Received Date : 06-May-2016

Revised Date : 18-Jul-2016

Accepted Date : 02-Aug-2016

Article type : Original Article: Biotechnology and in vitro Diagnostics

Application of recombinant antigen 5 allergens from 7 allergy-relevant Hymenoptera species in diagnostics

Short title: Recombinant Hymenoptera antigens 5 in diagnostics

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.13000

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Abstract

Background: Hymenoptera stings can cause severe anaphylaxis in untreated venom-allergic patients. A correct diagnosis regarding the relevant species for immunotherapy is often hampered by clinically irrelevant cross-reactivity. In vespid venom allergy, cross-reactivity between venoms of different species can be a diagnostic challenge. To address immunological IgE cross-reactivity on molecular level 7 recombinant antigens 5 of the most important *Vespoidea* groups were assessed by different diagnostic setups.

Methods: The antigens 5 of yellow jackets, hornets, European and American paper wasps, fire ants, white-faced hornets and *Polybia* wasps were recombinantly produced in insect cells, immunologically and structurally characterized and their sIgE reactivity assessed by ImmunoCAP, ELISA, cross-inhibition and basophil activation test (BAT) in patients with yellow jacket or *Polistes* venom allergy of two European geographical areas.

Results: All recombinant allergens were correctly folded and structural models and patient reactivity profiles suggested the presence of conserved and unique B cell epitopes. All antigens 5 showed extensive cross-reactivity in sIgE analyses, inhibition assays and BAT. This cross-reactivity was more pronounced in ImmunoCAP measurements with venom extracts than in sIgE analyses with recombinant antigens 5. Dose-response-curves with the allergens in BAT allowed a differentiated individual dissection of relevant sensitization.

Conclusions: Due to extensive cross-reactivity in various diagnostic settings, antigens 5 are inappropriate markers for differential sIgE diagnostics in vespid venom allergy. However, the newly available antigens 5 from further vespid species and the combination of recombinant allergen-based sIgE measurements with BAT represents a practicable way to diagnose clinically relevant sensitization in vespid venom allergy.

Key words

antigen 5, basophil activation test, cross-reactivity, Hymenoptera venom allergy, *in vitro* sIgE testing

Abbreviations

BAT; basophil activation test

CCD; cross-reactive carbohydrate determinants

CD; circular dichroism

GNA; *Galanthus nivalis* agglutinin

HBV; honeybee venom

PV; *Polistes* venom

SD; standard deviation

sIgE; specific IgE

VIT; venom immunotherapy

YJV; yellow jacket venom

Author contributions

MS: performed the experiments, analyzed the data, wrote the manuscript

BE: coordinated the recruitment of hymenoptera venom-allergic patients, supervised basophil activation tests, analyzed the data, wrote the manuscript

CM-A: coordinated the recruitment of PV-allergic patients, collected and analyzed the data

GP: recruited YJV-allergic patients, performed basophil activation tests, collected data

PS: recruited PV-allergic patients, collected data

MM: collected sera, collected and analyzed the data

LS: performed experiments, analyzed data

DR: established recombinant expression of allergens

TB: contributed to the interpretation of data, revised the final version of the manuscript

ES: initiated the study, revised the final version of the manuscript

UD: was responsible for the diagnostic work-up of hymenoptera venom-allergic and control patients

MO: supervised the study, analyzed the data, wrote the manuscript

CS-W: supervised the study, contributed to the interpretation of data, revised the final version of the manuscript

SB: initiated and supervised the study, analyzed the data, designed the figures, wrote the manuscript

Introduction

Insect venoms of Hymenoptera species such as bees, hornets, wasps, yellow jackets and ants can cause severe anaphylactic reactions with potentially fatal outcome. The only curative treatment which is effective in reducing the risk of subsequent systemic reactions is venom immunotherapy (VIT) (1). Therefore, identification of the species that provoke the allergic reaction is a prerequisite for a successful therapy. Unnecessary treatment with more than one or even with the wrong venom leads to incomplete protection, higher costs, increased risk of side effects and possible de novo

sensitizations (2).

Differentiation between cross-reactivity and clinically relevant multiple sensitization is a major problem in the diagnosis of Hymenoptera venom allergy. Cross-reactivity in Hymenoptera venom-allergic patients can be due to cross-reactive carbohydrate determinants (CCDs) or to sequence homology between allergens (3-10). CCD-based positive results in sIgE analyses or in cellular tests seem to be clinically irrelevant and often hamper the correct diagnosis of Hymenoptera venom allergy with regard to the relevant venom (11).

