel of recombinant bee and wasp allergens expressed as glycosylated and nonglycosylated proteins to analyze sera from patients with bee and wasp allergy as well as sera from patients with pollen allergy with false-positive IgE test results to natural bee and wasp allergen extracts for IgE reactivity. Furthermore, we performed IgE inhibition studies with the recombinant venom allergens and glycosylated plant allergens/proteins to identify cross-reactive structures. Cross-reactive carbohydrate epitopes on Api m 1, Api m 2, Ves v 2, Phl p 4, and

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Abbreviations used

n: Natural
r: Recombinant
VIT: Venom immunotherapy

bromelain were found to interfere with the serologic discrimination of bee and wasp allergy and are thus responsible for false IgE serologic test results to bee and wasp in certain patients allergic to pollen and plant products. Recombinant (r) Api m 1, rApi m 2, and rVes v 5 expressed as nonglycosylated allergens in *E coli* were shown to distinguish bee from wasp allergy.

METHODS**Patients**

Forty-three patients with bee and/or wasp venom allergy (29 from Slovenia, 14 from Austria) were included in this study. Patients from Slovenia with a history of at least 1 systemic reaction of Mueller grade II to IV (see this article's Table E1 in the Online Repository at www.jacionline.org) showed CAP-FEIA double-positivity for honey bee and wasp venom (Phadia, Uppsala, Sweden). The sensitization was confirmed by skin prick testing (10 and 100 μ g/mL). Fourteen of the double-positive patients were diagnosed as having bee venom allergy (Table I, patients 1-14, Sb) and 13 patients as having wasp venom allergy because the culprit insect has been identified (Table I, patients 15-27, Sw). Two patients were diagnosed as having bee and wasp venom allergy because they have experienced reactions to both insects (Table I, patients 28 and 29, Sb+w). Patients were asked whether they had been stung after VIT to determine tolerance (Table I). Of the 14 Austrian patients, 7 were CAP-FEIA-positive only for bee venom (Table I, patients 30-36, Ab), and 7 were CAP-FEIA-positive only for wasp venom (Table I, patients 37-43, Aw). In addition, sera from 6 Austrian patients with pollen allergy without bee or wasp allergy but positive IgE reactivity to natural bee and/or wasp extracts (CAP) were studied. These patients contained IgE antibodies to nApi m 1 and natural (n) Phl p 4 when tested by ImmunoCap Immuno Solid-phase Allergen Chip (ISAC) (Table I, Ap 44-49). Sera from 3 individuals without allergy from Austria were also studied as negative controls (Table I, An 1-3). In the case of patients who had received immunotherapy, only sera obtained before treatment were included in the serologic analysis (Table I). For the anonymized analysis of the sera, approval was obtained by the ethical committee of the Medical University of Vienna.

Venoms, allergens, protein expression, and purification

Honey bee (*Apis mellifera*) venom, wasp (mixture of *Vespa germanica* and *Vespa vulgaris*) venom, nApi m 1, and bromelain from pineapple stem were purchased from Sigma (St Louis, Mo). nPhl p 4 was purified from timothy grass pollen by chromatography as previously described.¹⁹

Recombinant Bet v 1 was obtained from BIOMAY (Vienna, Austria). rApi m 1, rApi m 2, and rVes v 5 were expressed in *E coli* BL21 (DE3) as his-tagged proteins and purified by inclusion body preparation and Ni²⁺-affinity chromatography (see this article's Fig E1 in the Online Repository at www.jacionline.org). rApi m 2 was also produced as a glycosylated protein by baculovirus-infected insect cells and purified by Ni²⁺-affinity chromatography (Fig E1).²⁰

Purity of proteins was checked by SDS-PAGE and Coomassie blue staining (Fig E1).²¹

Protein concentrations were determined by Micro BCA Protein Assay (Pierce, Rockford, Ill).

IgE immunoblotting, IgE inhibition experiments, IgE ELISA

Comparable amounts of bee or wasp venom (Sigma) were subjected to SDS-PAGE and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany).²²

Patients' sera (dilution 1:10) were preincubated at 4°C overnight with rApi m 1, glycosylated rApi m 2, rVes v 5, or rBet v 1 (10 μ g/mL). Preabsorbed sera were then exposed to nitrocellulose-blotted bee or wasp venom, and bound human IgE antibodies were detected with ¹²⁵I-labeled antihuman IgE antibodies and visualized by autoradiography.²³ Densitometry of signals was performed on scanned immunoblot images by using the Java ImageJ analysis tool (<http://rsbweb.nih.gov/ij/>). In addition, IgE competition studies were performed by ELISA inhibition assay. Patients' sera were diluted 1:5 in PBS containing 0.5% wt/vol BSA and 0.05% vol/vol Tween 20 and were preincubated with nPhl p 4, bromelain, nApi m 1, or insect cell-expressed rApi m 2 (10 μ g/mL serum dilution) and, for control purposes, with an unrelated nonglycosylated allergen, rBet v 1, or buffer alone overnight at 4°C. Then sera were incubated with ELISA plate-bound nApi m 1 and insect cell-expressed rApi m 2, and bound IgE was detected. IgE reactivity to rApi m 1, glycosylated and nonglycosylated rApi m 2, and rVes v 5—as well as to bromelain and nPhl p 4—was measured by direct ELISA.²⁴

RESULTS**IgE testing based on natural bee and wasp allergen extracts does not always allow identification of the sensitizing venom**

We studied a group of 29 patients from Slovenia for whom the serologic and skin test diagnosis based on IgE reactivity testing to venom extracts gave double-positive test results with bee and wasp venom extracts. Double allergy could be confirmed by clinical diagnosis only for 2 patients (Table I, Sb+w). Fourteen patients were sensitized to bee venom but not wasp venom (Table I, Sb) and 13 patients seemed to be sensitized to wasp venom but not to bee venom because the culprit insect was known (Table I, Sw).

In addition, we have included in our study sera from 7 Austrian patients (Table I, Ab) who showed exclusive IgE reactivity to bee venom extracts as well as sera from additional 7 Austrian patients with exclusive IgE reactivity to wasp venom extracts (Table I, Aw). The IgE profiles to venom extracts were in agreement with clinical data in these patients and allowed the unambiguous identification of the culprit insect. Finally, we studied a group of 6 Austrian patients with pollen allergy who did not have allergic symptoms to bee or wasp but gave positive IgE test results with bee and/or wasp extracts. These patients showed also IgE reactivity to purified nApi m 1 and nPhl p 4 in ImmunoCap ISAC chip testing.

Glycosylated Api m 2 shares IgE epitopes with allergens in bee and wasp venom extracts

We searched for the presence of cross-reactive IgE epitopes in bee and wasp venom extracts by IgE-immunoblot inhibition experiments using purified bee and wasp allergens as well as glycosylated plant allergens (Fig E1). The major bee venom allergen, Api m 1, was expressed as nonglycosylated allergen in *E coli* (Fig E1, lane 1). In addition, nonglycosylated variants of Api m 2 and the major wasp allergen, Ves v 5, were produced in *E coli* (Fig E1, lanes 3 and 4). *E coli*-expressed Api m 2 migrates at a lower molecular weight than glycosylated rApi m 2, which had been expressed in insect cells (Fig E1, lanes 2 and 3). Depending on the insect cells used and the conditions used for expression, we have obtained rApi m 2 molecules that were glycosylated to different degrees (data not shown). In addition, we used purified nPhl p 4, a highly glycosylated grass pollen allergen, and carbohydrate-containing bromelain (Fig E1, lanes 5 and 6).