Over the last decade the increased knowledge about the molecular composition of Hymenoptera venoms and the development of component-resolved diagnostics has added clinical value especially for, but not limited to, the discrimination between honeybee and vespid venom allergy. Molecular diagnostics has demonstrated to have the potential to discriminate between clinically significant and irrelevant sensitization, to increase the specificity and sensitivity of diagnostics and to monitor immunotherapeutic intervention (12-19).

However, due to the structural similarity and, therefore, cross-reactivity of the so far identified important allergens of vespid venoms, the differentiation between allergies to the stings of different species is still challenging. A particular problem is the distinction between yellow jacket venom (YJV) and *Polistes* venom (PV) allergy (5, 20). In addition to their established importance in North America and Mediterranean regions of Europe, paper wasps, especially *Polistes dominula*, increasingly spread all over Europe as well as in the US from the warmer to the more moderate climate zones. Although we recently demonstrated that paper wasp venom is devoid of CCD-based cross-reactivity (21), cross-reactivity between *Polistinae* and *Vespinae* (especially YJV) venoms is frequently observed (5, 20).

The antigens 5 are the most abundant proteins and most potent allergens in vespid venoms (22-25). These proteins of unknown function were found in the venoms of nearly all *Vespoidea* species e.g. social wasps of the *Polistes, Dolichovespula, Vespa, Vespula and Polybia* genera (26) and show a varying degree of sequence homology.

It was the aim of this study to address the extent of immunological IgE cross-reactivity of recombinantly produced, CCD-free antigen 5 proteins of the most allergy-relevant hymenoptera groups (yellow jackets, hornets, European and American paper wasps, fire ants, white-faced hornets and Polybia wasps) on a molecular level in patient groups of two different geographical areas (South Bavaria, Germany; Cordoba, Spain). This is a prerequisite for developing new strategies for advanced next generation diagnostic and therapeutic options.

Methods

Patients

Blood and/or sera of 63 patients with anaphylactic reactions to either YJV or PV were analyzed. 43 patients were from the area of South Bavaria (Munich) in Germany and 20 patients were from the area of Cordoba in Spain. The German patients were primarily sensitized to YJV according to their history. Since *Polistes dominula* is virtually not present in this area, allergic reactions to this species can be excluded with high probability. The 20 patients from Spain were primarily allergic to PV. Since European paper wasps and yellow jackets coexist in this area and are difficult to discriminate, systemic reactions due to stings of both insects cannot be excluded. 2 patients with honeybee venom (HBV) allergy and sIgE only to HBV and 9 non-allergic individuals served as controls.

The diagnosis of venom allergy was based on a combination of clinical history of anaphylactic sting reactions, a positive intradermal skin test and/or positive sIgE levels to YJV or/and PV venom extract (i3 or/and i77). All patients had given informed written consent to draw additional blood samples and the local ethics committees approved the study.

Cloning and recombinant production of antigens 5

The coding regions of antigens 5 were amplified either from venom gland cDNA or synthesized genes by PCR and recombinantly produced in *Spodoptera frugiperda* (Sf9) insect cells. Cloning and expression in insect cells is described in the supplemental methods.

Immunoreactivity of patient sera with recombinant antigens 5

sIgE immunoreactivity of all sera with the antigens 5 was assessed by ELISA. A detailed description of the ELISA and of cross-inhibition experiments is given in the supplemental methods. The lower end functional cut-off, indicated as dotted lines, was calculated as the mean of the negative controls plus 3 SDs plus 10%.

ImmunoCAP measurements

sIgE antibodies to the different allergen extracts, allergen components, MUXF as well as total IgE was determined using the UniCAP250 platform (Thermo Fisher Scientific, Uppsala, Sweden) according to the recommendations of the manufacturer.

Basophil activation test

BATs were performed in 21 YJV-allergic patients, 2 HBV-allergic patients and 2 non- allergic controls as described previously (11), using the Flow CAST (Bühlmann Laboratories AG, Schönenbuch, Switzerland). Allergen concentrations were 2, 10, 50 and 250 ng/mL. A short description is given in the supplemental methods. One patient (patient 9) was a non-responder and excluded from further analysis.