TABLE I. Demographic, serologic, and clinical characterization of patients

	Patient no.	Sex	Bee slgE (kUA/L)	Wasp slgE (kUA/L)	Mueller grade	Skin prick test Bee/Wasp	Culprit insect	VIT venom	Tolerance to field resting
Sb	1	M	21.9	3.03	IV	neg/neg	Bee	Bee	+
	2	M	47.9	1.98	IV	100/100	Bee	Bee	Bee + Wasp NK
	3	M	14.2	1.64	III	10/neg	Bee	Bee	+
	4	M	25.1	0.7	II	10/100	Bee	Bee	+
	5	F	6.57	0.5	IV	ND/ND	Bee	Bee	+
	6	F	6.52	1.13	III	10/neg	Bee	Bee	+
	7	F	8.4	0.56	IV	10/neg	Bee	Bee	+
	8	F	12.1	0.77	III	10/100	Bee	Bee	+
	9	F	0.96	8.44	IV	100/neg	Bee	Bee	Bee NK Wasp NK
	10	M	1.64	0.5	IV	10/neg	Bee	Bee	+
	11	F	3.92	0.39	IV	10/neg	Bee	Bee	+
	12	M	2.45	0.79	III	10/100	Bee	Bee	+
	13	M	0.6	0.92	IV	neg/neg	Bee	Bee + Wasp	Bee NK Wasp +
	14	M	1.92	0.57	IV	10/neg	Bee	Bee	+
Sw	15	M	8.43	5.89	III	neg/10	Wasp	Wasp	+
	16	M	5.73	1.54	III	neg/10	Wasp	Wasp	+
	17	M	14.5	3.96	IV	100/100	Wasp	Wasp	Bee NK Wasp NK
	18	F	3.1	7.22	III	neg/neg	Wasp	Wasp	+
	19	F	3.11	2.83	IV	100/100	Wasp	Wasp	+
	20	M	1.02	1.72	IV	neg/10	Wasp	Wasp	+
	21	F	0.57	0.47	III	100/100	Wasp	Wasp	+
	22	M	2.34	2.09	IV	neg/neg	Wasp	Wasp	+
	23	M	0.83	5.39	III	neg/neg	Wasp	Wasp	+
	24	M	0.377	4.6	II	100/10	Wasp	Wasp	+
	25	F	0.97	1.02	IV	neg/neg	Wasp	Wasp	+
	26	M	3.33	3.69	IV	100/100	Wasp	Wasp	+
Sb + w	27	F	0.57	1.11	IV	10/10	Wasp	Wasp	+
	28	F	>100	10.3	IV/III	100/100	Bee + Wasp	Bee + Wasp	Bee + Wasp NK
Ab	29	F	20	3.65	II/III	10/100	Bee + Wasp	Bee + Wasp	Bee- Wasp +
	30	M	4.34	<0.35	I	ND	Bee	No	NK
	31	M	14.3	<0.35	II	ND	Bee	Bee	NK
	32	M	7.48	<0.35	I	ND	Bee	No	NK
	33	F	4.42	<0.35	II	ND	Bee	Bee	NK
	34	M	4.12	<0.35	II	ND	Bee	No	NK
	35	M	2.04	<0.35	II	ND	Bee	No	NK
Aw	36	M	0.7	<0.35	I	ND	Bee	No	NK
	37	M	<0.35	17.4	III	ND	Wasp	No	NK
	38	M	<0.35	4.46	II	ND	Wasp	No	NK
	39	F	<0.35	3.9	II	ND	Wasp	No	NK
	40	M	<0.35	7.57	II	ND	Wasp	No	NK
	41	F	<0.35	6.67	I	ND	Wasp	No	NK
	42	M	<0.35	5.38	NK	ND	Wasp	No	NK
	43	F	<0.35	16.4	II	ND	Wasp	No	NK
Ap	44	M	9.07	5.32	0	ND	0	No	0
	45	M	12.2	3.9	0	ND	0	No	0
	46	M	3.36	0.64	0	ND	0	No	0
	47	F	17.4	4.31	0	ND	0	No	0
	48	M	6.98	1.59	0	ND	0	No	0
	49	M	5.37	2.31	0	ND	0	No	0

F, Female; M, male; ND, not done; neg, no positive reaction in skin prick test; NK, not known; +, tolerated; -, not tolerated; SlgE, specific serum IgE antibody levels. Patient groups, symbols, and abbreviation: Ab, Austria, bee allergy; Ap, Austria, pollen allergy; Aw, Austria, wasp allergy; Sb, Slovenia, bee allergy; Sb + w, Slovenia, bee and wasp allergy; Sw, Slovenia, wasp allergy; specific serum IgE antibody levels (kUA/L), severity of the clinical reaction (Mueller grade I-IV), skin prick test results (allergen extract concentrations in µg/mL eliciting a positive reaction), clinically identified culprit insect, venom extract used for immunotherapy (VIT venom), and the tolerance to field resting.

Immunoblot inhibition experiments were performed with sera from 3 patients with bee venom allergy (P3, P4, P9) and from 3 patients with wasp venom allergy (P15, P19, P23) who were all double-positive in serologic extract-based tests. Preabsorption of sera with the glycosylated rApi m 2 led to an almost complete loss (P3, 72%; P4, 85%; P9, 81%; P19, 99%; P23, 88%) or strong reduction (P15, 63%) of IgE binding to nApi m 2 (43 kd) in bee

venom (Fig 1, A) and also to a complete loss (P3, 95%; P9, 92%; P19, 91%) or a strong reduction (P4, 65%; P15, 75%) of IgE binding to bands corresponding to the homologous protein (nVes v 2) in wasp venom (43 kd; Fig 1, B). In addition, IgE binding to the glycosylated fraction of nApi m 1 in bee venom at approximately 18 kd was reduced (P4, 25%; P9, 22%; P15, 50%) or almost completely abolished (P19, 89%; P23, 78%) after preincubation with

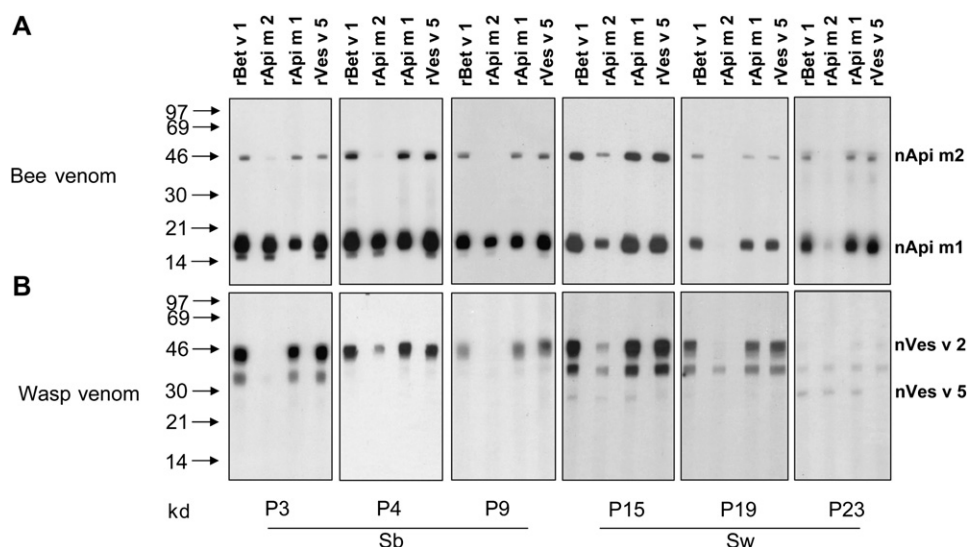


FIG 1. Cross-reactivity between natural bee and wasp venom allergens as demonstrated by IgE-immunoblot inhibitions. Nitrocellulose-blotted bee (A) or wasp (B) venom extracts were exposed to sera from patients with bee venom allergy (Sb: P3, P4, P9) or from patients with wasp allergy (Sw: P15, P19, P23) that had been preincubated with rBet v 1, rApi m 2–insect cells, rApi m 1–*E coli*, or rVes v 5–*E coli*. Molecular masses are indicated on the left margins.

rApi m 2 (Fig 1, A). Preadsorption of sera P3 and P9 with rApi m 2 resulted in a complete inhibition of IgE binding to wasp venom allergens (Fig 1, B).

We also found that nPhl p 4 and bromelain could inhibit IgE reactivity to nApi m 2, to bands in venom extract corresponding to Ves v 2, and to Api m 1, as was observed with glycosylated rApi m 2 (data not shown). The degree of inhibition of IgE cross-reactivity obtained with highly glycosylated rApi m 2 was stronger than that obtained with rApi m 2 with less glycosylation (data not shown).

Preabsorption of sera from patients with bee venom allergy with the recombinant *E coli*–expressed Api m 1 led to a reduction of IgE binding to the nonglycosylated form of nApi m 1 in bee venom (16 kd)²⁵ (Fig 1, A: P3, 95%; P4, 68%; P9, 78%) but had no effect on IgE binding to wasp venom allergens. Preadsorption of sera from patients with wasp venom allergy with *E coli*–expressed rApi m 1 had no effect on the IgE reactivity to bee as well as wasp venom allergens (P15, P19, P23). When sera were preincubated with rVes v 5, no effect on patients' IgE binding to honey bee venom was observed, but binding to nVes v 5 was reduced (24 kd; Fig 1, B: P15, 55%; P23, 77%). Preincubation of sera with the unrelated major birch pollen allergen Bet v 1 did not inhibit IgE reactivity to any bee or wasp allergen (Fig 1, A and B).

IgE cross-reactivity between nPhl p 4, plant glycans, and glycosylated Api m 1 and Api m 2

Glycosylated Api m 2 inhibited IgE binding to a band corresponding to Api m 1 in bee venom extract and to a band corresponding to Ves v 2 in wasp extract (Fig 1). Further, we had observed that nPhl p 4 and bromelain inhibited IgE binding to the very same bands. To identify the cross-reactive structures, ELISA inhibition experiments with purified nApi m 1 and glycosylated insect cell–expressed Api m 2 were performed (Table II). By using sera from patients with bee allergy (Sb5, Sb9), we found that

nPhl p 4 and bromelain caused a partial inhibition of IgE binding of approximately 30% to nApi m 1 and an even more pronounced inhibition of approximately 50% to glycosylated Api m 2 (Table II). Furthermore, we found that glycosylated Api m 2 partially inhibited IgE binding to nApi m 1 and vice versa.