Other methods

SDS-PAGE, Western blotting, CD spectroscopy and structural modeling are described in detail in the supplemental methods.

Results

Recombinant Expression and characterization of Hymenoptera antigens 5

In order to address the antigen 5-based IgE cross-reactivity of Hymenoptera venoms, the antigens 5 of 7 allergy-relevant species were recombinantly produced. The antigens 5 of the yellow jacket *Vespula vulgaris* (Ves v 5), the hornet *Vespa crabro* (Vesp c 5), the European paper wasp *Polistes dominula* (Pol d 5), the American paper wasp *Polistes annularis* (Pol a 5), the white-faced hornet *Dolichovespula maculata* (Dol m 5), the fire ant *Solenopsis invicta* (Sol i 3) and the wasp *Polybia scutellaris* (Poly s 5) were cloned either from venom gland cDNA or synthetic genes. The baculovirus-mediated expression in *Spodoptera frugiperda* (Sf9) insect cells after purification yielded recombinant proteins with an expected molecular weight of approximately 25 kDa (Figure 1A, upper and middle panel). While 6 of the antigens 5 are unglycosylated the sequence of Sol i 3 contains 3 potential N-linked glycosylation sites. The glycosylation was confirmed using *Galanthus nivalis*

agglutinin (GNA) (Figure 1A, lower panel) which detects terminal mannose residues and, therefore, indicates the presence of N-linked glycans in general. However, the protein was devoid of CCD-based cross reactivity (data not shown) as described previously for other allergens produced in Sf9 cells (14, 27-29).

Since X-ray crystallography structures are available only for Ves v 5 and Sol i 3 (30, 31) we have built structural models of the remaining 5 allergens by molecular modeling (32). As expected from sequence alignments (Figure S1) all 7 antigens 5 show a very similar fold as demonstrated by the overlay of all 7 structures (Figure 1B). The correct and similar folding of the recombinantly produced antigens 5 was confirmed by CD-spectroscopy (Figure S2). The identity on protein level between the investigated allergens ranges from 45% to 85% and is the highest between Pol d 5 and Pol a 5 (Figure 1C). Although the identity among the antigens 5 belonging to the same subfamily (*Vespula*, *Vespa* and *Dolichovespula* in the *Vespinae* and *Polistes* and *Polybia* in the *Polistinae* subfamily) is the highest (64-85%), the identity between the antigens 5 belonging to different subfamilies still ranges between 45% and 63%. However, despite of the identical fold, the surface charge is different between all investigated allergens (Figure 1D) and hints to the presence of shared as well as unique B cell epitopes.

sIgE cross-reactivity of antigens 5 in Hymenoptera venom-allergic patients

The sIgE reactivity with the 7 recombinant antigens 5 of 63 patients with systemic reactions to YJV and/or PV was addressed by ELISA. In the patient group from Germany 41/43 (95.5%) were reactive with Ves v 5 (Figure 2A). Except for patient 41 (sIgE to Ves v 5 of 6.87 kU_A/L in ImmunoCAP measurement and negative sIgE to Ves v 5 in the ELISA) ELISA and ImmunoCAP results were comparable for all patients (Table S1). Additionally, the sIgE reactivity with all other antigens 5 was very pronounced: cross-reactivity was highest with the hornet allergen Vesp c 5 (67.4%), which

shows 68% identity on protein level but less pronounced (27.9%) with Dol m 5 (64% identity). sIgE reactivity with the European and American paper wasp as well as with the fire ant antigen 5 was 44.2%, 37.2% and 32.6%, respectively. The reactivity with Poly s 5 was 58.1%.

In the group of patients from Spain, all sera were reactive with Pol d 5 and 75% with Ves v 5 (Figure 2A). sIgE reactivity with Pol a 5 and Poly s 5 was 65 and 70%, respectively, and lower (50%) with Vesp c 5, Dol m 5 and Sol i 3. In the control group, consisting of non-allergic patients or patients with HBV allergy, none of the sera showed any reactivity with the antigens 5 (Figure S3).

Looking at the sIgE reactivity of individual patients, the profiles demonstrated to be very diverse without discernible pattern (Figure 2C). In both groups some patients reacted only to the antigen 5 of the allergy-eliciting species (e.g. patient 35 and 57) and others showed sIgE to all of the investigated allergens (e.g. patients 22, 26, 29 and 60). Again other patients exhibited reactivities with the various antigens 5 in different combinations which are not connected to their degree of homology on protein level. The unpredictable cross-reactivity of the different antigens 5 is also reflected by the weak association of reactivity of the individual patients to the particular allergens, as observed in correlation and regression analyses (Figure S4).