We then studied IgE cross-reactivities using sera from patients with pollen allergy without bee or wasp allergy who had shown IgE reactivity to natural venom extracts, nPhl p 4, and nApi m 1 and found that nPhl p 4 caused an almost complete inhibition of IgE reactivity to nApi m 1 and glycosylated rApi m 2. Also, bromelain strongly inhibited IgE binding to the glycosylated bee allergens. These sera contained cross-reactive IgE antibodies to nApi m 1 and glycosylated rApi m 2 (Table II).

Nonglycosylated rApi m 1, rApi m 2, and rVes v 5 allow IgE-based diagnosis of bee and wasp venom allergy

Next we tested all patients sera for IgE reactivity to purified recombinant and natural allergens (ie, nonglycosylated rApi m 1, rApi m 2, rVes v 5; glycosylated rApi m 2 and plant glycoallergens, nPhl p 4, bromelain; Table III; see this article's Fig E2 in the Online Repository at www.jacionline.org).

We found that nonglycosylated, *E coli*–expressed rApi m 1 was exclusively recognized by patients with bee venom allergy (Sb, Sb+w, Ab), but not from patients with wasp venom allergy or patients with pollen allergy who had reacted with glycosylated bee and wasp allergens (Table III; Fig E2, A). The only patient with bee venom allergy, P8, who did not react with rApi m 1, showed IgE reactivity to rApi m 2 in its glycosylated and not glycosylated form. One of the patients with bee venom allergy also had specific IgE antibodies to Ves v 5 (Sb, P2; Table III). IgE binding to rVes v 5 was found in each of the patients with wasp venom allergy (Sw, Sb+w, Aw; Table III; Fig E2, B). Hence, a combination of *E coli*–expressed rApi m 1, rApi m 2, and rVes v 5 allowed diagnosing bee and wasp venom allergy in each of the tested patients. rApi

TABLE II. Inhibition of IgE binding to nApi m 1 or insect cell-expressed rApi m 2 by nPhl p 4, bromelain, rApi m 2, or nApi m 1

Sera	rBet v 1	nPhl p 4		Bromelain		rApi m 2		nApi m 1	
	OD	OD	%	OD	%	OD	%	OD	%
nApi m 1									
Sb5	0.221	0.149	33	0.175	21	0.171	23	0.01	95
Sb9	0.146	0.101	31	0.097	34	0.095	35	0.011	92
Ap44	0.199	0.002	99	0.025	87	0.005	97	0.001	99
Ap48	0.144	0.003	99	0.024	83	0.006	96	0.001	99
Ap49	0.158	0.008	95	0.013	92	0.002	99	0.008	95
rApi m 2									
Sb5	0.169	0.064	62	0.075	57	0.019	89	0.088	48
Sb9	0.224	0.133	41	0.141	37	0.018	92	0.119	47
Ap44	0.353	0.008	98	0.112	68	0.018	95	0.036	90
Ap48	0.187	0.008	96	0.071	62	0.03	84	0.015	92
Ap49	0.271	0.043	84	0.051	81	0.024	91	0.043	84

Displayed are the OD values corresponding to the levels of bound IgE antibodies of sera (*Sb5*, *Sb9*, *Ap44*, *Ap48*, *Ap49*) to nApi m 1 or insect cell-expressed rApi m 2 after preincubation with a non-cross-reactive allergen (rBet v 1) or with glycosylated allergens (nPhl p 4, bromelain, rApi m 2, nApi m 1). The percentages of inhibition of IgE binding are shown.

I served as a specific diagnostic marker for bee venom allergy and rVes v 5 as a marker for wasp allergy.

Glycosylated rApi m 2 was recognized by all bee and wasp venom double-positive patients (*Sb*, *Sb+w*, *Sw*; Table III), by 5 of 7 patients with CAP-FEIA positivity to bee venom (*Ab*; Table III), and by all patients with pollen allergy with false-positive IgE test results to bee and wasp allergens (*Ap*; Table III). One patient with IgE reactivity exclusively to wasp venom also showed IgE reactivity to glycosylated rApi m 2 (*Aw*, *P40*; Table III). Glycosylated rApi m 2 therefore cannot be used to distinguish between patients with bee and wasp allergy and may give rise to false-positive IgE test results. Even *E coli*-expressed nonglycosylated rApi m 2 was recognized by certain patients who were sensitized only against wasp venom (eg, *P16*, *P19*, *P20*), perhaps because of the presence of peptide epitopes similar to those on the homologous Ves v 2 allergen (Table III).

IgE reactivity to nPhl p 4, a highly glycosylated allergen from timothy grass pollen, and the glycoprotein bromelain from pineapple stem were found for 23 (Phl p 4) and 25 (bromelain) patients with bee or wasp venom allergy, respectively. None of the patients with pollen allergy with false-positive IgE test results to glycosylated bee or wasp allergens showed IgE reactivity to the nonglycosylated *E coli*-expressed bee and wasp allergens (*Ap*; Table III). Persons without allergy did not show any IgE reactivity to the proteins tested (*An*; Table III; Fig E2, A-F).

DISCUSSION

Diagnosis of the culprit allergens in patients with bee and wasp allergy is important because VIT represents an effective treatment for these patients and allows prevention of severe anaphylactic reactions.^{1,2} The identification of the culprit insect can be made when the insect causing allergic reactions has been identified and when unambiguous results can be obtained by skin testing and IgE serology. However, skin tests may often give false-negative results because of poor quality and low concentration of the allergen extracts used for skin testing.⁴ Using higher concentration may result in false-positive reactions because of the toxic properties of the venom allergens. Furthermore, patients with venom allergy and even patients without clinical sensitivity to venoms often exhibit false-positive IgE test results to venom extracts. The presence of cross-reactive

carbohydrates without clinical relevance has been suggested as a possible reason for such false-positive IgE test results.^{5-10,15,17,26} It has been suggested to use IgE inhibition assays with allergen extracts to address the problem of false-positive IgE test results, but also, in these assays, cross-reactive carbohydrates often do not allow the diagnosis of the culprit insect.^{10,16} Here we used a panel of recombinant bee and wasp allergens as well as glycosylated plant allergens for IgE testing in well defined patient groups. We demonstrate that an allergen panel made up of *E coli*-expressed nonglycosylated rApi m 1, rApi m 2, and rVes v 5 allowed improvement of IgE-based diagnosis of bee and wasp allergy. Patient *Sb2*, who had been classified as a patient with bee venom allergy because the culprit insect has been recognized, showed IgE reactivity to rVes v 5 and therefore would fall into a group of true double-positive patients. However, it has not been tested whether patient *Sb2* has a clinically relevant sensitization to wasp because only a resting event to bee was known. Thus, when the combination of the *E coli*-expressed allergens was used, patients with false double-positivity to bee and wasp venom extracts because of shared carbohydrate cross-reactivity could be identified. Accurate diagnosis was also possible for patients with clear extract-based serology, and no false-positive test result was obtained when patients with allergy to plant glycoproteins and false-positive IgE serology to bee and wasp allergen extracts were tested. IgE inhibition experiments with insect cell-expressed Api m 2 and glycosylated natural Api m 1, the major grass pollen glycoallergen, Phl p 4, and bromelain identified cross-reactive carbohydrate epitopes as causes for the false serologic test results.

It is possible that other bee and wasp allergens may also play an important role in bee and wasp allergy.^{27,28} Furthermore, it is possible that there are cross-reactive peptide epitopes in bee and wasp venoms such as the hyaluronidases.²⁹ Currently we do not have information about the biological role of carbohydrate epitopes in eliciting allergic reactions because only limited data regarding the *in vivo* relevance of the individual bee and wasp allergens are available. In fact, our results indicate that the IgE reactivity of the insect cell and thus glycosylated bee allergens is higher than that of the *E coli*-expressed allergens.

This difference in IgE reactivity can possibly be a result of changes in protein structure, because one function of glycosylation may be stabilization or different protein folds.