Cross-inhibition experiments using Ves v 5 and Pol d 5 were performed for selected patients who showed reactivity to both allergens (Figure 2D). Thereby, for two patients with YJV allergy (patients 26 and 30) the inhibition of reactivity to Pol d 5 by Ves v 5 was 91% and 100%, respectively, and the other way round approximately 60%. In the group of patients with PV and/or YJV allergy the results again were very diverse, ranging from comparable inhibition by both allergens (patients 53 and 61) over inhibition by only one allergen (patients 54 and 60) up to stronger inhibition by either Pol d 5 (patients 44 and 48) or Ves v 5 (patients 47, 50 and 59).

Activation of basophils from hymenoptera venom-allergic patients by antigens 5

In BAT the YJV-allergic patients showed diverse activation profiles in response to the different antigens 5 (Figure 3). 6/20 (30%) patients exhibited basophil activation in response to Ves v 5 and/or Vesp c 5 only (patients 2, 3, 5, 6, 8 and 12). The basophils of further 11 patients (55%) were activated by either all (patients 7 and 11) or different combinations (patients 1, 10, 13, 15, 16, 18, 19, 20 and 21) of antigens 5. However, in most of these patients the basophil activation was more pronounced in response to Ves v 5 and/or Vesp c 5. Only for patients 4 and 14 the activation pattern was more distinct in response to other allergens than Ves v 5 and/or Vesp c 5. Also Poly s 5 was able to activate patient-derived basophils (e.g. patients 4, 11 and 20) in this assay. Patient 17, who also exhibited low activation in the positive control as well as the non-allergic (patient 73 and 74) and HBV-allergic (patients 71 and 72) controls showed no basophil activation in response to the antigens 5.

sIgE cross-reactivity in ImmunoCAP measurements

For the diagnosis of hymenoptera venom allergy, only the recombinant antigens 5 Ves v 5 and Pol d 5 are available on immunoassay platforms for routine sIgE determination. Therefore, we analyzed the sIgE reactivity of YJV-allergic patients assessed in BAT for their reactivity with the allergen components Ves v 5, Pol d 5 and Ves v 1 as well as with various available hymenoptera allergen extracts and MUXF in ImmunoCAP measurements (Figure 4 and Table 1).

Solenopsis invicta whole body extract (i70) showed the lowest sIgE reactivity (9.5%) followed by *Vespa crabro* venom (i75) with 52.5%. Using the cut-off of 0.35 kU_A/L, sIgE reactivity with the other venom extracts ranged between 66.7% (*Dolichovespula* venom, i2) and 100% (*Vespula* spp. venom, i3) (Figure 4). Lowering the cut-off to 0.1 kU_A/L, a threshold that was shown to be applicable on the UniCAP platform (33), increased the reactivities with the venom extracts ranging between 81

and 100% (Figure 4). There was a high reactivity (71.4% using the 0.35 kU_A/L cut-off) of the sera with recombinant Ves v 1, and some sera (28.6%) reacted with the CCD-marker MUXF.

However, the extent of cross-reactivity between Ves v 5 and Pol d 5 was slightly higher compared to the ELISA measurement, most likely due to the higher sensitivity of the UniCAP platform. Using the cut-off of 0.35 kU_A/L, 95.2% and 81% of sera exhibited sIgE to Ves v 5 and Pol d 5, respectively. Intriguingly, except for patient 20 in this group of YJV-allergic patients, sIgE to Ves v 5 was always higher compared to Pol d 5 (Table 1). In the group of patients with PV and/or YJV allergy this was also true for 4/16 (25%) patients and the other way round for 13/16 (81.3%) of patients (Table S1).

Discussion

In order to address the extent of antigen 5-based IgE cross-reactivity in different *in vitro* diagnostic settings and to develop proper tools for an advanced diagnosis, the antigens 5 of the most allergy-relevant Hymenoptera subfamilies were recombinantly produced in insect cells. All 7 allergens showed a similar and proper folding which was comparable to their native counterparts (34, 35). As expected from amino acid sequences, 6 of the antigens 5 were unglycosylated. Only the fire ant allergen Sol i 3 was recombinantly produced carrying N-linked glycans. Although the natural Sol i 3 does not show any glycosylation, it was demonstrated that some of the allergen produced recombinantly in insect cells can be glycosylated at Asn124 (35). However, Sol i 3 did not show any CCD-based cross-reactivity as demonstrated previously for other allergens produced in Sf9 insect cells (14, 27-29).

First, sIgE cross-reactivity of the 7 antigens 5 was assessed by ELISA in two groups of Hymenoptera venom-allergic patients. The first group was from the area of South Bavaria in Germany and, therefore, primarily sensitized to YJV and the second from the area of Cordoba in

Spain and selected for allergy to PV. However, in the second group allergy to YJV cannot be fully excluded (see methods section). In both groups the degree of cross-reactivity with all antigens 5 was very pronounced and ranged from approximately 30% to 75%. In the individual patients the sIgE reactivity profiles were very diverse and showed various combinations of recognition without discernible pattern. In both groups the degree of cross-reactivity and the reactivity profiles were not obviously connected to the degree of homology between the antigens 5.

Independent from the degree of identity on protein level, ranging from 47 to 85%, all investigated antigens 5 show very similar 3-dimensional structures. However, the structural analysis demonstrated that surface charges of the allergens differ independent from the degree of homology. Since charged amino acids are important factors to determine the shape of an epitope and the quality of the epitope-paratope-interaction (36), these differences might explain the variations in cross-reactivity. The diverse reactivity profiles of patients hint at the presence of epitopes which are conserved between all investigated antigens 5 and others which are unique for one or shared between particular of them.

Interestingly, the *Polybia* antigen 5 Poly s 5, which according to murine studies was suggested to be a hypoallergenic variant (37), showed strong reactivity in sIgE analyses as well as potent activation of basophils. This observation and the fact that the closely related species *Polybia paulista* in Brazil causes allergic reactions (38, 39) suggest that IgE immune responses to particular allergens in mice and humans might differ dramatically.

Since only two of the antigens 5 (Ves v 5 and Pol d 5) are commercially available for routine diagnosis, sIgE cross-reactivity with various venom extracts was tested in ImmunoCAP measurements using sera of YJV-allergic patients. Thereby, the degree of cross-reactivity was even higher compared to using the antigens 5 as molecular allergens. This can be explained by the presence of other cross-reactive allergens such as phospholipases, hyaluronidases and other not yet identified allergens present in all venoms (40). In this analysis CCD-based cross reactivity represents

only a minor interfering factor since only few patients exhibited sIgE to CCDs. Furthermore, we could recently demonstrate the absence of CCDs in European and American PV (21). The only ImmunoCAP which showed reduced reactivity was of the fire ant. However, this is the only ImmunoCAPs which is loaded with whole body instead of venom extract and, therefore, might show low sensitivity due to the underrepresentation of relevant allergens in the whole protein content.

Admittedly, the clinical relevance of the observed cross-reactivities and sensitization profiles on a symptom-based level remains unclear due to the fact that most of the patients probably were only stung by one Hymenoptera species and that diagnostic sting provocations are ethically not justifiable. Definitely, in the future an extension of this study to patients experiencing field stings by different Hymenoptera species would be of particular interest.

For discrimination between YJV and PV allergy CAP-inhibition experiments with both venoms were found to be helpful for the correct prescription of immunotherapy (5, 41). We performed cross-inhibition experiments in patients with primary sensitization either to YJV or to PV and/or YJV, applying Ves v 5 and Pol d 5. In most cases where the disease-eliciting insect was identified by clear clinical history or intradermal skin testing (e.g. patients 26, 30, 44 or 48) the results of the cross-inhibition corresponded to this diagnosis. In other cases where the disease-eliciting insect was unknown (e.g. patients 47, 54 and 60) cross-inhibition gave good hints to the relevant venom, although the clinical relevance of these results remains unclear. However, for two patients (patients 53 and 61) with negative intradermal skin test to YJV and low sIgE to YJV, Pol d 5 and Ves v 5 showed the same inhibitory capacity. Nevertheless, inhibition experiments are helpful to identify the patient-relevant insect, however, they are expensive, time consuming, difficult to interpret, and thus rarely used in clinical routine.