TABLE III. IgE reactivities to purified allergens as tested by ELISA

	Patient	IgE rApi m 1 <i>E coli</i>	IgE rApi m 2 Insect cells	IgE rApi m 2 <i>E coli</i>	IgE rVes v 5 <i>E coli</i>	IgE nPhl p 4	IgE Bromelain	Culprit insect
Sb	1	0.342	1.346	0.485	0.082	0.542	0.673	Bee
	2	0.143	0.869	0.183	0.258	0.236	0.187	Bee
	3	0.124	0.389	0.126	0.086	0.655	0.568	Bee
	4	0.212	0.539	0.286	0.084	0.069	0.304	Bee
	5	0.472	0.279	0.103	0.082	0.173	0.165	Bee
	6	0.257	0.186	0.092	0.079	0.075	0.097	Bee
	7	0.599	0.135	0.201	0.084	0.148	0.094	Bee
	8	0.089	0.244	0.121	0.081	0.142	0.156	Bee
	9	0.178	0.179	0.119	0.081	0.238	0.166	Bee
	10	0.109	0.245	0.188	0.078	0.122	0.099	Bee
	11	0.326	0.123	0.204	0.082	0.074	0.108	Bee
	12	0.367	0.121	0.089	0.097	0.081	0.101	Bee
	13	0.221	0.123	0.134	0.084	0.079	0.094	Bee
	14	0.146	0.144	0.085	0.084	0.081	0.101	Bee
Sw	15	0.084	0.756	0.096	0.324	0.348	0.456	Wasp
	16	0.067	0.399	0.128	0.191	0.329	0.207	Wasp
	17	0.088	0.576	0.098	0.254	0.307	0.411	Wasp
	18	0.071	0.345	0.092	0.501	0.203	0.102	Wasp
	19	0.076	0.357	0.124	0.222	0.478	0.262	Wasp
	20	0.088	0.339	0.126	0.276	0.104	0.107	Wasp
	21	0.081	0.262	0.094	0.175	0.132	0.116	Wasp
	22	0.079	0.108	0.096	0.137	0.087	0.097	Wasp
	23	0.088	0.142	0.092	0.629	0.126	0.093	Wasp
	24	0.075	0.201	0.095	0.279	0.126	0.129	Wasp
	25	0.084	0.456	0.092	0.202	0.089	0.184	Wasp
	26	0.074	0.147	0.091	0.196	0.089	0.084	Wasp
	27	0.074	0.171	0.087	0.208	0.13	0.141	Wasp

Sb+w	28	0.323	1.027	0.206	0.236	0.954	0.879	Bee/wasp
	29	0.186	0.411	0.352	0.219	0.458	0.346	Bee/wasp
Ab	30	0.971	0.243	0.184	0.059	0.196	0.168	Bee
	31	0.305	0.159	0.153	0.062	0.085	0.079	Bee
	32	0.318	0.085	0.083	0.067	0.091	0.082	Bee
	33	0.189	0.242	0.158	0.078	0.085	0.083	Bee
	34	0.227	0.086	0.088	0.064	0.078	0.086	Bee
	35	0.472	0.176	0.273	0.066	0.082	0.081	Bee
	36	0.522	0.463	0.302	0.062	0.156	0.129	Bee
Aw	37	0.072	0.042	0.081	0.203	0.055	0.082	Wasp
	38	0.072	0.047	0.048	0.414	0.063	0.073	Wasp
	39	0.073	0.055	0.066	0.312	0.064	0.074	Wasp
	40	0.071	0.166	0.079	0.188	0.244	0.105	Wasp
	41	0.089	0.052	0.075	0.695	0.076	0.072	Wasp
	42	0.059	0.049	0.066	0.321	0.063	0.084	Wasp
	43	0.072	0.056	0.065	0.149	0.062	0.062	Wasp
Ap	44	0.063	0.421	0.081	0.084	0.976	0.458	0
	45	0.066	0.188	0.075	0.078	0.247	0.142	0
	46	0.058	0.152	0.072	0.064	0.221	0.132	0
	47	0.068	0.455	0.068	0.072	0.847	0.285	0
	48	0.072	0.289	0.073	0.068	0.745	0.186	0
	49	0.073	0.331	0.076	0.082	0.994	0.514	0
An	1	0.062	0.068	0.075	0.038	0.048	0.063	0
	2	0.084	0.072	0.067	0.066	0.084	0.076	0
	3	0.092	0.066	0.056	0.084	0.085	0.051	0

Ab, Patients with bee allergy, Austria; An, persons without allergy; Ap, patients with pollen allergy, Austria; Aw, patients with wasp allergy, Austria; Sb, patients with bee allergy, Slovenia; Sb+w, patients with bee and wasp allergy, Slovenia; Sw, patients with wasp allergy, Slovenia.
The mean OD values (*bold and grey*, positive above cutoff) are shown.

Nevertheless, we think that the diagnosis of bee and wasp allergy by IgE serology can be made only using nonglycosylated allergens. The diagnostic test based on the 3 *E coli*-expressed

allergens may improve IgE-based diagnosis and thus should allow better prescription of VIT with bee and wasp allergen extracts.

Clinical implications: A recombinant allergen-based test without cross-reactive carbohydrates using Api m 1, Api m 2, and Ves v 5 should be useful for the serologic diagnosis of bee and wasp allergy.

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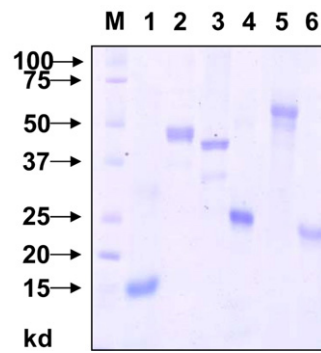


FIG E1. Coomassie blue-stained SDS-PAGE containing purified allergens used for IgE testing. *Lane 1*, rApi m 1-*E coli*; *lane 2*, glycosylated rApi m 2-insect cells; *lane 3*, rApi m 2-*E coli*; *lane 4*, rVes v 5-*E coli*; *lane 5*, nPhlp 4; *lane 6*, bromelain; *lane M*, molecular weight marker. Molecular weights are indicated at the left side in kilodaltons.