Furthermore, it was proposed that YJV and PV allergy should be discriminated by measurement of the level of sIgE to antigens 5 and phospholipases (20, 41). Most of the patients in this study, for whom the allergy-relevant venom was clearly identified, showed higher levels of sIgE

to the appropriate antigen 5 and for the others the clinical relevance of the results cannot be figured out with absolute certainty. Nevertheless, at least one patient with clear history of YJV allergy had a higher sIgE level to Pol d 5 than to Ves v 5 and for many other patients the sIgE levels were in a very comparable range. Moreover, the amount of sIgE to particular allergens is a factor which can depend on other reasons than primary sensitization such as quality of the allergen used for sIgE testing, and in many cases, results will be difficult to interpret.

Additionally, a subgroup of patients with YJV allergy was assessed for their response to the 7 antigens 5 in BAT, which was proven to be a powerful method to complement accurate diagnostics and to elucidate clinically relevant sensitizations (42). Although the antigens 5 showed likewise extensive cross-reactivity and various activation profiles, most patients exhibited the strongest response to the antigen 5 they are primarily sensitized to. Therefore, in most patients BAT would have been superior in identifying the clinically relevant Hymenoptera species by the recording of dose-response curves and the calculation of half-maximum stimulation as described previously for the dissection of HBV and YJV double-sensitization (11).

Taken together our results extend former findings, demonstrating cross-reactivity of vespid venoms, on a molecular basis by assessing interference of the antigens 5 of the most important Hymenoptera groups in different diagnostic settings. Although useful as marker allergens for the discrimination between HBV and vespid venom, antigens 5 are inappropriate markers for the dissection of vespid venom allergy showing cross-reactivity in various diagnostic settings assessing sIgE. This clearly demonstrates the need for the identification of novel marker allergens and better solutions for a more reliable diagnosis. However, due to their relevance as most important major allergens in vespid venoms, omitting antigens 5 in diagnostics is hardly feasible. Our results indicate that the combination of recombinant allergen-based sIgE measurements, skin tests, basophil activation testing and careful recording of patient history (including distinct knowledge of the physician about distribution and behavior of relevant Hymenoptera species) might represent a

practicable way to dissect clinically relevant sensitization in vespid venom allergy.

Acknowledgements

We gratefully acknowledge the technical assistance of Stefanie Etzold, Franziska Martin and Erika Arnold. Moreover, we thank Domingo Barber for critical discussion of the topic.

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Table 1. Clinical and serological data of YJV-allergic patients analyzed in basophil activation test.

Patient ID	Sting reaction grade*	Skin test[1] (i.c.) YJV	tIgE [kU/L]	sIgE Ves spp. (i3) [kU_A/L]	sIgE Ves v 1 (i211) [kU_A/L]	sIgE Ves v 5 (i209) [kU_A/L]	sIgE Vesp c (i75) [kU_A/L]	sIgE Pol d (i77) [kU_A/L]	sIgE Pol d 5 (i210) [kU_A/L]	sIgE Pol spp. (i4) [kU_A/L]	sIgE Dol m (i2) [kU_A/L]	sIgE Sol i (i70) [kU_A/L]	sIgE MUXF (o218) [kU_A/L]
1	IV	0.001	113	1.85	0.16	1.64	0.42	0.47	0.73	0.43	0.50	0.16	0.40
2	I	0.01	952	12.8	45.4	67.1	7.56	8.49	14.5	7.01	8.32	1.77	1.72
3	II	0.01	89.0	1.90	0.09	2.12	0.21	0.97	1.21	0.99	0.31	0.00	<0.1
4	II	0.1	108	3.78	0.89	4.10	0.17	2.17	2.98	2.24	0.78	0.00	<0.1
5	I	0.01	17.7	4.45	0.60	1.96	0.30	0.19	0.21	0.15	0.39	0.03	0.26
6	III	0.0001	130	4.03	2.42	6.82	2.51	2.04	2.96	1.88	2.84	0.19	0.10
7	III	0.001	315	6.74	0.55	7.09	1.40	3.10	4.46	3.04	2.49	0.16	1.12
8	II	0.001	18.6	5.30	1.33	0.33	0.07	0.05	0.01	0.01	0.12	0.00	<0.1
9	III	0.001	177	21.5	1.10	10.0	0.43	4.60	6.49	4.89	1.24	0.13	<0.1
10	I	0.0001	10.5	5.10	0.39	4.31	0.15	0.75	1.02	0.84	0.55	0.00	<0.1
11	I	0.0001	31.9	17.1	8.65	4.13	1.23	1.31	1.44	1.10	0.58	0.12	<0.1
12	II	0.001	273	6.32	1.03	1.83	0.13	0.04	0.05	0.01	0.11	0.08	<0.1
13	III	0.01	29.6	5.43	0.85	2.51	0.06	0.35	0.40	0.30	0.30	0.06	<0.1
14	IV	0.001	119	6.03	0.54	1.70	0.38	0.47	0.55	0.42	0.32	0.09	1.10
15	III	0.01	32.0	1.52	0.01	1.45	0.55	0.63	0.69	0.58	1.20	0.10	<0.1
16	II	0.0001	582	0.92	4.16	47.6	8.17	25.2	36.8	27.9	12.7	1.60	6.24
17	I	0.001	28.9	8.42	1.40	0.62	0.03	0.07	0.08	0.04	0.05	0.05	<0.1
18	III	0.0001	29.1	3.18	1.10	1.87	0.10	0.60	0.90	0.59	0.18	0.06	<0.1
19	IV	0.001	82.5	4.61	0.12	1.33	0.72	0.85	0.78	0.84	1.42	0.14	<0.1
20	I	0.01	101	8.59	0.22	0.54	0.37	0.76	1.50	0.76	0.58	0.08	0.45
21	II	0.0001	122	7.56	0.16	5.72	0.13	2.18	2.75	1.82	0.48	0.01	<0.1