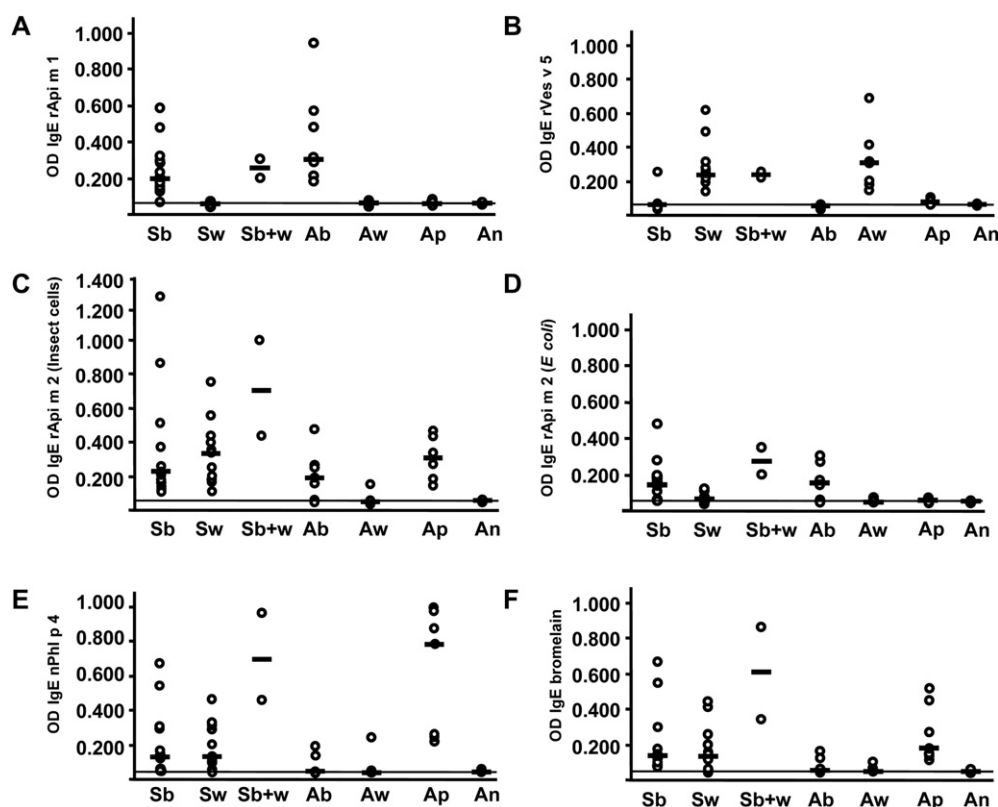


FIG E2. Diagnostic dissection of bee and wasp allergy using *E coli*-expressed bee and wasp venom allergens. Scatter plots showing specific serum IgE levels to rApi m 1-*E coli* (A), rVes v 5-*E coli* (B), glycosylated rApi m 2-insect cells (C), rApi m 2-*E coli* (D), nPhl p 4 (E), and bromelain (F) in sera from patients with bee and/or wasp venom allergy and patients with pollen allergy. Sb, Patients with bee venom allergy, Slovenia (n = 14); Sw, patients with wasp allergy, Slovenia (n = 13); Sb+w, patients with bee and wasp allergy, Slovenia (n = 2); Ab, patients with bee allergy, Austria (n = 7); Aw, patients with wasp allergy, Austria (n = 7); Ap, patients with pollen allergy, Austria (n = 6); An, persons without allergy (n = 3). Mean OD values corresponding to the amount of bound IgE antibodies are displayed. The cutoff levels of the ELISAs (median OD for individuals without allergy) are displayed as horizontal lines.

TABLE E1. Definition of Mueller grades I to IV

Mueller grade I	Urticaria, pruritus, nausea, anxiety
Mueller grade II	Any grade I reaction + angioedema, vomiting, diarrhea, abdominal pain, dizziness
Mueller grade III	Any grade I + II reaction + shortness of breath, dyspnea, wheezing, stridor, dysphagia, hoarseness, general weakness, fear of death
Mueller grade IV	Any grade I, II, or III symptom + drop in blood pressure, collapse, loss of consciousness, incontinence, cyanosis