sIgE and tIgE levels were determined using the UniCAP 250 system (Thermo Fisher Scientific). Red: sIgE ≥ 0.35 kU_A/L; orange: sIgE between 0.1 and 0.35 kU_A/L; green: sIgE <0.1 kU_A/L

*According to Ring and Messmer (43)

[1]For intradermal skin tests the lowest venom concentration [µg/mL] that gave a positive result is displayed.

Figure legends

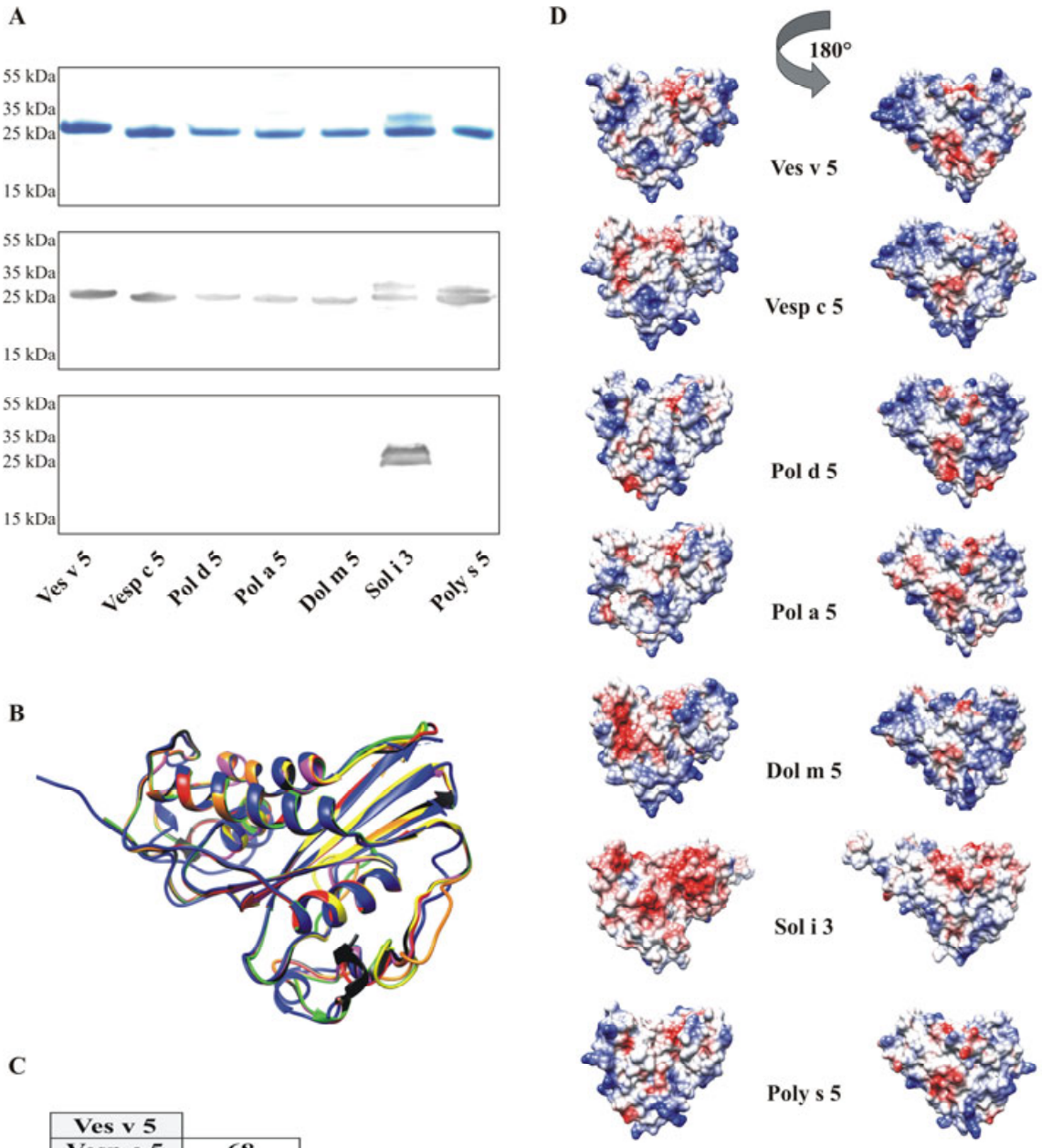
Figure 1. Recombinant expression and characterization of antigens 5. A, SDS-PAGE and immunoblot of recombinant antigens 5 visualized by either Coomassie blue staining (upper panel) or anti-V5 epitope antibody (middle panel) and *Galanthus nivalis* agglutinin (lower panel). B, Overlay of the structural ribbon diagrams of the investigated antigens 5, generated either by x-ray diffraction(30, 31) or structural modeling. C, Sequence identity between the different antigens 5 on protein level (in %). D, Electrostatic potential of the antigens 5. Coulombic surface coloring indicates the electrostatic potential, ranging from basic (blue) to acidic (red) surface properties.

Figure 2. s IgE reactivity of individual hymenoptera venom-allergic patients with recombinant antigens 5 in ELISA. A and B, sIgE immunoreactivity of patients from Germany (n=43) with a primary sensitization to YJV (A) or of patients from Spain (n=20) with a primary sensitization to PV and/or YJV (B). C, sIgE reactivity of selected representative patients. The lower end cut-off of the ELISAs is represented by dotted lines. D, sIgE cross-inhibition with Ves v 5 and Pol d 5 for selected patients with primary sensitization to either YJV (patients 26 and 30) or PV and/or YJV.

Figure 3. Basophil activation tests with recombinant antigens 5. Human basophils from YJV-allergic patients from Germany were exposed to different concentrations of the seven antigens 5 (Ves v 5 (*Vespula vulgaris*), Vesp c 5 (*Vespa crabro*), Pol d 5 (*Polistes dominula*), Pol a 5 (*Polistes annularis*),

Dol m 5 (*Dolichovespula maculata*), Sol i 3 (*Solenopsis invicta*) and Poly s 5 (*Polybia scutellaris*)). Additionally, stimulation with anti-FcεRI antibody (positive control) and plain stimulation buffer (negative control) is shown. Patients 71 and 72 and 73 and 74 represent HBV-allergic patients and non allergic controls, respectively. Activation is shown as percentage of $CD63^+$ out of total basophilic cells.

Figure 4. sIgE reactivity of YJV-allergic patients (n=21) in ImmunoCAP measurements. The sIgE immunoreactivity with the different hymenoptera venom extracts, individual allergens and MUXF was determined using the UniCAP250 platform (Thermo Fisher). Dotted lines indicate the 0.1 and 0.35 kU_A/L cut-offs. Numbers in bold on top of the graph indicate the percentage of IgE reactivity using the cut-off of 0.35 kU_A/L and and in regular writing of 0.1 kU_A/L, respectively



Ves v 5							
Vesp c 5	68						
Pol d 5	58	60					
Pol a 5	56	62	85				
Dol m 5	64	72	63	61			
Sol i 3	45	51	46	45	47		
Poly s 5	59	61	77	76	61	48	
	Ves v 5	Vesp c 5	Pol d 5	Pol a 5	Dol m 5	Sol i 3	Poly s 5